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Bacterial and osteoblast behavior on titanium, cobalt–chromium alloy and stainless steel treated with alkali and heat: A comparative study for potential orthopedic applications

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ABSTRACT

Hypothesis: Anatase-modified titanium (Ti) substrates have been found to possess antibacterial properties in the absence of ultraviolet irradiation, but the mechanism is not known. We hypothesize that this is due to the bactericidal effects of reactive oxygen species (ROS) generated by the surface anatase.
Experiments: Alkali and heat treatment was used to form anatase on Ti surface. The generation of ROS, and the behavior of bacteria and osteoblasts on the anatase-modified Ti were investigated. Cobalt–chrome (Co–Cr) alloys and stainless steel (SS) were similarly treated with alkali and heat, and their surface properties and effects on bacteria and osteoblasts were compared with the results obtained with Ti.
Findings: The anatase-functionalized Ti substrates demonstrated significant bactericidal effects and promoted apoptosis in osteoblasts, likely a result of ROS generated by the anatase. The alkali and heat-treated Co–Cr and SS substrates also reduced bacterial adhesion but were not bactericidal. This effect is likely due to an increase in hydrophilicity of the surfaces, and no significant ROS were generated by the alkali and heat-treated Co–Cr and SS substrates. The treated Co–Cr and SS substrates did not induce significant apoptosis in osteoblasts, and thus with these properties, they may be promising for orthopedic applications.

1. Introduction

Titanium (Ti) and its alloys, cobalt–chromium (Co–Cr) alloys and stainless steel (SS) are the three types of commonly-used metals for orthopedic implants. The failure of metallic implants after implantation is a clinical challenge [1], and bacterial infection is a major cause of such failures. Implant-related infections usually require prosthesis removal and replacement which cause attendant patient trauma and prolonged hospitalization with high health and social costs [2]. Therefore, surface modification of implants to inhibit bacterial contamination without compromising its bulk mechanical properties has attracted much attention [3]. Different strategies have been developed such as immobilization of anti-adhesive or bactericidal agents [4], fabrication of various surface topographies [5], and ion implantation [6]. However, these strategies require complex reactions or expensive equipment, which may not be advantageous for large-scale production.

Titanium dioxide (TiO_2) has been reported to have antibacterial effect and this antibacterial effect can be enhanced by ultraviolet (UV) irradiation due to the production of electron–hole pairs on its surface, which result in the generation of reactive oxygen species (ROS) [7]. TiO_2 exists in different polymorphs, and the two most common ones are rutile and anatase. Rutile is more stable than anatase, and anatase exhibits higher photocatalytic activity under UV irradiation due to its high-energy [001] facet [8], and is more widely used for antibacterial applications [9,10]. However, since UV light may not be appropriate for orthopedic implants, and is harmful to the human body, some recent studies have focused on the modification of the spontaneously-formed TiO_2 layer on Ti to achieve antibacterial properties without UV irradiation. Surface TiO_2 has been converted into anatase via anodization of Ti in strong acid followed by heat treatment, and these modified Ti substrates exhibit antibacterial effect against many types of bacteria including *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* and *Porphyromonas gingivalis* [11–13]. However, the mechanism responsible for the antibacterial effect of the anatase is still far from certain.

Recently, it has been reported that anatase nanoparticles can generate ROS in the absence of UV irradiation [14,15]. However, whether ROS can be generated on the anatase-modified Ti is not known because nanoparticles may have different properties from bulk materials of the same composition [16]. In addition, how the concentration of the generated ROS changes with time has

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not been investigated. In this study, we hypothesize that the above-mentioned antibacterial effect of anatase-modified Ti is due to ROS generated by the surface anatase. To verify this hypothesis, anatase was first prepared on Ti via immersion of Ti in alkali solution followed by heat treatment [17], and surface ROS density as well as bacterial adhesion and viability on the pristine and anatase-functionalized Ti was then investigated. The cytotoxicity of the anatase-functionalized substrates towards mammalian cells was also investigated in order to evaluate their potential biomedical applications.

Since Co–Cr and SS are also important materials for orthopedic implants, Co–Cr and SS substrates were similarly treated with alkali and heat to investigate whether this surface treatment can reduce bacterial adhesion while retaining biocompatibility towards mammalian cells. According to our knowledge, bacterial and osteoblast behavior on Co–Cr and SS substrates treated with a combination of alkali and heat has not been studied. Thus, a comparison of the responses of bacteria and osteoblasts to the treated Ti, Co–Cr and SS substrates would provide a useful assessment of their potential orthopedic applications.

2. Materials and methods

2.1. Materials

Ti foils (purity 99.6%, annealed, 0.52 mm thick), Co–Cr foils (Co50/Cr20/W15/Ni10/Fe3/Mn2, 0.6 mm thick) and SS foils (AISI-316, 0.5 mm thick) were purchased from Goodfellow Inc., London, UK. *S. aureus* 25923, *Escherichia coli* (*E. coli*) DH5α and osteoblasts (MC3T3-E1 subclone 14) were obtained from American Type Culture Collection (Manassas, USA). Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma–Aldrich Chemical Co, Singapore. All the other chemicals if not specified were purchased from Sigma–Aldrich Chemical Co, Singapore.

2.2. Substrate preparation

Ti, Co–Cr and SS foils were cut to squares of 1×1 cm^2. The Ti substrates were cleaned ultrasonically for 10 min in Kroll's reagent (4.0% HF, 7.2% HNO_3, 88.8% water). 1 M NaOH was added to stop the reaction, and the substrates were then cleaned sequentially by sonication in dichloromethane, acetone and water (10 min each). The treated substrates were placed in 40% HNO_3 for surface passivation (40 min), and then rinsed thoroughly with water. The TiS substrate was prepared by soaking the washed Ti in 1 M NaOH aqueous solution for 24 h at 60 °C, and the TiH and TiSH substrates were obtained by heating the Ti and TiS substrates, respectively, for 1 h at 600 °C in air. The Ti and TiS substrates were also heated for 10 h at 600 °C in air to obtain the TiH-10 and TiSH-10 substrates, respectively. The Co–Cr and SS substrates were cleaned by sonication in dichloromethane, acetone and water for 10 min each. The washed Co–Cr and SS substrates were subsequently soaked in 1 M NaOH for 24 h at 60 °C followed by heating at 600 °C for 1 h, to obtain the Co–CrSH and SSSH substrates, respectively. The freshly prepared substrates were used for subsequent experiments. In this paper, the suffixes "S", "H" and "SH" were used to indicate the substrates that had been treated by soaking in NaOH solution, heating and their combination, respectively.

2.3. Characterization

The chemical composition of the surfaces was analyzed by X-ray photoelectron spectroscopy (XPS) on a Kratos AXIS Ultra^{DLD} spectrometer (Kratos Analytical Ltd, Manchester, UK) with a monochromatized Al Kα X-ray source (1486.7 eV photons). All binding energies were referenced to the C 1s (C–C) peak at 284.6 eV. X-ray diffraction (XRD) analysis of the surfaces was conducted using a D8 Advance diffractometer (Bruker AXS, Billerica, USA). Surface morphology of the prepared substrates was investigated using a field emission scanning electron microscope (FESEM, JEOL JSM-6700, Tokyo, Japan), and surface roughness (Ra) was measured using an atomic force microscope (AFM, Bruker Dimension ICON, Billerica, USA). Static water contact angles were measured at room temperature with a telescopic goniometer (Rame-Hart, Succasunna, USA).

2.4. Measurement of surface ROS density

The ROS density on the substrate surface was determined by luminol-based chemoluminance assay [18]. In brief, the substrates were placed in a 24-well microplate, and 1 ml of 0.2 M NaOH solution containing 5 mM luminol was added to each substrate in the dark. The chemoluminance was immediately measured with a microplate reader (Tecan Infinite® M200, Männedorf, Switzerland). To investigate the generation of ROS as a function of time, each substrate was immersed in 1 ml of water for 1, 2, 4, 8 and 24 h. The water was then removed and 1 ml of the luminol solution was added to each substrate in the dark, and the chemoluminance was measured. The ROS density was calculated based on a standard curve prepared by using the Fenton reaction (addition of predetermined amounts of 50 mM hydrogen peroxide and 0.2 M ferrous sulfate into the 5 mM luminol solution).

2.5. Bacterial culture and adhesion assay

S. aureus and *E. coli* were cultured in tryptic soy broth (TSB) and nutrient broth (NB), respectively, overnight at 37 °C. An aliquot of bacterial suspension was then taken out and incubated in the broth for another 8–10 h at 37 °C. The bacterial suspension was centrifuged at 2700 rpm for 10 min, and after removal of the supernatant, the bacterial pellet was washed with phosphate buffered saline (PBS, pH 7.4), and resuspended at a concentration of 5×10^7 cells/ml in PBS by sonication. The bacterial concentration in the suspension was estimated from the OD at 540 nm (an OD of 0.1 at 540 nm is equivalent to $\sim 10^8$ cells/ml based on a standard calibration from spread plate counting). The substrates were placed in a 24-well microplate, and each piece was exposed to 1 ml of the bacterial suspension for 4 h at 37 °C. After the incubation period, the bacterial suspension was removed by aspiration, and the substrates were washed twice with PBS. The viability of the bacteria on the substrates was assessed by staining with the LIVE/DEAD® Baclight™ bacterial viability kit (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. The substrates were stained with 50 μl of the combination dye for 15 min, and then observed under an Eclipse Ti inverted microscope system with C-HGFIE Intensilight fiber illuminator (Nikon, Tokyo, Japan). Quantification of bacterial adhesion was carried out by using the spread plate method [19]. In brief, the adherent bacteria were removed by sonication for 7 min, and then resuspended in PBS. 100 μl of the bacterial suspension was taken out, and after decimal serial dilutions with PBS, 100 μl of each dilution was then spread onto a growth agar plate for determination of the viable cell number after incubation overnight at 37 °C.

2.6. Cell culture and cytotoxicity assay

To evaluate the potential application of the treated substrates for orthopedic implants, the effects of such surfaces on osteoblasts were evaluated. The cells were cultured in MEM alpha medium (Life Technologies, Carlsbad, USA) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin