Antibacterial activities and cytocompatibility of zinc-contained strontium phosphate coating on titanium

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Abstract

Periprosthetic joint infection (PJI) is one of the most pivotal issues accounting for clinical failure of titanium (Ti)-based implants. Therefore, it is of great significance to develop an antibacterial coating with high biocompatibility on the Ti implant surface. In this article, a novel zinc-doped strontium phosphate coating, denoted as Sr-Zn-PCC, was deposited on Ti substrates by phosphate chemical conversion (PCC) method. The texture characterizations indicated that continuous coatings containing SrHPO\textsubscript{4}, Sr\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} and SrZn\textsubscript{2}(PO\textsubscript{4})\textsubscript{2} phases were formed on the Ti surface. It is found that the doping of Zn could tune the surface microstructure, roughness, wettability and corrosion resistance of the coatings. The Sr-Zn-PCC coating showed the same bacterial adhesion property at short time (2 h), but tended to hinder biofilm formation on coatings after incubation for up to 24 h. Up to 80% of Staphylococcus aureus (S. aureus) were killed within 24 h by contact with the Sr-Zn-PCC surface. Moreover, cytocompatibility assay indicated that MC-3T3 cells had good adhesion, spreading properties on the coatings, suggesting that it is a promising alloy with both excellent antibacterial ability and high biocompatibility for the applications on orthopedic implants.

1. Introduction

Titanium (Ti) and Ti-based alloys are preferred in total joint arthroplasty (TJA) ascribing to their distinguished mechanical properties and improved biocompatibility [1–3]. Despite that, periprosthetic joint infection (PJI) was an inevitable risk of complications and etiologies of implant failure after total joint arthroplasty (TJA) using Ti, even though many advances in preventive techniques [4–6]. Staphylococcus aureus (S. aureus) is the most common pathogen isolated from PJI, where up to 50% of cases are caused by hard-to-treat methicillin resistant S. aureus (MRSA) strains [7–9]. Since S. aureus has multiple mechanisms to promote immune evasion, including biofilm formation, antibiotic resistance and the ability to persist in necrotic bone, it has made successful treatment of PJI more challenging and overall less successful after clean surgery [10–12]. Recently, various strategies were devoted to the development of new antimicrobial substances and applications that efficiently prevent the bacterial colonization and improve the infection resistance of biomaterials. For implant materials, a functional coating with a special micro-nano structure is usually constructed on the surface to give the implant the desired antibacterial ability [13–16]. Another strategy is to load antimicrobial substances directly on the surface of biomaterials, including quaternary ammonium ions, litsea cubeba essential oil, diatomaceous earth
and nitric oxide that have shown significant antibacterial activity against a wide variety of bacteria [17–21]. Surface modification is an effective way to improve the antibacterial property of Ti implants [2]. For medical applications, the metals widely applied in coatings are silver (Ag), copper (Cu) and zinc (Zn) that have strong antimicrobial properties [22–25]. Ag and Cu were found to result in much better antibacterial activity, but the associated mechanism could result in cytotoxicity. It is well known that Zn is an essential mineral component for hundreds of biological enzymes and transcription factors [26]. Besides, as a nutritionally required metal, Zn is easily handled and excreted by the human body. Moreover, it has been reported that Zn exhibits an antibacterial effect on Gram-positive and Gram-negative bacterial strains and possesses minimal toxicity to host cells [27–29].

In the recent research, a variety of physical or chemical methods have been applied to modify the surface of implants [30]. Among them, the phosphate chemical conversion (PCC) method stands out as suitable choices for surface modification due to its dual controllable preparations in terms of morphology and composition [31]. Nowadays, the PCC method has been widely used in the surface modification of common biomedical metal materials such as magnesium, titanium and zinc alloys [32–34]. Some biofunctional metal cations such as calcium (Ca), magnesium (Mg), zinc (Zn), strontium (Sr), etc were used as PCC coating materials [28, 35]. Sr is an essential trace element in human body that could stimulate bone formation and inhibit bone resorption [36]. The modification of Ti surface via simultaneous introduction of Sr and Zn could offer a novel strategy to enhance antibacterial performance and maintain biocompatibility for immediate clinical application. Quan-ming Zhao et al prepared zinc/srontium-doped titanium dioxide microporous coating on titanium surface by micro-arc oxidation method. Their results showed that the Zn/Sr coating could promote the proliferation and differentiation of osteoblasts, enhance bone integration in the rabbit femurs, and improve bacteriostatic and biological properties [37]. Chen XB et al prepared a Sr apatite coating on Mg substrate by chemical conversion method. Their results demonstrated that Sr apatite coating has good biocompatibility and can promote bone marrow mesenchymal stem cells (BMMSCs) proliferation and differentiation [34]. Hai-shan Shi et al prepared SrHPO4 microsphere particles using a homogeneous precipitation method, their results showed the β-SrHPO4 clusters exhibited significantly better affinity, enhanced proliferation and osteogenic differentiation of BMSCs [38]. Liu Bing et al used PCC method to prepare scholzite (CaZn3[(PO4)2·2H2O]) and hopeite (Zn3(PO4)2·4H2O) coatings on Ti surfaces that exhibited favorable bone formation ability and good antibacterial activity [39–41]. However, the preparation of strontium phosphate and strontium zinc phosphate coatings on Ti has not been studied.

Inspired by the above efforts, we firstly prepared Sr-PCC and Sr-Zn-PCC coating on Ti substrates by PCC method. The surface microstructure, phase and element composition, wettability, corrosion resistance were systematically characterized. We also focused on their antimicrobial properties and biocompatibility. S. aureus was used to test the antibacterial property of the experimental coatings since it is the main representative organism in PJI. The coating exhibited good antibacterial activity against S. aureus and prevented biofilm formation. Additionally, Sr-Zn-PCC coating showed good biocompatibility on MC3T3 osteoblast cells. Thus, the Sr-Zn-PCC coating presents a promising application for the development of antibacterial Ti-based orthopedic implants.

2. Materials and methods

2.1. Materials and coating processes

Commercially pure Ti (cp-Ti, grade 2, composition in wt.%: Fe: 0.12; C: 0.08; N:0.01; Ti: balance) rod was machined to disks with a dimension of 9.10 × 3 mm and used as substrates. The surfaces of all Ti disks were ground by a series of SiC papers with incremental coarseness up to #1000 to eliminate the cutting grooves, and subsequently rinsed in the ultrasonic cleaner. Before PCC treatment, the as-treated Ti disks were successively acid etched by 2 wt.% hydrofluoric acid (HF) for 15 s and surface activated by 3 g l⁻¹ colloidal titanium phosphate solution for 30 s. After that, the pretreated Ti disks were connected with pure iron (Fe) clips to form a galvanic coupling system to promote coating formation, and subsequently immersed in PCC solution with designed compositions (as listed in table 1) at 70 °C for 60 min. All chemical reagents including strontium chloride hexahydrate (SrCl₂·6H₂O, AR), sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O, AR), Zinc dihydrogen phosphate dihydrate (Zn(H₂PO₄)₂·2H₂O, AR) and Sodium nitrate (NaNO₃, AR) were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). The pH of PCC solution was adjusted to 3.0–3.25 using H₃PO₄ and the reaction process was conducted in an ultrasonic field produced by the ultrasonic cleaner with an output power of 240 W. Finally, the coated Ti disks were washed with deionized water and dried at 40 °C for further characterization. The bare Ti disks were used as control.
2.2. Coatings characterization

The surface morphologies and elements compositions of bare and coated Ti disks were observed by field emission scanning electron microscope (FE-SEM, SU-70, Hitachi, Japan) equipped with an energy dispersive spectrometer (EDS). The phase composition of the coatings was examined by a Rigaku D/max-γB x-ray diffractometer (XRD, Japan) using Cu-Kα radiation operated at 40 kV and 100 mA, with a scan rate of 4° min⁻¹ and a scan step of 0.02° from 10° to 80°. Atomic force microscopy (AFM, Dimension Icon, Veeco Instruments Inc., USA) was used to evaluate the surface three-dimensional (3D) topography and roughness of the bare and coated Ti disks, which was operated in the tapping mode in air. Images were recorded using a SiNi tip with a nominal spring constant of 42 Nm⁻¹ within a scan area of 10 × 10 μm. The measurement was repeated three times (n = 3), and the surface Average roughness (Sa), RMS roughness (Sq) and Peak-Valley roughness (Sz) values were calculated using the NanoScope Analysis software (version 1.5).

2.3. Wettability tests

To evaluate the wettability of the coatings, the sessile drop method was used to detect the contact angles by dropping a deionized water droplet on the bare and coated Ti disks surface at room temperature in the air with relative humidity of 36%–40%, operated by an automatic contact angle instrument (DSA100S, KRUSS, Germany). The measurement was repeated three times (n = 3) for stable value and the contact angle values were averaged.

2.4. Electrochemical measurements

The corrosion behavior characteristics of bare and coated Ti disks were investigated by electrochemical workstation (CHI660E, China) using a three-electrode setup that consisted of a saturated calomel electrode (SCE) as the reference electrode, a platinum sheet as counter electrode and the bare or coated Ti disks with 1 cm² exposed area as the working electrode. Electrochemical measurements including potentiodynamic polarization tests and electrochemical impedance spectroscopy (EIS) tests were operated in the simulated body fluid (SBF) at 37 ± 1 °C. The Tafel polarization curves were recorded at a constant voltage scan rate of 1 mV s⁻¹ in the potential range of ±250 mV to determine the corrosion potential (Ecorr) and the corrosion current density (Icorr). And EIS tests were conducted at an open circuit potential (OCP) after reaching a steady state up to 1800s, with a voltage perturbation amplitude of 5 mV and a frequency range from 100 kHz to 0.01 Hz. The EIS data were fitted with the Zview software. All the electrochemical measurements were repeated three times (n = 3) to ensure the reliability of the results.

2.5. Bacteria culturing

S. aureus was chosen as it is considered to be one of the main causes of infection in orthopedic and dental implants. S. aureus (ATCC25923) was routinely cultured in brain heart infusion (BHI) broth (Thermo Scientific) in a shaker at 220 rpm, 37 °C for 16 h and then diluted to OD600 = 0.30 with a spectrophotometer (Nano drop 2000). Prior to seeding, the Ti substrates were autoclaved at 120 °C for 15 min, and added to wells of a 24-well culture plate. To assay bacterial adhesion onto surfaces, 1.0 ml of the diluted bacterial culture was incubated with titanium substrates in 24-well plates for 2 h at 37 °C and then removed for visualization. To examine the effect of Zn on growth and biofilm formation, we cultured the bacteria for 24 h.

2.6. Antibacterial assessments

As described in the previous literature by Bailong Tao et al [42], after incubation, samples were lightly rinsed to remove any non-adherent bacteria, and vortexed in 5 ml of sterile PBS for 1 min to remove all bacteria from the samples. This bacterial solution was then diluted serially and plated on BHI agar plates. These plates were incubated overnight, and colonies were counted to calculate the Colony Forming Units (CFUs) of bacteria that had grown onto the samples. The adhesion and antibacterial rate were tested by the following formula.

\[
\text{Adhesion rate(%) =} \frac{N_{\text{sample}}}{N_{\text{control}}} \times 100\% 
\]
Antibacterial rate(%) = \frac{N_{control} - N_{sample}}{N_{control}} * 100% 

where \( N_{control} \) corresponds to the number of bacteria colonies with the bare Ti and \( N_{sample} \) corresponds to the number of bacteria colonies with the coating samples.

### 2.7. Bacterial morphology

Samples were taken out and fixed with 4% paraformaldehyde at 4 °C for 30 min. After that, they were washed three times for 10 min. All the samples were transferred into a new plate and dehydrated sequentially through a series of ethanol solutions (30%, 50%, 70%, 95% and 100%) for 10 min each, the dehydration process in 100% ethanol was repeated twice. Finally, the surfaces were air dried in a fume hood. After gold (Au) sputter coating, the adhesion and biofilm growth of the bacterial on the samples were observed by FE-SEM (SU-70, HITACHI, Japan) operated at 5 kV.

### 2.8. Confocal laser scanning microscope analysis

Ti substrates were removed from wells with sterile forceps and gently rinsed three times with Phosphate Buffered Saline (PBS, \( pH = 7.4 \)) to remove non-adherent bacteria. The bacteria were then stained with a solution of ViaQuant™ Viability/Cytotoxicity Kit for Bacteria Cells (GeneCopoeiaTM). In this study, 1.5 \( \mu l \) NucBeacon Green stock solution and 1.5 \( \mu l \) of propidium iodide stock solution were added to 1.0 ml of NaCl solution (0.85% w/v) to derive 1X dye mixture solution. After that, 50 \( \mu l \) of the mixture dye was gently added to each substrate and then they were incubated at room temperature in the dark for 15 min. Fluorescent bacteria were imaged using a confocal laser scanning microscope (CLSM 800, Leica, Germany) with 40 objectives. After incubation for 2 h and 24 h, the surface coverage of bacteria under each field of view was determined by calculating the surface area of live and dead bacteria cells with Image J.

### 2.9. Cell viability and proliferation assays

A live/dead assay was performed to investigate the cytotoxicity of different samples. MC-3T3 cells (5 \times 10^4 cells/ml) were seed on various samples surface and incubated for two days. Cells were then stained with LIVE/DEAD Cell Imaging Kit (488/570) (Invitrogen) as described by the manufacturer’s protocol and imaged on Keyence BZ-9000 to determine whether they were live (green) or dead (red). The proliferation rates of MC-3T3 cells grown on different samples were assessed using a cell counting kit-8 (CCK-8 kit, Dojindo Molecular Technologies). The MC-3T3 cells with three replicates were seeded into a 24-well plate at a density of 5 \times 10^4 cells ml⁻¹ and pre-incubated for 24 h to allow for complete adherence before conducting CCK-8 assay. At the scheduled time period (24 h and 48 h), 500 \( \mu l \) of DMEM containing 10% (v/v) CCK-8 was added. After incubating for 2 h, the optical density (OD) was measured at \( \lambda = 450 \) nm using a spectrophotometer (SPECTROstar Nano, BMG Labtech Inc.). All the CCK-8 values were normalized to the control, which represents 100% cell viability.

The cell morphologies grown on different samples were examined by staining with rhodamine–phalloidin labeling dye (Thermo Fisher Scientific). Trace amounts of cells cultured at 2 days were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Next, cell cytoskeleton was treated with rhodamine–phalloidin and cell nucleus were stained with Hoechst 33258 at ambient temperature. Both red and blue fluorescent images were taken with 40 × magnification using CLSM and then merged afterwards.

### 2.10. Statistical analysis

All results were shown as means ± standard deviation for at least three replicates. One-way ANOVA with post hoc contrasts by Student–Newman–Keuls test or Student’s t-test was used to test the statistical significance using the SPSS 16.0 Software Package (SPSS Inc., Chicago, IL, USA). Values of \( p < 0.05 \) were considered statistically significant.

### 3. Results and discussion

#### 3.1. Surface morphology

In order to investigate the details of the structure of the surface morphologies and microstructures of bare Ti and coated Ti after PCC treatment, FE-SEM was employed. As shown in figures 1 (a) and (d), bare Ti has a relatively smooth surface while presents typical parallel scratch groove morphologies that are generated in the pre-treatment process of sanding. After PCC treatment, the surface morphologies of Ti changed dramatically, forming a continuous, homogeneous and complete coating covering the whole surface of Ti disks in both PCC solutions. The Sr-PCC and Sr-Zn-PCC coatings have similarities in the shape of a single crystal, both of which are composed of bulk-shaped crystals, but differ in the grain size and crystal aggregation morphology on the
coating surface. The Sr-PCC coating is formed by micron-scale rectangular columnar crystals interwoven with each other, and the crystals grow close to vertically perpendicular to the surface of Ti substrate (figures 1(b) and (e)). The Sr-Zn-PCC coating is composed of square bulk crystals with different dimension sizes, and nanoscale crystallites are sporadically distributed on the surfaces of square bulk crystals, as shown in figures 1(c) and (f). In addition, the Sr-Zn-PCC coating crystals assembled tightly with no obvious gap between adjacent crystals, while the surface of Sr-PCC has a relatively loose aggregation crystalline structure.

3.2. Phase composition
As shown in figure 2, the EDS spectrum of the two PCC coatings and the relative elemental composition were determined by EDS. The main element components of Sr-PCC coating are O, P and Sr, by contrast, the main elements of Sr-Zn-PCC coating includes not only O, P and Sr, but also Zn, indicating that Zn element was successfully doped into the Sr-containing PCC coating. Ti element was not detected by EDS, indicating that Ti ion in the substrate was not participated in the coating formation process. On the other hand, it also indicates that both Sr-PCC and Sr-Zn-PCC coatings have large thickness values. Actually, the thickness of the two coatings is about 20–30 μm, and can be changed by adjusting some reaction parameters, such as reaction time, temperature or ultrasonic power, which will be researched in the future work. In addition, the Zn: Sr atomic ratio in Sr-Zn-PCC is close to 2: 1, which indicated that the Zn atoms were not simply doped into the strontium-containing phosphate, but formed zinc phosphate or strontium-zinc phosphate together with Sr.

As shown in figure 3, the XRD patterns of Sr-PCC and Sr-Zn-PCC coating reveal that the two kinds of coatings possess completely different phase compositions. Sr-PCC coating mainly consists of strontium hydrogen phosphate (SrHPO₄, JCPDS #70–1215), accompanied by a minor phase of strontium phosphate (Sr₃(PO₄)₂, JCPDS #85-0502) (figure 3(a)), while Sr-Zn-PCC coating mainly consists of strontium zinc phosphate (SrZn₂(PO₄)₂, JCPDS #50-0159) (figure 3(b)), which is consistent with the ratio of Sr:P and Sr:Zn of Sr-PCC and Sr-Zn-PCC coating as shown in the EDS results, respectively. In addition, the narrow and intense diffraction peaks indicate that various phosphates in both Sr-PCC and Sr-Zn-PCC coating have a high crystallinity.

Generally, the corrosion dissolution of metal substrate in acidic PCC solution is the first step to initiate chemical conversion reactions, such as treatment of Fe, Mg, Zn alloys [29, 43, 44]. Unlike the above alloys such as Fe, Mg, Zn, etc, Ti alloys are generally chemically inert in PCC solution, because the presence of a stable TiO₂ passivation film on the surface of Ti alloy can prevent corrosion dissolution of the surface in a short time. As a result, Ti ions cannot be released to participate in the formation of PCC coating. This characteristic makes it difficult to prepare PCC coating on the surface of Ti alloy [32]. However, after coupling pure Fe clips with Ti disks in this study, Ti/Fe galvanic coupling system can be considered as a whole to participate in chemical reactions. Therefore, the formation and phase composition of Sr-PCC and Sr-Zn-PCC coating can be explained by the following reaction equations (equations (1)–(7)). Since the corrosion potential difference between Ti and
Fe in acidic solution, the pure Fe clip can be seen as a coupling anode for corrosion dissolution in acidic PCC solution to provide initial power for phosphating reaction. On the other hand, Ti as a coupling cathode undergoes hydrogen evolution reactions on its surface (equations (1) and (2)) [45]. Besides, the mechanical vibration effect generated by the external ultrasonic field will accelerate the hydrogen overflow, leading to an increase in the pH value adjacent to Ti surface, consequently, the ionization balance of H$_2$PO$_4^-$ is broken and PO$_4^{3-}$ is generated, as shown in (equation (3)). Meanwhile, the transient high-temperature (up to 5000 K) and high-pressure (up to 2000 atm) generated by ultrasound will cause local oversaturation of the ion concentration near the Ti surface, resulting from the heterogeneous nucleation of SrHPO$_4$ and Sr$_3$(PO$_4$)$_2$ (equations (4) and (5)) [41]. Furthermore, as the main strontium-containing phosphate, SrHPO$_4$ is also susceptible to further reactions in aqueous solutions to generate Sr$_3$(PO$_4$)$_2$ (equation (6)) [46]. As for Sr-Zn-PCC coating, since a sufficient amount of Zn$^{2+}$ are contained in the PCC solution, Zn$^{2+}$ and Sr$^{2+}$ jointly participate in the reaction to generate a zinc strontium phosphate compound ((SrZn$_2$(PO$_4$)$_2$), as shown in (equation (7)).

Fe $\rightarrow$ Fe$^{2+}$ + 2e$^-$

(1)
3.3. Roughness and wettability

The surface topology features and roughness evolution of the Ti disks before and after PCC treatment were determined by AFM. The 3D images are presented in figure 4, and their corresponding roughness results that provide quantitative information about the height and depth of the coating surface structure are listed in table 2. The AFM 3D topographical images demonstrate micromorphology characteristics of bare and coated Ti samples (figures 4(a)–(c)) that are consist with the observation results of SEM (figure 1). Due to the existence of phosphate crystals, the roughness values of Sr-PCC and Sr-Zn-PCC coating surfaces are distinctly higher than that of bare Ti. In particular, there are obvious gaps between crystals on the surface of Sr-PCC coating, resulting in a relatively large roughness. While the addition of Zn changes the phase composition and orientation of the coating crystals, making the surface of Sr-Zn-PCC coating consists of dense tiled crystals, and the roughness is smaller than that of Sr-PCC coating.

As illustrated in figure 5(a), the digital photos of water droplet shapes on the surface of bare and coated Ti were obtained by testing the wettability according to the droplet method. The contact angle value of bare Ti is 105.0° ± 5.4°, indicating that the surface of Ti substrate after sanding has hydrophobicity and poor wettability. For the coated samples, the contact angle value of Sr-PCC coating decreases to 86.3° ± 4.9°, while the contact angle value for Sr-Zn-PCC coating is 66.8° ± 5.2°, indicating that the phosphate coating formed after PCC treatment, especially the Sr-Zn-PCC coating, can significantly improve the hydrophilicity of Ti substrate (figure 5(b)). Although bare-Ti has a relatively smooth surface state with the minimum roughness, its wettability is the worst. This is because on the premise of no specific micro-nano structure, the inherent thin oxide layer on its surface has a lower solid surface free energy, making the liquid difficult to spread and exhibiting poor wettability [47]. As for the two PCC coatings, the crystals on the surface of Sr-PCC coating are relatively dispersed, and there are interspaces between the crystals with relatively high roughness. Such a surface state is not conducive to liquid spreading and wetting, making its wettability weaker than that of Sr-Zn-PCC coating with dense tiled crystals and lower roughness. For implants, a hydrophilic surface with excellent wettability is more conducive to the adhesion and growth of cells and proteins in the surrounding tissue environment, thus promoting bone integration [32]. But other works in the literature state that hydrophobicity is required to achieve antibacterial behavior [48, 49]. Importantly, a strong hydrophobic layer cannot be used for coating of

![Figure 4. Representative AFM images of bare and coated Ti samples. (a) bare Ti, (b) Sr-PCC coating, (c) Sr-Zn-PCC coating.](image-url)

![Table 2. Surface roughness characteristic values of bare and coated Ti samples. Values are showed as mean ± SD, n = 3.](table-url)
fixation surfaces of total joint arthroplasty because it could also prevent host bone osseointegration and lead to early mechanical failure [30]. The solution lies in a coating technology that retains required host cell interactions while selectively inhibiting bacterial adhesion. Sr-Zn-PCC coating could open new avenues for prevention of bacterial attachment while simultaneously enhancing bone osseointegration.

3.4. Corrosion characteristics

The internal of the living body is a chemically and physiological harsh environment for metallic implants [51]. Components such as water, chloride ions, and proteins contained in body fluids will have a continuous erosion effect on the implant surface [52]. Meanwhile, the biomechanics of the implants will also facilitate the corrosion [51]. Despite the presence of a protective passive film on the surface of Ti alloys with good corrosion resistance, the peel and dissolution of the passive film will also occur under the above-mentioned combined effects, resulting in weakening of the implant strength and releasing of harmful metal ions, which eventually makes a sharply increased risk of infection and failure around the Ti implants [53]. Therefore, excellent corrosion resistance is one of the key characteristics of the Ti implant surface coatings.

Figure 6 shows the potentiodynamic polarization curves of bare and coated Ti samples. The corresponding electrochemical parameters calculated by Tafer extrapolation method are listed in table 3. Generally speaking, the corrosion tendency is reflected by the corrosion potential ($E_{\text{corr}}$) from the perspective of thermodynamics while the corrosion current density ($I_{\text{corr}}$) reflects the corrosion rate from the perspective of dynamics. More positive $E_{\text{corr}}$ represents weaker corrosion tendency and lower $I_{\text{corr}}$ represents slower corrosion rate [54].
According to Table 3, the $E_{\text{corr}}$ values of bare Ti, Sr-PCC coating and Sr-Zn-PCC coating increase orderly, while the $I_{\text{corr}}$ values decrease orderly, indicating that the corrosion resistance of coated Ti is significantly better than that of bare Ti, and Sr-Zn-PCC coating has the best corrosion resistance.

In order to further analyze the electrochemical behavior of Sr-PCC and Sr-Zn-PCC coating, EIS tests were conducted on bare and coated Ti. The typical Nyquist plots of bare and coated Ti samples obtained after OCP stabilization in SBF are exhibited in figures 7(a) and (b). It can be seen that bare and coated Ti samples show different corrosion characteristics, where bare Ti has a typical incomplete single semicircular capacitive loop with one time constant, while coated Ti illustrates double semicircular capacitive loops with two-time constants. Meanwhile, the capacitance loop diameter of Sr-Zn-PCC coating is significantly larger than that of Sr-PCC coating and bare Ti, indicating that Sr-Zn-PCC coating has higher corrosion resistance, which is consistent with the results of polarization curves (figure 6). Correspondingly, EIS results of bare and coated Ti can be fitted with two different equivalent electrical circuits (EEC), as shown in figure 7(c). $R_c$ stands for electrolyte resistance, $R_{ct}$ for charge transfer resistance, and $Q_{dl}$ for double-layer capacitance. In addition, $R_c$ and $Q_{dl}$ in the EEC of coated Ti represent coating resistance and coating capacitance, respectively [55]. And $Q$ is the constant phase element (CPE) that can be defined by $Q = C_ω^{n-1}$, where $C$ is the pure capacitor, $ω$ is the angular frequency and $n$ is the experimental exponent of CPE representation varying between 0 and 1 ($n = 1$ for a pure-capacitor behavior) [56]. Table 4 lists the fitted parameters obtained from the EEC of bare and coated Ti. From data of Table 4, PCC coatings can increase the impedance resistance of Ti substrate owning to their higher $R_c$ values, and Sr-Zn-PCC coating has superior corrosion resistance for its higher $R_c$ value compared with Sr-PCC coating.

The PCC coating composed of metallic phosphates can be seen as an electrical insulator, and the whole Ti substrate surface is covered by PCC coating in this research, which means that corrosion only occurs on the surface of PCC coating during electrochemical testing [41]. Therefore, the coated Ti samples reveal excellent corrosion resistance. Comparing the two coatings, the dense surface structure with lower porosity of the Sr-Zn-PCC coating makes it serve as a better protective barrier.

### Table 3. The electrochemical parameters calculated from polarization curves by Tafel extrapolation method of bare and coated Ti samples. Values are showed as mean ± SD, n = 3.

| Sample       | $E_{\text{corr}}$ (V) | $I_{\text{corr}}$ ($\times 10^{-9}$, A cm$^{-2}$) | $\beta_q$ (V/decade) | $\beta_i$ (V/decade) | $R_p$ (kΩ·cm$^2$) |
|--------------|-----------------------|-----------------------------------------------|---------------------|---------------------|-------------------|
| Bare Ti      | $-0.46 \pm 0.03$      | $33.0 \pm 4.6$                               | $3.7 \pm 0.1$       | $5.9 \pm 0.1$       | $3.0 \pm 0.5$     |
| Sr-PCC       | $-0.22 \pm 0.04$      | $1.2 \pm 0.3$                                | $3.7 \pm 0.6$       | $6.6 \pm 0.4$       | $85.5 \pm 12.1$   |
| Sr-Zn-PCC    | $-0.16 \pm 0.04$      | $0.24 \pm 0.05$                              | $4.8 \pm 0.1$       | $5.1 \pm 0.3$       | $453.4 \pm 27.1$  |

**3.5. Antibacterial activity**

Bacterial attachment to surfaces is an early stage in biofilm formation. It is assessed that the adherence of *S. aureus* to the different surfaces after 2 h incubation by performing live/dead visualization with CLSM in which live bacterial cells were stained green with NucBeacon Green and dead cells were stained red with propidium iodide. The total attachment (of live and dead cells) on the three surface types showed no significant difference as seen from figure 8(a). All three surfaces had a similar coverage of live bacteria in the field of view (figure 8(b)). Next we quantified the attached bacteria to the surface of the substrates (figures 8(c) and (d)). A conventional detection and enumeration method for viable bacteria is to detach them from the samples and count CFUs on BHI solid culture medium. No statistically significant difference was detected between these three groups. Results are in line with those observed on live/dead visualization.

To visualize the number and morphology of the adhered bacteria surfaces, SEM observation was performed and the results are depicted in figures 8(e) and (f). It was found that the number of bacteria on the Zn coating surface exhibited no significant difference when compared with that of bare Ti and Sr-PCC groups and the bacteria maintained their perfect sphere-shaped after incubation for 2 h. This result indicated that the differences in surface microstructure of bare and coated Ti did not have statistically significant influence on the growth and survival state of *S. aureus* during short periods, which may be attributed to the scratches on the surface of bare Ti and the phosphate crystal constituent units of Sr-PCC and Sr-Zn-PCC coating are all on the micrometer scale. Furthermore, both Sr-PCC and Sr-Zn-PCC coating prepared in this study have high crystallinity and superior corrosion resistance, which means that the coating surfaces still maintain a relatively stable state in a short period of 2 h, and the amount of Zn$^{2+}$ released is so less that failing to exert significant bacteriostatic effect.

A later stage of biofilm development (24 h) exhibits resistance to destruction by the host immune system and antimicrobials, which is more challenging in clinical practice. To determine whether the Sr-Zn-PCC coating possessed antibacterial effect, *S. aureus* biofilm was grown on it for 24 h and then visualized using CLSM, CFU.
and SEM, respectively. Of the three different samples, the bare Ti and Sr-PCC surfaces had a similar coverage of live bacteria in the field of view (figures 9(a) and (b)). However, coverage of live bacteria for the Sr-Zn-PCC surface exhibited a sharp drop which was only one fifth of that on the bare Ti ascribed to the Zn$^{2+}$ released. Moreover, the coverage of dead bacteria increased on the Sr-Zn-PCC surface.

Next, the antibacterial activities were investigated using a plate-counting method (figures 9(c) and (d)). It could be observed that there was a large number of bacterial colonies on bare Ti and Sr-PCC group. While the amount of S. aureus colonies adhering onto the Sr–Zn-PCC substrates was relatively decreasing comparing with that of other samples. After the quantitative analysis, the results indicated that the Sr–Zn-PCC had a higher antibacterial activity with the antibacterial rates of around 80%.

Figure 7. (a) Nyquist plots of EIS spectra for the bare and coated Ti samples in SBF after a steady of OCP, (b) Enlarged image of the yellow rectangular area in (a) for a better view, and (c) Equivalent electrical circuits used to model the impedance behavior of bare and coated Ti samples.
Table 4. EIS fitted parameters of the equivalent electrical circuits for bare and coated Ti samples. Values are showed as mean ± SD, n = 3.

| Sample       | $R_s$ ($\Omega \cdot \text{cm}^2$) | $Q_{dl}$ ($\times 10^{-7} \Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{S}^n$) | $n_{dl}$ | $R_i$ ($\times 10^3 \Omega \cdot \text{cm}^2$) | $Q_s$ ($\times 10^{-7} \Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{S}^n$) | $n_c$ | $R_c$ ($\times 10^3 \Omega \cdot \text{cm}^2$) |
|--------------|-----------------------------------|-------------------------------------------------|---------|--------------------------------------------|--------------------------------------------|-------|--------------------------------------------|
| Bare Ti      | 16.7 ± 2.0                        | 4.6 ± 0.1                                       | 0.89 ± 0.01 | 5.6 ± 0.8                                  | —                                          | —     | —                                          |
| Sr-PCC       | 34.7 ± 3.7                        | 226.2 ± 17.7                                    | 0.67 ± 0.06 | 97.6 ± 10.3                                | 1.7 ± 0.2                                  | 0.80 ± 0.05 | 8.5 ± 0.5                                  |
| Sr-Zn-PCC    | 39.5 ± 1.8                        | 32.7 ± 4.3                                      | 0.75 ± 0.05 | 359.6 ± 12.8                               | 54.1 ± 6.3                                 | 0.74 ± 0.04 | 17.6 ± 1.8                                  |
To further analyze the antibacterial behavior, SEM was also used to investigate the number and shape of *S. aureus* (figures 9(e) and (f)). As expected, the bacteria on bare Ti and Sr-PCC substrates survived and grew over the whole surface. In contrast, Sr-Zn-PCC surface showed very few bacteria, the total biomass on the Sr-Zn-PCC surface was found to be only one-sixth than that on bare Ti and Sr-PCC surfaces indicating that Zn treatments retained antimicrobial properties (figure 9(f)). As shown in figure 9(e), *S. aureus* was sphere-shaped with smooth surface depicting the perfect healthy morphology on bare Ti and Sr-PCC surfaces. When exposure to Sr-Zn-PCC surfaces for 24 h, much of the attached *S. aureus* became partially shrunkled and some bacteria dissolved at the edges indicating cell death (red arrows).

### 3.6. Cytocompatibility

Sr, Zn are all essential metal elements for human daily metabolism. Previous studies had already shown that these alloying elements possess good biocompatibility [57, 58]. The cytotoxicity of the Sr-Zn-PCC arrays is qualitatively assayed using the LIVE/DEAD Cell Imaging Kit and the results are presented in figure 10(a). Almost no dead cell can be observed from the samples since the release of Zn$^{2+}$ display acceptable biocompatibility. Additionally, the average cells numbers on the Sr-Zn-PCC surface exhibit no significant difference when comparing to that of bare Ti and Sr-PCC group (figure 10(c)). We also tested the cell toxicity of Sr-Zn-PCC arrays using a CCK-8 kit (figure 10(d)). After 24 h and 48 h incubation, Zn alloys exhibited no obvious toxicity to MC3T3 cells and the relative viability of MC-3T3 cells remained above 90% consistent with the live/dead staining results. Our results indicate that Sr-Zn-PCC has good potential biocompatibility and can be considered a viable implant material.

After 2 days of cell culture, the morphologies of MC-3T3 were observed via CLSM (figure 10(b)). In order to identify all the subtle structural changes, we used trace amounts of cells that maintained a large space between the cells. The cells seeded onto different substrates exhibited normal morphology. Besides, their actin

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**Figure 8.** Adherence of *S. aureus* on different surfaces after incubation for 2 h. (a) Representative CLSM images with LIVE/DEAD staining of *S. aureus* on bare Ti, Sr-PCC, Sr-Zn-PCC. (b) Surface area covered by both live and dead bacteria in the field of view for each titanium surface. (c) Recultivation of *S. aureus* colonies previous dissociated from bare Ti, Sr-PCC and Sr-Zn-PCC coatings after 2 h of incubation. (d) Adhesion rate of *S. aureus* cultured with various substrates. (e) SEM images showing the distribution of bacteria on bare Ti, Sr-PCC and Sr-Zn-PCC surface (upper) and morphology of *S. aureus* after co-incubation with different samples (lower). (f) Quantitative measurements of the number of adhered bacteria on various samples after 2 h of incubation. Data are shown as the mean ± SD, n = 3. *Statistically significant difference (p < 0.05).*
cytoskeleton was well-organized. Moreover, when comparing to bare Ti substrates, the pseudopodia significantly increased on Sr-Zn-PCC and Sr-PCC groups. It has been proved that micro-nano dual scale structures exhibit rough surface characteristic and favourable wettability, which enhance hydrophilicity and cell adhesion \[59, 60\]. In this study, both Sr-PCC and Sr-Zn-PCC coatings consisted of micron-scale bulk-shaped crystals. Their rough surfaces formed by the interconnection of bulk crystals are conducive to the extension of the cell pseudopodia, because the protruding crystal edges provide more acting points for MC3T3 cell adhesion.

4. Conclusion

In summary, continuous and homogeneous Sr-PCC and Sr-Zn-PCC coatings were formed on the whole surface of Ti substrate by PCC method. SrHPO₄, Sr₃(PO₄)₂, and SrZn₂(PO₄)₂ were identified on the coatings. The doping of Zn could change the crystal structure of PCC coatings from rectangular columnar to square bulks and also lead to the compacted assembly of crystals with compared with the pristine Sr-PCC coating. It was found that the Sr-Zn-PCC coating could significantly improve the hydrophilicity of Ti alloys. Furthermore, PCC coatings improved the corrosion resistance of Ti, with Sr-Zn-PCC coating outperforming Sr-PCC coating and bare Ti substrate. In vitro tests revealed that Sr-Zn-PCC surface coating possess high antimicrobial abilities against S. aureus as well as satisfying cytocompatibility properties. Antibacterial activity assay revealed that the Sr-Zn-PCC coating had the same bacterial adhesion as Sr-PCC coating and bare Ti substrate at short time (2 h) but higher antibacterial activity up to 24 h incubation. Moreover, Sr-Zn-PCC provided good cells attachment and spreading due to the rough surface with good hydrophilicity. This work could lead to breakthroughs in the prevention of TJII and displays great clinical prospects for orthopedic applications.
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Authors’ contributions

Kangqing Zuo and Yixin Yin performed most of the experiments, collected and analyzed the data, and prepared the figures. Liping Yao, Kai Wang, Yuanyuan Yan, Bing Liu performed some of the experiments. Zongliang Ma performed AFM experiment. Yupeng Lu provided advice and revised the manuscript for intellectual content. Xiaoyan Li, Guiyong Xiao designed the experiments, interpreted the experimental results, and drafted the manuscript. All authors approved the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.
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