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GROWTH OF MC3T3-E1 PRE-OSTEOBLASTS ON BORATE CONTAINING GLASSES & GROWTH OF OSTEOBLASTIC CELLS ON 13-93 GLASS SCAFFOLDS

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Content

Abstract

Three borate-based glasses, compositions designated 1B, 2B, and 3B, with B_2O_3 levels of 15, 31, and 46 mole percent (**Table 1**), respectively, were tested for their effects on the growth of MC3T3-E1 mouse pre-osteoblastic cells under physiological fluid conditions. Silicate type 45S5 glass was used as a control for comparison. Independently, the biocompatibility of recently developed silicate-based porous 13-93 bioactive glass scaffolds was tested; Saos-2 human osteosarcoma cell line was used. Tests performed for the two studies included: contact assays of cell growth at the glass interface, chemical analysis of borate released into the culture medium, quantitative fluorescence DNA assay of cell proliferation in the presence of the test glasses, and scanning electron microscope imaging. The results obtained suggest that 1B and 2B glasses permit a satisfactory level of MC3T3-E1 cell growth while the porous 13-93 bioactive glass scaffolds appear highly promising for possible use in bone tissue engineering.

1. Introduction

Porous bioactive glass scaffolds are a key component in the process of rebuilding a broken bone. These scaffolds can be utilized for a wide variety of situations, including joint or bone repair in: accident patients, wounded soldiers, and osteoporosis sufferers. Previously tested materials designated for this purpose, included: metallics, ceramics, polymers, glass, donor bone. However, the materials mentioned each pose their own challenge. Many individuals are allergic to metallic compounds, ceramics do not readily absorb and have a long degradation time *in vivo*, polymers are structurally weak, cells do not grow well in glass, and patient's bodies may reject donor marrow. Optimally, the patients own bone tissue would be utilized for restoring a damaged region [1].

Our first research objective was to find a material with the benefits of silicate type bioactive glass that is additionally easy to work with. Developed in the 1970's, silicate type bioactive glasses, such as 45S5 glass of the SiO_2-Na_2O-CaO-P_2O_5 system (**Table 1**), are an established glass for bone repair purposes. These were the first glasses to demonstrate bioactivity; ability to bond with bone. Glasses based on the 45S5 composition are an appealing material due to their rapid bonding with bone which provides mechanical stability. The glasses are also biocompatible and promote osteoprogenitor cell function [2]. The recently developed borate-based glasses of a Na_2O-CaO-B_2O_3-SiO_2-P_2O_5 system (**Table 1**), derived from the silicate based 45S5 glasses, have also demonstrated bioactivity. The borate-based glasses are an attractive material because they have exhibited important benefits that the silicate type glasses lack. Borate-based glasses are easier to construct and mold into the desired shapes and separate tests have shown that the glasses readily convert into hydroxyapatite; important for effective bonding with bone tissue [3, 4]. Due to their considerable novelty, the effects of the borate on cell growth are largely unknown. In this project, three of the borate-based glasses, compositions designated 1B, 2B, and 3B (**Table 1**) were tested to assess the growth of the MC3T3-El mouse pre-osteoblast cells in the presence of and on these three glasses under physiological fluid conditions. Silicate type 45S5 glass was used as a control for comparison [5, 6].

Our second major objective was to find a relatively simple and efficient scaffold production method that would maintain the glass's bioactive properties. Silicate-based porous 13-93 bioactive glass scaffolds of the SiO_2-Na_2O-CaO-P_2O_5-K_2O-MgO system were recently fabricated using a sintering technique by Mr. Qiang Fu, Material Science and Engineering Department at the University of Missouri-Rolla. Porous 45S5 bioactive glass has shown the ability to support *in vitro* growth and development of osteoblast-like cells. The difficulty is the preparation of the 45S5 glass. The technique employed requires temperatures of about 1000°C. This causes the glass to crystallize and decreases its bioactivity or even neutralizes it completely. Due to this, an alternative scaffold has been developed. Tests have shown that 13-93 glass maintains its bioactive levels and requires a less strenuous process to manufacture. The glass forms a dense sintering neck at a temperature of 700°C. To obtain a highly porous three-dimensional scaffold a polymer sponge method, which is both fast and cost efficient, appears most promising [3,7].

The silicate-based 13-93 bioactive porous scaffolds are 85 ± 2% porous with a pore size between 200μm and 500μm (**Fig. 7**). This pore size permits vascularization; the in growth of veins and arteries to supply nutrients and eliminate waste products from the cells proliferating within the scaffolds [8]. This allows a clean and healthy environment for the growth of the cells.

The scaffolds are similar to bone not only in structure but also in strength. The scaffolds have a compressive strength of 3.0 ± 0.5MPa, while bones compression strength is between 2MPa and 12MPa [9]. The 13-93 porous scaffolds are designed to be implanted into a broken bone region with the purpose to stimulate the regeneration of the damaged bone [10]. Overtime, the scaffolds fully transform into hydroxyapatite and incorporate into the individuals own bone tissue [3, 7, 11].

2. Materials and Methods

2.1 *Cell Line and Culture*

The MC3T3-E1 mouse pre-osteoblast cell line and Human Osteosarcoma (Saos-2) cell line were both grown in α-MEM medium supplemented with 10% fetal calf serum (FCS), 2ml L-Glutamine, and 1ml penicillin streptomycin(PS), for every 100ml medium [12, 13]. Cells were maintained in 35 mm and 60 mm culture dishes in 2.5ml and 5ml complete MEM medium, respectively. Cells were incubated at 37 C in a humidified atmosphere containing 5% CO_2 and passaged every three to four days using PBS - to washing wastes out the plates, and 1xTrypsin - to detach the cells from the plates [14, 15].

2.2 *Borate-Based Glass Preparation*

In total, four different glasses were used in the borate-glass experiments. Silicate-based 45S5 glass was used as a control for comparison (also designated 0B). A borate equivalent of the 45S5 glass was developed (designated 3B). The 3B glasses had the same molar composition as the 45S5 except that all of the SiO_2 was substituted with B_2O_3. Additionally, two other borate containing glasses (designated 1B and 2B) were fabricated. The 1B and 2B glasses contained 1/3 and 2/3 of the B_2O_3 concentration of the 3B glass, respectively. SiO_2 accounted for the remaining portions.

To prepare borate glasses, glass particles were crushed in a hardened steel mortar and pestle, and sieved through stainless steel sieves allowing sizes between 150μm and 300μm. The prepared circular wafers with flat surfaces were cut into sheets with a diamond-coated blade. Dry wafers were smoothed by grinding with SiC paper, first with a 240 grit and next a 600 grit. Both 85 mg and 33 mg size wafers were prepared. The 85mg glass samples dimensions were: 10mm x 6mm x0.5mm. The 35mg glass samples dimensions were: 6mm x 4mm x 0.5mm [3].

2.3 *Porous 13-93 Glass Scaffold Preparation*

A mixture of analytical grade Na2CO3, K2CO3, MgCO3, CaCO3, SiO2, and NaH2PO4.2H2O was melted in a platinum crucible at 1300°C and quenched between cold stainless steel plates. Glass particles were crushed in a hardened steel mortar and pestle, and sieved through stainless steel sieves allowing sizes less than 150μm. The average particle size was reduced further to d50=2μm by attrition milling the particles for two hours with ethanol solvent.

The highly porous three-dimensional scaffolds were assembled using a polymer sponge technique. A glass slurry was prepared - dispersion and viscosity of the slurry were monitored. These parameters were important for proper porous network formation. The glass slurry was

carefully poured into a polyurethane sponge, in such a way that the slurry coated the walls; squeezed out of the polyurethane sponge excess slurry was decanted. The aqueous slurry with powder loading of 35vol% was prepared using 1wt% polyvinyl alcohol (PVA) (DuPont Elvanol® 90-50). Ball milling was performed for 24 hours in polypropylene bottles using alumina grinding media in order to ensure a homogeneous slurry mixture. The polyurethane sponges were submerged in the slurry and where compressed and released numerous times to allow the slurry to adhere to the sponge walls. The newly coated sponges were dried at room temperature for 24 hours. To burnout the binder and sponge, the samples were heated at 1 C/min to 500 C. Finally, heating up the samples at 5 C/min to 700 C (holding 10 minutes) allowed for sintering of the scaffolds without loss of their bioactivity (without crystallization of the glass) [9].

2.4 *Glass Maintenance*

Prior to usage, the 45S5, 1B, 2B, and 3B bioactive glasses and the 13-93 porous bioactive glass scaffolds were ultrasonically washed four times. The glasses were first washed twice with distilled water and then twice with 100% ethanol before being placed in a dry-heat sterilizer for a minimum of 2 hours.

2.5 *ICP Analysis*

Using inductively-coupled plasma mass spectrometry (ICP-MS) the concentration of borate ion released into the culture medium by each of the borate glasses was measured. Dry-heat sterilized duplicate samples of the four glasses were placed in serum-free alpha-MEM (100 mg glass/2.87 ml) in capped plastic tubes and incubated for four days at 37 C. The medium was withdrawn, diluted 25-fold with water in capped 60 ml conical tubes, and shipped to Acme Analytic Lab (Vancouver BC) for ICP-MS analysis. ICP values of 34 elements were reported in units of parts per billion (ppb) (**Fig. 1**) [3].

2.6 *Contact Assays*

To qualitatively assess the biocompatibility of the borate containing glass samples the morphology and density of the MC3T3-E1 cells near the glass interface were visually observed by conducting a contact assay. Into twelve 35mm plates, 2.5 ml complete MEM medium with 10% FCS containing 15,000 MC3T3-E1 cells per cm^2 was pipetted. Cells were allowed to attach to the plate for 2 hours before triplicate samples of 45S5, 1B, 2B, and 3B glasses (either 33 mg or 85 mg size pieces) were placed into the plate and immobilized with silicone stopcock lubricant. The plates were incubated at 37 C for periods of one and three days. Pictures of the glass interface were taken on day one and three. The plates were placed on the mechanical stage of a phase contrast microscope fitted with a CCD camera (Olympus Model DP70) and the *x*- and *y*- coordinates of the mechanical stage were recorded to assure repeat viewing of the same location of each glass interface. For the intermittent mixing assay the plates were prepared the same way except that they were placed on a mobile platform that moved every 5 minutes for 1 minute (**Fig. 3**) [3]

2.7 *Fluorescent DNA Assay of Borate-Based Glasses*

Total DNA of the stationary and intermittent mixing samples were monitored by a fluorescent DNA assay. Triplicate samples of each glass (either 33 mg or 85 mg size pieces) were placed into 35mm dishes containing 2.5 ml complete MEM medium with 10% FCS and 15,000 MC3T3-E1 cells per cm^2 as described in the Contact Assay section. The samples were incubated for four days before cell growth was inhibited. The culture MEM medium was extracted from each plate, the plates were rinsed with PBS, and cells were fixed with 1.5ml ice-cold 70% ethanol for 45 minutes. The glass pieces were removed and the ethanol was replaced by 2 ml of ice-cold 10mM tris (pH 7.5)/1mM EDTA (TE) solution. Plates were kept on ice to prevent enzyme degradation and the cells were lysed by sonic disruption using an ultrasonic oscillator (Branson Model 250 Sonifier). The lysates were centrifuged at 6000 RPM for 10 minutes at 4 C. Aliquots of the lysates were pipetted into a 96-well plate. PicoGreen® dsDNA reagent (Molecular Probes) was added to fluorescently measure the amount of dsDNA in the cell lysates [16]. A BMG Optistar plate reader (485nm ex/520nm em) measured the fluorescence intensities of the samples, lambda DNA was used as standard for comparison (**Fig. 4 and 5**) [3, 17].

2.8 *Fluorescent DNA Assay of Porous 13-93 Glass Scaffolds*

Silicate-based 13-19 porous glass test scaffolds were inoculated with Saos-2 cells at a density of 100,000 cells per scaffold and incubated at 37 C. At intervals of 2, 4, and 6 days the four scaffolds each were removed, washed with PBS, fixed with 70% ethanol and frozen for subsequent measurement of DNA content. On day six, 1.5ml of ice-cold 10mM tris (pH 7.5)/1mM EDTA (TE) solution was added to each scaffold. Using the technique described above the scaffolds were crushed into powder to release the cellular DNA and lysates were collected. To assess the amount of cell growth, aliquots of the lysate were pipetted into a 96-well plate and 100µl of PicoGreen® dsDNA reagent (Molecular Probes) was added to the wells to measure the amount of dsDNA in the cell lysates [16] the same fluorescent intensity recording technique as described above was used (**Table 2**) [3, 17].

2.9 *Scanning Electron Microscopy Imaging of Borate-Based Glasses*

Scanning electron microscopy (SEM) of the glass surfaces was performed to visually observe cell morphology, their adherence to the glass, and their overall growth pattern on the four glasses (**Fig. 6**). Duplicates of 45S5, 1B, 2B, and 3B glasses and two blanks were tested. A cell density of 15,000 cells per cm^2 in complete MEM medium containing 10% FCS was inoculated into 10 wells of a 24 well plate. Plates were incubated at 37 C. The experiment was run for both a two and a four day period. At the end of the incubation period, the glasses were washed twice with PBS pre-warmed to 37 C and fixed in 2% glutaraldehyde in PBS for 60 min at 37 C. The glasses were washed 7 times with PBS for 2 minutes each. To dehydrate the cells, 2ml of 50%, 70%, 80%, and 90% ethyl alcohol was added to each well and kept for 10 minutes. 2ml of 100% ethyl alcohol was added to the wells and kept for 10 minutes; this last step was performed three times. Under a fume hood the glasses were transferred into glass dishes containing 100% hexamethyldisilazane (HMDS) and soaked for 3-5 minutes. The HMDS was

replaced once and the glasses were soaked for another 3-5 minutes. Using watch maker forceps, the glasses were lifted out of the HMDS and the edge was blotted on tissue paper to drain off excess HMDS. The glasses were allowed to air dry before being placed back into the 24 well plate and into a desiccator to avoid re-hydration of samples. The prepared glasses were then gold and platinum (Au/Pt) coated for scanning electron microscopy observation [18].

2.10 *Scanning Electron Microscopy Imaging of Porous 13-93 Glass Scaffolds*

A similar procedure, as described above, was utilized to observe Saos-2 cells under a scanning electron microscope (**Fig. 7**). Scaffold samples were inoculated with 100,000 Saos-2 cells per scaffold. Scaffolds were incubated, fixed at intervals of 2, 4, and 6 days and prepared for examination by scanning electron microscopy using the same procedure as described above (Section III. J) [18].

3. Results and Discussion

3.1 *Borate-Based Glasses*

The ICP analysis, chemical analysis of borate released into the medium, has revealed that glasses 1B, 2B, and 3B release 1.296mM, 2.886mM, and 16.524mM borate ions, respectively (**Figure 1**). It was expected that 3B glass would release a substantially greater amount of borate than the other glasses because it contains 46.1 mole percent B_2O_3, whereas 1B and 2B contain 15.4 and 30.7 mole percent, respectively. The correlation between borate-ion concentrations and growth of MC3T3-E1 cells was clearly verified to be an inverse relationship. Increase in borate-ion concentration, especially of amounts greater than 5mM, showed considerably low concentrations of cell density of less than 35% (**Figure 2**).

To visually observe the effects of the borate on the MC3T3-E1 cell growth immobile and mobile contact assays were performed. The pictures from day one illustrate similar cell concentrations in the plates containing 45S5, 1B, 2B, and 3B. However, by day three the concentrations in the plates containing 45S5 glass were considerably greater than the concentration in the 3B plates. The 1B and 2B plates showed satisfactory growth (**Figure 3**). The fluorescent DNA analysis confirmed the concentrations in each plate. The total DNA (relative to the 45S5 control glass) for the 85 mg samples were 100%, 77.3%, 45.3%, and 22.4% cell proliferation for the 45S5, 1B, 2B, and 3B samples, respectively. The plates containing 33 mg samples seemed to show a lower degree of borate-ion effect on MC3T3-E1 cell growth; this may have been due to an insignificant amount of borate release (**Figure 4**).

Interestingly, mobility appeared to decrease borate-ion effect. In a secondary fluorescent DNA assay between static and dynamic conditions differences in cell proliferation were 7.7%, 37.7%, and 41.6% for 1B, 2B, 3B samples, respectively (**Figure 5**). This apparent decrease in borate-ion effect may be due to the dilution of borate in the medium as it was intermittently mixed. In the stationary samples borate released into the medium stayed in close proximity to the glass and to the cells bound to the glass.

Scanning electron microscope imaging of MC3T3-E1 cells on the borate glasses and the 45S5 glass exhibited the same morphology. The images showed the same pattern; cell concentration decreased from 45S5 to 3B (**Figure 6**).

3.2 *Porous 13-93 Bioactive Glass Scaffolds*

The fluorescent DNA assay revealed an approximate linear increase in Saos-2 cell growth over the six day incubation. The average amounts of DNA measured at intervals of 2, 4, and 6 days were 82 ± 61, 409 ± 158, and 1114 ± 191 ng/scaffold, respectively (**Table 2**). The scanning electron microscopy images confirmed the fluorescent assay observed pattern. Saos-2 cells showed an increase in cell density over the six day incubation period. The cells showed same cell morphology and appeared well adhered to the glass scaffolds (**Figure 7**).

4. Conclusion

The first major objective of our research was to find a material with the benefits of silicate type bioactive glass that is also easy to work with. The second objective was to find a relatively simple and efficient scaffold production method that would maintain the glass's bioactive properties.

The three borate-based glasses, compositions designated 1B, 2B, and 3B, demonstrated the additional qualities 45S5 lacks and seemed to have high potential to serve as an alternative for the popular silicate-based bioactive glass. The growth of MC3T3-E1 mouse pre-osteoblast cells was assessed in the presence of and on these three glasses under physiological fluid conditions. The results gathered from the contact assays, quantitative fluorescence assays, and scanning electron microscope imagings suggest that the borate-ion released into the medium has an inhibitory effect on MC3T3-E1 cell growth. It appears that mobility decreased the effects of the borate-ion; the quantitative difference in DNA concentrations between 45S5 glass and 3B were significantly lower in the mobility test than in the stationary contact assays. The scanning electron microscope imaging illustrated same cell morphology on all the glasses. In summary, the results obtained suggest that only 1B and 2B glasses permit a satisfactory level of MC3T3-E1 cell growth, but still are not the most attractive alternative for the silicate-based 45S5 bioactive glass.

The biocompatibility of the 13-93 bioactive porous glass scaffolds produced using a sintering technique by Mr. Qiang Fu were tested by a fluorescent DNA assay and scanning electron microscopy imaging. The results of the fluorescent DNA assay have shown an approximately linear increase in cell density during the six-day incubation. Additionally, the SEM images obtained showed same cell morphology, an increase in cell density with incubation time, and the cells well adhered to the scaffolds. Collectively, the results obtained suggest that the porous 13-93 glass scaffolds appear promising for possible use in bone tissue engineering.

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Table 1. Compositions of the four glasses used in the borate–based glass experiments

Glass	Mole Percent				
	Na_2O	CaO	B_2O_3	SiO_2	P_2O_5
0B (45S5)	24.4	26.9	0	46.1	2.6
1B	24.4	26.9	15.4	30.7	2.6
2B	24.4	26.9	30.7	15.4	2.6
3B	24.4	26.9	46.1	0	2.6

Table 2. Average amounts of DNA measured from the Fluorescent DNA Assay of Porous 13-93 Glass Scaffolds

Day	Average Total DNA (ng/scaffold)
2	82 ± 61
4	409 ± 158
6	1114 ± 191

Figure Captions

Figure 1. ICP analysis of borate-ion released (from 45S5, 1B, 2B, and 3B glasses) into serum-free alpha MEM after a four day incubation at 37 C.

Figure 2. Effect of Borate-ion concentrations (mM) with respect to MC3T3-E1 cell density.

Figure 3. Contact assay images of 45S5, 1B, 2B, and 3B glass with MC3T3-E1 pre-osteoblastic cells after one day and three days of culture.

Figure 4. Quantitative measurement of DNA content in MC3T3-E1 cultures incubated for 4 days with 33mg and 88mg glass samples.

Figure 5. Quantitative measurement of DNA content in MC3T3-E1 cultures incubated for four days at 37 C with 85mg glass samples under static and intermittent mixing conditions.

Figure 6. Scanning electron microscopy imaging of MC3T3-E1 pre-osteoblastic cells on 45S5, 1B, 2B, and 3B glass (400X).

Figure 7. Scanning electron microscopy imaging of plain porous 13-93 bioactive glass scaffold (50X) compared with porous 13-93 bioactive glass scaffolds seeded with Saos-2 human osteosarcoma cells (400 X).

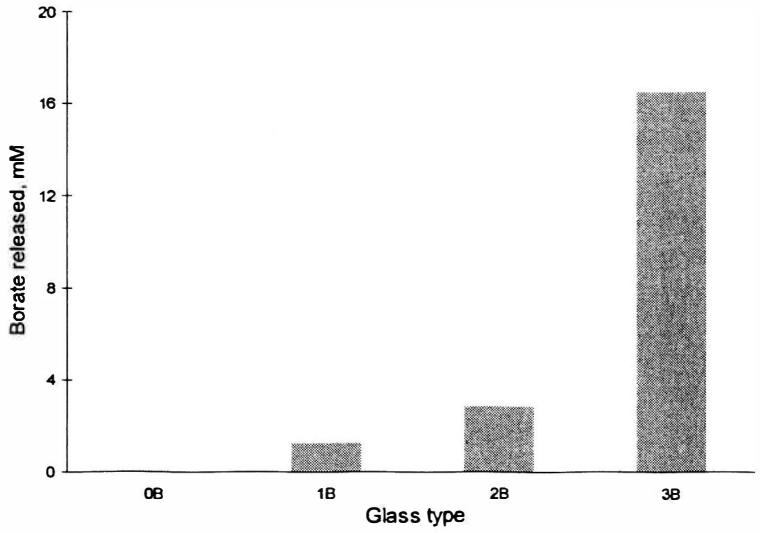
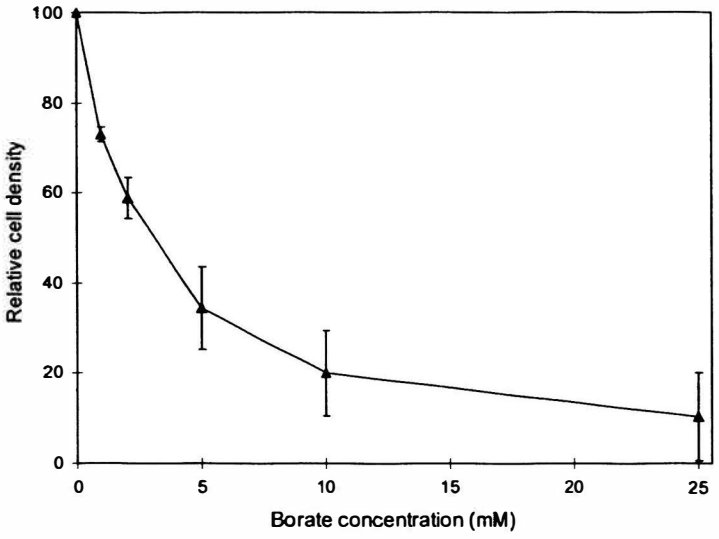


Figure 1.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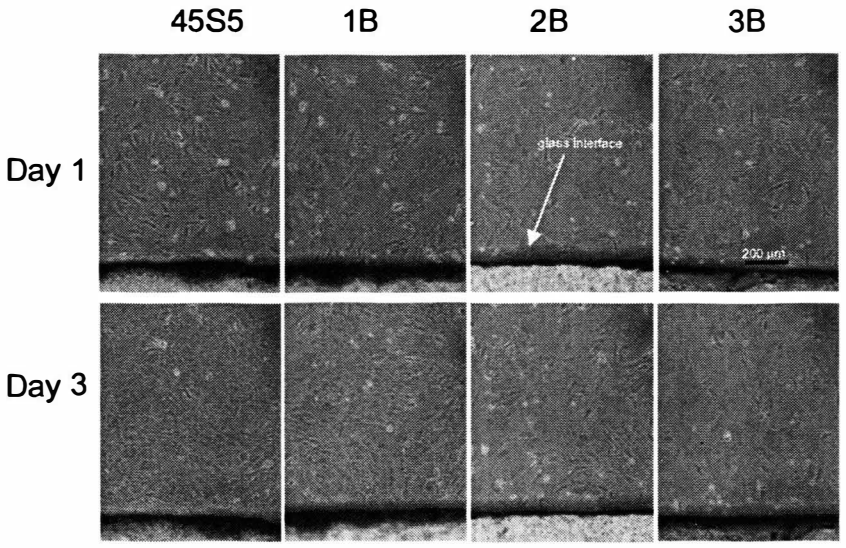
60
40
20
0
Relative cell density
0
5
10
15
20
25
Borate concentration (mM)

Figure 2.



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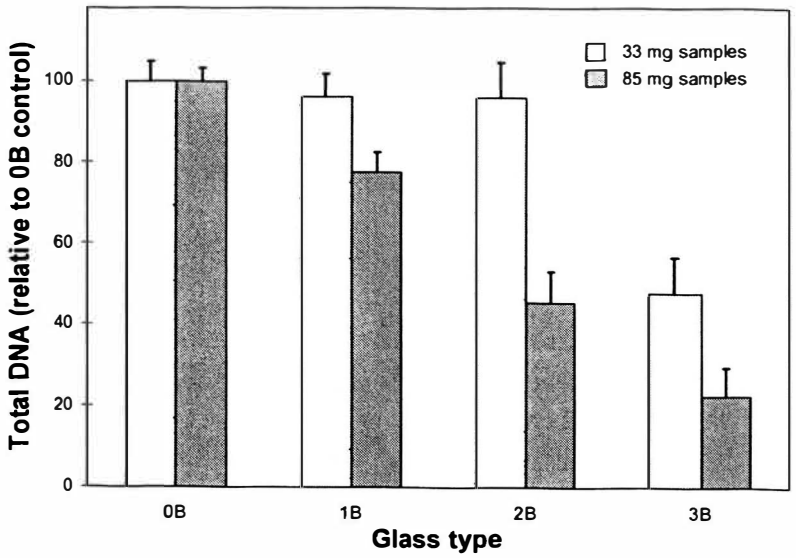
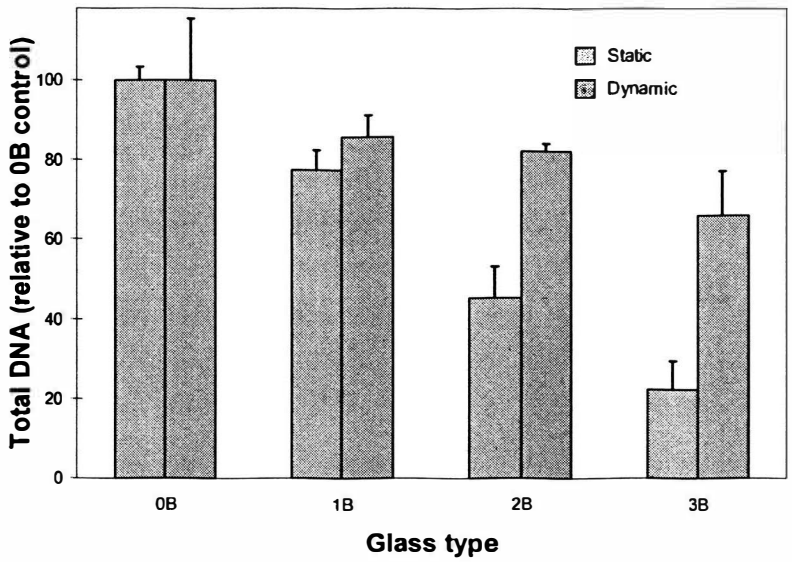

33 mg samples
85 mg samples
Total DNA (relative to 0B control)
100
80
60
40
20
0
0B
1B
2B
3B
Glass type

Figure 4.


Total DNA (relative to 0B control)
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60
80
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Static
Dynamic
0B
1B
2B
3B
Glass type

Figure 5.

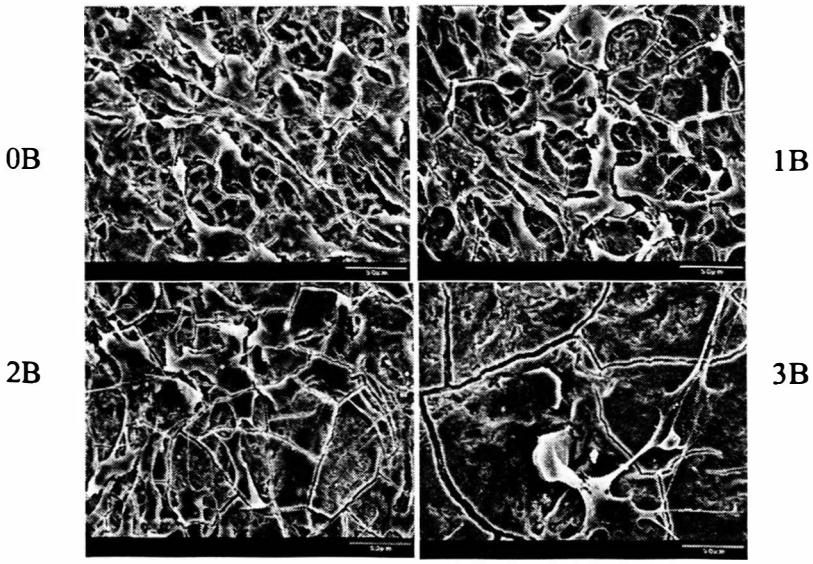
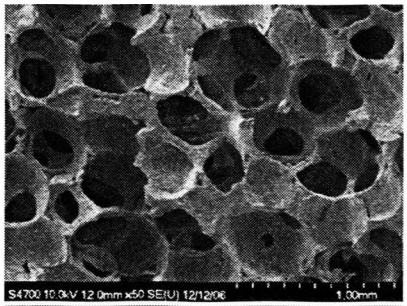
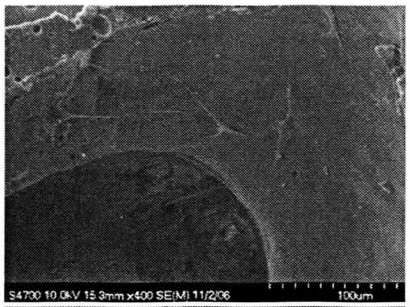


Figure 6.

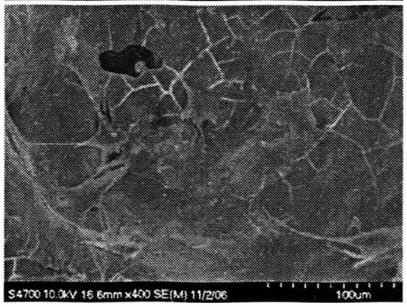
Porous Scaffold

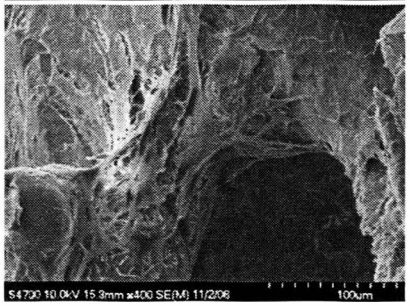




Day 2

Day 4





Day 6

Figure 7.