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Protocols

Mn-containing titanium surface with favorable osteogenic and antimicrobial functions synthesized by PIII&D

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ABSTRACT

Reasonable incorporation of manganese into titanium is believed to be able to enhance the osteogenic and antibacterial activities of orthopedic implants. However, it is still a challenge to compromise Mn-induced cytotoxicity and better develop its biocompatibility and antimicrobial ability. To pinpoint this issue, a stable Mn ion release platform was created on Ti using plasma immersion ion implantation and deposition (PIII&D) technique. Compared with as-etched titanium, as a result, promoted antibacterial abilities against gram-negative bacteria species and enhanced osteogenic-related gene expressions on rBMMSC were observed on Mn-containing sample. Meanwhile, the Mn-containing samples showed no obvious cytotoxicity. Our results here provide insight to be better understanding the relationships between additives-induced biological performance and the dose, state, and stability of the doped element.

1. Introduction

It is popularly accepted that the interactions between implant surface and host cells have a significant effect on determining the efficiency of surgical implant operation [1]. Increasing research has focused on surface modification of the most wildly used Ti-based orthopedic materials [2,3]. Among which, our group has paid considerable attention on the surface loading of inorganic additives such as Mn [4], Fe [5], Cu[6], Zn [7,8], Ag [9], or combined dual elements [10,11] on Ti to promote the osteogenic ability and better compromise the cytotoxicity and antibacterial activities of the additives using various techniques. One suggestion in common from these researches is that the biological response to material depends more or less on the concentration, existence form, and stability of the additives.

Particularly, the coatings with reasonable incorporation of Mn that synthesized through Micro Arc Oxidation (MAO) technique on Ti showed good biological performance overall. Whereas adverse rat bone marrow mesenchymal stem cells (rBMMSC) proliferation rate and ALP expression were still observed from the surface containing relatively high dosage of Mn [4]. In addition, there are plenty researches verified that biomaterials overdosed by Mn could be detrimental to cells. Luthen et al. [12] revealed obvious cytotoxicity on human osteoblast of high dosage $MnCl_2$-containing culture medium, while no obvious adverse effect can be detected from the relatively lower one. Park et al. [13] investigated Mn-induced cytotoxicity on nanostructured Mn-incorporated Ti oxide layer with 1.65 mg/L Mn ions released within first 24 h. Stredrick et al. [14] demonstrated that the Mn concentrations resulted in a half viability (EC_{50}) of CATH.a cells and SK-N-SH cells are 3.3 mg/L and 11.0 mg/L respectively.

There was a case compared the biological behaviors of bulk-incorporated Zn-containing Ti synthesized by MAO technique and subsurface-incorporated Zn-containing Ti obtained via PIII&D method. Up-regulated *in vitro* osteogenic-related gene expressions and improved *in vivo* bone growth stimulation were detected from the subsurface-implanted Zn-containing Ti compared with the other [15]. It enlightens us on implanting Mn onto the very subsurface of Ti using PIII&D to stabilize the additive and further reduce the ion release ratio, thus to yield better biological benefit of manganese without introducing any Mn-induced cytotoxicity.

PIII&D is a versatile technique, which is capable of modifying its surface characteristics without changing the bulk properties of substrates, and allows yielding suitable surface chemical composition through predetermining the metallic cathode and bias parameters [16]. Herein, we fabricated and characterized two groups of Mn-containing titanium using PIII&D technique. The *in vitro* antimi-

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Table 1
Important instrumental parameters used in Mn-PIII&D.

	Target	Cathodic arc
Pulsing frequency (Hz)	5	5
Voltage pulse duration (μs)	500	500 (Mn5); 800 (Mn8)
Implantation voltage (kV)	−30	
Implantation time (h)	1	
Pressure (Pa)	5×10^{-3}	

crobial behaviors against gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*), and osteogenic activities responded to rBMMSC of Mn-PIII&D samples compared with as-etched Ti, were investigated.

2. Materials and methods

2.1. Mn-PIII&D

Commercial pure Ti plates (Cp Ti, Grade 2) with dimensions of $10 \times 10 \times 1\,mm^3$, or $20 \times 20 \times 1\,mm^3$ were pretreated by etching against a mixed acid solution (HF: HNO_3: H_2O = 1: 5: 34) in sonicator for 10 min twice, followed by sonicating against ethanol and deionized water for several times, and then dried in room temperature for further use (as-etched Ti). Mn-containing modification layer were fabricated on as-etched Ti by PIII&D equipment (PIIIS-700, Southwest Institute of Nuclear Industry, China) with pure manganese cathode. To avoid burst transport during PIII&D process, recirculation cooling water system was applied to help the temperature around sample stage maintains to be 30–40 °C. In addition, a magnetic duct with a curved shape was inserted between the plasma source and main chamber in our instrument to remove macro-particles produced from the cathodic arc. The key processing parameters of Mn-PIII&D are listed in Table 1. The samples prepared with voltage pulse duration of 500 μs were labeled as Mn5, and the samples prepared with that of 800 μs were labeled as Mn8.

2.2. Surface characterization and manganese release

The surface topographies of various samples were observed by field emission scanning electron microscopy (FESEM; Magellan-400, FEI, USA). The surface and interior layer elemental compositions, binding states and manganese depth profiles of various specimens were investigated by X-ray photoelectron spectrometry (XPS; Physical Electronics PHI 5802, USA) with a monochromatic Al Kα source. The manganese-containing samples were soaked in 5 ml of physiological saline solution at 37 °C without stirring for 2 weeks. At each time point, the leaching liquid was collected and the concentration of Mn ions released into solution was analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES).

2.3. rBMMSC responses

2.3.1. Cell culture

The cytocompatibility of various samples to rBMMSC (Stem Cell Bank, Chinese Academy of Sciences, China) was determined by investigating the cell attachment, proliferation, alkaline phosphate (ALP) activity, collagen secretion and osteogenic-related gene expressions. In a humidified incubator (STIK, China) that maintains constant temperature at 37 °C and supplied with 5% CO_2, cells were cultured in a petri dish (Thermo Scientific, USA) containing 8 ml of α-minimum essential medium (α-MEM; Gibco, Invitrogen, Inc, USA), which was supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Inc, USA) and 1% penicillin-streptomycin (Gibco, Invitrogen, Inc, USA). The culture medium was refreshed every 3 days. *In vitro* experiments were carried out between passages 3 and 6 of rBMMSC, and quantitative assays were conducted in triplicate.

2.3.2. Cell attachment and viability

The rBMMSC were seeded on various samples in 24-well plates at a density of 2×10^4 cells per well supplemented with 1 ml culture medium to observe the spreading status of rBMMSC on various samples at different time point. After incubated for 1, 4, and 7 days respectively, samples attached with cells were fixed with 2.5% glutaraldehyde solution (Gibco, Invitrogen, USA) for 30 min, followed by dehydrated against a series of ethanol solutions (30, 50, 75, 90, 95, and 100% (v/v)) for 10 min each. After the final dehydration immersed in absolute ethanol twice, samples were primarily dried with a series of hexamethyldisilizane (HMDS) solution solved in ethanol (33, 50, 67, and 100% (v/v)). The samples were observed via SEM (S-3400, Hitachi, Japan) after finally being air-dried.

Similarly, the rBMMSC were seeded on various samples with four replicates for each group in 24-well plates at a density of 2×10^4 cells per well and supplemented with 1 ml of culture medium to observe the cell proliferation and viability according to alamarBlue (AbD Serotec Ltd, UK) assay. After being incubated for 1, 4, and 7 days, samples attached with cells were transferred to a new 24-well plate and 1 ml of phenol red free fresh medium supplemented with 5% alamarBlue was added to each well. After cultured for another 4 h, 100 μl supernatant of culture medium from each well was transferred to a 96-well plate to measure the absorbance at 570 nm and 600 nm by a plate reader (MK3, Thermo Scientific, USA). Then the cell proliferation ratio was calculated following the protocol of the alamarBlue assay.

2.3.3. ALP activity

The ALP activity of rBMMSC seeded on Mn8 and as-etched Ti were measured. After being cultured on various specimens with size of $10 \times 10 \times 1\,mm^3$ for 7 and 14 days in 24-well plates at a density of 1×10^4 cells per well and supplemented with 1 ml culture medium, cells were transferred to a new 24-well plate with samples. The cells were dissociated from samples with lysis buffer for 60 min at 4 °C, and incubated with 100 μl of *p*-nitrophenyl phosphate (*p*NPP) and 20 μl of glycine buffer. The mixed solution was reacted for 30 min at 37 °C, and then quenched by adding 20 μl of 1 M NaOH. The ALP activity of rBMMSC was determined by measuring the absorbance at a wavelength of 405 nm, while the total protein content was determined using a Bio-Rad protein assay kit (Bio-Rad, USA) at a wavelength of 570 nm. The relative activity of ALP was normalized to the counterpart of total protein concentration.

2.3.4. Collagen secretion

The collagen secretion from the surface of Mn8 compared with as-etched Ti were measured on rBMMSC according to the methods reported in literature [17]. Briefly, after being cultured for 7 and 14 days respectively, samples attached with cells were washed twice against PBS, fixed with 4% paraformaldehyde for 20 min and stained for collagen secretion with 0.1 wt% sirius red that saturated in picric acid for 20 h. Afterwards, cells were washed against 0.1 M acetic acid for multiple times and images were taken under brightness field. To be quantitative, the stain on the specimens was dissolved in 500 ml of de-stain solution (0.2 M NaOH: methanol = 1:1) and then the absorbance of solution at 492 nm was measured on plate reader.

2.3.5. Real-time quantitative PCR (RT-qPCR) analysis

To investigate Mn-induced osteogenic activity on rBMMSC from gene level, osteogenic-related gene expressions including alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP-2),