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# Effects of copper nanoparticles in porous $TiO_2$ coatings on bacterial resistance and cytocompatibility of osteoblasts and endothelial cells

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ABSTRACT

Copper (Cu) has garnered increasing interest due to its excellent antimicrobial activity and important roles in human metabolism. Although the biological effects of Cu have been studied, the effects of Cu nanoparticles (NPs) on cell behavior are not well understood. In this study, porous $TiO_2$ coatings doped with different amounts of Cu NPs (designated as 0 Cu, 0.3 Cu, and 3.0 Cu) are deposited on titanium by micro-arc oxidation (MAO). The Cu NPs coated samples exhibit excellent antibacterial activity against *Staphylococcus aureus* (*S. aureus*). *In vitro* cytocompatibility evaluation discloses that 0 Cu and 0.3 Cu have no toxicity to osteoblasts but 3.0 Cu shows cytotoxicity. 0.3 Cu promotes proliferation and adhesion of osteoblasts and enhances extracellular matrix mineralization (ECM), but has little effects on the alkaline phosphatase activity (ALP) and collagen secretion. Surprisingly, the Cu NPs coated samples show a different behavior with endothelial cells. Both 0.3 Cu and 3.0 Cu show no cytotoxicity on endothelial cells and promote cell proliferation. Production of nitric oxide (NO) and secretion of vascular endothelial growth factor (VEGF) by the endothelial cells are observed from the Cu NPs doped $TiO_2$ coatings.

## 1. Introduction

Copper (Cu) is one of essential trace elements and serves a number of important roles in human beings [1–3]. For instance, Cu takes part in enzyme-based processes for bone metabolism, stimulates new vessel formation, and accelerates early skin wound healing [4–9]. To overcome the problems caused by bacterial infection, various antibacterial materials were developed for biomedical application [10–15]. Cu also has excellent antibacterial properties against a broad spectrum of pathogens including gram-positive and gram-negative bacteria by interfering with DNA replication and disrupting cell membranes [16–19]. Hence, incorporation of Cu into biomaterials can enhance the osteogenic and angiogenic activity as well as antibacterial activity to mitigate implant-associated infections.

Nanoparticles (NPs) with a small size and large surface-to-volume ratio have more interactions with biological targets such as cell and bacteria than conventional micrometer-sized particles [20–23]. Therefore, many inorganic NPs such as Ag and Cu ones have been investigated. Ag has attracted more interest due to its more potent effects on bacteria and cells [24–28], but there have been fewer studies on Cu NPs. Jia et al. prepared Cu NPs coated cellulose films with efficient antibacterial activity [29]. Villanueva et al. synthesized silica-coated Cu NPs and the materials could maintain the antimicrobial activity for at least four cycles [30]. In addition to the inherent antibacterial activity of Cu, Cu NPs can easily penetrate micrometer-sized bacterial membranes and interact closely with the membranes of microorganisms to enhance the biocidal effect. Their antimicrobial activity is due to not only release of metal ions to the solution, but also generation of reactive oxygen species (ROS) which produce subsequent oxidative damage to cellular structures [31].

Micro-arc oxidation (MAO) is an effective technique to form a microporous and adherent $TiO_2$-based coatings on titanium (Ti) implants. During MAO, functional elements such as Ca, P, Zn, Ag, Si, and Cu can be incorporated into the $TiO_2$ coatings to enhance the apatite forming ability, biocompatibility, osteoconductivity and antimicrobial activity of Ti implants by using suitable electrolytes [32–35]. In this work, $TiO_2$ coatings doped with Cu NPs are prepared by MAO in electrolytes with different Cu concentrations and the effects on the behavior of osteoblasts and endothelial cells including the antibacterial activity against *S. aureus* are studied systematically. The results can provide guidance

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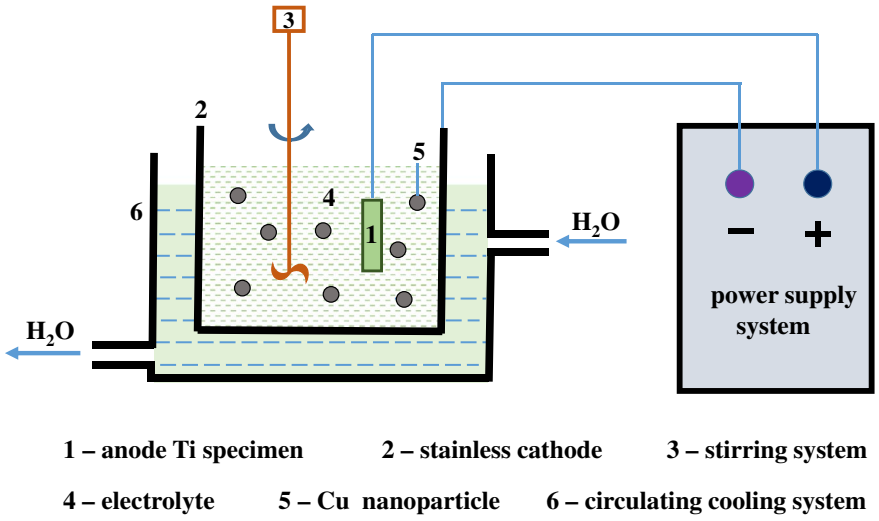


**Fig. 1.** Schematic diagram of the MAO experimental device.

for the orthopedic and dental implants with antibacterial ability and cytocompatibility for medical applications.

## 2. Experimental details

### 2.1. Preparation of Cu NPs incorporated $TiO_2$ coatings

Commercial pure titanium plates (Cp Ti, TA2, purity > 99.85%) with a diameter of 14 mm and thickness of 2 mm were used in this study. The samples were ground with 300, 600, 800, and 1000 grit SiC paper and ultrasonically cleaned with acetone, ethanol, and distilled water prior to MAO. During MAO, a pulsed DC power supply was employed. The Ti plate served as the anode and a double wall stainless steel electrolytic cell as the cathode. The cell was filled with an electrolyte comprising sodium dihydrogen phosphate ($NaH_2PO_3$), sodium hydroxide (NaOH), and Cu nanoparticles. The schematic diagram of the MAO experimental process is shown in Fig. 1. The main processing parameters are listed in Table 1. The electrolyte was kept below 30 °C with circulating water and magnetic stirring at 35 rpm. After MAO, the samples were gently washed with deionized water and air dried.

### 2.2. Characterization

The surface morphology and cross-sectional microstructure of the MAO coatings were examined by Nano 430 scanning electron microscope (SEM) and the elemental composition was determined by energy-dispersive X-ray spectrometry (EDS). The phase components in the coatings were analyzed on a Rigaku DyMax 2500 X-ray diffractometer (XRD) and the chemical states were determined on a VG ESCALAB Mark II X-ray photoelectron (XPS) instrument with an Al $K_\alpha$ source. The XPS spectra were calibrated with the C 1s peak at a binding energy of 284.8 eV.

### 2.3. Cu ion release

The specimens were immersed in 20 ml of PBS and kept at 37 °C for 14 days. The liquid was collected after 1, 3, 7 and 14 days and replaced with fresh PBS. The Cu concentration leached to the PBS was determined by inductively-coupled plasma mass spectrometry (ICP-MS, Agilent 7500, Agilent) in triplicate for each immersion time.

### 2.4. Antibacterial activity

The antibacterial activity of 0 Cu, 0.3 Cu, and 3.0 Cu was evaluated *in vitro* by the bacteria counting method with *S. aureus* as the model bacteria. The samples were sterilized in an autoclave at 121 °C for 40 min. A suspension containing bacteria at a concentration of $10^7$ CFU $ml^{-1}$ was dripped onto the sample surfaces to a density of 60 μl $cm^{-2}$ and incubated at 37 °C for 24 h. Afterwards, serial dilutions in 10-fold steps were made and the diluted bacterial suspension was inoculated onto a standard agar culture medium. After incubation at 37 °C for 24 h, the live bacteria were counted according to the National Standard of China GB/T 4789.2 protocol.

Prior to SEM observation, a suspension containing the bacteria at a concentration of $10^7$ CFU $ml^{-1}$ was dripped onto the sample. After incubation at 37 °C for 24 h, the bacteria were fixed with a 2.5% glutaraldehyde solution and dehydrated in a series of ethanol solutions (30, 50, 75, 90, 95 and 100%) for 15 min. After sputter-coating a thin platinum layer onto the sample surface to prevent charge, the morphology was observed by SEM.

Fluorescence microscopy was utilized to investigate the antibacterial activity of the coatings. After incubation at 37 °C for 24 h, the attached bacteria were removed. The samples were rinsed twice with a physiological saline solution and a Live/Dead BacLight staining reagent mixture was added. After staining for 15 min, the adherent bacteria on the samples were observed by fluorescence microscopy.

**Table 1**
Main processing parameters and electrolyte components used in the MAO process.

| Sample | Electrolyte components ($g \cdot l^{-1}$) | | | Process parameters | | |
|---|---|---|---|---|---|---|
| | NaOH | $NaH_2PO_4$ | Cu nanoparticles | Current density, $A \cdot dm^{-2}$ | Final voltage, V | Duration, min |
| 0 Cu | 2 | 15 | 0 | 20 | 476 ± 3 | 5 |
| 0.3 Cu | 2 | 15 | 0.3 | 20 | 470 ± 3 | 5 |
| 3.0 Cu | 2 | 15 | 3.0 | 20 | 465 ± 3 | 5 |