Biomaterials 35 (2014) 6882–6897

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Stimulation of bone growth following zinc incorporation into biomaterials

Yuqin Qiao [a,1], Wenjie Zhang [b,c,1], Peng Tian [a], Fanhao Meng [a], Hongqin Zhu [a], Xinquan Jiang [b,c,**], Xuanyong Liu [a,*], Paul K. Chu [d]

[a] State Key Laboratory of High Performance Ceramics and Superfine Microstructure, Shanghai Institute of Ceramics, Chinese Academy of Sciences, Shanghai 200050, PR China
[b] Department of Prosthodontics, College of Stomatology, Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, 639 Zhizaoju Road, Shanghai 200011, PR China
[c] Oral Bioengineering Lab, Shanghai Research Institute of Stomatology, Ninth People's Hospital Affiliated to Shanghai Jiao Tong University, School of Medicine, Shanghai Key Laboratory of Stomatology, 639 Zhizaoju Road, Shanghai 200011, PR China
[d] Department of Physics & Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, PR China

ARTICLE INFO

Article history:
Received 22 April 2014
Accepted 28 April 2014
Available online 23 May 2014

Keywords:
Zinc
Titanium
Plasma immersion ion implantation and deposition
Osteogenic activity
Osseointegration

ABSTRACT

Rapid development of zinc biology has broadened the applications of Zn-incorporated biomaterials to tissue engineering but also raised concerns about the long-term safety of released Zn^{2+} ions. Clinical success hinges on the amount of incorporated zinc and subsequent optimized release sufficient to stimulate osseointegration. In this study, zinc is incorporated into the sub-surface of TiO_2 coatings by plasma immersion ion implantation and deposition (PIII&D). The Zn-implanted coatings show significant improvement compared to the "bulk-doped" coatings prepared by plasma electrolyte oxidation in terms of osteogenesis *in vitro* and *in vivo*. Molecular and cellular osteogenic activities demonstrate that rBMSCs cultured on the Zn-implanted coatings have higher ALP activity and up-regulated osteogenic-related genes (OCN, Col-I, ALP, Runx2) compared to the bulk-doped Zn coatings and controls. *In vivo* osseointegration studies conducted for 12 weeks on the rat model show early-stage new bone formation and the bone contact ratio (12 week) on the Zn-implanted coating is larger. The ZnT1 and ZIP1 gene expression studies demonstrate that the Zn-implanted coatings can better stimulate bone growth with reduced Zn release than those doped with zinc throughout the coatings.

1. Introduction

In spite of rapid development in molecular biology and biotechnology, satisfactory osseointegration is still a major challenge for orthopedic and dental implants [1]. There have been increasing efforts to address this issue by fabricating new bioactive materials that can promote the osseointegration process [2,3] and incorporation of biological essential elements such as calcium, phosphorus, magnesium, and zinc into biomaterials has been observed to enhance bone formation and mineralization [4,5]. The effects are particular obvious for Zn-incorporated biomaterials including bone cements [6,7], bioglasses [8,9], ceramics [10,11], and coatings [12,13]. Zinc is an essential trace element involved in many metabolic and cellular signaling pathways and important to normal growth, immune functions, and neuro-behavioral development [14]. Bone growth retardation has been correlated with dietary zinc deficiency/deprivation based on animal and human subject studies [15–17] and zinc has been considered essential to skeletal development [18,19]. In addition, there is cellular and molecular evidence that zinc supplementation or incorporation into biomaterials can stimulate osteoblast differentiation by up-regulating the expression of bone marker genes such as alkaline phosphatase (ALP), collagen type I (Col-I), osteocalcin (OCN), and osteopontin (OPN) further promoting extracellular matrix mineralization *via* increased collagen secretion synthesis and calcium deposition for bone nodule formation [20,21].

* Corresponding author. State Key Laboratory of High Performance Ceramics and Superfine Microstructure, Shanghai Institute of Ceramics, Chinese Academy of Sciences, Shanghai 200050, PR China. Tel./fax: +86 21 52412409.
** Corresponding author. Department of Prosthodontics, College of Stomatology, Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, 639 Zhizaoju Road, Shanghai 200011, PR China.
E-mail addresses: xinquanjiang@aliyun.com (X. Jiang), xyliu@mail.sic.ac.cn (X. Liu).
[1] Both Wenjie Zhang and Yuqin Qiao are equal contribution to this work.

http://dx.doi.org/10.1016/j.biomaterials.2014.04.101
0142-9612/

Despite the established roles of zinc in bone metabolism, the feasibility of Zn-incorporated biomaterials in clinical applications relies on many factors especially safety issues associated with the zinc content and release kinetics. Uncontrolled fast release of Zn^{2+} can disrupt zinc homeostasis, alter the concentrations of other trace metals such as calcium, iron, and copper causing deficiency, and bind to low affinity sites leading to protein dysfunction [22–24]. It has been reported that the influence of Zn^{2+} on the osteogenic differentiation of mesenchymal stem cells (MSCs) is dose-dependent [25,26]. Therefore, it is crucial to determine the tolerable and safe upper intake level and much work has been done to investigate the optimal zinc contents in various biomaterials. For instance, Oh and colleagues have shown improved cellular mineralization of MSCs on 2% Zn-doped bioactive glass compared that with 5% Zn [27] and Li et al. have reported cytotoxicity to MSCs due to locally high concentrations of zinc on titanium nanotubes incorporated with zinc [28]. The dose-dependent effect of zinc has also been demonstrated on osteoblasts. For example, Valentina et al. have reported that insertion of zinc (≤5%, w/w) in Hench's 45S5 bioactive glasses can improve the bonding ability without cytotoxic effects to human MG-63 osteoblasts [29], while Saino et al. have demonstrated that 58S-Zn0.4 (0.4 wt% ZnO) exhibits better cytocompatibility and enhanced osteoblast differentiation for human osteosarcoma cell SAOS-2 compared to 58S, but increasing the zinc content to 2% decreases the proliferation rate [9]. As mentioned above, the optimal zinc contents appears to depend on many factors such as the physicochemical properties of materials, cell lines, and zinc release kinetics in different physiological surroundings thus hampering large-scale commercial applications. All in all, the ideal solution to ensure the safety of Zn-incorporated biomaterials is to maximize osteogenesis *in vitro* and *in vivo* while keeping the amount of Zn to a minimum. Although the mechanism underlying the toxicity caused by excessive zinc is still not well understood, there is evidence that the expression of zinc transporters is highly responsive to changes of the extracellular zinc levels and dietary zinc supplementation/deficiency [30,31]. Zinc transporters of the ZnT (cation diffusion facilitator, SLC30) family and ZIP (Zrt- or Irt-like protein, SLC39) family play critical roles in zinc homeostasis and have tissue-specific expression patterns [32]. Succinctly speaking, the ZnT family decreases cytoplasmic zinc by either transporting zinc out of the cells or sequestering zinc into the intracellular compartment, whereas the ZIP family functions in an opposite manner as a pathway for zinc influx through the plasma membrane or from vesicles [33,34]. The zinc transporter 1 (ZnT1), which is a ubiquitous zinc exporter predominantly located in the plasma membrane, is also expressed in bone marrow mesenchymal stem cells [35]. Recent studies demonstrate that cells exposed to high concentrations of zinc or rats fed with a dietary zinc supplement exhibit an up-regulated expression of ZnT1 to enhance zinc excretion [36,37]. Moreover, Tang et al. have shown that overexpression of ZIP1 in MSCs results in an increased gene expression of Runx2 which promotes osteoblast differentiation [38]. Hence, differential expression of ZnT1 and ZIP1 can be utilized to detect the status of zinc in rBMSCs [39].

Compared to the effects of dietary zinc supplements, the osteogenic response to Zn-incorporated biomaterials is more complex due to the interactions between cells/tissues and biomaterials [40,41]. In the latter case, it is possible that the osteogenic response triggered by Zn released into the cell–material interface is distinguishable from that released into the physiological environment but there has been no direct verification. In this respect, a technique that introduces Zn to only the surface and sub-surface in a controlled fashion should be more effective than wet chemical methods such as hydrothermal synthesis, sol–gel process, spray pyrolysis, and electrochemical technique like plasma electrolytic oxide because these techniques introduce a uniform concentration of Zn throughout the entire materials or coatings. The objective of this study is to investigate the osteogenic capability of different amounts of Zn incorporated into the surface by means of plasma electrolytic oxidation (PEO) and plasma immersion ion implantation and deposition (PIII&D). PEO is an efficient way to prepare ceramic coatings with a micrometer-scale porous structure, large thickness, high hardness, superior wear resistance, as well as good adhesion to the substrate [42–44]. In this method, different amounts of zinc can be incorporated by varying the composition of the electrolytes while the surface morphology and microstructure remain relatively unchanged [12]. PIII&D can be used to tailor the surface properties of a variety of materials including metals, ceramics, polymers, as well as biomedical implants and components with a complex shape while the favorable bulk attributes of the materials can be preserved [45,46]. The efficiency of these two zinc incorporation strategies is compared by examining the *in vitro* osteogenic response by the ALP activity assay, immunofluorescence staining of OCN, and osteogenic-related gene mRNA expression. Implants modified by these two techniques are inserted intramedullary into femurs of rats for 12 weeks to evaluate the bone response *in vivo* and the effects are compared to those on Ti and Zn-free PEO coatings as controls.

2. Materials and methods

2.1. Preparation of Zn-incorporated TiO_2 coatings

To extract sufficient nucleic acid and protein samples in the cellular and molecular experiments, commercial pure titanium plates (Cp Ti, TA1, purity > 99.85%) with dimensions of 20 mm × 20 mm × 1 mm were used to provide a large surface area for cell adhesion and proliferation. In the animal experiments, pure medical titanium rods (Grade 1) with an external diameter of 2.0 mm and length of 7.0 mm were employed.

Two strategies were employed to introduce zinc into the TiO_2 coatings (Fig. 1). PEO was conducted in electrolytes composed of 0.1 mol/L calcium acetate monohydrate (CA, $C_4H_6O_4Ca \cdot H_2O$), 0.05 mol/L glycerophosphate disodium salt pentahydrate (GP, $C_3H_7Na_2O_6P \cdot 5H_2O$) and various amounts of zinc acetate dihydrate (ZA, $Zn(CH_3COO)_2 \cdot 2H_2O$) to prepare Zn-free (Z0) and bulk-doped coatings (PEO-Zx) [12]. By adjusting the concentrations of zinc acetate dihydrate (0.02 and 0.06 mol/L) in the electrolytes, coatings designated as PEO-Z1 and PEO-Z2 were produced. In cathodic arc PIII&D, the cathode (10 mm in diameter made of 99.99% zinc) was used to implant Zn into the Z0 coating (Z0-PIII-Zn) at 15 kV, 450 μs pulse duration, and 6 Hz pulsing frequency for 2 h. During Zn PIII, the vacuum chamber pressure was 2.5×10^{-3} Pa and the sample stage was cooled by circulating water to keep the sample temperature at 25 °C.

2.2. Characterization of coatings

Scanning electron microscopy (S-3400N, Hitachi, Japan) was used to examine the surface morphology of the coatings and energy-dispersive X-ray spectrometry [EDS, equipped on the electron probe X-ray microanalysis system (EPMA, JAX-8100, Japan)] was performed to determine the elemental compositions and cross-sectional mappings of titanium (Ti), phosphorous (P), calcium (Ca), and zinc (Zn) in the Zn-free and Zn-incorporated TiO_2 coatings. X-ray photoelectron spectroscopy (XPS) was performed on the Physical Electronics PHI 5802 equipped with a monochromatic Al K_α source to determine the surface (~10 nm) elemental compositions and chemical states. To obtain the cross-sectioned samples, the specimens were ground with successive grades of SiC paper, followed by polishing to 1 μm diamond finish. All the specimens were coated with carbon to avoid charging.

The surface wettability of the coatings was assessed using a contact angle instrument (SL200B, Solon, China) according to the method published in the literature [47]. Since the sterilization process might affect the surface wettability, the contact angles before and after sterilization were measured.

2.3. Ion release from modified TiO_2 coatings

The release rates of Zn ions from the Zn-incorporated coatings to PBS, FBS-free and 10% FBS-containing DMEM were determined by ICP-AES (inductively-coupled plasma atomic emission spectrometry, Varian Liberty 150). The samples were placed in sterile microcentrifuge tubes (15 ml), rinsed with 10 ml of new medium, and incubated at 37 °C for 1, 4, 7 and 14 days, respectively. At the end of each incubation period, all the leachates were removed and replaced with fresh medium aliquots.