In vitro application of drug-loaded hydrogel combined with 3D-printed porous scaffolds

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Abstract
Titanium mesh and three-dimensional titanium alloy scaffolds play a key role in addressing oral and maxillofacial bone defects, which can provide a specific environment and structure for bone growth and development. The two main causes of implant surgery failure are aseptic loosening and bacterial-induced implant-associated infections. To make bone defect implants effective for a long time, the ideal scaffold should take into account the two functions of osseointegration and anti-infection. Therefore, on the basis of the low-elastic-modulus Ti-10Ta-2Nb-2Zr (TTNZ) alloys developed by the research group in the early stage, this study intends to combine the vancomycin-loaded hydrogel with the 3D-printed through-hole porous titanium alloy scaffold to endow 3D-printed TTNZ scaffolds with antibacterial properties. The antibacterial properties of the complex were investigated by the zone of inhibition test and the adhesion/free antibacterial test. The effects of the composite system on osseointegration were investigated from the aspects of cell adhesion, cell proliferation and osteogenesis-related gene expression. The results showed that loading 2.5 wt.% and 5 wt.% vancomycin did not affect the structure of chitosan–hyaluronic acid hydrogel. The properties of the hydrogels were examined by scanning electron microscopy, Fourier-transform infrared, degradation experiment in vitro and vancomycin release experiment in vitro. When combined with porous scaffolds, the drug-loaded hydrogels exhibited slower drug release rates and longer release times. In addition, in vitro studies found that the TTNZ scaffolds loaded with 5 wt.% vancomycin had a certain effect on the expression of osteogenesis-related genes in cells, but the antibacterial effect was the best. The porous scaffolds loaded with 2.5 wt.% vancomycin hydrogel TTNZ scaffolds did not inhibit cell proliferation, adhesion, alkaline phosphatase activity, and osteogenesis-related gene exPRESSION, but the antibacterial effect on free bacteria was not as good as that of TTNZ scaffolds loaded with 5 wt.% vancomycin. This study, complementing the advantages of the two and controlling the local release rate of vancomycin, provides a new idea for future 3D printing of titanium alloy stents for anti-infection.

1. Introduction
Titanium and titanium alloys have become common materials for implants due to their good mechanical properties, biocompatibility, and corrosion resistance [1], and they play a key role in addressing oral and maxillofacial bone defects [2–4]. Cell-seeded porous structures combine the advantages of autograft and allograft bone, and they do not have the problems of scarcity of donor sites or immune rejection [5]. Bacterial infection and aseptic loosening are two major causes which give rise to implant failure [6]. Porous titanium alloys are bioinert materials, and it is hard to prevent implant-associated infection [7]. Implant-associated infections caused by bacteria can lead to osteomyelitis and implant failure [8]. Many bacteria can infect the regions on and around internal implants, but only a few genera are common pathogens around internal implants. Staphylococci, including Staphylococcus aureus and...
Staphylococcus epidermidis, account for 70% of all orthopedic implant infections [9]. In addition, the emergence and prevalence of methicillin-resistant *S. aureus* (MRSA) has exacerbated the severity of implant-associated infections [10, 11]. MRSA is listed as a ‘high-priority pathogen’ by the World Health Organization and poses a significant threat to public health [11].

Treatment of implant-associated infection is very limited due to biofilm formation and antibiotic resistance. It is difficult for drugs to penetrate into the porous titanium stent to completely remove bacteria. Systemic use of antibiotics can easily lead to low concentrations of antibiotics around the implant, making it difficult to effectively fight against bacteria; in addition, high concentrations of systemic antibiotics can also cause damage to other tissues in the body. However, the topical delivery system of titanium implants allows higher concentrations of antibiotics to penetrate the biofilm and bone tissue, solving the above problems to some extent [12, 13]. Compared with systemic drug delivery systems, the advantages of topical drug delivery systems on titanium implants are that they can provide better drug concentrations in the bone microenvironment, improve bioavailability, and provide more personalized disease treatment [12].

In this field, much research work is devoted to the development of various implant coatings, most of which are only osseointegration without anti-infective [14–16] or have anti-infection effects without osseointegration [17, 18]. However, for implants to be effective in the long term, an ideal coating should balance the two functions of osseointegration and anti-infection.

Among degradable biomaterials, hydrogels are excellent drug carriers. Antibacterial hydrogels loaded with antibiotics can continuously and slowly release antibiotics at the implantation site as the hydrogel degrades, which is an ideal way to apply antibiotics locally [19]. However, the hydrogel is soft and cannot play a good mechanical supporting role [20]. Wu et al [21] combined a gelatin–chitosan hydrogel with titanium discs to make up for the deficiencies in mechanical properties of antibacterial hydrogels. Croes et al [17] combined chitosan coating containing vancomycin and nano-silver with porous titanium, but the cytotoxicity of nano-silver strongly affected the antibacterial effect of the composite system. Zhang et al [16] fabricated 3D-printed porous Ti-6Al-4V with simvastatin-loaded hydrogel, which achieved the effects of sustained drug release and osseointegration.

Aseptic loosening is usually caused by a variety of factors, such as stress shielding effects, implant surface structures that are not conducive to bone ingrowth and new bone deposition, etc [22]. Making the titanium alloy scaffold into a porous shape with interpenetrating pores can simulate the structure of cancellous bone, thereby effectively reducing the elastic modulus of the scaffold. It is conducive to the growth of osteoblasts, the exchange of nutrients, and the promotion of new bone formation [23]. At present, it is considered that the pore size suitable for the growth of bone cells is 200–500 µm. In order to promote the formation of capillaries and the transport of metabolites, the pore size is required to be no less than 300 µm [24]. A scaffold with a triply periodic minimal surface (TPMS) structure can meet these requirements. The porous structure constructed using the TPMS is similar to the porous structure of human bone tissue, which is an advantage that other porous structures do not have [25, 26]. In addition, the TPMS has many advantages, such as smooth surfaces, interconnected pores and controllable fabrication, which is beneficial to the growth, reproduction and metabolism of bone cells in the scaffold [27]. Due to the high geometric complexity of the three-period minimal surface, such structures can currently only be fabricated by 3D printing [28].

In our previous work, we developed a new type of medical Ti-10Ta-2Nb-2Zr (TTNZ) metal powder, and our 3D-printed TTNZ scaffolds have pro-osseogenic functions with the same biosafety as pure titanium at the cellular and animal levels [29]. In addition, our research group carried out research on the surface modification of micro-arc titanium oxide in the early stage, which can form macro-, nano-, and micron-scale coatings on the surface of porous TTNZ scaffolds, and the coating can effectively promote the formation of hydroxyapatite, providing suitable conditions for the growth and differentiation of osteocytes, providing them potential for application in bone tissue engineering [30]. However, the use of these scaffolds as bone implants does not prevent the occurrence of implant-associated infections [31]; therefore, combining titanium alloy scaffolds with antibiotics appears to be a better option than systemic administration. By local administration, high concentrations of antibiotics can be targeted precisely at the implant site, avoiding potential adverse side-effects and systemic toxicity.

In this study, aiming at the specific composition and micro/nanostructure of human bone tissue, we used a TPMS structure (Gyroid structure) to fabricate the porous scaffolds. We innovatively combined a three-dimensional through-structured TTNZ scaffold with vancomycin-loaded hydrogel to produce a new type of sustained-release antibacterial. Vancomycin has a significant antibacterial effect against Gram-positive bacteria, and we added vancomycin to the hydrogel to fight implant-related infections caused by *Staphylococcus aureus* and MRSA [20]. Vancomycin is less toxic to osteoblasts and chondroblasts than ciprofloxacin and tobramycin [32]. We designed a titanium scaffold with a through-hole structure and a micro-arc oxidation surface coating, and filled the pores and surface of the titanium scaffold.
with antibacterial hydrogel. This study investigated its osseointegration and antibacterial effects, and provided a theoretical basis for obtaining a multifunctional complex with both osseointegration and anti-infection effects.

2. Materials and methods

2.1. Porous TTNZ alloy scaffold preparation

Ti-10Ta-2Nb-2Zr powder, with a particle size of 15–70 µm, was used with a spherical shape, which we presented elsewhere [29, 30, 35].

The manufacturing parameters were set as follows: the laser scanning speed was 700 mm s⁻¹ with a layer thickness of 25 µm, the laser power was set at 170 W, and the spot size was 70 µm in diameter. TTNZ powder was melted in an argon atmosphere with less than 80 ppm of oxygen. The thickness of each layer was 25 µm. This process was repeated layer by layer until the entire porous scaffold was complete. The unit cell of the porous structure was Gyroid type, the porosity was 75%, the wall thickness was 250 µm, and the pore size was 450–650 µm. The mechanics experiments used 3D-printed cylindrical specimens (Ø 10 mm, height of 12 mm), and the in vitro cell and bacteria experiments used disc-shaped specimens (Ø 14 mm, thickness of 3 mm). Dimetal-100 machine (Laseradd Technology Co., Ltd, Guangzhou, China) was used to produce porous 3D-printed samples (3DP). After printing, all the experimental samples were ultrasonically cleaned in acetone, ethanol, and deionized water for 10 min to remove the unsintered TTNZ powder.

Microarc oxidation (MAO) was used to modify the surface of the 3DP TTNZ scaffold. After printing, all the experimental samples were ultrasonically cleaned in acetone, ethanol, and deionized water for 10 min to remove residual TTNZ powder. Each printed sample was immersed in an aqueous solution of 18% HCl and 48% H₂SO₄ for 30 min, and the temperature was controlled at 60 °C in a constant temperature water bath. Then, the samples were ultrasonically cleaned with acetone, ethanol, and deionized water for 30 min to prepare them for MAO. The 3D-printed scaffolds treated by MAO (3DM) were prepared as previously mentioned [30]. After treatment, they were washed with distilled water ultrasonically for 5 min and dried at room temperature.

2.2. Synthesis and properties of the vancomycin hydrogel

2.2.1. Synthesis of the vancomycin-loaded hydrogel

The hydrogel was synthesized on the basis of the research of Song et al [34, 35]. Hyaluronic acid was dissolved in ultrapure water, sodium periodate solution was added, and the mixture was stirred for 24 h at 25 °C in the dark. Ethylene glycol was added to stop the reaction, and the obtained mixture was placed into a dialysis bag (MWCO 7000) and dialyzed in ultrapure water for 3 d; the water was changed constantly, and 1% silver nitrate solution was used to detect the sodium periodate content in the dialyzed external environment water. When there was no precipitation in the reaction solution, the dialysis was terminated. The dialyzed solution was frozen and dried to obtain aldehyde-based hyaluronic acid.

Phosphate-buffered saline (PBS) solution was used to prepare 6 wt.% carboxymethyl chitosan and aldehyde hyaluronic acid, and vancomycin (VA) was added to the aldehyde hyaluronic acid solution to make mass concentrations of 2.5 wt.% and 5 wt.%, respectively. After it was completely dissolved, the drug-loaded hyaluronic acid solution and the carboxymethyl chitosan solution were mixed in equal volumes to prepare the vancomycin hydrogel.

Hyaluronic acid, carboxymethyl chitosan, and sodium periodate were purchased from Aladdin (Shanghai, China); VA was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2.2. Properties of the vancomycin-loaded hydrogel

The hyaluronic acid, aldehyde hyaluronic acid, carboxymethyl chitosan, and drug-free hydrogel freeze-dried samples were ground into powder, and potassium bromide was used to press the tablets. Fourier-transform infrared (FT-IR) (Thermo Scientific Nicolet iS5, Thermo, Waltham, MA, USA) was used to investigate whether the hydrogel was successfully crosslinked. A field-emission scanning electron microscope (Zeiss Sigma 300, Zeiss, Germany) was used to investigate the morphology and microstructure of the drug-free hydrogel and vancomycin hydrogel.

The swelling ratios of hydrogels containing different concentrations of vancomycin were determined gravimetrically [36]. The prepared samples (0 wt.% VA hydrogel, 2.5 wt.% VA hydrogel, 5 wt.% VA hydrogel) were soaked in PBS solution with pH = 7.4 at 37 °C to fully absorb water and reach a swelling equilibrium state. After 48 h, the free water on the surface of the sample was wiped off, and the wet weight (Mt) of the sample was accurately determined. After that, the samples were lyophilized in a freeze-dryer, and its dry weight (M0) was accurately determined. The swelling ratio (SR) of the hydrogel was calculated by the following formula:

\[
SR(\%) = \left( \frac{Mt - M0}{M0} \right) \times 100\%.
\]

According to the study of Song et al [37], an in vitro degradation experiment of hydrogel was designed. We Added 50 mg (W0) hydrogel samples (n = 3) containing 0 wt.%, 2.5 wt.% and 5 wt.% vancomycin to each 15 ml centrifuge tube after lyophilization, and placed them in 5 ml PBS, 1 mg l⁻¹ of lysozyme/PBS solution or 10 units ml⁻¹ hyaluronidase/PBS at 37 °C. The hydrogel samples were
2.4.1. Cell proliferation and viability
Mouse embryo osteoblast precursor cells (MC3T3-E1) were used to evaluate the cell proliferation and differentiation of the five groups of samples. MC3T3-E1 cells were a gift from the College of Biology, Hunan University. The initial density of MC3T3-E1 cells was 4 × 10^4 cells/100 μl. The 3DP, 3DM, 3DM@0 wt.% VA, 3DM@2.5 wt.% VA, and 3DM@5 wt.% VA (n = 3) were placed in a 24-well plate, and 50 μl of cell suspension was inoculated per well. The scaffolds were cultured in minimum essential medium alpha containing 10% fetal bovine serum (FBS), 100 μM penicillin, and 100 μg ml⁻¹ streptomycin, all of which were from Gibco, at 37 °C in 5% CO₂ and 95% air. After culturing for 1, 3, 5, and 7 d, Cell Counting Kit-8 (CCK-8, Biosharp, Hefei, China) was used to evaluate the number of viable cells for cell proliferation, and a microplate reader (Epoch, Biotek, VT, USA) was used to measure the absorbance at 450 nm.

2.4.2. Alkaline phosphatase (ALP) activity
MC3T3-E1 cells were seeded on a 24-well plate at a density of 2 × 10^4 cells well⁻¹, incubated with the five sets of scaffolds (n = 3) mentioned in section 2.4.1 in minimum essential medium alpha for 24 h, and then placed in osteogenic differentiation medium (100 nM dexamethasone, 10 mM β-glycerophosphate, 50 g ml⁻¹ ascorbic acid) for 7 d. After using the ALP kit (Beyotime, Shanghai, China), the optical density at 405 nm was detected, and the ALP activity of the MC3T3-E1 cells was determined.

2.4.3. Morphologies of cells
MC3T3-E1 cells were seeded on the surface of five groups of samples at a density of 5 × 10^4 cells sample⁻¹ and cultured in minimum essential medium alpha containing 10% FBS, 100 U ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin, all of which were from Gibco, at 37 °C in 5% CO₂ and 95% air. After 24 h of incubation, the scaffold was washed three times with PBS, fixed with 4% paraformaldehyde for 1 h, and permeated with 0.1% Triton. Then, we used 2-(4-Aminophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (Solarbio, Beijing, China) and fluoresceinisothiocyanate–phalloidin (FITC-phalloidin) (Solarbio, Beijing, China) to label cellular nuclei and F-actin proteins. A laser confocal microscope (Olympus FV1200, Olympus, Japan) was used to observe the adhesion morphology of MC3T3-E1 cells on each group of samples.

2.4.4. Quantitative real time polymerase chain reaction (qRT-PCR)
MC3T3-E1 cells were seeded on a 24-well plate at a density of 2 × 10⁴ cells well⁻¹, incubated with the five sets of scaffolds (n = 3) mentioned in section 2.4.1 in minimum essential medium alpha for 24 h, and then placed in osteogenic differentiation...
medium for 14 d. The expression levels of osteocalcin (OCN), osteopontin (OPN), collagen type I (COL-I), and runt-related transcription factor-2 (Runx2) were detected. Total ribonucleic acid (RNA) was extracted according to the instructions of the RNA extraction kit (TRIzol reagent). The obtained RNA was reverse-transcribed by a PrimeScript Real Time reagent Kit (Takara) to form complementary DNA (cDNA). The Bio-Rad CFX Manager system was used for qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acted as a housekeeping gene. The primer sequences are shown in table 1.

### 2.5. In vitro antimicrobial activity

####  2.5.1. Inhibition zone experiment

The standard strain ATCC43300 of MRSA (vancomycin minimum inhibitory concentration, 0.5–2 μg ml⁻¹) was selected to evaluate the in vitro antibacterial properties of each group of scaffolds. Bacteria were purchased from Shanghai Biore-source Collection Center. Sterile trypticase soy broth was used to adjust the bacterial concentration to 1 × 10⁶ CFU ml⁻¹ by the McFarland turbidimetric method. Then, 100 μl of bacteria were spread on Müller–Hinton agar medium, and 3DP, 3DM, 3DM@0 wt.% VA, 3DM@2.5 wt.% VA, and 3DM@5 wt.% VA samples were placed after drilling. After culturing for 24 h, the diameter of the inhibition zone was measured using a Vernier caliper.

#### 2.5.2. Adhesion/free antimicrobial performance analysis

After sterilization, the five groups of scaffolds were placed in 24-well plates, and 1000 μl of 0.5 × 10⁶ CFU ml⁻¹ MRSA suspension was added to each well. The 24-well plate was placed in a bacterial incubator and incubated at 37 °C for 4 h and 24 h. After incubation, the culture medium was aspirated, and the surface of the scaffolds was washed three times with PBS. Each group of samples was set to a volume of 9 ml of PBS, the free bacteria in the nonadherent state on the surface of the samples were washed and removed, and trypticase soy agar medium was diluted in equal proportions. The cells were then placed in a bacterial incubator at 37 °C for 24 h and counted for analysis. After washing with PBS, ultrasonic vibration was used to treat each group of titanium sheets, and all the bacteria adhering to the surface were eluted as much as possible, diluted, and plated in equal proportions in the same way, before counting and analysis after culturing for 24 h.

### 2.6. Statistical analysis

All quantitative results are expressed as the mean ± standard deviation. One-way variance analysis (ANOVA) was used for statistical analysis. Statistical product service solutions (SPSS) version 22 (IBM, Chicago, IL, USA) was used for statistical analysis, and the least significant difference (LSD)
method was used for further multiple comparisons. A $p$-value $<0.05$ was considered statistically significant.

3. Results

3.1. Properties of vancomycin-loaded hydrogel

We used FT-IR to verify the success of hyaluronic acid modification and hydrogel crosslinking, and we observed the microstructure of each group of hydrogels by SEM. We soaked the hydrogels in PBS for 48 h to reach swelling equilibrium and calculated the swelling ratio. In order to observe the in vitro degradation of hydrogels, we evaluated the in vitro degradation behavior of hydrogel samples crosslinked by equal volume mass fractions of carboxymethyl chitosan and aldehydized hyaluronic acid under simulated physiological conditions (PBS, hyaluronidase solution or lysozyme solution) at 37 °C.

Figure 2(a) shows the FT-IR spectra of the carboxymethyl chitosan powder, hyaluronic acid powder, aldehyde hyaluronic acid powder, and drug-free hydrogel. Compared with the hyaluronic acid powder, the spectrum of the aldehyde hyaluronic acid formed an absorption peak for the aldehyde carbonyl C=O at 1728 cm$^{-1}$, indicating that its aldehyde-based modification was successful. Compared with carboxymethyl chitosan and aldehyde hyaluronic acid, the original absorption peak of aldehyde carbonyl C=O at 1728 cm$^{-1}$ disappeared, as well as that at 1079 cm$^{-1}$, in the FT-IR spectrum of the vancomycin-free hydrogel. The disappearance of the absorption peak of C-O-C indicated that the aldehyde group in the aldehyde hyaluronic acid was involved in the reaction and, at the same time, affected the spatial structure of the aldehyde hyaluronic acid. Since the formation of the hydrogel takes a certain amount of time, we can use this property to load some bioactive molecules and drugs into the hydrogel and then inject it into the target site through a syringe.

The swelling property of the hydrogel was tested by measuring the change in wet weight during the 48 h incubation period in PBS at 37 °C. According to the swelling curve (figure 2(b)), the 0 wt.% VA hydrogel, 2.5 wt.% VA hydrogel, and 5 wt.% VA hydrogel showed a swelling equilibrium state after 48 h of incubation, and the swelling rates were $47.8 \pm 0.7\%$, $44.9 \pm 1.5\%$, and $44.2 \pm 1.7\%$, respectively. The addition of vancomycin reduced the swelling rate of the hydrogel. The findings were consistent with those of Luppi et al. [38]. This relatively low SR can limit the amount of water penetration and the degree of volume change, which is beneficial to maintaining the morphology and mechanical compatibility of the hydrogel. It can also extend the release time of the loaded drug to cause an effective sustained release. In recent years, many studies have shown that hydrogels have become important modern drug delivery sustained-release materials due to their high water content (70%–99%), good biocompatibility, plasticity, toughness, and similarity to natural matrices [39, 40].

Figure 2(c) shows the microscopic morphology of vancomycin hydrogels with different concentrations observed under SEM. The freeze-dried hydrogel under the electron microscope was loose and porous with a pore size of 10 μm–100 μm. The addition of vancomycin did not affect the structure of the hydrogel. This kind of network structure was formed by crosslinking carboxymethyl chitosan and hyaluronic acid through a Schiff base reaction. This porous structure was conducive to drug release and cell growth.

Chitosan and hyaluronic acid can be degraded by lysozyme and hyaluronidase, respectively. In the degradation test, the size of each group of hydrogel samples decreased steadily. As shown in figure 2(d), the hydrogel samples were degraded in about 16 d in the in vitro environment containing lysozyme or hyaluronidase. The degradation rate was relatively uniform throughout the whole process, and there was almost no sudden acceleration or sudden deceleration. In the presence of enzymes, the hydrogels were degraded by about 50% at day 5. The hydrogels degraded slowly in the absence of lysozyme or hyaluronidase, and the hydrogels degraded about 60% by day 18. During the experiments, the hydrogels were partially degraded even in the absence of enzymes in PBS. This indicates that the degradation of the hydrogel is the result of the combined action of enzymatic hydrolysis and hydrolysis. This degradation is likely through surface and internal erosion processes. The amount of vancomycin loaded did not significantly affect the degradation rates of the hydrogel samples [41]. This also reflects from the side that the hydrogel material can exist as a relatively stable drug carrier.

3.2. Physicochemical properties of composite scaffold

We observed the microstructure of each group of scaffolds by SEM, detected the surface chemical composition of the scaffolds before and after MAO by EDS, and tested the elastic modulus and yield strength of each group of scaffolds using a universal chemical testing machine. The scaffolds were compressed at a rate of 2 mm min$^{-1}$ at room temperature, and each scaffold was subjected to an increasing load until the deformation rate reached 80%. The 0.2% offset method was used to derive the elastic modulus and the yield strengths of the scaffolds. To characterize the in vitro release of vancomycin, we collected PBS used to soak each group of hydrogels and complexes at 30 min and 1, 3, 7, 14, 21, 28, 35, and 42 d, and we measured its absorbance at 280 nm.

3.2.1. Surface characterization

Figures 3(a) and 4(a) show the gross view and the SEM images of 3DP and 3DM after gold spraying, respectively. The surface of the 3DP scaffolds was very rough, and a large amount of granular TTNZ powder
was sintered and cast on the surface of the stent. The surface of the 3DM scaffold was evenly covered with micron/nano diameter ‘volcanic pores’, and the large pores were wrapped in the small pores and connected to each other. This rough surface and porous structure are conducive to cell adhesion and growth [24, 42]. According to EDS analysis (figure 4(b)), Ca and P were effectively immobilized on the MAO coating, and the Ca/P ratio (1.6869) of the 3DM group was close to the chemical composition of human bone tissue (1.67). Figures 3(b) and 4(c) show the gross view and the SEM image of the antibacterial hydrogel–3DM titanium alloy microporous scaffold composite system after the antibacterial hydrogel was injected into the 3DM scaffold, respectively. It was observed under the electron microscope that the layered hydrogel material was wrapped and covered the metal trabeculae of the 3DM stent. The distribution was uniform in the composite system, and hydrogel filling was observed in the pores.

### 3.2.2. Mechanical properties

Figure 5(b) shows the static compression test results of the porous scaffold. Table 2 shows that the yield strengths of the 3DP group, 3DM group, 3DM@0 wt.% VA group, 3DM@2.5 wt.% VA group, and 3DM@5 wt.% VA group were 36.813 ± 3.009, 35.657 ± 3.082, 45.593 ± 0.780, 46.947 ± 0.422, and 47.763 ± 1.667 MPa, respectively. The elastic modulus were 1.213 ± 0.167, 1.277 ± 0.261, 1.710 ± 0.318, 1.937 ± 0.255, and 1.970 ± 0.442 GPa, respectively. Figures 5(b) and (c) are column charts of yield strength and elastic modulus of each group, respectively. MAO had no effect on the elastic modulus and yield strength of the 3D-printed TTNZ scaffolds, which was consistent with previous research by our research group. The elastic modulus and yield strengths increased after adding the hydrogel, but the mechanical properties of the scaffold were not changed by changing the concentration of vancomycin.

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**Figure 2.** (a) FT–IR of carboxymethyl chitosan, hyaluronic acid, aldehydated hyaluronic acid, and the 0 wt.% VA hydrogel; (b) swelling curves of hydrogels with three different vancomycin concentrations; (c) SEM images of three concentrationsof thevancomycin hydrogels; (d) degradation curves of hydrogels with three different vancomycin concentrations in PBS, lysozyme and hyaluronidase. (*)P < 0.05, ns: no significance.)
3.2.3. In vitro release of vancomycin

Figure 5(d) shows the vancomycin release profile of the 3DM@2.5 wt.% VA, 3DM@5 wt.% VA, 2.5 wt.% VA hydrogel, and 5 wt.% VA hydrogel. We collected PBS at 30 min and 1, 3, 7, 14, 21, 28, 35 and 42 d, and we used a visible spectrophotometer to measure the absorbance of the collected solution at 280 nm. The 2.5 wt.% and 5 wt.% VA hydrogels showed a burst release phenomenon after 24 h of release; the release amounts of VA were 46.281% and 45.735%, respectively, and the release amount did not change after 14 d ($p < 0.05$). In contrast, 3DM@2.5 wt.% VA and 3DM@5 wt.% VA released 8.675% and 9.731%, respectively, in the first 24 h, and there was no obvious burst release. The release amount did not change after 35 d of release ($p < 0.05$), and the final release amounts were 90.890% and 90.210%, respectively. Inspired by Mircoiu et al. [43], we further analyzed the in vitro release mechanism of vancomycin. In order to determine this mechanism, we used origin 8.0 software to fit the drug release behavior of 3DM@2.5 wt.% VA and 3DM@5 wt.% VA in PBS combined with Ritger–Peppas model. The release index of 3DM@2.5 wt.% VA in PBS was 0.458, and the correlation coefficient was 0.982; the release index of 3DM@5 wt.% VA in PBS was 0.473, and the correlation coefficient was 0.971. Usually, the release index in the Ritger–Peppas model can be regarded as the main parameter for judging the drug release mechanism: when $n \leqslant 0.45$, the drug release mechanism is Fickian diffusion; when $0.45 < n < 0.89$, the drug release mechanism is non-Fick diffusion, i.e. the combined effect of drug diffusion and matrix corrosion; when $n \geqslant 0.89$, the drug release mechanism is matrix corrosion [44]. It can be seen that the drug release mechanisms of 3DM@2.5 wt.% VA and 3DM@5 wt.% VA in PBS were both non-Fick diffusion, i.e. the sustained release behavior of the vancomycin was the result of the combined action of the diffusion of the drug and the corrosion of the hydrogel. This result is in line with expectations and consistent with the study by Zhang et al [45].

3.3. In vitro cell cytotoxicity and differentiation

MC3T3-E1 cells were cocultured with each group of scaffolds for 24 h. Next, we used DAPI and FITC-phalloidin to label cellular nuclei and F-actin proteins, and then used a confocal microscope to observe the cell adhesion morphology. Following 1, 3, 5, and 7 d of coculture, the optical density at 450 nm was detected after adding CCK-8 reagent to the culture plate and incubating for 2 h, in order to detect the cell proliferation. After coculturing with the scaffolds in each group for 14 d, the expression of osteogenesis-related genes in MC3T3-E1 cells was detected by qRT-PCR method.

3.3.1. Cell proliferation and viability

After the MC3T3-E1 cells were cocultured with the four groups of scaffolds for 1, 3, 5, and 7 d, the proliferation of cells in each group was detected by CCK-8, and the cell viability was evaluated (figure 6(a)). On the first day, there was no difference between the groups containing 0, 2.5, and 5 wt.% VA, and they were all higher than those in the 3DM group. There was no difference between the groups containing different concentrations of VA on the third and fifth days; on the seventh day, there was no difference in cell viability between the 3DM@0 wt.% VA and 3DM groups, while it was significantly higher than that in the 3DM@5 wt.% VA group. However, the cell viability of the 3DM@5 wt.% VA group was no different from that of the 3DM and 3DM@2.5 wt.% VA groups. The 2.5 wt.% and 5 wt.% vancomycin hydrogels loaded on the 3DM scaffolds had no inhibitory effect on the viability of MC3T3-E1 cells, which is consistent with the findings of Zhang and Liao et al [46, 47].
3.3.2. ALP activity
ALP is an early marker of osteogenic differentiation. Using this as an indicator, we evaluated the results of osteogenic differentiation of MC3T3-E1 cells on each group of scaffolds (figure 6(b)). After 7 d of cocultivation, the ALP activity of the four groups of scaffolds was significantly higher than that of the blank group. Although the ALP activity of the 3DM, 3DM@0 wt.% VA, 3DM@2.5 wt.% VA, and 3DM@5 wt.% VA gradually decreased, there was no significant difference between the four groups. MAO treatment significantly improved the early osteogenic differentiation ability of cells, and loading 2.5 wt.% and 5 wt.% VA hydrogels on the 3DM scaffolds did not affect the early osteogenic differentiation ability of cells.

3.3.3. Morphologies of cells
The cell morphologies of MC3T3-E1 cells on the scaffolds of each group were observed by confocal microscopy. As shown in figure 7, the cells in the 3DP group and the blank group presented less pseudopodia, while the cells in the other groups spread well on the surface of the scaffold and presented more pseudopodia. MC3T3-E1 cells had a good adhesion shape and high cell density on the surface of scaffolds containing 0 wt.%, 2.5 wt.%, and 5 wt.% VA hydrogels, and the
cytoplasm bulged obviously, indicating that the vancomycin composites would not affect the early adhesion of cells.

3.3.4. qRT-PCR

We detected the effect of each group of scaffolds on the expression of osteogenesis-related genes in MC3T3-E1 cells by qRT-PCR. OPN is a mid-stage marker of osteogenic differentiation, OCN is a late marker, and COL-I is secreted when the cell matrix begins to mature and mineralize. Runx2 can effectively regulate the transcription of factors related to osteocyte differentiation and proliferation. MC3T3-E1 cells were cocultured with each group of scaffolds for osteogenic induction for 14 d. As shown in figure 8, the expression levels of OPN, OCN, Runx2, and COL-I in the 3DM@0 wt.% VA group were higher than those in the 3DP group on the 14th day after osteogenic induction. The expression of OPN in the 3DM@2.5 wt.% VA group was not significantly different from that in the 3DM@0 wt.% VA group and the 3DM@5 wt.% VA group, but the expression levels of OCN, Runx2, and COL-I were significantly higher than those in the 3DM@5 wt.% VA group; that is, the addition of the 2.5 wt.% VA hydrogel to the 3DM scaffolds did not have a negative effect on the osteogenic differentiation of MC3T3-E1 cells.

3.4. In vitro antimicrobial activity

We tested the antibacterial properties of each group of scaffolds by the inhibition zone method and spread plate method. Each group of scaffolds was cocultured with bacteria-coated plates for 24 h, and the size of the inhibition zone around the scaffolds was measured; each group of scaffolds and MRSA were cocultured in
Figure 6. (a) Cell activity after coculture of cells with scaffolds; (b) ALP activity after coculture of cells with scaffolds ($^{ap}p < 0.05$ vs. 3DM, $^{bp}p < 0.05$ vs. 3DM@0 wt.% VA, $^{cp}p < 0.05$ vs. 3DM@2.5 wt.% VA, $^{dp}p < 0.05$ vs. 3DM@5 wt.% VA, $^{ep}p < 0.05$ vs. blank).

Figure 7. Adhesion morphology of MC3TE-E1 cells cocultured with scaffolds in each group for 24 h.

Figure 8. Relative messenger RNA (mRNA) expression of osteogenesis-related genes (OPN, OCN, Runx2, and COL-I) in MC3T3-E1 cells ($^{ip}p < 0.05$ vs. 3DM, $^{ip}p < 0.05$ vs. 3DM@0 wt.% VA, $^{dp}p < 0.05$ vs. 3DM@2.5 wt.% VA, $^{ep}p < 0.05$ vs. 3DM@5 wt.% VA).

liquid medium for 4 h and 24 h, and free bacteria and bacteria adhered to the scaffolds were diluted, spread, and counted.

3.4.1. Inhibition zone experiment
We cocultured five groups of scaffolds with MRSA in the punched wells for 24 h and showed the antibacterial properties of the scaffolds by measuring the size of the inhibition zone on solid agar medium (figure 9). The radius of the inhibition zone was from the center of the scaffold to the medium without colony formation. The 3DP, 3DM, and 3DM@0 wt.% VA groups did not exhibit inhibition zones, whereas the 3DM@2.5 wt.% VA and 3DM@5 wt.% VA groups showed inhibition zones, and the diameters of the inhibition zones were $3.400 \pm 0.245$ cm and $3.867 \pm 0.112$ cm, respectively. There was no viable flora in the bacterial cycle. The diameter of the inhibition zone in the 3DM@5 wt.% VA group was greater than that in the 3DM@2.5 wt.% VA group and was statistically significant ($p < 0.05$).

3.4.2. Adhesion/free antimicrobial performance analysis
Topical application of VA provides an ideal antibacterial effect, especially for MRSA. The focus of this experiment was to determine whether porous TTNZ scaffolds loaded with VA can achieve local
antibacterial effects by locally releasing antibiotics. Bacteria were incubated with the five groups of scaffolds for 4 h and 24 h. The results are shown in figure 10. For MRSA, the 3DM@5 wt.% group completely inhibited the growth of free/adherent bacteria at each timepoint. After cocultivation for 4 h, the 3DM@2.5 wt.% group completely inhibited the growth of free/adherent bacteria. However, after 24 h of cocultivation, the 3DM@2.5 wt.% group could not completely inhibit the growth of adherent/free bacteria, and the colony number of adherent bacteria was not significantly different from that for the 3DM@0 wt.% group.

4. Discussion

The micropore size of 3DP and 3DM scaffolds was approximately 400 µm. According to the literature, in order to promote the formation of capillaries and the transport of metabolites, the pore size is required to be no less than 300 µm, and 400 µm micropores are conducive to the growth of bone tissue cells [48]. The uniform filling of the hydrogel is beneficial to the comprehensive antibacterial performance of the composite system and will not leave a local antibacterial ‘blind zone’. At the same time, the 3DM microporous structure provides a favorable place for the colonization of bacteria; thus, filling the hydrogel into the scaffold can effectively prevent bacteria from entering the pores of the 3DP scaffold and causing implant contamination.

Aseptic loosening and implant-associated infections are the two main causes of implant failure. Aseptic loosening is usually caused by a variety of factors, such as stress shielding effects, as well as implant surface structures that are not conducive to bone ingrowth and new bone deposition [49]. Making the titanium alloy scaffold into a porous shape with a through-hole can effectively reduce the elastic modulus of the scaffold, and using the new Ti-10Ta-2Nb-2Zr alloy with a lower elastic modulus to prepare the scaffold can further reduce the elastic modulus. The elastic modulus of type III cortical bone is 12.6–21.0 GPa, and the elastic modulus of type III cancellous bone is approximately 1.37 GPa [50]. The scaffolds in this study can reduce the ‘stress shielding’ effect caused by mismatched mechanical properties and avoid aseptic loosening of the implant.

The in vitro degradation of hydrogels was determined by weight loss method at 37 °C. Chitosan-hyaluronic acid is a polysaccharide-based hydrogel. Chitosan can slowly degrade in vitro. However, in the presence of lysozyme, the rate of degradation can be accelerated [51]. During the degradation process, we can see a gradual reduction in the sample volume, which may be due to sufficient shrinkage between the degradation solution and the sample surface. The degradation effects of hyaluronidase and lysozyme on hydrogels were almost synchronous, and the results we obtained were consistent with those of Song et al [37]. This may be related to the ratio of carboxymethyl chitosan and aldolized hyaluronic acid in the preparation of this hydrogel [52].

According to the research of Xia et al [53], the chitosan-hyaluronic acid hydrogel had no obvious accumulation in Sprague Dawley rats and would not bring a huge burden to the them. Li et al [52] showed that chitosan-hyaluronic acid hydrogel samples were completely degraded 14 d after subcutaneous injection into the back of mice without massive fibroblast invasion. Therefore, the chitosan-hyaluronic acid hydrogel has good biodegradability and biosafety.

During the observation period, the vancomycin hydrogel achieved initial controlled drug release locally after being combined with the scaffold. The porous surface of the through-hole and the interconnected pores hidden inside can increase the drug release time and play a role in controlling the release dose. Studies have shown that 6 h after surgery (the so-called tie-breaker period) is the key to preventing implant-associated infections when the pathogen is still metabolically inactive, ensuring that the amount of drug released is critical for preventing infection at the implant site [54]. The average release of 3DM@2.5 wt.% VA and 3DM@5 wt.% VA within 24 h was greater than the minimum inhibitory concentration (MIC) of MRSA. Carboxymethyl chitosan-hyaluronic acid hydrogel is a biodegradable nontoxic hydrogel that can be degraded in vivo in 2 weeks [37, 52]. The vancomycin release rate of the hydrogel bound to the metal scaffold was significantly reduced,
Figure 10. (a) The number of adhered/free bacteria on the scaffold surface of each group was quantified by the plate counting method at 4 h and 24 h; (b) logarithmically normalized the number of adhered/free bacteria at 4 h and 24 h \((p < 0.05\) vs. 3DM, \(p < 0.05\) vs. 3DM@0 wt.% VA, \(p < 0.05\) vs. 3DM@2.5 wt.% VA, \(p < 0.05\) vs. 3DM@5 wt.% VA).

which may be due to the complex porous structure of the metal scaffold blocking the contact between the hydrogel and the solvent, and part of the hydrogel may remain inside the scaffold, resulting in a reduced release rate.

Studies have shown that vancomycin has better biocompatibility with osteoblasts and skeletal cells than other commonly used antibiotics, such as ciprofloxacin and tobramycin [32]. The vancomycin-loaded hydrogel in this study did not produce significant cytotoxicity due to the low local release concentration. At the same time, hyaluronic acid is a natural extracellular matrix that promotes cell attachment and growth. MAO can prepare
nanoscale porous titanium dioxide bioactive coatings on the surface of titanium alloys, which can better simulate the structure of the natural bone extracellular matrix and provide better biological properties for the adhesion of bone cells [55].

Loading 2.5 wt.% and 5 wt.% VA hydrogels on the 3DM scaffolds did not affect the ALP activity of cells. This may be related to the micropatterned structure and through-hole structure of the titanium coating. The patterned coating structure with through-holes is the key to promoting osteogenic differentiation. At present, most titanium meshes have right-angle pores, and the pore type is a non-through type, which is not conducive to cell growth and adhesion [23]. The Gyroid-type structure is a TPMS structure, and the porous structure constructed using the tri-periodic Gyriod-type structure is a TPMS structure, and the two stages. In the first stage, the cells proliferate and the matrix matures. Specific proteins associated with bone cell phenotype, such as ALP, can be detected during the process of cell proliferation and matrix maturation on days 10–15. During the second stage, the matrix is mineralized and the markers of post-osteogenesis are expressed, such as OCN, at days 10–15 and 25–30. Finally, multiple anabolic signaling pathways are actively involved in the control of bone formation [58, 59]. The expression of ALP and COL-I during extracellular matrix formation and maturation, and the expression of OCN and OPN during bone matrix mineralization, are major markers of osteogenic differentiation. On day 7, the ALP activity analysis showed a smaller but no significant difference in ALP expression in the 3DM@5 wt.% VA group compared with 3DM@0 wt.% VA group and 3DM@2.5 wt.% VA group. On day 21, the expression of osteogenic genes (OCN, Runx2 and COL-I) in the 3DM@5 wt.% VA group was lower than that in the 3DM@2.5 wt.% VA group. One possible reason is the differential expression of different genes in the early and late stages of osteogenesis. Another possible explanation is that osteoblast-derived cells are more sensitive to vancomycin, and the involved release of vancomycin is too high, which may affect the adhesion and differentiation of osteoblasts [60, 61]. These results show that the MAO coating can significantly promote the osteogenic differentiation of cells. After 2.5 wt.% VA was loaded, the local drug effect did not significantly affect the osteogenic differentiation of cells.

Bacterial adhesion and bacterial biofilms on implant surfaces are closely related to the formation of implant-associated infections. Infections around implants are often closely associated with bacterial adhesion, proliferation, and bacterial biofilm formation within host tissues and around the implant surface [62]. The enumeration study of the in vitro experiments showed that the 3DM scaffold loaded with 5 wt.% VA could inhibit the adhesion of MRSA on its surface, the locally released VA could kill free bacteria, and the ability of 3DM scaffolds loaded with 2.5 wt.% VA to inhibit bacterial adhesion decreased after coculture with bacteria for 24 h. However, the scaffolds modified by MAO did not inhibit the antibacterial nanoeffect, but the number of bacteria that adhered to the surface increased after coculture for 24 h. This may be because MAO will form a dense oxide layer on the surface of the scaffold, which greatly increases the number of bacteria attached to the surface. The surface area of the material increased, and the range of adhesion to bacteria increased. In addition, the titanium dioxide layer formed by MAO can trap bacteria and form a trap killing system on the implant surface. Therefore, the typical porous structure formed by MAO is beneficial to the development of antibacterial properties [63].
After coculturing with bacteria for 4 h, the 3DM@0 wt.% VA group showed a transient antibacterial adhesion effect, which may have been caused by the chitosan component in the hydrogel. Chitosan and its derivatives are cationic surfactants; the cations they carry can adsorb negatively charged bacteria, and the high surface activity can make them aggregate on the surface of the bacteria to form a polymer film, changing the permeability of the bacterial cell membrane, affecting bacterial metabolism and physiological functions, and causing microbial death [55]. Yu et al conjugated gentamicin with hyaluronic acid and then combined chitosan through layer-by-layer assembly technology. The study found that when gentamicin was completely released, the system could also continue to be antibacterial by virtue of the antibacterial function of chitosan itself [6]. After cocultivation for 24 h, the 3DM@2.5 wt.% group could not completely inhibit the growth of adherent/free bacteria, which may have been due to the early local release of less antibiotics than the 3DM@5 wt.% group. Although the 3DM@2.5 wt.% group released more than the MIC of MRSA within 24 h, the amount of bacteria grown in the in vitro study was significantly larger than that of the pathogenic bacteria in implant-related infections in humans. The pathological mechanism of implant-associated infection is relatively complex, and various factors such as the internal environment, pathogenic bacteria, and material electrolytic layer will affect the antibacterial effect. Therefore, the in vitro antibacterial results still need to be verified by animal experiments in vivo under the complex internal environment.

5. Conclusions

In this study, we used a hydrogel to load vancomycin, and then combined the drug-loaded hydrogel with a MAO-modified 3D-printed TTNZ scaffold. Vancomycin was loaded and released in a controlled manner to prevent implant-associated infections. Our experiments confirmed that the vancomycin hydrogel–TTNZ scaffold complex could reduce the early burst release phenomenon of the vancomycin, and it had a significant inhibitory effect on the adhesion and colonization of MRSA on the implant surface, while it did not affect the MC3T3-E1 cell adhesion, proliferation, osteogenic differentiation, and other behaviors. The drug-loaded hydrogel–TTNZ scaffold complex is a new type of titanium alloy stent with both antibacterial and osteopromoting properties. Therefore, the complex may become a novel scaffold for preventing implant-associated infection.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Author contributions

Conceptualization, X X and M C; methodology, H H, Z W and Z Y; software, X F and S B; validation, X X and M C; data curation, H H; writing—original draft preparation, H H; writing—review and editing, H H; visualization, H H; supervision, J L; project administration, X X and M C; funding acquisition, X X. All authors read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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