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Improved *in vitro* and *in vivo* biocompatibility of dual plasma modified titanium alloy

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ABSTRACT

Dual carbon and nitrogen (C–N) plasma immersion ion implantation (PIII) is conducted to modify Ti–6Al–4V alloy. A graded implanted layer containing TiN and TiC is formed and in comparison with the C-implanted and un-implanted sample, the C–N implanted sample exhibits improved surface roughness and *in vitro* cell adhesion and proliferation. Micro-CT evaluation conducted after 1, 6, and 12 weeks of implantation shows the least amount of average bone volume on the un-implanted Ti pin, whereas that on the C–N implanted sample is more than that on the C-implanted at every time point. The biocompatibility enhancement after dual C–N PIII is attributed to the synergistic effects rendered by TiN and TiC as well as altered surface roughness. Our results suggest that dual C and N PIII is the preferred technique for Ti–6Al–4V alloy in clinical applications.

1. Introduction

Ti–6Al–4V alloy is widely used in orthopedic applications because of the good mechanical properties and excellent corrosion resistance. However, long-term success of Ti–6Al–4V implants, especially osteointegration, still needs to be evaluated in order to reduce patient suffering [1]. Since the biofunctionality of a biomedical implant is strongly affected by its surface characteristics, the use of surface modification to promote osteointegration is viable. In particular, plasma immersion ion implantation (PIII) has been employed to incorporate new biofunctional groups on titanium alloy surface [2]. Since the implanted layer is only confined to the near-surface region, the materials can be selectively modified to enhance the surface bioconductivity while the favorable bulk attributes can be retained [3,4]. In this work, we investigate the effects of dual carbon–nitrogen PIII on the biocompatibility of Ti–6Al–4V alloy and determine the relationship between surface characteristics and biocompatibility.

2. Materials and methods

Medical grade Ti–6Al–4V alloys with dimensions of $\Phi 5 \times 2$ mm^3 were mechanically polished to a mirror finish and ultrasonically washed in acetone and ethanol prior to ion implantation. PIII was conducted on a multi-purpose plasma immersion ion implanter equipped with a graphite cathodic arc source in the Plasma Laboratory in City University of Hong Kong [5–10]. During carbon PIII, Ar was bled into the chamber at a flow rate of 4 standard cubic centimeters per minute (sccm) and the carbon plasma was created by vacuum arc discharge. In dual carbon–nitrogen PIII, nitrogen was introduced at 15 sccm and the plasma was triggered by 1000 W radio frequency (RF). Both sets of PIII experiments were performed for 2 h at −30 kV (sample bias), 500 μs (pulse width), and 10 Hz (pulsing frequency).

X-ray photoelectron spectroscopy (XPS, Physical electronics PHI 5802) with Al K_α irradiation was used to determine the chemical states and elemental depth profiles of the implanted samples. The sputtering rate was estimated to be about 5.67 nm·min^{-1} based on that calculated from a SiO_2 standard sputtered under similar conditions. The binding energies were referenced to the C 1s line at 285.0 eV. A Gaussian–Lorentzian peak fitting model was adopted to deconvolute the Ti2p spectra. The surface morphology was investigated by atomic force microscopy (AFM) (Auto-Probe CP, Park Scientific Instruments) in the contact mode.

The *in vitro* tests included evaluation of the adhesion and proliferation of mouse MC3T3-E1 pre-osteoblasts. The cells were cultivated in the DMEM medium with 10% fetal calf serum in a CO_2 incubator supplied with 5% CO_2 at 37 °C. In the cell adhesion assay, the MC3T3-E1 pre-osteoblasts were seeded on each sample in 96-well tissue culture plates at a density of 1×10^4 cells per well and cultured for 4 h. Afterwards, the seeded samples were rinsed twice with sterile phosphate buffered saline (PBS), fixed with 2% polyoxymethylene solution, and stained with the LIVE/DEAD staining kit. The cell numbers in three random fields were counted using a fluorescence microscope (Carl Zeiss Axio Observer). Cell viability and cell proliferation were assessed using MTT (3-[4,5-dimethylthiahiazo-2-yl]-2,5-diphenytetrazolium bromide) assay. The MC3T3-E1 pre-osteoblasts were cultured for 2, 4, and 7 days at an initial density of 5×10^3 cells/well. The absorbance at 570 nm was used as an indicator of cell proliferation determined by a microplate

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reader (Bio-Tek, USA). The results of the *in vitro* cell experiments were statistically analyzed by the one-way analysis of variance (ANOVA) and a p value less than 0.05 was considered to indicate statistical significance.

The animal experiments were approved by The Department of Health and The Committee on the Use of Live Animals in Teaching and Research (CULATR). 12-week old male ICR mice were used in this study. A hole 1.25 mm in diameter was prepared at the distal femurs using dental drill until the hole reached 4 mm in depth and then a Ti alloy pin ($\Phi 1.5 \times 4$ mm^3) was implanted into the prepared hole in both femurs. After the operation, the mice underwent micro-computer tomography (Micro-CT) evaluation directly using a micro-computed tomography device (SKYSCAN 1076, Skyscan Company) and more micro-CT examinations were conducted every week up to the 12th week. After scanning, the 2D planes were reconstructed using the NRecon (Skyscan Company) and the bone volume around the implant was determined by the CTAn program (Skyscan Company).

3. Results and discussion

The AFM images over a scanned area of 1×1 μm^2 in Fig. 1 provide evidence that the relatively smooth surface on the un-implanted sample (Fig. 1a) is altered by PIII and nanosized potholes can be observed after the plasma treatment (Fig. 1b and c). The average roughness values increase from 8.79 Å on the un-implanted sample to 13.6 Å on the C-implanted sample and 16.4 Å on the C–N implanted sample. In general, a proper surface roughness bodes well for cell attachment [2].

Fig. 2 displays the elemental depth profiles, high resolution XPS Ti2p spectra, and corresponding fitted peaks of the C-implanted and C–N implanted samples. The near surface of the implanted sample shows graded Ti, O, C and N concentrations. As shown in Fig. 2(a), there is a thin C-containing layer about 340 nm thick on the C-implanted sample. After removal of the top surface materials contaminated with carbon and oxygen (sputtering for 4 min) [11,12], the high resolution XPS Ti spectrum in Fig. 2(b) reveals that the layer is composed of TiC, TiO_2, Ti_2O_3, TiO, and Ti, with the binding energies at 454.9 eV, 458.9 eV, 456.9 eV, 455.1 eV and 454.3 eV, respectively [13]. The formation of titanium oxide is due to non-UHV (ultra-high vacuum) implantation conditions in our PIII instrument [14,15]. In comparison with the C-implanted sample, higher relative contents of C and N are observed from the C–N implanted sample [Fig. 2(c)]. The thickness of the implanted layer is about 400 nm. The high resolution XPS Ti spectrum in Fig. 2(d) shows that besides TiC, TiO_2, Ti_2O_3, TiO, and Ti, a certain amount of TiN (455.8 eV binding energy for Ti2p3/2 [13]) is present in the implanted layer.

The adhesion results of MC3T3-E1 pre-osteoblasts cultured on the un-implanted, C-implanted, and C–N implanted samples are presented as histograms in Fig. 3. Compared to the un-implanted sample, more MC3T3-E1 pre-osteoblasts adhere on the PIII treated samples and the C–N implanted sample exhibits significantly enhanced cell adhesion. Cell proliferation is an important factor when evaluating cell response to the materials. The cell proliferation results on the samples after 2, 4 and 7 days of incubation are illustrated in Fig. 4. At each time point, the cell numbers on both implanted samples are larger than those on the un-implanted one. In particular, the C–N implanted sample shows the highest degree of MC3T3-E1 pre-osteoblasts. The cell adhesion and cell viability assay indicates that cytocompatibility is evidently enhanced after dual C–N PIII and that the implanted layers are nontoxic as reflected by the favorable cell behavior.

The bone behavior around the Ti alloy pin in the same living ICR mice is examined by continuous micro-CT monitoring. Fig. 5 depicts the micro-CT reconstruction images of the Ti alloy pin after 1, 6 and 12 weeks of implantation and Fig. 6 shows the corresponding change

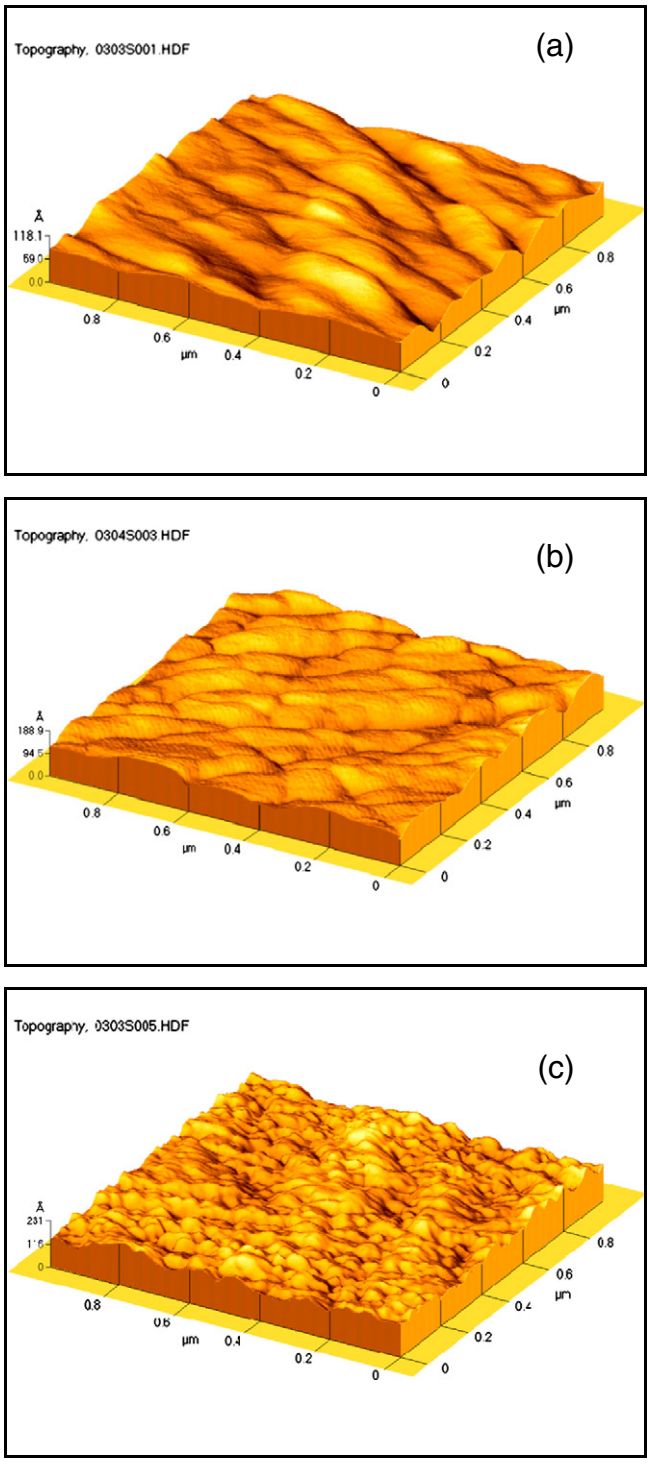

Fig. 1. AFM images of (a) un-implanted Ti alloy, (b) C-implanted Ti alloy and (c) dual C–N implanted Ti alloy.

in the average bone volume from week 1 to week 12. During the entire implantation period, all the samples are stable and show direct contact with the surrounding bone. An overall comparison among 1, 6, and 12 weeks of implantation shows that the average volume is the largest on the C–N implanted sample, followed by the C-implanted sample with the un-implanted Ti pin showing the least at every time point. The *in vivo* study confirms that dual C–N PIII yields the most favorable bone behavior.

A surface structure containing TiN and TiC is produced on titanium alloy surfaces by dual C–N PIII and this structure induces more