Stimulation of bone growth following zinc incorporation into biomaterials

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A B S T R A C T

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 Zn2+ 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 TiO2 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.

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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].

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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 4555 bioactive glasses can improve the bonding ability without cytotxic 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 and cellular environments 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 Irtr-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 intra-medullary 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 TiO₂ 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 TiO₂ coatings (Fig. 1). PEO was conducted in electrolytes composed of 0.1 mol/L calcium acetate monohydrate (CA, C₆H₅O₃Ca·H₂O, 0.05 mol/L glycerophosphate disodium salt pentahydrate (GP, C₆H₁₁Na₂O₇P·5H₂O) and various amounts of zinc acetate dihydrate (ZA, Zn(CH₃COO)₂·2H₂O) 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 ZO 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 × 10⁻⁷ Pa and the sample stage was cooled by circulating water to keep the sample temperature at 25°C. Zn-incorporated TiO₂ coatings were characterized by XPS, SEM, and EDX.

2.2. Characterization of coatings

Scanning electron microscopy (5–3400N, 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 TiO₂ coatings. X-ray photoelectron spectroscopy (XPS) was performed on the Physical Electronics PHI 5800 equipped with a monochromatic Al Kα 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 1μm 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. Zn release from modified TiO₂ 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.
2.4. Isolation and culturing of rat bone mesenchymal stromal cells

Primary cultures of BMSCs were isolated from 6-week-old male Fisher 344 rats at equivalent ages and approximate weights as described by Jiang et al. [48] and the procedures were approved by the Ninth People’s Hospital Animal Care and Use Committee (Shanghai, China). After isolation of the primary culture by frequent medium change, the rat BMSCs (rBMSCs) were expanded in basal medium composed of Dulbecco’s modified Eagle’s medium (DMEM, Gibco), 10% FBS (Hyclone, Australia origin), and 1% antibiotics (pencillin 100 U/ml, streptomycin 100 μg/ml), at 37 °C under 5% CO₂. The culture medium was changed every 3 days. The conventional surface markers used to identify the rBMSCs were CD90 and CD105 positive while CD34 and CD45 were negative.

2.5. Cell proliferation assay

AlamarBlue assay was performed to evaluate cell proliferation on the samples. Briefly, rBMSCs were seeded at a density of 1 × 10⁵/well. And after culturing for 1, 4, and 7 days, the cells were incubated in basal medium supplemented with 10% AlamarBlue (Invitrogen, USA) for 4 h at 37 °C. Post reaction medium aliquots (100 μl) were transferred to 96 well plates and the absorbance values at 570 nm and 600 nm were recorded. The relative cell reduction was calculated according to the manufacturer’s instruction.

2.6. ALP activity

The rBMSCs at passage 3 were seeded at a density of 1 × 10⁵/well on 6-well plates for different samples. The ALP activity was determined in rBMSCs cultured in the basal medium using a colorimetric endpoint assay. At day 4, 7, and 14, the total cellular protein prepared from the cells was lysed in the ALP lysis buffer (1% Igepal CA-630, 10 mM Tris-HCl and 1 mM MgCl₂, pH 7.5). The aliquot with the protein sample was dissolved in a glycine buffer (pH 10.5) and the reaction commenced by adding p-nitrophenyl phosphate disodium. After incubation at 37 °C for 30 min, the reaction was stopped by adding of 50 μl of 1 M NaOH to each well and production of p-nitrophenol from p-nitrophenyl phosphate disodium was measured at 405 nm on a microplate reader (Multiskan MK3, Thermo). The samples were analyzed in triplicates and referenced against the p-nitrophenol standard curve to determine the amount of released p-nitrophenol. The cellular ALP activity was normalized against the total protein concentration measured by a BCA protein assay kit (Pierce, Rockford, USA) and expressed as μM/μg protein.

2.7. Measurement of osteocalcin secretion

The secretion levels of osteocalcin were analyzed using immunofluorescence and Enzyme-linked immunosorbent assay (ELISA), respectively. For immunofluorescence, the cells on different samples were fixed with 4% paraformaldehyde, washed in PBS, and blocked/permealized (3% bovine serum albumin (BSA) and 0.1% triton X-100, in PBS) for 30 min. The samples were incubated with primary antibody anti-osteocalcin (1:800) for 1 h at room temperature. After washing in PBS, the samples were incubated in Alexa Fluor 488-conjugated secondary antibody (1:1000) for 1 h at room temperature. The specimens were mounted with Fluor Shield (Sigma–Aldrich) and images were acquired on an Olympus fluorescence microscope.

Quantification of the extracellular matrix amount of osteocalcin was performed according to the manufacturer’s instruction (Takara). Briefly, the samples were washed extensively with PBS and incubated with lysis buffer at 15 min on ice to generate a whole cell lysate. Lysates were then centrifuged at 10,000 rpm for 30 min and the supernatant was collected as total protein. The total protein concentration was evaluated by a BCA protein assay kit (Pierce, Rockford, USA). To obtain the calibration and standard curves, rat osteocalcin full length peptide was used. The captured proteins were detected with Peroxidase-labeled antibody and the substrate 3,3′,5,5′-tetramethylbenzidine solution. The reaction was stopped with sulfuric acid and measured at 450 nm on a microplate reader.

Table 1

<table>
<thead>
<tr>
<th>Gene (rat)</th>
<th>Primer sequences (F, forward; R, reverse)</th>
<th>Amplicon (bp)</th>
<th>Accession number</th>
</tr>
</thead>
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<tr>
<td>GAPDH</td>
<td>F: GGCAAGTTCAAGGCACAGT R: GCCAGTAGTCACCAAGCAGCAT</td>
<td>76 bp</td>
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<td>OCN</td>
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<td>172 bp</td>
<td>NM_013414.1</td>
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<tr>
<td>ALP</td>
<td>F: GCAGTCCATGTGTTATATCGT R: CCAAGACTGGCTGCAAG</td>
<td>209 bp</td>
<td>NM_013059.1</td>
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<tr>
<td>Runx2</td>
<td>F: TCTTCCAAGGCTGAGGCC R: TGCCATCTCCAGGTCG</td>
<td>154 bp</td>
<td>NM_053470.1</td>
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<tr>
<td>Col-I</td>
<td>F: TCTGACCAGAGATATGTGTTAC R: GAAGCAAGTTTCTCTCCAAAGG</td>
<td>198 bp</td>
<td>XM_213440</td>
</tr>
<tr>
<td>ZnT1</td>
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<td>269 bp</td>
<td>NM_022853.2</td>
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<tr>
<td>ZIP1</td>
<td>F: CGCTGGCTGTGTCAGGTCG</td>
<td>175 bp</td>
<td>NM_001134577.1</td>
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</table>
2.8. RNA extraction and real-time quantitative RT-PCR analysis

The total RNA was extracted using the Trizol reagent (Life Technologies) at days 1, 4, 7, and 14 according to the manufacturer's protocols. The RNA concentration and purity were determined spectrophotometrically by Lambda 750 UV/Vis/NIR spectrophotometry (Perkin Elmer) and the quality was controlled on a 1% agarose gel stained with SYBR. In the CDNA synthesis, 1 μg of total RNA was reversely transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) and oligo d(T) primers (Roche). The real-time polymerase chain reaction (RT-PCR) was used to quantify the gene expression of the osteogenic markers. In brief, the 10-fold diluted cDNA was used as the template in the 10-μl qRT-PCR reactions using the SYBR Green Maternix and Roche LightCycler 480 System (Roche). The level of each target gene was calculated by the ΔΔCt method and GAPDH gene expression was used as an endogenous control for normalization. Each sample was analyzed in triplicates and the reaction mixture without the cDNA was used as a negative control in each run. The PCR primer sequences used in this project are listed in Table 1.

2.9. Western blotting analyses of ZnT1

Rat BMSCs were lysed in the ice-cold RIPA buffer containing protease inhibitor cocktail (Pierce) and the protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, USA). The protein samples (20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF, Bio-rad, USA). After blocking in phosphate-buffered saline (PBS-T) (0.01M PBS/0.1% Tween-20) containing 5% nonfat dry milk in phosphate-buffered saline (PBS) for 1 h at room temperature, the membranes were incubated overnight with anti-ZnT1 (Santa Cruz Biotechnology, USA; 1:5000) and anti-GAPDH (Santa Cruz Biotechnology, USA; 1:10000) primary antibodies in the blocking buffer. Specific protein bands were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA; 1:5000) and developed using enhanced chemiluminescent cocktail (Pierce) and the protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, USA). The 10-fold diluted cDNA was used as the template in the 10-μl qRT-PCR reactions using the SYBR Green Maternix and Roche LightCycler 480 System (Roche). The level of each target gene was calculated by the ΔΔCt method and GAPDH gene expression was used as an endogenous control for normalization. Each sample was analyzed in triplicates and the reaction mixture without the cDNA was used as a negative control in each run. The PCR primer sequences used in this project are listed in Table 1.

2.10. In vivo osseointegration study

2.10.1. Surgical implantation

All the animal experiments were reviewed and approved by the Animal Ethical Committee at the Ninth People’s Hospital affiliated to Shanghai Jiaotong University, School of Medicine (Shanghai, China). A rat femoral model was used in this study and divided into 5 groups of 2 each based on the modification of the implants.

2.10.2. Sequential fluorescent labeling

Polychrome sequential fluorescent labeling was performed postoperatively to assess the time course of bone formation and mineralization as reported previously [30,51]. At 3, 6, and 9 weeks after operation, different fluorochromes were administered intraperitoneally at a sequence of 30 mg/kg alizarin red S (Sigma–Aldrich, USA), 25 mg/kg tetracycline hydrochloride (Sigma–Aldrich), and 20 mg/kg calcine (Sigma–Aldrich).

2.10.3. Histological analysis

The animals were sacrificed after 12 weeks of implantation by intraperitoneal injection of an overdose of pentobarbitone sodium. Immediately thereafter, the femurs containing the implants were fixed in neutral buffered formalin, dehydrated by ascending concentrations of ethanol, and finally embedded in poly-methylmethacrylate (PMMA). The embedded specimens were cut into 150 μm thick sections perpendicular to the bone using a saw microtome (Leica SP1600, Hamburg, Germany), ground, and polished to a final thickness of about 40 μm. The sections were observed for polychrome fluorescent labeling by confocal laser scanning microscopy (CLSM, Olympus). The excitation/emission wavelengths used to visualize the chelating fluorochromes were 543/600-640 nm (Alizarin Red S, red), 405/560-590 nm (tetracycline hydrochloride, yellow), and 488/500-550 nm (calcine, green) [52].

After fluorescence microscopy, the same sections were counter-stained with Van Gieson’s picrofuchsin and examined microscopically to visualize the mineralized bone tissue (red). The images were captured by a fluorescence microscope (Olympus IX 71, Olympus, Japan) and the panoramic images were acquired by Multiple Image Alignment (MIA), an advanced image capturing process enabled by cellSens Dimension software. Histogram analysis for evaluation of the percentage of bone to implant contact (BIC) was performed on 3 to 4 sections on each implant and all the areas of interest in this study were within the endosseous part of each.

**Fig. 2.** Effects of different zinc incorporation strategies on TiO2 coatings examined by SEM-EDS: (a) SEM top-view of surface microstructures, (b) EDS spectra of corresponding SEM images, and (c) elemental compositions in the interior detected by EDS maps: Ti, titanium; P, phosphorus; Ca, Calcium; Zn, zinc.
implant. The images were captured by a fluorescence microscope (Olympus IX 71, Olympus, Japan) and analyzed using Image-ProPlus software.

2.11. Statistical analysis

The data were expressed as means ± standard deviations (SD) from three independent experiments performed in triplicate. Statistical analysis was performed using the one-way analysis of variance (ANOVA) with Tukey–Kramer Multiple comparison post-test using GraphPad Instant Software (GraphPad Software, Inc., USA) with \( p < 0.05 \) being considered to be statistically significant.

3. Results

3.1. Zinc contents and distributions

Thorough physicochemical characterization of the two different types of Zn-incorporated TiO\(_2\) coatings is conducted to distinguish the contribution of zinc incorporation from other factors including the surface microstructure, roughness, surface wettability. Fig. 2 shows the SEM images of the Zn-free and Zn-incorporated TiO\(_2\) coatings. Zinc incorporation does not alter the main structural characteristics including the porous morphology, size (<5 \( \mu \)m), and homogeneous distribution of pores. The strong Zn signals in the EDS spectra of PEO-Z1 and PEO-Z2 indicate successful zinc incorporation by PEO. However, the absence of the zinc signal from the EDS spectrum of Z0-PIII-Zn does not mean that PIII&D fails. PIII&D introduces zinc into the top surface resulting in a shallow and nonuniform layer (~100 nm) beyond the measurable range by EDS [53]. According to the analytical depth (~1 – 2 \( \mu \)m), EDS only provides the relative elemental composition in the interior of the coatings (as summarized in Fig. 1). The zinc contents in the PEO-Zx coatings increase with increasing concentrations of zinc acetate in the electrolytes (PEO-Z1 < PEO-Z2). The cross-sectional EDS maps for the major elements (Ti, Ca, P and Zn) depicted in Fig. 3 clearly illustrate that the coatings have uniform distributions of Ca and P. That is, zinc is present throughout the entire PEO-Zx coatings but only exists in the near surface on the Z0-PIII-Zn coating (no contrast in the EDS map).

XPS is conducted to determine the surface chemical compositions (~10 nm) of the Zn-free and Zn-incorporated TiO\(_2\) coatings (Fig. 4). The Zn 2p peaks can be detected from all the Zn-incorporated TiO\(_2\) coatings (Fig. 4a). As shown by the high-resolution spectra of Zn of the Z0-PIII-Zn coating in Fig. 4b, the Zn 2p\(_{3/2}\) peak is at 1021.0 eV which is in good agreement with Zn\(^{2+}\) in ZnO. Our previous studies have also shown that zinc in the PEO-Zx coatings exists as ZnO [12]. To examine the zinc distribution in

![Fig. 3. Effects of different zinc incorporation strategies on elemental distributions throughout the coatings: cross-sectional mapping results.](image-url)
depth, the elemental depth profiles are obtained from ZO-PIII-Zn. As shown in Fig. 4c, Zn penetrates to a depth of nearly 50 nm which is much smaller than the thickness of the coating on PEO-Zx produced by PEO. XPS combined with EDS confirms the distribution of zinc in the PEO-Zx coatings and superficial distribution of zinc in ZO-PIII-Zn. Additionally, the chemical composition of the coatings calculated from the XPS spectra show that ZO-PIII-Zn prepared by PIII&D has larger Zn atomic ratio than the PEO-Zx coatings (Fig. 4d) on the surface.

3.2. Surface wettability

The relative surface wettability of the coatings is determined according to the water contact angles. Zinc incorporation by PEO does not alter the contact angles on PEO-Zx coatings appreciably compared to ZO, but PIII introduces larger changes, that is, increased contact angles from about 40° (ZO) to 120° (ZO-PIII-Zn) (Fig. 5). However, after sterilization by ethanol immersion, the contact angle on ZO-PIII-Zn drops to the same value as those of PEO-Zx and ZO which nearly remain constant (Fig. 5) suggesting that surface wettability makes negligible contribution to the difference in the osteogenic response on the PEO-Zx and ZO-PIII-Zn coatings.

3.3. Zn release kinetics

During our observation for 14 days, all the cumulative profiles (Fig. 6a and c–d) show similar release characteristics, that is, burst release followed by low level continuous release. Moreover, larger values from the PEO-Zx coatings than ZO-PIII-Zn are observed and the amounts of leached zinc increase with zinc contents in the PEO-Zx coatings (PEO-Z1 < PEO-Z2). The non-cumulative release (Fig. 6b) demonstrates that both PEO-Zx coatings give rise to smaller sustained release profiles with time while ZO-PIII-Zn shows nearly constant Zn release at each time point. To a certain extent, the Zn release profiles are direct reflections of the zinc contents.

**Fig. 4.** Effects of different zinc incorporation strategies on the chemical distributions in the superficial surfaces of coatings detected by XPS: (a) Typical survey scan of coatings, (b) high-resolution XPS spectra of Zn 2p, (c) depth profile of Zn in ZO-PIII-Zn, and (d) XPS chemical component analysis of all coatings.

**Fig. 5.** Effects of different zinc incorporation strategies and sterilization on the surface wettability: Contact angles (deg.) before sterilization and after sterilization.
Fig. 6. Zn ion release kinetics from the Zn-incorporated TiO₂ coatings: (a) Cumulative profiles in PBS; (b) non-cumulative profiles in PBS; (c) cumulative profiles in FBS-free DMEM medium and (d) cumulative profiles in 10% FBS-containing DMEM medium.
among coatings with the following order: Z0-PIII-Zn < PEO-Z1 < PEO-Z2. The trend is consistent with the aforementioned analysis about the zinc contents in the different coatings (Section 3.1).

3.4. Effects of zinc incorporation strategies on cell proliferation

AlamarBlue assay is performed after 1, 4, and 7 days to investigate cell proliferation on the samples (Fig. 7). Cell proliferation is significantly influenced by zinc incorporation (Z0 vs. PEO-Z1, \( p < 0.05 \)) and varies among the different Zn-incorporated coatings prepared by the two strategies (PEO-Z1 vs. PEO-Z2, ns; PEO-Z1 vs. Z0-PIII-Zn, \( p < 0.01 \)). However, the results do not correlate well with the Zn release rate as a result of the biggest proliferation rate and smallest Zn release from Z0-PIII-Zn among the Zn-incorporated coatings.

3.5. Effects of zinc incorporation strategies on ALP activity

As an early marker of MSC differentiation, ALP activity is measured at days 4, 7, and 14 to assess the osteogenic differentiation potential of rBMSCs cultured on the controls and Zn-incorporated coatings. The results are calculated by normalizing to protein contents. As shown in Fig. 8, rBMSCs cultured on Z0-PIII-Zn display significantly higher ALP activity throughout the observation period compared to both the PEO-Zx coatings and controls (pure Ti and Z0). With regard to the PEO-Zx coatings, higher ALP activity is observed from the rBMSCs cultured on PEO-Z1 than on PEO-Z2 at day 4. At later time points (days 7 and 14), the rBMSCs on PEO-Z1 exhibit comparable ALP activity as those on PEO-Z2. This observation shows that the ALP activity of rBMSCs on different coatings does not positively correlate with the Zn contents and the largest zinc amount in PEO-Z2 appears to be excessive giving rise to reduced ALP activity in the early stage (days 4).

3.6. Effects of zinc incorporation strategies on OCN protein expression

In addition to the ALP activity, expression of the osteogenic-related protein OCN is assessed from the rBMSCs cultured on the samples by immunofluorescence and ELISA assay, respectively. As shown in Fig. 9(a), the rBMSCs on all the samples elicit recognizable OCN-positive staining after 14 days, although the expression levels of rBMSCs on the controls (Ti and Z0) are weak. In comparison, OCN exhibits a higher expression on all the Zn-incorporated coatings and a significantly enhanced expression is observed from the rBMSCs on Z0-PIII-Zn relative to the PEO-Zx coatings. Using the ELISA assay (Fig. 9(b)), the level of OCN on Z0-PIII-Zn is found to be about 2.3, 1.7, and 1.5 times (\( p < 0.001 \), \( p < 0.01 \) and \( p < 0.5 \)) higher than those on the controls (Ti and Z0), PEO-Z1 and PEO-Z2, respectively.

3.7. Quantitative real-time PCR analysis

3.7.1. mRNA expression of osteogenic-related genes

To further investigate the influence on rBMSC differentiation on the molecular level, the expressions of osteogenic-related genes including the runt-related transcription factor 2 (Runx2), ALP, osteocalcin (OCN), and collagen I (Col-I) are quantified using real-time RT-PCR in the basal medium after culturing for 4, 7 and 14 days. Among these genes, ALP and Col-I are selected as early markers and osteocalcin as a later marker for osteoblastic differentiation. Runx2, a key bone-specific transcription factor playing an essential role in the commitment of pluripotent mesenchymal cells to the osteoblastic lineage is also examined. The expression pattern of the key osteogenic genes shows dramatic differences in the response to different Zn-incorporated TiO2 coatings at 4, 7 and 14 days (Fig. 10). In the early stage (day 4), no statistically significant mRNA variations are observed from different samples. At day 7, PEO-Z1 prepared by PEO with smaller zinc concentrations induce statistically higher expressions of ALP and Col-I but similar levels of OCN and Runx2 compared to the controls (Ti and Z0). A similar trend is observed from PEO-Z2 with a larger zinc concentration compared to the controls. Moreover, the expression levels of Col-I on the PEO-Zx coatings increase with zinc contents whereas those of ALP, OCN and Runx2 do not respond to changes in the zinc contents. At day 14, all these osteogenic-related genes display initial upregulation on PEO-Z1 with a smaller zinc concentration and then they are down-regulated on PEO-Z2 with a larger zinc concentration. Therefore, the amount of zinc in PEO-Z2 appears to be excessive and does not benefit osteogenic gene expression. In comparison, the transcription level of these four genes is significantly up-regulated on Z0-PIII-Zn compared to the controls and PEO-Zx at both days 7 and 14. Specifically, at day 7, the expression levels on Z0-PIII-Zn are about 1.5/1.4 times (ALP), 2.0/1.4 times (Col-I), 1.7/1.3 times (OCN), and 1.7/1.4 times (Runx2) higher than those on PEO-Z1 and PEO-Z2, respectively while at day 14, the expression levels on Z0-PIII-Zn are about 1.4/1.8 times (ALP), 1.2/1.5 times (Col-I), 1.6/2.3 times (OCN), and 1.3/1.6 times (Runx2) higher than those on PEO-Z1 and PEO-Z2, respectively. These results indicate the advantage of superficial zinc incorporation compared to a uniform coating.

3.7.2. Differential expressions of ZnT1 and ZIP1 genes

The response of ZnT1 gene expression to the various Zn-incorporated TiO2 coatings during proliferation and differentiation of rBMSCs is examined to assess the cellular zinc status (Fig. 11). The ZnT1 mRNA on Z0-PIII-Zn is similar to those on Ti and Z0 during the test period. However, the mRNA abundance of ZnT1 in rBMSCs on PEO-Zx coatings shows a significantly higher level than the controls (Ti and Z0) and Z0-PIII-Zn coating, suggesting that long-term exposure of rBMSCs to PEO-Zx coatings may result in direct and enhanced Zn\(^{2+}\) influx. In addition, the mRNA levels of ZIP1 in MSCs seeded on different Zn-incorporated TiO2 coatings are determined (Fig. 11). At the early time points (1 and 4 days), zinc incorporation obviously stimulates the gene expression of ZIP1 except Z0-PIII-Zn. However, over a longer period of cell differentiation (7 and 14 days), the mRNA expression of ZIP1 is significantly up-regulated on Z0-PIII-Zn but remains unchanged on PEO-Zx compared to the controls.
3.8. Western blotting analyses of ZnT1

As shown in Fig. 12, a general trend towards the enhanced expression of ZnT1 is observed from PEO-Z2 with a larger Zn amount compared to Z0 (Zn-free), Z0-PIII-Zn, and PEO-Z1 (with smaller Zn amount) coatings. This finding is consistent with mRNA expression levels of ZnT1 in response to different Zn-incorporated TiO₂ coatings.

3.9. Effects of different zinc contents on in vivo implant osseointegration

3.9.1. Sequential fluorescent labeling analysis

The new bone formation and mineralization process around the Zn-free or Zn-incorporated implants is monitored by three types of fluorochrome labeling at specific time intervals of 3, 6, and 9 weeks (Fig. 13). After 3 weeks, alizarin red is found to be deposited onto a broader area adjacent to the implants with zinc, especially that adjacent to Z0-PIII-Zn which exhibits the most intense and widely distributed red fluorescence. Furthermore, alizarin red is found to extend along the Z0-PIII-Zn surface indicating a larger bone formation rate than the controls and PEO-Zx coatings. The similar tendency is observed for tetracycline (yellow) which represents the bone formation process after 6 weeks. At a later time point (9 weeks), calcein (green) is incorporated into the bone closest to the implant coatings demonstrating similar patterns. According to the combined images collected at different time points, the newly formed bone spreads throughout the entire region from the implant surface towards existing bones and the most distinctive fluorescent lines are produced by labels deposited adjacent to Z0-PIII-Zn.
Fig. 10. Expression of osteogenic-related genes in MSCs on cp Ti, Z0, PEO-Z1, PEO-Z2 and Z0-III-Zn was measured by quantitative real-time RT-PCR. The results were normalized to GAPDH and expressed as fold increase relative to cp Ti values. *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significant statistical difference (p > 0.05).
Fig. 11. Effects of different zinc incorporation strategies on mRNA level of ZnT1 and ZIP1 determined by quantitative real-time RT-PCR. The results are normalized to GAPDH and expressed as fold increase relative to cp Ti values. *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significant statistical difference (p > 0.05).
PIII-Zn (Fig. 13). In general, sequential fluorescent labeling shows that Z0-PIII-Zn stimulates more new bone formation, especially at early time points.

3.9.2. Histological analyses

To evaluate the bone response on the implants and healing process, histological analysis is performed on the tissues/biomaterials interface to characterize the bone regenerating capacity of the different Zn-incorporated coatings. The implant sections stained with Van Gieson’s picrofuchsin are displayed in Fig. 14. Both the controls (Ti and Z0) and Zn-incorporated implants exhibit new bone induction within 12 weeks and the newly formed bones extend from the surface of the parent bone into the cavity. Histological images illustrate that the Ti implants are surrounded by a thin layer of fibrous connective tissue (yellow) with a thickness of 10–50 μm separating the bone tissues from the implant. For the TiO2-coated implants (Z0, PEO-Zx and Z0-PIII-Zn), microtomographic images display tight and direct bone bonding to the implants without intervening fibrous layers and the Zn-incorporated coatings are observed to stimulate the formation of larger amounts of mineralized and newly formed bones in the cortico-cancellous bone than the Zn-free coating (Z0). The bone-to-implant contacts (BIC) are measured and shown in Fig. 15. There is no difference in the response to larger zinc contents in the PEO-Zx coatings. The BIC index of Z0-PIII-Z120 is 66.16 ± 5.67%, that is significantly higher than those of PEO-Z2 (46.04 ± 11.80%, p < 0.01), PEO-Z1 (39.71 ± 7.49%, p < 0.01), Z0 (30.31 ± 5.29%,

![Fig. 12. The effect of different Zn-incorporated TiO2 coatings on the expression of ZnT1.](image)

![Fig. 13. Sequential polychrome labels observed for 9 weeks in rat models: alizarin red S (red, 3 weeks), tetracycline (yellow, 6 weeks) and calcein (green, 9 weeks). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)
p < 0.001), and Ti (21.23 ± 2.54%, p < 0.001). In comparison, Z0-PIII-Zn induces better bone-to-implant contact than the controls and PEO-Zx coatings.

To validate and extend the results derived from the histological sections, SEM and EDS are performed to examine the bone formation on the Z0-PIII-Zn implant in the bone marrow cavity. As shown in Fig. 16, newly formed bone characterized by intense distribution maps of calcium (green) and phosphorous (red) bond tightly to the implant without showing gaps or fibrous tissue interposition. The combined histological analysis confirms the markedly better bone regenerating capacity of Z0-PIII-Zn than the controls and PEO-Zx coatings.

4. Discussion

Zinc acts as a local regulator of bone cells and has a stimulatory effect on bone metabolism in vitro and in vivo [18]. Therefore, there is significant research to explore the use of zinc in biomaterials as a therapeutic agent to induce rapid bone healing and bone growth particularly pertaining to dentistry and orthopedics [54]. A major factor impeding widespread application of Zn-incorporated biomaterials is safety. Unlike zinc supplements in which Zn ions play a determining role in bone formation and can be carefully controlled, the situation on Zn-incorporated biomaterials is more complex. In this study, the effectiveness of two zinc incorporation strategies in
relation to osteogenesis of rBMScs and osseointegration of experimental defects in long bones of rats is assessed. Despite smaller Zn release at all defined time points, the rBMScs cultured on Z0-PIII-Zn show a higher proliferation rate, enhanced ALP activity, and more up-regulated bone-specific gene expressions (such as ALP, OCN, type I collagen and Runx2) than the controls (Ti and Z0) and PEO-Zx coatings. Furthermore, after inserting in the cortical and cortico-cancellous bone in an in vivo femur implantation model, Z0-PIII-Zn demonstrates superior osseointegration to the PEO-Zx coatings and controls as manifested by firm abutments between the implants and surrounding skeletal tissues. The histological analysis confirms that new bones are formed and fill the gaps between the implants and bones on the PEO coatings (Z0, PEO-Zx and Z0-PIII-Zn). The in vitro and in vivo results show that zinc incorporation (Z0-PIII-Zn) achieved by PIII&D delivers greater osteogenic potential than that produced by PEO. The degree of accelerated osteogenesis appears to be in contrast to the released Zn concentrations and it somewhat conflicts former belief that a larger Zn concentration within the safety limits generally results in better osteogenesis in a given system. Our results show clear differences between these two types of Zn-incorporated coatings.

The roles of surface properties such as surface topography, wettability, and chemistry in osteoblast differentiation, extracellular matrix production, local factor production, and stimulation of an osteogenic microenvironment have been recognized [55–57]. Therefore, characterization of the surface properties of different Zn-incorporated coatings is essential to elucidating osteogenesis. With regard to the PEO-Zx coatings, our previous studies have demonstrated that zinc can be incorporated into the entire TiO2 coating (about 5–10 μm) by adding zinc acetate into a Ca-P containing mixed electrolyte and the main features such as the surface micro-/nano-structure, surface roughness, and wettability are the same as those of the Zn-free TiO2 coating (Z0) [12]. In the case of the Z0-PIII-Zn layer, only the microstructure remains unchanged. After zinc plasma ion implantation, the surface wettability changes significantly from being more hydrophilic (Z0) to more hydrophobic (Fig. 3). It is useful to eliminate the variation in the surface wettability between Z0-PIII-Zn and PEO-Zx or Z0 by sterilization via ethanol immersion (Fig. 3) [58]. As discussed above, the coatings after zinc incorporation by either PEO or PIII&D show similar surface microstructures and wettability (after sterilization) as Z0. It offers a major advantage to compare the osteogenic responses of rBMScs to the different samples.

In the Zn-incorporated biomaterials, the action of zinc on osteogenesis is more complicated not only due to the complex zinc metabolism but also in influenced by differences in the cells–materials interface. It is noted that the difference in released Ca and P ions can be neglected compared to the large amounts of additives (204 ppm of Ca and 125 ppm of P) in the DMEM medium and nutrients in the bone marrow cavity. Moreover, the Zn-free TiO2 coating (Z0) which contains similar amounts of Ca and P with the Zn-incorporated TiO2 coatings is used as a negative control in all in vitro and therefore, the focus of discussion is the role of Zn2+. Considerable evidence has revealed that zinc transporters, in response to variations in extracellular Zn2+ availability in vitro or in vivo, can adjust the rate of zinc influx and efflux through differentially regulating the expressions [38,59]. It is likely that this expression represents a steady state that reflects the regulation outcome, which obviously influences the Zn-related biological processes. There is increasing evidence showing the correlation...
between ZnT1 upregulation and cytotoxicity caused by excess zinc as well as ZnT1 down-regulation and zinc deficiency [36,60]. The ability of ZnT1 to reflect zinc bioavailability under Zn-deprived and -excess conditions is shown and in this study, ZnT1 is utilized as a marker to detect whether Zn released from the TiO2 coatings is excessive. There is a trend towards up-regulated ZnT1 mRNA and protein expression when the cells are cultured on the PEO-Zx coatings with larger zinc concentrations and Zn release than the controls. The expression of ZnT1 is unchanged or slightly increased, but there is no statistical significance on Z0-PIII-Zn. Overall, the results indicate that superficial incorporation of zinc limits the release of Zn to an acceptable level.

Different from ZnT1, previous studies show that the ZIP1 mRNA level is not highly regulated by zinc supplements or restriction [61]. A recent study shows that overexpression of ZIP1 in MSCs can induce up-regulated expression of Runx2 which further promotes osteogenic differentiation [38]. As shown in Fig. 11, although the mRNA levels of PEO-Zx are significantly up-regulated at the earlier time points (1 and 4 days), they fall on the same level as the mRNA expression on the controls (Ti and Z0) during the cell differentiation period. In contrast, the mRNA level remains the same as the controls at 1 day, but increases on Z0-PIII-Zn at all later time points (4, 7 and 14 days). The different expression patterns of ZIP1 on the Zn-incorporated coatings can be correlated to the mRNA expression of osteogenic markers at 7 and 14 days.

Collectively, the Zn-dependent patterns of ZnT1 and ZIP1 reflect the different sensitivity of zinc transporters towards extracellular zinc availability. It may be explained by the close contact between the MSCs and porous TiO2 coatings in the isolated extracellular microenvironment, where dissolution of Zn raises the local ionic concentration in the biological fluid at the interface with the biomaterials. This locally high concentration of Zn on the surface may be an underlying factor as it can be uptaken more efficiently than a high concentration Zn\(^{2+}\) outside. It is therefore possible that both Zn release and presence on the surface are important. In comparison, it is believed that cells are more sensitive and efficient to uptake Zn ions located in the cells—biomaterials interface rather than Zn ions diffusing into the fluids. Despite the smaller Zn release from Z0-PIII-Zn compared to the PEO-Zx coatings, the presence of larger zinc concentrations on the surface produces superior biological responses both in vitro and in vivo.

5. Conclusion

A better understanding of the roles played by zinc in regulating bone formation is crucial to better design and fabrication of biomaterials in tissue engineering. Herein, a systematic investigation is performed on two different modes of Zn introduction, namely, PEO and PIII\&D, and detailed in vitro osteogenesis studies and in vivo osseointegration are conducted. The Zn-implanted coating (Z0-PIII-Zn), which possesses the smallest total zinc concentration, shows the best osteogenic characteristics and bone regenerating capacity. By monitoring the mRNA expressions of the zinc transporters, ZnT1 and ZIP1, zinc implantation more efficient. This comparative study suggests a practical strategy to efficiently minimize the incorporated zinc quantity while retaining the enhanced biological responses and safety plugging excessive zinc introduction.

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