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Electrically polarized HAp-coated Ti: *In vitro* bone cell–material interactions

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ABSTRACT

Electrically polarized bulk sintered hydroxyapatite (HAp) compacts have been shown to accelerate mineralization and bone tissue ingrowth *in vivo*. In this work, a comprehensive study has been carried out to investigate the influence of surface charge and polarity on *in vitro* bone cell adhesion, proliferation and differentiation on electrically polarized HAp-coated Ti. Uniform and crack free sol–gel derived HAp coatings of 20 ± 1.38 μm thickness were polarized by application of an external d.c. field of 2.0 kV cm^{-1} at 400 °C for 1h. *In vitro* bioactivity of polarized HAp coatings was evaluated by soaking in simulated body fluid, and bone cell–material interactions were studied by culturing with human fetal osteoblast cells (hFOB) for a maximum period of 11 days. Scanning electron microscopic observation showed that accelerated mineralization on negatively charged surfaces favored rapid cell attachment and faster tissue ingrowth over non-polarized HAp coating surfaces, while positive charge on HAp coating surfaces restricted apatite nucleation with limited cellular response. Immunochemistry and confocal microscopy confirmed that the cell adhesion and early stage differentiation were more pronounced on negatively charged coating surfaces as hFOB cells expressed higher vinculin and alkaline phosphatase proteins on negatively charged surface compared to cells grown on all other surfaces. Our results in this study are process independent and potentially applicable to any other commercially available coating techniques.

1. Introduction

Today, the occurrence of musculoskeletal disorders, such as osteoporosis and osteoarthritis, have been recognized as among the most common human health problems, and afflicting more than 33% of US adults. In parallel, a range of musculoskeletal metallic implants are also available in the market to treat or even prevent these diseases, which cost society an estimated $20 billion in the US alone in 2008, an expenditure that is projected to grow by 11.8% by 2012 [1]. However, the major problems concerning the success and lifetime of these implants in physiological conditions is their low degree of osseointegration and/or tissue integration properties at the implant–tissue interface, which delay the bone reconstruction process as well as prolong the healing time [2]. To address these limitations, hydroxyapatite (HAp)-coated implants have been thoroughly investigated as a therapeutic approach to promote osseointegration of metallic implants [3]. The bioactive HAp coating has the ability to form an interfacial bond with living osseous tissue and can potentially enhance tissue ingrowth with dental and orthopedic implants [4]. Nevertheless, the inferior osteogenic capacity of commonly available HAp-coated implants with a lower degree of osteogenesis compared to living bone tissue presents a significant challenge to orthopedic and reconstructive surgeons. After implantation, synthetic HAp coating may take days or even weeks to start a new bone mineralization process. This can be a concern for elderly patients or patients with immune deficiency. Therefore, various methods have been attempted to improve osseointegration over the last decade, including micro- and nanostructuring of the surface via various lithographic techniques [5], anodization of Ti [6] and inducing a surface charge in organomolecular self-assembled monolayer coatings [7]. The success of these implants is still a subject of debate, and therefore continuous investigations of new therapeutic approach are in progress.

In recent years, the electrostatic charge storage capacity of polarized sintered HAp has attracted the attention of many biologists and materials scientists [8–13]. According to these studies, a range of polarization charge between 0.08 μC cm^{-2} and 1.2 mC cm^{-2} can be stored within sintered HAp compacts by applying a d.c. electric field at elevated temperatures. Interestingly, these induced bulk polarization charges has been found to influence the mineralization process as well as cell adhesion and growth on HAp compact surfaces both *in vitro* [14–17] and *in vivo* [18,19]. The original concept of using polarized HAp as bone graft material was first introduced by Yamashita et al. [14]. It has been demonstrated that apatite formation accelerates on the negatively polarized HAp ceramic surfaces in simulated body fluid (SBF), while positively charged HAp surfaces inhibit apatite formation [14,15]. The dependence of cell adhesion on the induced electrostatic charge of these polarized HAp compact surfaces was also

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evaluated in previous studies [16]. In our previous report, we demonstrated that tailoring the combined effect of stored surface charge, surface wettability and charge polarity on the sintered polarized HAp surface enables the differential binding of inorganic ions (e.g. Ca^{2+}, Cl^-, Na^+, HCO_3^-) and organic cell adhesive proteins (e.g. fibronectin, vitronectin) with different surface properties, resulting in accelerated mineralization and improved cell adhesion, proliferation and differentiation on a negatively charged HAp surface, while causing the opposite effect on positively charged surfaces [17]. However, most of the investigations to date were carried out on electrically polarized sintered HAp compacts; very few reports are available on polarized HAp-coated implants [20,21]. However, HAp-coated metal implants are of great interest since coated implants are widely used in actual load-bearing clinical applications to enhance tissue–material interactions.

Here, we report on a comprehensive study of polarized HAp-coated Ti, in an attempt to understand the efficacy of polarization treatments on *in vitro* bone cell–material interactions. We have investigated the hypothesis that the combined influence of stored surface charge and charge polarity of polarized HAp coatings can stimulate tissue integration, enhancing early stage mineralization and bone cell–material interactions. To study this hypothesis, HAp coating was processed on a Ti substrate with a HAp slurry using a spin coater. Our results in this study are process independent and potentially applicable to any other commercially available coating technique, e.g. plasma spray. *In vitro* bioactivity of polarized HAp coatings was evaluated by soaking in SBF, and bone cell–material interaction was studied by culturing with human fetal osteoblast cells (hFOB). The present investigation is also intended to provide data on early stage cell anchorage as well as differentiation by vinculin and alkaline phosphatase (ALP) proteins expression for a range of incubation periods to unveil the bone cell–material interactions.

2. Materials and methods

2.1. Processing of HAp coatings

HAp powder was prepared in-house by the sol–gel route, using calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) and diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) as Ca and P precursors, respectively [17]. After calcinations at 1000 °C for 1 h, HAp powder with 4.55 ± 0.01 $m^2\ g^{-1}$ specific surface area was obtained as characterized by a BET Surface Area Analyzer (TRISTAR 3000, Micromeritics, GA, USA). Commercially pure Ti (99.6 at.%) (Alfa Asear, MA, USA) discs, 12 mm in diameter and 0.5 mm in thickness, were used as the substrate. Samples were prepared by first abrading with silicon carbide paper in successive grades from 600 to 1200 grit (Leco Corporation, MI, USA), then cloth polishing with a 1 μm alumina suspension and finally ultrasonically cleaning in distilled water. A 40 wt.% solid loaded HAp slurry was prepared by dispersing sol–gel synthesized HAp powder in HAp sol. The slurry was used in the HAp coating preparation using a spin coater (Laurell Tech. Corp., PA, USA). Slurry-based thick film preparation was done to gain greater control over the coating's thickness and homogeneity [22]. After depositing, coating was heat treated in a muffle furnace (Thermo Fisher, MA, USA) at 700 °C for 15 min. For phase identification of calcined HAp powders and heat-treated coated samples, X-ray diffraction (XRD) was carried out (Siemens Corporation, NY, USA) using copper K_α radiation ($\lambda = 1.541874$ Å) at 30 kV in the 2θ range between 20° and 60°. Coating surface morphology was observed under a field emission scanning electron microscope (FESEM; FEI, OR, USA). To determine the coating thickness, the coated samples were first mounted in epoxy resin and then the cross-sections were prepared for FESEM observation.

2.2. Polarization and thermally stimulated depolarization current (TSDC) measurement on HAp coatings

Polarization was carried out on sintered HAp-coated samples in air, using platinum electrodes, with a Keithley 6487 picoammeter (Keithley Instruments, OH, USA) and a ceramic strip heater (OMEGA, CT, USA). During polarization, the samples were heated from room temperature to polarization temperature (T_p) with a controlled heating rate of 5 °C min^{-1} and soaked at T_p for 1 h before applying a constant d.c. electrical field (E_p). At T_p, an external d.c. electric field of 2.0 kV cm^{-1} was applied and kept for 1 h at T_p, then maintained until the sample cooled down to room temperature. Two different polarization temperatures (T_p), i.e. 300 and 400 °C, were used to evaluate the effect of temperature on electrical polarization. The polarity of induced surface charges depends on the applied external d.c. field polarity, while the magnitude of the stored charge depends on the polarization temperature. In order to estimate the stored static charge during polarization, a thermally stimulated depolarization current (TSDC) technique was used [23]. A polarized HAp-coated sample was heated at a rate of 5 °C min^{-1} from room temperature to 550 °C and the thermally stimulated released depolarization current was measured using a Keithley 6487 picoammeter. The stored electrical charge density was calculated from the TSDC spectra using the following equation [9]:

$$Q_p = \frac{1}{\beta}\int J(T)dT \quad (1)$$

where Q_p denotes the stored charge density, and β and $J(T)$ are the heating rate and current density, respectively.

2.3. In vitro study

In vitro mineralization and cell–material interactions were evaluated for both positively and negatively charged HAp coating surfaces. Previous reports on polarized sintered HAp used a threshold charge density of more than 1.0 μC cm^{-2} for samples to show any significant differences in biological response *in vitro* [14,17]. Therefore, based on the TSDC results, we selected only 400 °C poled HAp-coated samples for *in vitro* studies as the samples at this temperature exhibited a sufficiently higher stored charge density (1.69 μC cm^{-2}) in comparison with that of the 300 °C poled coated samples (~0.365 μC cm^{-2}). For comparison, unpoled HAp-coated Ti and uncoated commercially pure (cp)-Ti samples were used as controls in all of our studies. Triplicate samples per group were evaluated for all experiments. From here on, the negatively polarized coating surface will be termed the "N" poled surface and the positively polarized coating surface will be termed the "P" poled surface.

2.3.1. In vitro mineralization study in SBF

The combined influence of the stored surface charge and the charge polarity on *in vitro* mineralization behavior was evaluated by immersion in SBF, which has an ionic concentration similar to that of human blood plasma. The solution used has the following ionic concentrations [24]: 2.5 mM Ca^{2+}, 1.5 mM Mg^{2+}, 142.0 mM Na^+, 5.0 mM K^+, 148.5 mM Cl^-, 4.2 mM HCO_3^-, 1.0 mM HPO_4^{2-} and 0.5 mM SO_4^{2-}. Each sample was immersed in 15 ml of SBF solution and kept in a static condition inside a biological thermostat at 37 °C. After immersion for 5 days, samples were carefully washed in deionized (D.I.) water for a very short duration to ensure minimal dissolution of precipitated minerals. After washing, samples were dried in an oven at 50 °C for 4 h, coated with gold then observed under a FESEM. To quantitatively analyse the mineralization process, the weight change of the samples was measured after immersion in SBF for 1, 5 and 7 days. To measure the weight change, the samples were removed from the SBF solution at the