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.

Keywords: manganese, titanium, PIII&D, antibacterial, osteogenesis

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 widely 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 MnCl₂-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. Stedrick 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* antimicrobial 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 $10 \times 10 \times 1 \text{ mm}^3$, or $20 \times 20 \times 1 \text{ mm}^3$ were pretreated by etching against a mixed acid solution ($\text{HF}:\text{HNO}_3:\text{H}_2\text{O}=1: 5: 34$) 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 $30\sim 40\text{ }^\circ\text{C}$. 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 $500\text{ }\mu\text{s}$ were labeled as Mn5, and the samples prepared with that of $800\text{ }\mu\text{s}$ 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 $K\alpha$ 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. rBMMS C responses

2.3.1 Cell culture

The cytocompatibility of various samples to rBMMS C (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% CO₂, 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 rBMMS C, and quantitative assays were conducted in triplicate.

2.3.2. Cell attachment and viability

The rBMMS C 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 rBMMS C 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 hexamethyldisilazane (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 μ l 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 $10 \times 10 \times 1 \text{ mm}^3$ 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 1M 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 570nm. 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 rBMSC from gene level, osteogenic-related gene expressions including alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP-2), osteopontin (OPN), and runt-related transcription factor 2 (Runx2) were analyzed on Mn8 and Ti with β -actin as the housekeeping gene for normalization and relatively quantified using RT-qPCR. The rBMSC at density of 1×10^5 cell/well were introduced on three identical specimens with dimension of $20 \times 20 \times 1 \text{ mm}^3$ in a petri dish for 7, and 14 days, respectively. At the end of each incubation point, samples attached with cells were washed twice against PBS and the total RNA was extracted by 1 ml of the TRIzol reagent (Invitrogen, USA). The complementary DNA (cDNA) was reverse -transcribed from 1 mg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit. Afterwards, RT-qPCR was executed on the Roche LightCycler480 system (Roche, Switzerland) using an SYBR Green I PCR Kit. The relative expressions for the target genes were normalized to that of the reference gene β -actin and the primers for RT-PCR are listed in Table 2.

2.4. Antibacterial ability

The antibacterial activity of sample that containing relative higher dose of Mn (Mn8) compared with as-etched Ti against gram-negative both *E. coli* and *P. aeruginosa* was investigated by statistical investigations, including bacterial counting method and alamarBlue assay, and further confirmed via SEM observation.

Specimens with size of $10 \times 10 \times 1 \text{ mm}^3$ were sterilized against 75% Ethanol for 4 times (30 min each), followed by air dried in hood. Then samples were transferred

to a new 24-well culture plate. A drop of 60 µl bacteria solution at a density of 10^7 cfu/ml was introduced on each sample. After incubated at 37 °C in dark for 24 h, samples were rinsed with physiological saline solution twice. Then each sample and attached bacteria solution was transferred into a tube containing 4 ml of physiological saline. The tubes were vigorously shaken for 60 s using a vortex mixer to dissociate bacteria from sample surfaces. Subsequently, the detached bacterial suspension was diluted in 10-fold step by step with sterile physiological saline. 100 µl of diluted bacterial solution was re-cultured on a standard Luria-Bertani (LB) agar gel culture medium at 37 °C in dark for another 16 h. Then the active bacterial colonies were counted according to the National Standard of China GB/T 4789.2 protocol and the antibacterial ratio was calculated as follows,

$$\frac{(A-B)}{A} \times 100\%$$

in which, A is the average number of bacterial colonies detached from as-etched Cp Ti (CFU/sample); B is the total number of bacterial colonies detached from Mn-containing sample (CFU/sample).

The samples for alamarBlue assay and SEM observation were obtained following the procedures described in section 2.3.2 on rBMMS; the bacterial concentration seeding for alamarBlue assay was 10^7 cfu/ml.

2.5. Statistical analysis

Statistically significant differences (P) between various groups were measured using one-way analysis of variance or Tukey's multiple comparison tests that executed

through GraphPad Prism 5.

3. Results and discussions

3.1. Surface characterization and Mn release

As shown in Fig. 1a-c, the surface morphologies of as-etched Ti and Mn-containing samples have no obvious difference; all showing gully-like structures that arose from the pretreatment of acid etching. In addition, it can be found that numerous nano-grains with diameters between 5~20 nm appear on the surface of as-etched Ti (Fig. 1d), which was reported as obtained from pickling process as well [6, 18]. While after Mn-PIII&D, the nano-grains disappeared and the surfaces of Mn-containing samples (Fig. 1e and 1f) became smooth compared with as-etched Ti. It possibly results from the high energy bombardment of the Mn plasma that generated from cathode arc and accelerated by the high negative bias during Mn-PIII&D [19].

Mn-PIII&D samples were analyzed by XPS to assess the chemical states and Mn elemental distributions along with depth profile. Fig. 2a-d show the Mn 2p spectra obtained from the surface and interior layer of Mn-PIII&D specimens. The Mn 2p doublet obtained from the surfaces of Mn5 and Mn8 both located at 653.4 eV and 641.2 eV (Fig. 2a and 2b), corresponding with the binding energies of Mn 2p_{1/2} and Mn 2p_{3/2} in Mn₂O₃ respectively [20, 21]. The Mn 2P spectrum that examined from 20 nm cleaned layer of Mn8 located around 649.5 eV and 638.5 eV, indicating that the interior layer of Mn8 consists only of pure metallic Mn [22]. In addition to the same

metallic Mn doublet compared with the interior spectrum of Mn 8 that located at 649.5 eV and 638.5 eV, characteristic peaks that located at 653.7 eV, 642.8 eV, and 641.2 eV, which all corresponded with the binding energies of Mn₂O₃ [21, 23, 24], were detected from that of Mn5.

As shown in Fig. 2e, the depth profiles between 15 and 100 nm of Mn element from Mn5 and Mn8 samples resemble Gaussian distribution, with the peak distribution of Mn all at 30-50 nm away from surface. The surface enrichment of Mn on Mn5 and Mn8 compared with those in interior layer about 15 nm deep arises from deposition during Mn-PIII&D process, indicating a Mn containing thin film was formed on the surfaces of both Mn5 and Mn8 [11]. The deposition depths were all about 15 nm. The surface and peak Mn concentration of Mn5 were 6.28 at% and 10.00 at%, respectively. In comparison, those of Mn8 were 13.43 at% and 17.00 at%, respectively.

To understand the role of Mn ion plays in controlling the biological effects on cells and bacteria, the ion release characteristics from each Mn-containing sample that soaked in physiological saline for 2 weeks were collected. As shown in Fig. 2f, small amount of Mn ions could be continuously detected from Mn-containing samples all the time. Specifically, the total amounts of Mn ions leached from Mn5 and Mn8 within first 24 h, which were 0.0073 mg/L (ppm) and 0.01 mg/L respectively, are much lower than those from the Mn-doped Ti synthesized by MAO technique in our previous study that caused adverse ALP activity and from the nanostructured Mn-doping Ti that caused obvious *in vitro* disadvantages on cell, which were 0.48

mg/L and 1.65 mg/L, respectively [4, 13].

3.2. *In vitro* cytocompatibility

Fig. 3 shows the cell attachment on each sample after being incubated for 4 days. Well-extended and homogenously distributed cell clusters with entangled filopodia and lamellipodia can be observed both from Mn-free and Mn-containing samples, indicating that no obvious cytotoxicity was introduced during Mn incorporation. To determine the cell viability and proliferation of rBMSC seeded on various samples culturing for different days, alamarBlue assay was applied and the result is shown in Fig. 4a. No statistical difference ($P > 0.05$) can be seen among the three groups of surfaces within 7 days, further confirming that Mn-P111&D samples introduced no cytotoxicity to rBMSC. In addition, the ALP activity of rBMSC cultured on as-etched Ti and Mn8 that contained relatively higher Mn content for 7 and 14 days showed no statistical differences either (Fig. 4b; $P > 0.05$). As a major marker that determines the quantity and quality of bones formed around the implant, collagen secretions of rBMSC that cultured on Mn8 compared with Ti were measured. Fig. 4c shows that the collagen deposition of rBMSC on Mn8 was statistically higher than that on Ti on day 14, while there was no detectable difference between these two groups on day 7. RT-qPCR analysis on osteogenesis-related gene expressions including ALP, BMP-2, OPN and Runx2 of rBMSC seeded on Ti and Mn8 for 7 and 14 days are displayed in Fig. 4d-g, respectively. Significantly up-regulated gene expressions were detected on most of the investigated markers within 14 days and

none showed any unfavorable effect.

In combination, Mn-PIII&D samples showed no adverse effect on the proliferation and osteogenic differentiation of rBMMSC. Instead, Mn8 that contained relatively higher Mn content and be able to release more Mn ion imparts excellent osteogenic activity with enhanced collagen secretion and osteogenesis-related gene expressions of rBMMSC. It is popularly accepted that manganese plays key role in osteogenesis and osteoinduction activities of cells through interfering carbohydrate metabolism and mucopolysaccharides synthesis processes in bone [25, 26]. In addition, numerous studies have verified that manganese deficiency *in vivo* could cause dysfunction of bone metabolism and bone growth [27, 28]. Therefore, we believe that the favorable effects on rBMMSC observed from Mn8 can be attributed to the presence of Mn that implanted in the supernatant of Ti and the subsequently gentle release of Mn ion, which was widely accepted to be capable of altering the local micro-environment around biomaterials and consequently contributes to *in vitro* or *in vivo* cell functions [29, 30].

3.3. Antibacterial activity

Mn ion or Mn oxide has been verified to be able to inhibit bacterial proliferation or adhesion especially against gram-negative species in certain circumstances [4, 31, 32]. Therefore, in the present work, the bacterial morphology observation, alamarBlue assay, and the bacteria counting methods were carried out on Mn8 and Ti against *E. coli* and *P. aeruginosa*, respectively. As presented in Fig. 5a and b, the viabilities of

both species assessed by alamarBlue on Mn8 were significantly lower than those on as-etched Ti, verifying that Mn induced unfavorable effects against the proliferation of *E. coli* and *P. aeruginosa*. To quantify the antibacterial efficacies, the attached bacteria were detached and re-cultivated on agar plates according to the bacterial counting method. As shown in Fig. 5e, the total amounts of both kinds of colonies that detached from Mn8 and re-attached on agar plate were less than those from as-etched Ti. The statistical results are presented in Fig. 5c and d, showing that the antibacterial ratios against *E. coli* and *P. aeruginosa* of Mn8 compared with Ti were $20\pm6\%$ and $\sim 58\pm4\%$, respectively. The morphologies of bacteria seeded on various surfaces were observed via SEM and the results are shown in Fig. 6. The total amounts of both bacterial species attached on Mn8 and Ti showed no obvious differences. Whereas, it was found with high magnification SEM images that the cell membranes of *E. coli* and *P. aeruginosa* seeded on Mn8 (insets in Fig. 6c and d) all showed shriveled and deformed status. In comparison with the relatively intact and rod-like bacterial membranes that spread on the surface of Ti (insets in Fig. 6a and b), it is further confirmed that Mn8 sample was at least cytostatic to *E. coli* and *P. aeruginosa*. As mentioned in section 3.1, the Mn ion release rate within first 24 h from Mn8 is only 0.01 mg/L, which is much less than the minimum concentration of 12.5 mg/L that caused antibacterial activity according to reference [31]. Therefore, Mn ion release itself in the present study seems like not sufficient to generate Mn-induced antimicrobial activity. One possible source that Mn8 reduced the viability of both gram-negative bacteria could attribute to the existence form of implanted Mn as

Mn₂O₃, which was reported possessing catalytic activity and being able to generate ROS groups that could inhibit the germination of bacteria [4, 33].

4. Conclusions

Mn was successfully implanted onto the supernatant of as-etched Ti substrate using PIII&D technique. Thus, a stable Mn release platform that contributed to the up-regulated biological performance Ti implants was created. On one hand, the Mn-PIII&D sample that contains relatively higher Mn content and possesses faster Mn release rate showed increased collagen deposition and significantly enhanced osteogenesis-related gene expressions, without introducing any detrimental effect to rBMMSC. On the other hand, Mn-PIII&D sample was able to decrease the viability of gram-negative *E. coli* and *P. aeruginosa*.

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Notes and references

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Tables

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

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

Table 2. Primer sequences used in RT-qPCR.

Gene (rat)	Primer sequences (F, forward; R, reverse; 5'-3')	Product size (bp)
β -actin	F: CACCCGCGAGTACAACCTTC R: CCCATACCCACCATCACACC	207
ALP	F: CGTCTCCATGGTGGATTATGCT R: CCCAGGCACAGTGGTCAAG	209
BMP-2	F: ATGGGTTTGTGGTGGAAAGTG R: TGTTTGTGGAGTGGATGTC	167
OPN	F: CAAGCGTGGAACACACAGCC R: GGCTTTGGAAGTCGCCTGACTG	165
Runx2	F: TCTTCCCAAAGCCAGAGCG R: TGCCATTCGAGGTGGTCG	154

Figure captions

Fig. 1. Surface morphologies of various samples under low (a~c) and high (d~f) magnification detected by FE-SEM.

Fig. 2. Mn 2p high-resolution spectrum obtained from the surface (a/b) and the interior in 20 nm depth (c/d) of various samples detected by XPS; Mn depth profiles of various samples detected by XPS (e); and Mn release characteristics soaked in 0.9 wt% NaCl solution (f). (error bars represent mean $\pm$ SD; n=3)

Fig. 3. SEM morphologies of rBMSC cultured on various samples for 4 days.

Fig. 4. Cell responses on Mn-PH&D and Ti samples: (a) alamarBlue reduction of rBMSC cultured for 1,4 and 7 days; (b) Relative ALP activity of rBMSC seeded on Mn8 and Ti for 7 and 14 days; (c) collagen secretion of rBMSC cultured for 7 and 14 days; osteogenesis-related gene expressions of rBMSC cultured for 7 and 14 days: (d) ALP, (e) BMP-2, (f) OPN, and (g) Runx2. (error bars represent mean $\pm$ SD; n=3; *P < 0.05, **P < 0.01, ***P < 0.001)

Fig. 5. Antibacterial activity of Mn-PH&D and Ti samples: alamarBlue reductions of *E. coli* (a) and *P. aeruginosa* (b) cultured for 24 h; percent reductions of bacteria re-cultivated on agar: *E. coli* (c) and *P. aeruginosa* (d) colonies were previously

dissociated from various samples; (e) re-cultivated *E. coli* and *P. aeruginosa* colonies on agar for 16 h: bacterial colonies were previously dissociated from Mn8 and Ti that cultured for 24 h in dark. (error bars represent mean $\pm$ SD; n=3; *P < 0.05, **P < 0.01, ***P < 0.001)

Fig. 6 SEM morphologies of *E. coli* (a/c) and *P. aeruginosa* (b/d) cultivated on Mn8 and Ti for 24 h in dark.

E. coli

P. aeruginosa

Ti

Mn8

rBMMSC proliferation

Ti

Mn5

Mn8

Ti **Mn5** **Mn8**

A stable Mn ion release platform was created on Ti by PIII&D technique, and enhanced osteogenic and antibacterial abilities were observed.