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Effect of grain size on mechanical, surface and biological properties of microwave sintered hydroxyapatite

Sudip Dasgupta, Solaiman Tarafder, Amit Bandyopadhyay, Susmita Bose *

W. M. Keck Biomedical Materials Research Laboratory, School of Mechanical and Materials Engineering, Washington State University, Pullman, WA 99164-2920, USA

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ABSTRACT

Hydroxyapatite (HA) compacts having average grain sizes of 168 ± 0.086 nm, 1.48 ± 0.627 μm and 5.01 ± 1.02 μm are processed from synthesized HA powder by microwave sintering at varying sintering temperature for different times. Superior mechanical and biological properties are shown by nano-grain HA compacts as compared to their micron grained counterparts. Compressive strength, indentation hardness, and indentation fracture toughness are increased with the decrease in HA grain size. The highest surface energy and maximum wettability are exhibited by nano-grain HA. HA compacts are assessed for cell–material interaction by SEM, MTT and immunochemistry assays using human osteoblast cell line for 1, 5 and 11 days. MTT assays showed higher number of living cells and faster proliferation on nano-grain HA surface. Osteoblast cells on nano-grain HA surface expressed significantly higher amount of vinculin and alkaline phosphatase (ALP) protein markers for cell adhesion and differentiation respectively. This study shows the effect of grain size on physical, mechanical and *in vitro* biological properties of microwave sintered HA compacts.

1. Introduction

Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), the structural prototype of tooth and bone mineral, has been used as an important implant and scaffold material, and drug delivery agent, with significant clinical potential in medicine, dentistry and orthopedics [1–3]. From extensive *in vitro* and *in vivo* studies, synthetic HA has been shown to lack local or systemic toxicity, to stimulate bone growth without immune response complications [4,5] and to spontaneously integrate into the host hard tissue [1]. Biological and mechanical properties of synthetic HA are largely determined by its particle size, morphology, crystallinity, and composition, which depend on the synthesis precursors and processing.

Bone is a composite material consisting of nanoscale mineral particles and a matrix of collagen fibers. Nanocrystalline HA (nano HA) would be more interesting than micro-sized HA from a biological and medical viewpoint because of its similarity to minerals in natural bone. Compared to conventional microscale HA, which lacks phase purity and homogeneity, nano HA offers the possibility to enhance the rate of bone-bonding formation and to have excellent mechanical properties due to its high surface area to volume ratio, superior chemical homogeneity and microstructural uniformity [6]. Furthermore, nano HA was shown to be able to inhibit the growth of certain kinds of cancer cells, such as the liver, throat and bone cancer cells, while having little side effect on normal cells [7]. The rate of HA bonding to bone was demonstrated to be dependent not on the composition but on the release of calcium and phosphate ions from HA, determining the development of implant–bone interfacial strength [8]. Consequently, sufficient dissolution of calcium and phosphate species is necessary to form bone-like apatite and bone bonding. The dissolution of nano HA has been proven to be very different from that of microscale HA. The dissolution of nano HA is dominated by its particle size [1]. The particle-size effect is explained by the fact that small sized particles of HA may be degradable and stimulate bone ingrowth as they dissolve in the physiological environment [9]. Although nano HA has been extensively studied, most work has been focused on its synthesis procedures, structure analysis and applications [6,10]. Until now, to the best of our knowledge, no detailed investigation has been carried out dealing with the effects of the synthesis parameters on the structure of nano HA.

It has been reported that surface properties, such as surface area, charge, and topography, depend on the grain size of a material [11]. In this respect, nanostructured materials possess higher surface area with increased portions of surface defects and grain-boundaries [12]. Meanwhile, hydroxyapatite (HA) has been considered as a good candidate for designing hard tissue implants because of its excellent biological properties such as nontoxicity, lack of inflammatory response and immunological reactions, and is able to intimately bond to new bone [13,14]. Webster et al. have reported that osteoblast adhesion and proliferation were significantly greater on nanostructured alumina and HA than on conventional formulations

* Corresponding author. Tel.: +1 509 335 7461; fax: +1 509 335 4662.
E-mail address: sbose@wsu.edu (S. Bose).

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of the same ceramic after 3 and 5 days [15,16]. More importantly, synthesis of alkaline phosphatase and deposition of calcium containing mineral was significantly greater by osteoblasts cultured on nanostructured ceramics than conventional ceramics after 21 and 28 days [11,17].

The efficiency of HA ceramics as orthopedic implant greatly depends on its grain size. Not only the osteoblast cells show grain size dependent activities on HA compacts, but mechanical properties of HA compacts also greatly vary with change in grain size in sintered HA microstructure. Particularly, orthopedic implant exhibits remarkably different bioactivities and mechanical reliability at nanoscale. Thus it is interesting to investigate how one can monitor the biological as well as mechanical properties of HA ceramics by changing its grain size. Microwave sintering of ceramics to achieve improved mechanical properties has widely been used by the scientific community [18]. Notable advantages of microwave sintering are rapid volumetric heating rate, shorter processing time and cost effectiveness in terms of energy savings [18–20]. Heating uniformity caused by volumetric heating, and thus, shorter sintering time of microwave sintering leads to achieve finer grain. Controlled grain growth and finer microstructure due to microwave sintering result in high mechanical properties in sintered ceramics.

The objective of this study was to understand the influence of grain size on mechanical properties and bioactivity of sintered HA. In the present study, we processed HA compacts with different grain size using microwave sintering. The mechanical properties of these HA compacts are compared with those of HA compacts reported previously [19]. Surface energy of nano and micron size HA compacts is also compared in this study. *In vitro* bone cell–material interaction of HA compacts was investigated for adhesion, proliferation and differentiation of human osteoblast cells using SEM, MTT assay and confocal microscopy.

2. Materials and methods

2.1. Synthesis of HA nanopowders

HA nanopowder was synthesized using an emulsion synthesis rout [21]. Briefly, 5 M aqueous solution of Ca^{2+}-ion was prepared by dissolving 0.01 mol (2.362 g) of $Ca(NO_3)_2 \cdot 4H_2O$ in 2 ml distilled water. 0.006 mol (0.686 g) of phosphoric acid (H_3PO_4) (85.7%) was added to the system to maintain Ca to P molar ratio 1.67. Organic phase of the emulsion was prepared by the addition of 10 vol.% surfactant, poly(oxyethylene)$_{12}$ nonylphenol ether (NP12), in cyclohexane with vigorous stirring. An aqueous to organic ratio (A/O) of 1:15 was maintained for HA nanopowder synthesis. The pH of the emulsion was adjusted to 9 with dropwise addition of NH_4OH to initiate reaction between $Ca(NO_3)_2 \cdot 4H_2O$ and H_3PO_4 to form HA nanocrystals. All reactions were aged for 24 h at room temperature to grow non-agglomerated HA nanocrystals with high crystallinity. After aging, the emulsion was evaporated on the hot plate at 150 °C followed by complete drying at 450 °C. Dry precursor powder was calcined at 650 °C for 4 h to get carbon free crystalline HA nanopowder.

2.2. Consolidation of calcined HA powders

As synthesized HA nanopowders were processed following the procedure as described in our previous work [19]. To investigate the effect of calcination temperature on particle size, the as synthesized HA nanopowders were calcined at 800 °C for 4 h and 900 °C for 10 h. From here on, we will denote the as synthesized HA nanopowders after processing as HA (I), and as synthesized HA nanopowders calcined at 800 and 900 °C as HA (II) and HA (III), respectively. Particle size distribution was measured using dynamic light scattering (DLS) technique (NICOMPTM 380, Santa Barbara, CA). Disks with approximate dimensions of 12 mm in diameter and 2 mm in height were prepared by uniaxial pressing at 50 MPa, followed by cold isostatic pressing at 345 MPa. Cylindrical compacts with approximately 6 mm in diameter and 9 mm in height were also prepared using the same technique for mechanical testing.

2.3. Sintering and characterization of HA

The disks and cylindrical HA were sintered in a 3 kW microwave furnace [MW-L0316V, LongTech Co., Ltd, ChangSha, HuNan, P.R. China] at different temperature for different time interval as described in Table 1. The constituent phases of sintered HA (I), HA (II) and HA (III) compacts were analyzed using a Philips fully automated X-ray diffractometer with CuK_α radiation and a Ni filter. The diffractometer was operated at 35 kV and 30 mA over the 2θ range of 20 to 60° at a step size of 0.02° and a count time of 0.5 s per step. Microstructure was characterized using a field-emission scanning electron microscope (FESEM) (FEI Inc., OR, USA). Sintered HA grain sizes were determined from SEM images *via* a linear intercept method [22] using the equation G = (L / N)C, where G is the average grain size (μm), L is the test line length (cm), N is the number of intersections with grain boundaries along test line L; and C is the conversion factor (μm/cm) of the picture on which the test lines were drawn as obtained from the scale bar.

The sintered HA pellets were characterized for microhardness and indentation fracture toughness. Fracture toughness was calculated from the radial crack length that appeared after the indentation test. The equation for the calculation of fracture toughness is given below [23].

$$\text{Fracture toughness } (K_{IC}) = 0.016 \times (E/H)^{1/2} \times P/C^{3/2}$$

where, E is Young's modulus of the sample, H is microhardness in GPa, P is the applied load and C is the half of the crack length. Compressive strength analysis was performed with cylindrical HA compacts.

2.4. Contact angle measurement and surface energy calculation

Contact angles of different liquids on sintered HA disk surface were measured using the sessile drop method on a face contact angle set-up equipped with a microscope and a camera [Model VCA Optima, AST products, Billerica, MA, USA]. Contact angles were determined with liquids such as water, formamide, glycerol, and cell culture media. The liquid surface tension and their components were used in the following equation [24] to calculate the surface energy:

$$\gamma_L(1+\cos\theta) = 2\left(\gamma_S^{LW}\gamma_L^{LW}\right)^{1/2} + 2\left(\gamma_S^{+}\gamma_L^{-}\right)^{1/2} + 2\left(\gamma_S^{-}\gamma_L^{+}\right)^{1/2}$$

where, θ is the contact angle of liquid L and solid S, γ^{LW} is the apolar component of the surface energy, γ^+ is the Lewis acid component (electron acceptor) and γ^- is the Lewis base component (electron donor).

2.5. In vitro cell–material interactions

In vitro cell–material interactions were studied for a maximum incubation period of 11 days using human fetal osteoblast (hFOB) cell (ATCC, VA, USA). Triplicate samples per group were evaluated for all experiments. Each sample was sterilized by autoclaving at 121 °C for 20 min prior to the cell culture experiment. Following this, cells were seeded onto the sample surfaces, placed in a 24-well plate. Cells were seeded at a density of 1×10^4/well. Then 1 ml of McCoy's