Bioinspired anchoring AgNPs onto micro-nanoporous TiO₂ orthopedic coatings: Trap-killing of bacteria, surface-regulated osteoblast functions and host responses

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The therapeutic applications of silver nanoparticles (AgNPs) against biomedical device-associated infections (BAI), by local delivery, are encountered with risks of detachment, instability and nanotoxicity in physiological milieus. To firmly anchor AgNPs onto modified biomaterial surfaces through tight physicochemical interactions would potentially relieve these concerns. Herein, we present a strategy for hierarchical TiO₂/Ag coating, in an attempt to endow medical titanium (Ti) with anticorrosion and antibacterial properties whilst maintaining normal biological functions. In brief, by harnessing the adhesion and reactivity of bioinspired polydopamine, silver nanoparticles were easily immobilized onto peripheral surface and incorporated into interior cavity of a micro/nanoporous TiO₂ ceramic coating in situ grown from template Ti. The resulting coating protected the substrate well from corrosion and gave a sustained release of Ag⁺ up to 28 d. An interesting germicidal effect, termed "trap-killing", was observed against Staphylococcus aureus strain. The multiple osteoblast responses, i.e. adherence, spreading, proliferation, and differentiation, were retained normal or promoted, via a putative surface-initiated self-regulation mechanism. After subcutaneous implantation for a month, the coated specimens elicited minimal, comparable inflammatory responses relative to the control. Moreover, this simple and safe functionalization strategy manifested a good degree of flexibility towards three-dimensional sophisticated objects. Expectedly, it can become a prospective bench to bedside solution to current challenges facing orthopedics.

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1. Introduction

Titanium (Ti) is standard implant material used worldwide in orthopedics and dentistry for its unique characteristics in mechanics, biocompatibility, and cost. However, biomedical device-associated infections (BAI) remain major issue as it is responsible for delayed healing, implant failure and repeated surgeries [1–3]. The potential of employing AgNPs as constitutes of implant coatings in situ on Ti to control BAI locally, through either their release-killing or contact-killing capabilities has encouraged an emerging area worthy of serious consideration for the following reasons. First, despite its counterparts (e.g. antibiotics and antimicrobial peptides), nanosilver, dating from 120 years ago [4], remains a lively disinfectant of great interest for its good stability (long-lasting release), broad antibacterial spectrum, strong oligodynamic effect (especially against “super-bugs”), and low incidence of antibiotic resistance [5–8]. Second, immobilizing a bio-acceptable amount of AgNPs on implant surfaces or incorporating them into biomaterials is likely to relieve their toxicological concerns for mammalian cells and bodies [8–11]. The proposed cytotoxic mechanisms, whether described by cellular uptake, oxidative stress or rupture of cell membranes, are based in general on freeform particles in the solution. However, the cellular
responses to the superficial presence of nanosilver could be different due to the controlled extracellular liberation, restricted movability, and reduced internalization probability [7,12]. Third, by coating-enabled designing of surface topography and composition (with additional Ag) of Ti, one can anticipate, from the osteogenic point of view, not only depressed microbial attachment and biofilm colonization but also encouraged osteoblast functions and osseointegration [6]. This is especially crucial to the stable and functional biomaterial-tissue contact over the long term since an implant fails by looseness if the material lacks osteogenic activity.

More specifically, we highlight a kind of biomimetic hierarchical titania/silver (TiO$_2$/Ag) coatings for Ti implants where particulate silver is combined with the nano/micro-structured TiO$_2$. The latter can derive conveniently from Ti templates via single or combined solution reactions in acidic/alkaline electrolytes, such as anodic oxidation, acid etching and micro-arc oxidation (MAO) [5,6,13,14]. From the compositional aspect, TiO$_2$ is bioactive and biocompressible per se [15]; on the other hand, the resulting micro-/ultra-topographies can mimic the hierarchically defined environment of extracellular matrix (ECM) so as to biologically instruct the behaviors of osteoblasts [16]. Intriguingly, the coating structure (tubes, pores, etc.) can act simultaneously as reservoirs of extra silver to attain sustainable release of bactericidal Ag$^+$. It is envisioned that such simple TiO$_2$/Ag surfaces may be sufficient to selectively guide cell behaviors but inactive bacterial adherence [5,6,13,14].

In this study, we shall first spontaneously grow micro/nano-porous ceramic TiO$_2$ coatings on substrate Ti via a rapid one-step MAO reaction. By adjusting the electrical parameters and time, it is easy to acquire desirable dimensional configuration of pores and topology concurrently. These physical cues can be perceived simultaneously but disparately by osteoblasts (10$^5$ m$^{-2}$) and their behaviors [17]. In regard to the immobilization of AgNPs, a mussel inspired self-polymerized polydopamine (PD) anchor shall be employed. The formation of this nanolayered coating (<100 nm) from dopamine monomer at pH 8.5 is facile and substrate-independent [18,19]. Specifically, PD can be developed in situ onto TiO$_2$ via covalent cohesion with dissociation forces as high as 805 pN [20–22]. It is also capable of chelating noble ions like Ag$^+$, reducing them to particles and stabilizing them, a process involving metal coordination, electron and electrostatic interactions [23,24]. By this means, a rigid TiO$_2$/PD/Ag coating can be readily built on Ti. Besides, the system may have extra merits including enhanced corrosion and wear resistance conferred by MAO, reduced Ag$^+$ loss due to the metal-fixation ability of PD [25], and better bioactivity and cell adhesion from the catecholic moieties [19,26].

The aims of the present work were: (1) to develop and characterize the foregoing AgNP-modified micro/nano-porous TiO$_2$ coatings; (2) to assess the durability of Ag$^+$ released from the antimicrobial coating and its potency in preventing the attachment and colonization of *Staphylococcus aureus* (*S. aureus*), a causative strain for BAI, as well as its anticorrosion efficacy in Hank’s solution; (3) to investigate in vitro how osteoblast-like MG-63 cells react with the nanosilver-containing interfaces in terms of attachment, proliferation, alkaline phosphatase activity, collagen secretion, and ECM mineralization; and (4) to evaluate in vivo the physiological responses of subdermal tissues upon this coating exposure in a rabbit animal model. In addition, preliminary studies would be performed, utilizing complex-shaped samples to illustrate the flexibility and adaptability of our method toward clinical applications, thereby offering an opportunity for the batch manufacturing of silver-coated titanium implants.

## 2. Materials and methods

### 2.1. Preparation and characterization of silver-deposited MAO coatings

Commercially available pure titanium (cTi) discs with a size of 10 x 10 x 0.5 mm were mechanically polished up to 1200 grit and sequentially rinsed by ultrasonication in acetone, ethanol, and deionized water (DI). The mussel-inspired strategy to immobilize AgNPs onto the surface of micro-arc-oxidized Ti is depicted in Fig. 1a. First, MAO was conducted using a homemade setup, as was previously described [27], in 0.5 M NaOH solution with a pulsing power supply (JHMAO-6H; Environmental Golden-Arc, Beijing China) at 220 V for 5 min. The frequency and duty cycle were maintained at 500 Hz and 4% respectively. The obtained titania-rich samples were ultrasonically rinsed in DI and air dried. Secondly, a thin film of PD was applied to anodized titanium onto which Ag$^+$ was able to be immobilized via chelation to the catechol groups of PD [18]. In brief, the MAO-treated samples were subjected to 2 mg/mL dopamine hydrochloride (Alfa Aesar) in Tris–HCl buffer (10 mM, pH = 8.5; Sigma) for 24 h under constant vibration in darkness at 37 ℃, then thoroughly ultra-sonicated to detach excess monomer and particles, and further immersed in AgNO$_3$ (1 mM; Beijing Chemical Reagent Co., Ltd., China) solution for another 6 h. Next, the substrates were rinsed for 5 min to remove the residual silver ions before being exposed to 1-h ultraviolet (UV, λ = 254 nm, 2.5 W/m$^2$) to further reduce AgNPs. Unless otherwise stated, the irradiation upon the resulting samples sterilized and activated their surfaces at the same time before cell culture and antibacterial experiments. The samples after MAO process, PD treatment and silver immobilization are designated as MAO, MAO/PD and MAO/Ag in the subsequent discussion.

The morphology and microstructure of coatings were investigated by field emission scanning electron microscopy (FE-SEM, S-4800, Hitachi) and transmission electron microscopy (TEM, H-9000NAR, Hitachi). The pore distribution was calculated from random SEM images for triplicate specimens using the popular freeware ImageJ. The topological structure and surface roughness were obtained using an in-situ Nanomechanical Test System (TI-900 Tribolindenter, Hysitron Inc.) with a diamond scanning probe. The crystal structure of the coatings was analyzed by X-ray diffractometer (XRD, D8 Focus, Bruker) equipped with Cu-Kα radiation source (λ = 1.54 Å) between 2θ of 10° and 80°. For compositional analysis, energy-dispersive X-ray spectrometry (EDS), X-ray photoelectron spectroscopy (XPS, Kratos, UK), and microscopic Fourier transform infrared spectroscopy (Micro-FTIR, Thermo Fisher) were employed. Raman spectra were obtained with an excitation laser of Ar⁺ 514 nm in the range of 500—2000 cm$^{-1}$ using a confocal Raman microscope (Renishaw 1000).

### 2.2. Ag$^+$ release into phosphate buffer solution (PBS)

To investigate the release behavior of Ag$^+$ ions from the MAO/Ag coatings, specimens (n = 3) were immersed in 6 mL of PBS each at 37 ℃ in darkness for 4 weeks, and the entire volume was collected at predetermined time points, and fresh PBS was refilled accordingly. After analysis using inductively coupled plasma-atomic emission spectrometry (ICP-AES, Leeman, USA), the release profiles and rates of Ag$^+$ were determined. Moreover, the total Ag content was measured by sampling (n = 3) in 4% dilute nitric acid.

### 2.3. Electrochemical experiments

The anticorrosion properties of the samples were evaluated electrochemically in vitro, employing a Metrohm Autolab...
electrochemical workstation (German) with three electrodes: working electrode, platinum counter electrode and saturated calomel (SCE) reference electrode. All measurements were performed in Hank’s solution (8.00 g/L NaCl, 0.14 g/L CaCl2, 0.40 g/L KCl, 0.35 g/L NaHCO3, 0.12 g/L Na2HPO4-12H2O, 0.06 g/L KH2PO4, 0.03 g/L MgSO4, 1.0 g/L C6H12O6; pH = 7.4) to simulate the biocorrosive environments around an implant. The specimens (exposed area: 0.385 cm²) were immersed for 7200 s to achieve balanced open circuit potentials (OCP). The electrochemical impedance spectroscopy (EIS) measurements were conducted using an alternating current voltage of 10.0 mV within the frequency range of 10\(^{-2}\)–10\(^{5}\) Hz. Potentiodynamic polarization curves (Tafel plots) were measured at a scanning rate of 0.5 mV/s. Data fitting and analysis were performed with the help of the software Nova 1.8.

2.4. Contact angle (CA) measurements

Static CAs were measured on a SL200B contact angle system (Kino, USA) at ambient humidity and temperature. Distilled water was used as the media and 6 measurements were performed on each specimen for statistical accountability.

2.5. Protein adsorption

Bovine serum albumin (BSA, 1 mg/mL in PBS) was used as a model protein in this study. Aliquots of 200 µL were deposited by pipette carefully onto the substrata in 24-well tissue culture plates (TCPS, Corning, USA), and were incubated in humidified atmosphere at 37 °C for 2 h. Afterward, the nonadherent protein was detached by PBS, and 200 µL of 2% sodium dodecyl sulfate (SDS, Sigma) were added per well. The plates were subsequently shaken at 37 °C overnight to extract adhered protein. Protein concentrations were determined at 570 nm on a microplate reader (Bio-RAD, USA) using micro Bicinchoninic Acid (BCA, Thermo scientific) kits following the guidelines from the manufacturer. As an alternative, samples, after removing the nonadherent proteins, were fixed by 4% (w/v) paraformaldehyde (PFA) and labeled with FITC (0.5 mg/mL, Sigma). The adsorbed proteins were thus visualized as green fluorescence under excitation at 488 nm on a laser scanning confocal microscope (CLSM, Nikon ALR-SI; identical apparatus throughout the experiments).

2.6. In vitro bactericidal assessment

The antibacterial activity of the cTi, MAO, MAO/PD, and MAO/Ag samples (n = 3) was determined by co-culture experiments utilizing model S. aureus mutans in the Luria Bertani (LB) medium at 37 °C. Aliquots of 500 µL of bacterial suspension at adjusted concentration (OD\(_{600}\) ~0.5, corresponding to ~2 × 10\(^8\) colony forming units (CFU)/mL) were introduced uniformly onto the sterilized sample surfaces and allowed to incubate in aerobic conditions for 24 h. The samples were then rinsed with PBS to remove the nonadherent bacteria and stained with SYTO 9 (Life Technologies). The adherent bacteria were visualized under a fluorescent microscope (Olympus IX71) and quantified using ImageJ software.
conditions for 6- and 24-h for anti-attachment assay, and 7 d for anti-biofilm assay. Afterwards, the discs were gently washed with sterile PBS to eliminate non-adherent bacteria and then fixed. For independent titanium discs, at least 5 random low and high magnification SEM images were taken to count the amount of retained bacteria and observe their morphological changes. The CFU counting on agar plates was employed to see whether the bacteria on MAO/Ag surfaces were killed. The PBS-rinsed specimens were transferred to sterile tubes containing 1 mL of fresh broth, and subsequently sampled via ultrasonic vibration for 10 min. After being serially diluted, the number of viable bacteria in the sample suspensions was determined as the units of colonies formed. The antibacterial rate was calculated as: \[((A−B)/A)\times100\%\], where A, B are the CFU counts for cTi and MAO/Ag, respectively. In addition, fluorescence staining was also utilized in situ to distinguish viable/dead bacteria cells as well as to visualize biofilm formation on the samples, using Live/Dead® BacLight™ bacterial viability kits (Molecular Probes™, Invitrogen), which contain two dyes SYTO™ 9 and propidium iodide (PI). Because of their different abilities to penetrate bacterial cells and integrate with nuclei, only disrupted cell membranes are susceptible to PI whereas SYTO permeates both intact and disrupted membranes [28]. Therefore, a similar staining protocol were added to each sample surface after rinsing with 0.85% physiological saline. Each sample was stained for 15 min in darkness, and then examined under CLSM at 5 random positions. Data analysis was performed using ImageJ. If necessary, XYZ tomo-scanning mode was applied to take a series of sliced images. They were reconstructed to create the 3-D field of view, by software package NIS-Elements AR 3.0.

2.7. Cell culture and seeding

The human osteoblast-like cell line MG-63 was used. Cells were routinely cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. At 80–90% confluence, cells were trypsinized and harvested by centrifugation, resuspended and diluted to the desired density in culture medium, and finally inoculated onto the as-sterilized specimens in 24-well TCPS at a density of 1 \times 10^5 cells/mL. The medium was refreshed every two days.

2.8. Cytocompatibility studies

For cell attachment and spreading, the discs, after 6 h in culture, were collected with caution, and gently rinsed in PBS. For the SEM examination, cells on the samples were fixed with 2.5% (v/v) glutaraldehyde (GA) in PBS at 4 °C overnight. Subsequently, the fixed cells were dehydrated in graded ethanol. For the immunofluorescence study, cells were fixed in 4% PFA/PBS for 15 min, and permeabilized with 0.1% (v/v) Triton X-100 for 5 min and blocked in 1% BSA for 1 h at 37 °C. Then, Dylight 488-conjugated Anti-FAK antibody kit (Abcam, USA) targeting human focal adhesion kinase was added at 1:100 dilutions and co-incubated overnight at 4 °C. The focal adhesions were observed by CLSM technique. Additionally, FITC-conjugated phalloidin (5 µg/mL) and DAPI (2 µg/mL) double staining was utilized to count cells and observe the cytoskeletal development after 6 h by a similar staining protocol.

For cell proliferation and survival, assays using Cell Viability Kit-8 (CCK-8, Dojindo, Japan) and Lactate Dehydrogenase Kit (LDH, abcam, USA) were performed respectively in triplicate according to the manufacturers’ instructions. In CCK-8 test, 10% CCK-8 solution in medium was added at defined points, and incubated at 37 °C for 2 h, and then the OD values at λ = 450 nm (with reference to 630 nm) were read. The viability of cells was calculated by this formula: Cell viability = [(ODsample − ODblank)/(OPnegative − ODblank)] \times 100\%, where the negative refers to TCPs. For LDH assay, the culture media was collected and centrifuged, and the diluted supernatants (1:25) were mixed with LDH reaction solution and incubated at 37 °C for 30 min, followed by spectrophotometric quantification at λ = 450 nm. The results were expressed as the unit of LDH enzyme activity per min (U/min) when catalyzing the production of NADH (reduced form of nicotinamide-adenine dinucleotid). In addition, at 1 d, the cells’ survival ability on the samples was assessed by staining living cells with 2 µM Calcein AM and the dead with 4 µM PI (Live/Dead Cell Stains, Dojindo, Japan). For cell culture morphology, cells on samples were also fixed for SEM studies and fluorescence (actin) observation.

2.9. Osteogenic differentiation studies

For alkaline phosphatase (ALP) activity, after 3-, 7- and 14-d, the cells on the specimens were lysed in 1% Triton X-100 using standard freeze-thaw cycles. The ALP activity in the lysis was was probed spectrophotometrically by measuring the colorimetric production of p-nitrophenol (p-NP) via p-nitrophenyl phosphate (p-NPP) endogenously by ALP enzymatic reaction, as per manufacture instructions (Nanjing-jian-cheng, China). The results were normalized against the total intracellular protein content measured by the Micro BCA protein kit and expressed as mM production of p-NP by each gram of protein (mM p-NP/mgprotein). Qualitatively, the cells on the specimens were fixed in 4% PFA, and stained with BCIP/NBT ALP Color Development Kit (Beyotime, China).

The collagen secretion and ECM mineralization assays were carried out at 28 d after fixing samples in 4% PFA for 15 min. For the degree of collagen secretion, the material-cell constructs were stained in a 0.1% solution of Sirius Red (Sigma) in saturated picric acid overnight. After rinsing with 0.1 M acetic acid until no more color appeared, optical images for each sample were acquired. Quantitatively, the stain was eluted in 400 µL of the destain solution (0.2 M NaOH/methanol 1:1) before optical density at 570 nm was measured using a microplate reader. The ECM mineralization degree was visualized by Alizarin Red S (ARS, Sigma) staining. Briefly, specimens were stained with ARS (2%, pH = 4.3) for 10 min at 4 °C and thoroughly rinsed with DI to eliminate unbound stain. Finally, snapshots were taken. Also, the samples were photographed using CLSM with excitation/emission wavelengths at 543/617 nm [29]. The bound Ca was extracted using 10% cetylpyridinium chloride for 2 h and the absorbance of the resulting solution was measured at 562 nm.

2.10. The feedback of cellular activities on Ag⁺ release in culture

In culture environment, cells adhere to and spread on a biomaterial’s surface, and metabolize for energy so as to maintain their normal activities such as migration, replication, and differentiation. For AgNP-containing surfaces, this means that the cells would settle their large body on the nanosilver, followed by secretion of ECM and metabolic products. In order to reveal what would happen to those underlying particles (remain or rather disappear), the MG-63 cells in culture were segregated at scheduled times from the material surface by trypsinization (Fig. 11a). The obtained samples were thoroughly washed in DI, dried in air, and investigated under SEM (fixed material-cell constructs at the same time point were used as control). Furthermore, the repercussion effect of cellular activities on the release rate of Ag⁺ during culture was investigated through an in-situ culture of MG-63 cells on materials for up to 28 d. Six MAO/Ag samples were randomized into two groups: one
seeded with cells, and the other without, and they were incubated typically at 37 °C with media change every 2–3 d (identical to 2.7). The Ag⁺ in the collected media was detected by ICP-AES after digestion using concentrated HNO₃.

2.11. Subcutaneous implantation

Authorized by the animal ethics committee of the Peking University Health Science Center, we used 6 male adult New Zealand

Fig. 2. Morphological and microstructural characterization of MAO/Ag coatings. (a) SEM image with EDS mapping (inset); (b) A magnified image of circled area in image (a), showing AgNPs (arrows) formed both on surface and inside pores; (c) SEM image of the cross-section; (d) Diameter distribution of the micro/nanopores; (e) Quantitative results of EDS; (f) Cross-section EDS linear scan curve of image (c); SEM side view (g) and TEM observation (h, i) of scratched off coatings. (insets in i) SAED pattern and lattice space (10 fringes counted) of silver; (j) The surface topographical roughness of cTi (j1), MAO (j2), MAO/PD (j3) and MAO/Ag (j4).
white rabbits (weight: 3–3.5 kg) in this study to evaluate and compare the biological responses of surrounding tissues towards Ti plates and MAO/Ag coated ones. After anesthetized with 0.2 mL/kg of Ketamine, the rabbits were placed in a prostrate position with the dorsal skin shaved and disinfected. Afterward, a 10-mm longitudinal skin incision was made to expose the superficial plane of the deep fascia. A pocket was then built by blunt dissection with scissors in which one piece of implant was inserted. Specifically, MAO/Ag plates were placed having the modified face towards the outward side of pockets. Lastly, each subcutaneous pocket was sutured using absorbable thread (PDS II, ETHICON). By repetition of this procedure, each animal received 4 implants (2 Ti and 2 MAO/Ag coated plates), and hence, a total of 24 implants (12 for each group) were inserted. Intramuscular injection of cephazolin (10 mg/kg) was performed for the first 3 d post-operatively. After 1 month, the animals were euthanized and the plates with surrounding tissues were harvested for further histological analysis.

2.12. Histological evaluation

The specimens were fixed in 10% formalin, dehydrated in gradient ethanol solutions (40–100%) and then embedded in paraffin after the inside implants were carefully removed. Five histological transverse sections (6 μm thick) from each sample were prepared and stained with hematoxylineosin (H-E) and photographed under fluorescence microscopy (DP71, Olympus) for evidence of inflammation. The images were analyzed using Imagej software to determine the thickness of the fibrotic capsule around each implant and the number of fibroblasts. In addition, surfaces of the withdrawn implants were investigated by SEM to evaluate the extent of fibrosis and nanosilver degradation.

2.13. Clinical adaptability studies

To testify the adaptability of the coating procedure to sophisticated implants, we modified two kinds of these surfaces: (1) wire-shaped pure titanium specimens (Φ 0.5 mm, L > 10 mm), and (2) cylindrical Ti6Al4V alloy scaffolds (Φ 10 × H 5 mm, for characterization and cell culture; Φ 4 × H 6 mm, for mechanical testing). The Ti wires were commercially available and frequently used, while the scaffolds were fabricated layer-upon-layer by electron beam melting (EBM; Arcam AB, Sweden) from ultrafine Ti6Al4V alloy powders (p = 4.3 g/cm³). Briefly, 3-D samples with hexagonal periodic macropores, formed by 400-μm interconnected struts, were

Fig. 3. Chemical composition of the MAO/Ag coatings: (a) XRD patterns; (b) Raman spectra; (c) Micro-FTIR spectra; (d) XPS results comprising a survey spectrum (d1) and corresponding core-level spectra for Ti 2p (d2), O 1 s (d3) and Ag 3d (d4); (e) a simplified schematic of the anticipated titania-catechol-silver coordinations, partly adapted from literature [20].
designed by CAD software and converted into STL data that was then transferred to the Arcam system for "printing" using scanning electron beams with ultrahigh energy. The average pore size and porosity were 682 μm and 73%, respectively. The coating procedure was identical to that of Ti plates, except that the voltage in MAO was adjusted from 220 to 320 V for the EBM scaffolds. After MAO/Ag coating, the microstructures of the wires and scaffolds were characterized by FE-SEM. Specially, the scaffolds were sawed in half to expose the interior wall structures before SEM. Compressive tests of the MAO-treated scaffolds (n = 3) were performed on a universal mechanical tester (Instron5969, USA) at a displacement rate of 0.2 mm/min at room temperature. It had been stipulated that a scaffold failed at the point when its first strut broke. The 0.2% offset yield strength (YS) and the ultimate compressive strength (UCS) were obtained from the stress–strain curves. Also, we evaluated ex vivo the cytocompatibility of the engineered Ti wires and Ti6Al4V parts by seeding MG-63 cells for a subsequent cultivation of 5 d and 14 d, respectively. For seeding, the samples (n = 3) were pre-wetted and Ultra low-attachment TCPS (Corning, USA) were used to guarantee cell attachment to the specimens rather than to the bottom of plates.

2.14. Statistical analysis

All the quantitative data were presented as means ± standard deviations. The statistical analysis was done using either one-way analysis of variance (ANOVA) or Student's t-test, wherever necessary, and the significance was regarded at p value < 0.05.
Results

3.1. Fabrication and characterization of the micro/nano-porous MAO/Ag coatings

In a typical process, as schemed in Fig. 1a, MAO in NaOH solution by anodic plasma sparking led to the in-situ growth of porous TiO$_2$ coatings on titanium (Fig. S1). The resulting surfaces, rich in Ti-O species, were subsequently subjected to a dopamine solution at pH 8.5 to spontaneously form the PD layer on them. Although this facile, mild coating is widely used, the precise molecular mechanism for dopamine polymerization has been too complicated to be fully disclosed [30]. Generally, the covalent oxidative polymerization and crosslinking by pH induced oxidization should constitute an important part (Fig. 1b). It is an “eumelanin-like” model where DA molecules suffer from oxidization to form dopamine-o-quinone, followed by intra-molecular cyclization through 1,4-Michael addition to yield leucodopaminechrome that is further oxidized and rearranged to produce 5,6-dihydroxyindole (DHI) and 5,6-indolequinone. These products then undergo branching reactions to generate multiple isomers of dimers and, subsequently, higher oligomers, which self-assemble via the reverse dismutation reaction between catechol and o-quinone to give the cross-linked PD. Owing to the nanoscale reactive nature of PD, the native porous morphology (diameter from less than 200 nm to over 1.2 mm, Fig. 2d) of MAO coatings was not destroyed. Further, the MAO/PD samples were impregnated into silver nitrate. Here, catechols served as a chelating agent of silver ions. Simultaneously, they reduced a part of Ag$^+\$ to nanosilver (Fig. S2a, c). However, the surface density of AgNPs was not satisfactory. In order to thoroughly reduce the remaining free Ag$^+$ ions (especially in the pores), one more step of UV exposure (1 h) was considered. As a result, uniformly dense nanosilver, compared to that on the surface, was incorporated within the pore (Fig. S3), as had been anticipated in Fig. 1a. On the other hand, particles were hardly detectable on the MAO coatings without applying the PD layer even after identical UV irradiation (Fig. S2e), suggesting the necessary role of catechol-Ag chelation. In Fig. 2c, the thickness of

Fig. 5. (a) Quantitative counts of adhered bacteria cells and their fraction (% total adhered ones) in “trap”. (b) The attachment and viability of S. aureus on MAO/Ag specimens after 24 h incubation: representative low- (b1) and high- (b2) magnification SEM images; (b3) CFU counting using the detached bacteria; (b4) in-situ Live/Dead visualization for the living status of retained bacteria. (Inset in b2) Three-dimension reconstruction of a series of projections (636.4 × 636.4 × 17 μm$^3$) by XYZ scanning (See also Fig. S6). (c) Representative SEM images (c1–c4 are surface views; c5, c6 are side views) highlighting the “trap-killing” phenomena. For better visualization, nanosilver bound to membrane is false-colored pink (others blue), and some bacteria are false-colored yellow; disintegrating/dissintegrated cells are indicated by stars. The dashed line in (c6) highlights the contour of two adjacent pores. (d) Schematic illustration of the possible killing modes involved during adhesion stage: (d1) the majority of planktonic bacteria were repulsed from the surface by the releasing Ag$^+$; (d2) some of the landed bacteria were disrupted via contact with AgNPs on the surface; (d3) other survivors with negative membrane charge were attracted into the micropores (positively-charged by interior silver) and were killed by the so-called “trap-killing” principle, which might include: 1: down-falling and collision with pore walls; 2: nanosilver binding via electrostatic attraction or specific interactions such as Ag-thiol bond; 3: membrane distortion and destruction by nanosilver and local Ag$^+$ for one or more bacteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the MAO/Ag coating is 2.26 μm. The major surface/cross-sectional constituents were found to be Ti, O, C, Ag and Na (Fig. 2e, f). Under TEM in Fig. 2h, the dark particles with high electron cloudy density and diffraction rings were assigned to AgNPs, while the surrounding gray substance should be PD coating. A lattice fringe of 2.43 Å obtained from the HRTEM image (Fig. 2i) is indexed to (111) facet of metallic silver [31]. After MAO, MAO/PD and MAO/Ag processes, the surface roughness was 61.67, 53.40 and 65.55 nm, respectively (Fig. 2j).

According to the XRD results (Fig. 3a), the major surface constituents of the MAO coatings were TiO₂ in the form of anatase (A) and rutile (R). The polymerization of dopamine on TiO₂ did not alter the patterns. Moreover, in the diffractogram of MAO/Ag samples, three characteristic peaks at 2θ = 38.0°, 44.4° and 77.5° were clearly observed, corresponding to the (111), (200) and (310) planes of cubic silver crystal (JCPDS No. 65-2871) [32], respectively. The Raman spectra (Fig. 3b) also verified the formation of anatase (bands at 638, 518, 396, and 150 cm⁻¹) and rutile (bands at 612 and 446 cm⁻¹) in the MAO coatings [33]. In the case of MAO/Ag samples, new broad bands existing at approximately 1603 and 1368 cm⁻¹ should originate from stretching and deformation of aromatic rings, indicating PD formation [26,32]. In Fig. 3c, for both MAO/PD and MAO/Ag, the broad absorption around 3437 cm⁻¹ was assigned to phenolic hydroxyl stretching vibrations of catechol groups, whereas the peaks at 1580 and 1176 cm⁻¹ corresponded to the stretch vibrations of aromatic rings [34] and C–C groups [35], respectively. The N–H shearing vibration of the amide group was evident at 1519 cm⁻¹ [36]. After AgNPs deposition, a new strong absorption appeared at 620 cm⁻¹, which could be ascribed to the chelating between PD and silver ions. XPS was performed to determine the Ag chemical states. Again, ~368.0 and 374.0 eV binding energies in Fig. 3d corroborated Ag (0) 3d⁵/₂ and Ag (0) 3d³/₂, respectively [5]. Besides, the nitrogen signal (12.76 at.%) was due to dopamine’s amine groups, and the intrinsic substrate peaks weakened as the TiO₂ oxide layer was generated (Fig. 3d2). The O 1 s at 522.9 (Ti–O), 531.4 (C=O) and 532.9 (C–OH, C=O–C) eV corresponded to TiO₂ and PD, respectively (Fig. 3d3). Anderson et al. [20] have demonstrated that catechol could form bidentate binuclear surface complexes on TiO₂ surfaces (each OH group from the catechol binds to a separate Ti molecule on the exposed surface) with covalent and ionic bond characteristics (Fig. 3e). Hence, the PD film should be chemically adhered rather than adsorbed to the MAO coating, which resulted in a complete firm inorganic-polymer matrix. One the other side, PD and silver are associated via forces of metal coordination, electron and electrostatic interactions [23,24]. Correlating these with the as-mentioned micro/nanoscale structure and nanoscale roughness, one can conclude that a TiO₂/PD/Ag composite coating with rigid character and hierarchically micro/nano-textured surface topography was successfully developed.
3.2. Ag\(^+\) release in PBS

Fig. 4(a1–a3) displays the Ag\(^+\) release time profiles from MAO/Ag in PBS. The total Ag content was 8.57 ± 1.01 mg/cm\(^2\). As a control, AgNP-deposited smooth Ti (Ti/Ag) was fabricated in the same way, but the content of silver was only 3.08 ± 0.24 mg/cm\(^2\). This further verifies the high loading efficiency by porous matrix due to particle incorporation (Fig. 4a4). In the initial 6 h, ~2.65 mg/cm\(^2\) (a fraction of 30.9%) of Ag\(^+\) was leached into PBS, at an average rate of 0.44 mg/cm\(^2\)/h. The value then neared 4.75 mg/cm\(^2\) (55.4% of total) upon 1-d immersion, and kept gaining slowly over an extended period of time. After 28 d, there was still 5.1% of remnant, showing good durability. Further evidence is given by SEM-EDS in Fig. 4(a5–a8). The initial 0.55 at.% (Fig. 2e) of Ag declined gradually with time. The remaining atomic content was merely 0.28 at.% after 28 d, but smaller particles were still retained around the cavities (Fig. 4a8, arrows).

3.3. Corrosion resistance

For biomaterials, corrosion is inevitable and is responsible for prosthetic instability and then looseness. It can be avoided or alleviated through improving the anticrosion properties of materials. The corrosion resistance of the coatings was determined, as shown in Fig. 4(b). After coating, the OCP values of the samples (MAO, MAO/PD, MAO/Ag) shifted positively (despite fluctuations) and reached final potentials at −0.18, −0.21, and −0.13 V (vs. SCE) after 2 h, respectively (Fig. 4b1). This may reflect the existence of passive components in the coatings. The MAO/Ag samples had the highest OCP values and thus the best thermodynamic stability. By Tafel plots (Fig. 4b2), all modifications resulted in more positive corrosion potential (E\(_{corr}\)), lower corrosion current density (I\(_{corr}\)), and greater polarization resistance (R\(_p\)), as also outlined in Table 1. Particularly, MAO/Ag treatment attained the best substrate protection by decreasing I\(_{corr}\) of cTi by over one order of magnitude. The stability of the coatings was estimated via EIS spectra at E\(_{corr}\). In Nyquist plots in Fig. 4b3, the resistive semicircle’s diameter ranked as: MAO/Ag > MAO > MAO/PD > cTi, which fit well with the OCP and Tafel measurements. Additionally, having linear slopes around −1 in Bode-log|Z| plots, all the coated samples displayed nearly capacitive responses across a wide range of frequencies in Bode-Phase angle plots (Fig. 4b4), disclosing the coatings’ passive inherence.

3.4. Hydrophilicity

In biological systems, the hydrophilicity of the implant surface plays a pivotal role in mediating protein adsorption and cell adhesion. The wettability of bare and modified titanium 24 h and 1 week subsequent to preparation was determined by CA measurements of sessile water drop, as presented in Table 2. All the treatments dramatically improved hydrophilicity of cTi according to the 24-h measurement, with the MAO samples being the most wettable ones (nearly complete spreading of water droplets). The hydrophilicity of a modified surface often changes with time, so its stability should also be studied. After one week’s standing in air, the CA of cTi dropped slightly whilst that of the MAO increased drastically by 35.6° compared with MAO/PD (+11.3°) and MAO/Ag.
Nevertheless, the coated ones were still hydrophilic enough compared to cTi (55.7\%). The above deterioration might be a result of ubiquitous environmental contamination by adsorbing carbonaceous compounds (e.g. oil) at ambient conditions. The polar groups (e.g. OH, NH2) of the PD layer can confer excellent anti-fouling ability to the substrates to better resist such pollution [37,38].

3.5. Protein adsorption

The amount of BSA adsorbed on the surface after 2 h of incubation was assayed, and Table 3 displays the results, which are: MAO/Ag > MAO/PD > MAO > cTi. Notably, the protein content of the PD layer and MAO/Ag specimens was more than doubled in comparison to that of cTi. It could possibly originate from the fact that BSA can be covalently grafted to PD surfaces via the coupling between o-benzoquinone and amine [37]. Also, the enhanced fluorescence intensity from the FITC-labeled protein revealed similar trend as thicker protein layers were formed (Fig. S3).

3.6. Antibacterial assessment

The prohibition of initial bacterial adhesion onto the surfaces, which later could evolve into biofilms, is recognized as the first and pivotal step in combating BAI [17]. In this study, the antibacterial activity against the early adherence of S. aureus was first evaluated by morphological observation after cultivation on various surfaces for 24 h. Active bacteria challenged cTi surfaces by undergoing the binary or multiple fission stages. They were characterized by cell aggregates with smooth intact walls (Fig. S4a). Conversely, the number of bacteria on the MAO/Ag samples was reduced by over 64.2\% (Fig. 5a, left y-axis). More interestingly, some of the attached S. aureus cells were entrapped within the pores (circled, Fig. 5b1); their membranes were rough and disrupted, having random nanosilver particles (false-colored pink, Fig. 5b2) clung. The trapping efficiency was determined statistically to be ~24.9\% (Fig. 5a, right y-axis). Actually, similar phenomena existed in the MAO and MAO/PD groups (Fig. S4b, c), but their efficiency was significantly lower (~14.7\% and 10.9\%, respectively). In order to see whether the above trapped bacteria were killed by silver, a CFU counting assay was run with the detached bacteria from the surfaces (Fig. 5b3). The antibacterial rate is over 99.85\%, with reference to cTi. Since only those active bacteria can develop colonies, the microbes as a whole should be effectively poisoned. Besides, in-situ Live/Dead fluorescent staining of the retained S. aureus on MAO/Ag was performed (Fig. 5b4), and nearly all cells were stained red. Particularly, the 3-D visualization of the samples, through tomo-scanning, provided more direct evidence for that (inset in Fig. 5b4; Fig. S6).
Fig. 9. (a) Cell morphologies on various surface types (cTi, MAO, MAO/PD, MAO/Ag) at different stages. The insets are magnified areas showing the body of cells interacting with flat substrates or those with micro/nanostructures. Arrows indicate the round, dead cells. (b) The coloration of ALP activity (deep blue), the collagen (COL) secretion (purplish red), and calcium (CAL) deposition (red or purplish red) on different specimens. Both optical images (top) for the macroscopic appearance and fluorescent ones (down) for the local characters were taken for the CAL groups. All optical pictures are 10 mm × 10 mm in area, and the fluorescent images are 600 μm × 600 μm. The circles highlight those especially positively-stained spots (rich in calcium nodules). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 10. Cell–Ag interactions: (a) the schemed experimental protocol; (b) the in-situ release profiles and release rates of MAO/Ag samples with and without cells; (c) the silver-deposited surfaces before and after decellularization during the culture periods. Here, we observed an interactive cause–effect relationship between MAO/Ag surfaces and cells. First, nanosilver-decorated micro/nanoporous surfaces had apparent affinity for MG-63 cells and they were gradually covered by the incrassated ECM from the latter. Second, the coverage above in return slowed down the release of Ag⁺ into media.
envisioned that the above physical entrapment effect associated with AgNPs' biocidal capability would mostly likely produce an alternative antibacterial mode—"trap & kill". For better understanding, the MAO/Ag surfaces with bacteria at the initial adhesion stage (6 h) were specifically investigated by SEM. Six typical areas are presented (Fig. 5c): (c1) two bacteria were prone to fall down into a pore, with a ruptured one on the side (the yellow star); (c2) a microbe was captured with distorted membrane where AgNPs (red arrows) were bound; (c3) one more cell was almost trapped aside from the existing one in the "trap"; (c4) a flattened bacterium was being attacked and disintegrated by nanosilver/Ag⁺; (c5) side-view of a falling bacterium adhered with nanosilver, through collision contact with the peripheral porous walls; (c6) side-view of a nanosilver-ruptured cell within the pore. Note also that some particles had become smaller in these fields, suggesting the co-

Table 1  
Electrochemical parameters of the uncoated and coated Ti samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ecorr/V</th>
<th>Icorr/A cm⁻²</th>
<th>Rpt/O cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>cTi</td>
<td>−0.39041</td>
<td>1.16E-05</td>
<td>30239</td>
</tr>
<tr>
<td>MAO</td>
<td>−0.35934</td>
<td>8.68E-06</td>
<td>45618</td>
</tr>
<tr>
<td>MAO/PD</td>
<td>−0.36513</td>
<td>9.23E-06</td>
<td>38609</td>
</tr>
<tr>
<td>MAO/Ag</td>
<td>−0.35411</td>
<td>1.58E-06</td>
<td>48287</td>
</tr>
</tbody>
</table>

Table 2  
The effects of surface modification on contact angles.

<table>
<thead>
<tr>
<th></th>
<th>cTi</th>
<th>MAO</th>
<th>MAO/PD</th>
<th>MAO/Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within 24 h</td>
<td>59.7 ± 3.8</td>
<td>10.3 ± 5.7*</td>
<td>34.4 ± 5.0*</td>
<td>29.3 ± 3.9*</td>
</tr>
<tr>
<td>After a week</td>
<td>55.7 ± 3.8</td>
<td>45.9 ± 5.8*</td>
<td>45.7 ± 3.1*</td>
<td>42.2 ± 4.0*</td>
</tr>
</tbody>
</table>

Asterisks (*) indicate significant difference compared to cTi. p < 0.05.
existence of releasing killing.

Furthermore, we examined the anti-biofilm efficacy of the coatings through a prolonged culturing of 7 d, by means of Live/Dead staining and SEM studies. As depicted in Fig. 6a, large areas of strong green fluorescence (a1) as well as a confluent, dense layer of mature biofilm (a2) indicate that bacteria propagated uncontrollably on cTi. Subsequently, they would encase themselves in slimy matrices. Furthermore, they would adhere tightly to the substrates. In contrast, those on other surfaces were homogeneously-distributed and spread wide. In particular, the MAO/PD and MAO/Ag surfaces had more broad-shaped cells. After 3 d, cells on all coated surfaces proliferated readily and spread out with a healthy spindle osteoblastic shape, and they had numerous elongated lamellipodia overlapping their neighboring ones. Round cells were rarely detectable. All cells were already sub-confluent at 5 d, and they adhered tightly to the substrates. In contrast, those on cTi were inferior in terms of quantity and morphology throughout the observation periods.

### 3.7. Effects of Ag coating on cytocompatibility

The effects of AgNPs on cell attachment were investigated through F-actin/FAK staining and SEM observation after 6 h of incubation, as displayed in Fig. 7, S7. Most cells seeded on cTi already began to adhere, by assembling focal adhesions and stress fibers and by outstretches lamellipodia to sense the substrate (Fig. 7a1; Fig. S7a). Nonetheless, it was too early for them to fully spread out. By comparison, those cultured on MAO had a greater amount and larger area of focal contact, and exhibited fast, tight adhesion and favorable spreading (Fig. 7a2; Fig. S7b). Interestingly, the anchorage of MG-63 was guided by the micropits and in return the cells blanketed the pores using their entire bodies. This was true for all the coated groups with pore structures (Fig. S7b–d). Quantitatively, the adhesion was improved by 18.4% relative to cTi (Fig. 7b). Stimulated by both the porous topography and adhesive nature of PD, the amount of MG-63 on MAO/PD was remarkably augmented by over 41.6% with elongated morphologies. Despite that the anchoring of AgNPs postponed the adhesion process by reducing the number of cells by about 7.9% compared with MAO/PD, the MAO/Ag group was still much better than cTi in terms of cell number and spreading. To our delight, MG-63 could adhere to and spread on both the porous and planar areas with nanosilver (Fig. 7c). In the first 6 h, cells’ pseudopodia (red stars) outstretched in a contact-guided way to feel the surface, but they appeared to circumvent nanosilver. Upon 24 h in culture, almost all cells had been closely-anchored and well-extended with pore-associated filopodia. Besides, they clad AgNPs (white arrows) by overlaying a layer of ECM (Fig. 7c3, c4).

To be frank, a few near-round dead cells were detected on MAO/Ag at 1 d, as indicated by cellular Live/Dead staining (Fig. 8c). It suggested that individual cells varied in survivability upon even identical MAO/Ag exposure. Furthermore, the test of LDH enzyme release was conducted. Similarly, the MAO/Ag group showed slightly higher LDH activity, although the values on a daily basis (i.e., LDH release rate) dropped with time (Fig. 8b). It verified that silver release indeed contributed to the increase of intracellular stress and thus apoptosis. However, we could not simply conclude from this that the coatings are not safe. According to the viability data in Fig. 8a, cell multiplication on silver-coated materials was normally favorable with respect to that on cTi, particularly at longer incubation durations. We then examined the capacity of MG-63 cells to develop cytoskeleton on these surfaces, as demonstrated in Fig. 8d. After culturing for 3 d, relatively weak stress fibers were formed by F-actin in the cTi groups, compared to the modified ones. The assembly of stress fibers continued in the following days, accompanied by replication, for all the observed groups. Particularly, MAO and PD treatment together led to networked cytoskeletons distributing throughout the cell bodies after 5 d on MAO/PD and MAO/Ag. These suggested that silver immobilization by MAO/Ag treatment did not disturb the organization of the cytoskeleton. The SEM images in Fig. 9a further disclosed the morphology of MG-63 cells. At 1 d, the cells on cTi were sparsely distributed and less spread (some even with a near-round shape). Except that, those on other surfaces were homogeneously-distributed and spread wide. In particular, the MAO/PD and MAO/Ag surfaces had more broad-shaped cells. After 3 d, cells on all coated surfaces proliferated readily and spread out with a healthy spindle osteoblastic shape, and they had numerous elongated lamellipodia overlapping their neighboring ones. Round cells were rarely detectable. All cells were already sub-confluent at 5 d, and they adhered tightly to the substrates. In contrast, those on cTi were inferior in terms of quantity and morphology throughout the observation periods.

### 3.8. Effects of Ag coating on osteo-differentiation functions

First, in vitro ALP activity at 3-, 7- and 14-d was measured as an early hallmarker for osteogenic differentiation potential of MG-63. ALP activity in osteoblasts is an important factor in promoting bone mineral formation and it shows a scale of changes during osteoblastic differentiation. As illustrated in Fig. 9a, the normalized ALP activity for all groups increased from 3 d to 7 d. When cells were cultured on MAO, they displayed high ALP values only next to TCPS. Compared with that on cTi, the ALP activity expression at 7 d was upshifted on MAO/PD but comprised a bit on MAO/Ag. Nevertheless, the MAO/Ag group caught up in the following week, ending with its ALP values pronouncedly higher than that of cTi. Additionally, BCIP/NBT based ALP staining for the overall ALP activity on the entire surface was performed at 7 d (Fig. 9b). The deeper the blue color, the stronger the expression of ALP. Note also that the MAO groups displayed the best staining feature and thus the highest osteo-differentiation activity, while the intensities of stains on other coatings were akin to (MAO/Ag) or stronger than (MAO/PD) those on the cTi. The collagen secretion and mineral deposition, being intrinsic characteristics of bone-like structures, were further estimated as osteogenic markers at the late stage. Larger areas of bright red color were observed on MAO plates from both Sirius red (for collagen) and ARS staining (for calcium), implying that ECM collagen was secreted and calcified by differentiating osteoblasts. Besides that, no significant difference could be distinguished among the collagen stains for cTi, MAO/PD and MAO/Ag. Again, the three groups in general were colored positive (red) by ARS staining. However, the MAO/PD and MAO/Ag groups demonstrated more locally better-stained spots/nodules (dashed circles), according to the optical/fluorescence images. Analysis of the corresponding images in Fig. 9a further demonstrated that ECM collagen staining was more extensive on MAO/PD and MAO/Ag, compared to cTi.
3.9. The feedback of cellular activities on Ag⁺ release in culture

According to our former results that nanosilver was almost completely consumed during a period of 28 d in PBS. However, after seeding MG-63 cells onto MAO/Ag samples, it was found that cells clad the AgNP-decorated micro/nano-porous surfaces as they spread (Fig. 5B; cells enclosed surrounding silver in their way of elongation, for 1 d) and multiplied (Fig. 9a; seemingly no silver could be observed, for 5 d). Following the methods illustrated in Fig. 10a (also see experimental section 2.10), we investigated the sample surfaces after segregating cells. As is evident in Fig. 10c, cells covered the surface gradually through the secreted ECM components; whereas, after trypsinizing cells, we rediscovered remnants of silver particles underneath, but with reduced size and amount. Afterwards, whether the coverage accelerated or decelerated the degradation of AgNPs was probed, and the results are presented in Fig. 10b. Generally, the release of Ag⁺ was slower with the presence of MG-63 cells and this trend was more apparent after a 2-week incubation.

3.10. Subcutaneous tissue compatibility in vivo

In addition to in vitro tests, in vivo assays were implemented to evaluate the subcutaneous tissue compatibility of MAO/Ag. Histological sections at 30 d post-implantation are given in Fig. 11a (left), with pure Ti as control. The “encapsulation” of the materials, a typical foreign-body reaction [40], was observed in both groups. Beside a few fusiform fibroblasts (red stars), neither phagocytosis nor inflammation in the ambient connective tissues was found, an implication that silver should have been released locally without any adverse reactions. Further, the fibroblast number and capsule thickness was used as a quantitative measure (Fig. 11b). No significant discrepancy (p > 0.05) was found for the amount of fibroblasts between the two groups. The average thickness of the fibroblast membrane was 193.8 ± 30.8 and 187.4 ± 23.5 μm for MAO/Ag and cTi, respectively. However, there was no statistical difference either.

Besides, surfaces of the encapsulated samples were examined (Fig. 11a, right). Nanosilver was undetectable on MAO/Ag (circled section), and the regions of adhered fibrous tissues (yellow arrows) were micro-sized, like those of cTi. Altogether, the in vivo results reflect a tissue reaction as mild as that elicited by the pristine titanium towards the Ag-releasing implant surfaces. According to the release test (Fig. 4a), over 50% of nanosilver was released after 1 d, and the Ag⁺ concentration was relatively high earlier but quite low later (after 1 week). Compared to cTi, the MAO/Ag surface was likely to evoke only slightly higher acute foreign-body reaction, but with its impact negligible in the long term (almost no silver after 4 weeks). Besides, topical Ag⁺ in adjacent cells was improved considerably by the biological adhesive nature of PD[26]; (6) it can enable relatively easy yet uniform loading of AgNPs onto different surfaces of complicated 3-D constructs, e.g. porous scaffolds. Utilizing the as-fabricated MAO/Ag coatings, we managed to balance the dilemma between bacteria-killing and regular cell functions/compatible tissue responses, and to concomitantly attain significantly enhanced corrosion resistance of titanium. Moreover, the procedures as a whole were good in facility, repeatability and adaptability toward clinical applications. The three pivotal elements of our TiO₂/Ag system are: the micro/nano-porous titania, adhesive PD, and antimicrobial AgNPs. Any two of them are associated directly or indirectly through specific chemical interactions to make an integrated robust coating. They contribute to the aforementioned multiple functionalities via their physical—chemical properties solely or a combination of them. For instance, the improved corrosion resistance is most likely due to the compact titania inner layer that underlays close to the substrate (Fig. S3); while the loading of silver could also exert an impact, possibly through the sacrifice of Ag before Ti, considering their different metal activities. For another, the dramatically enhanced hydrophilicity can be ascribed to the micro/nano-pore structure. It collaborates with the chemical reactivity and bioadhesion property of PD, and leads to an elevated amount and better conformation of absorbed protein, eventually stimulating osteoblasts to assume fast, elegant attachment and stretching. Most interestingly, an efficient antibacterial mode of “trap-killing” is enabled by the coordinated combination of the physical trap effect of micropores and the chemical killing property of silver (particles + ions), as illustrated in Fig. 5d and Fig. 6d.

4. Discussion

The experimental design, purpose, and summary of this study are briefly included in Fig. 13. In this work, we demonstrated a facile biomimetic strategy of designing hierarchical TiO₂/Ag coatings with multi-functionalities for application in bone related implants. Assisted by the PD anchor, AgNPs (~50 nm) were successfully tethered onto surfaces and impregnated within cavities of micro-nanoporous ceramic TiO₂ coatings, which grew spontaneously during MAO and bound tightly to the substrate. The use of PD had yielded a range of benefits: (1) it was necessary for the chelation of Ag⁺ and contributed partly to its reduction and high surface density; (2) it acted as a “bridge” between the underlying TiO₂ and superficial Ag feature, involving multiple interactions that made the TiO₂/Ag coating system robust; (3) it conjugated identically to both the peripheral surfaces and the interior porous walls due to its good hydrophilicity and high affinity towards metal ions, thus resulting in surface-immobilized and pore-embedded AgNPs at the same time; (4) the long-standing release of Ag⁺ was tailored by PD via the active interactions between the catechol groups and the Ag⁺. The inherent reductive capacity of PD can also resist the oxidative dissolution of AgNPs [42]; (5) the adhesion state of MG-63 cells was improved considerably by the biological adhesive nature of PD [26]; (6) it can enable relatively easy yet uniform loading of AgNPs onto different surfaces of complicated 3-D constructs, e.g. porous scaffolds. Utilizing the as-fabricated MAO/Ag coatings, we managed to balance the dilemma between bacteria-killing and regular cell functions/compatible tissue responses, and to concomitantly attain significantly enhanced corrosion resistance of titanium. Moreover, the procedures as a whole were good in facility, repeatability and adaptability toward clinical applications. The three pivotal elements of our TiO₂/Ag system are: the micro/nano-porous titania, adhesive PD, and antimicrobial AgNPs. Any two of them are associated directly or indirectly through specific chemical interactions to make an integrated robust coating. They contribute to the aforementioned multiple functionalities via their physical—chemical properties solely or a combination of them. For instance, the improved corrosion resistance is most likely due to the compact titania inner layer that underlays close to the substrate (Fig. S3); while the loading of silver could also exert an impact, possibly through the sacrifice of Ag before Ti, considering their different metal activities. For another, the dramatically enhanced hydrophilicity can be ascribed to the micro/nano-pore structure. It collaborates with the chemical reactivity and bioadhesion property of PD, and leads to an elevated amount and better conformation of absorbed protein, eventually stimulating osteoblasts to assume fast, elegant attachment and stretching. Most interestingly, an efficient antibacterial mode of “trap-killing” is enabled by the coordinated combination of the physical trap effect of micropores and the chemical killing property of silver (particles + ions), as illustrated in Fig. 5d and Fig. 6d.
For AgNP-bearing antibacterial coatings, the satisfactory loading density of silver and its active, prolonged release are two essential factors determining their success in practice. Owing to the porous structure (natural reservoir) and the metal-binding catechol anchor (controlled liberation [25,43]), we have first achieved a relatively high Ag density of over 8.57 mg/cm² as well as a dynamic yet durable liberation, i.e., 2.65, 3.81 and 8.14 mg/cm² (or 30.9, 55.4, 94.9% of total) released within 6 h, 1 d and 28 d, respectively (Fig. 4a). This is desirable as orthopedic/dental implants are particularly vulnerable to bacterial colonization during the first 6 h [17], while it also allows surgical traumas to heal and the implant to integrate with tissues, which empirically takes weeks. Compared with data in literature, the surface particle density as well as the released concentrations of Ag⁺ is appreciably high enough to eliminate potential infections. Necula et al. found that TiO₂/Ag coatings with ca. 2.1 μg/cm² of AgNPs on the surface could inactivate 98.0 ± 2% of S. aureus in 24 h [44]. Kim et al. [45] proposed the minimal inhibitory Ag⁺ dose to S. aureus to be 3.56 ng/mL after 24 h using 13.5-nm particles. Ho et al. suggested an initial release of only 0.0125–0.0167 μg/cm²/h to effectively kill S. aureus (over 99.5%) on PGA surgical sutures [46]. Gao et al. embedded Ag₂O particles into TiO₂ nanotubes, and proved that released Ag⁺ at concentrations ranging from 0.027 to 0.049 mg/cm² (at 1 d) was able to generate absolute antibacterial rate for both S. aureus and Escherichia coli (E. coli) bacterial strains [6]. Exceptionally, AgNPs here were charged both on the surface and inside the pores (Fig. 2; Fig. S3). Locally, the Ag⁺ concentrations surrounding or within the micro/nanopores should be much higher than that for the planar counterparts (see discussion below). Now, consider the bacterial crowds were approaching the MAO/Ag surface, most of the planktonic pioneers should be repelled and eliminated by surrounding ionized Ag⁺ (“release killing”; Fig. 5d1). Some of the settled cells directly touched the nanosilver-bearing surface, and underwent membrane damage by AgNPs per se (i.e. “contact killing” via permeabilization effects and their secondary products like ROS; Fig. 5d2). In actuality, these two often work together through interacting with the thiol group proteins, turning the DNA into a condensed form and disabling its replication ability [44]. The other survivors having a tendency for the micropores [47,48], are expected to be entrapped (Fig. 5d3; step 1). This trend may be encouraged by the electrostatic

Fig. 12. Adaptability of the coating procedure to be applied to wire-shaped Ti and openporous Ti6Al4V specimens. (a) Microstructures of the AgNP-deposited micro/nanoporous cTi wires (a1) and Ti6Al4V scaffolds (a2). Insets in top right and bottom left are zoomed-in SEM graphs, and optical images, respectively (see also Fig. S9). (b) Compressive properties of scaffolds with and without MAO treatment at 320 V for 5 min. (c) Fluorescent images of nuclei (blue)/cytoskeleton (green) for MG-63 cells cultivated on modified wires (5 d) and scaffolds (14 d) revealing the cells migrating along the wires (c1) and their relatively even spatial growth into the porous surfaces of the 3-D scaffolds (c2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 13. Schematic diagram of the biomimetic strategy of devising hierarchical TiO$_2$/Ag coatings with multi-functionalities for application in bone tissue engineering.

Fig. 14. The proposed mechanism of MG-63 activities on MA0/Ag surfaces, inducing minimal toxicity and maintaining normal osteoblast functions.
attraction between the bacterial membrane (negatively charged) and silver-containing pores (AgNPs with chemisorbed Ag⁺ were positively charged [42]). Besides, that process can be further accelerated by hydrodynamic effects arising from the suction of solution into porous materials. In our study, most of the MAO pores (400–1000 nm, Fig. 2d) were near-custom made for one or two cells of S. aureus, and their interior walls were similarly charged with particulate silver (Fig. 5c). Thus, for the captured spherical bacteria, they were likely to assume multiple-point contact with those pore-presented nanoparticles (contrarily, only single-point contact was available to bacteria adhered to planar surfaces) (Fig. 5d3; step 2). This greatly enhanced the contact between bacterial membrane and the AgNPs and thus improved the killing efficiency via contact mode of actions (Fig. 5c5). In addition to that, the above solution suction increased the collision frequency between the porous walls and bacteria [49], promoting some nanosilver to bind with the peptidoglycan membrane via electrostatic force and specific interaction such as Ag-thiol bonds. Meanwhile, water infiltrated through the pits to oxidize the nanoparticles inside, thus giving off Ag⁺ continually. The metabolism of bacteria has also been shown to acidify the environment which quickens Ag⁺ release [50]. Consequently, locally high Ag⁺ concentration was produced within the pores. Therefore, when the “prey” was engulged, it had little chance to escape, eventually going through persistent, harsh particulate/ionic attacks that destroyed the membrane integrity (Fig. 5d3; step 3, 4). On the other hand, subsequent exponential bacterial growth under the as-mentioned confined room can be spatially restrained, too. Besides, those in deeper pores are likely to be biologically disconnected from their environment and may have difficulty consuming glucose for propagation [51]. These physical factors, along with the durable silver liberation, may have played a role in controlling biofilm development (Fig. 6d).

As we can see, either in the adhesion stage or in the long run, the physical entrapment and chemical silver coordinated with each other to engender the so-called “trap & kill” mode. Any attempt to strictly separate their contributions, to the best of our current knowledge, may simply fail. More to be sure, the chemical effects from nanosilver are crucial. Without silver, the micropores can never realize their full entrapping ability. More precisely, they could not actively inactivate or kill bacteria, and those as-mentioned effects are too weak to prevent bacterial colonization on the surface during prolonged culture (e.g. MAO and MAO/PD groups in Fig. 6a, b). In a scientific sense, however, such “trap-killing” phenomenon deserve special emphasis as they provide a different approach to the design of advanced devices with customized pores or channels (e.g. size and shape) and functionalized germicides that can selectively detect and remove, or capture and kill micrograms for biomedical care and environment management [47,48,52]. It is also noteworthy that only shape-sphere-shaped S. aureus were utilized in our experiment, primarily for their pathogenicity in orthopedics and partially for their ease of being trapped within the cavities. Nevertheless, the application of the as-demonstrated TiO₂/Ag coatings goes beyond that strain. Silver has a broad antibacterial spectrum against gram-positive (S. aureus) and gram-negative (E. coli) strains both at very low concentrations (ppb) [6,45,53]. Due to the thinner peptidoglycan wall, gram-negative bacteria have higher vulnerability to nanosilver than gram-positive bacteria [50,54], a valid phenomenon in literature [6]. The silver concentrations from our coatings were potent enough to kill the S. aureus with a rigid network-structured cell wall. The released Ag⁺ ions should face a much smaller barrier, in principle, on the way diffusing and penetrating into the inner membrane of gram-negative bacteria, ending with stronger toxic effects. Similarly, the contact-killing efficacy by particles can be enhanced. 

Dispersed nanosilver in suspension/colloid is prevalently believed to be eukaryote toxic, with analogous risks of cellular uptake, oxidative stress, and membrane damage compared to that for bacteria [8,9]. However, our knowledge about the biological outcomes of mammalian cells/tissues contacting directly with AgNP-bearing surfaces is thus far limited. Intriguingly, our results revealed that (1) MG-63 cells were eventually tightly-adsorbed (good focal adhesion assembly) and wide-spread in the presence of AgNPs, though the process was hindered in part; (2) the majority of cells proliferated well, whereas apoptosis/death of individuals did happen in the early stage; (3) silver-releasing was associated with the short-term, slight increase in LDH activity (cell stress), but it exerted no inhibition on the osteogenic differentiation properties in the long term, which were actually preserved or even promoted; (4) the subcutaneous research in vivo demonstrated minimal inflammatory reactions for up to a month. These things are especially encouraging since an ideal biomaterial for bone implantation, aside from the ability to lower the risk of infection, must first have biocompatibility to interact safely with host tissues, and then, they should not hinder and are even expected to restore or optimize the implant functionalities.

Gao et al. fabricated NT-Ag2O arrays with good cytocompatibility, osteoblast spreading, proliferation and differentiation [6]. However, the particles existed actually in the nanotube wall rather than on the surface, making contact and response by osteoblasts difficult. Qin et al. prepared silver-ion-implanted stainless steel with cytocompatible surfaces that can further enhance osteogenic differentiation [7]. Still, the particles were too small (7 nm) to be effectively perceived by osteoblasts, yet the authors did not look closely at the nanosilver–cell interfaces to see what had happened. So what interests us is how the AgNP-presented surfaces interplay with osteoblasts, which as far as we know, has rarely been reported. As illustrated in Fig. 14, the surface initiated self-regulation of MG-63 cells (the way cells “feel” MAO/Ag surfaces) and its feedback on silver release (the way AgNPs “feel” cells) may constitute a part of that. On one hand, the physio–chemical nature of material surfaces where AgNPs are deposited is crucial for eliciting favorable cellular activities. An envisioned material for biomedical applications, should help initiate correctly a cascade of biological events at material/cell interfaces, which briefly encompass hydration-shell formation, serum protein adsorption, ligand-receptor recognition between serum and cell membrane, and the following functional cell behaviors (i.e. adhesion, spreading, proliferation, migration and differentiation) [17]. Normally, a number of surface properties, such as topography, chemical groups and free energy all exert potent impacts. For example in our work, the MAO/Ag surface with high specific surface area, good hydrophilicity, and abundant adhesive residues was chosen, and it proved to have great capability of retaining proteins and presenting them accurately for cell adhesion and spreading. Besides, the ECM-mimic nano-micro topography/structure is known to stimulate osteoblasts to proliferate, differentiate and maturate, via contact-directed integrin clustering and intracellular signaling [55]. This has also been evidenced in our study with the obviously increased cell viability, ALP activity, and collagen/calcium contents for coated groups (Fig. 8).

On the other hand, cells are generally stress-responsive to surfaces decorated with silver, so they, in most likelihood, would give feedback to material interfaces and affect their properties. As soon as they anchor to spread, metabolizing cells secrete ECM, a cross-linked network of fibrous macromolecules (majorly collagen) assembled with tissue specific compositions (proteoglycans and glycoproteins) [55], and these ECM components would have high wrapping tendency around nanoparticles to form multi-layered collagen/protein “coronas” [56] by which means AgNPs are entombed. This entombment could yield potentially three
extremely important benefits (Fig. 14): (1) the chance of particulate nanosilver shedding into medium to be internalized is further minimized; (2) the contact induced cytotoxicity from the particle itself could be excluded; (3) the release rate of Ag\(^{+}\) might be reduced due to the blocking effect of outermost ECM “coating” especially as it thickens later on. As to silver’s effects on cells, an over-dose harms while a small one may profit [6]. It is well-confirmed that AgNPs accelerate the proliferation of hepatoma cell at low doses (0.5 μg/ml) [11], which possibly accounts for the promoted cell viability in our study (Ag\(^{+}\): average 0.48 μg/well/d in the first 5 d). K. Comfort et al. also inferred that eukaryocytes are able to tackle prolonged, low levels (in the sub–ppm range) of AgNP treatment [57]. Meanwhile, Pauksch et al. found that even a dose of 1 μg/g AgNO\(_3\) has no influence on osteoblast differentiation [8]. In that way, the exponential growth of cells induces continuing production of collagen and comparatively high ALP values (indicative of an alkaline micro-environment favorable for bone formation), and it facilitates the generation of a mineralized matrix in middle and late cellular stage. Expectedly, this in return would further retard the liberation of Ag\(^{+}\). Hereto, an interesting but tough question concerns the molecular mechanism of how the foregoing cell-Ag interactions occur, and its universality for other surfaces/cells remains unknown. However, the issues are clear—bacteria fail to colonize the MAO/Ag surfaces while MG-63 cells attach, spread, proliferate, and differentiate normally on them, which is essential to guarantee the safe, high quality implantation.

Promising biomaterials are usually built into biomedical implants or devices with specific shapes and complex architectures (e.g. dental implant, hip and knee prostheses) to customize the anatomy of individual patients. In light of this, any candidate technique for surface engineering should allow a suitable degree of flexibility to be applied to materials of complicated geometries. For instance, it is difficult to touch the non-planar surfaces or internal parts of three-dimensional constructs via technologies such as plasma spraying and magnetron sputtering [6,16]. Contrarily, MAO process, PD formation and AgNP immobilization, as demonstrated, are inherent wet-chemical treatments. This enables them adaptive to virtually any type and shape of Ti-based implants/devices (Fig. 12a). Besides, they are relatively simple, facile, and green, and bear good scalability, hence boding well for widespread clinical use. A recent clinical study using commercial MAO-coated Ti-allow hip stems (Bencox, Korea) to treat displaced femoral neck fractures has proven a satisfactory outcome [58]. Further, the MAO modification did not alter much the intrinsic mechanical property of the material (Fig. 12b). After decoration of silver, the implants could still sustain normal biological activity of osteoblasts (Fig. 12c). However, there are some limitations: (1) MAO only works on valve metals Al, Ti, Mg, Ta, W, Zn, and Zr and their alloys; (2) the impact of MAO on materials’ mechanical properties can vary with specimen type and size and treatment condition; (3) given the difficulty to homogeneously irradiate light to 3-D complicated structures, it is better to seek alternatives (e.g. other reductants, like ascorbic acid) for further reduction of silver. Thus, further improvement of the technique itself as well as more comprehensive animal studies for the in-vivo efficiency should be carried out before we can move forward with clinical application.

5. Conclusions

In this paper, we demonstrated a simple, controllable and cost-effective approach to produce biomimetic hierarchical TiO\(_2\)/Ag coatings by combining the MAO technique with mussel-inspired metallization. The resultant coatings showed “trap-killing” antimicrobial potency whilst they elicited neither repressive effect on cell functions of osteoblast-like MG-63 in vitro nor serious inflammatory response in a rabbit model in vivo. The coating procedures are good in maneuverability, repeatability and practicability regarding biological and medical devices with complicated shapes and architectures. Taken together, our findings provide preliminary insights into the development of alternative antibacterial strategies, and into the issue of how mammal cells interact with AgNP modified surfaces. This study should be helpful guidance to implant developers/regulators on the widespread and safe use of silver-coated medical implants in the future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.10.035.

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