Processing of Biomaterials

PREPARATION AND BIOACTIVE CHARACTERISTICS OF POROUS BORATE GLASS SUBSTRATES

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ABSTRACT

Whereas silicate-based bioactive glasses and glass-ceramics have been widely investigated for bone repair or as scaffolds for cell-based bone tissue engineering, recent data have demonstrated that silica-free borate glasses also exhibit bioactive behavior. The objectives of this study were to fabricate porous, three-dimensional substrates of a borate glass and to investigate the biocompatibility of the borate glass substrates by *in vitro* cell culture with human mesenchymal stem cells (hMSCs) and hMSC-derived osteoblasts (hMSC-Obs). Borate glass particles with sizes 212-355 μm were loosely compacted and then sintered at 600°C to form porous disc-shaped substrates (porosity ≈ 40%). Partial or nearly complete conversion of the glass substrates to a calcium phosphate (Ca-P) material was achieved by soaking the substrates for 1 day or 7 days in a 0.25 molar K_2HPO_4 solution at 37°C and at pH of 9.0. Bone marrow derived hMSCs and hMSC-Obs seeded in the samples both adhered to the porous constructs whereas hMSC-Obs markedly synthesized alkaline phosphatase, an early osteogenic marker. These data indicate strong bioactive characteristics for the borate glass constructs and the potential use of the constructs for bone tissue engineering.

INTRODUCTION

Certain compositions of glasses, glass-ceramics, and ceramics, referred to as bioactive ceramics, have been widely investigated for healing bone defects, due to their ability to enhance bone formation and to bond to surrounding tissue [1-5]. Cell-seeded bioactive ceramics are also of interest as potential scaffolds for bone tissue engineering [6,7]. Hydroxyapatite and tricalcium phosphate ceramics, composed of the same ions as bone, are biocompatible and produce no systemic toxicity or immunological reactions, but they resorb slowly or undergo little conversion to a bone-like material after implantation [8,9]. Many bone regeneration applications require gradual resorption of the implanted biomaterials and concurrent replacement of the biomaterials by the host bone.

Bioactive glasses are superior to the less reactive ceramics in that they are osteoinductive as opposed to osteoconductive. Furthermore, the dissolution and conversion of bioactive glasses to a calcium phosphate (Ca-P) material seems to induce bone cell differentiation [10]. A characteristic feature of bioactive glasses is the time-dependent modification of the surface, resulting in the formation of a calcium phosphate (Ca-P) layer through which a bond with the surrounding tissue is established [11,12]. It has been suggested that the formation of a Ca-P layer *in vitro* is indicative of a material's bioactive potential *in vivo* [4,5,13].

Since the report of its bone bonding properties in 1971 by Hench *et al.* [14], the bioactive glass codenamed 45S5, referred to as Bioglass®, with the composition of 45% SiO_2, 6% P_2O_5, 24.5% Na_2O, and 24.5% CaO (by weight), has received most interest for biological applications [4,5]. Bioactive glasses based on the 45S5 composition are attractive scaffold materials because

their rapid bonding to bone provides early mechanical stability, in addition to stimulating osteoprogenitor cell function, and biocompatibility [15-17]. *In vivo* studies have shown that 45S5 glass can stimulate bone regeneration [18-20], whereas *in vitro* studies have shown that the glass itself and the soluble ionic species released by dissolution have an osteoinductive effect [21-24]. Porous bioactive silicate glass constructs based on the 45S5 composition have been developed as possible tissue engineering scaffolds [25,26]. Cell culture experiments indicated that the porous glass can function as a template for generating mineralization *in vitro* [25].

The low chemical durability of some borate glasses has been known for decades but the potential of borate glasses in biomedical applications has not been explored until recently [27,28]. A borate glass, designated 45S5B1, with the same composition as 45S5 bioactive glass but with all the SiO_2 replaced by B_2O_3, was investigated by Richard [29]. *In vitro* experiments indicated that a Ca-P layer forms on the surface of the borate glass upon immersion in a K_2HPO_4 solution at 37°C and that the Ca-P layer forms more rapidly on the borate glass than on 45S5 bioactive glass [29]. As a first *in vivo* experiment, 45S5B1 borate glass particles (partially reacted in a K_2HPO_4 solution to produce a surface Ca-P layer) and 45S5 glass particles were separately implanted into defects (0.6–1.2 mm in diameter) in the tibia of rats [29]. Histological examination of the harvested constructs indicated that the partially converted borate glass particles promoted bone growth more rapidly than the 45S5 glass particles. Both types of glass particles promoted sufficient bone growth for closure of the implant site after 60 days [29].

The more rapid conversion of borate glass to Ca-P at near body temperature and the favorable *in vivo* reaction of particles to produce bonding with bone warrant additional investigations of the value of borate glass as bone replacement materials and as scaffolds for bone tissue engineering. However, little is known about the fabrication of the borate glass into porous, three-dimensional constructs or the effects of the borate glass on cell attachment, growth and differentiation. The objectives of this study were to produce porous, three-dimensional substrates of a borate glass intended for bone tissue engineering and to investigate the effects of the fabricated borate glass constructs on attachment and differentiation of human mesenchymal stem cells (hMSCs) and hMSC-derived osteoblasts (hMSC-Obs).

EXPERIMENTAL PROCEDURE

Fabrication of Borate Glass Substrates

Particles of borate glass (Na_2O-CaO-B_2O_3) were prepared by melting reagent grade chemicals in a platinum crucible, quenching the melt, and crushing the glass in a hardened steel mortar and pestle. After removing the metallic impurities magnetically, the particles were sieved through stainless steel sieves to produce sizes in the range of 212–355 μm. Porous disc-shaped substrates (15 mm diameter × 2–3 mm thickness) were produced by pouring the glass particles into vibrating graphite molds, followed by sintering for 10 min at 600°C. The structure of the porous substrates was examined using X-ray diffraction and optical microscopy. The porosity of the substrates was estimated from the computer imaging of optical micrographs and from the measured density.

Conversion of the porous borate glass substrates to Ca-P was investigated by immersing the substrates in 0.25 molar K_2HPO_4 solution with a starting pH value of 9.0 at 37°C and measuring the weight loss as a function of time. The structural characteristics of the converted material were observed using scanning electron microscopy (SEM; Hitachi S-4700). Some glass substrates used in cell culture experiments were partially or fully converted to Ca-P to determine the most favorable condition of the borate glass for supporting cell growth and differentiation. The par-

tially converted borate glass substrates (denoted pBG) and the fully converted substrates (denoted Ca-P) were prepared by immersing the porous glass substrates for 1 day and 7 days, respectively, in the K_2HPO_4 solution.

Cell Culture on Porous Borate Glass Substrates

Human bone marrow derived mesenchymal stem cells (hMSCs) were isolated from bone marrow samples (AllCells, Berkeley, CA) using a RosetteSep kit (Stem Cell Technologies, Inc., Vancouver, BC, Canada). The hMSCs were grown in monolayer in cell culture media consisting of 89% DMEM, 10% FBS, 1% penicillin – streptomycin (basal cell culture media). After 4 days non-adherent cells were removed and the media was changed every 4 days. Cells were passaged up to four times each time upon confluency. Upon the 4th passage, 50% of the hMSCs were exposed to osteogenic supplemented medium (basal cell culture media, 100 nM dexamethasone, 50 μg/mL L-ascorbic acid-2-phosphate). Upon exposure to osteogenic supplement, hMSCs differentiated into osteoblastic cells (hMSC-Obs) [30-32], whereas the other 50% hMSCs continued incubation in basal culture complete media without osteogenic supplement.

The hMSCs and hMSC-Obs were seeded (30,000 cells per cm^3) on porous substrates of the unconverted borate glass (BG), the partially converted borate glass (pBG), or the completely converted borate glass (Ca-P), and incubated for an additional 14 days. Live cell assay was then performed using Promega (Madison, WI) CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, which quantified cell viability through NADH activity using 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). The absorbance values for MTS correlate with a live cell number as documented in the product information sheet. Alkaline phosphatase activity (AP) was assayed by Napthol as-biphosphate, fast red violet salt, and N,N dimethylformamide solution (Sigma-Aldrich Co., St. Louis, MO).

RESULTS AND DISCUSSION

Figure 1 shows an optical micrograph of the surface of a porous borate glass substrate produced by sintering. The touching particles are bonded at the necks, providing enhanced strength without significant flow of the glass into the pores. The reduction of the porosity of the substrates during sintering was negligible. Computer imaging of optical micrographs indicated that the substrates had a porosity of 40-45% and a median pore size of 100-150 μm. The porosity estimated by computer imaging was in agreement with the value determined from the measured density of the substrate and the density of the fully dense glass (2.58 g/cm^3). X-ray diffraction showed that the glass in the porous substrate remained amorphous after sintering.

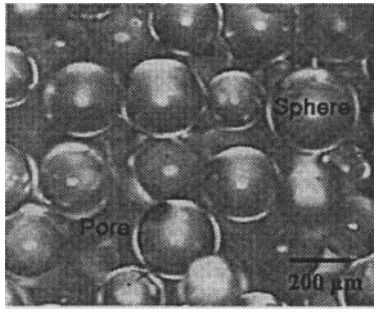

Figure 1. Optical micrograph of the surface of a porous borate glass substrate produced by sintering a loosely compacted mass of particles (212-355 μm) for 10 min at 600°C.

The weight loss data for the porous borate glass substrates during their conversion to Ca-P in K_2HPO_4 solution are shown in **Fig. 2** as a function of time. Conversion of the glass to Ca-P, as indicated by the maximum weight loss (60-65%), was completed after approximately 7 days. The conversion of the borate glass to Ca-P is believed to involve dissolution of the glass into the surrounding liquid and precipitation of calcium and phosphate ions onto the surface of the substrate [33]. Assuming that all of the sodium and borate ions from the glass go into solution and all of the calcium ions go into the formation of a Ca-P material with the composition of stoichiometric hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, then the theoretical weight loss should be 69%. The discrepancy between the maximum measured weight loss and the theoretical weight loss may be due to incomplete conversion of the glass, some calcium ions remaining in solution, the formation of a nonstoichiometric hydroxyapatite with a Ca/P ratio lower than the stoichiometric value of 1.67, or a combination of all three factors. Chemical analysis of the Ca-P material formed by the conversion of similar borate glasses under the same conditions indicated that the Ca/P ratio was well below 1.67 [34].

Conversion of the borate glass to Ca-P starts at the surface and moves inward [33]. By controlling the time of reaction in the K_2HPO_4 solution, substrates with different ratios of the surrounding Ca-P layer to the borate glass core can be produced. Constructs reacted for 1 day consisted of an interconnected mass of composite particles, with a thin surface layer of the glass converted to Ca-P. The thickness of the Ca-P layer, estimated from the weight loss data was 40-50 μm. Substrates reacted in the K_2HPO_4 solution for 7 days were almost fully converted to Ca-P, and consisted of an interconnected mass of Ca-P particles. **Figure 3** shows SEM micrographs of the surfaces of the three types of porous substrates used in the present work in cell culture experiments. The unconverted borate glass (BG) substrate has smooth surfaces characteristic of the spheroidized glass particles, whereas the constructs of the partially converted glass (pBG) and the fully converted glass (Ca-P) have less smooth surfaces. High resolution SEM, performed in related work [35], indicated that the Ca-P material was highly porous, with fine pores on the order of several tens of nanometers.

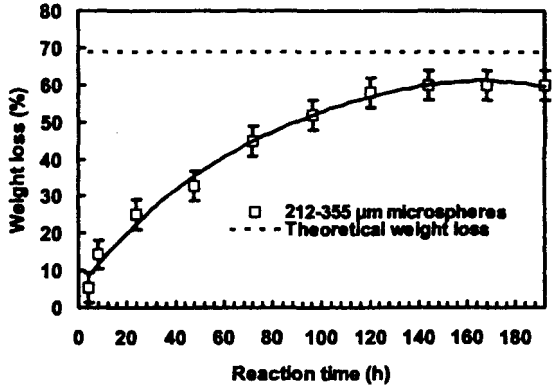

Figure 2. Weight loss of porous borate glass substrates as a function of time in 0.25 molar K_2HPO_4 solution at 37°C and a pH value of 9.0. Conversion of the glass to a calcium phosphate (Ca-P) material in the solution is accompanied by a weight loss. The estimated theoretical weight loss is shown by the horizontal dotted line.

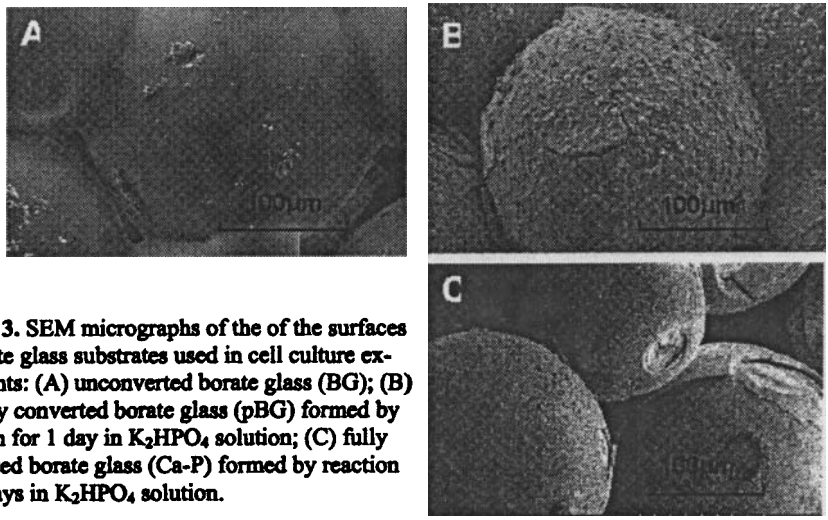

Figure 3. SEM micrographs of the of the surfaces of borate glass substrates used in cell culture experiments: (A) unconverted borate glass (BG); (B) partially converted borate glass (pBG) formed by reaction for 1 day in K_2HPO_4 solution; (C) fully converted borate glass (Ca-P) formed by reaction for 7 days in K_2HPO_4 solution.

The differences in the condition of the borate glass substrates may influence the interaction with cells. However, the most favorable condition of the borate glass for cellular interaction is, at present, unclear. The unconverted borate glass (BG) with its smooth surface initially may not provide favorable sites for cell attachment and significant dissolution of calcium, sodium and borate ions will occur initially into the surrounding fluid as the glass surface reacts with the fluid. For constructs of the partially converted glass (pBG), the porous Ca-P surfaces may provide more favorable sites for cell attachment. Dissolution of calcium, sodium and borate ions into the surrounding fluid is still expected to occur but at a lower rate than for the unconverted glass. The fully converted constructs (Ca-P) provide surface sites similar to those of the pBG constructs, but almost no dissolution of sodium and borate ions into the surrounding fluid will occur due to the absence of any significant quantity of borate glass in the substrate.

The unconverted borate glass substrates (BG) disintegrated during cell culture experiments, presumably due to reactions of the glass with the cell culture medium. However, the partially converted substrates (pBG) and the fully converted substrates (Ca-P) remained intact and maintained their original cylindrical shape throughout the experiments. Live cell number (MTS) assayed after 14 days verified the cell viability of both hMSCs and hMSC-Obs cultured on the pBG and Ca-P substrates. The hMSCs seeded on the pBG templates had significantly higher cell viability than hMSCs seeded on the Ca-P templates (Fig. 4). The data show a similar trend for hMSC-Obs seeded on the pBG and Ca-P templates but the difference is not significant due to the wider variability of the data for Ca-P templates. The higher cell viability of the hMSC on the pBG substrates may indicate that pBG stimulates cell function. As outlined earlier, a key difference between the pBG and Ca-P substrates is the potential for dissolution of calcium, sodium, and borate ions from the underlying borate glass core of the pBG templates into the culture medium. The mechanism by which these ions may influence cell function is not clear at present but may be important for determining the optimum condition of the borate glass substrates for tissue engineering applications. For cells seeded on the pBG substrates, the data in Fig. 4 also indicated

that the osteogenic cells had significantly higher cell viability than hMSCs. The hMSCs were initially seeded at a higher density than the hMSC-Obs (9,000 cells per construct, versus 3,000 cells per construct, respectively) due to the fact that MSCs proliferated more rapidly than MSC-Obs during the pre-treatment. The data presented in **Fig. 4** plots the MTS absorbance per seeded cell number normalized to MSCs grown on Ca-P. Future studies will investigate apoptosis and proliferation of MSCs and their differentiated osteoblasts on the borate glass substrates.

Active alkaline phosphatase was produced by the cells within the borate glass substrates as indicated by the dark red stain (**Fig. 5**). Higher alkaline phosphatase activity was seen in hMSC-Obs samples (**Fig. 5C, D**) as compared to the undifferentiated, hMSCs (**Fig. 5A, B**). This indicates that a combination of osteogenic supplements with a bioactive borate glass substrate will have a positive effect on osteogenic differentiation.

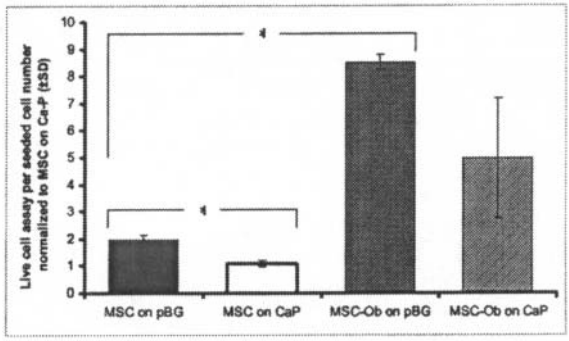

Figure 4. Live cell assay using light absorbance as an metabolic indicator of hMSCs and hMSC-Obs on partially converted borate glass (pBG) and Ca-P substrates. n=4; *= Students' T-test $p < 0.05$.

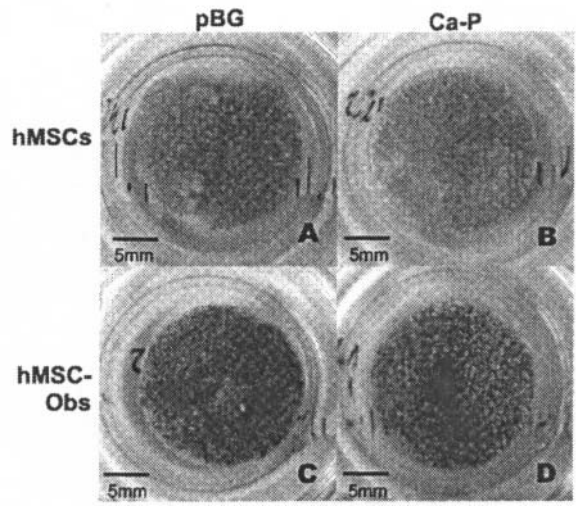

Figure 5. Photographs of (A) human mesenchymal stem cells (hMSCs) cultured on substrates of partially converted borate glass (pBG), (B) hMSCs cultured on substrates of fully converted borate glass (Ca-P), (C) hMSC derived osteoblasts (hMSC-Obs) cultured on pBG substrates, and (D) hMSC-Obs cultured on Ca-P substrates. All samples stained for alkaline phosphatase activity using the same protocol. Red (dark) stain indicates high alkaline phosphatase activity.

CONCLUSIONS

Porous bioactive borate glass substrates, prepared by sintering a loosely compacted mass of particles, were conditioned in 0.25 molar K_2HPO_4 solution at 37°C to convert a controlled amount of the glass to a calcium phosphate (Ca-P) material. The cytocompatibility of porous substrates consisting of the unconverted glass (BG), the partially converted glass (pBG), and the fully converted glass (Ca-P), was investigated by *in vitro* cell culture with human mesenchymal stem cells (hMSCs) and hMSC derived osteoblasts (hMSC-Obs). The hMSCs seeded on pBG substrates had a higher metabolic activity and cell viability than on Ca-P substrates. For pBG substrates, hMSC-Obs had significantly higher cell viability than hMSCs. Alkaline phosphatase activity on the pBG and Ca-P substrates with hMSC-Obs revealed the ability of these materials to support osteogenic cells. The data suggest the necessity for additional *in vitro* and *in vivo* investigations of the potential of bioactive borate glass as a cell-accommodating scaffold for bone tissue engineering. In particular, the pBG construct, consisting of a network of borate glass particles surrounded by a Ca-P layer, had the highest cell viability for both cell types and may represent a more favorable condition of the borate glass for bone tissue engineering.

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