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Divalent ion encapsulated nano titania on Ti metal as a bioactive surface with enhanced protein adsorption

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ABSTRACT

A novel approach on incorporation of divalent species such as Mg, Ca and Sr into the titania nanostructures formed on Ti metal surface and their comparative study on enhancement of bioactivity, protein adsorption and cell compatibility is reported. When treated with hydrogen peroxide, Ti metal forms hydrogen titanate. On subsequent treatment with Mg or Ca or Sr nitrate solutions, respective ions are incorporated into hydrogen titanate layer, and heat treatment leads to titania decorated with these ions. The resultant heat-treated samples when soaked in simulated body fluid form bone-like apatite which indicates the present surface modification enhances the bioactivity. Further, enhanced protein adsorption in bovine serum albumin is an indication of suitability of these divalent species to form chelate compounds with amino acids, and Ca containing titania nanostructure favours more protein adsorption compared to the others. Cytocompatibility studies using MG-63, human osteosarcoma cell lines shows these divalent ion containing titania nanostructure favours the cell attachment and did not show any cytotoxicity. Bioactivity, enhanced protein adsorption along with cytocompatibility clearly indicates such surface modification approach to be useful to design hard tissue replacement materials in orthopaedic and dental field.

1. Introduction

Development of bioactive titanium (Ti) and Ti alloys with rapid osteointegration demand novel surface modification approaches wherein modified surface not only improves the bonding between the implant and living bone through the apatite layer and also accelerate the protein adsorption on its surface. These adsorbed proteins allow the bone cells to attach and proliferate on the metal surface. Although Ti and Ti alloys are well known biomedical grade hard tissue replacement materials used in fractured bone, they lack bioactivity (i.e., direct bonding between implant and living bone) [1].

Compared to metals, bioactive ceramics such as hydroxyapatite, apatite wollastonite and various types of bioglasses enhance the new bone formation in human body and bonds to living bone [2,3]. However, lack of mechanical strength of these bioactive ceramics is a serious concern to be used as orthopaedic devices in order to withstand the body weight. This necessitates developing various bioactive metals where surface is either modified chemically or coated with the above mentioned bioactive ceramics [4,5]. Coated metallic implants developed by plasma spray, dip coating, sol-gel coating are well established methods already available in the literatures [6]. However, each methodology has its own merits and demerits as well.

Among various chemical treatment methods discussed in the literatures, NaOH or KOH, H_2O_2 or mixed acid treatment to develop bioactive Ti and Ti alloys are well recognised [7–9]. NaOH treatment as proposed by Kokubo and co-workers found that a porous network structure formed on Ti and Ti alloys at an elevated temperature accelerate the bone-like apatite formation through Ti-OH groups on metal surface [10–12]. This apatite formation in simulated body fluid solution (SBF) is further evidenced by direct bone formation in the animal experiments [12]. However, presence of excess Na^+ ions on metal surface and its release into the human body may cause cell death. Subsequently, several groups reported that partial Na^+ ion release by water treatment or completely replaced by dilute HCl solution and heat treatment form titania surface layer that induces apatite formation in physiological body fluid solution [13,14]. This titania surface shows both osteoconductivity as well as osteoinductivity when implanted in various

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animal model such as beagle dog or rabbit [15]. One of the authors recently reported that the osteointegration was further improved when NaOH treated Ti metal were subsequently soaked in concentrated HCl or HNO_3 or H_2SO_4 or simply using mixed acid and heat treatment [16–19]. Results showed that heat treatment forms titania surface layer that enhances the bioactivity compared to without heat treatment samples. Surface positive charge due to the trapped Cl^-, $NO_3{}^-$, $SO_4{}^{2-}$ ions in titania are the driving force for calcium and phosphate ions to deposit on metal surface to form apatite and the same was reported by animal study [14,16,19]. Recently, Pattanayak et al. reported that the Na^+ ions from NaOH treated Ti alloy can be replaced by Ag^+ ions and this Ag containing titania layer provides antimicrobial activity as well as bioactivity. Optimum Ag concentration that shows antibacterial, bioactivity and cell compatibility was also discussed [20]. Besides, Kokubo et al. also proposed that apatite formation can be enhanced when Na^+ ions are replaced by Ca^{+2} and/or Sr^{+2} ions on Ti and Ti alloys [21–24].The release of these ions into the body fluid is believed to be a prerequisite condition for enhancement of bioactivity of Ti metal [24].

Apart from the above mentioned chemical treatment methods, bioactive Ti metal can also be prepared by H_2O_2 or $H_2O_2/TaCl_5$ treatment at an elevated temperature form gel like hydrated titania layer that form anatase when heated above 400 °C [9,25–27]. This anatase network structure provides bioactivity. Pattanayak et al. recently reported that H^+ ions from hydrated titania layer can be replaced by Ag^+ ions to form Ag containing titania that shows good antibacterial, bioactivity and cell compatibility [28].

In general, when an implant is placed in human body, blood first comes in contact with it and some of the blood proteins are adsorbed on the surface. Literature says fibronectin is the first protein that adsorbs on surface of the implant [29]. Bovine serum albumin (BSA) is an abundant protein with approximate concentration of 50 mg/mL in blood plasma. The main function of BSA is to maintain the p^H and osmotic pressure of blood and it also plays a vital role in transportation of fatty acids in blood [30]. Thus adsorbed protein subsequently enhances the osteoblast (bone forming cells) cell attachment, proliferation, differentiation and bone tissue regeneration. This protein adsorption is largely depending on the surface roughness, temperature, cationic site, electrostatic interaction, hydrophobicity and crystalline nature of the metal surface. Literature reports that the protein adsorption is mainly due to the electrostatic attraction between a charged adsorbent and oppositely charged amino acid side chains that lead to significant change in free energy favouring the adsorption process [31]. However, the mechanism of protein adsorption is still remained unclear.

In the present study, we made an attempt to investigate the surface morphology and surface chemical composition on protein adsorption where porous titania layer on the surface of the Ti metal is encapsulated with divalent species such as Mg, Ca or Sr and quantify the presence of these elements on extent of protein adsorption. These divalent species incorporated porous titania layer was prepared by simple chemical and thermal treatment approach. Further, the bioactivitiy, cytotoxicity and cell attachment studies were carried out to understand the effect of surface morphology and surface chemical composition to develop an artificial implant with rapid bone integration.

2. Materials and methods

2.1. Preparation of the samples

Commercially pure Ti metal (CP Ti, Grade 2, Nilaco, Japan) was cut into rectangular samples of dimensions of $10 \times 10 \times 1\ mm^3$, abraded with #400 SiC paper, washed with acetone, 2-propanol, and ultra pure water for 15 min each in an ultrasonic cleaner, and then dried overnight in an oven at 40 °C. Samples were soaked in 10 mL of 27% H_2O_2 (Alfa aesar) solution at 70 °C in a water bath, at 120 strokes/min for 3 h and then gently washed with ultra pure water. The H_2O_2 pre-treated samples were subsequently treated with 10 mL of 100 mM Mg/Ca/Sr nitrate solution at 40 °C for 3 h and then gently washed with ultra pure water and allowed to dry. These samples were subjected to heat treatment at 600 °C for 1 h in a muffle furnace in air atmosphere. The rate of heating was maintained at 5 °C/min. For TEM observation, Ti grids were directly analysed after treatment with H_2O_2 solution and subsequently in 100 mM Mg/Ca/Sr nitrate solution and heat treatment in the similar manner as described above. All the notations of chemical treatments used in the present study are listed in Table 1 and the same notations will be used in the manuscript.

2.2. Surface analysis of the treated Ti metals

The surfaces of the Ti metals subjected to various chemical and thermal treatments described in Section 2.1 were observed using scanning electron microscope (SEM, TESCAN, Czech Republic). The composition of chemically treated Ti metal surfaces were analysed by energy dispersive X-ray analysis (EDX) attached to the SEM at an acceleration voltage of 15 kV. This analysis was carried out in three different location of each sample and averaged to quantify the amount of Mg/Ca/Sr incorporated into the Ti metal surface.

X-ray diffraction (XRD) analysis was carried out to set the differences in the crystallographic structure for the heat-treated samples and compared with that of untreated Ti metal. XRD measurements were made on a Bruker D8 Advance diffractometer with Cu Kα radiation and detected using a Bruker Lynx Eye detector. XRD spectra were recorded in the range of 20–50° 2θ in a step size of 0.02°.

In order to identify the phases present in the modified surface, laser Raman spectroscopy (RENISHAW Co., UK) was used. For this measurement He-Ne Laser with a wavelength of 630 nm was used. Elements such as Mg, Ca, Sr on the surface of the Ti metal samples subjected to these chemical and heat treatments was analysed using X-ray photoelectron spectroscopy (XPS, Thermo V G Scientific, UK). In this analysis, Al-Kα radiation line 1486.6 eV was used as the X-ray source. The XPS take-off angle was set at 45°, which enabled the system to detect photoelectrons to a depth of 1–5 nm from the surface. The binding energy of the measured spectra was calibrated by reference to the C1s peak of the surfactant CH_2 groups on the substrate occurring at 284.6 eV.

Morphology of Ti metal subjected to various chemical and heat treatments were observed under transmission electron microscope (TEM; Tecnai 20 G2 FEI, The Netherland). SAED pattern were also taken for each conditions.

2.3. Protein adsorption study

Protein adsorption study is considered as an important parameter for the evaluation of biomaterials to be useful in various biomedical fields. Surface phase composition, hydrophilicity, and surface roughness of a material are the key factors that affect the protein adsorption. In the present study, bovine serum albumin (BSA) was used as a model protein in order to understand the adsorption of protein on various chemically and heat treated titanium metal surfaces. Stock solution of BSA was prepared at a concentration of 1 mg/mL in phosphate buffer solution (PBS) at pH 7.4. From this stock solution, 100 μL (contains 100 μg of protein) was pipetted onto the Ti-HP-H, Ti-HP-Mg-H, Ti-HP-Ca-H and Ti-HP-Sr-H samples in a 24 well culture plate and it was incubated at 37 °C for 3 h. Ti metal is used as the control for this study. After the specified time period, samples were removed from the solution and washed for 1 min using distilled water to remove any unadsorbed