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# Glass Bone Implants: The Effect of Surface Topology on Attachment and Proliferation of Osteoblast Cells on 45S Bioactive Glass

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## ABSTRACT

Bioglass 45S is a promising bone implant material with superior biocompatibility. Past research showed that adhesion of bone cells to titanium is strongly affected by its surface architecture. However, little is known about the role of surface topology of glass on its use as an implant. Thus, we systematically investigated the effect of surface roughness ($R_a$ ~ 0.01 – 1.2 μm) on cell adhesion and proliferation on 45S Bioglass *in vitro*. MG63 osteosarcoma and MC3T3 osteoblast precursor cells were seeded on the glass samples, and incubated for up to 6 days. The attachment, morphology and proliferation of cells were investigated using fluorescence microscopy. Our results show that cell attachment (as indicated by cell spreading and number of focal adhesion sites), and proliferation rate decrease with increasing roughness of bioactive glass surface. These findings provide important insight for improving surface characteristics of bioactive glass bone implants.

## INTRODUCTION

Although bone implants date back to 600 A.D., the search for an ideal bone-replacement material continues [1]. In fact, with increasing life expectancy destructive lesions of bones, whether due to disease or trauma, are becoming increasingly common. Some such conditions include benign and malignant bone tumors, bone fractures, middle ear deafness and periodontal diseases. In all cases, the ultimate goal is to replace the defective or missing bone tissue with a functioning material that will last a patient's lifetime. In 1952, the Swedish orthopedic surgeon, Brånemark, in his research on titanium implants observed that bone grew into such close proximity with the titanium that it effectively adhered to the metal [2]. However, titanium implants have been found to cause scar tissue to form on the implant surface over time requiring implant replacement after a 10-20 year period.

The field of bone implant research was revolutionized by materials scientist Larry Hench's discovery of Bioglass in the 1960's [3]. Bioglass® of composition $24.5Na_2O$- $24.5CaO$-$6P_2O_5$-$45SiO_2$ (wt%), was shown to develop a layer of hydroxyapatite ($Ca_5(PO_4)_3(OH)$) *in vivo*, the major mineral constituent of bone, making the material bioactive. Furthermore, bioactive glass resorbed over time in the body and was replaced by natural bone; and the ions (especially silicon) leaching from the Bioglass have been shown to stimulate bone precursor cells to differentiate into mature, calcified matrix secreting osteoblasts [4]. These unique characteristics of Bioglass are not shared by any other currently used bone replacement material, indicating its superior osseointegration [5].

Studies on titanium metal implants have shown that its surface microarchitecture (roughness and texture) influences cell behavior [6]. In a preliminary study Levy et al. [7]

compared two glass samples and found greater cell proliferation on smoother samples. We have performed a systematic investigation on the effect of surface roughness on cell adhesion and proliferation on classical melt-quench prepared 45S Bioglass samples *in vitro*.

## MATERIALS AND METHODS:

### *Fabrication and Surface Preparation of Glass Samples*

Following the chemical composition of 45S Bioglass, 13.7 g of $SiO_2$, 12.8 g of $Na_2CO_3$, 9.1 g of $CaCO_3$, and 4.3 g of $Ca_5(OH)(PO_4)_3$ powders were mixed to fabricate bioactive glass. The mixture was calcined for 6 hours in a platinum crucible at 750°C and melted at 1500°C. The melt was cast into a cylindrical stainless steel mold preheated to 400°C and annealed at 500°C to remove internal stress. Following cooling to room temperature, the cast was cut into ~2 mm thick discs using a diamond-coated circular saw blade.

Samples were polished to various surface roughnesses using a series of silicon carbide abrasive papers with decreasing particle size (d) (paper #120 with d=125 μm; #240 with d=58 μm; #800 with d=8 μm; #1200 with d=3 μm) on an automatic polishing machine (ATA, Sapphir 500, Germany). In order to obtain the smoothest, optically flat surface, selected glass samples were polished additionally with 1 μm size $CeO_2$ powder. Sample surface roughness was measured and quantified using a Taylor Hobson profilometer (Taylor Hobson Form Talysurf, UK). Five different regions of a given sample were measured to give average surface roughness, $R_a$ (area under the surface profile divided by the length traversed by the stylus) of 1.16, 0.433, 0.0381, 0.0114, 0.0112 μm for the samples polished with120, 240, 800 and 1200 papers, and $CeO_2$ powder. Images of sample surfaces were recorded with a Reichert MeF3 optical microscope (Dewley, New York).

### *Cell Lines and Culture Conditions*

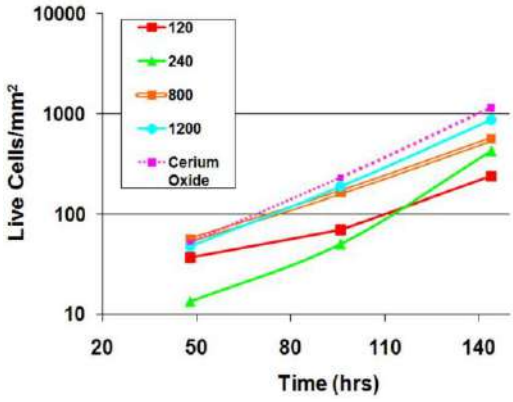
MG63 human osteosarcoma cells (ATCC CRL 1427), and MC3T3-E1 subclone E-4 newborn mouse calvarial bone pre-osteoblasts (ATCC CRL-2593) were cultured under standard conditions in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 100 U/ml penicillin/streptomycin in a cell culture incubator at 37˚C, 5% $CO_2$-atmosphere, and 100% humidity. Polished glass samples were rinsed in 70% ethanol, air-dried, exposed to UV light for sterilization, and placed into cell culture dishes. Cells were trypsinized and seeded onto the glass samples.

### *Fluorescence Microscopy and Quantitative Image Analyses*

Samples were examined on a Nikon TE2000 inverted fluorescent microscope and imaged with 10x and40x long distance air objectives (cell proliferation assays), or a 60x high numerical aperture (NA 1.4) oil immersion objective (cell adhesion assays). A set of images (10 images/sample) was taken using a SPOT RT CCD camera (Diagnostic Instruments Inc, Sterling Heights, MI), live and dead cells counted, and average numbers calculated and plotted using Excel software. Cell morphology, number and location of focal adhesion sites was quantified and correlated with sample surface roughness. Image z-stacks of cells were taken using a Nikon LiveScan Swept Field Confocal Microscope System (Nikon Instruments Inc., Melville, NY), and 3D-volume reconstructions were created using the Nikon NIS Elements imaging software.

## RESULTS AND DISCUSSION

***Proliferation of bone cells on 45S Bioglass with variable surface topology***



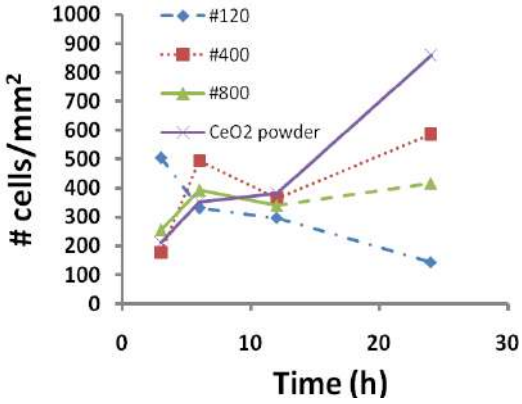




Figure 1 (left). MG63 cell density on 45S glass of different roughness vs. incubation time.
Figure 2 (right). Early stage MC3T3 cell density vs. time on 45S glass of various roughness.

To investigate the growth and proliferation of bone cells on 45S Bioglass implants of variable surface roughness, MG63 osteoblast-like, and MC3T3 precursor osteoblast cells were seeded onto the samples (at ≤500 cells/mm$^2$). At various time points post seeding (2, 48, 96, 144 hours) selected samples were stained using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen, Carlsbad, CA). Cells were imaged after staining (5 images/sample), live (green) and dead (red) cells counted manually, and their average numbers calculated. Alternatively, cells that adhered to the glass were formaldehyde fixed (after 3, 6, 12, 24 hours) and nuclei stained with DAPI. Quantitative analyses showed that cell number on smooth surfaces ($R_a$ ~0.011, polished with #1200 abrasive paper or $CeO_2$-powder) on average increased more than 4-fold, while cell numbers on rough surfaces ($R_a$ ~1.16, polished with #120-paper) increased less than 3-fold, suggesting that cells adhered and proliferated more efficiently on smoother Bioglass surfaces (Fig. 1). Cells growing on Bioglass samples with intermediate surface roughness ($R_a$ ~ 0.038, polished with #800-paper) proliferated intermediately (more than 3-fold, Fig. 1). After 6 days (144 hours) a well-defined correlation between cell number and decreasing surface roughness was observed on all samples (Fig. 1). Similar results were obtained when MC3T3 cells were seeded onto the glass samples, or when cell number was determined by counting DAPI-stained cell nuclei after cells were formaldehyde-fixed on the samples (Fig. 2). Standard deviation and standard error of mean for the data in Figs. 1 and 2, taken from different regions of a sample, varied from ~ 30% and 8% in early stages to ~15% and 5% at late stages, respectively. Cells proliferated faster after an initial lag period caused by the negative effect of trypsinization, and after cells had re-established necessary cell-surface adhesions.

Taken together, our results demonstrate that the Bioglass used in this study is biocompatible, and that a smoother surface topology appears to increase bone cell proliferation.

***Adhesion of bone cells on 45S Bioglass with variable surface topology***

Cell adhesion is a complex and dynamic process, and acts as the first step in the bonding between implant and surrounding tissue. Since cell adhesion is a prerequisite for successful cell

proliferation in 2D in-vitro cell culture, a slower proliferation rate of MG63 and MC3T3 cells on rougher Bioglass surfaces observed in this study, suggests that cells do not adhere as well to the rougher surfaces, and thus may contribute to the slower proliferation rate. To further test this hypothesis, Live/Dead-stained MG63 cells growing for various times on the samples were imaged at higher magnification to resolve basic morphological features of the cells. In Figure 3, representative example images of MG63 cells growing for 48 hours on Bioglass samples with rough (polished with #120 paper) and smooth ($CeO_2$-powder polished) surface topology are shown. As expected, cells on smooth Bioglass samples spread out flat on the surface exhibiting more typical epitheloid morphology with clear lamellipodial extensions (Fig. 3, left panel), while cells on the rough samples remained round, or spherical and were forming cell clusters (Fig. 3, right panel); this suggests that cells on the smooth Bioglass surfaces adhered better and formed more and stronger cell-adhesion sites.

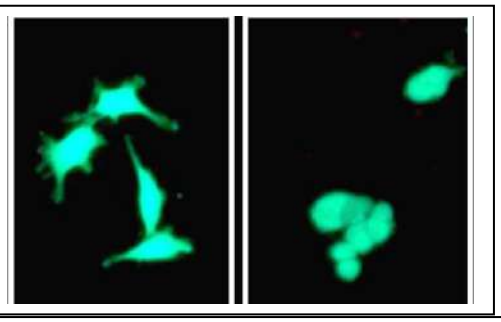


Figure 3. High magnification images of MG63 cells on 45S glass polished with $CeO_2$ powder (left) and #120 paper (right).

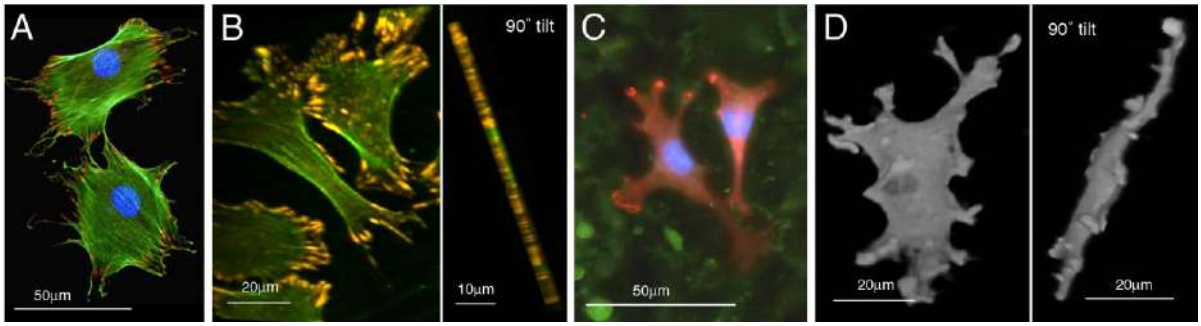



Figure 4. MC3T3 cells growing for 12-24 hours on smooth ($R_a$ ~0.1 µm; A and B), and rough ($R_a$ ~1.2 µm; C and D) 45S Bioglass surfaces. Cells on smooth surfaces spread out flat and form typical focal adhesions (vinculin, red and orange in A, B), and actin stress fibers (phalloidin, green in A, B), while cells on rough surfaces spread out less, and do not form typical focal adhesions (vinculin, red remains cytoplasmically localized in C) and actin stress fibers (no green stress fibers in C). Nuclei in (A) are stained with DAPI (blue). Confocal 3D-volume reconstructions of cells viewed onto the cell surface, and tilted 90˚ are shown in the right part of panels (B) and (D).

To examine cell-adhesion to Bioglass with variable surface roughness in greater detail, MC3T3 bone precursor cells were seeded on the Bioglass samples and incubated for increasing time periods. After 3, 6, 12, and 24 hours, selected dishes were removed from the incubator and transferred to an orbital shaker (30 seconds at 250 rpm) to dislodge poorly adhered cells. Cells remaining on the samples were fixed with 2% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 1% bovine serum albumin (BSA)/PBS in preparation for indirect immuno-fluorescence labeling. Cells were stained using monoclonal anti-human vinculin

antibodies (clone hVin-1, Sigma, Saint Louis, MO) (a marker protein for cellular focal adhesions) followed by Cy3-conjugated AffinitiPure goat anti mouse IgG antiserum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), Alexa488-conjugated phalloidin (Molecular Probes, Invitrogen) to decorate the filamentous actin (F-actin) cytoskeleton, and DAPI (4',6-diamidino-2-phenylindole) (Molecular Probes, Invitrogen) which binds to nuclear chromatin. Representative, high-resolution micrographs of stained cells growing for 12-24 hours on 45S Bioglass with smooth surface ($R_a$ ~0.1 μm, $CeO_2$-powder polished samples), and rough surface texture ($R_a$ ~1.2 μm, #120-paper polished samples) are shown in Fig. 4 (A, B) and Fig. 4 (C, D), respectively.

As expected, cells on smooth Bioglass surfaces ($R_a$ ~0.21 – 0.01 μm), in general formed typical bundles of more or less parallel oriented actin filaments (stress fibers, green) that extended throughout the cell cytoplasm, as is typical for cultured cells growing on stiff plastic, glass, or matrigel surfaces [8]. As expected, these cells also formed numerous, well-organized focal adhesions as indicated by the robust, spotted localization of the focal adhesion marker protein, vinculin (red), preferentially located on the peripheral ends of actin filament bundles (Fig. 4A). Confocal 3D-volume reconstructions of cells viewed onto the cell surface, and tilted by 90˚ indicated that cells were strongly attached and spread out flat with a cell thickness of only a few micrometers, again typical for cultured cells grown on stiff surfaces (Fig. 4B).

In contrast, cells growing for 12-24 hours on rough ($R_a$ ~1.2 -0.4) Bioglass surfaces, in general did not form pronounced actin filament bundles (stress fibers) as indicated by the homogenous, diffuse green Alexa488-phalloidin labeling of the entire cytoplasm (Fig. 4C). These cells also seemed not to establish solid focal adhesions as indicated by the diffuse, cytoplasmic localization of the marker protein, vinculin (red, Fig. 4C). Vinculin is known to assemble at cellular focal adhesion sites, and to generate a physical link between the cytoplasmic portion of integrin trans-membrane receptors and actin filaments [9]. 3D-volume reconstructions of cells growing on Bioglass samples that were polished with #120-grain abrasive paper and viewed onto the cell surface, and tilted by 90˚ indicated that cells were not as flat, and not as well spread out as observed for cells growing on smooth Bioglass surfaces, and that cells adapted their morphology to follow the rough surface topology of the samples (Fig. 4D). In agreement with these findings, following proliferation of MC3T3 cells for 24 hours on these samples indicated that initially, cells appeared to attach to the rough surfaces, however over time were not able to establish and maintain robust cell adhesions, and thus were lost when samples were agitated to dislodge loosely attached cells (see Materials and Methods) (Fig. 2, sample polished with #120 paper). These findings support our hypothesis expressed above that bone cells growing on smooth Bioglass surfaces attached and proliferated better compared to cells growing on rough Bioglass surfaces.

## CONCLUSIONS

We have performed a detailed investigation of the influence of surface roughness ($R_a$ ~1.2 to ~0.01 μm) of Bioglass implants on the attachment and proliferation of bone cells using cell culture models *in vitro* using fluorescence cell labeling and high-resolution fluorescence microscopy. Preliminary results presented here indicate that: (I) Our Bioglass is biocompatible and supports cell growth. (II) Cells sense and respond to the surface roughness via cell adhesion and cellular proliferation, and (III) the cells attach and proliferate better on smoother Bioglass surfaces compared to cells growing on rougher surfaces. These results are in agreement with an

earlier study investigating cell/surface interactions and adhesion on 45S bioactive glass [7], however, contradict some findings of cell attachment to titanium implant surfaces, where cells adhere and proliferate better on rougher surfaces as compared to smoother surfaces [10].

The cells exhibit a flatter and more pronounced spread-out morphology and form numerous solid, well defined focal adhesions linked to well-assembled bundles of actin filaments (stress fibers) on smoother surfaces. On rougher surfaces, we find diffuse cytoplasmic localization of the focal adhesion marker protein, vinculin, and the actin-filament decorating peptide-drug, phalloidin. The morphology of cells growing on titanium and on soft matrices is also known to be modulated in response to surface roughness or matrix stiffness thereby affecting the localization and morphology of cell adhesion sites. These morphological changes are believed to directly influence cell proliferation, cell migration, and cellular signaling events. Altogether, our findings provide important insight into the design of bioactive glass bone-implants in general. Since cell proliferation and differentiation are cross-correlated, in future, it will be interesting to investigate whether bioactive glass implants or scaffolds can be designed specifically, to either favor cell proliferation or differentiation, depending on their intended use.

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