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Antibacterial effects of titanium embedded with silver nanoparticles based on electron-transfer-induced reactive oxygen species

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ABSTRACT

Although titanium embedded with silver nanoparticles (Ag-NPs@Ti) are suitable for biomedical implants because of the good cytocompatibility and antibacterial characteristics, the exact antibacterial mechanism is not well understood. In the present work, the antibacterial mechanisms of Ag-NPs@Ti prepared by plasma immersion ion implantation (PIII) are explored in details. The antibacterial effects of the Ag-NPs depend on the conductivity of the substrate revealing the importance of electron transfer in the antibacterial process. In addition, electron transfer between the Ag-NPs and titanium substrate produces bursts of reactive oxygen species (ROS) in both the bacteria cells and culture medium. ROS leads to bacteria death by inducing intracellular oxidation, membrane potential variation, and cellular contents release and the antibacterial ability of Ag-NPs@Ti is inhibited appreciably after adding ROS scavengers. Even though ROS signals are detected from osteoblasts cultured on Ag-NPs@Ti, the cell compatibility is not impaired. This electron-transfer-based antibacterial process which produces ROS provides insights into the design of biomaterials with both antibacterial properties and cytocompatibility.

1. Introduction

Although titanium and titanium alloys with good stability and biocompatibility in the physiological environment are widely used in dental and orthopedic implants [1–3], the materials do not have intrinsic antibacterial ability. Bacterial infection is one of the most serious complications after surgery leading to not only implant failure, but also complication, morbidity, and mortality [4–6]. Several strategies have been proposed to endow titanium implants with antibacterial characteristics. In particular, titanium embedded with silver nanoparticles (Ag-NPs@Ti) by plasma ion immersion implantation (PIII) has drawn much attention because of the broad antibacterial spectrum and capability of tuning the surface morphology of the biomaterials [7,8]. However, the exact antibacterial mechanism of Ag-NPs@Ti is still not well understood.

The antibacterial mechanism of Ag-NPs in liquids have been investigated. Sondi et al. assumed that accumulation of Ag-NPs on the cell wall and bacteria membrane increased the permeability leading to cell death [9]. Free silver radicals were detected from the bacteria solution after treating with Ag-NPs [10] and the cell metabolism was disrupted by Ag^{+} leached from the Ag-NPs [11–13]. However, the Ag-NPs embedded in the substrate are different from those dispersed in liquids because of interactions with both the substrate and bacteria in physiological liquids upon contact. The micro-galvanic effects between Ag and titanium evoke electron transfer in the surrounding medium and in fact, electron transfer is a universal behavior in bacteria and cell respiration. However, there have been few studies on the effects by considering both the materials and biological aspects and concomitant interactions between the biomaterials and biological system. What's more, whether reactive oxygen species (ROS) is produced or not is a fundamental issue in antibacterial processes. There have been recent studies on the role of ROS in antibacterial processes suggesting practical approaches to enhance our current antibiotics

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arsenal [14–16]. In fact, Nathan et al. have suggested that a better understanding of how to manipulate ROS production can spur medical advances in immunology and combination therapy [17]. Thus, as potential bone implant materials, it is important to study whether Ag-NPs@Ti induces ROS during bacteria killing and a better understanding will aid the design and fabrication of future biomaterials.

In this work, Ag-NPs are incorporated into titanium by PIII and the antibacterial characteristics and mechanism are investigated. The antibacterial effect of Ag-NPs on different substrates is determined. Our results show that electron transfer between the Ag-NPs and titanium produces a large amount of ROS which play a key role in bacteria killing by inducing physiological changes in the bacteria such as intracellular oxidation, protein and DNA/RNA release, as well as membrane potential variation.

2. Experimental section

2.1. Sample preparation and characterization

2.1.1. Sample preparation

Titanium (Ti, 99.99%) samples with dimensions of 10 mm × 10 mm × 1 mm were ground with SiC abrasive paper (from 400 grit to 2000 grit), mounted on a sample holder, introduced into a plasma immersion ion implanter, and evacuated to a base pressure of 2.5×10^{-3} Pa. Ag (99.99%) produced by a filtered cathodic arc plasma source was implanted into the polished Ti samples which were pulse-biased to −30 kV (pulse duration of 450 μs and frequency of 6 Hz). The samples were plasma-implanted for 1 h, 2 h, or 3 h (samples designated as 1 h-Ag-NPs@Ti, 2 h-Ag-NPs@Ti, and 3 h-Ag-NPs@Ti, respectively). More details about the PIII process can be found elsewhere [8].

2.1.2. Surface characterization

Atomic force microscopy (AFM, Veeco's MultimodeV, Veeco, USA) and scanning electron microscopy (SEM, JSM 7001F, JEOL, Japan) were conducted to characterize the surface morphology. The X-ray diffraction (XRD) spectra were acquired on a Philips Siemens D500 X-ray diffractometer and X-ray photoelectron spectroscopy (XPS, K-Alpha, Thermo Fisher Scientific, USA) was conducted to determine the chemical states (sputtering rate of about 7 nm/min). Energy dispersive X-ray spectroscopy (EDS) on the SEM were used to determine the silver concentration. The experimental procedures are illustrated schematically in Fig. S1.

2.1.3. Electrochemical measurements

The electrochemical properties of the samples were determined on an electrochemical workstation (Zennium, Zahner, Germany) using a three-electrode configuration comprising a saturated calomel electrode (SCE) as the reference electrode, a platinum rod as the counter electrode, and the sample (area of 0.5 cm^2) as the working electrode. The electrochemical measurements were performed at room temperature. The open circuit potential (OCP) measurements were carried out before the Tafel curves were acquired at a scanning rate of 1 mV/s from −0.5 V to 0.5 V with respect to OCP.

2.2. Antibacterial effects

2.2.1. Antibacterial effects of Ag-NPs@Ti

Four groups of samples, namely Ti with 0.1 mM H_2O_2 (ROS positive group), 1 h-Ag-NPs@Ti, 2 h-Ag-NPs@Ti, and 3 h-Ag-NPs@Ti, and the unprocessed Ti control were studied. The samples were immersed in 75% ethanol for 15 min and dried in a biosafety cabinet. Afterwards, each sample was put on the well of a 24-well plate and the biofilm forming strains of *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) were introduced. *S. aureus* and *E. coli* were cultured in the Lysogeny broth (LB) medium overnight at 37 °C in an incubator shaken at 220 rpm. The bacteria solution was diluted to $OD_{600} = 0.1$ with the fresh medium and cultivated for another 3 h for reactivation. The bacteria solution was diluted to a concentration of $2–3 \times 10^5$ CFU/mL and 100 μL were spread on the surface of the samples. The gaps between the wells were filled with autoclaved water in order to prevent evaporation of the medium. At time points of 1, 3, 6, 18 and 24 h, the adhered bacteria were detached from the surface with 900 μL of phosphate buffered saline (PBS), diluted to the proper concentration, spread on a solid agar plate, and cultivated for another 16 h to count the colony forming unit (CFU). The antibacterial rate was determined by the following formula:

$$\text{Antibacterial rate} = \left(1 - \frac{\text{CFU}_{\text{experimental group}}}{\text{CFU}_{\text{control group}}}\right) \times 100\%$$

2.2.2. Antibacterial effects of Ag-NPs on different substrates

The antibacterial effect of 2 h-Ag-NPs on substrates with different conductivity, namely Ti (conductor), Si (semiconductor), and SiO_2 (insulator) was assessed. Based on the antibacterial results of Ag-NPs@Ti, an incubation time of 6 h was chosen and CFU counting was performed to compare the antibacterial rates according to session 2.2.1 above.

2.2.3. Live/dead staining

In the viability assay, after rinsing in PBS twice for 5 min, the sample with bacteria was stained with LIVE/DEAD® *Ba*cklight Bacterial Viability Kit (L7007, Invitrogen, USA) for 15 min in darkness. The samples were then examined on an inverted microscope (BM-20AYC, BM) with 420–480 nm and 520–580 nm as the excitation and emission wavelengths (green fluorescence) and 480–550 nm and 590–800 nm as the excitation and emission wavelengths (red fluorescence). Representative images were obtained and merged.

2.3. Intracellular oxidative stress measurement

2.3.1. Intracellular ROS level evaluation

The intracellular ROS level was investigated by fluorescence imaging. At time points of 1, 3, 6, 18, and 24 h, the samples were washed with PBS twice and 100 μL of 2′, 7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime, China) were added to the surface and reacted for 15 min. The excess dye was washed with PBS and the samples were observed by inverted fluorescent microscopy as mentioned above. Flow cytometry (FCM, BD FACS Calibur, USA) was employed to determine quantitatively the intracellular ROS level using 488 nm and 522 nm as the excitation and emission wavelengths. The X Geo mean data of FL1-H was used to evaluate the fluorescence intensity of each group.

2.3.2. Intracellular superoxide concentration detection

The intracellular superoxide was quantitatively measured with the Superoxide Assay Kit (S0060, Beyotime, China) to evaluate the intracellular oxidative stress level after culturing for 3 and 6 h. The bacteria solution was collected and diluted to a proper concentration before the assay kit was added. After cultivation for another 3 min at 37 °C, OD_{450} and OD_{600} were monitored to quantitatively determine the superoxide.

2.3.3. Protection effect of antioxidant

To confirm the oxidative stress rendered by Ag-NPs@Ti, N-