Antibacterial Surface Design of Titanium-Based Biomaterials for Enhanced Bacteria-Killing and Cell-Assisting Functions Against Periprosthetic Joint Infection


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Supporting Information

ABSTRACT: Periprosthetic joint infection (PJI) is one of the formidable and recalcitrant complications after orthopedic surgery, and inhibiting biofilm formation on the implant surface is considered crucial to prophylaxis of PJI. However, it has recently been demonstrated that free-floating biofilm-like aggregates in the local body fluid and bacterial colonization on the implant and peri-implant tissues can coexist and are involved in the pathogenesis of PJI. An effective surface with both contact-killing and release-killing antimicrobial capabilities can potentially abate these concerns and minimize PJI caused by adherent/planktonic bacteria. Herein, Ag nanoparticles (NPs) are embedded in titania (TiO₂) nanotubes by anodic oxidation and plasma immersion ion implantation (PIII) to form a contact-killing surface. Vancomycin is then incorporated into the nanotubes by vacuum extraction and lyophilization to produce the release-killing effect. A novel clinical PJI model system involving both in vitro and in vivo use of methicillin-resistant Staphylococcus aureus (MRSA) ST239 is established to systematically evaluate the antibacterial properties of the hybrid surface against planktonic and sessile bacteria. The vancomycin-loaded and Ag-implanted TiO₂ nanotubular surface exhibits excellent antimicrobial and antibiofilm effects against planktonic/adherent bacteria without appreciable silver ion release. The fibroblasts/bacteria cocultures reveal that the surface can help fibroblasts to combat bacteria. We first utilize the nanoarchitecture of implant surface as a bridge between the inorganic bactericide (Ag NPs) and organic antibacterial agent (vancomycin) to achieve total victory in the battle of PJI. The combination of contact-killing and release-killing together with cell-assisting function also provides a novel and effective strategy to mitigate bacterial infection and biofilm formation on biomaterials and has large potential in orthopedic applications.

KEYWORDS: antibiotics, silver nanoparticles, titania nanotubes, cells/bacteria coculturing, antimicrobial properties

1. INTRODUCTION

As the demand for total joint arthroplasty (TJA) is increasing due to the aging population, periprosthetic joint infection (PJI) is becoming a formidable and recalcitrant complication requiring costly treatment, multiple operations, and extended hospitalization. PJI can be categorized as early (less than 3 months after surgery), delayed (3 to 24 months), or late (more than 2 years). Early PJIs are generally caused by high virulent bacteria such as Staphylococcus aureus (S. aureus), especially methicillin-resistant S. aureus (MRSA). MRSA-PJI involves the interactions among bacteria, biomaterials, and tissues, and the formation of a biofilm composed of bacteria and extracellular matrix plays a vital role in the pathogenesis of PJI. After a mature biofilm has developed, bacteria can leave the biofilm penetrating the biomaterials surface into surrounding fluids and tissues. Although radical debridement, empirical
antibiotics, and prosthesis retention have been utilized, MRSA-PJI in the early stage is difficult to treat and can affect the prognosis.10,11 Therefore, it is critical to develop practical prophylactic strategies to mitigate early MRSA-PJI.

Antimicrobial coatings are useful in early MRSA-PJI prevention, and anti-infective surfaces can be primarily classified into antimicrobial-agent-releasing or contact-killing ones,12–15 and the relevant animal models have mainly concentrated on implant and bone infection to mimic the pathogenesis of implant-related osteomyelitis.16,17 Nevertheless, early PJI caused by hospital-associated (HA) pathogens occurs in the special intra-articular internal environment that is completely different with osteomyelitis in the bone medullary cavity.18 Furthermore, the impaired soft tissue surrounding the prosthesis makes the joint susceptible to exogenous infection during perioperation.18 Bacteria can be cultured from peri-prosthetic soft tissues in PJI patients,19,20 and it is essential to establish an effective animal model for early MRSA-PJI to systematically evaluate the intra-articular antibacterial effects of the coatings. The properties of antimicrobial biomaterials are usually assessed by single cultures with tissue cells or bacteria in vitro. However, considering that about 30% of orthopedic surgeries are in a bacterially contaminated state21 and early PJI are generally induced during the insertion of prostheses,22 coculturing with both tissue cells and bacteria needs to be performed on anti-infective coatings to better mimic the physiological conditions.

Silver nanoparticles (Ag NPs) that are immobilized on titanium by plasma immersion ion implantation (PIII) produce long-lasting in vitro and in vivo antibiofilm activity against surface-adhered bacteria without producing drug-resistant bacteria and cytotoxicity.16,22,23 Nonetheless, MRSA has recently been found to form strong free-floating biofilm-like aggregates in human synovial fluid (SF),24 and colonization of S. aureus in peri-implant tissues may also be a general phenomenon in implant infection.9,14,25 Consequently, a simple contact-killing surface may have limited or no effects on free-floating biofilm-like aggregates or bacterial colonization in peri-implant tissues,16,26 and such antiadhesive strategy may not satisfy clinical needs. Hence, surface functionalization should combine contact-killing and release-killing activities for prophylaxis of early MRSA-PJI. Anodically oxidized titania nanotubes (TiO2–NT) have been used as the reservoir to load antibacterial agents to provide short-term antimicrobial effects due to the large specific surface area, “half-open” nanostructures, and excellent biocompatibility.27,28 Traditionally, owing to the lack of better alternatives, vancomycin with powerful activity against all staphylococci is the main treatment for MRSA infection.29 It can penetrate biofilms and decrease the number of bacteria.14,30 Meanwhile, Ag NPs are also the most hopeful candidate for controlling bacterial infection due to their broad antimicrobial spectrum,31 and synergetic combination of them with antibiotics by means of the nanotechnology may be a new and powerful approach to overcome bacterial infection.

In this work, by considering the complex pathogenic process of MRSA-PJI, we design and prepare a sophisticated anti-infective coating on titanium. Ag NPs are embedded in TiO2 nanotubes by PIII to attain the long-term contact-killing ability and vancomycin is loaded into the TiO2 nanotubes by vacuum extraction to provide short-term release-killing. The antibacterial properties against planktonic and sessile clinical epidemic MRSA are determined in vitro and the biological effects with fibroblasts and MRSA simultaneously on the surface are studied by coculturing in vitro. Furthermore, the antibiofilm capacity, anti-infection efficiency, and cellular biological effects in the peri-implant soft-tissues are evaluated by the intra-articular rabbit PJI model in vivo.

2. MATERIALS AND METHODS

2.1. Sample Preparation and Characterization. The specimens used in the in vitro experiments were commercial pure Ti plates (10 mm × 10 mm × 1.0 mm), and in the in vivo experiments, the samples were Ti screws (3.5 mm in diameter and 11 mm long) with Ti washers (3.5 mm inner diameter, 10.5 mm external diameter, and 1.0 mm thick). The Ti samples were ultrasonically pretreated in an acidic solution (HNO3:HF = 5:1 v/v) at room temperature for 10 min, ultrasonically cleaned in ethanol and deionized water several times, and dried in air. The TiO2 nanotubes were prepared by anodic oxidation of titanium in an electrolyte with 0.5 vol % hydrofluoric acid at 20 V for 30 min.32 Afterward, by performing filtered cathodic arc PIII, Ag NPs were implanted and immobilized in situ in the TiO2 nanotubes. The important processing parameters are listed in Table 1.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Anodic Oxidation</th>
<th>Silver Plasma Immersion Ion Implantation (Ag PIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>20 30</td>
<td>6 2.5 × 10−3 90 −30</td>
</tr>
<tr>
<td>NT-Ag</td>
<td>20 30</td>
<td>6 2.5 × 10−3 90 −30</td>
</tr>
<tr>
<td>NT-V</td>
<td>20 30</td>
<td>6 2.5 × 10−3 90 −30</td>
</tr>
<tr>
<td>NT-Ag-V</td>
<td>20 30</td>
<td>6 2.5 × 10−3 90 −30</td>
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Table 1. Important Parameters in Anodic Oxidation and Plasma Immersion Ion Implantation (PIII)

All the specimens were thoroughly cleaned in water and sterilized in 75% (v/v) ethanol.

Vancomycin (Sigma-Aldrich, St Louis, MO, USA) was introduced to the TiO2 nanotubes by lyophilization and vacuum drying27,33, with a vancomycin solution (100 mg/mL) in phosphate-buffered saline (PBS; Sigma).33 After cleaning with deionized water and drying, 20 μL of the vancomycin solution were pipetted onto the specimen surface and spread evenly by gentle shaking. The samples were freeze-dried under vacuum at −75 °C for 2 h and rinsed quickly with 1 mL of PBS to remove superfluous vancomycin. The flushed fluid was collected for subsequent analysis. Four groups were tested: TiO2 nanotubes (control, denoted as NT), Ag PIII TiO2 nanotubes (denoted as NT-Ag), vancomycin-loaded TiO2 nanotubes (denoted as NT-V), and vancomycin-loaded NT-Ag (designated as NT-Ag-V).

The surface morphology and chemistry were investigated by field-emission scanning electron microscopy (FESEM; Magellan 400, FEI, USA) equipped with energy-dispersive X-ray spectroscopy (EDS). The crystallinity was studied by X-ray diffraction (XRD; Ultima IV, Rigaku, Japan) with Cu Kα (λ = 1.541 Å) at a glancing angle of 1°. The phase was identified by referencing to the standard JCPDS database. The Raman spectra were acquired on a Raman microscope (LabRAM, Horiba Jobin Yvon, France) equipped with an Ar ion laser at 20 mW (514.5 nm). X-ray photoelectron spectroscopy (XPS; PHI 5800, Physical Electronics Inc., Eden Prairie, MN) was employed to determine and field-emission transmission electron microscopy (TEM; JEM-2100F, JEOL Ltd, Japan) was performed at 200 kV. Materials were scraped from the samples and dispersed in ethanol ultrasonically, and a droplet of the suspension was placed on a holey carbon grid covered with porous carbon film.
2.2. Bacterial Isolate and Characterization. One epidemic clinical isolate (MRSA, ST239) originated from the inpatient with implant infection at The Orthopedics Department of Shanghai Jiao Tong University Affiliated Sixth People’s Hospital, the largest orthopedics trauma clinical medical center in eastern China. Our study was approved by Shanghai Jiao Tong University Affiliated Sixth People’s Hospital Ethics Committee (Ethical code, 2014-63).

2.3. Ag and Vancomycin Release Tests. The NT-Ag and NT-Ag-V samples were soaked in 10 mL of phosphate buffered saline (PBS) or trypticase soy broth (TSB) for 1, 4, 7, 14, 21, and 28 days at 37 °C without agitation. The leachates were collected at each time point for quantitative analysis by inductively coupled plasma optical emission spectrometry (ICP-OES, Nu Instruments, Wrexham, U.K.). The protocol to determine the release of vancomycin from the NTs followed previous studies. In brief, the NT-V and NT-Ag-V samples were individually immersed in 1 mL of PBS on a 24-well plate at room temperature and agitated at 70 rpm. After the specific time intervals, a 500 μL of the sample were taken to determine the release kinetics, and then 500 μL of fresh PBS were added to the remaining solution each time. The extracted solutions were collected periodically for up to 4 h and analyzed for vancomycin concentration by ultraviolet spectrophotometry (Inesa Analytical Instrument Co., LTD, Shanghai, China) at 237 nm.33 A standard curve obtained with known vancomycin concentrations was adopted to determine the vancomycin concentrations in the PBS.

2.4. In Vitro Cytotoxicity Evaluation. The fibroblast-like cell line (HT1080, Shanghai Institute of Biological Science, Chinese Academy of Sciences, China) was employed to evaluate the cytotoxicity of the specimens, and the cytotoxicity was examined by the Cell Counting Kit-8 assay (CCK-8, Beyotime Bio-Tech, China). First, the four different specimens were placed on 24-well plates. Cells with a density of 2 × 10^4/well and 1 mL of the growth medium composed of DMEM (Dulbecco’s modified Eagle’s medium, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) were added to the wells containing the samples. They were cultured for 1 and 4 days in a humidified atmosphere of 5% CO2 at 37 °C. After each period, the culture medium was completely removed, and 1.0 mL of fresh medium containing the samples. They were cultured for 1 and 4 days in a humidified atmosphere of 5% CO2 at 37 °C. In this assay, each Raα value of the adhered bacteria on the surface was calculated as aforementioned, and the procedure was repeated three times.

2.5. In Vitro Antibacterial Assay. In the in vitro antibacterial tests, the clinical epidemic MRSA (ST239) was cultivated in TSB medium overnight at 37 °C and the concentration of inocula was adjusted to 10^9 colony forming units (CFU)/mL through McFarland in TSB supplemented with 1% (w/v) glucose (TSBG) or 1% TSBG supplemented with synovial fluid obtained from outpatients (TSBG +SF). One mL of the bacterial suspension was added to the 24-well plates on which each foil specimen was placed and statically incubated for 1, 6, 12, and 24 h at 37 °C. At a given time point, the specimens were removed from the suspension and nonadherent bacteria on the surface were gently dislodged with PBS three times. The viable counts of planktonic bacteria in the original culture medium were determined by serial dilution and the spread-plate method (SPM).16 The number of CFU on the sheep blood agar (SBA) was counted according to the National Standard of China GB/T 4789.2 protocol. In addition, each specimen was put into 1 mL of sterile PBS followed by ultrasonic vibration (150 W, B3505S-2T, Branson Ultrasound Co., Shanghai, China) for 10 min to dislodge the adhered bacteria and rapid vortex mixing (Vortex Genie 2, Scientific Industries, Bohemia, NY) for 1 min. The suspension was taken and living bacteria were counted by SPM. Each test was repeated three times.

The antibacterial rates (Ra) for planktonic and adherent bacteria were calculated based on the following formulas: (1) the antibacterial ratio of planktonic bacteria in the culture medium (RAP) = (A-B)/A × 100%, and (2) the antibacterial ratio of adherent bacteria on the sample (Raα) = (C-D)/C × 100%, where B is the mean number of viable bacteria in the culture medium inoculated with NT, NT-Ag, NT-V, or NT-Ag-V, A is the mean number of viable bacteria in the culture medium inoculated with NT sample, D is the mean number of viable bacteria attached on the surface of NT, NT-Ag, NT-V, or NT-Ag-V, and C is the mean number of viable bacteria attached on the surface of NT. The number of planktonic bacteria was also estimated by improved SPM. 100 μL of the bacteria suspension from the bacteria-specimen coculturing system was uniformly spread on the SBA and incubated at 37 °C overnight before typical pictures were taken.

2.6. Evaluation of in Vitro Bacterial Biofilm by SEM and CLSM. To assess the antiadhesive effect, the biofilms on the foils were qualitatively examined by scanning electron microscopy (SEM; JEOL JSM-6310LV, Japan) and confocal laser scanning microscopy (CLSM CLS10 meta; Zeiss, Germany). The samples with adherent bacteria were fixed with 2.5% glutaraldehyde overnight at 4 °C and dehydrated with a series of gradient alcohol (50, 70, 80, 90, 95, and 100 v/v %). After dehydration, the specimens were critical-point dried, coated with gold in a sputter coater, and examined by SEM. In the CLSM test, the specimens were placed on a 24-well plate, stained with 1 mL of combined dye (LIVE/DEAD BacLight bacteria viability kits, Invitrogen) for 20 min, and imaged by CLSM. The living bacteria with intact membranes were stained green whereas dead ones with impaired membranes were stained red.

2.7. Longevity of the Antiadhesive Ability on NT-Ag. In order to evaluate the long-term antiadhesive effect of Ag–PiH, NT and NT-Ag were incubated separately in sterile PBS for 28 days. At given time points (1, 7, 14, 21, and 28 d), 1 mL of the bacteria suspension (10^6 CFU/mL) was put on the specimens overnight at 37 °C. In this assay, each Raα value of the adhered bacteria on the surface was calculated as aforementioned, and the procedure was repeated three times.

2.8. Bacteria-Tissue Cells Cocultures. According to the previous report,34 in vitro coculture was carried out with MRSA (ST239) and fibroblast cells on the specimens. Twenty μL of the bacterial suspensions (10^6 CFU/mL) were distributed on the samples and incubated at 37 °C for 2 h. Afterward, fibroblast cells suspended in the modified culture medium which included the cell growth medium and 2% (v/v) TSB were seeded on the bacteria-contaminated samples with a density of 2 × 10^4 cells/well on a new 24-well plate. MRSA (ST239) and fibroblast cells were cocultured in a humidified 5% CO2 at 37 °C for 3 days. Finally, the fibroblast cells were fixed in 4% paraformaldehyde, imaged by SEM after serial dehydration, dried, gold coated, and examined by fluorescence microscopy (Leica Microsystems LTD, Germany) after staining with TRITC-phalloidin and DAPI. The quantitative results were expressed as the surface coverage by fibroblast cells in the presence of adherent bacteria and analyzed according to the aforementioned reference. All the tests were performed in triplicate.

2.9. Animal Surgery. 2.9.1. New Rabbit PJI Model. The animal experimental protocol was approved by the Animal Care and Ethics Committee of Sixth Peoples Hospital affiliated to Shanghai Jiao Tong University, School of Medicine. 32 New Zealand White rabbits weighing an average of 3.2 kg (2.5–4.0 kg) were used. The rabbit PJI model was described previously5,56 and Zhai et al.35 found that 1 mL of inoculum with 10^6 CFUs S. epidermidis injected into the knee joint formed a stable PJI model. Considering that the virulence of MRSA surpassed that of the S. epidermidis, 10^6 CFUs could be the appropriate dose to make the improved PJI model. The rabbits were randomly divided into 4 groups (8 in each group, NT, NT-Ag, NT-V, and NT-Ag-V).

Surgery was performed under general anesthesia with 3% pentobarbital (1 mL/kg). After shaving the hairs around left knee, the lower extremity was sterilized and draped with sterile surgical drapes. A 3 cm longitudinal skin incision was made over the lateral aspect of the left knee. Arthrotomy was implemented fully exposing the lateral femoral condyle and lateral collateral ligament (LCL). With a 3.5 mm sterile drill bit, a hole parallel to the coronal axis was drilled in the lateral femoral condyle anterior to the LCL. A metal screw and metal washer were implanted into the knee joint. The joint capsule, deep fascia, and skin were closed by 4–0/3–0 monofilament nylon suture. After surgery, 100 μL of inoculum with 10^6 CFUs of MRSA were injected into the knee joint near the washer. Every animal received acetaminophen 30 mg/kg/day for analgesia for 3 days after...
operation. After surgery, the body temperature and weight of each rabbit were recorded and the rabbits were not found to have fever or weight loss.

2.9.2. Gross Observation and Radiographic Evaluation of Inflammatory Reaction. Fifteen days after surgery, high-resolution lateral radiographs were acquired when the rabbits were under anesthesia with 3% pentobarbital. In the following days, the rabbits were euthanized with an overdose of pentobarbital and the skin of the knee joint and joint capsule were successively sectioned under sterile conditions. The inflammatory condition was graded on an improved four point scale. The grade one articular cavity included no purulent effusion and necrotic tissue. The synovial fluid was clear and the screw tail and washer were covered by synovial tissue. The grade two articular cavity showed mild inflammation with little necrotic tissue and no obvious pus. The synovial fluid was slightly muddy and the screw tail and washer were covered by synovial tissue. The grade three articular cavity exhibited moderate inflammation with necrotic tissue and more purulence. The synovial fluid was turbid and the screw tail and washer were covered by infectious synovial membrane and necrotic tissue. The grade four articular cavity showed severe inflammation with white sticky pus and a large number of necrotic tissue. The joint fluid was not visible and joint capsule was full of purulence. The screw tail and washer were covered by white sticky tissue. The gross appearance of each treated knee was given a score as follows: 0 = grade one, 1 = grade two, 2 = grade three, and 3 = grade four. Three independent observers assessed the inflammatory reaction and calculated the corresponding score in a blind fashion.

2.9.3. CFU Counting of Metal Washer and Surrounding Tissues In Vivo. After harvesting from the knee joint, the washers were washed gently three times with normal saline (NS) to dislodge the planktonic bacteria. The peri-prosthetic soft tissues were weighed and calculated the corresponding score in a blind fashion.

2.9.4. SEM of Metal Washer In Vivo. After harvesting from the knee joint, the washers were washed gently three times with normal saline (NS) to dislodge the planktonic bacteria and then immersed in tubes containing 1 mL of NS. The samples were sonicated at 150 kHz for 15 min and vortexed rapidly for 1 min in order to break the biofilm aggregates and detach the adhered bacteria. The peri-prosthetic soft tissues were weighed and homogenized using a high-speed homogenizer (Jingxin Industrial Limited Company, Shanghai, China) in tubes with 1 mL of NS and 100 μL of the diluted were spread on SBA in duplicates. The plates were incubated overnight at 37 °C and the viable bacterial colonies were counted as mean ± standard deviation (S.D.) and expressed by log_{10} CFU/per metal washer and log_{10} CFU/g of peri-implant soft tissue.

2.9.5. Histological Analysis. The experimental knee joints were isolated and peri-implant soft tissues were dissected. The tissues were fixed in 10% neutral buffered formalin for 72 h, dehydrated in serial alcohol solutions, and infiltrated with xylene and paraffin before paraffin embedding. Sagittal sections of the soft tissues were cut into sections (approximately 5 μm thickness) by a sliding microtome (Leica, Hamburg, Germany), deparaffinized in xylene, and stained with Mayer’s Hematoxylin and Eosin (H&E) and May-Grünwald Giemsa for histological evaluation using an optical microscope.

2.10. Statistical Analysis. Each experiment was repeated three times, and the results were expressed as means ± standard deviations. The one-way analysis of variance (ANOVA) was combined with a Student–Newman–Keuls (SNK) post hoc test to determine the statistical significance. The differences in the numbers of CFU were analyzed by the two-tailed Mann–Whitney rank sum test. In all the tests, P values of <0.05 or <0.01 were considered statistically significant.

3. RESULTS

3.1. Sample Preparation and Characterization. In a typical process as shown in Figure 1, the precleaned Ti underwent anodic oxidation leading to in situ growth of the TiO₂ nanotube arrays on the surface. After PIII, Ag nanoparticles were immobilized in the TiO₂ nanotubes and the samples were subsequently loaded with vancomycin by vacuum extraction. As shown in Figure 2a-b, a homogeneous nanoporous topography composed of sparsely distributed TiO₂ nanotubes appears on the Ti surface after anodic oxidation, with a tube diameter of less than 100 nm and tube spacing of less than 50 nm from each other. This type of nanotube arrays is different from the closely distributed nanotubes because they have a larger specific surface area bonding well for loading of antibiotics. The inset in Figure 2b shows the typical morphology of an individual TiO₂ nanotube. Figure 2c exhibits the secondary electron (SE) image of the Ag PIII TiO₂ nanotubes (NT-Ag). The pristine nanotube structure is sputtered to some extent during Ag PIII and no significant Ag nanoparticles are observed from the nanotube surface, although there are a few individual ones (indicated by red arrow). The backscattered electron (BSE) image acquired from the same region is displayed in Figure 2d. Tiny Ag nanoparticles are observed on the nanotube surface (white dots) indicated by not only the red arrow but also the green one. The image before Ag nanoparticles “breaking through the soil” is captured. Here, a vacuum extraction method is implemented to load vancomycin into the TiO₂ nanotubes with/without Ag PIII. With regard to the NT-V sample and as shown in Figure 2e, the TiO₂ nanotubes are covered/loaded with a homogeneous layer of vancomycin showing a topography different from that of the TiO₂ nanotubes. This is further evidenced by the high-magnification image of the vancomycin-loaded TiO₂ nanotubes in Figure 2f, in which the purple arrow shows that the tube space is filled with vancomycin. This is corroborated by the higher magnification inset. A layer of vancomycin is also vacuum-extracted onto the inner wall of the TiO₂ nanotubes (indicated by purple arrows). With regard to the vancomycin-loaded Ag-implanted TiO₂ nanotubes (i.e., NT-Ag-V), most of the Ag nanoparticles are not on the nanotubes surface in the SE image, despite the presence of a few individual exposed nanoparticles (red arrow). The corresponding BSE image
shows these tiny Ag nanoparticles (green arrow) which are confirmed by the high-magnification images in the corresponding insets. Meanwhile, the TiO₂ nanotubes are covered by a homogeneous layer of vancomycin, even on the tube inner wall (purple arrow).

The TiO₂ nanotubes are split to investigate the cross sections. Figure 3a shows the cross-section of the pristine TiO₂ nanotubes showing a bamboo-like morphology. Microarea elemental analysis is conducted by EDS (indicated by yellow arrow) and only Ti, O, and C are detected (Figure 3b,c). The cross-sectional SE image of NT-Ag is displayed in Figure 3d. Combining the corresponding BSE image (Figure 3e), there are both embedded Ag nanoparticles (green arrow) and exposed Ag nanoparticles (red arrow) in/on the TiO₂ nanotubes. EDS shows the presence of Ag (Figure 3f). Concerning sample NT-V, the obvious topography contrast of the tube inner wall (Figure 3g) implies successful loading of vancomycin. The elemental contents after the vancomycin treatment increase (C, 3.48 at. % to 13.40 at. %; O, 36.95 at. % to 45.90 at. %; Cl, 0 to 0.32 at. %), accompanied by the corresponding decrease in the Ti concentrations from 59.57 at% to 39.45 at%. With regard to NT-Ag-V, the vacuum extraction process enables vancomycin to flow into the TiO₂ nanotubes (indicated by purple arrow in Figure 3j). The inner wall of the tube is coated with a layer of vancomycin (indicated by yellow arrow in Figure 3k). Based on Figure 3l, there is no appreciable difference in the amounts of vancomycin difference between NT-V and NT-Ag-V.

Figure 4a shows the XRD patterns of samples NT, NT-Ag, NT-V, and NT-Ag-V. A layer with the amorphous TiO₂ nanotubes array on the Ti surface is indicated by the diffraction peaks assigned to anatase and rutile phases and Ag PIII does not alter the phase structure. After vacuum extraction to load vancomycin, new diffraction peaks emerge from the XRD patterns of NT-V and NT-Ag-V and can be indexed to the vancomycin phase in the TiO₂ nanotubes. As shown in Figure 4b, the Raman bands at 150 and 612 cm⁻¹ of the inset correspond to the surface anatase and rutile phases, respectively. After vancomycin introduction, new Raman bands appear from the spectra of NT-V and NT-Ag-V. The Raman bands at 876 and 986 cm⁻¹ are assigned to the C–C symmetrical stretch and C–C antisymmetrical stretch, respectively. The 1125 cm⁻¹ Raman band is associated with the CH₁ symmetrical twist and that at 1236 cm⁻¹ corresponds to C–N bending. The 1606 cm⁻¹ Raman band originates from stretching of aromatic rings. All these Raman bands are characteristic of vancomycin indicating successful incorporation of vancomycin into TiO₂ nanotubes. In comparison, the vancomycin molecules show stronger Raman bands on NT-Ag than NT, with a Raman intensity ratio of around 4 due to surface-enhanced Raman scattering (SERS) from the Ag nanoparticles. XPS is carried out to investigate the elemental chemical states. The Ti 2p XPS spectrum shows two peaks at 464.6 and 458.8 eV (Figure 4c) characteristic of Ti 2p₁/₂ and Ti 2p₃/₂ in TiO₂, respectively. Figure 4d shows the Ag 3d doublets at 368.3 eV (Ag 3d₃/₂) and 374.3 eV (Ag 3d₅/₂) with a spin energy separation of 6.0 eV stemming from metallic silver (Ag⁰). After vancomycin introduction, the C 1s XPS spectrum shows three peaks at 288.4, 286.6, and 285.0 eV, which are assigned to imide C, amide – C, and C–C/C–H, respectively, as shown in Figure 4e-f. Figure 4g-h show that N 1s contains two subpeaks at 401.0 and 400.0 eV corresponding N–C₃ and N–C/N–C=O, respectively. Figure 4i shows the schematic illustration of loading and interactions of vancomycin molecules in the Ag nanoparticles modified TiO₂ nanotubes. Based on these characterization results, a layer containing TiO₂ nanotubes, Ag nanoparticles, and vancomycin is fabricated.

3.2. Drug Loading/Release and Cytotoxicity Evaluation. The loading efficiency of vancomycin in NT-V and NT-Ag-V is evaluated according to a previous method. After an initial wash, more than 80% of vancomycin is retained in the two groups of NTs as shown in Figure 5a. Release of vancomycin from the two kinds of NTs is investigated and shown in Figure 5c-d. As reported previously, the release process has two time phases: high initial release and slow subsequent release. In the first half an hour, over 50% of vancomycin is released from NT-V and NT-Ag-V and in the next 0.5–4 h, these two groups show a relatively slow and constant elution process. As shown in Figure 5c-d, the concentrations of vancomycin released are approximately 9.16 (NT-V) and 8.09 (NT-Ag-V) µg/mL at 240 min, which are still higher than those from the MIC of MRSA in our study (1 µg/mL). NT-Ag and NT-Ag-V are immersed in PBS or TSB for one month but no detectable silver ion (Ag⁺) release is observed (below the detection limit of ICP-OES). Figure 5b shows that compared to NT (control), NT-Ag, NT-V, and NT-Ag-V have no cytotoxicity.

3.3. In Vitro Antibacterial Property Against planktonic Bacteria. The antibacterial rate against planktonic bacteria
(Rap) is presented in Figures 6 and S1. With respect to the Rap value, although the culture medium is TSBG (Figure S1a) or TSBG+SF (Figure 6a) and compared with NT and NT-Ag which exhibit little or no lethality against planktonic bacteria in the culturing period, NT-V and NT-Ag-V have stronger antibacterial ability with the Rap values exceeding 90%. The antibacterial effects of different samples against planktonic bacteria are qualitatively investigated by SPM as shown in Figure 6b and S1b. During the culturing period, the amount of bacteria in NT and NT-Ag increase but there is no obvious statistical significance between them. In comparison, NT-V and NT-Ag-V show fewer bacteria at every time point indicating that the latter two samples have better antibacterial ability.

3.4. In Vitro Antiadhesive Property Against Sessile Bacteria. In the short and long-term experiments, the antibacterial rates against adherent bacteria (Raa) are presented in Figures 7 and S2. For the short-run Raa value, in contrast with NT, the modified samples (NT-Ag, NT-V and NT-Ag-V) have stronger antibacterial ability with the Raa values exceeding 90%. The antibacterial effects of different samples against planktonic bacteria are qualitatively investigated by SPM as shown in Figure 6b and S1b. During the culturing period, the amount of bacteria in NT and NT-Ag increase but there is no obvious statistical significance between them. In comparison, NT-V and NT-Ag-V show fewer bacteria at every time point indicating that the latter two samples have better antibacterial ability.

Figure 3. Cross-sectional morphology and composition by SEM and EDS: (a) SE image of NT with the EDS spectrum of the spot indicated by the yellow arrow (b); (d,e) SE and BSE images of NT-Ag with the EDS spectrum of the spot indicated by the yellow arrow (f); (g) SE image of NT-V with the EDS spectrum of spot indicated by the yellow arrow (h); (j,k) SE images of NT-Ag-V with the EDS spectrum of the spot indicated by the yellow arrow (l); (c, i) EDS results of the cross sections of various samples. Note: green arrow, embedded Ag nanoparticles; red arrow, exposed Ag nanoparticles; purple arrow, vancomycin.
surface shows an opposite tendency. Figures 8b and S3b show the similar bacterial adhesion trend by SEM. As the test is extended from 1 to 24 h, little bacteria colonization is found on NT-V and NT-Ag-V. In contrast, the number of bacteria on NT increases and finally a sea of bacteria conglomerates into colonies to form a recalcitrant biofilm. Although NT-Ag has the antiadhesive ability, some bacteria are observed from the surface at the end of the experiment. Additionally, the growth status of MRSA in the TSBG+SF is different from that in TSBG and there are some fibrous proteins on NT-V and NT-Ag-V after 12 and 24 h.

3.5. In Vitro Cell-Bacteria Cocultures. Compared to single-cultures with either bacteria or cells, our cells-bacteria coculture better simulates the clinical conditions of early PJI. The adhesion, spreading, and growth behavior of fibroblast cells are assayed by immunocyto-stained fluorescent image (ISFI) and SEM. As shown in Figure 9a, SEM reveals the cell morphology on the different surfaces. After 3 days, instead of adherent cells, clustered bacteria considered as biofilms are present on NT, suggesting that it is almost impossible for cells to survive in this hostile environment. The number of bacteria on the surface of NT-Ag decreases significantly compared to NT and a few odd-shape cells surrounded by bacteria are found on the surface. However, robust cells survive well on NT-V and NT-Ag-V, especially NT-Ag-V on which the cells stretch better with a few filopodia and establish good intercellular connections. As shown in the fluorescent images (Figure 9b), NT shows no viable cells and a few cells without filopodia extensions on NT-Ag sample have a spherical and wizened morphology. NT-V has more fibroblasts adhesion to the surface than the former two groups and the largest number of cells with a polygonal shape is observed from NT-Ag-V. As shown in Figure 9c, after 3 days, there is no cell surface coverage on NT, indicating that fibroblast cells cannot survive in the presence of adhering MRSA. NT-Ag shows little surface coverage and the percentage reaches 12.7%. In contrast, the percentages of
surface coverage on NT-V and NT-Ag-V are 27.8% and 47.1%, respectively, and in comparison with the other three groups, NT-Ag-V shows the most significant statistical difference.

These results demonstrate that only in the presence of sufficient anti-infective agents can cells win the race against attacking bacteria. This can be observed from NT-Ag-V which indicates the superiority of the combined release-killing vancomycin and contact-killing Ag nanoparticles with TiO₂ nanotubes platform as illustrated in Figure 9d.

3.6. In Vivo Antibacterial Performance Evaluation. In our model, the effect of different samples is assessed by observing the inflammatory symptoms of the knee joint and

Figure 5. Vancomycin loading, release, and sample cytotoxicity: (a) Loading efficiency of vancomycin in the nanotubes; (b) Cytotoxicity of fibroblast cells cultured with various samples for 1 and 4 days; (c-d) Release kinetics of vancomycin from NT-V and NT-Ag-V, respectively.

Figure 6. Antibacterial efficacy against planktonic bacteria in vitro after 1, 6, 12, and 24 h: (a) Antibacterial rates of planktonic bacteria (Rap) for four specimens in the TSBG+SF medium. ***P < 0.001. (b) Representative photos of recultivated planktonic bacteria colonies on SBA for four specimens in the TSBG+SF medium.
biofilm formation on the metal washers, counting the quantity of bacteria on the washers and peri-implant soft tissues, and evaluating the knee joint imageological features and histopathological changes.

3.6.1. Radiographical and Gross Evaluation for Peri-Implant Soft Tissues and CFU Counting of Soft Tissues. After 2 weeks, the rabbits are examined by X-ray, and the radiographic characteristics of the peri-implant tissue are revealed (Figure 10a). The soft tissue swelling of NT and NT-Ag is more severe than that of NT-V and NT-Ag-V. The inflammatory response of the knee is also evaluated. As shown in Figure 10b, the trend of the inflammatory reaction in articular cavity is the same as the imaged features. Compared to NT and NT-Ag showing thick and white suppurative covering the whole joint cavity, NT-V and NT-Ag-V show little or no serosanguineous exudation and necrosis tissue. The average gross scores of NT, NT-Ag, NT-V, and NT-Ag-V are 2.63 ± 0.52, 2.13 ± 0.64, 0.5 ± 0.53, and 0.25 ± 0.46, respectively (Figure 10c). NT and NT-Ag are not statistically significant and there is also no significant difference between NT-V and NT-Ag-V, but there are differences among NT, NT-Ag, NT-V, and NT-Ag-V (P < 0.05). The trend is NT ≈ NT-Ag > NT-V ≈ NT-Ag-V and consistent with that determined by radiology.

In addition, soft tissues surrounding the metal washers are harvested for bacterial load derivation from the homogenates of soft tissues. With respect to the average CFU counts per gram of peri-washer soft tissues, the trend is as follows: NT ≈ NT-Ag > NT-V ≈ NT-Ag-V (Figure 10d). The differences are not statistically significant between NT and NT-Ag as well as between NT-V and NT-Ag-V but statistically significant among other groups.

3.6.2. Evaluation of In Vivo Biofilm on the Metal Washers and CFU Counting of Washers. In the knee PJI model, the modified metal washers are removed and the in vivo biofilm formation and bacteria number are determined. According to Figure 11a, there is obvious in vivo biofilm formation on the NT and NT-Ag washers, whereas no biofilm can be observed from the NT-V and NT-Ag-V washers. SEM is conducted to further examine biofilm formation and bacterial aggregation from random regions. As shown in Figure 11b, NT shows biofilms and dense clustered bacteria. NT-Ag shows reduced biofilm formation and several bacteria are detected. In contrast, NT-V and NT-Ag-V show no biofilms and different quantities of fibroblast cells adhere and grow on the washers. The cell adhesion, growth, and accumulation reveal better outcome from NT-Ag-V. The bacteria detected by SEM is quantified by subsequent bacterial cultures. Figure 11c shows the quantitative bacterial counts from the sonicated eluates of the washers for each group. The average counts of CFU per washer display the following trend: NT > NT-Ag > NT-V ≈ NT-Ag-V. There is no statistical difference between group NT-V and NT-Ag-V but there are statistical differences among the other groups.

3.6.3. Histological Evaluation. The morphological change of the peri-implant soft tissues is assessed by H&E staining and the bacterial residue is evaluated by Giemsa staining. As shown in Figure 12, the typical feature of soft tissue infection is shown by NT including significant acute inflammation, exudation, synovial tissue necrosis, and neutrophils infiltration into tissues. Moreover, there are many bacteria in the slices of Giemsa staining. NT-Ag shows a relatively mild inflammation reaction and a certain amount of neutrophils through H&E staining, and a few bacteria are observed from the slices of Giemsa staining. However, on NT-V and NT-Ag-V, there is little or no sign of infection and the amount of bacteria decreases significantly. There is no significant difference between the two groups. Furthermore, according to the evaluation of soft-tissue condition, there are fresh granulation tissues with mature fibroblasts and newly formed vessels on NT-Ag-V and NT-V.
indicating that the survival state of fibroblast cells improve in the presence of released antimicrobial agents. As shown in Figure 10b and Figure 12d3-d4, NT-Ag-V shows more thick and robust fibrotic capsules with fibroblast cells and angiogenesis in the granulation tissues than NT-V (Figures 10b and 12c3-c4), implying NT-Ag-V promotes early restoration of soft tissues around the prosthesis.

4. DISCUSSION

A qualitative relationship between the Ag nanoparticles and release behavior of Ag ions is first established. The classical nucleation theory describes the nucleation and growth behavior of Ag nanoparticles in nano-TiO$_2$. When the Ag concentration exceeds the solubility limit in nano-TiO$_2$, the system relaxes via nucleation and growth of Ag nanoparticles. During Ag PIII, energetic Ag ions bombard the nano-TiO$_2$ with sufficient energy and come to rest at a distance from the surface. The energy lost of the incoming ions is partially transformed into atomic scale heating (ASH), which plays a significant role in Ag cluster evolution. Here, the total energy gain by an individual Ag ion in Ag PIII is determined by

$$E = E_k + QeV_{bias} + E_i + E_p$$

Considering the mean charge state $Q \approx 2$ and the mean work function of nano-TiO$_2$ $W \approx 5$ eV, the kinetic energy $E_i$ is calculated to 2.55 eV by the equation.

Figure 9. Antibacterial evaluation in the bacteria/cells coculture model: (a) SEM morphology of the attached bacteria and fibroblasts on the four samples. The images below have higher magnification than the areas in the red boxes. The blue arrow and green arrow mark the bacteria and fibroblast, respectively. (b) Fluorescent images of fibroblasts on the four samples after staining with DAPI (blue) and TRITC-phalloidin (red). (c) Surface coverage of the four different surfaces after adhesion, spreading, and growth of fibroblast cells for 3 days. **P < 0.01, ***P < 0.001. (d) Schematic illustration of the process of bacteria, fibroblasts, and samples.
The energy gain components of the total energy of an individual Ag ion reaching the nano-TiO₂ are listed in Table 2. The total energy gain of an individual Ag ion over a wide bias voltage (V_{bias}) is much greater than the minimum displacement energy in the range of 10⁻⁴⁰ eV.⁴³ Hence, during Ag PIII, the stationary Ag atoms under the nano-TiO₂ surface can be removed and dislodged. Accordingly, the Ag PIII process can be divided into two regimes. At a relatively small bias voltage, for example, V_{bias} = 0.5/1.0 kV (Table S1), the implanting depth of Ag is shallow and Ag nanoparticles can migrate to the surface with ease. In this case, the longer Ag PIII is carried out on the nano-TiO₂, the more Ag nanoparticles gather on the surface. They gradually undergo Ostwald ripening, coalesce from being discrete to continuous, and even form a nanothick Ag thin film on the nano-TiO₂ surface (Figure 13a). At a relatively high bias is used in Ag PIII, for instance, 30 kV as used in our experiments here, since the penetration depth is larger, the Ag nanoparticles have a long path to migrate out of the interior of nano-TiO₂. The majority of the Ag nanoparticles are embedded in the interior of nano-TiO₂ beneath the surface, although some of them can still migrate into the surface. As a consequence, Ag ions cannot be released quickly from the Ag-embedded TiO₂ and the released amount is smaller than 10 ppb⁴⁸,⁴⁹ which is less than the minimum bactericidal concentration of 100 ppb.⁵⁰ Prolonged Ag⁺ leaching renders the release-killing ability of the Ag-implanted surface while limited Ag⁺ release contributes to a contact-killing as illustrated in Figure 13b. To further verify the above theoretical analysis and give a direct experimental evidence, high resolution TEM analysis was carried out to investigate the presence of Ag nanoparticles on/in TiO₂ nanotubes. As shown in Figure 13c, in a randomly selected area, prevalent nanoparticles can be seen along the nanotube wall from the inside to the nanotube top, which include the partially exposed ones (the minority, indicated by “1”) and the embedded ones (the majority, indicated by “2”). Furthermore, the corresponding elemental analysis results of microregions “1” and “2” by EDS were given in Figure 13g, which demonstrated that the above nanoparticles were Ag nanoparticles. Likewise, Figure 13d–f also demonstrated the existence of Ag nanoparticles, consistent with the above analysis (Please see supplementary details). In this case, direct contact of adherent bacteria with Ag nanoparticles-embedded TiO₂ nanotubes will cause the disruption of their...
normal behaviors contributed by electron transfer and free radical formation.51,52

A growing number of TJAs accelerate the development of PJI so that their prevention is a continuous and arduous challenge.13,14 Implant-associated infections are commonly caused by surface-adhered bacteria that form thick and multilayered biofilms.53,54 In terms of pathogens, compared with S. epidermidis which produces less virulence factors, S. aureus is more toxogenic and once infected, multiple virulence factors of MRSA enable it to invade and undermine the host tissues and the infection tends to be more destructive.53,55 However, although MRSA invades host tissues surrounding prostheses or forms a biofilm on implant surfaces, it is crucial that MRSA should survive in the local surgical site and the postoperative impaired host immune defense cannot resist the strong etiological agent. If MRSA has enough time and space to colonize the prosthesis and peri-prosthesis tissues and attack them, early PJI will be difficult to eradicate. Therefore, we should take advantage of a high concentration of antibacterial agents to sterilize as many bacteria as possible by means of release from the antimicrobial coatings. However, a small amount of residual bacteria still have the chance to populate the implant surface. It has been demonstrated that S. aureus biofilm formation can be induced by subminimal inhibitory concentrations of some antibiotics such as cephalothin56 and vancomycin,57 and this is thought to arise from a global gene regulation in response to cell stress.58 Hence, an antiadhesive surface is also needed. With regard to systematic prophylaxis of early MRSA-PJI, a single release-killing or contact-sterilizing surface is not good enough and the most efficacious method is to construct an implant coating that combines a controlled release system that kills nonadherent bacteria during the short-term and a permanent antiadhesive layer that inhibits bacterial colonization in the long term.

Clinically, a 6-h postimplantation period is the “decisive period” to prevent bacterial adhesion.59,60 For the prophylaxis of surgical site infections (SSIs), a guiding document summarizing the clinical practice has been published by the U.S. Department of Health and Human Services and preoperative intravenous administration of antimicrobial agents should be provided for a few hours after the surgery.61 However, local delivery with fewer side effects is superior to systematic administration which brings small topical drug concentrations but high intravenous concentrations and may induce systemic toxicity.14 Certainly, our anti-infective coatings play a good role in the prophylaxis of early MRSA-PJI. As shown in Figures 6–8 and S1–S3, the released vancomycin can kill planktonic and sessile MRSA in the early stage. As time goes on, the quantity of released vancomycin diminishes (Figure 5c-d) and the leached vancomycin can be gradually metabolized in vivo. With regard to the adhered bacteria, NT-Ag shows some antibacterial effects during the course of 24 h and the immobilized Ag NPs can also provide long-term and stable antiadhesive effects up to 28 days. Specifically, unlike the coatings prepared by Cheng et al.17 and Mei et al.62 which have good antibacterial properties based on the release of silver ions, there is insignificant Ag⁺ leaching from NT-Ag thus minimizing the cytotoxicity induced by Ag⁺.

In our in vitro study, we first evaluate the antibacterial properties ex vivo with human SF. With regard to the choice of

Figure 11. Systematic evaluation of the metal washers in vivo in the four groups: (a) General observation of in vivo biofilm formation on the four washers. (b) Representative SEM images of the attached bacteria on the four washers. The images in the blue boxes are higher magnification of the areas in the red boxes. (c) Survival of bacteria on the washes. The results are expressed as the actual amounts of CFUs retrieved and the horizontal line demonstrates the median value. Each group includes eight animals. *P < 0.05, **P < 0.01, ***P < 0.001.
experimental strains, previous studies for reproducible results use the well-known standard strain (MRSA, ATCC433000), but such strain cannot represent the clinical prevalent strain type. Campoccia et al. has suggested that the reference strains should be of the major clinical prevailing strain type and the selection should be based on updated studies of molecular epidemiology. In our experiments, for better simulating clinical conditions, we choose the clinical MRSA (ST239), an epidemic HA-MRSA in China, and it enhances the validity of our experiment. As for the animal model that closely models early MRSA-PJI, we can directly visualize in vivo biofilm formation, find the intravital biofilm-forming characteristics of clinical MRSA, and detect bacterial accumulation on the washers by SEM. Moreover, our protocols also have the following two advantages. One is that MRSA is introduced directly into the knee joint when the incision is closed, but previous models import bacteria directly into the bone marrow cavity prior to biomaterial insertion or immerse implants in a bacterial culture suspension prior to intra-articular implantation. The second one is that it includes the essential fixed type presented in total knee arthroplasty.

The use of in vitro coculturing model is also in keeping with the clinical situation that intraoperative bacterial contamination of primary TJA is common, and the dissected periprosthetic soft-tissue with the impaired host immune defense makes the joint susceptible to bacteria. Gristina has suggested that there is a race to the implant surface between the host cells from the peri-implant tissue and invading bacteria. The modified surface can afford the projection of cell-to-substratum events and for the host, mammalian cells can rapidly inhabit the surface prior to incursive pathogens, which will greatly decrease PJI. As shown in Figure 9, in contrast with NT, NT-Ag-V in vivo presents the best peri-implant soft tissues growth without infective inflammation (Figure 10b) and demonstrates burly fibroblasts on the washer (Figure 11b4). During culturing, there is a fierce competition between MRSA and fibroblast cells in the race to the implant surface, and fibroblasts cannot survive or adhere to the surface when the number of bacteria is high. Although NT-Ag possesses the antibacterial ability against adhered MRSA, it cannot kill the floating bacteria so that fibroblast cells cannot anchor on the surface. Nevertheless, as the amount of bacteria is reduced by the action of released

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**Table 2. Energy Gain of an Individual Silver Ion during PIII**

<table>
<thead>
<tr>
<th>( E_i ) (eV)</th>
<th>( Q ) (( \times 10^4 ))</th>
<th>( E_v ) (eV)</th>
<th>( E_i ) (eV)</th>
<th>( E_v ) (eV)</th>
<th>( E_i ) (eV)</th>
<th>( E_v ) (eV)</th>
<th>( V_{bias} ) (kV)</th>
</tr>
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<tr>
<td>69.00</td>
<td>6.00 ( \times 10^4 )</td>
<td>2.55</td>
<td>2.95</td>
<td>( \sim ) 0</td>
<td>29.1</td>
<td>30.0</td>
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*From ref 43. †From ref 69.
vancomycin, fibroblast cells have the opportunity to stick to the NT-Ag-V surface to resist bacteria. The intricately modified surface not only kills planktonic bacteria directly and prevents bacterial adhesion, but also better supports the cell-to-substratum events to reduce infection, which suggests the synergetic superiority of the combined release-killing vancomycin and contact-killing Ag nanoparticles with TiO2 nanotubes platform. A robust fibrous interface around the implants also serves as a phylactic barrier to keep implants from microorganic invasion. It is of vital importance that early postoperative rehabilitation and the gradually recuperative immune system, albeit attenuated in the peri-implant environment, can protect the space from bacterial colonization. The combination of release-killing and contact-killing properties on the designed surface is illustrated in Figure 14a, which also shows the trap-killing ability contributed by Ag nanoparticles making surface charge shift positively to electrostatically attract negatively charged bacteria. Figure 14b summarizes the plausible antibacterial and antibiofilm mechanisms on the titanium surface in a cells-bacteria coexisting system.

5. CONCLUSIONS

Silver nanoparticles are incorporated into TiO2 nanotubes by plasma immersion ion implantation (PIII) to produce a contact-killing antimicrobial surface with limited silver ion release. Vancomycin is subsequently loaded into the nanotubes by vacuum extraction and lyophilization to establish a release-killing surface. The release profile of vancomycin exhibits two time phases: initially large release followed by slow subsequent release, but no detectable silver ions are lixiviated from the Ag−

Figure 13. (a) Schematic illustrating the basic physical processes (from left to right) involved in the generation of Ag nanoparticles (purple balls) in nano-TiO2 by Ag plasma immersion ion implantation (Ag PIII). (b) Schematic illustrating the contact-killing mode and release-killing mode of Ag nanoparticles. (c−f) TEM images showing the existence of Ag nanoparticles in the TiO2 nanotubes together with the corresponding EDS results acquired from spots "1" to "8" (g).
PIII nanotubes during a long immersion time period. As verified by our in vitro and in vivo models, the vancomycin-loaded Ag-implanted TiO<sub>2</sub> nanotubes show excellent antibacterial activity against both planktonic and sessile bacteria during the 24 h culturing period and bacterial adhesion can be inhibited up to 28 days. This bodes well for prophylaxis of early PJI during the perioperative period and perhaps even inhibited up to 28 days. This bodes well for prophylaxis of early PJI. Owing to the dual antibacterial properties and cell-assisting functions, the materials and methods are very attractive to the prevention of PJI and promotion of soft tissue reparation and Challenges. A Review of Molecular Mechanisms and Implications for Biofilm-Resistant Materials. Biomaterials 2012, 33, 5967–5982.

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11. Soriano, A.; Garcia, S.; Bori, G.; Almela, M.; Gallart, X.; Macule, F.; Sierra, J.; Martinez, J. A.; Suso, S.; Mensa, J. Treatment of Acute PJI during the perioperative period and perhaps even inhibited up to 28 days. This bodes well for prophylaxis of early PJI. Owing to the dual antibacterial properties and cell-assisting functions, the materials and method are very attractive to the prevention of PJI and promotion of soft tissue reparation especially for prosthesis implants.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b02803.
Additional materials as discussed in the text. (PDF)


Supporting Information

Antibacterial Surface Design of Titanium-Based Biomaterials for Enhanced Bacteria-Killing and Cell-Assisting Functions Against Periprosthetic Joint Infection

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S-1. Supporting Information

Figure S1. Antibacterial efficacy against planktonic bacteria in vitro after 1, 6, 12, and 24 hours: (a) Antibacterial rates of planktonic bacteria (Rap) for four specimens in the TSBG medium. ***P<0.001. (b) Representative photos of recultivated planktonic bacteria colonies on sheep blood agar for four specimens in the TSBG medium.
Figure S2. Quantitative antibacterial evaluation for sessile bacteria *in vitro* in the short-term and long-term tests: (a) During the course of 24 hours, the antibacterial rates of sessile bacteria (Raa) of the four specimens in the TSBG medium. (b) Over the course of 28 days, the antibacterial rates of sessile bacteria (Raa) for the four specimens in the TSBG medium. **P<0.01, ***P<0.001.
Figure S3. Qualitative antibacterial evaluation of the sessile bacteria in vitro after 1, 6, 12, and 24 hours: (a) In the TSBG medium, fluorescent images illustrating biofilm formation on the four samples after staining with the Baclight live/dead bacteria. Magnification is ×200 and the scale bar is 30 µm. (b) In the TSBG medium, the SEM morphology showing biofilm formation on the four samples. Magnification is ×2000 and the scale bar is 10 µm.
S-2. Supplementary Analysis

Herein, we firstly try to build a qualitative relationship between the presence form of Ag nanoparticles and the release behavior of Ag ions. Back to the source, firstly, the classical nucleation theory can give us a qualitative comprehension of the nucleation and growth behavior of Ag nanoparticles in nano-TiO$_2$. The equation, 
\[
\Delta G = 4\pi r^2 \gamma - 4\pi r^3 RT \ln S \frac{S}{3V_m},
\]
expresses the factors that determine the excess free energy ($\Delta G$) during crystallization process, including cluster radius (r), cluster surface tension ($\gamma$), ideal gas constant (R=8.314 Jmol$^{-1}$K$^{-1}$), absolute temperature (T), molar volume of bulk crystal ($V_m$). Besides, “S” represents the ratio of solute concentrations at saturation and equilibrium conditions. According to this, under supersaturated condition (S>1), “r” increase will lead to “$\Delta G$” decrease to stabilize the clusters, with a nucleus critical radius ($r^*$) determined by 
\[
r^* = \frac{2\gamma V_m}{RT \ln S}.
\]
From this, ascending “lnS” will induce descending “r*”, which indicates that upon Ag atoms concentration exceeding the solubility limit in nano-TiO$_2$, a system will relax by Ag nanoparticles nucleation and growth.

During Ag PIII process, energetic Ag plasma arriving at nano-TiO$_2$ have sufficient energy to come to rest under the surface. Since they deliver kinetic and potential energy, blocking by nano-TiO$_2$ transforms their energy into the atomic scale heating (ASH), which plays a significant role in Ag clusters evolution. Here, three acceleration zones are taken into consideration for cathodic-arc Ag ions, including: (1) Zone I near cathode spot forms the Ag ion kinetic energy $E_k$; (2) Zone II in space-charge sheath between Ag plasma and nano-TiO$_2$ surface endows Ag ion of charge state Q with the kinetic energy $QeV_{bias}$ ($e$, elementary charge) by applying negative bias voltage ($V_{bias}$) to nano-TiO$_2$ before impacting the surface. (3) Zone III in nanoscale vicinity of nano-TiO$_2$ produces image charge acceleration and the kinetic energy $E_i$. Meanwhile, Ag ion owns substantial potential energy $E_p$, determined by the equation $E_p = E_c + E_e + E_Q$, where $E_c$, $E_e$, $E_Q$ represent cohesive energy, excitation energy and ionization energy, respectively. The ionization energy $E_Q$ of the above
triply charged Ag ion is the summation of three ionization steps as $E_{Q} = \sum_{i=0}^{Q-1} E_{Q}^{i}$. As a result, the total energy gain of an individual Ag ion during Ag PIII is determined by the equation, $E = E_k + QeV_{bias} + E_i + E_p$. Considering the mean charge state $Q \approx 2^4$ and the mean work function of nano-TiO$_2$ $W \approx 5$ eV, the kinetic energy $E_i$ was calculated to 2.55 eV by the equation $E_i = \frac{W}{2} \sum_{j=0}^{Q-1} \frac{2(Q-j)}{\sqrt{8(Q-j)+2}}$. According to references and calculations, the energy gain components of total energy of an individual Ag ion upon reaching nano-TiO$_2$ were listed in Table 2. From this table, the total energy gain of an individual Ag ion over a wide bias voltage ($V_{bias}$) is much greater than the minimum displacement energy in the range of 10~40 eV$^3$. This means that, during Ag PIII, the stationary Ag atoms under nano-TiO$_2$ surface can be removed and dislodged out of interior. On this basis, the Ag PIII process can be divided into two cases. Under a relatively low bias voltage, for example, $V_{bias}$=0.5/1.0 kV (Table S1), the implanting depth of Ag plasma is shallow under the surface of nano-TiO$_2$. As a result, Ag nanoparticles can migrate to the surface with ease. In this case, the longer Ag PIII lasts on nano-TiO$_2$, the more Ag nanoparticles gather on the surface. Then they gradually undergo Ostwald ripening, coalescence from discrete to continuous, even to form a nano-thick Ag thin film on nano-TiO$_2$ surface (Figure 13a). The majority of exposed Ag nanoparticles (even Ag thin film) on surface make it easy to release Ag ions (Ag$^+$) from nano-TiO$_2$ in a physiological pH environment. So, recently, Mei et al$^7$ designed the Ag-implanted TiO$_2$ nanotubes by low bias voltage Ag PIII technique (0.5/1.0 kV) that can release Ag$^+$ from surface to kill bacteria. The second case is to adopt a relatively high bias voltage for Ag PIII, for instance, 30 kV used here. In this case, for one thing, the Ag nanoparticles have a long path to migrate out of the interior of nano-TiO$_2$; for another, the majority of Ag nanoparticles are embedded in the interior of nano-TiO$_2$ beneath the surface, although some of them can migrate onto the surface in a budding form. As a consequence, Ag ions cannot be released from the Ag-embedded TiO$_2$ or the release amount is lower
than 10 ppb\textsuperscript{8,9}, much less than the minimum bactericidal concentration of 100 ppb\textsuperscript{10}. All in all, feasible Ag\textsuperscript{+} leaching can impart a release-killing ability to the Ag-implanted surface while limited Ag\textsuperscript{+} releasing can contribute to a contact-killing property of the Ag-embedded surface via a direct interaction mode, as illustrated in Figure 13b.

Randomly selected another area, as seen in Figure 13d, it clearly shows a set of lattice fringes, in which the lattice fringe spacing of 2.04 Å can be indexed as the (200) plane of Ag nanoparticles\textsuperscript{8}, further evidenced by the corresponding fast Fourier transform (FFT) electron diffraction pattern of the inset. Moreover, the EDS results further confirmed the existence of Ag nanoparticles in regions “3” and “4” (Figure 13g). Likewise, the majority of embedded Ag nanoparticles came to eyes in another random field, as seen in Figure 13e. Together with the lattice fringe spacing of 2.04 Å of the partially exposed nanoparticle and the corresponding EDS results of regions “5” and “6”, they further demonstrated the presence and distribution of Ag nanoparticles within TiO\textsubscript{2} nanotubes. More interestingly, in Figure 13f, the twin crystal of Ag nanoparticles was formed when the interior tiny ones migrating to surface during PIII. The lattice fringe spacing of 2.35 Å was assigned to the (111) plane of the partially exposed Ag nanoparticles\textsuperscript{8}, and the EDS analysis of regions “7” and “8” further evidenced the existence of Ag nanoparticles. As a result, the tiny nanoparticles in dark color, including the embedded ones and the partially exposed ones, were attributed to the Ag nanoparticles generated in Ag PIII process. The size development and distribution of Ag nanoparticles from the inside to the top along the TiO\textsubscript{2} nanotubes accorded well with the above theoretical analysis of Ag nanoparticles formation during Ag PIII process.
Table S1. Energy gain of an individual silver ion during PIII.

<table>
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<tr>
<th>E_k (eV)</th>
<th>Q eV_{bias} (eV)</th>
<th>E_i (eV)</th>
<th>E_c (eV)</th>
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Notes: ^a From reference 3, ^b From reference 11.
REFERENCES


