Advances in Bioceramics and Biocomposites

A collection of papers presented at the 29th International Conference on Advanced Ceramics and Composites, January 23-28, 2005, Cocoa Beach, Florida

Editor
Mineo Mizuno

General Editors
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Preface

A "Bioceramics and Biocomposites" session was started in 2002 in the 26th International Conference on Advanced Ceramics and Composites. The session was raised to a Bioceramic symposium in 2005. We appreciate the program chair for the decision. It was timely since bioceramics have been recognized to be one of the most important materials in order to overcome problems of an aging society in the near future.

The use of ceramics in biological environments and biomedical applications is of increasing importance, as is the understanding of how biology works with minerals to develop strong materials. Bones and teeth are composed of inorganic (calcium phosphate) and organic (protein) materials. They are ultimate composites, being skillfully tailored to show both structural and bioactive functions. Therefore this symposium contained several topics, such as biomimetics, processing of materials for biomedical applications, interactions of ceramics in biological/biomedical applications, performance issues in biomedical ceramics, orthopaedic replacements, and dental ceramics.

A total of 45 papers were presented in this Symposium: including 10 invited papers. Authors, from academia, national laboratories, industries, and government agencies, gathered in Cocoa Beach in Florida in 2005, from 9 countries around the world.

The symposium organizers would like to thank all of the participants in the symposium and the staff at the ACerS. We appreciate ACerS for their efforts in organizing the review process and coordinating the production of this volume of Ceramic Engineering and Science Proceedings. The symposium organizers hope that this symposium will promote the quality of life for humanity.

Mineo Mizuno
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Processing of Biomaterials
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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 K2HPO4 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% SiO2, 6% P2O5, 24.5% Na2O, 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

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their rapid bonding to bone provides early mechanical stability, in addition to stimulating osteo-
progenitor 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 poten-
tial 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 SiO2 replaced by B2O3, 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 K2HPO4 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
K2HPO4 solution to produce a surface Ca-P layer) and 45S5 glass particles were separately im-
planted 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 favor-
able 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 engi-
neering. 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 differen-
tiation. 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 (Na2O-CaO-B2O3) were prepared by melting reagent grade chemi-
cals 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 K2HPO4 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 K₂HPO₄ 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% 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 cm²) 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® AQueous 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/cm³). 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.](image-url)
The weight loss data for the porous borate glass substrates during their conversion to Ca-P in K$_2$HPO$_4$ 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$_2$HPO$_4$ 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$_2$HPO$_4$ 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](image-url) Figure 2. Weight loss of porous borate glass substrates as a function of time in 0.25 molar K$_2$HPO$_4$ 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.
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