Sustainable release of vancomycin from micro-arc oxidised 3D-printed porous Ti6Al4V for treating methicillin-resistant Staphylococcus aureus bone infection and enhancing osteogenesis in a rabbit tibia osteomyelitis model

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Elimination of infection and enhancement of osteogenesis by orthopaedic implants are two critical factors in the treatment of complex bone infections. A prolonged and expensive procedure requiring two surgical steps and a 6–8-week period of joint immobilisation is utilised as a primary treatment for revision arthroplasty of an infected prosthesis, greatly affecting long-term patient care for the ageing population. Here, we evaluated the effects of vancomycin-loaded in micro-arc oxidised (MAO) three-dimensional (3D) printed porous Ti6Al4V scaffolds on osteogenesis. This system showed a high loading capacity and sustained vancomycin release kinetics, as demonstrated using high-performance liquid chromatography. In vivo, 0.1 mL of 10^8 colony forming units (CFU) methicillin-resistant Staphylococcus aureus was injected into the tibias of rabbits to induce severe osteomyelitis. Physical, haematological, radiographic, microbiological, and histopathological analyses were performed to evaluate the effects of treatment. Rabbits with vancomycin-loaded in MAO scaffolds showed the inhibition of bone infection and enhancement of osteogenesis, resulting in better outcomes than in the other groups. Overall, these findings demonstrated the potential of this 3D printed porous Ti6Al4V, with good osteogenesis and sustained vancomycin release properties, for application in the treatment of complex bone infections.

Introduction

One of the most common forms of prosthetic joint infections (PJIs) is osteomyelitis, which can cause implant revision or removal or, in extreme cases, amputation.1,2 On the inert surface of the prosthetic implant, bacteria can anchor, multiply, and develop a biofilm, i.e., a hydrated polysaccharide matrix secreted by bacteria, resulting in PJI.3–6

Biofilm development can be systematically divided into four stages: bacterial adhesion, bacterial aggregation, biofilm maturation, and cellular detachment. Biofilms can protect inner microorganisms from host immune responses, causing implant loosening, poor osteointegration, peri-implant infection, and prosthesis revision. One treatment option to remove the infected tissue is antibiotic therapy; however, even a 10-fold increase in antibiotic concentrations cannot consistently inhibit the development of biofilms, and detached bacteria can further induce additional infections.5,7,8 Accordingly, it is difficult to eliminate mature biofilms, even after several cycles of antibiotic therapy.

Effective treatments must also induce a rapid regrowth of new bone and maturation of the bone–implant interface.9,10 Among the current treatment strategies, costly methods involving a two-step surgical process and a 6–8-week period of immobilisation and regrowth of new bone are widely used.11,12 However, such approaches are painful and are associated with the risk of PJI, resulting in a tremendous financial burden and dramatically prolonging the healing processes.

Accordingly, an alternative approach to this two-stage treatment strategy is a single-step surgical method using a drug-device combination system.13–16 This approach has been
attempted using antibiotic-impregnated polymeric bone cement; however, this system is limited with regard to the amount of antibiotics that can be loaded without compromising mechanical properties, thus limiting the ability of the approach to address existing infections. Drug-loaded bone cement has several other disadvantages, including harsh setting conditions required within patient tissues; weak control of release kinetics, leading to primary bolus release and potential adverse effects at high doses; suppression of bone repair; reduced remodelling of bone; and slow and painful patient recovery. These issues, particularly with bone repair, are exacerbated in obese patients and patients with diabetes taking immunosuppressive therapy. Additionally, in elderly patients, the risk of brittle fractures owing to osteoporosis and slower wound healing highlights the complexity of such procedures. In recent years, three-dimensional (3D) printed porous Ti6Al4V implants (the most popular alloy used in orthopaedic implant fabrication) have been widely used owing to their potential in patient-specific design and excellent osseointegration properties. However, implant surfaces that promote host cell adhesion, spreading, and growth are also favourable to bacteria, which share many of the same adhesive mechanisms as host cells. Hence, the development of a new drug loading system on the surface of 3D-printed porous Ti6Al4V implants is urgently needed to overcome these disadvantages of other strategies.

Based on our previous study, we established a novel vancomycin-loaded coating with a multilevel structure, showing good biological performance in vitro. In our design, polydopamine (PDA) served as an adherent anchor for heparin immobilised onto the underlying microporous micro-arc oxidised (MAO; TiO2/CaP) layer. H. Lee et al. firstly used dopamine self-polymerization to form surface-adherent polydopamine films onto a wide range of inorganic and organic materials, on the basis of which a variety of functional coatings were created. Therefore, the development of a new drug loading system on the surface of 3D-printed porous Ti6Al4V implants is urgently needed to overcome these disadvantages of other strategies.

Experimental

Scaffold fabrication and modification

3D printed macroporous Ti6Al4V scaffolds (the diameter is 5 mm and the height is 28 mm) were designed using computer-assisted design software (Magics, Materialise, Belgium) based on rabbit marrow cavity size and were fabricated using electron beam machining. The pore size of the porous scaffolds was 640 μm, the strut diameter was 400 μm, and the porosity was 73%; these features have been shown to be beneficial for the in-growth of bone and blood vessels. All specimens were blown with air to eliminate metal dust; successively washed with acetone, alcohol, and deionised water in an ultrasonic cleaner; and dried at 60 °C overnight. All parameters of the MAO process were carried out using a JH-10 pulsing power supply (Jinhu-lv-bao Co., Ltd, Beijing, China). For vancomycin immobilization, scaffolds were first soaked in 10 mL of freshly prepared dopamine hydrochloride solution (2 mg mL−1, pH adjusted to 8.5) in 10 mmol L−1 Tris buffer for 24 h to form a PDA layer on the surfaces. Afterward, the samples were oscillated in a mixed solution of vancomycin and heparin for 6 h. Ti6Al4V scaffolds (TSs) after MAO and PDA/heparin/vancomycin treatments were denoted as TS-M and TS-M/P/V, respectively.

The superficial and cross-sectional morphologies of the outer and inner surfaces of MAO-treated scaffolds were characterised using field-emission scanning electron microscopy (FE-SEM; Hitachi, Japan). The functional groups in TS-M/P/V were analysed employing Fourier transform infrared spectroscopy (FTIR; ECTOR22; Nicolet, USA). In vitro vancomycin release tests of TS-M/P/V and TS-P/V were done using high performance liquid chromatography (HPLC) analysis (HPLC on an Agilent 1200 series system equipped with a diode array detector DAD, Agilent Technologies) at room temperature. Three samples of each type were taken in 10 mL centrifuge tubes and incubated at room temperature under constant rotation (100 rpm) over a 30-day period, respectively. The PBS was removed and replaced with the same volume of fresh one at given time intervals. The mobile phase of the column was 0.0043 + 0.0103, R2 = 0.9989, y represents the OD value and x represents the concentration of vancomycin) obtained with known vancomycin concentrations was adopted to determine the vancomycin concentrations in the PBS. Each test was repeated three times to ensure the reproducibility of the results. In order to investigate the effects of MAO on the loading capacity of vancomycin, we also tested the release kinetics of vancomycin loading scaffolds without MAO treatment (TS-P/V). The specific procedures used were
described previously. Furthermore, to determine the effect of the pH value on the vancomycin release, the studies of TS-M/P/V in PBS of pH 5 were also performed under the same conditions to be in comparison with the one submersed in PBS of pH 7.4. Each test was repeated three times to ensure the reproducibility of the results.

**Bacterial culture**

The strain of methicillin-resistant *Staphylococcus aureus* (MRSA) used in this study was isolated from a human patient with bacteremia (based on swab culture and antibiotic sensitivity tests), at a Beijing Hospital. Strains of fresh single colonies were diluted with normal saline until the turbidity reached 0.5 McFarland standard (1 × 10^8 CFU mL^−1^) and were then used for the development of the experimental rabbit model.

**Surgery and animal welfare and health**

All surgical procedures were approved by the Chinese Center for Disease Control and Prevention. The study animals were bred at the Department of Laboratory Animal Science of Chinese Center for Disease Control and Prevention. All animal procedures were performed in accordance with the principles of the Guide for the Care and Use of Laboratory Animals after obtaining the approval from the Animal Ethics Committee of Peking University Health Science Center (approval no. LA2014214). Eighteen specific pathogen-free male New Zealand white rabbits (average weight: 3.7 ± 0.2 kg) were used in this study. The animals were randomly assigned to three groups: TS, TS-M, and TS-M/P/V. The capacity of TS-M/P/V to cure osteomyelitis and enhance osseointegration was evaluated in a rabbit tibia osteomyelitis model, as described previously.18 Isoflurane inhalation anaesthesia was used to anaesthetise the rabbits, hair was shaved from around the proximal part of the right tibia, and the lower extremities were disinfected and draped with sterile surgical drapes. Next, a 3 cm longitudinal skin incision was made over the proximal tibia; the boundary was manually delineated. A 1 cm longitudinal incision was made over the proximal tibia. Using a surgical drill, we created a 5 mm diameter defect in the tibial plateau, and 0.1 mL (10^8 CPU) sample was injected into the rabbit tibial medullary cavity. Previous studies have shown that this dosage resulted in a 100% infection rate of cement bodies. Finally, the scaffolds sterilized with cobalt-60 were inserted into the proximal part of the tibial medullary cavity (Fig. 1), and the incision was closed in layers with an absorbable thread (PDS II; Ethicon).

**Physical examination and haematological analysis**

Using an electronic scale and infrared thermometer, body weights and temperatures were measured pre-operatively on the day of surgery and every week for 6 weeks after surgery. Additionally, 0.5 mL blood was collected from the ear vein to evaluate white blood cell counts in peripheral blood (PBWBC) every week for 6 weeks after surgery.

**Specimens and radiography**

At the day of sacrifice, lateral X-rays were performed to identify the osteomyelitis grade according to the modified osteomyelitis scoring system (Table 1). Furthermore, 3 weeks after the surgery, all 18 rabbits were anaesthetised for lateral X-rays of tibiae to evaluate the antibacterial efficacy.

**Micro-CT analysis**

The ex vivo micro-CT imaging of the scaffolds and surrounding area was performed on the excised tibiae after the 6-week follow-up. The micro-CT images were acquired on an Inveon MM system (Siemens, Munich, Germany) to measure the amount and distribution of bone in each scaffold. Each specimen was analysed with segmentation software, and the analysed regions of interest included bone within scaffolds plus the tibiae proximal to this bone; the boundary was manually defined. Blinded scoring of the micro-CT images (Table 2)

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**Table 1 Osteomyelitis scoring system: X-ray analysis**

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
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<tbody>
<tr>
<td>0</td>
<td>No radiological abnormalities</td>
</tr>
<tr>
<td>1</td>
<td>Mild periostial reaction</td>
</tr>
<tr>
<td>2</td>
<td>Mild osteolysis directly around the implant</td>
</tr>
<tr>
<td>3</td>
<td>Evident osteolysis around the implant</td>
</tr>
<tr>
<td>4</td>
<td>More extensive metaphyseal osteolysis</td>
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**Table 2 Osteomyelitis scoring system: micro-CT**

<table>
<thead>
<tr>
<th>Osteomyelitis grade</th>
<th>Morphological changes</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>No radiological abnormalities</td>
</tr>
<tr>
<td>1</td>
<td>Mild periostial reaction</td>
</tr>
<tr>
<td>2</td>
<td>Mild cortical thickening</td>
</tr>
<tr>
<td>3</td>
<td>Evident periostial reaction</td>
</tr>
<tr>
<td>4</td>
<td>Evident cortical thickening</td>
</tr>
<tr>
<td>5</td>
<td>Mild osteolysis</td>
</tr>
<tr>
<td>6</td>
<td>Extensive cortical thickening</td>
</tr>
<tr>
<td>7</td>
<td>Focal loss of cortical wall</td>
</tr>
<tr>
<td>8</td>
<td>Evident osteolysis</td>
</tr>
<tr>
<td>9</td>
<td>Extensive cortical thickening</td>
</tr>
<tr>
<td>10</td>
<td>Loss of cortical morphology</td>
</tr>
<tr>
<td>11</td>
<td>Loss of spongy morpholgy</td>
</tr>
<tr>
<td>12</td>
<td>Extensive osteolysis</td>
</tr>
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</table>
enabled the quantification of the osteomyelitic condition of the rabbit tibia after the 6-week follow-up period. Furthermore, the ratio of the total amount of bone was designated as the bone volume fraction (BVVF), which was calculated and presented as bone volume/total volume (BV/TV). The bone volume fraction (BV/TV) is the volume of mineralised bone per unit volume of the sample. Appropriate mineralised bone phases were calculated by adjusting the threshold value (1000–3885). For a visual illustration, 3D reconstructions of the scaffolds were constructed with 2D images using a 3D visualisation system (Inveon Research Workplace; Siemens).

**Microbiological examination**

**Examination of the implant.** After sacrificing, coagulase testing was used to identify the strains inoculated in the rabbits: a staphylococcal colony was emulsified in a drop of water on a clean glass slide, a drop of healthy rabbit plasma was placed into the staphylococcal suspension on the slide and mixed with it. A coarse clumping of cocci visible to the naked eye was read as positive within 10 seconds and the one that has clumping indicated a positive coagulase test. Similar suspensions of control positive and negative strains were prepared to confirm the proper reactivity of the plasma. The scaffolds and tibias were collected separately, and implant-adhering bacteria were detached from the implant into 1 mL phosphate-buffered saline (PBS) using an ultrasonic cleaning treatment (Sonorex Digital 10P; Bandelin, Berlin, Germany; 10 min at 80% intensity). The tubes were then centrifuged at 4000 rpm for 10 min at 4 °C. The supernatants were collected and resuspended in 300 μL PBS. Ten-fold serial dilutions of samples were incubated on 5% sheep blood agar plates (Thermo Fisher, Beijing, China) at 37 °C for 48 h, and the culture dishes were then quantified for specific bacterial growth. Specifically, the number of inoculated viable cells in the culture dishes were counted and calculated.

**Examination of peri-implant bone.** The tibias of rabbits were mechanically homogenised (Omni Tissue Homogenizer and Hard Tissue Homogenizing tips; Omni International, GA, USA) to fully release the bacteria within bone. Bacteria were then quantified using serial dilutions and viable counts on 5% sheep blood agar plates.

**Histological evaluation.** Scaffolds and tibias were harvested, fixed in 10% formalin for 1 week, and dehydrated in a graded ethanol series (50%, 70%, 80%, 90%, 95%, and 100%) under vacuum conditions for 2 days. Samples were embedded in methyl methacrylate, and thin slices (200–300 μm) were cut from the blocks and ground to a thickness of 100–150 μm using transverse saw cuts and a polishing machine (exact band saw; Exakt Apparatebau, Norderstedt, Germany). The slices were then stained with toluidine blue dye solution. Using a BIOQUANT Image Analysis System (BIOQUANT Image Analysis Corp., Nashville, TN, USA), the cortical thickening or destruction of cortical bone and other infection signs were visualised and evaluated by three blinded, independent observers according to an osteomyelitis scoring system29 (Table 3). Furthermore, cross-sections of the scaffolds were normalised to 100%, and the percentages of the bone in-growth (BI) and bone-implant contact ratio (BICR) were calculated for each section. The BI of the entire scaffold, defined as the percentage of new bone within the defined area, was evaluated. The BICR was measured as the fraction of the surface area of the implant in contact with the bone. The BI and BICR were measured to evaluate osseointegration in each group.

**Statistical analysis**

All data are expressed as mean ± standard deviations (SD). Statistical significance was analysed by one-way analysis of variance (ANOVA) or Student’s t-tests, and the level of significance was set at p values <0.01 or 0.05.

**Results**

**Sample preparation and characterisation**

In this study, 3D-printed porous Ti6Al4V scaffolds functionalised with micro-/nanoporous CaP and vancomycin were obtained by MAO treatment and PD/Hep-assisted chemistry, respectively. The microstructures and surface morphologies of the scaffolds at different functionalisation stages were studied by SEM (Fig. 2A–C). Based on the macroporous TS (Fig. 2A), microporous CaP coatings (pore sizes: 0.1–2 μm) were grown in situ from a titanium substrate after MAO treatment (Fig. 2B), as previously described.22 The self-coating of the PD layer and subsequent conjugation of heparin and vancomycin only weakly altered the primary surface morphology (Fig. 2C).

![Fig. 2 Sample characterisation. SEM images (1000x and 5000x magnification) of the surface morphologies of TS (A), TS-M (B), and TS-M/P/V (C) scaffolds. (D) Representative FTIR spectrum of the TS-M/P/V scaffolds. (E) In vitro release kinetics of vancomycin from TS-M/P/V scaffolds. (F) The release profiles of vancomycin from TS-M/P/V in PBS of pH 5.0 and pH 7.4 (values were presented as mean ± standard deviation, n = 6).](image-url)

### Table 3 Osteomyelitis scoring system: histology

<table>
<thead>
<tr>
<th>Morphological abnormality</th>
<th>Score (per abnormality)</th>
</tr>
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<tbody>
<tr>
<td>Cortical thickening</td>
<td>0: absent</td>
</tr>
<tr>
<td>Presence of micro-abscesses</td>
<td>1: mild to moderate</td>
</tr>
<tr>
<td>Periosteal elevation</td>
<td>2: moderate to severe</td>
</tr>
<tr>
<td>Enlarged haversian canals</td>
<td></td>
</tr>
</tbody>
</table>

### Notes

1. The percentages of the bone in-growth (BI) and bone-implant contact ratio (BICR) were calculated for each section.
2. The BI of the entire scaffold, defined as the percentage of new bone within the defined area, was evaluated.
3. The BICR was measured as the fraction of the surface area of the implant in contact with the bone.
4. The BI and BICR were measured to evaluate osseointegration in each group.
5. All data are expressed as mean ± standard deviations (SD).
6. Statistical significance was analysed by one-way analysis of variance (ANOVA) or Student’s t-tests.
7. The level of significance was set at p values <0.01 or 0.05.
FTIR spectrometry (Fig. 2D) showed that the CaP coating was induced by MAO treatment, as demonstrated by the typical peak of PO$_3$$^\text{3-}$ (triply degenerate $\nu$- vibration of O–P–O bonds) vibrations, which emerged at 562 and 604 cm$^{-1}$, along with another two phosphate peaks at 874 ($\nu$1 nondegenerate symmetric P–O stretching band) and 1041 cm$^{-1}$ (triply degenerate $\nu$3 antisymmetrical P–O stretching modes). Additionally, the peaks at 1329 and 1234 cm$^{-1}$ corresponded to the N–H and S=O antisymmetrical stretching vibrations, suggesting the successful immobilisation of heparin mediated by polydopamine. Furthermore, two characteristic peaks of vancomycin, i.e., aromatic C=C (1481 cm$^{-1}$) and C=O (1726 cm$^{-1}$) stretching vibration bands, were also identified, verifying the feasibility of this drug functionalisation strategy. Combined with the results of SEM observations, we concluded that vancomycin-loaded composite coatings were successfully constructed on the porous titanium alloy.

Next, the release behaviours of vancomycin from TS-M/P/V were evaluated (Fig. 2E). Similar to our results in vitro,$^{22}$ the release process of TS-M/P/V in vivo comprised two time phases: burst release and slow stable release. The total release time was approximately 19 days. About 48% of vancomycin was released from TS-M/P/V within the first hour, and the remaining amount was eluted within 450 h. Finally, 1.8 mg vancomycin was released in 19 days. In contrast, vancomycin release from TS-P/V remained low, reaching approximately 0.3 mg.

**Surgery and follow-up**

In total, 21 rabbits received implants by transpatellar incision, among which 18 received intramedullary inoculation with 0.1 mL MRSA ($10^8$ CFU). Owing to respiratory depression, two rabbits did not survive anaesthesia (one rabbit from the TS group and the other from the TS-M/P/V group). Another rabbit (from the TS-M group) had to be sacrificed during follow-up owing to humane endpoint complications. Finally, there were six rabbits in each group during follow-up.

**Physical examination and haematological analysis**

Weight loss was obvious in the first week after surgery in all rabbits. Rabbits in the TS and TS-M groups showed significantly more weight loss than those in the TS-M/P/V group at the last three time points before sacrifice ($p < 0.05$; Fig. 3A). The temperature ranges in rabbits in the TS and TS-M groups were 38.7–39.6 °C and 38.5–39.4 °C, respectively, whereas that in the TS-M/P/V group was 37.5–39.1 °C. The body temperature in the TS-M/P/V group was significantly lower than that in the other groups beginning at the second time point after surgery ($p < 0.05$; Fig. 3B). Compared with pre-operative levels, all rabbits showed a distinct increase in PBWBC after surgery. During follow-up, all groups showed a relative decrease in PBWBC; however, PBWBC in the TS-M/P/V group was significantly lower at nearly all postoperative time points ($p < 0.01$; Fig. 3C).

**Specimen morphology and radiography analysis**

Bone morphological changes and clinical radiological parameters for infection, including periosteal elevation and osteolysis, were observed. We used a modified scoring system for osteomyelitis to evaluate the antibacterial capability of the scaffolds (Table 1). As shown in Fig. 4C and F, rabbits in the TS-M/P/V group showed no signs of osteomyelitis or abnormal bone morphology. In contrast, rabbits in the other groups showed signs of osteomyelitis, including periosteal reactivity with subperiosteal calcification (Fig. 4E, black arrow), as well as abnormal bone morphology (Fig. 4A and B). Metaphyseal and diaphyseal osteolyses were also observed in the other groups. The results produced by the modified osteomyelitis scoring system revealed that scores in the TS-M/P/V group were significantly lower than those in other groups ($p < 0.01$; Fig. 4G). These results indicated that the antibacterial effects of the TS-M/P/V scaffolds were obvious.

**Micro-CT analysis**

Micro-CT imaging of the excised tibiae was carried out at 6 weeks to observe the scaffolds and surrounding area, focusing on bone remodelling of the scaffolds. The TS-M/P/V group showed clearly mineralised cortical bone apposition around the implant and on its surface (Fig. 5C and F). No signs of infection were observed in the TS-M/P/V group; however, the TS group showed distinctive osteolysis on micro-CT (Fig. 5A and D). Furthermore, as shown in Fig. 5F, the intraporous BVF (bone volume fraction within the pores of the scaffolds) in the TS-M/P/V group (39.2% ± 3.8%) was significantly higher than that in the TS group (18.6% ± 2.2%; $p < 0.05$). The peri-implant BVF in the TS-M/P/V group (54.3% ± 4.6%) was significantly higher than that in the TS group (26.8% ± 3.5%; $p < 0.05$). 3D images are shown in Fig. 6. Ti, bones in the peri-implant region or within the scaffolds are labelled gray and green respectively. More green region means more bone growth. From these images, we concluded that the osseointegration of scaffolds was better in the TS-M/P/V group than in the TS group.
Microbiological examination

Before quantitative determination, coagulase/catalase testing and MALDI-TOF mass spectrometry revealed that the same strains were inoculated into all rabbits and that 100% of cultures from bones and implants were positive. Average bacterial counts from the tibia of rabbits in the TS-M/P/V group were significantly lower than those from the tibias of rabbits in the TS and TS-M groups (\(p < 0.01\); Fig. 7A). In fact, little bacterial growth was detected in the tibias of rabbits in the TS-M/P/V group. Average bacterial counts from the implants in the TS-M/P/V group were also significantly lower than those in implants in the TS and TS-M groups (\(p < 0.01\); Fig. 7B). Furthermore, for all groups, the average bacterial counts from the tibia were significantly lower than those from the implant sites.

Histological evaluation

Toluidine blue staining revealed that the tibial cortex morphology was normal in sections from the TS-M/P/V group and that there were no indications of infection (Fig. 8C). In contrast, the TS and TS-M groups showed signs of infection, including cortical discontinuity, cortical thickening, and absence of implant in-growth (Fig. 8A and B). The modified scoring system (Table 2) allowed quantitative discrimination of the osteomyelitic status between the TS-M/P/V group and the other two groups. As shown in Fig. 8D, the osteomyelitis scores in the TS and TS-M groups were significantly higher than that in the TS-M/P/V group, indicating that the infection status in the TS-M/P/V group was milder. Representative histological images of the osseointegration of scaffolds are shown in Fig. 8A–C. Fig. 8E shows a quantitative analysis of BI and BICR in scaffolds from the TS, TS-M, and TS-M/P/V groups. Compared with the TS-M group, significantly higher BI and BICR were found in the TS-M/P/V group (\(p < 0.05\)), indicating that the elimination of infection contributed to bone ingrowth of the scaffolds. Moreover, compared with the TS
group, the BI in the TS-M group was enhanced by 126% (7.19% ± 1.2% versus 16.25% ± 2.03%; \( p < 0.05 \)), and the percentage of bone in contact with the TS-M group was enhanced by 96.75% (11.49% ± 1.78% versus 5.84% ± 0.79%; \( p < 0.05 \)).

**Discussion**

From an orthopaedic perspective, effective orthopaedic implant-tissue interfaces should possess the following qualities. First, the interface should enhance the proliferation and differentiation of human mesenchymal stem cells (hMSCs) for osteoinduction by providing active biological factors.\(^{27}\) Second, the interface should support blood vessel in-growth and new bone formation, resulting in acceptable osteoconductive ability.\(^{31}\) Finally, the interface should efficiently kill the attached and surrounding bacteria to reduce the risk of biofilm formation and long-period infection.\(^{32,33}\) These are essential abilities through which biological interfaces inhibit and kill bacteria while promoting host cell adhesion and proliferation and providing haemocompatibility and absorbability. 3D-printed customised porous Ti6Al4V implants with interconnected macropores and high porosity can meet the requirements of osteoconduction and stability owing to their good flexibility, reproducibility, BI ability, and cost-effectiveness. Nevertheless, compared with conventional solid implants, porous implants with large specific surface areas can be infected by bacteria and result in long-term infection, indicating a need to decorate the implant with a functional coating to overcome this limitation.

Based on previous research,\(^{22}\) we have proposed an original, feasible method for multiple functionalisations of 3D-printed Ti6Al4V scaffolds. First, TS was treated with MAO to create micro-/nanoporous TiO2/CaP coatings in situ on the TS macro-porous strut walls. Next, vancomycin and heparin were immobilised on the macro-/nanoporous surfaces premodified by a PDA layer originating from the self-polymerisation of dopamine and bound tightly to CaP. The different constituents provided various biofunctions and showed synergistic effects. For example, the PDA layer acted as an anchor platform, with high stability and biocompatibility. Additionally, in our previous study,\(^{22}\) heparin was proved to be able to improve the blood compatibility in vitro. Vancomycin, which is widely used for the prevention and treatment of postoperative orthopaedic
infections, was charged and delivered through multiple interactions with PDA/heparin for sustained controlled release, endowing our implants with excellent antibacterial and anti-biofilm functions. Our results showed that improvements in cell attachment, proliferation, and differentiation may be related to the synergistic effects of surface topography and chemical composition. Both the micro-/nanoporous topology of MAO and the functional groups of PDA and heparin have been shown to direct protein adsorption and cell adhesion and further promote osteoblast spreading, migration, and proliferation.34

To determine the mechanism mediating the sustained release of vancomycin, it is first necessary to elucidate the mechanism of vancomycin immobilisation on the implant surface. In this drug loading system, heparin was immobilised on hydroxylapatite/PDA via a covalent bond.35 The Schiff base reaction between PDA and amino groups of vancomycin resulted in immobilisation.36 In addition, the negatively charged sulphur group on heparin can adsorb the positively charged vancomycin.37 Moreover, the nanoporous surface created by MAO can store vancomycin and lead to longer release times.38 On the one hand, the pores created by MAO increase the superficial area for drug delivery. On the other hand, the calcium phosphate coating created by MAO is also a critical factor for drug loading and release.39 When implanted in vivo, the bonds between vancomycin and heparin were broken owing to changes in the charge in the environment. Furthermore, bacteria can produce lactic acid during their metabolic activity, resulting in acidification of the microenvironment40 and causing the Schiff bases to be cleaved to PDA and amino groups. Thus, vancomycin can be released according to the level of bacterial growth. This process is shown in Fig. 9.

The release of Ca2+ from the scaffold into the extracellular environment promotes the osteogenic differentiation of hMSCs on the scaffold. Moreover, the higher alkaline phosphatase activity, an early phase indicator of the osteoblastic differentiation of hMSCs, observed in the treated group supported this point. However, the performance of this mechanism in our animal infection model should be further evaluated in future studies.

For the induction of osteomyelitis, we used a rabbit model because of the cost-effectiveness of such a model and the frequent use of rabbits in studies of surgical trauma and administration of high-dose antibiotics. To induce 100% infection in osteomyelitis models, 106–108 CFU is typically used to stimulate osteomyelitis.41–43 According to our previous experimental experience, we used 1 × 106 CFU to develop severe osteomyelitis. Based on body temperature changes and PBWBC tests, all groups showed significant temperature and PBWBC increases, implying that extensive destructive lesions may have formed in the soft tissues around the injured site. During the following days, all of these indices were decreased in the TS-M/P/V group, indicating the effective control of infection. The body weights of animals decreased in all bacteria-treated groups, whereas the PBWBC increased after bacterial injection. Additionally, in the drug-loaded group, weight loss was slightly lower than that in other groups, and the survival rates were higher. In order to evaluate the antibacterial efficacy of the developed drug delivery system in vivo, histopathological and radiological tests were performed. Notably, these evaluations showed that infection rates were lower in the tibias of animals treated with vancomycin-loaded scaffolds. Moreover, no improvement in the infection status was detected in animals implanted with MAO-treated scaffolds or in the control group (bare scaffold). Bone abscesses were reduced in the TS-M/P/V group in comparison with that in other groups, suggesting the preservation of vancomycin activity in vivo. However, signs of osteomyelitis were more detectable in other groups than in the vancomycin-loaded scaffold. All these phenomena may be attributed to the micro-/nanoporous topography of MAO and the functional groups of vancomycin, highlighting the significant role of the scaffold as a matrix for the preservation of antibiotics. Because osteomyelitis has a high incidence of recurrence with S. aureus strains, it is essential to optimise the characteristics of the drug-loaded scaffold in terms of high drug loading and time-related release behaviours in order to prepare a system that completely removes bacteria from the site of infection. However, it is also necessary to design an appropriate scaffold with the ability to enhance osteogenesis at the defect site, while also controlling the infection. Unfortunately, biomaterial surfaces that enhance bone cell adhesion, spreading, and growth also favour bacterial colonisation and share many of the same adhesive mechanisms as bone cells, such as extracellular matrix protein fibronectin.44 Hence, one important issue in the field of orthopaedics is to create a stable bone-material interface with implants, particularly when faced with bone infections. We have recently come to understand the significance of addressing infection during the bone wound healing process; however, to apply this understanding to develop effective treatments will require the ability to locally eliminate infection and induce new bone formation during the appropriate timeframe. To illustrate the clinical translational potential of our well-designed coating for optimal single-stage revision, we developed an animal model with...
induced osteomyelitis and used radiography and microbiological analyses to quantify antibacterial efficiency and bone formation. The animal model showed quantifiable differences in intraosseous bacterial survival and bone remodelling for multiple weeks, making it a valuable tool for the study of later-stage assessment and the development of new therapies.

Furthermore, the surgical techniques used in this study were easily adaptable to other bacterial species, enabling the study of new therapies using a variety of bone pathogens. After 100% isolation of bacteria from animals and reculture, little bacterial growth was observed in the tibia of rabbits in the TS-M/P/V group, indicating that the infection was completely controlled and eliminated during the initial stage of infection. Thus, these findings are promising for long-term bone-implant integration, demonstrating improved interfacial strength when compared with uncoated implants.

In summary, this study focused on the development of a multifunctional coating owing to the urgent need for suppression of infection and for tissue-integrating strategies in orthopaedic applications. Although further preclinical testing is needed using a larger animal model, the data shown here collectively suggested that the MAO-treated vancomycin-loaded coating developed in this study may be a promising approach to enable single-stage revision with minimal risk of re-infection and loosening, thereby extending the use of implants. This simple, safe, and economical technology has the potential to be applied in broad orthopaedic implant applications, directly benefiting patients who rely on prosthetic joints and other implants. Although the present method can realise a sustainable release of vancomycin, the accurate regulation of vancomycin release time and rate must be implemented in the future.

Conclusions

We successfully prepared the anti-infection and osteogenic multi-functional 3D printed porous Ti6Al4V implants by MAO together with loading vancomycin and further evaluated the effects of treatment in a rabbit tibia osteomyelitis model. In comparison with the blank control group, the treatment group displayed a remarkable inhibition of bone infection and enhancement of osteogenesis. Furthermore, the pH-responsive release of vancomycin was greatly promoted by MAO treatment, the amount of which can reach 1.8 mg within 19 days. In total, this multi-functional 3D printed porous Ti6Al4V implant can play a role in the treatment of the infective bone defects.

Conflicts of interest

There are no conflicts to declare.

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