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Covalent immobilization of antimicrobial agents on titanium prevents *Staphylococcus aureus* and *Candida albicans* colonization and biofilm formation

Running title: Anti-infective titanium prevents microbial biofilm formation

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Synopsis

Objectives: Biofilm-associated implant infections represent a serious public health problem. Covalent immobilization of antimicrobial agents on titanium, thereby inhibiting biofilm formation of microbial pathogens, is a solution to this problem.

Methods: Vancomycin (VAN) and caspofungin (CAS) were covalently bound on titanium (Ti) substrates using an improved processing technique adapted to large-scale coating of implants. Resistance of the VAN-coated Ti (VAN-Ti) and CAS-coated Ti (CAS-Ti) substrates against *in vitro* biofilm formation of the bacterium *Staphylococcus aureus* and the fungal pathogen *Candida albicans* was determined by plate counting and visualized by confocal scanning laser microscopy. The efficacy of the coated titanium substrates was also tested *in vivo* using an adapted biomaterial-associated murine infection model, in which control-Ti, VAN-Ti or CAS-Ti substrates were implanted subcutaneously and subsequently challenged with the respective pathogens. The osseointegration potential of VAN-Ti and CAS-Ti was examined *in vitro* using bone marrow derived stromal cells, and for VAN-Ti also in a rat osseointegration model.

Results: *In vitro* biofilm formation of *S. aureus* and *C. albicans* on VAN-Ti and CAS-Ti substrates, respectively, was significantly reduced as compared to biofilm formation on control-Ti. *In vivo*, we observed over 99.9 % reduction in biofilm formation of *S. aureus* on VAN-Ti substrates and 89 % reduction in biofilm formation of *C. albicans* on CAS-Ti substrates, as compared to control-Ti substrates. The coated substrates supported osseointegration *in vitro* and *in vivo*.

Conclusions: These data demonstrate the clinical potential of covalently bound vancomycin and caspofungin on titanium to withstand microbial biofilm formation without jeopardizing osseointegration.

Key words: Titanium, *Staphylococcus aureus*, vancomycin, *Candida albicans*, caspofungin

Introduction

Implant-related infections are among the most important challenges in modern orthopedic surgery.[1] These infections can occur perioperatively, as a result of direct microbial contamination during the operation, or postoperatively by hematogenous spread of microorganisms from a distant source of infection.[2] Removal of the infected implants, followed by excision of infected tissues and bone and a long-term antimicrobial treatment is currently the only possibility to cure those infections.[3] This leads to patient discomfort and creates a significant economic burden to society.[4] It is estimated that the medical costs associated with such infections can range from $40,000 - $70,000 per patient.[5] In recent years, the use of orthopedic devices has increased significantly, with a rising number of implant-related infections as a result.[6] Thus, there is a pressing need to develop new implants that are less susceptible towards infections.

About 80 % of implant-related infections are caused by staphylococci, with the nosocomial pathogen *Staphylococcus aureus* accounting for 34 % of all cases.[7] Fungal pathogens including *Candida* spp. are also able to colonize implants, such as orthopedic joint or hip implants.[8-10] In addition, there are various reports on colonization of dental implants by *Candida albicans*.[11] Both *S. aureus* and *C. albicans* are notorious for their ability to form biofilms, a key step in the development of implant-related infections.[12] Biofilms are microbial communities enclosed within an extracellular polysaccharide matrix and adhered to a biotic or abiotic surface.[13] Biofilm-associated infections are difficult to eradicate since they exhibit decreased sensitivity to host immunological defense and increased resistance to antibiotic and antimycotic treatments.[14]

Biofilm-related infections are associated with a high mortality rate and therefore, the ESCMID advises to remove infected implants when possible.[15] However, removal of infected devices in patients with reduced health condition, or in less accessible locations, as is the case for heart valves or orthopedic joints, might be impossible or life-threatening.[16,17] In addition, treatment of e.g. fungal prosthetic joint infections with standard antimycotics or two-stage replacement with an antibiotic-impregnated interim spacer is associated with a high failure rate.[9] Hence, the use of anti-infective implant coatings based on antimicrobial agents, thereby preventing biofilm formation of microbial pathogens, is an important antibiofilm strategy.[18] Substantial research has been performed on coatings based on the controlled release of antimicrobials.[19] Despite the efficacy of these coatings, limitations such as increased local toxicity and reduced long-lasting protection have stimulated research towards the design of coatings based on the covalent attachment of antimicrobial agents to the implant. [3,20-25]

In this study, we examined colonization and biofilm formation of *S. aureus* and *C. albicans* on titanium (Ti) implant substrates, which were functionalized by covalent immobilization of the antibiotic vancomycin (VAN) or the antimycotic caspofungin (CAS). We report that vancomycin- and caspofungin-functionalized Ti substrates significantly impair *in vitro* and *in vivo* biofilm formation of *S. aureus* and *C. albicans*, respectively. Furthermore, these functionalized titanium substrates support the osseointegration potential *in vitro* and *in vivo*, demonstrating the clinical applicability of such anti-infective coatings.

Materials and Methods

Strains and media

S. aureus SH1000 cells[26] were grown in Trypticase Soy Broth (TSB, Becton Dickinson Benelux) or on solid TSB medium containing 1.5 % agar at 37 °C. *C. albicans* strain SC5314[27] was routinely grown on YPD (1 % yeast extract, 2% peptone (International Medical Products, Belgium) and 2 % glucose (Sigma-Aldrich, USA)) agar plates at 37 °C. An overnight culture of *C. albicans* was prepared in liquid YPD medium and grown at 30 °C. RPMI 1640 medium (with L-glutamine and phenol red) without bicarbonate was buffered with MOPS (Sigma, USA). The pH of the RPMI 1640 medium was adjusted to 7.0 with 1 M NaOH.

Covalent immobilization of vancomycin or caspofungin on titanium substrates

Sterile round titanium (Ti) discs (commercially pure titanium, grade 2; height: 2 mm, diameter: 0.5 cm; beadblasted and etched, washed in isopropanol) were obtained from Biotech Dental (Salon-de-Provence, France). This clinically relevant Ti surface with an average surface roughness, S_a, of 0.78 ± 0.14 µm, further referred to as control-Ti, was functionalized by treatment with Fmoc-protected 3-aminopropyl-triethoxy silane, followed by deprotection.[28]

The immobilization of vancomycin or caspofungin to the amino-group functionalized discs was carried out by the following procedure: the functionalized Ti discs were placed in a hydrolysis vessel containing a solution (1 mL/disc) of n-heptane/hexamethylene diisocyanate (85:15) for 3 h at room temperature. Next, samples were rinsed with n-heptane and placed in a vessel containing 520 mg of vancomycin (Sigma, St Louis, US) or 305 mg of caspofungin (Merck & Co, Kenilworth, US) dissolved in 50 mL saturated sodium hydrogen carbonate buffer (9,6 g sodium hydrogen carbonate/100 mL, pH 8.4). After 16 h, the discs were rinsed

with demineralized, pyrogen-free water and subsequently, with acetone, after which the VAN-Ti and CAS-Ti discs were allowed to dry.

Quantification of vancomycin on VAN-Ti discs and caspofungin on CAS-Ti discs by high-performance liquid chromatography (HPLC)

Quantification of the amount of immobilized caspofungin or vancomycin on the Ti discs was assessed via HPLC upon hydrolysis, resulting in release of bound compound. To this end, VAN-Ti and CAS-Ti discs were immersed in vessels containing demineralized water (1 mL), isopropanol (0.5 mL) and triethylamine (1 mL). The vessels were closed and heated at 60 °C for 1 h in a drying cabinet. Next, the solvents were removed from the vessels by evaporation at 60 °C. Finally, the residues were dissolved in demineralized water (0.5 mL) and analyzed by HPLC on a C18 column (50 x 2.1 mm). The coated titanium discs were stable for at least 12 months upon storage at 4 °C.

***In vitro* activity testing of VAN-Ti discs**

Control-Ti discs and VAN-Ti discs were incubated overnight in Fetal Bovine Serum (FBS, Life Technologies, Europe). Next, the discs were placed on the bottom of sterile silicon tubes (9 mm OD x 5 mm ID × 15 mm L) (VWR International) to exclude the sides and bottom, and transferred to the wells of a 24-well plate. The discs were incubated with 0.2 mL of *S. aureus* cells (~1 x 10^4 cells/mL) in 1/20 TSB for 24 h at 37 °C under static conditions. Subsequently, the discs were removed from the tube and washed with PBS to remove non-adherent bacteria. Discs were transferred to centrifuge tubes containing PBS and adherent bacteria were detached from the discs by sonication at 45,000 Hz in a water bath sonicator (VWR USC 300-T) for 10 min, followed by vortexing for 1 min. Resulting bacterial suspensions were diluted and plated on TSB agar plates in duplicate. After 24 h of incubation at 37 °C, the number of colony-forming units (cfu) per mL was determined by plate counting.

In vitro activity testing of CAS-Ti discs

Control-Ti and CAS-Ti discs were placed in the wells of a 24-well plate and infected with 1 mL of *Candida* cells (final concentration $5x10^4$ cells/mL). *Candida* cells were allowed to adhere to the disc during the period of adhesion (90 min, 37 °C, static). Non-adherent cells were removed by washing steps with PBS and the discs were subsequently submerged in fresh RPMI 1640 medium for the next 24 h (mature biofilm development). Afterwards, non biofilm-associated cells were removed by washing with PBS. Quantification of biofilm formation was performed as previously described.[29] Briefly, discs were sonicated for 10 min at 40,000 Hz in a water bath sonicator (Branson 2210) and vortexed for 30 s in PBS. Samples were diluted and plated on YPD agar plates in duplicate. After 48 h of incubation at 37 °C, the amount of adhered and biofilm forming cells was quantified by cfu counting. Note that we checked whether potential protein coverage upon immersing the CAS-Ti discs overnight in serum is not hampering *in vitro* activity. We could indeed confirm that this is the case: the *in vitro* antibiofilm activity is still present if the discs were immersed in serum overnight prior to testing.

Confocal Laser Scanning microscopy (CLSM)

S. aureus and *C. albicans* biofilms grown on uncoated (control-Ti) or coated Ti discs were investigated with a CLSM (Leica TCS SP5, Heidelberg, Germany) in an inverted microscope, by using the LIVE/DEAD®BacLight™ stain (Molecular Probes, USA). This stain consists of a mixture of the SYTO®9 (green) and propidium iodide (red) nucleic acid fluorescent stains. Using this stain, live cells are stained green, whereas dead cells are stained red. During CLSM evaluation 25 digital images were taken with X-Y scan a few µm above the surface plane. The objective used was a HCX PL APO CS with magnification 63 x and numerical aperture 1.20. The thickness of the optic sections was 577 nm at full width half maximum and the image size of the micrographs was 2048 x 2048 pixels. The light sources were HeNe lasers using λ_{ex} =

594 nm (propidium iodide) and Ar laser using λ_{ex} = 488 nm (SYTO®9). Signals were captured at wavelength of 605-650 nm and 500-530 nm for propidium iodide and SYTO®9, respectively. Image analysis was performed in Matlab using a software macro developed at SP Food and Bioscience, Sweden, to calculate the area fraction of live and dead cells in a thin optical section close to the titanium surface.

Scanning electron microscopy (SEM)

Qualitative analysis of coated and non-coated Ti discs was done by scanning electron microscopy (SEM, Nova NanoSEM 450, FEI) with associated energy dispersive X-ray spectroscopy (EDX, EDAX), operated at standard high-vacuum settings. In order to avoid beam damage of the organic coatings, low-energy imaging was performed by applying a 3-4 keV stage (and sample) bias field resulting in an effective landing energy of 0.5 keV.

***In vivo* quantification of biofilm formation on VAN-Ti discs and CAS-Ti discs**

All animal experiments performed in this study were approved by the Animal Ethical Committee of the KU Leuven (project number P125/2011). Pathogen-free BALB/c female mice (20 g, 8 weeks old) were used. Animals were housed in groups of 4 in individually ventilated cages. Mice were provided with sterile food and water *ad libitum*.

A schematic overview of the *in vivo* experimental procedures is shown in Supplementary Figure 1 and 2. This model was originally developed to study *Staphylococcus epidermidis* biomaterial-associated infections.[30] In this study, the model was adapted to investigate *in vivo* *S. aureus* and *C. albicans* biofilm development on titanium discs.

One day prior to the surgery all animals were immunosuppressed by adding dexamethasone (0.4 mg/L) to the drinking water. Based on our previous work, immunosuppression results in higher reproducibility of the number of biofilm-forming cells retrieved from implanted devices.[29] Suppression of the immune system was carried out throughout the entire

experiment (4 days in total). At the day of implant, animals were anesthetized using an intraperitoneal injection of a mixture of ketamine (Ketamine1000®; Pfizer, Puurs, Belgium) and medetomidine (Domitor®; Pfizer) (45 mg/kg ketamine and 0.6 mg/kg medetomidine). The lower back of the animals was shaved and disinfected with iodine isopropanol (1 %). Prior to the incision, local anesthesia was performed with xylocaine gel (2 %, AstraZeneca, Zoetermeer, the Netherlands) directly on the skin. A small incision was made and the subcutis was carefully dissected to create a space (approximately 2 cm long and 1 cm wide) for 1 disc. The incision was closed with surgical staples, disinfected and locally anesthetized with xylocaine gel. Anesthesia was reversed with intraperitoneal injection of atipamezole (Antisedan® (Pfizer), 0.5 mg/ kg for mice). Twenty four h post implant, animals were anesthetized with a mixture of ketamine and medetomidine as indicated above and inoculated with the pathogens.

For inoculation of the discs with *S. aureus* or *C. albicans*, microbial overnight cultures were washed and resuspended in sterile saline (0.9 %) to a concentration of 1 x 10^8 cells/mL. 100 µl of the bacterial or fungal inoculum was injected subcutaneously into the area around the disc.

Subsequently, anesthesia was reversed with an intraperitoneal injection of atipamezole as indicated above. Bacterial and fungal biofilms were left to develop for 4 or 2 days, respectively. For discs explant, the animals were euthanized by cervical dislocation. The skin was disinfected with 0.5 % chlorhexidine in 70 % alcohol; discs were removed from under the subcutaneous tissue and washed twice with PBS before further quantification of biomass. The tissue surrounding the discs was collected in microcentrifuge tubes.

The bacterial and fungal burden was assessed by cfu quantification. Biofilms formed on the discs were washed twice with PBS, sonicated for 10 min at 40,000 Hz in a water bath sonicator (Branson 2210) and vortexed for 30 s in PBS. In addition, tissue samples were

weighed and homogenized. The resulting bacterial and fungal suspensions (discs and surrounding tissues) were diluted and plated in duplicate on TSB or YPD agar plates, respectively. The plates were incubated at 37 °C and cfu were counted after 24 h of incubation for *S. aureus* and 48 h of incubation for *C. albicans*.

In vitro osseointegration testing

To assess the osseointegration potential of VAN-Ti and CAS-Ti discs, primary osteogenic and vasculogenic cells were employed. Human bone marrow derived stromal cells (MSC) and human microvascular endothelial cells (HMVEC) were cultured in Advanced DMEM supplemented with 10 % FBS, 1x GlutaMAX, and 0.05 mg/mL gentamicin and medium 131 supplemented with MVGS (Life Technologies), respectively. MSC and HMVEC were seeded at cell density of 5000 cells/cm^2 and cultured in 5 % CO_2 at 37 °C for one passage. After reaching 95 % confluence, cells were trypsinized (Trypsin-EDTA, Sigma Aldrich), counted with a hemacytometer and used for experiments. Cells of the 4th passage were used for the experiments.

Coated or uncoated discs were placed onto culture plates: each disc was placed into one well of a 24-well culture plate (TPP, Switzerland). Subsequently, the top areas of the discs were seeded with MSC or HMVEC at a cell density of 9000 cells/disc: approximately 50 µL of cell suspension was placed on the top of the disc, and distributed evenly so that a drop covered the whole area of the disc. Discs were then kept in the incubator, allowing cells to attach. After 30 min, additional culture medium was added. Cells were cultured for 5 days, fixed with formalin (15 min), and then washed three times with PBS (5 min). Next, samples were incubated with Phalloidin (Sigma, P5282) at room temperature in the dark (30 min). For stock solutions, 1 mg of Phalloidin was dissolved in 10 mL methanol. For working solutions, the stock solution was diluted 1:20 with PBS. Samples were washed with PBS (10 min) for three times, mounted with Vectashield/DAPI (Vector Laboratories, USA) and inspected using a

fluorescent microscope (Nikon T300).

***In vivo* osseointegration testing**

The *in vivo* osseointegration of VAN-Ti implants was tested in Wistar rats. Rats were purchased from Charles River Laboratories (Italy) and weighed around 300 g. Following shipment, rats were allowed 2 weeks to habituate. Rats were housed in standard conditions with food and water *ad libitum* and 12:12 day/night cycles. All animal experiments were approved by the Veterinary Commission of the Republic of Slovenia (permit number 34401-31/2012/8). An open porous grade 2 titanium coating (OpTi) was applied to Ti6Al4V cylindrical implants (ø 1.5 mm, l = 6 mm) by vacuum plasma spraying (control-OPTi) and coated with vancomycin as described above for VAN-Ti (VAN-OPTi). The vancomycin load on VAN-OPTi implants was 82 pmol/cm^2. Before surgery, rats received a subcutaneous injection of butorphanol (1mg/kg BW; Butomidor, Ritcher Pharma AG, Austria). After 5 min rats were anesthesized by the inhalation of isoflurane (Forane, AbbVie Ltd., United Kingdom). An approximately 2 cm long incision was made on the lateral side of the left knee. The knee was opened laterally from the patella. After moving the patella and attached ligaments medially, a Ø 1.5 mm hole was drilled in the intercondylar space. The titanium implants (VAN-OpTi and control-OpTi) were placed into the drilled hole in a submerged position. The synovial membranes and skin were sutured with biodegradable sutures. Rats were given tramadol (0.5 mg/kg BW; Tramal, Stada, Germany) as pain alleviation immediately after the surgery and meloxicam (1 mg/kg; Loxicom, Norbrook Laboratories Ltd., United Kingdom) daily for five days post-surgery. One to 5 days after surgery, X-ray imaging (AXION Iconos R100, Simens, Germany) was done to check the position of the titanium implants (Supplementary Figure 3). Eight weeks after surgery, rats were euthanized by CO_2 inhalation. In total, 13 rats were used, 5 rats received a VAN-OPTi implant, 8 were

implanted with a control-OPTi sample. After sacrifice, femurs were collected and placed into 4 % paraformaldehyde for 2 days, and then stored in PBS at 4 °C until further processing.

Bone growth onto and surrounding the implants was analyzed using microfocus X-ray computed tomography (µCT). Image acquisition was carried out on a submicrometre resolution CT device (Phoenix Nanotom S, GE Measurement and Control Solutions, Germany) with an X-ray source equipped with a tungsten target. Both a 0.1 mm thick Cu filter and a 0.3 mm thick Al filter were placed in front of the X-ray source to avoid beam hardening and metal artifacts. Scanning was performed over 360° with a step size of 0.15°, a total of three radiographs was acquired on each position and the average radiograph was saved. An operating voltage of 110 kV using a current of 60 µA with a 1000 ms exposure time was applied, this led to an approximate isotropic voxel size of less than ca. $(1.75\ \mu m)^3$. The resulting radiographs were reconstructed in cross-sectional images using the Phoenix datos|x 2.0 software package with a beam hardening correction of 8 and a Gaussian filter of 6. Image processing of the reconstructed datasets was done with CTan (Bruker micro-CT, Kontich, Belgium). Firstly, the Ti implant was identified using a standardized global threshold and after despeckling by removing white speckle noise smaller than 10 voxels and black speckle noise smaller than 50 voxels, the resulting binarized data set was used to create a 3D implant model. Next, a region of interest for bone growth analysis was defined based on a dilation of the implant surface with 20 voxels. Finally, a global threshold was chosen manually to select the bone phase within the defined region of interest. After despeckling by removing white and black speckle noise smaller than 50 voxels, the resulting binarized data set was used to create a 3D model for the bone phase. 3D visualization of the combined implant and bone models was done using CTvol (Bruker micro-CT, Kontich, Belgium).

Statistical analyses and reproducibility of the results

Statistical analyses were performed using Student's *t*-test (GraphPad Prism Software). Differences were considered significant if $*p \leq 0.05$. All *in vitro* experiments performed in this study were repeated at least three times, always using two discs per tested group. Three independent discs were used for microscopy analyses. The *in vivo* experiment with *S. aureus* was performed using 11 BALB/c mice implanted with control-Ti discs and 8 mice implanted with VAN-Ti discs. The *in vivo* experiment with *C. albican*s was carried out using 8 mice implanted with control-Ti discs and 10 mice carrying CAS-Ti substrates. Experiments using MSC and HVMEC were performed in triplicate.

Results

Vancomycin- and caspofungin-coated titanium reduce *in vitro* biofilm development of *S. aureus* and *C. albicans*, respectively

After covalent immobilization of vancomycin or caspofungin to Ti discs, the amount of vancomycin bound to the discs was determined chromatographically using HPLC as 35 pmol/cm^2, whereas the amount of caspofungin bound to the discs was 2191 pmol/cm^2. These results are supported by the qualitative observations by SEM top view images of control-Ti, VAN-Ti or CAS-Ti discs (Figure 1). As the amount of vancomycin detected by HPLC is only sufficient to establish a monolayer of the molecule, this coating could not be visualized by SEM. On the other hand, the high amount of caspofungin results in a multilayered coating that could clearly be observed by SEM as a diffuse organic layer, indicative for the caspofungin coating, filling up the surface cavities of CAS-Ti samples.

To evaluate *S. aureus* and *C. albicans* colonization and biofilm formation on VAN-Ti and CAS-Ti discs, respectively, relative to control-Ti discs, coated and control discs were inoculated with a bacterial or a fungal cell suspension and biofilms were allowed to develop for 24 h. We found significantly less colonization of bacterial and fungal cells on VAN-Ti and CAS-Ti discs, respectively, compared to the control-Ti discs (*p<0.05) (Figure 2A and 2B), as measured by cfu counts. To further confirm these observations, biofilms formed on the discs were visualized by CSLM. CSLM imaging showed a significant reduction of the number of viable (green) cells on the VAN-Ti and CAS-Ti discs, respectively compared to the control-Ti discs (Figure 3A and 3B). In a next step, CSLM images were analyzed using an in-house developed software routine to calculate the area fraction covered by biofilms. As evidenced from Figure 3C, a 93 % reduction in viable area fraction was observed on the VAN-Ti discs, whereas 100 % reduction in viable fungal cells was documented on CAS-Ti discs (Figure 3D).

To assess whether CAS-Ti can affect adhesion of *C. albicans* cells, we assessed the number of viable *C. albicans* cells on CAS-Ti or control-Ti discs after adhesion for 90 min. We found that the number of *C. albicans* cells recovered from CAS-Ti discs after 90 min of adhesion was 10-fold lower as compared to control-Ti discs (data not shown), indicating that CAS-Ti discs can inhibit adhesion of *C. albicans* as well as reduce further biofilm formation by this pathogen.

VAN-Ti and CAS-Ti discs reduce *in vivo S. aureus* and *C. albicans* biofilm formation, respectively

An *in vivo* model of biomaterial-associated infections was originally developed to study *S. epidermidis* biofilm formation on silicone and titanium substrates.[30] In this study, we adapted this model to assess *S. aureus* and *C. albicans* biofilm development on Ti substrates. Coated or non-coated Ti discs were implanted subcutaneously on the back of mice. The next day, *S. aureus* or *C. albicans* cells were injected subcutaneously near the discs. First, we established the appropriate *S. aureus* and *C. albicans* inoculum to inject in the mice, resulting in biofilm formation on control-Ti discs *in vivo*. To this end, animals were challenged with a subcutaneous injection of different amount of *S. aureus* cells (1×10^5 cells, 1×10^6 cells and 1×10^7 cells) or *C. albicans* cells (2.5×10^4 cells, 2.5×10^5 cells and 1×10^7 cells), alongside the implant after which microbial colonization was quantified using cfu counting. Only in the mice challenged with the highest inoculum, biofilms developed on control-Ti discs (data not shown).

Next, we assessed whether VAN-Ti and CAS-Ti discs could resist *S. aureus* and *C. albicans* biofilm formation, respectively, using this *in vivo* model. We found that *S. aureus* biofilm formation on the VAN-Ti discs was reduced by approximately 99.9 % (Figure 4A), whereas *C. albicans* biofilm development on the CAS-Ti was inhibited by 89 %, as compared to the

359 control-Ti discs (*p<0.05) (Figure 4B). The numbers of *S. aureus* and *C. albicans* cells
360 recovered from VAN-Ti and CAS-Ti discs, respectively (Supplementary Figure 4).

361 Finally, we analyzed the amount of *S. aureus* and *C. albicans* cells in the peri-implant tissue.
362 No significant difference was found between the number of bacterial or fungal cells
363 colonizing the tissue surrounding the VAN-Ti or CAS-Ti discs, respectively, as compared to
364 control-Ti (Figure 4C and 4D), indicating that release of vancomycin or caspofungin from the
365 discs to the peri-implant tissue is negligible.

366 **Osseointegration potential**

367 For future applications of titanium implants coated with therapeutics, it is important to
368 examine whether such coated discs negatively affect growth of osteogenic and vasculogenic
369 cells, as those are highly relevant cell types in the bone tissue turnover and regeneration
370 processes[31]. Therefore, we first tested *in vitro* whether control-Ti, VAN-Ti and CAS-Ti discs
371 could support attachment and growth of both types of cells (MSC and HMVEC) as observed
372 by Phalloidin (staining of actin cytoskeleton) and DAPI stain (staining of nuclei) at day 5. We
373 found that control-Ti, as well as VAN-Ti and CAS-Ti discs fully supported attachment and
374 growth of both types of cells (Figure 5), indicating no cytotoxic effects of coated Ti discs for
375 both types of cells. Next, we analyzed the *in vivo* osseointegration of VAN-OPTi and
376 compared it to control-OPTi substrates in a rat model. Upon surgery, X-ray analysis was
377 performed to determine the localization of the implant (Supplementary Figure 3). All implants
378 were correctly positioned. MicroCT analysis was used to assess the 3D bone growth within
379 150 µm (ca. 20 voxels) around the implant surface. After 8 weeks of implantation, the volume
380 and distribution of the bone phase directly in contact with the VAN-OPTi surface was
381 comparable to control-OPTi surfaces (Figure 6), indicating that the vancomycin-coating does
382 not hamper osseointegration.

Discussion

In the past decades, the use of various types of medical devices has increased. On one hand, the use of these devices is often compulsory in hospitalized patients; on the other hand they may serve as a niche for microorganisms resulting in biofilm-associated infections. Such implant-related infections have become a serious problem worldwide.[1] Currently, the only existing successful therapy is a burdensome revision surgery, and in worst-case scenarios, amputation of the infected limb is necessary.[3,32] In addition to severe patient discomfort, such treatment procedures exert a significant financial burden on the health sector.[4] Over the last years, several measures have been taken to reduce the amount of implant-related infections by, for example, incorporating strict hygienic routines and perioperative antibiotic prophylaxis.[33-35] Nevertheless, since the incidence of implant-related infections continues to rise[6], research has been focusing on the development of new strategies to combat these infections.

In this study, we explored the potential of an improved antimicrobial coating technique to limit bacterial and fungal colonization of titanium implants. Titanium was chosen as the target material because of its extensive use in the field of orthopedic and dental implants.[36-37] Lately, substantial research has been performed to develop titanium implants on which antimicrobial molecules are covalently immobilized.[20-25] However, the described coating techniques make use of an argon atmosphere, making it difficult to coat large amounts of titanium implants simultaneously. Here, we used an improved technique to covalently coat antimicrobials on titanium implants that is amenable to large-scale production. In addition, given the highly reactive hexamethylene diisocyanate used for the reaction of the 3-aminopropyl-triethoxy silane with the antimicrobial agents, this coating technique has the potential to covalently link titanium surfaces with a plethora of structurally distinct, antimicrobial molecules.

Since Gram-positive bacteria such as *S. aureus* play a major role in biofilm-associated implant infections[7], we here developed titanium surfaces functionalized with the commonly used antibiotic vancomycin at a concentration of 35 pmol/cm^2, which is substantially higher than vancomycin loads described in previous studies (i.e. 4.17 pmol/cm2).[23] Moreover, the use of an FMOC-protected silane reagent in this study, rather than a silane reagent with a free amino group[38], prevents crosslinking of the silane. In addition, also fungal species can cause biofilm-associated implant infections, although these infections are less common. Therefore, we also investigated the antibiofilm activity of covalently bound caspofungin (at a concentration of 2191 pmol/cm²), an antimycotic with documented antibiofilm activity against the fungal pathogen *C. albicans*.[39-40]

Our results demonstrate that VAN-Ti and CAS-Ti substrates can limit colonization and biofilm formation of bacterial and fungal pathogens, respectively, under *in vitro* conditions. We found that *in vitro S. aureus* biofilm formation on VAN-Ti discs was decreased by approx. 50 % and that CAS-Ti discs completely prevented *C. albicans* biofilm formation. These findings were confirmed by microscopic analysis. Our results are consistent with earlier reports on the antimicrobial activity of titanium-bound vancomycin.[23,38] To the best of our knowledge, covalent immobilization of caspofungin to biomaterials or plastics has never been reported. However, one report exists regarding the non-covalent association of caspofungin to polystyrene by incubating the drug overnight in the wells of a polystyrene plate, which was effective in preventing *C. albicans* biofilm formation.[41]

Since silane-based titanium coatings can be unstable under physiological conditions[42], it is important to assess their efficacy under *in vivo* conditions. To evaluate the antibiofilm activity of VAN-Ti and CAS-Ti discs *in vivo*, an existing murine infection model of biomaterial-associated infection was used.[30] Our results show that this model can be adapted to examine biofilm formation by *S. aureus* or *C. albicans* on titanium discs. Strikingly, *in vivo*

staphylococcal biofilm formation was reduced by more than 99 % on the VAN-Ti discs, demonstrating the clinical potential of such coated discs to resist bacterial biofilm formation. It is likely that such reduction in staphylococcal colonization on the titanium discs is sufficient to allow further clearance of the infection by the host defense mechanism. In addition, also *C. albicans* biofilm development was significantly decreased on CAS-Ti discs (89 % inhibition). It should be noted that in our experimental setup, *S. aureus* and *C. albicans* biofilm formation on the discs was assessed after 4 and 2 days, respectively. Longer incubation periods were not tested at this stage. However, according to the study of Botequim and colleagues[43], non-releasing coatings are thought to produce longer-lasting antimicrobial or antibiofilm effects because they can ensure local on-site high concentrations of the bio-active molecules. In addition, analysis of the tissue surrounding the discs showed that there was a similar tissue burden in the mice in which control-Ti, VAN-Ti or CAS-Ti was implanted, suggesting that potential release of vancomycin or caspofungin is minimal. However, further research should address potential release of the coated compounds over a longer time period.

Finally, we tested the osseointegration potential of the VAN-Ti and CAS-Ti discs *in vitro* as such modified Ti substrates should still fully support osseointegration. The osseointegration potential of the discs was determined by visualizing the adhesion and proliferation of bone marrow derived stromal cells and microvascular endothelial cells, two cell types that are highly relevant in bone tissue turnover and regeneration processes.[31] No differences were observed when comparing growth of both cell types on control-Ti discs, VAN-Ti or CAS-Ti discs. These results were further corroborated *in vivo* in a rat osseointegration model for a clinically relevant orthopaedic implant surface coated with vancomycin, as the early bone response at the bone/implant interface of VAN-OPTi implants did not differ from the one at pristine OPTi implants. However, there are still extended *in vivo* analyses necessary, in the

implant osseointegration set-up, to further substantiate the current *in vitro* and *in vivo* data. In a follow-up study we envisage a thorough qualitative and quantitative analysis by combining gold standard 2D histology and histomorphometry with 3D µCT image analysis and this for both VAN-Ti and CAS-Ti coatings at various time points in infection models.

Taken together, we developed titanium substrates on which vancomycin or caspofungin was covalently linked, using a coating technique that has the potential to simultaneously coat large amounts of titanium implants. Furthermore, our results demonstrate that VAN-Ti and CAS-Ti substrates can significantly reduce colonization of *S. aureus* and *C. albicans* under both *in vitro* and *in vivo* conditions, illustrating the clinical usefulness of such anti-infective surfaces. However, note that such coatings do not necessarily protect for delayed hematogenous infections. This has to be further investigated in the future.

In nature, most biofilms consists of multiple species. As bacterial tolerance to antibiotics can be altered due to the presence of another species such as *C. albicans*[44-46], an efficient strategy to prevent biofilm-related infections will be to combine different antibiotics and antifungals in one coating, using the same coating technique.[41]

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Transparency declarations

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Author contribution

Ti discs were produced by F.I., while J.F.G was responsible for the production of OPTi implants; compound coating and analysis was performed by M.E. *In vitro* experiments were designed by S.K, E.G, K.D.B, K.T, N.D, N.V, H.T, P.V.D., B.P.A.C, J.M. and performed by S.K, N. D. and E.G. Microscopic analysis was performed by A.K, M.L, A.B. and J.V; i*n vivo* experiments by S.K, H.T., K.C., G.M., T.S., E.P., W.J.S., K.V. and P.V.D; and subsequent µCT analysis by A.B. and J.V. *In vitro* osseointegration potential was assessed by M.Fr., K.C. and M.K. K.D.B and K.T. coordinated the study. The manuscript was written by S.K. and

E.G. and revised by N.V, K.D.B, K.T, M.Fa, K.V., P.V.D. and J.M. All authors have read and approved the final manuscript.

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Figure legends

Figure 1: Scanning electron microscopy images of the titanium surface. The beadblasted and acid-etched titanium reference substrate (control-Ti) and Ti substrates coated with vancomycin (VAN-Ti) or caspofungin (CAS-Ti).

Figure 2: *In vitro* analysis of *Staphylococcus aureus* and *Candida albicans* biofilm formation on VAN-Ti and CAS-Ti discs, respectively. (A) Percentage of *S. aureus* and (B) *C. albicans* biofilm cells present on VAN-Ti discs and CAS-Ti discs, respectively, calculated relative to the amount of microbial cells on control-Ti discs. Data represent means ± standard errors of the means (SEM) from 3 independent experiments (*p<0.05).

Figure 3. Visualization of *in vitro Staphylococcus aureus* and *Candida albicans* biofilms on VAN-Ti and CAS-Ti discs, respectively. (A) *S. aureus* and (B) *C. albicans* biofilm cells grown on control-Ti, VAN-Ti and CAS-Ti discs, respectively, were stained with the LIVE/DEAD®BacLight™ viability kit and visualized by confocal laser scanning microscopy (CLSM). Viable cells were stained green and dead cells were stained red. Quantification of the viable and dead area fraction of (C) *S. aureus* and (D) *C. albicans* biofilm cells on VAN-Ti and CAS-Ti discs, respectively. Means and SEM of 3 independent experiments are shown (*p<0.05).

Figure 4: *In vivo* efficacy of VAN-Ti and CAS-Ti discs against *Staphylococcus aureus* and *Candida albicans* biofilm formation, respectively. (A) Quantification of *S. aureus* and (B) *C. albicans* biofilm formation developed on control-Ti, VAN-Ti and CAS-Ti in a murine model of biomaterial-associated infection (*p<0.05), via cfu counting. Quantification of the amount of (C) *S. aureus* and (D) *C. albicans* cells present in the tissue surrounding the control-Ti, VAN-Ti and CAS-Ti discs, respectively. All data represent means ± SEM from 2 independent experiments.

631 **Figure 5: *In vitro* analysis of the cytotoxicity of coated and uncoated Ti discs for bone**
632 **marrow derived stromal cells and microvascular endothelial cells.** Visualization of bone
633 marrow derived stromal cells and microvascular endothelial cells grown on control-Ti, VAN-
634 Ti and CAS-Ti discs. After 5 days of incubation, actin cytoskeleton was visualized by
635 phalloidin staining (green fluorescence) and nuclei were stained with DAPI (blue
636 fluorescence).

637 **Figure 6: *In vivo* analysis of the osseointegration of vancomycin coated open porous Ti**
638 **implants in a rat model.** µCT-based 3D visualization of the bone growth (red) within a
639 region of interest of 150 µm from the implant surface (grey) for control-OPTi and VAN-OPTi
640 implants following 8 weeks of implantation.

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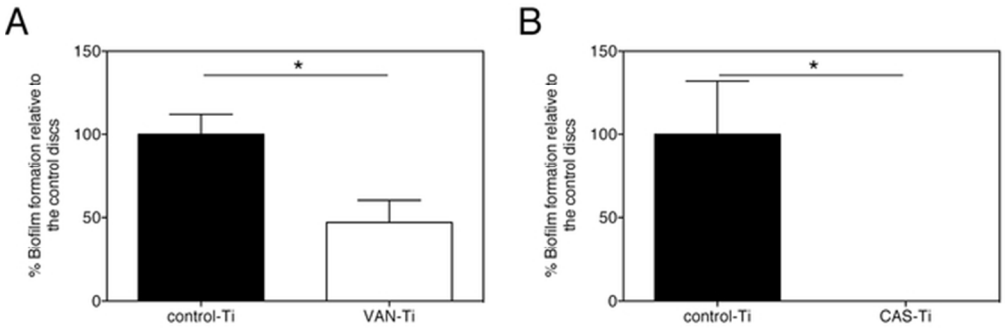

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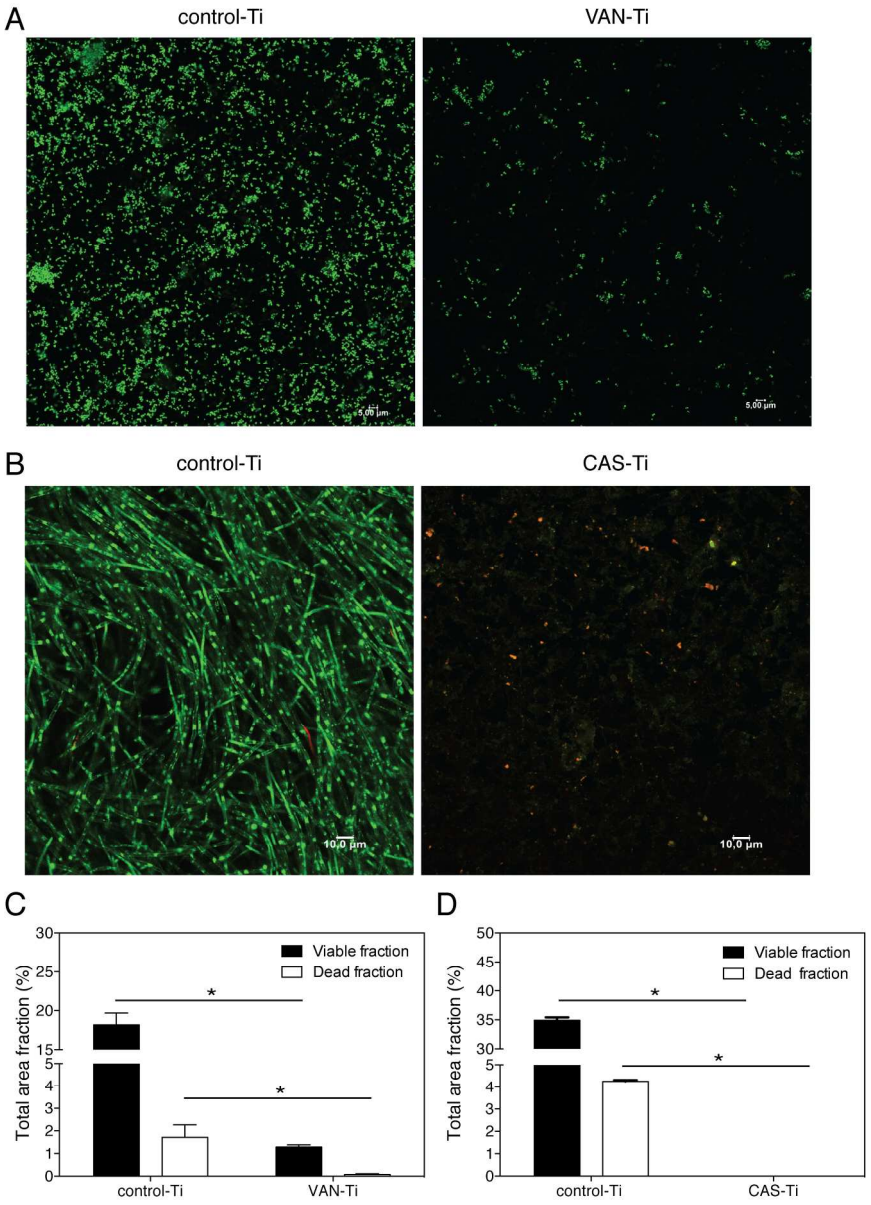

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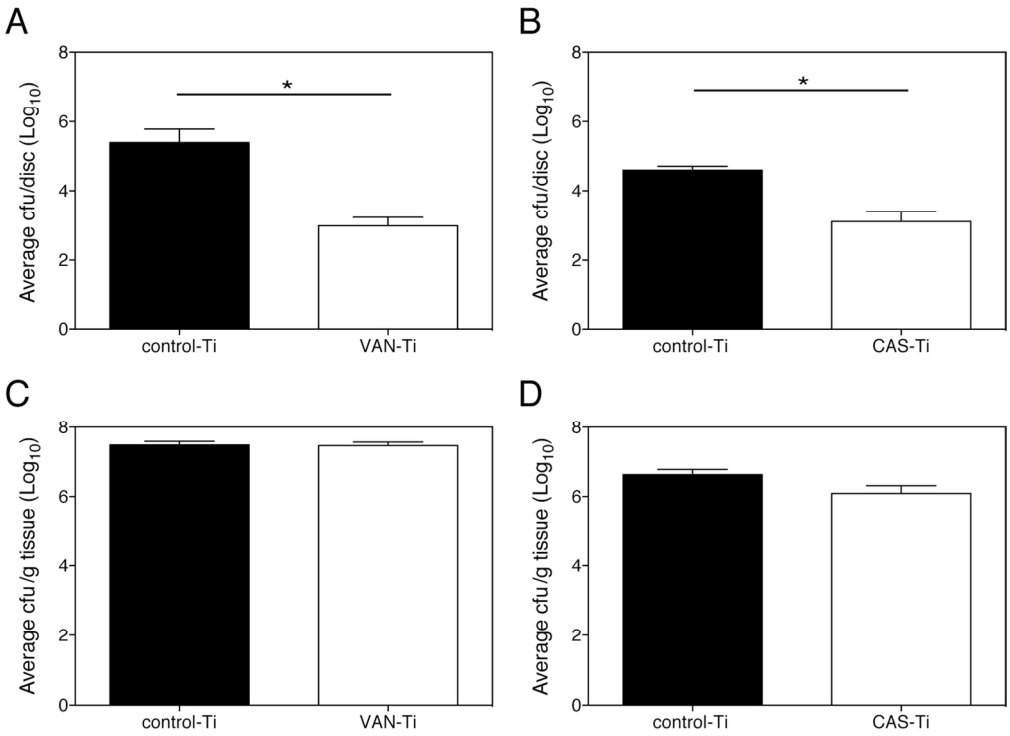

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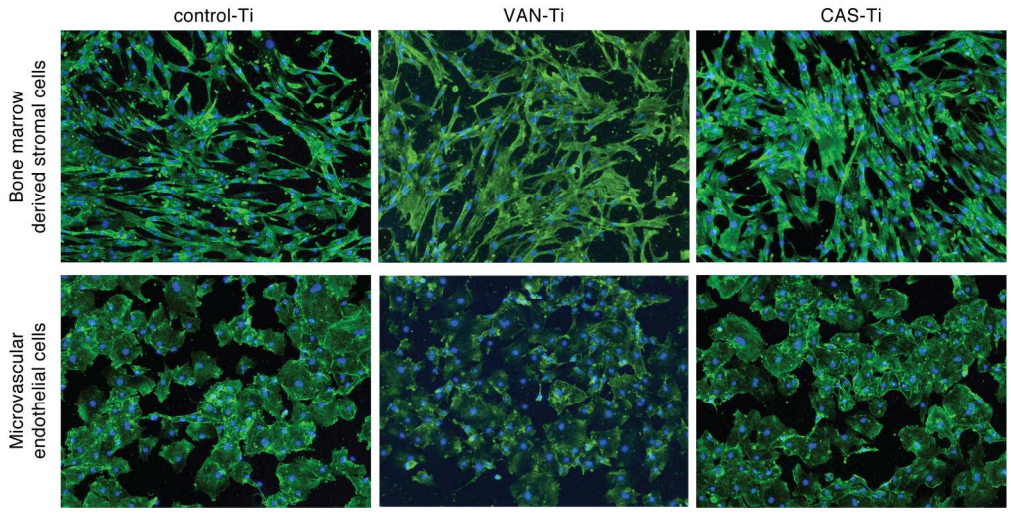

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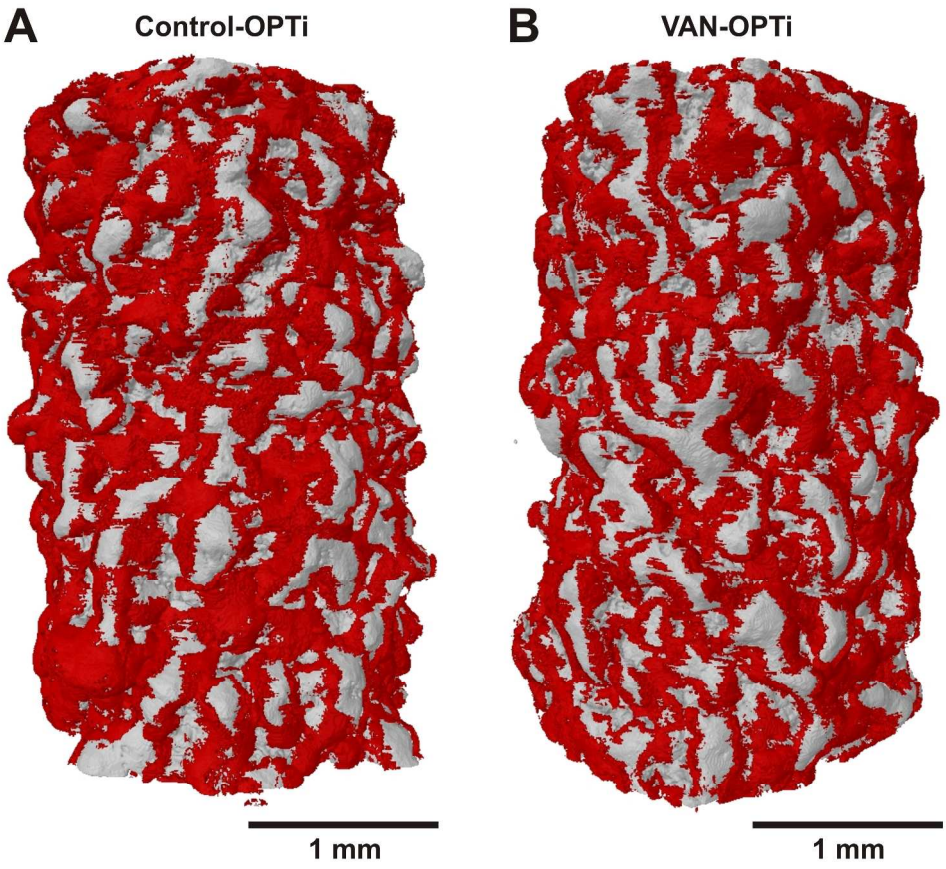

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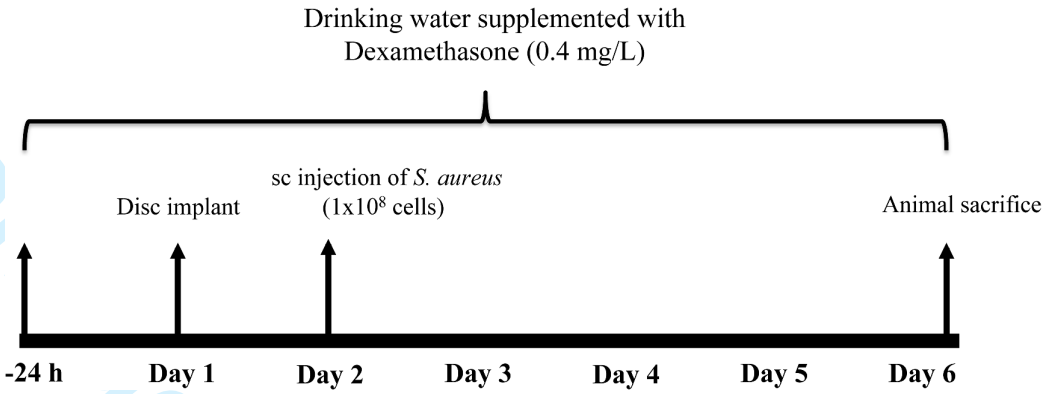
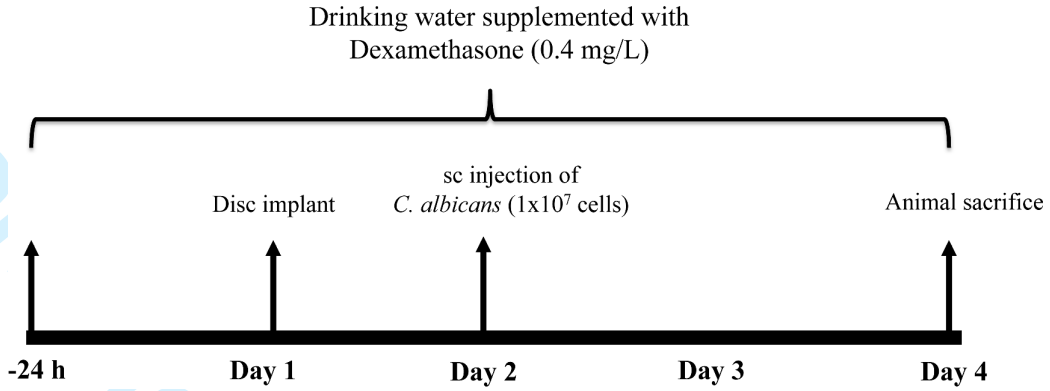
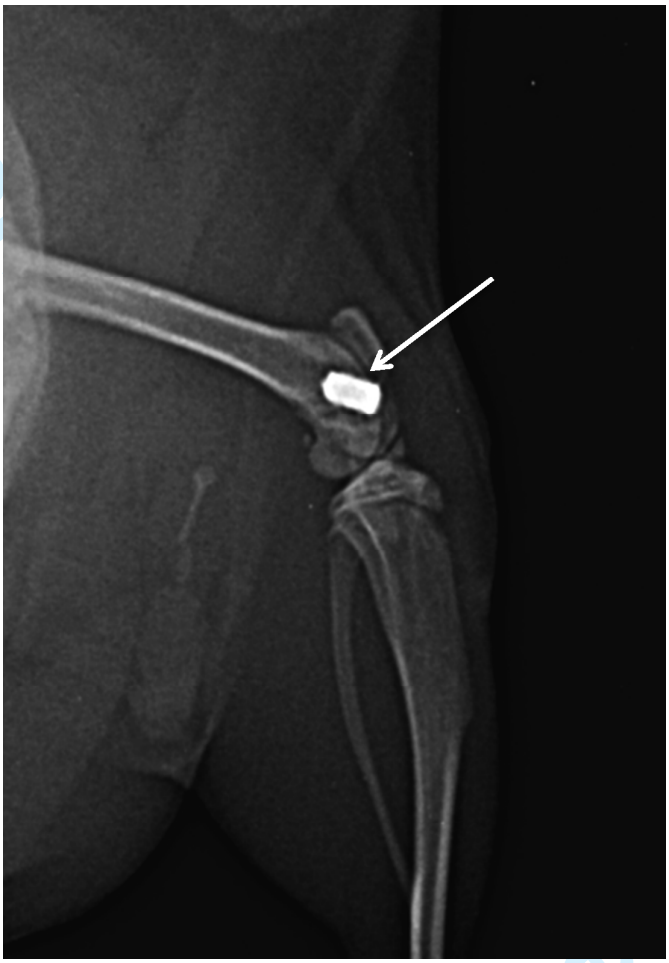


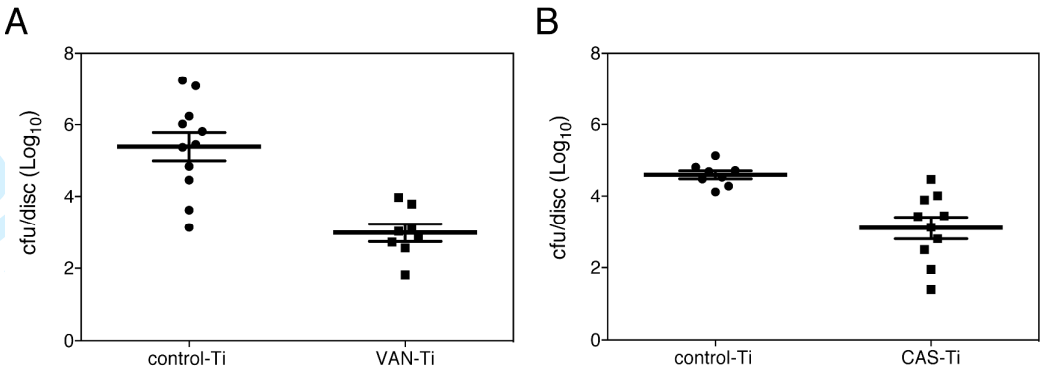
Supplementary Figure 1. Experimental time line of a novel biomaterial-associated murine model to test vancomycin-coated titanium discs.



Supplementary Figure 2. Experimental time line of a novel biomaterial-associated murine model to test caspofungin-coated titanium discs.



Supplementary Figure 3. X-ray imaging (AXION Iconos R100, Simens, Germany) was performed to check the position of the titanium implant (white arrow). This is usually done one to five days after operations.



Supplementary Figure 4. The number of *Staphylococcus aureus* and *Candida albicans* cells recovered from *in vivo* biofilms developed on vancomycin-coated (VAN-Ti) and caspofungin-coated titanium (CAS-Ti) discs, respectively.