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Title: Preventing implant-associated infections by silver coating

Running title: Effect of silver in implant-associated infections

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Abstract

Implant-associated infections (IAI) 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 aluminium niobium (TiAlNb) alloy. The surface elicited a strong, inoculum-dependent activity against *Staphylococcus* (*S.*) *epidermidis* and *S. 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 peri-operative infections in an inoculum-dependent manner, and led to a 100 % prevention rate after challenge with 2 x 10^6 CFU *S. epidermidis*/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 in 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 µg Ag/ml was reached in the cage fluid corresponding to almost 6 times the minimal inhibitory concentration of the staphylococci. Cytotoxicity towards 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 exposure. In conclusion, our data demonstrate that silver-coated TiAlNb is potent for preventing IAI and thus can be considered for clinical application.

Introduction

The number of implanted medical devices is steadily rising, and has become an effective intervention to substitute anatomical structure or biological function. Despite the rapid advancement in material science and surgical techniques, the burden of implant-associated infections (IAI) has grown ranging between 2% to 40% depending on the type of surgical implant (6). This creates an immense economic and health care problem (24), (30),(44). Infecting microorganisms can be introduced either intra-operatively via direct contact, post-operatively via continuous spread through the wound or in a later stage via the hematogenous or lymphogenous route (48). *Staphylococcus* (*S.*) *aureus* and coagulase-negative staphylococci including *S. epidermidis* account for 50 to 60 % of the causative bacteria (49). 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 (9). Effective treatment of IAI therefore involves surgery, often resulting in the removal of the non-salvageable biomaterial followed by a long course of antibiotic therapy (37, (55).

The significant difficulties in treatment of established biofilms prompted research on engineering 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 (3). Many promising *in vitro* data are available, yet, studies *in vivo* 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 multi-resistant bacteria (10) (14) (39). Recently, silver coating of central venous catheters (46) (53), of urinary catheters (47) and of ventilator endotracheal tubes (28) has been shown to reduce the infection rate. In orthopedic hardware, silver coated external fixation pins (34), proximal femur or tibia megaprostheses (19), and tumor endoprostheses (54) showed a trend towards a reduction of infections. Despite this broad clinical use, little is known about the stability of silver-coated alloys, the 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 (7, 8, 11, 16) and has been shown to induce resistance in Gram-negative bacteria and toxicity in eukaryotic cells (33, 42).

Here, we examine pre-clinical silver-coated titanium aluminium niobium (TiAlNb) alloys 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 IAI, its possible toxicity, and emergence of resistance upon silver exposure.

Methods

Media and chemical substances. For bacterial growth tryptic soy broth (TSB) and Mueller-Hinton broth and agar (MHB and MHA) were obtained from Becton Dickinson (Allschwil, Switzerland). Saline 0.9 % and sterile water ("aqua ad iniectabilia") were obtained from Bichsel (Interlaken, Switzerland). $AgNO_3$ was acquired from Sigma and dissolved as stock solution in sterile water. For *in vitro* and *in vivo* experiments the following antibiotics were used: daptomycin (DAP) (Cubicin®, Novartis) and vancomycin (VAN) (Vancocin®, Teva Pharma). Stock solutions of DAP were prepared in 0.9% saline and always supplemented *in vitro* with 50 mg/L (1.25 mmol/L) calcium ions ($CaCl_2$) according to the CLSI guidelines (5). 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 (Ra) of 3 microns**.** For the *in vivo* assays cylindrical cages (so-called tissue cages; 8.5 x 1 x 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 pre-treated with a 5 mM isonicotinic acid linker mixture [CH_2Cl_2-EtOH (1:1)]. After 24 h, each specimen was immersed twice into the mother liquor [EtOH-THF (1:1)] of 2 mM solution of silver(I) compound based on silver nitrate and ethanediyl bis(isonicotinate) ligand (see also (4)). 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* (SE) 1457 kindly provided by Prof. Mack (University of Swansea, UK), methicillin-resistant (MR) SE ATCC 35984, copper-resistant *S. aureus* (SA) ATCC 12600, MRSA ATCC 43300, MRSA USA300 JE2 wildtype and its putative ΔSilE mutant (transposon mutant knock out of gene locus SA USA300_1847) kindly provided by Prof. Schrenzel (University of Geneva, Switzerland) originally from the Nebraska Transposon Mutant Library (12), methicillin-susceptible (MS) SA 113 ATCC 35556, MSSA "Newman" ATCC 13420, *Enterobacter cloacae* (EC) ATCC 23355, and EC 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 (23), (37). Bacterial numbers were determined by plating aliquots from appropriate dilutions on MHA, followed by colony counting after 24 h of incubation at 37°C.

Minimal inhibitory concentration (MIC). According to the CLSI guidelines a standard inoculum of 1 x 10^5 to 5 x 10^5 CFU/ml was used. The MIC was determined by using 2-fold dilutions of $AgNO_3$ in MHB (5). The MIC was the lowest $AgNO_3$ concentration that inhibited

visible bacterial growth (36).

Induction of silver resistance. Bacteria (all strains mentioned above used except of SA 113) were seeded in flat-bottom 96-well plates at 10^5 CFU/ml in serially diluted $AgNO_3$ (concentrations ranging from 1000 µg/ml to 7.1 µg/ml) in TSB and incubated for 24 h at 37°C. Thereafter the bacteria at the highest $AgNO_3$ concentration with visible growth were used to inoculate and incubate a next 96-well plate in exactly the same manner. This procedure was repeated for 50 times. Development of resistance was considered, if growth occurred at a $AgNO_3$ concentration of at least 3 serial dilution steps higher as the step before.

Agar inhibition assays (Kirby-Bauer-Assay). The agar inhibition assay was performed as previously published (4). Briefly, MHA was inoculated with SE 1457 or SA 113 (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 diameter of the inhibition zones around the discs were measured.

Quantification of biofilm and adherent staphylococci after treatment with silver and antibiotics. MRSA 43300 at 10^5 CFU/ml were seeded into flat-bottom 96-well plates (Becton Dickinson and Company, Allschwil, Switzerland) and incubated with or without silver nitrate at 15.6 µg/ml (i.e. 91.8 µM) or 31.2 µg/ml (i.e. 183.6 µM) combined with 30 µg/ml DAP (i.e. 18.5 µM) or 50 µg/ml VAN (i.e. 34.5 µM) for 24 h at 37°C. After incubation, non-adherent bacteria were removed and each remaining biofilm was washed twice with PBS. Then the CFU of adherent bacteria as well as biofilm mass were quantified by plating and crystal violet staining respectively as described previously (23).

Animal model. In the Animal House of the Department of Biomedicine, University Hospital Basel, 12 to 15 weeks 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 xylazinum (Xylasol®; Graeub AG, Bern, Switzerland). Sterile tissue cages were subcutaneously implanted under aseptic conditions into an air pouch made in the back of each mouse (29). 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. peri-operative infection) or two weeks later (i.e. post-operative infection). After surgery, mice were treated with 0.05 mg/kg buprenorphine (Temgesic®, Essex Chemie AG, Luzern). The minimal infective dose was defined as the count of CFU/tissue cage which was required to induce a persistent infection (15 days) in 100 % of the tissue cages (see also (23)). For the experiments with pre-operative antibiotic prophylaxis, we intraperitoneally administered DAP 3 h or VAN 30 min before cage implantation at concentrations previously determined (23) 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 SE 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 24h 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 non-infected mice were determined after a single intraperitoneal administration of VAN at either 50 mg/kg or 100 mg/kg body weight. Divided in groups of three mice for every time point, TCF was aspirated after 0, 30, 60, 120, 240, 360, 480, 720 and 1440 min. Concentrations of VAN were determined by using a high-performance liquid chromatography (HPLC) method. The C_{max} (mg/l), the area under the concentration-time curve (AUC) (mg · h/l), and the terminal half-life ($t_{1/2}$) (h) were calculated by a computer-assisted method as previously described (51). 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 hours (q12h) (454 mg · h/l) (21).

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 (8, 23).

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). 1.5 ml of water was added to each TCF sample followed by the addition of 150 µl 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 grinded 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®, power level 9-10) for 30 min followed by centrifugation at 1'500 rpm for 30 min. Supernatants of the samples were collected, filtrated and measured by ICP-OES.

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 µm sections were prepared for haematoxylin-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 activity 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 SE 1457 and SA 113. 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 10^4 CFU/ml to 10^7 CFU/ml SE 1457. For SA 113, we observed slightly smaller inhibition zones with 22.6 to 13.9 mm for 10^4 CFU/ml to 10^7 CFU/ml bacteria. This is in line with previous findings that silver is less effective in the agar inhibition assay against *S. aureus* (16) as compared to *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 SE 1457 at the time of surgery (i.e. peri-operatively) or 14 days after implantation (i.e. post-operatively).

The inocula ranged from the minimal infective dose of 2 x 10^6 CFU/cage up to 1 x 10^8 CFU/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 x 10^7 CFU/cage, silver still managed to significantly decrease the planktonic bacteria after 14 days compared to uncoated controls (5.8 x 10^3 CFU/ml vs. 6.6 x 10^4 CFU/ml, p-value 0.04) (Fig. 2B). However, at the highest inoculum (Fig. 2C) none of the silver-coated cages was able to control bacterial growth (p-value 0.10). In accordance, the infection prevention rate was inoculum-dependent (Fig. 2D). In the peri-operative setting, the low inoculum resulted in a 100 %, the middle inoculum in a 20 % and the high inoculum in a 0% prevention rate. We subsequently investigated whether this effect was also reproducible in a post-operative infection with a low inoculum of 2 x 10^6 CFU/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 peri-operative than in post-operative infections, we were interested in the dynamics of silver release. Therefore, we measured the silver concentration in the TCF in peri- and post-

operative infections with SE 1457.

The silver concentration in the TCF showed a similar decrease over time with or without infection of SE 1457 (Fig. 3). The second day after implantation the silver concentration was 82 ± 50 µg/ml with infection versus 88 ± 36 µg/ml without infection, after 6 days 28 ± 18 µg/ml versus 18 ± 6 µg/ml and 11 ± 5 µg/ml versus 4 ± 1 µg/ml at day 14. When infecting the cages 14 days post-operatively the silver concentration was only 3 ± 1 µg/ml at day 28. Between day 6 and 10 the silver concentration fell below the previously determined MIC ($AgNO_3$) of 15.6 µg/ml for SE 1457 (see 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 post-operative 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 IAI. Therefore, we next applied the silver-coated cages against SA 113 in a peri-operative infection using the minimal infective dose of 1 x 10^3 CFU/cage. Silver coating of the cages was able to control the infection resulting in 2.7 x 10^3 CFU/ml in the TCF after 9 days, whereas the uncoated cages led to a growth up to 8.3 x 10^6 CFU/ml (p-value 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 post-operative infection setting (data not shown). In conclusion, silver coating showed a trend of controlling a peri-operative infection with *S. aureus*, but was not capable of preventing infection. Further, the silver remaining in the cages after 14 days did not affect growth or adherence of post-operatively inoculated *S. aureus*.

Silver increases bactericidal activity of vancomycin and daptomycin against adherent MRSA *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 µg/ml daptomycin (DAP) or 50 µg/ml vancomycin (VAN) *in vitro* and measured the reduction of biofilm by using crystal violet staining (Fig. 5A) and surface-adherent MRSA 43300 by CFU counting (Fig. 5B). At a concentration of 31.25 µg/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 were reduced by approximately 3 $\log_{10}$ by VAN or DAP alone (see dotted line Fig. 5B). By addition of silver at MIC the adherent bacteria decreased by approximately 1 $\log_{10}$ in 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 a MRSA infection *in vivo*. The timing of the antibiotics was chosen according to the

pharmacokinetic (PK) profile (see supplemental Figure 1) to achieve maximal effect. The dosing of the antibiotics was correlated to the clinically used ones. As single agents, neither pre-operative DAP nor VAN nor silver coated cages were sufficient to prevent a persistent infection with MRSA. Remarkably, in combination with pre-operatively applied DAP, silver coating prevented the growth of planktonic as well as adherent MRSA resulting in a 100 % prevention rate. The additive effect of pre-operative VAN led to a significant reduction of planktonic growth and prevented adherence in 33%. The control group was exposed to uncoated cages and saline and showed no bacterial growth inhibition (Fig. 6). Thus, the additive effect of pre-operative DAP and silver coating was highly efficacious *in vivo*.

No inducible silver resistance in staphylococci. Due to these promising results, we asked the question 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 (22) ,(25), (43). Hence, we exposed seven staphylococcal clinical isolates and ATCC strains (MSSE 1457, MRSE ATCC 35984, MSSA ATCC 13420, MRSA ATCC 43300, copper-resistant SA ATCC 12600, MRSA USA300 and its putative ΔSilE mutant; all exhibiting a MIC ($AgNO_3$) of 15.6 or 31.2 µg/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* (EC ATCC 13047 harboring the chromosomal silver resistance cassette SilPABCRSE and EC ATCC 23355 without known silver resistance). EC 13047 showed initially the same sensitivity to silver as the staphylococcal strains (MIC 31.2 µg/ml), but became highly resistant after the 5^{th} passage (MIC > 1000 µg/ml). On the other hand, EC 23355 did not develop a silver resistance during 50 passages. These results reinforce the available evidence that Gram-positive bacteria seem to lack the potential of a *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 (18). We therefore examined the tissue surrounding the cages microscopically for signs of silver induced damage. This was done for 28 mice with uncoated and for 29 mice with coated cages. There was consistently no difference between silver coated and uncoated cages. Both exhibited a lymphocytic pseudo-capsule 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.

Further we quantified the percentage of viable leucocytes in the TCF. 2 days after implantation of silver coated cages 90 % of leucocytes were viable. At day 6 already 96 % were alive and after 9 days the viability further increased to 98 %. The uncoated cages always showed approx. 98 % viability (Fig. 8).

Discussion

IAI are difficult to treat due to their strongly reduced susceptibility to antibiotics and to host immune defenses. Furthermore, resistance 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 IAI (19, 40). Little is known about the efficacy of silver on biofilm-forming staphylococci *in vivo*. Here, we show that silver-coated TiAlNb cages effectively prevented IAI by *S. epidermidis in vivo.* In combination with DAP or VAN the silver coating also thwarted MRSA infections. Additionally, the silver coated cages demonstrated a low toxicity against directly exposed cells and in the surrounding tissue. Emergence of silver resistance was not observed in several strains of staphylococci, which have been exposed during 50 passages. Thus, silver coating is a promising strategy for lowering the risk of IAI.

TiAlNb was chosen as base for the silver coating, since titanium and its alloys have documented good biocompatibility in patients with orthopedic implants (20). In the present study, silver coating was stable and kept its activity after gamma-sterilization and disinfection with alcohol.

Silver has a broad antimicrobial spectrum (10). Recent *in vitro* data demonstrate that different silver compounds are efficacious in eradicating bacterial biofilms (32). 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 a considerable variance within the genus of staphylococci. *S. epidermidis* was more susceptible to $AgNO_3$ 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 (45), or to a not yet recognized protective factor. In line, the higher recalcitrance of *S. aureus* compared to *S. epidermidis* in IAI was also demonstrated in an analysis of clinical data (50).

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 x 10^6 CFU/cage was cleared *in vivo* to 100%, whereas higher inocula of 10^7 CFU/cage were only partially eradicated and 10^8 CFU/cage 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 a 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 µg/ml on the second day after implantation to 3 - 4 µg/ml after two weeks. This corresponded to initial concentrations of almost 6 times the MIC (15.6 µg/ml for *S. epidermidis*) to much lower levels after two weeks. Nevertheless, 33 % of the cages were cleared in an infection two weeks after implantation with 2 x 10^6 *S. epidermidis* CFU/cage supporting the additional effect of the host immune defense.

Peri-operative antibiotic prophylaxis – correctly timed and administered – is known to reduce the risk of IAI (13, 35, 38, 52). However, in the last decade the infection rate after implantation of prosthesis has remained unchanged ranging between 1 – 2 % for hip and knee prostheses in primary arthroplasty and increasing up to 10 % in secondary revisions after IAI (2). Previous *in vitro* data demonstrate that antibiotics fail to eradicate surface-adherent bacteria independent of biofilm formation (23) (27), (41) suggesting that peri-operative antibiotics may only reduce the bacterial load around the implant but not eradicate already implant-attached bacteria. In accordance, in our *in vivo* model, peri-operative prophylaxis alone did not prevent any infection with MRSA. Remarkably, in combination with surface coated silver a prevention rate of 100% with DAP and 33% with VAN was achieved. This effect presumably resulted from additional killing of the planktonic bacteria through released silver as well as killing upon direct contact to the silver coated surface.

Fifty-seven to 85 % of prosthetic joint infections occur peri-operatively ((15), (26)). Therefore the rapid release of silver in the first days after implantation makes these coatings attractive for maximizing the efficacy of peri-operative prophylaxis and simultaneously minimizing a prolonged toxic effect. Especially in case of one-stage exchange of a device for IAI, re-implantation 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 endoprotheses (54). Moreover, other silver compounds such as silver oxynitrate may be more efficacious against biofilm as recently shown (32). The presented coating has probably no effect in late hematogenous IAI 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 is so far only known in Gram-negative bacteria. Especially in staphylococci, being the most frequent pathogen in IAI, we intended to induce silver resistance by performing serial exposure to subinhibitory silver concentrations (43). Among others, we chose staphylococci with putative potential of silver resistance. One harbored chromosomally a 60% homologue of SilE, a protein belonging to the silver resistance mechanism in Gram-negatives. Another possessed a copper-resistance (1) 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* (ATCC 13047) with genomic encoded silver resistance cassette became resistant after 5 cycles whereas another randomly chosen *Enterobacter cloacae* (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 periplasmatic silver-sequestration protein (42). 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.

Cytotoxicity of silver is debated. We found in the TCF only a modest initial decrease of leukocyte viability (minimal 90 %) and a rapid recovery after approximately 6 days. These data are further strengthened by the fact that the surrounding tissue was 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 of clinically used silver-coated megaprostheses for patients with bone sarcoma (17), (18, 19). In our setting, as high silver concentrations are only present within the first days after implantation, a long term exposure and toxicity of the surrounding cells is 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, bone). Compared to published levels of silver concentrations of up to 16'750 µg/g dry weight in the liver of e.g. sheep without any visible toxicity (31) 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 –clasts *in vivo*.

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

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References

1. **Baker, J., M. Sengupta, R. K. Jayaswal, and J. A. Morrissey.** 2011. The Staphylococcus aureus CsoR regulates both chromosomal and plasmid-encoded copper resistance mechanisms. Environmental Microbiology **13:**2495-2507.
2. **Beswick, A. D., K. T. Elvers, A. J. Smith, R. Gooberman-Hill, A. Lovering, and A. W. Blom.** 2012. What is the evidence base to guide surgical treatment of infected hip prostheses? Systematic review of longitudinal studies in unselected patients. Bmc Med **10**.
3. **Campoccia, D., L. Montanaro, and C. R. Arciola.** 2013. A review of the biomaterials technologies for infection-resistant surfaces. Biomaterials **34:**8533-8554.
4. **Chevrier, I., J. L. Sague, P. S. Brunetto, N. Khanna, Z. Rajacic, and K. M. Fromm.** 2013. Rings, chains and helices: new antimicrobial silver coordination compounds with (iso-)nicotinic acid derivatives. Dalton transactions **42:**217-231.
5. **CLSI.** 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. CLSI document M07-A9. . **9th ed.**
6. **Darouiche, R. O.** 2004. Current concepts - Treatment of infections associated with surgical implants. New Engl J Med **350:**1422-1429.
7. **Dibrov, P., J. Dzioba, K. K. Gosink, and C. C. Hase.** 2002. Chemiosmotic mechanism of antimicrobial activity of Ag+ in Vibrio cholerae. Antimicrobial agents and chemotherapy **46:**2668-2670.
8. **Dominguez-Herrera, J., F. Docobo-Perez, R. Lopez-Rojas, C. Pichardo, R. Ruiz-Valderas, J. A. Lepe, and J. Pachon.** 2012. Efficacy of Daptomycin versus Vancomycin in an Experimental Model of Foreign-Body and Systemic Infection Caused by Biofilm Producers and Methicillin-Resistant Staphylococcus epidermidis. Antimicrobial agents and chemotherapy **56:**613-617.
9. **Donlan, R. M., and J. W. Costerton.** 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev **15:**167-193.
10. **Eckhardt, S., P. S. Brunetto, J. Gagnon, M. Priebe, B. Giese, and K. M. Fromm.** 2013. Nanobio Silver: Its Interactions with Peptides and Bacteria, and Its Uses in Medicine. Chemical Reviews **113:**4708-4754.
11. **Feng, Q. L., J. Wu, G. Q. Chen, F. Z. Cui, T. N. Kim, and J. O. Kim.** 2000. A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. Journal of Biomedical Materials Research **52:**662-668.
12. **Fey, P. D., J. L. Endres, V. K. Yajjala, T. J. Widhelm, R. J. Boissy, J. L. Bose, and K. W. Bayles.** 2013. A Genetic Resource for Rapid and Comprehensive Phenotype Screening of Nonessential Staphylococcus aureus Genes. Mbio **4**.
13. **Fogelber.Ev, E. K. Zitzmann, and Stinchfi.Fe.** 1970. Prophylactic Penicillin in Orthopaedic Surgery. J Bone Joint Surg Am **A 52:**95-&.
14. **Fromm, K. M.** 2013. Silver coordination compounds with antimicrobial properties. Applied Organometallic Chemistry **27:**683-687.
15. **Giulieri, S. G., P. Graber, P. E. Ochsner, and W. Zimmerli.** 2004. Management of infection associated with total hip arthroplasty according to a treatment algorithm. Infection **32:**222-228.
16. **Gordon, O., T. Vig Slenters, P. S. Brunetto, A. E. Villaruz, D. E. Sturdevant, M. Otto, R. Landmann, and K. M. Fromm.** 2010. Silver coordination polymers for prevention of implant infection: thiol interaction, impact on respiratory chain enzymes, and hydroxyl radical induction. Antimicrobial agents and chemotherapy **54:**4208-4218.
17. **Gosheger, G., J. Hardes, H. Ahrens, A. Streitburger, H. Buerger, M. Erren, A. Gunsel, F. H. Kemper, W. Winkelmann, and C. von Eiff.** 2004. Silver-coated megaendoprostheses

in a rabbit model - an analysis of the infection rate and toxicological side effects. Biomaterials **25:**5547-5556.

18. **Hardes, J., H. Ahrens, C. Gebert, A. Streitbuerger, H. Buerger, M. Erren, A. Gunsel, C. Wedemeyer, G. Saxler, W. Winkelmann, and G. Gosheger.** 2007. Lack of toxicological side-effects in silver-coated megaprostheses in humans. Biomaterials **28:**2869-2875.
19. **Hardes, J., C. von Eiff, A. Streitbuerger, M. Balke, T. Budny, M. P. Henrichs, G. Hauschild, and H. Ahrens.** 2010. Reduction of periprosthetic infection with silver-coated megaprostheses in patients with bone sarcoma. Journal of surgical oncology **101:**389-395.
20. **Head, W. C., D. J. Bauk, and R. H. Emerson.** 1995. Titanium as the Material of Choice for Cementless Femoral Components in Total Hip-Arthroplasty. Clin Orthop Relat R**:**85-90.
21. **Healy, D. P., R. E. Polk, M. L. Garson, D. T. Rock, and T. J. Comstock.** 1987. Comparison of steady-state pharmacokinetics of two dosage regimens of vancomycin in normal volunteers. Antimicrobial agents and chemotherapy **31:**393-397.
22. **Hendry, A. T., and I. O. Stewart.** 1979. Silver-resistant Enterobacteriaceae from hospital patients. Can J Microbiol **25:**915-921.
23. **John, A. K., M. Schmaler, N. Khanna, and R. Landmann.** 2011. Reversible daptomycin tolerance of adherent staphylococci in an implant infection model. Antimicrobial agents and chemotherapy **55:**3510-3516.
24. **Kapadia, B. H., M. J. McElroy, K. Issa, A. J. Johnson, K. J. Bozic, and M. A. Mont.** 2014. The Economic Impact of Periprosthetic Infections Following Total Knee Arthroplasty at a Specialized Tertiary-Care Center. J Arthroplasty **29:**929-932.
25. **Kaur, P., and D. V. Vadehra.** 1986. Mechanism of resistance to silver ions in Klebsiella pneumoniae. Antimicrobial agents and chemotherapy **29:**165-167.
26. **Kessler, B., P. Sendi, P. Graber, M. Knupp, L. Zwicky, B. Hintermann, and W. Zimmerli.** 2012. Risk factors for periprosthetic ankle joint infection: a case-control study. J Bone Joint Surg Am **94:**1871-1876.
27. **Knobloch, J. K.** 2008. Biofilm formation is not necessary for development of quinolone-resistant "persister" cells in an attached Staphylococcus epidermidis population. Int J Artif Organs **31:**752-760.
28. **Kollef, M. H., B. Afessa, A. Anzueto, C. Veremakis, K. M. Kerr, B. D. Margolis, D. E. Craven, P. R. Roberts, A. C. Arroliga, R. D. Hubmayr, M. I. Restrepo, W. R. Auger, R. Schinner, and N. I. Grp.** 2008. Silver-coated endotracheal tubes and incidence of ventilator-associated pneumonia - The NASCENT Randomized Trial. Jama-Journal of the American Medical Association **300:**805-813.
29. **Kristian, S. A., T. Golda, F. Ferracin, S. E. Cramton, B. Neumeister, A. Peschel, F. Gotz, and R. Landmann.** 2004. The ability of biofilm formation does not influence virulence of Staphylococcus aureus and host response in a mouse tissue cage infection model. Microb Pathogenesis **36:**237-245.
30. **Kurtz, S. M., E. Lau, H. Watson, J. K. Schmier, and J. Parvizi.** 2012. Economic Burden of Periprosthetic Joint Infection in the United States. J Arthroplasty **27:**61-65.
31. **Langanki, D., M. E. Ogle, J. D. Cameron, R. A. Lirtzman, R. E. Schroeder, and M. W. Mirsch.** 1998. Evaluation of a novel bioprosthetic heart valve incorporating anticalcification and antimicrobial technology in a sheep model. Journal of Heart Valve Disease **7:**633-638.
32. **Lemire, J. A., L. Kalan, A. Bradu, and R. J. Turner.** 2015. Silver Oxynitrate, an Unexplored Silver Compound with Antimicrobial and Antibiofilm Activity. Antimicrobial agents and chemotherapy **59:**4031-4039.

33. **Maillard, J. Y., and P. Hartemann.** 2013. Silver as an antimicrobial: facts and gaps in knowledge. Crit Rev Microbiol **39:**373-383.
34. **Masse, A., A. Bruno, M. Bosetti, A. Biasibetti, M. Cannas, and P. Gallinaro.** 2000. Prevention of pin track infection in external fixation with silver coated pins: Clinical and microbiological results. Journal of Biomedical Materials Research **53:**600-604.
35. **Meehan, J., A. A. Jamali, and H. Nguyen.** 2009. Prophylactic Antibiotics in Hip and Knee Arthroplasty. J Bone Joint Surg Am **91A:**2480-2489.
36. **Murray, P. R.** 2003. Manual of clinical microbiology. American Society of Microbiology **8th edition**.
37. **Nowakowska, J., H. J. Griesser, M. Textor, R. Landmann, and N. Khanna.** 2013. Antimicrobial properties of 8-hydroxyserrulat-14-en-19-oic acid for treatment of implant-associated infections. Antimicrobial agents and chemotherapy **57:**333-342.
38. **Pavel, A., R. L. Smith, and I. J. Larsen.** 1974. Prophylactic Antibiotics in Clean Orthopedic Surgery. J Bone Joint Surg Am **A 56:**196-196.
39. **Percival, S. L., W. Slone, S. Linton, T. Okel, L. Corum, and J. G. Thomas.** 2011. The antimicrobial efficacy of a silver alginate dressing against a broad spectrum of clinically relevant wound isolates. International wound journal **8:**237-243.
40. **Politano, A. D., K. T. Campbell, L. H. Rosenberger, and R. G. Sawyer.** 2013. Use of Silver in the Prevention and Treatment of Infections: Silver Review. Surgical Infections **14:**8-20.
41. **Qu, Y., A. J. Daley, T. S. Istivan, D. A. Rouch, and M. A. Deighton.** 2010. Densely adherent growth mode, rather than extracellular polymer substance matrix build-up ability, contributes to high resistance of Staphylococcus epidermidis biofilms to antibiotics. The Journal of antimicrobial chemotherapy **65:**1405-1411.
42. **Randall, C. P., A. Gupta, N. Jackson, D. Busse, and A. J. O'Neill.** 2015. Silver resistance in Gram-negative bacteria: a dissection of endogenous and exogenous mechanisms. J Antimicrob Chemoth **70:**1037-1046.
43. **Randall, C. P., L. B. Oyama, J. M. Bostock, I. Chopra, and A. J. O'Neill.** 2013. The silver cation (Ag+): antistaphylococcal activity, mode of action and resistance studies. The Journal of antimicrobial chemotherapy **68:**131-138.
44. **Ratner, B. D.** 2007. A paradigm shift: biomaterials that heal. Polym Int **56:**1183-1185.
45. **Rosenstein, R., and F. Gotz.** 2013. What Distinguishes Highly Pathogenic Staphylococci from Medium- and Non-pathogenic? Curr Top Microbiol **358:**33-89.
46. **Rupp, M. E., S. J. Lisco, P. A. Lipsett, T. M. Ped, K. Keating, J. M. Civetta, L. A. Mermel, D. Lee, E. P. Dellinger, M. Donahoe, D. Giles, M. A. Pfaller, D. G. Maki, and R. Sherertz.** 2005. Effect of a second-generation venous catheter impregnated with chlorhexidine and silver sulfadiazine on central catheter - Related infections - A randomized, controlled trial. Ann Intern Med **143:**570-580.
47. **Saint, S., J. G. Elmore, S. D. Sullivan, S. S. Emerson, and T. D. Koepsell.** 1998. The efficacy of silver alloy-coated urinary catheters in preventing urinary tract infection: A meta-analysis. American Journal of Medicine **105:**236-241.
48. **Sendi, P., F. Banderet, P. Graber, and W. Zimmerli.** 2011. Periprosthetic joint infection following Staphylococcus aureus bacteremia. The Journal of infection **63:**17-22.
49. **Tande, A. J., and R. Patel.** 2014. Prosthetic Joint Infection. Clin Microbiol Rev **27:**302-345.
50. **Teterycz, D., T. Ferry, D. Lew, R. Stern, M. Assal, P. Hoffmeyer, L. Bernard, and I. Uckay.** 2010. Outcome of orthopedic implant infections due to different staphylococci. Int J Infect Dis **14:**E913-E918.

51. **Usansky, J. L., Desai A., Tang-Liu, D.** 2010. PK Functions for Microsoft Excel. Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, CA http://www.boomer.org/pkin/soft.html.
52. **van Kasteren, M. E. E., J. Mannien, A. Ott, B. J. Kullberg, A. S. de Boer, and I. C. Gyssens.** 2007. Antibiotic prophylaxis and the risk of surgical site infections following total hip arthroplasty: Timely administration is the most important factor. Clin Infect Dis **44:**921-927.
53. **Veenstra, D. L., S. Saint, S. Saha, T. Lumley, and S. D. Sullivan.** 1999. Efficacy of antiseptic-impregnated central venous catheters in preventing catheter-related bloodstream infection - A meta-analysis. Jama-Journal of the American Medical Association **281:**261-267.
54. **Wafa, H., R. J. Grimer, K. Reddy, L. Jeys, A. Abudu, S. R. Carter, and R. M. Tillman.** 2015. Retrospective evaluation of the incidence of early periprosthetic infection with silver-treated endoprostheses in high-risk patients CASE-CONTROL STUDY. Bone Joint J **97B:**252-257.
55. **Zimmerli, W., A. Trampuz, and P. E. Ochsner.** 2004. Prosthetic-joint infections. The New England journal of medicine **351:**1645-1654.

Figure legends

Figure 1. Agar inhibition assay with silver-coated discs (TiAlNb) before and after sterilization. Inhibition zones from different inocula of *S. epidermidis* (A) and *S. aureus* (B). Results are mean + SD from three independent experiments, * $p < 0.05$

Figure 2. Silver coated (open circles) and uncoated (closed circles) tissue cage infection with *S. epidermidis* 1457 in C57BL/6 mice. Planktonic CFU in the tissue cage fluid from different initial inocula in peri-operative infections: 2 x 10^6 CFU/cage (A), 1 x 10^7 CFU/cage (B) and 1 x 10^8 CFU/cage (C). Prevention rate for silver coated (open columns) and uncoated (closed columns) TiAlNb cages for peri-operative (D) and post-operative (E) infections. Results are mean ± SD from three independent experiments for 2 x 10^6 CFU/cage with n = 7 and 1 x 10^7 CFU/cage with n = 9 and one experiment for 1 x 10^8 CFU/cage with n = 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 3. Silver concentration in tissue cage fluid from peri-operative (A) or post-operative (B) infections. Results are mean ± SD from at least three independent experiments($n \geq 12$ for peri-operative infection, n = 11 for post-operative infection). Dotted line represents the MIC of *S. epidermidis* 1457 for $AgNO_3$.

Figure 4. Effect of silver against *S. aureus* 113 in C57BL/6 mice. Planktonic CFU in the tissue cage fluid (A) of silver coated (open circles) and uncoated (closed circles) TiAlNb cages and infection prevention rate (B) of silver coated (left column) and uncoated (right column) TiAlNb cages from initial inoculum of 1 x 10^3 CFU/cage in a peri-operative infection. Results are mean ± SD from one experiment with eight mice.

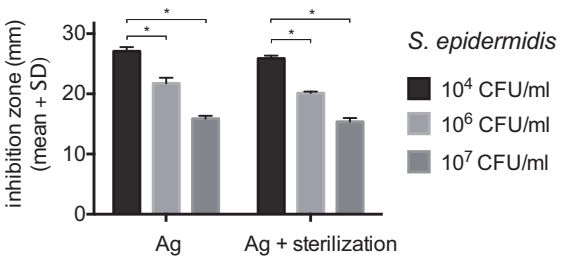
Figure 5. *In vitro* effect of silver alone or in combination with vancomycin (VAN) or daptomycin (DAP) on biofilm (A) and adherent CFU (B) of methicillin resistant *S. aureus* (MRSA) ATCC 43300. Dotted line in B represents the 3 $\log_{10}$ reduction compared to control with saline. Results are mean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

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

Figure 7. Representative microscopic images from tissue surrounding uncoated (A) or silver coated (B) tissue cages in C57BL/6 mice. 400 x magnification, staining with haematoxylin-eosin. * granulation tissue surrounding former cages, ** former space of tissue cage

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

A



B