

Preventing Implant-Associated Infections by Silver Coating

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Implant-associated infections (IAIs) are a dreaded complication mainly caused by biofilm-forming staphylococci. Implant surfaces preventing microbial colonization would be desirable. We examined the preventive effect of a silver-coated titanium-aluminum-niobium (TiAlNb) alloy. The surface elicited a strong, inoculum-dependent activity against *Staphylococcus epidermidis* and *Staphylococcus aureus* in an agar inhibition assay. Gamma sterilization and alcohol disinfection did not alter the effect. In a tissue cage mouse model, silver coating of TiAlNb cages prevented perioperative infections in an inoculum-dependent manner and led to a 100% prevention rate after challenge with 2×10^6 CFU of *S. epidermidis* per cage. In *S. aureus* infections, silver coating had only limited effect. Similarly, daptomycin or vancomycin prophylaxis alone did not prevent *S. aureus* infections. However, silver coating combined with daptomycin or vancomycin prophylaxis thwarted methicillin-resistant *S. aureus* infections at a prevention rate of 100% or 33%, respectively. Moreover, silver release from the surface was independent of infection and occurred rapidly after implantation. On day 2, a peak of 82 $\mu\text{g Ag/ml}$ was reached in the cage fluid, corresponding to almost $6 \times$ the MIC of the staphylococci. Cytotoxicity toward leukocytes in the cage was low and temporary. Surrounding tissue did not reveal histological signs of silver toxicity. *In vitro*, no emergence of silver resistance was observed in several clinical strains of staphylococci upon serial subinhibitory silver exposures. In conclusion, our data demonstrate that silver-coated TiAlNb is potent for prevention of IAIs and thus can be considered for clinical application.

The number of implanted medical devices is steadily rising and has become an effective intervention to substitute for defective anatomical structure or biological function. Despite the rapid advancement in material science and surgical techniques, the burden of implant-associated infections (IAIs) has grown, ranging between 2% and 40%, depending on the type of surgical implant (1). This creates an immense economic and health care problem (2–4). Infecting microorganisms can be introduced either intraoperatively via direct contact, postoperatively via continuous spread through the wound, or in a later stage via the hematogenous or lymphogenous route (5). *Staphylococcus aureus* and coagulase-negative staphylococci, including *Staphylococcus epidermidis*, account for 50 to 60% of the causative bacteria (6). Due to their ability to rapidly form biofilm on the inert surface, they manage to grow and evade not only the defensive mechanisms of the host but also the bactericidal activity of antibiotics (7). Effective treatment of IAIs therefore involves surgery, often resulting in the removal of the nonsalvageable biomaterial followed by a long course of antibiotic therapy (8, 9).

The significant difficulties in treatment of established biofilms prompted research on engineering of implant surfaces that could resist microbial colonization. Current approaches include physically active antiadhesive surfaces, coatings with various bactericidal materials and molecules, with quorum-sensing quenchers, or even with host immune modulators (10). Many promising *in vitro* data are available, yet *in vivo* studies or clinical evaluations are scarce.

Silver is promising for coating implants as it has a broad spectrum of antibacterial activity against planktonic and sessile Gram-positive and Gram-negative bacteria, including multiresistant bacteria (11–13). Recently, silver coating of central venous catheters (14, 15), urinary catheters (16), and ventilator endotracheal tubes (17) has been shown to reduce the infection rate. In ortho-

pedic hardware, silver-coated external fixation pins (18), proximal femur or tibia megaprotheses (19), and tumor endoprotheses (20) showed a trend toward reduction of infections. Despite this broad clinical use, little is known about the stability of silver-coated alloys, their efficacy on biofilm-forming bacteria—especially in combination with antibiotics—and the kinetics of release. Moreover, silver has a broad range of bacterial targets, including the respiratory chain (21–24), and has been shown to induce resistance in Gram-negative bacteria and toxicity in eukaryotic cells (25, 26).

Here, we examine the use of a preclinical silver-coated titanium-aluminum-niobium (TiAlNb) alloy against biofilm-forming *S. aureus* and *S. epidermidis* *in vitro* and *in vivo* in a subcutaneous tissue cage mouse model for its activity to prevent IAIs, its possible toxicity, and the emergence of resistance upon silver exposure.

MATERIALS AND METHODS

Media and chemical substances. For bacterial growth, tryptic soy broth (TSB) and Mueller-Hinton broth and agar (MHB and MHA) were ob-

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tained from Becton, Dickinson (Allschwil, Switzerland). Saline (0.9%) and sterile water (*aqua ad iniectionem*) were obtained from Bichsel (Interlaken, Switzerland). AgNO₃ was acquired from Sigma and dissolved as stock solution in sterile water. For *in vitro* and *in vivo* experiments, the antibiotics daptomycin (DAP) (Cubicin; Novartis) and vancomycin (VAN) (Vancocin; Teva Pharma) were used. Stock solutions of DAP were prepared in 0.9% saline and always supplemented *in vitro* with 50 mg/liter (1.25 mmol/liter) calcium ions (CaCl₂) according to CLSI guidelines (27). Stock solutions of VAN were prepared in water.

Surface alloys. The surfaces used in this study were comprised of titanium alloys such as TiAlNb, corresponding to ISO 5832-11. For the *in vitro* agar inhibition, we used discs of 10-mm diameter and 2-mm thickness. These were grit blasted on one side with alumina microspheres in order to obtain a roughness factor (R_a) of 3 μ m. For the *in vivo* assays, cylindrical cages (so-called “tissue cages”; 8.5 by 1 by 30 mm; volume, 1.9 ml) made of the same alloys were provided by Medacta, Schaffhausen, Switzerland.

Coating with silver. Discs and tissue cages were first cleaned with an alkaline solution according to the industrial process and pretreated with a 5 mM isonicotinic acid linker mixture (CH₂Cl₂-EtOH [1:1]). After 24 h, each specimen was immersed twice into the mother liquor (EtOH-THF [1:1]) of a 2 mM solution of silver(I) compound based on silver nitrate and ethanedyl bis(isonicotinate) ligand (see also reference 28). Each immersed specimen was incubated for 3 h in the dark, thereafter biologically washed with hydrofluoroether-isopropyl alcohol solution, and finally gamma sterilized (25 kGy).

Bacterial strains and growth conditions. The following bacterial strains were used: *S. epidermidis* 1457 (kindly provided by D. Mack, University of Swansea, United Kingdom), methicillin-resistant *S. epidermidis* (MRSE) ATCC 35984, copper-resistant *S. aureus* ATCC 12600, methicillin-resistant *S. aureus* (MRSA) ATCC 43300, the MRSA USA300 JE2 wild-type strain and its putative Δ SiIE mutant (transposon mutant knockout of *S. aureus* gene locus USA300_1847) (kindly provided by J. Schrenzel, University of Geneva, Switzerland) originally from the Nebraska Transposon Mutant Library (29), methicillin-susceptible *S. aureus* (MSSA) strain SA113 (ATCC 35556), MSSA Newman ATCC 13420, *Enterobacter cloacae* ATCC 23355, and *E. cloacae* ATCC 13047. The strains were stored at -80°C with the cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). The inoculum for *in vivo* and *in vitro* studies was prepared as previously published (8, 30). Bacterial numbers were determined by plating aliquots from appropriate dilutions on MHA, followed by colony counting after 24 h of incubation at 37°C .

MIC. According to the CLSI guidelines, a standard inoculum of 1×10^5 to 5×10^5 CFU/ml was used. The MIC was determined by using 2-fold dilutions of AgNO₃ in MHB (27). The MIC was the lowest AgNO₃ concentration that inhibited visible bacterial growth (31).

Induction of silver resistance. Flat-bottom 96-well plates were seeded with bacteria of all strains mentioned above, except *S. aureus* strain SA113 at 10^5 CFU/ml in serially diluted AgNO₃ (concentrations ranging from 1,000 μ g/ml to 7.1 μ g/ml) in TSB and incubated for 24 h at 37°C . Thereafter the bacteria at the highest AgNO₃ concentration with visible growth were used for inoculation and incubation of a subsequent 96-well plate in exactly the same manner. This procedure was repeated for 50 times. Development of resistance was considered if growth occurred at an AgNO₃ concentration of at least 3 serial dilution steps higher than the step before.

Agar inhibition assays (Kirby-Bauer assay). The agar inhibition assay was performed as previously published (28). Briefly, MHA was inoculated with *S. epidermidis* 1457 or *S. aureus* SA113 (1×10^4 , 1×10^6 , and 1×10^7 CFU/ml). TiAlNb discs were placed in this agar. The plates were incubated for 18 h at 37°C , and the diameters of the inhibition zones around the discs were measured.

Quantification of biofilm and adherent staphylococci after treatment with silver and antibiotics. Flat-bottom 96-well plates were seeded with MRSA ATCC 43300 cells at 10^5 CFU/ml (Becton Dickinson and Company, Allschwil, Switzerland) and incubated with or without silver

nitrate at 15.6 μ g/ml (i.e., 91.8 μ M) or 31.2 μ g/ml (i.e., 183.6 μ M) combined with 30 μ g/ml DAP (i.e., 18.5 μ M) or 50 μ g/ml VAN (i.e., 34.5 μ M) for 24 h at 37°C . After incubation, nonadherent bacteria were removed, and each remaining biofilm was washed twice with phosphate-buffered saline (PBS). Then the CFU of adherent bacteria as well as biofilm mass were quantified by plating and crystal violet staining, respectively, as described previously (30).

Animal model. In the Animal House of the Department of Biomedicine, University Hospital Basel, 12- to 15-week-old female C57BL/6 mice were kept under specific-pathogen-free conditions according to the regulations of the Swiss Veterinary Law and with approval of the University Hospital Basel Animal Ethical Committee. Mice were anesthetized via intraperitoneal injection of 65 mg/kg ketamine (Ketalar; Pfizer AG, Zürich, Switzerland) and 13 mg/kg xylazine (Xylasol; Graeb AG, Bern, Switzerland). Sterile tissue cages were subcutaneously implanted under aseptic conditions into an air pouch made in the back of each mouse (32). The inocula contained 5×10^2 to 1×10^8 CFU of bacteria, which were injected directly into the cage percutaneously either immediately after implantation (i.e., perioperative infection) or 2 weeks later (i.e., postoperative infection). After surgery, mice were treated with 0.05 mg/kg buprenorphine (Temgesic; Essex Chemie AG, Luzern, Switzerland). The minimal infective dose was defined as the count of CFU per tissue cage that was required to induce a persistent infection (15 days) in 100% of the tissue cages (see also reference 30). For the experiments with preoperative antibiotic prophylaxis, we intraperitoneally administered DAP 3 h or VAN 30 min before cage implantation at concentrations previously determined (30) and as determined in the pharmacokinetic/pharmacodynamic studies. Saline (0.9%) was administered in the same way as a control.

Tissue cage fluid (TCF) was collected by percutaneous aspiration at 2, 6, and 9 days after infection and for *S. epidermidis* 1457 additionally at 14 days. The numbers of planktonic bacteria in the TCF were determined by plating appropriate dilutions on MHA plates. Finally the cages were explanted and incubated in TSB for 48 h at 37°C . After 24 h, the tissue cages were vigorously vortexed. If no bacterial growth was detectable after plating the samples on MHA, the infection was considered prevented. The prevention rate was defined as the number of cages without growth divided by the total number in the individual treatment group.

Pharmacokinetic/pharmacodynamic studies. Antibiotic concentrations in the TCF of noninfected mice were determined after a single intraperitoneal administration of VAN at either 50 or 100 mg/kg body weight. Divided into groups of three mice for every time point, TCF was aspirated after 0, 30, 60, 120, 240, 360, 480, 720, and 1,440 min. Concentrations of VAN were determined by using a high-performance liquid chromatography (HPLC) method. The maximum concentration of drug in serum ($C_{\max}$ [milligrams per liter]), the area under the concentration-time curve (AUC [milligram-hours per liter]), and the terminal half-life ($t_{1/2}$ [hours]) were calculated by a computer-assisted method as previously described (33). A dose of 200 mg/kg body weight was chosen to target an AUC from 0 to 24 h (AUC_{0-24}) similar to that in humans after doses of 1 g every 12 h (q12h) (454 mg $\cdot$ h/liter) (34).

In case of DAP, a dose of 50 mg/kg body weight was chosen to obtain an AUC_{0-24} similar to that in humans after a dose of 6 mg/kg body weight (22, 30).

Measurement of silver concentration. The silver concentration in the TCF was measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES [PerkinElmer Optima 7000 DV]). Water (1.5 ml) was added to each TCF sample, followed by the addition of 150 μ l of 10% nitric acid in order to keep the silver ions in solution. The tissue samples were supplemented with 1 ml of water and then ground at 4°C with the Polytron. After a further addition of 2 ml of 20% nitric acid, the samples were sonicated in the ultrasonic bath (Transsonic digital; Elma) at power level 9 to 10 for 30 min, followed by centrifugation at 1,500 rpm for 30 min. Supernatants of the samples were collected, filtered, and measured by ICP-OES.

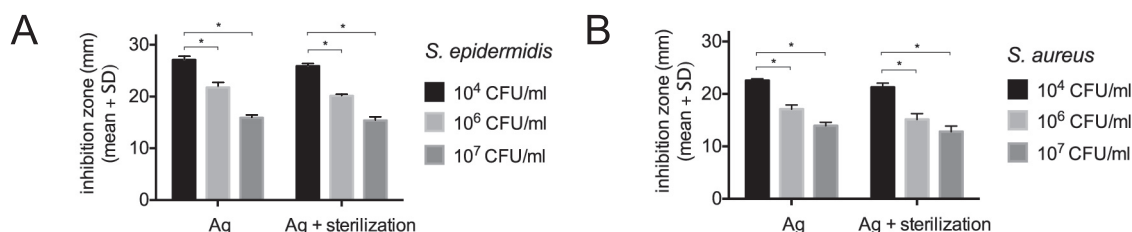


FIG 1 Agar inhibition assay with silver-coated discs (TiAlNb) before and after sterilization. Shown are inhibition zones from different inocula of *S. epidermidis* (A) and *S. aureus* (B). Results are means + standard deviations (SD) from three independent experiments. *, $P < 0.05$.

Leukocyte viability. Leukocyte number and viability in TCF were assessed by trypan blue exclusion. Cells were examined under high-power light microscopy.

Histological staining. After cage explantation, the subjacent tissue was cut out and transferred into 4% formaldehyde solution (Thermo Scientific) in PBS. After overnight fixation, samples were embedded in paraffin, and 5- μ m sections were prepared for hematoxylin-eosin (HE) staining.

Statistical analysis. Data were analyzed with Prism 6.0f (GraphPad Software, Inc.), and the Mann-Whitney test was used for statistical analyses.

RESULTS

Sterilization does not decrease antistaphylococcal activity of silver coating *in vitro*. TiAlNb alloys are widely used for orthopedic implants. In this study, we aim to increase its potential by coating the alloy with silver and thereby rendering it a source of antibacterial activity. For its clinical application, it is of great importance that it still retains its properties after the sterilization procedure. Hence we first investigated the antibacterial activities before and after gamma sterilization and alcohol disinfection in an agar inhibition assay (Fig. 1).

Silver coating resulted in an inoculum-dependent inhibition zone for *S. epidermidis* strain 1457 and *S. aureus* strain SA113. Gamma sterilization and alcohol disinfection did not decrease the activity. The inhibition zones of silver-coated TiAlNb alloys were 27 to 15.9 mm for 10⁴ to 10⁷ CFU/ml *S. epidermidis* 1457. For *S. aureus* SA113, we observed slightly smaller inhibition zones of 22.6 to 13.9 mm for 10⁴ to 10⁷ CFU/ml bacteria. This is in line with previous findings that silver is less effective in the agar inhibition assay against *S. aureus* (24) than against *S. epidermidis*. Thus, we confirmed in a first step that silver coating of TiAlNb alloy produces a strong antistaphylococcal effect and neither gamma sterilization nor alcohol disinfection influenced its outcome.

Silver coating of tissue cages prevents infection with *S. epidermidis* *in vivo*. Due to these promising results, we next wondered if the *in vitro* effects could be reproduced *in vivo* in the mouse tissue cage infection model. Therefore, we challenged subcutaneously inserted sterilized silver-coated TiAlNb cages with *S. epidermidis* 1457 at the time of surgery (i.e., perioperatively) or 14 days after implantation (i.e., postoperatively).

The inocula ranged from the minimal infective dose of 2×10^6 CFU per cage up to 1×10^8 CFU per cage. Silver coating resulted in a rapid initial decrease and complete clearance of the planktonic bacteria at day 9 with the lowest inoculum (Fig. 2A). With 1×10^7 CFU per cage, silver still managed to significantly decrease the planktonic bacteria after 14 days compared to uncoated controls (5.8×10^3 CFU/ml versus 6.6×10^4 CFU/ml; $P = 0.04$) (Fig. 2B). However, at the highest inoculum (Fig. 2C), none of the silver-

coated cages was able to control bacterial growth ($P = 0.10$). In accordance, the infection prevention rate was inoculum dependent (Fig. 2D). In the perioperative setting, the low inoculum resulted in a prevention rate of 100%, the middle inoculum in a rate of 20%, and the high inoculum in a rate of 0%. We subsequently investigated whether this effect was also reproducible in a postoperative infection with a low inoculum of 2×10^6 CFU per cage. Here, the prevention rate was 27% with the coated cages (Fig. 2E), whereas the uncoated cages did not prevent any infection.

In summary, the silver coating prevents an infection with *S. epidermidis* in an inoculum- and time-dependent manner.

Silver is released early after implantation. After documenting the stronger effect of silver in perioperative than in postoperative infections, we were interested in the dynamics of silver release. Therefore, we measured the silver concentration in the TCF in peri- and postoperative infections with *S. epidermidis* 1457.

The silver concentration in the TCF showed similar decreases over time with or without infection with *S. epidermidis* 1457 (Fig. 3). The second day after implantation, the silver concentration was 82 ± 50 μ g/ml with infection versus 88 ± 36 μ g/ml without infection. After 6 days, the rate was 28 ± 18 μ g/ml versus 18 ± 6 μ g/ml, and at day 14, the rate was 11 ± 5 μ g/ml versus 4 ± 1 μ g/ml. When the cages were infected 14 days postoperatively, the silver concentration was only 3 ± 1 μ g/ml at day 28. Between days 6 and 10, the silver concentration fell below the previously determined MIC (AgNO₃) of 15.6 μ g/ml for *S. epidermidis* 1457 (dotted line in Fig. 3). From these results, we can conclude that the release of silver into the TCF is mostly independent of an existing infection. Moreover, we can explain the lower prevention rate in a postoperative infection with the lower silver concentration at the time of infection.

Silver-coated tissue cages reduce planktonic *S. aureus* but are unable to prevent infection *in vivo*. Together with *S. epidermidis*, *S. aureus* is the leading cause of IAIs. Therefore, we next applied the silver-coated cages against *S. aureus* SA113 in a perioperative infection using the minimal infective dose of 1×10^3 CFU per cage. Silver coating of the cages was able to control the infection, resulting in 2.7×10^3 CFU/ml in the TCF after 9 days, whereas the uncoated cages led to a growth of up to 8.3×10^6 CFU/ml ($P = 0.07$) after 9 days (Fig. 4).

However, no bacterial clearance and no infection prevention were achieved. In accordance, no effect on the planktonic and adherent bacteria was detected in the postoperative infection setting (data not shown). In conclusion, silver coating showed a trend of controlling a perioperative infection with *S. aureus*, but it was not capable of preventing infection. Furthermore, the silver remaining in the cages after 14 days did not affect growth or adherence of postoperatively inoculated *S. aureus*.

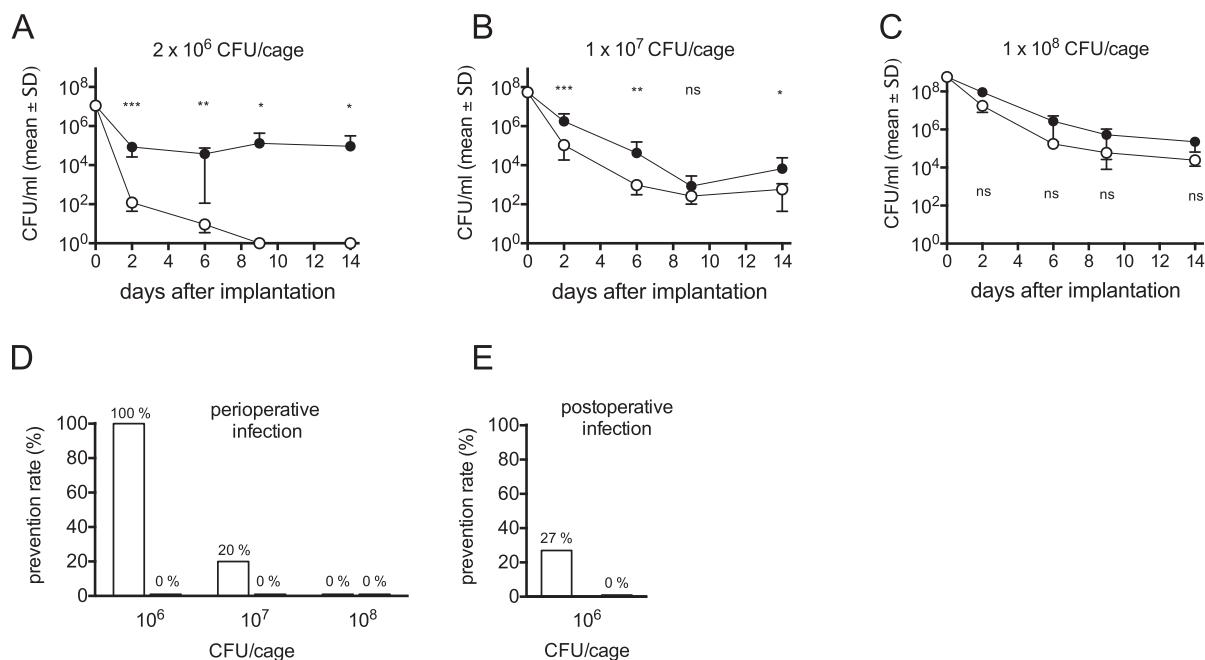


FIG 2 Silver-coated (open circles) and uncoated (closed circles) tissue cage infection with *S. epidermidis* strain 1457 in C57BL/6 mice. The amounts of planktonic CFU per cage in the tissue cage fluid from different initial inocula in perioperative infections are as follows: 2×10^6 (A), 1×10^7 (B), and 1×10^8 (C). (D and E) Prevention rates for silver-coated (open columns) and uncoated (closed columns) TiAlNb cages for perioperative (D) and postoperative (E) infections. Results are means $\pm$ SD from three independent experiments for 2×10^6 CFU per cage with $n = 7$ and 1×10^7 CFU per cage with $n = 9$ and one experiment for 1×10^8 CFU per cage with $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Silver increases the bactericidal activities of vancomycin and daptomycin against adherent MRSA cells *in vitro*. As silver alone was not able to prevent *S. aureus* infection, we questioned whether the addition of antibiotics would lead to an increased bactericidal activity. Therefore, we first combined silver with 30 $\mu\text{g}/\text{ml}$ daptomycin (DAP) or 50 $\mu\text{g}/\text{ml}$ vancomycin (VAN) *in vitro* and measured the reduction of biofilm by crystal violet staining (Fig. 5A) and surface-adherent MRSA ATCC 43300 by CFU counting (Fig. 5B). At a concentration of 31.25 $\mu\text{g}/\text{ml}$ AgNO_3 corresponding to the MIC, silver by itself reduced the biofilm by 90%. VAN and DAP by themselves reduced the biofilm nearly completely. The adherent MRSA cells were reduced by approximately 3 log₁₀ by VAN or DAP alone (dotted line in Fig. 5B). With addition of silver at the MIC, the adherent bacteria decreased by approximately 1 log₁₀ under all conditions. Taken together, silver exhibited a

strong reduction of biofilm and proved to have an additive effect against adherent MRSA.

Joint action of silver and daptomycin prevents MRSA infection *in vivo*. We subsequently investigated whether the observed combined action of silver and antibiotics would prevent an MRSA infection *in vivo*. The timing of the antibiotics was chosen according to the pharmacokinetic profile (see Fig. S1 in the supplemental material) to achieve maximal effect. The dosing of the antibiotics was correlated to the clinically used ones. As single agents, neither preoperative DAP nor VAN nor silver-coated cages were sufficient to prevent a persistent infection with MRSA. Remarkably, in combination with preoperatively applied DAP, silver coating prevented the growth of planktonic as well as adherent MRSA cells, resulting in a 100% prevention rate. The additive effect of preoperative VAN led to a significant reduction of planktonic growth

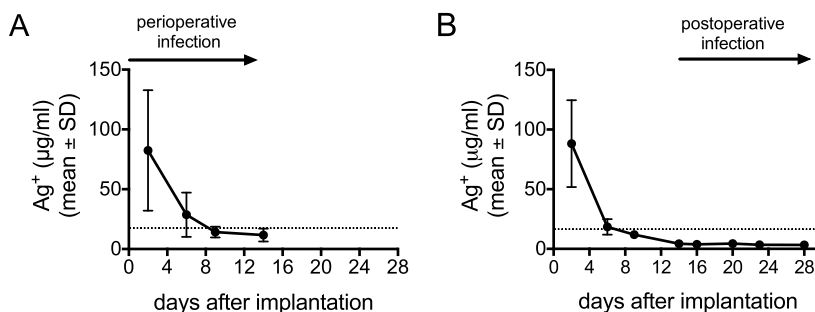


FIG 3 Silver concentration in tissue cage fluid from perioperative (A) or postoperative (B) infections. Results are means $\pm$ SD from at least three independent experiments ($n \geq 12$ for perioperative infection, $n = 11$ for postoperative infection). The dotted line represents the MIC of *S. epidermidis* strain 1457 for AgNO_3 .

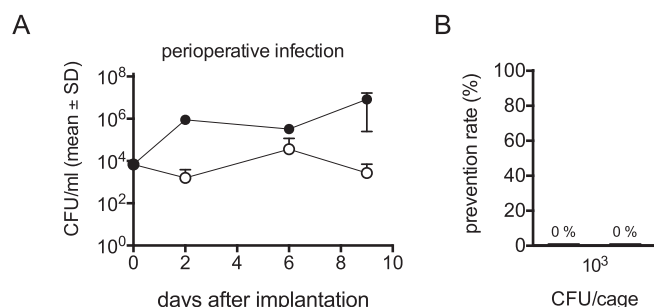


FIG 4 Effect of silver against *S. aureus* strain SA113 in C57BL/6 mice. Shown are planktonic CFU in the tissue cage fluid (A) of silver-coated (open circles) and uncoated (closed circles) TiAlNb cages and the infection prevention rate (B) of silver-coated (left column) and uncoated (right column) TiAlNb cages from the initial inoculum of 1×10^3 CFU per cage in a perioperative infection. Results are means $\pm$ SD from one experiment with eight mice.

and prevented adherence in 33% of cases. The control group was exposed to uncoated cages and saline and showed no bacterial growth inhibition (Fig. 6). Thus, the additive effect of preoperative DAP and silver coating was highly efficacious *in vivo*.

There is no inducible silver resistance in staphylococci. Due to these promising results, we asked the question of whether exposure to silver could induce resistance in staphylococci. If so, it would abolish the efficacy of silver-containing implants, but so far no silver resistance has been observed in Gram-positive bacteria (35–37). Hence, we exposed seven staphylococcal clinical isolates and ATCC strains (MSSE 1457, MRSE ATCC 35984, MSSA ATCC 13420, MRSA ATCC 43300, copper-resistant *S. aureus* ATCC 12600, and MRSA USA300 and its putative Δ SilE mutant—all exhibiting an AgNO_3 MIC of 15.6 or 31.2 $\mu\text{g}/\text{ml}$) to subinhibitory concentrations of silver and performed serial passaging via daily subculturing. Interestingly, no staphylococcal strain showed a significant increase in silver resistance during 50 passages. As a positive control, subculturing was also performed with two Gram-negative strains of *Enterobacter cloacae* (ATCC 13047 harboring the chromosomal silver resistance cassette *SilPABCRSE* and ATCC 23355 without known silver resistance). ATCC 13047 initially showed the same sensitivity to silver as the staphylococcal

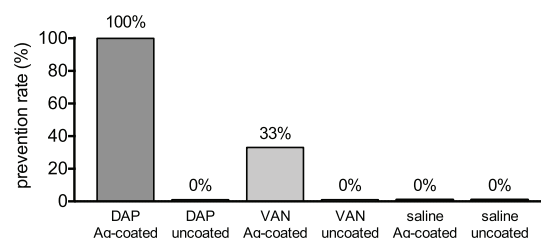


FIG 6 Infection prevention rate of silver-coated or uncoated tissue cages infected with methicillin-resistant *S. aureus* (MRSA) strain ATCC 43300 in C57BL/6 mice. Antibiotic prophylaxis was with daptomycin (DAP) or vancomycin (VAN). Saline (SAL) was used as a control. Results are from at least two separate experiments (DAP, $n \geq 5$; VAN, $n \geq 5$; saline, $n \geq 2$).

strains (MIC of 31.2 $\mu\text{g}/\text{ml}$) but became highly resistant after the 5th passage (MIC of $>1,000 \mu\text{g}/\text{ml}$). On the other hand, *E. cloacae* ATCC 23355 did not develop silver resistance during 50 passages. These results reinforce the available evidence that Gram-positive bacteria seem to lack the potential for *de novo* development of silver resistance.

Cytotoxicity of silver released from tissue cages. Cytotoxicity of silver is a major concern for its application on indwelling devices and orthopedic prostheses (38). We therefore examined the tissue surrounding the cages microscopically for signs of silver-induced damage. This was done for 28 mice with uncoated cages and 29 mice with coated cages. There was consistently no difference between silver-coated and uncoated cages. Both exhibited a lymphocytic pseudocapsule with sporadic polymorphonuclear neutrophils (representative images in Fig. 7), which was attributed to the normal wound healing process in response to the subcutaneous foreign body.

Furthermore, we quantified the percentage of viable leukocytes in the TCF. Two days after implantation of silver-coated cages, 90% of leukocytes were viable. At day 6, already 96% were alive, and after 9 days, the viability further increased to 98%. The uncoated cages always showed approximately 98% viability (Fig. 8).

DISCUSSION

IAIs are difficult to treat due to their strongly reduced susceptibility to antibiotics and to host immune defenses. Furthermore, re-

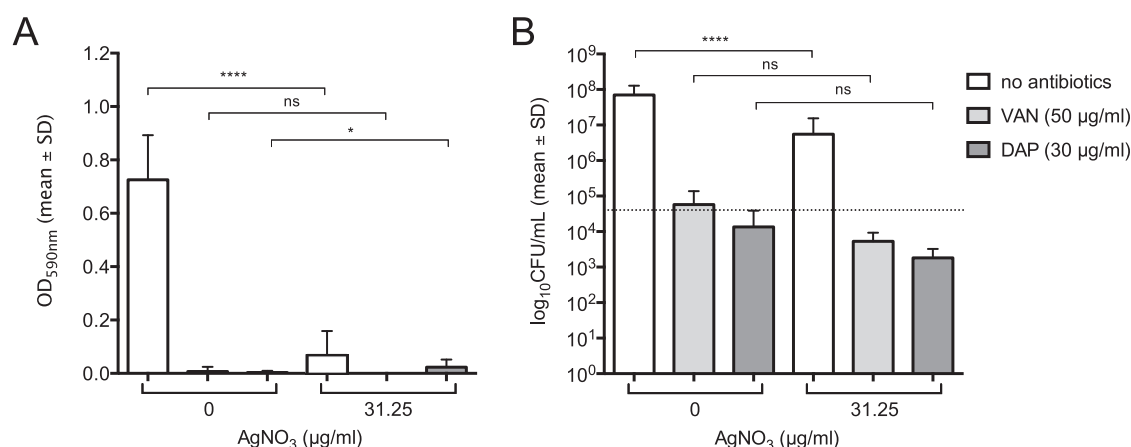


FIG 5 *In vitro* effect of silver alone or in combination with vancomycin (VAN) or daptomycin (DAP) on biofilm (A) and adherent CFU (B) of methicillin-resistant *S. aureus* (MRSA) strain ATCC 43300. The dotted line in panel B represents the 3- $\log_{10}$ reduction compared to the control with saline. Results are means $\pm$ SD from three independent experiments.

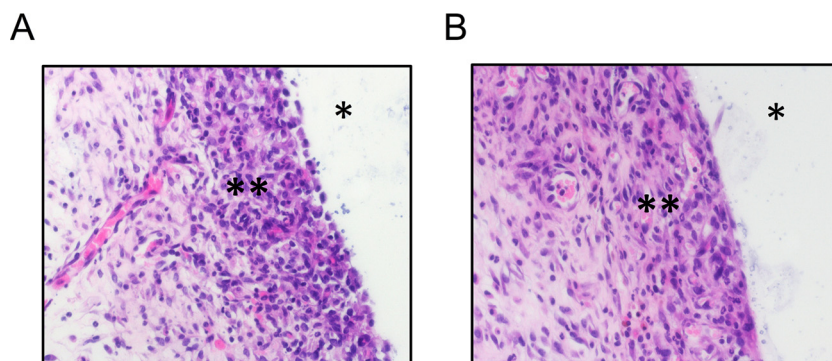


FIG 7 Representative microscopic images from tissue surrounding uncoated (A) or silver-coated (B) tissue cages in C57BL/6 mice. 400 $\times$ magnification, staining with hematoxylin-eosin. **, granulation tissue surrounding former cages; *, former space of tissue cage.

sistance against commonly used antibiotics has globally emerged in recent years. New antimicrobial coatings of implant surfaces may therefore be an attractive option to reduce the risk and improve the treatment of IAIs (19, 39). Little is known about the efficacy of silver against biofilm-forming staphylococci *in vivo*. Here, we show that silver-coated TiAlNb cages effectively prevented IAIs by *S. epidermidis* *in vivo*. In combination with DAP or VAN, the silver coating also thwarted MRSA infections. Additionally, the silver-coated cages demonstrated low toxicity against directly exposed cells and in the surrounding tissue. Emergence of silver resistance was not observed in several strains of staphylococci, which had been exposed during 50 passages. Thus, silver coating is a promising strategy for lowering the risk of IAIs.

TiAlNb was chosen as the base for the silver coating, since titanium and its alloys have demonstrated good biocompatibility in patients with orthopedic implants (40). In the present study, the silver coating was stable and kept its activity after gamma sterilization and disinfection with alcohol.

Silver has a broad antimicrobial spectrum (11). Recent *in vitro* data demonstrate that different silver compounds are efficacious in eradicating bacterial biofilms (41). Interestingly, this effect was strain and species dependent, with a stronger activity against Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* than against Gram-positive *S. aureus*. Similarly, we observed considerable variance within the genus of *Staphylococcus*. *S. epidermidis* was more susceptible to AgNO₃ than *S. aureus* *in vitro* and was eradicated by silver even without additional antibiotics *in vivo*. The lower susceptibility of *S. aureus* to silver may be due to its vast

armamentarium of virulence factors (42) or to a not yet recognized protective factor. In line, the higher recalcitrance of *S. aureus* compared to *S. epidermidis* in IAIs was also demonstrated in an analysis of clinical data (43).

In the present study, both *in vitro* and *in vivo* assays revealed an inoculum- and dose-dependent effect of silver. Accordingly, *S. epidermidis* at 2×10^6 CFU per cage was cleared *in vivo* to 100%, whereas a higher inoculum of 10^7 CFU per cage was only partially eradicated and an inoculum of 10^8 CFU per cage was eradicated not at all. Remarkably, the killing of low-inoculum *S. epidermidis* occurred over time and not at once. There was an initial rapid decrease of bacterial numbers, followed by a slower steady reduction until no viable bacteria could be detected at day 9 after infection. These findings were further supported when tissue cages were explanted on the second day after infection. Two out of 2 cages remained infected, whereas explantation on day 6 yielded 100% clearance (data not shown). This behavior may have various reasons: there may be two different bacterial populations, the first more susceptible to silver and the second more resistant, thus taking longer to be eradicated. Furthermore, the survival curve could merely reflect the decreasing concentration of silver in the TCF reaching a threshold where the eradication of the bacteria depends on the host immune defense. The silver concentration in the TCF declined rapidly from 88 μ g/ml on the second day after implantation to 3 to 4 μ g/ml after 2 weeks. This corresponded to initial concentrations of almost 6 times the MIC (15.6 μ g/ml for *S. epidermidis*) to much lower levels after 2 weeks. Nevertheless, 33% of the cages were cleared in an infection 2 weeks after implantation with 2×10^6 *S. epidermidis* CFU per cage, supporting the additional effect of the host immune defense.

Perioperative antibiotic prophylaxis—correctly timed and administered—is known to reduce the risk of IAIs (44–47). However, in the last decade the infection rate after implantation of prosthesis has remained unchanged, ranging between 1 and 2% for hip and knee prostheses in primary arthroplasty and increasing up to 10% in secondary revisions after IAIs (48). Previous *in vitro* data demonstrate that antibiotics fail to eradicate surface-adherent bacteria independent of biofilm formation (30, 49, 50), suggesting that perioperative antibiotics may only reduce the bacterial load around the implant but not eradicate bacteria already attached to the implant. In accordance, in our *in vivo* model, perioperative prophylaxis alone did not prevent any infection with MRSA. Remarkably, in combination with the silver-coated sur-

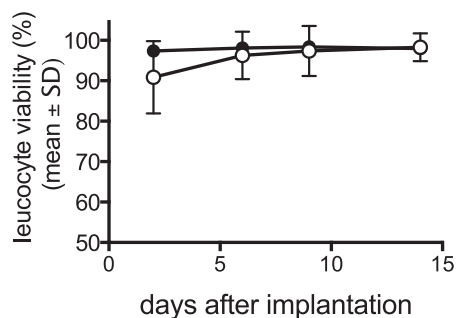


FIG 8 Leucocyte viability in the tissue cage fluid from silver-coated (open circles) and uncoated (closed circles) tissue cages. Results are means $\pm$ SD from at least three independent experiments ($n \geq 27$).

face, prevention rates of 100% with DAP and 33% with VAN were achieved. This effect presumably resulted from additional killing of the planktonic bacteria through released silver as well as killing upon direct contact with the silver-coated surface.

Fifty-seven to 85% of prosthetic joint infections occur perioperatively (51, 52). Therefore, the rapid release of silver in the first days after implantation makes these coatings attractive for maximizing the efficacy of perioperative prophylaxis and simultaneously minimizing a prolonged toxic effect. Especially in the case of one-stage exchange of a device for IAI, reimplantation of a silver-coated prosthesis would exert an additional local effect against the remaining bacteria in the tissue after surgical debridement. Clinical studies are needed to demonstrate the efficacy of the coating in this setting. In this regard, encouraging clinical results were seen in a retrospective study with another silver coating on endoprostheses (20). Moreover, other silver compounds such as silver oxynitrate may be more efficacious against biofilm, as recently shown (41). The presented coating probably has no effect in late hematogenous IAIs due to the fast release of silver. To overcome this issue, triggered-release platforms could be promising.

For a sustainable clinical utilization, an antimicrobial should have a low propensity to induce bacterial resistance. Silver resistance so far is only known in Gram-negative bacteria. Especially in staphylococci, which are the most frequent pathogen in IAIs, we intended to induce silver resistance by performing serial exposure to subinhibitory silver concentrations (37). Among others, we chose staphylococci with putative potential of silver resistance. One strain harbored chromosomally a 60% homologue of *SilE*, a protein belonging to the silver resistance mechanism in Gram-negative organisms. Another possessed copper resistance (53) as a possible cross-mechanism. None of the 7 tested staphylococcal strains gained silver resistance after 50 passages. In contrast, the Gram-negative *Enterobacter cloacae* strain (ATCC 13047) with a genomically encoded silver resistance cassette became resistant after 5 cycles, whereas another randomly chosen *Enterobacter cloacae* strain (ATCC 23355) did not acquire resistance. It has recently been shown in Gram-negative bacteria that silver resistance requires an efflux transport system and either a loss of outer membrane porin or an additional periplasmic silver sequestration protein (26). Our study strengthens the fact that this concerted action against intracellular silver is so far neither known to be inherent nor inducible for Gram-positive bacteria, which makes silver coatings interesting for clinical use.

The cytotoxicity of silver is debated. We found in the TCF only a modest initial decrease of leukocyte viability (minimal viability of 90%) and rapid recovery after approximately 6 days. These data are further strengthened by the fact that the surrounding tissues were comparable in coated and uncoated cages. These findings show that silver in this setting has a negligible effect on eukaryotic cells and appears to be safe for clinical use. This is in accordance with previously published results from studies with clinically used silver-coated megaprotheses for patients with bone sarcoma (19, 38, 54). In our setting, as high silver concentrations are only present within the first days after implantation, long-term exposure and toxicity of the surrounding cells are probably negligible. We did not measure the systemic exposure of silver in the blood and other distant tissues where silver potentially could accumulate (e.g., liver, kidneys, and bone). Compared to published levels of silver concentrations of up to 16,750 µg/g dry weight in the liver of sheep, for example, without any visible toxicity (55), we assume

our coating to result in lower levels. Nevertheless these silver coatings need to be investigated further for systemic and long-term toxicity, osseointegration, and their related toxicity to osteoblasts and osteoclasts *in vivo*.

In conclusion, our findings indicate that silver-coated TiAlNb implants are able to efficiently prevent postoperative infections, especially in conjunction with perioperative antibiotic prophylaxis. Particularly in high-risk, high-bacterial-burden situations such as in infection revision surgery, silver-coated TiAlNb implants may be good and safe candidates to reduce the infection rate.

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