Advances in Bioceramics and Biocomposites II

A Collection of Papers Presented at the 30th International Conference on Advanced Ceramics and Composites
January 22–27, 2006, Cocoa Beach, Florida

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Mineo Mizuno

General Editors
Andrew Wereszczak
Edgar Lara-Curzio
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Preface

Bones and teeth are composed of inorganic (calcium phosphate) and organic (proteins) materials. They are ultimate composites, being skillfully tailored to show both structural and bioactive functions. The role of biomaterials is important because life expectancy continues to increase in our society along with the needs of this segment of the population.

It was a timely fashion that a “Bioceramics and Biocomposites” session started in 2002 in the 26th International Conference on Advanced Ceramics and Composites, and growing interest in this topic resulted in the organization of a full symposium in 2005. At present, bioceramics are undoubtedly recognized to be one of most important materials in order to overcome problems on the 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.

These proceedings contain papers that were presented at the Bioceramics and Biocomposites symposium held at the 30th International Conference and Exposition on Advanced Ceramics and Composites, Cocoa Beach, Florida, January 22–27, 2006. Authors from 12 different countries, representing academia, national laboratories, industries, and government agencies, presented a total of 43 papers at this symposium.

This symposium covered topics such as processing of biomaterials, orthopaedic replacements and performance issue of biomaterials, biomimetics and biocomposites, drug delivery and dental ceramics.

The symposium organizers would like to thank all the participants in the symposium and the staff at The American Ceramic Society. The symposium organizers hope that this symposium will inspire the development of better biomaterials to contribute to improvements in the quality of life.

MINEO MIZUNO
JIAN KU SHANG
RICHARD RUSIN
WALTRAUD KRIVEN
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This book is one of seven issues that comprise Volume 27 of the Ceramic Engineering & Science Proceedings (CESP). This volume contains manuscripts that were presented at the 30th International Conference on Advanced Ceramic and Composites (ICACC) held in Cocoa Beach, Florida January 22–27, 2006. This meeting, which has become the premier international forum for the dissemination of information pertaining to the processing, properties and behavior of structural and multifunctional ceramics and composites, emerging ceramic technologies and applications of engineering ceramics, was organized by the Engineering Ceramics Division (ECD) of The American Ceramic Society (ACerS) in collaboration with ACerS Nuclear and Environmental Technology Division (NETD).

The 30th ICACC attracted more than 900 scientists and engineers from 27 countries and was organized into the following seven symposia:

- Mechanical Properties and Performance of Engineering Ceramics and Composites
- Advanced Ceramic Coatings for Structural, Environmental and Functional Applications
- 3rd International Symposium for Solid Oxide Fuel Cells
- Ceramics in Nuclear and Alternative Energy Applications
- Bioceramics and Biocomposites
- Topics in Ceramic Armor
- Synthesis and Processing of Nanostructured Materials
The organization of the Cocoa Beach meeting and the publication of these proceedings were possible thanks to the tireless dedication of many ECD and NETD volunteers and the professional staff of The American Ceramic Society.

ANDREW A. WERESZCZAK
EDGAR LARA-CURZIO
General Editors

Oak Ridge, TN (July 2006)
In Vitro Evaluation
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INITIAL IN VITRO INTERACTION OF HUMAN OSTEOBLASTS WITH NANOSTRUCTURED HYDROXYAPATITE (NHA)

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ABSTRACT

Nanostructured hydroxyapatite (NHA) was fabricated by Spark Plasma Sintering (SPS), while microstructured hydroxyapatite (MHA) by conventional method. Human Osteoblasts were cultured on both NHA and MHA and the cell attachment, proliferation and mineralisation were evaluated. After 90 min incubation the cell density on NHA surface is significantly higher than that of MHA and glass control, whereas average cell area of a spread cell is significantly lower on NHA surface compared to MHA and glass control after 4 h incubation. Mineralisation of matrix has been determined after 14 days culture by using alizarin red assay combined with cetylpyridinium chloride (CPC) extraction. NHA shows significant enhancement (~p<0.05) in mineralisation compared to MHA. Results from this study suggest that NHA is a much better candidate for clinical uses in terms of bioactivity.

1. INTRODUCTION

Interaction between cells and implanted materials depends on the physical and chemical characteristics of materials and particularly on its chemical composition, particle size and surface properties, which include their topography, roughness, surface energy, hydrophilicity and hydrophobicity. Such characteristics determine how biological molecules will adsorb to the surface. Specifically, maximum vitronectin (a protein contained in serum that is known to mediate osteoblast adhesion), fibronectin and albumin adsorption was noted on hydrophilic surface with high surface roughness and /or energies, such selected protein has been identified to mediate adhesion of specific anchorage-dependent cells (such as osteoblasts, fibroblasts, and endothelial cells) on substrate surfaces. Therefore, surface properties will affect cell adhesion, attachment on implanted materials in the first phase of cell/material interactions and thus further influence the cell's capacity to proliferate and to differentiate on contact with the implant. Design of materials with improved physical and chemical properties could enhance cell response to biomaterial implants and further extend their lifetime and, therefore, decrease the rate of revision surgery.

It has been reported that surface properties (such as surface area, charge, and topography) depend on the grain size of a material. In this respect, nanostructured materials possess higher surface area with increased portions of surface defects and grain-boundaries. Meanwhile, hydroxyapatite has been considered as a good candidate for designing hard tissue implants due to its excellent biological properties such as non-toxicity, lack of inflammatory response and immunological reactions, and is able to intimately bond to new bone. Consequently, It is extremely attractive to explore if and how nanostructured hydroxyapatite with enhanced surface properties (such as increased surface area and charge, as well as ability to alter adsorption of
chemical species) could be used to promote cell response and bonding of juxtaposed bone to an orthopaedic or dental implants composed of nanophase ceramics. However, only a few reports have been published till now. Webster has reported that osteoblasts adhesion and osteoblasts proliferation was significantly greater on nanophase alumina, titania, and hydroxyapatite than on conventional formulations of the same ceramic after 3 and 5 days and more importantly, compared to conventional ceramics, synthesis of alkaline phosphatase and deposition of calcium containing mineral was significantly greater by osteoblasts cultured on nanophase than conventional ceramics after 21 days and 28 days.\textsuperscript{5, 10, 11} In this article, a primary human osteoblast cell model has been used to study the cellular response to nanostructured HA (NHA) compared with microstructured HA (MHA).

2 EXPERIMENTS

2.1 Materials

HA powder has been prepared by precipitation at room temperature using Ca(OH)\textsubscript{2} and H\textsubscript{3}PO\textsubscript{4} as starting materials\textsuperscript{12}. Such powder has been characterized using XRD and FTIR as a hydroxyapatite phase with low crystallinity and with incorporation of carbonate ions. The shape of the HA crystals are acicular according to TEM images. Crystallite size calculated according to XRD results is about 20-40 nm, while the particle size measured by Mastersizer microplus (Malvern Ltd, UK) is several microns. All nanostructured HA (NHA) samples used for cell culture were sintered at 900°C for 3 min by the SPS process with a heating rate of 100°C min\textsuperscript{−1} and a pressure of 50 MPa at vacuum atmosphere. All microstructured HA (MHA) compacts were sintered by conventional method at 1200°C for 2 h with a heating rate of 5°C min\textsuperscript{−1} in air.

2.2 Surface characterisation

The sintered compacts were polished using Silica colloid (0.06\(\mu\)m) and chemically etched in 18.0 mM HCl solution to reveal the grain boundaries, and then microstructural observations of sintered ceramics were conducted using a high-resolution scanning electron microscope (FESEM-XL30, Philips). Topography and surface roughness of NHA and MHA has been evaluated by Atomic force microscopy (AFM). Five measurements were made on each sample with a scanning area of 5 x 5 \(\mu\)m. The classical mean surface roughness parameter \(R_s\) has been used to characterize surface roughness. Aqueous wettability of nanostructured HA and microstructured HA has been analysed by contact angle measurements on polished samples. Measurements were run in triplicate per sample and repeated at three different times.

2.3 Cell morphology

HOBs in complete Dulbecco’s modified Eagles medium (DMEM) containing 10% foetal bovine serum (FBS), 1% antibiotics and 0.85 mM ascorbic acid-2 phosphate were seeded onto microstructured HA (MHA) and nanostructured HA (NHA) discs at a density of 4x10\textsuperscript{4} cells/cm\textsuperscript{2}, then incubated at 37°C in a humidified incubator with 5% CO\textsubscript{2} for 90 min, 4 h, and 24 h. Glass coverslips purchased from Chance Glass Ltd were used as control materials. At the pre-determined time points, samples were rinsed in phosphate buffered saline (PBS) to remove any non-adherent cells and medium. The remaining cells were fixed in 1.5% glutaraldehyde for 30 min at 4°C, then dehydrated through a series of ethanol concentrations (50%, 70%, 90%, and 100%) and dried using hexamethyldisilazane (HMDS). Once dry, the samples were coated with gold and examined in a JEOL JSM-840 Scanning Electron Microscope at 10kV\textsuperscript{13, 14}.

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2.4 Cell proliferation

Cells were harvested with 0.1% trypsin-EDTA solution in phosphate-buffered saline (PBS, pH 7.4) from cell culture flasks and were resuspended in culture medium, then were seeded at a concentration of $4 \times 10^4$ cells/cm$^2$ onto disks of MHA and NHA. Tissue culture polystyrene was used as a control material. Cells were left to grow on the disks for 1, 3, 7 days in a 37°C incubator with 5% CO$_2$. At the pre-determined time points, each of the disks was transferred to new wells in a new 24-well plate and 1.5 ml medium were added to each disk. 150 μl of freshly prepared 5 mg/ml 3-(4,5-dimethylthiazolyl-2)-2,5- diphenyltetrazolium bromide (MTT) were added to each well containing the disks. The plates were placed in an incubator at 37°C for 3 h. Afterwards, the supernatant of each well were removed and acidified isopropanol (0.04 M HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark-blue crystals. After all crystals were dissolved, the plates were read on a Microplate reader (Ascent) with a test wavelength of 540 nm against a reference wavelength of 620 nm$^{15}$. 

2.5 Alamar Blue$^\text{TM}$ assay

Cells were harvested with 0.1% trypsin-EDTA solution in phosphate-buffered saline (PBS, pH 7.4) from cell culture flasks and were resuspended in culture medium, then were seeded at a concentration of $4 \times 10^4$ cells/cm$^2$ onto disks of MHA and NHA. Tissue culture polystyrene was used as a control material. After 1 day incubation in a 37°C incubator with 5% CO$_2$, samples were taken out into new wells and 1.25 μg/ml Alamar Blue$^\text{TM}$ were added into each well, then incubated for 3 h. The fluorescence was measured using a FLUOstar OPTIMA (BMG LABTECH) plate reader at 530ex-590em nm wavelengths. The suspension containing Alamar Blue$^\text{TM}$ was removed from sample wells and new medium added to incubate further$^{16,17}$. 

2.6 Mineralisation

Ability of cells to produce mineralised matrix is essential with regard to development of materials for bone regeneration. Whether mineralisation of matrix occurred was determined using alizarin red-S (AR-S) assay combined with cetylpyridinium chloride (CPC) extraction$^{18,19}$. Alizarin red is a dye which binds selectively to calcium salts and is widely used for calcium mineral histochemistry. AR-S binds ~2 mol of Ca$^{2+}$/mol of dye in solution. Briefly, Cells were seeded onto disks of MHA and NHA at a concentration of $4 \times 10^4$ cells/cm$^2$ by using medium, which was supplemented with 10 mM β-glycerophosphate and 100 nM dexamethasone. Tissue culture polystyrene was used as a control material. At the pre-determined time points (7, 14 days), samples were briefly rinsed with PBS followed by fixation (ice-cold 70% ethanol, 1 h). Samples then were rinsed with nanopure water and stained for 10 min with 40 mM AR-S, pH 4.2, at room temperature. Afterwards, samples were rinsed five times with water followed by a 15 min wash with PBS to reduce non-specific AR-S stain. Stained cultures were destained by using 10% (w/v) cetylpyridinium chloride (CPC) in 10 mm sodium phosphate, pH 7.0, for 15 min at room temperature. After destain, the plates were read on a Microplate reader (Ascent) with a test wavelength of 540 nm against a reference wavelength of 620 nm. Furthermore, for determination of the matrix mineralisation directly on HA samples, two groups of samples were used. Group 1 was cultured with HOBs, and Group 2 without HOBs as background. To calculate the mineral content of the extracellular matrix, group 2 values were subtracted from group 1. 

2.7 Statistical analysis