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The effects of annealing temperature on corrosion behavior, Ni^{2+} release, cytocompatibility, and antibacterial ability of Ni-Ti-O nanopores on NiTi alloy

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ABSTRACT

The present work investigated the effects of annealing on corrosion behavior, Ni^{2+} release, cytocompatibility, and antibacterial ability of nickel-titanium-oxygen (Ni-Ti-O) nanopores (NPs) anodically grown on nearly equiatomic NiTi alloy, aiming at optimizing annealing process to yield favorable comprehensive performance. The morphology and crystal structure of the NPs were characterized by scanning electron microscopy and X-ray diffraction respectively. It was found that when the annealing temperature was < 600 °C, the NP layer could be well preserved on the substrate surface, and annealed at 400 °C led to the transformation of amorphous phase to anatase. Annealing at 200 °C significantly enhanced the corrosion resistance and at 400 °C drastically reduced Ni^{2+} release of the NiTi alloy, but their cytocompatibility had no appreciable difference, indicating the release level of Ni^{2+} is well tolerated by osteoblasts. Although the release amount of Ni^{2+} is reduced after annealing especially the sample annealed at 400 °C, their antibacterial ability is even better when compared with that of the unannealed sample. These results suggest the NPs annealed as at 200–400 °C are promising as coatings of biomedical NiTi alloy.

1. Introduction

Over the past decades, nearly equiatomic nickel-titanium (NiTi) alloy has been extensively used as implant materials due to its unique shape memory effect and superelasticity as well as good biocompatibility [1–4]. After inserted into human body, the implants will immediately contact with body fluids, which is a complicated electrochemical system. Electrochemical corrosion will inevitably occur as long as it is in service, consequently degrading its mechanical properties. Naturally formed oxide film on the NiTi alloy is extremely thin (~4 nm) with poor self-healing ability [5], so its protective ability is very limited. Therefore, further improving its corrosion resistance through proper surface modification is highly required. In addition, infection is another potential risk for all kinds of implants, resulting in patient suffering, financial burden, and even fatality [6,7]. Accordingly, endowing the NiTi alloy with good corrosion resistance and antibacterial ability is highly desirable.

It is well known that corrosion resistance of biomaterials can be improved through proper surface treatments [8–10]. Anodic growth of oxide films on valve metals and their alloys has attracted much interests in recent years due to its economical, reproducible, and other desirable properties [11]. Our previous studies have shown constructing ordered Ni-Ti-O nanopore (NP) layer on the NiTi alloy surface through anodization could improve its corrosion resistance [12,13]. Following work demonstrated the NPs with length in the range of 1–11 μm showed favorable antibacterial ability due to proper release amount of Ni^{2+} [14]. More importantly, as reported in the literature, the release level could be well tolerated by human beings [15].

Annealing, a traditional heat treatment process, has been successfully applied to improve the corrosion and other desirable properties of Ti-based biomaterials [16,17]. Following works showed annealing of TiO_2 nanotubes (NTs) leads to better performances, including corrosion resistance, antibacterial activity and biocompatibility. For example, Mazare and co-workers have shown that an optimal annealing temperature (650–750 °C) of TiO_2 NTs yielded low corrosion current density combined with good antibacterial activity and enhanced haemocompatibility [18]. Yeniyol and co-authors reported that annealed TiO_2 NTs incorporated with Ag showed reproducible antibacterial ability on

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transmucosal parts of dental implants [19]. Based on the previous research results, we hypothesize annealing may influence the corrosion behavior, cytocompatibility and antibacterial ability of Ni-Ti-O NPs. In the present work, we fabricated Ni-Ti-O NPs on the NiTi alloy by anodization and annealed them at different temperatures, aiming at optimizing annealing process to yield desirable corrosion resistance, cytocompatibility, and antibacterial ability for better clinical application.

2. Experimental details

2.1. Sample preparation and characterization

Commercial NiTi (50.8 at.% Ni) rod (Φ9 mm) (Xi'an Saite, China) was cut into small pieces (2 mm in thickness), which were then grinded to a mirror finish with 0.25 μm diamond spray, and cleaned in acetone, ethanol and distilled water sequentially, finally drying in air. The samples were then subjected to anodization to produce Ni-Ti-O NPs. An ethylene glycol solution containing 0.3 M NaCl and 5.0 vol% H_2O was used as electrolyte. Anodization was carried out in a two-electrode configuration at 10 V and 37 °C for 10 min. After anodization, the samples were immediately washed with deionized water and ultrasonically cleaned for 5 min to remove the remaining electrolyte on the sample surface. The as-grown Ni-Ti-O NPs were then annealed at different temperatures (200, 400, and 600 °C, denoted as NP-200, NP-400, NP-600 respectively) for 2 h at a heating and cooling rates of 3 °C/min. Scanning electron microscopy (SEM, JSM-7100F, JEOL, Japan) was used to observe the surface, sub-surface and cross-sectional morphologies of the anodized and annealed samples. The crystalline structure of the samples was analyzed by X-ray diffraction (XRD, DX-2700, Haoyuan) using Cu K_α radiation at an incident angle of 2°.

2.2. Corrosion behavior

Corrosion behavior of the samples were tested on an electrochemical workstation (CS350, CorrTest, China) in phosphate buffered saline (PBS, pH 7.4) at 37 °C. The sample, platinum foil and saturated calomel electrode were used as working, counter and reference electrode, respectively. Open circuit potential (OCP) of the samples were continuously monitored for 1 h, followed by electrochemical impedance spectroscopy (EIS) at OCP over a frequency range of 0.01 Hz to 0.1 MHz with a sinusoidal perturbation potential amplitude of 10 mV. Potentiodynamic polarization was performed at a scan rate of 1 mV/s and recorded over a potential range of −0.8 to 1.5 V *vs.* SCE. Corrosion potential (E_{corr}), corrosion current density (I_{corr}) and cathodic Tafel slopes (β_c) was obtained by Tafel extrapolation method.

2.3. Ni^{2+} release

Each specimen was immersed in 3 ml of PBS at 37 ± 0.5 °C for 24 h. The PBS containing the released Ni^{2+} was analyzed by inductively-coupled plasma mass spectroscopy (ICP-MS, 7500, Agilent).

2.4. Cytocompatibility assay

Cytocompatibility of the samples was evaluated by live/dead fluorescence staining and cell proliferation. Before the experiments, all the samples were sterilized with 75 vol% alcohol for 40 min and rinsed with sterile PBS three times. Newborn mouse calvaria-derived MC3T3-E1 subclone 14 pre-osteoblastic cells were seeded and incubated on the sample surfaces at a density of 2×10^4 cells/cm^2.

The cytotoxicity of the samples was assayed by Live/Dead® viability/cytotoxicity kit (Invitrogen). After the cells were cultured for 1, 3, and 5 days on the sample surfaces, they were rinsed thrice with PBS. Then 30 μl of the work solution in the kit was immediately added to each sample surface and incubated in darkness at 37 °C for 40 min. Subsequently, all the samples were gently rinsed with PBS and observed by confocal laser scanning microscope (CLSM, C2 Plus, Nikon). Cell proliferation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After culturing for 1, 3, and 5 days, the samples were transferred to a new aseptic 24-well plate with 1 ml of the fresh medium containing 10 vol% MTT in each well and incubated for 4 h to form formazan, which were then dissolved with dimethylsulfoxide (DMSO) of 1 ml for each well. The resultant solution of 100 μl was transferred to a 96-well plate and the absorbance was measured on a microplate reader (Infinite, F50, TECAN) at 492 nm.

2.5. Antibacterial ability

Gram-positive *Staphylococcus aureus* (*S. aureus*, ATCC6538) was used to investigate the antibacterial ability of the samples. Prior to each experiment, *S. aureus* was collected and incubated on beef extract-peptone (BEP) overnight at 37 °C. The inocula of *S. aureus* was serially diluted to 1.0×10^5 CFU/ml in PBS for the antibacterial experiment. The antibacterial ability of the four groups (NiTi alloy, anodized sample and NP-200, NP-400) against *S. aureus* were evaluated. These specimens with 1 ml of the prepared bacterial suspension were incubated for 12 h in 48-well plates at 37 °C. At the end of incubation, viable bacteria in PBS were evaluated by standard serial dilution and plate-counting method. The blank control was the bacterial suspension without the sample. The antibacterial ability was determined by calculating the antibacterial rate (R) using the following formula:

$$R = (N_{blank} - N_{sample})/N_{blank} \times 100\% \quad (1)$$

Here, N_{blank} and N_{sample} indicate the average number of viable bacteria in the suspension inoculated without and with the samples respectively.

2.6. Statistical analysis

Each experiment was repeated three times and the data from a typical one were shown. The data were expressed as mean ± standard deviations. A one-way ANOVA followed by the Student-Newman-Keuls (NSK) test was used to determine the level of statistical different. The difference was considered to be significant and highly significant when $p < 0.05$ and 0.01, respectively.

3. Results and discussion

3.1. Sample characterization

Fig. 1 shows the surface, subsurface, and cross-sectional SEM images of NP, NP-200, NP-400, and NP-600. There is no uniform and complete film on NP-600, and only fragmentary fragments can be observed. In comparison, irregular NP layers on the surface and ordered nanoporous structure of sub-surface are clearly seen from all of the other groups. The cross-section of all the samples are observed from the high-magnification SEM images except for NP-600 because of the detachment of the NPs after annealing. One possible explanation is the growth and phase transform of oxides at the NP/substrate interface increases the internal stress and weaken the adhesion strength when the temperature is high enough [20]. The length of the NPs shows slightly decrease with increasing annealing temperature, decreasing from 2.78 μm for NP to 2.05 μm for NP-400. This is due to the high temperature results in sintering of the nanoporous structure [9] and the structure becomes relatively compact. NP-600 is omitted in subsequent experiments because of its fail in surface integrity.

Fig. 2 shows the XRD patterns of the NiTi alloy, NP, NP-200 and NP-400. Three prominent peaks at 42.8°, 61.3° and 78° correspond to (110), (200) and (211) planes of the NiTi substrate. There is no difference in the patterns between them except for that of NP-400, in which three additional peaks corresponding to (101), (004) and (112) planes of anatase phase can be observed. Our previous works have shown that