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The fabrication and *in vitro* properties of antibacterial polydopamine-LL-37-POPC coatings on micro-arc oxidized titanium

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ABSTRACT

Bacterial infection commonly occurs in clinical settings when the procedure involves a medical implant. Thus, the fabrication of antimicrobial medical materials has attracted much attention in recent years. To improve the antibacterial properties of titanium (Ti)-based biomedical materials, surface microporous structures, with antimicrobial peptide coatings, were employed in this study. Native Ti substrates were endowed with a certain level of antibacterial activity after treatment with the micro-arc oxidation (MAO). A multilayer consisting of polydopamine, cationic antimicrobial peptides LL-37, and phospholipid (POPC) was coated onto MAO substrates, leading to antibacterial activity against both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. The combination of polydopamine-LL-37-POPC was found to alleviate the burst release of LL-37 in the initial phase. This multilayer coated onto microporous Ti substrates also showed favorable cytocompatibility to both mesenchymal stem cells (MSCs) and osteoblasts. These findings illustrate a novel strategy for the development of antibacterial Ti-based implants.

1. Introduction

Surface engineering of titanium implants has gained attention in recent years for applications in prosthesis. The long-term use of these is often a challenge due to poor integration with the surrounding bone tissue and/or bacterial infection [1,2]. Implant infections resulting from perioperative and/or postoperative procedures, namely nosocomial infections, are principally caused by the colonization and proliferation of bacteria on the implant surface [3]. Contamination with bacteria concomitant with biofilm formation and surrounding tissue inflammation results in a high incidence rate of complications [4,5]. The development of antibacterial Ti-implants has made great strides in the past decades; however, no optimal solution has been obtained yet, mainly due to the discrepancy between effective antimicrobial activity and physiological environment coordination [6–9].

To improve the biocompatibility of Ti-implants, efforts are currently focused on the construction of micro-nanostructures, among which micro-arc oxidation (MAO) has proven to be a successful technique in facilitating cell adhesion and drug loading [10]. Principally, MAO deposits a micro-nano scale porous bioceramic layer on substrates by anodizing in an electrolyte with a high voltage plasma [11]. During the process of MAO, negatively charged particles are deposited on the surface of an anode material, depending on the electrolyte components. Recently, several electrolyte components and reaction parameters of MAO have been used to fabricate porous Ti-substrates with diverse compositions and surface structures, endowing them with distinctive properties such as cytocompatibility [12], immunomodulation [13], and corrosion resistant [14]. The MAO technique was applied here to address unmet clinical needs, including antibacterial activity.

As an effective approach for the fabrication of antibacterial materials, the use of antimicrobial surface coatings has achieved a certain level of success in pre-clinical studies [15]. The greatest advantage of this technique is the variety of possible antimicrobial agents and the feasibility of control release [16–22]. For instance, Taheri et al. [23] prepared a monolayer of 2-mercaptosuccinic acid to immobilize silver nanoparticles which showed striking antibacterial efficacy. Badar et al. [24] fabricated a layered double hydroxides (LDHs) coating loaded with ciprofloxacin to prolong the release of the antibiotic. However, regarding translational applications, both antibiotic and metal particles coatings have their own limitations for sustained use, specifically the fact that the rate of antibiotic-resistant bacteria far outpaces the development of alternative drugs [25]. Additionally, only a limited number of metal-based antimicrobials is able to meet market needs due to dose-related toxicity [26].

Cationic antimicrobial peptides (CAMPs) exist ubiquitously in almost all multicellular organisms and are a candidate for antimicrobial

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coatings [27–29]. CAMPs have broad-spectrum antibacterial activity and as a result could circumvent the issue of antibiotics resistance [30,31]. Moreover, with the bactericidal mechanism of CAMPs being gradually elucidated, employing CAMPs in the antimicrobial coating shows great advantages over other agents [32,33]. The sole human cathelicidin LL-37, one of the typical CAMPs, adopts several strategies to combat with both Gram-negative and Gram-positive bacteria [34,35]. These include disruption of the bacterial membrane by pore-forming [36], perturbing the bacteria-associated genes expression [37] and potentiating immune response [38]. In addition, LL-37 also behaves as a pleiotropic regulator orchestrating physiological activities, such as angiogenesis [39], immune modulation [40] and stem cells deployment [41]. Therefore, LL-37 would be a desirable antimicrobial agent because of its antibacterial essence and biocompatibility. Nevertheless, in the pericellular microenvironment, an inappropriate local concentration of LL-37 would attenuate its physiological functions [42]. Thus, it is essential to accurately control the release rate of LL-37 in the local environment, in order to ensure its effective biological functions.

Polydopamine, a common adhesive material, was proved to control drug release [43,44]. For example, Li et al. [45] deployed polydopamine to construct a hybrid coating composed of hydroxyapatite, Ag nanoparticles and chitosan on Ti substrates. However, it only functions by regulating the direct combined molecules, which still limits its accurate control of drug loading/release. To further pursue an alternative for release control, bioinspired phospholipid was employed in the present study. Phospholipid, one of the main ingredients of cytomembrane, has recently emerged as an outer coating to further manage sustained release of antimicrobials [46]; in that study, POPC was physically adsorbed onto the titania nanotubes to control the release of a functional peptides (HHC-36). Furthermore, Huong et al. [47] deployed polydopamine to improve the stability of POPC included liposomes and indicated that the improved stability could be attributed to the electrostatic interaction of polydopamine and phospholipid. This indicated that a multilayer consisted of polydopamine, LL-37, and that POPC could be constructed on titanium that could serve as an antibacterial coating. In addition, phospholipid coated on titanium was also proved to reduce bacteria adhesion [48] and support tissue integration [49]. Therefore, in this study, we set up an antibacterial system by preparing micro-arc oxidized titanium loaded with LL-37 and attempted to address the need of alleviating the burst release of LL-37 in the initial phase by the polydopamine-LL-37-POPC (palmitoyl-oleoyl phosphatidyl-choline) multilayer.

2. Materials and methods

2.1. Materials

Commercial bare titanium disks were provided by the Northwest Institute for Non-ferrous Metal Research (China). LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES, 95%) was synthesized by Top-peptide Co. (Shanghai, China). Fluorescein isothiocyanate (FITC) was provided by Solarbio Co. (Beijing, China). Hoechst 33258, 1-Palmitoyl-2-oleoyl-sn-*glycero*-3-phosphocholine (POPC), sodium hydroxide powder (97%), and 3-(4, 5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were supplied by Oriental Chemicals Co Ltd. (Chongqing, China).

2.2. MAO substrates preparation

Bare titanium disks were polished using sandpaper (400–2000 grit) and successively pre-cleaned by ultrasonication in acetone, ethanol, and distilled water for 15 min each. The cleaned bare titanium disks and a titanium foil acted as the anode and cathode, respectively (Fig. 1A). NaOH solution (10 g/L) was used as the electrolyte. The process was conducted under a pulsing power supply at 220 V for 5 min, while maintaining the electrolyte temperature steadily lower than 20 °C. After the MAO process, the samples obtained were washed with distilled water 6 times.

2.3. Construction and characterization of multilayer coatings

The prepared MAO substrates were immersed in 50 mL dopamine hydrochloride solution (2 mg/mL) containing 10 mM Tris buffer (pH 8.5) and then incubated overnight. Sequentially, the MAO substrates were rinsed with distilled water to remove any redundant polydopamine and then dried at 40 °C. According to the minimal inhibitory concentration (MIC) of LL-37 provided by a former study [35], the cleaned samples were then pipetted with 16 μM LL-37 solution and air dried. Next, POPC were dissolved in ethanol (2 mM) to coat the MAO substrates (50 μL per sample) and then dried at room temperature, denoted MAO-PD-L-P. The bare MAO substrates, MAO substrates coated with polydopamine, MAO substrates coated with polydopamine-LL-37, and MAO substrates coated with polydopamine-POPC were also prepared as control groups and denoted as MAO, MAO-PD, MAO-PD-L, and MAO-PD-P, respectively. The process of multilayer fabrication is shown in Fig. 1B.

The surface morphologies of all Ti-based samples were characterized using field emission scanning electron microscopy (FEI Nova 400 Nano SEM, Phillips Co, Holland) and atomic force microscopy (AFM; JPK Instruments AG, Germany-Nano Wizard II). The roughness of all samples was defined according to their root mean square roughness (Rq). The crystalline phases of different groups of samples were characterized by X-ray diffraction (D/Max 2500PC, Rigaku, Japan). The functional groups of the multilayer coatings were characterized by Fourier transform infrared spectroscopy (FTIR, model 6300, Bio-Rad Co. Ltd., USA) and X-ray photoelectron spectroscopy (XPS) (Axis Ultra, Kratos Analytical Ltd., England), respectively. The hydrophilicity/hydrophobicity of all samples was measured by contact angle tests (Model 200, Future Scientific, Taiwan, China).

2.4. Multilayer stability and LL-37 release profiles

The cumulative release amount of LL-37 from the MAO-PD-L and MAO-PD-L-P samples was measured using FITC labeling [50]. Briefly, 0.1 mg of FITC was dissolved in the LL-37 solution and stirred at 37 °C overnight. Then, the solution was purified by using dialysis tubes (1000 MWCO) and followed by lyophilization. The prepared LL-37-FITC powder was used to construct the targeted samples (MAO-PD-L and MAO-PD-L-P) as above. Then, three fluorescent samples of MAO-PD-L or MAO-PD-L-P were immersed into 1 mL PBS solution (pH 7.4) for different time intervals (0.5, 1.5, 2.5, 4.5, 24, 48, 72, 96, and 168 h) [46]. Thereafter, 500 μL of supernatant was collected to detect the LL-37 release and refreshed with equivolumetric new PBS solution. The cumulative release amount of FITC-labeled LL-37 was measured using an ultraviolet spectral photometer at 520 nm. Furthermore, soaked samples at time intervals of 0.5, 4.5, 24, 72, and 168 h were imaged with a Leica Upright Fluorescence Microscope (Wetzlar, Germany). Finally, the collected MAO-PD-L-P samples at 0.5, 4.5, 24, 72, and 168 h were further characterized by SEM to evaluate the POPC stability on the MAO-PD-L-P surface.

2.5. Bacterial morphology

As representatives of Gram-positive and Gram-negative bacteria, *Staphylococcus aureus* (ATCC29213) and *Escherichia coli* (ATCC25922) used in this study were supplied by the American Type Culture Collection. Both bacteria were cultured in Mueller–Hinton Broth (MHB) medium with shaking (150 rpm) at 37 °C. Then, 1 mL of *S. aureus* and *E. coli* (1×10^6 cells/mL) were seeded onto different samples and incubated at 37 °C for 6 h. The culture medium was then removed, and