A chlorhexidine-releasing epoxy-based coating on titanium implants prevents \textit{Staphylococcus aureus} experimental biomaterial-associated infection


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CHAPTER 4

ABSTRACT
Prevention of biomaterial-associated infections (BAI) remains a challenging problem, in particular due to the increased risk of resistance development with the current antibiotic-based strategies. Metallic orthopaedic devices, such as non-cemented implants, are often inserted under high mechanical stress. These non-cemented implants cannot be protected by e.g. antibiotic releasing bone cement or other antimicrobial approaches such as the use of bioactive glass. Therefore, we developed an antimicrobial coating for orthopaedic implants to prevent Staphylococcus aureus BAI with high mechanical stability in order to avoid abrasion during implantation procedures. We incorporated 5 and 10 wt% chlorhexidine in a novel mechanically stable epoxy-based coating, designated CHX$_5$ and CHX$_{10}$, respectively. The coatings displayed potent bactericidal activity in vitro against S. aureus, with over 80% of the release (19 µg/cm$^2$ for CHX$_5$ and 41 µg/cm$^2$ for CHX$_{10}$) occurring within the first 24 hours. In mice, the CHX$_{10}$ coating significantly reduced the numbers of CFU both on the implants and in the peri-implant tissues at 1 day after S. aureus challenge. The CHX$_{10}$-coated implants were well-tolerated by the animals, with no signs of toxicity observed in histology. Moreover, the coating significantly reduced the frequency of culture-positive tissues at 1 day, and of culture-positive implants at 1 and 4 days after challenge. In summary, the chlorhexidine-releasing mechanically stable epoxy-based CHX$_{10}$ coating prevented implant colonization and S. aureus BAI in mice and has good prospects for clinical development.
INTRODUCTION

Medical devices manufactured from titanium, such as prosthetic joints and dental implants, are widely used in modern medicine. Titanium’s lightness, good mechano-chemical and osseo-integrative properties make it an ideal material for bone implant application. Unfortunately, as with any inserted or implanted foreign body material, a significant problem with titanium implants is the risk of infection. These so-called biomaterial-associated infections (BAI) are feared complications in orthopaedic and trauma surgery with tremendous consequences for the patients. The majority of BAIs are caused by staphylococci, especially \textit{Staphylococcus epidermidis} and \textit{Staphylococcus aureus}. Formation of a biofilm consisting of bacteria, bacterial products and host proteins on the biomaterial surface is considered the major element in the pathogenesis of BAI. In addition, the presence of the foreign body material leads to colonization of tissue surrounding implants.

In order to prevent infection of medical devices, a variety of surface modifications have been developed. These include non-adhesive, contact-killing and tissue-integrating surfaces, as well as coatings for the controlled release of antimicrobial agents. To prevent both colonization of the implant itself and of the tissue surrounding the implant, an antimicrobial-releasing coating is preferred. However, the use of antibiotics on a medical device is discouraged by government regulatory agencies, especially the American Food and Drug Administration (FDA), in view of the increasing development of resistance. As an alternative for antibiotics, commonly used antiseptics such as chlorhexidine might be used. Chlorhexidine has broad antimicrobial activity and has been shown to prevent infection in patients and in animal models. It is used topically, for site preparation in surgical procedures, and also intracorporally, e.g. in rapid sterilization of exposed tissues in abdominal surgery prior to wound closure. Moreover, chlorhexidine is widely applied as an irrigant fluid in dentistry with low toxicity and good tolerability in soft tissue. In biomaterials, chlorhexidine has been applied in combination with silver-sulfadiazine in a coating on intravenous catheters, which decreased catheter-related infection in humans. Experimental titanium tibia intramedullary nails coated with chlorhexidine and chloroxylenol had reduced rates of orthopaedic device-related infection in rabbits.

In the present study we aimed to design an antimicrobial coating for titanium implants for general application in orthopaedics and trauma, with high mechanical stability in order to avoid abrasion during implantation procedures. Coatings and materials manufactured from epoxy resins are among the few available that meet this requirement. Epoxy resins can be catalytically cross-linked or they can be cured using reagents such as polyamines.
typically good adhesion to metallic substrates and good mechanical properties of epoxy-based coatings\textsuperscript{22–24} prompted us to investigate an epoxy-amine coating network containing chlorhexidine as the antimicrobial agent for orthopaedic implants. Dopamine is added to the coating because the catechol moiety of dopamine is known for its strong adhesion to a range of metallic substrates, including medical-grade titanium\textsuperscript{25–27}.

The purpose of the present study was therefore to develop an epoxy-based chlorhexidine-releasing coating for titanium implants with potent \textit{in vitro} as well as \textit{in vivo} antibacterial activity in a murine subcutaneous implant infection model. In this model, the release of chlorhexidine proved to be effective in preventing \textit{S. aureus} colonization of the implants and surrounding tissue without signs of toxicity.

**MATERIALS AND METHODS**

**COATING PREPARATION**

Coating formulations as described in Table 1 were prepared as follows: for solution A, isophorone diamine (IPD; Aldrich \textgeq 99\%) was dissolved in ethanol (EtOH; Merck pro analysis) at room temperature, while stirring. To this solution, dopamine hydrochloride (dopamine; Sigma) was added and left stirring until completely dissolved. Next, glycerol diglycidyl ether (epoxy resin; Aldrich technical grade) was added to the mixture. The resulting solution A was left to stand for 1 h at room temperature. For solution B, chlorhexidine diacetate salt hydrate (chlorhexidine (CHX); Sigma bis(biguanide) antimicrobial) was dissolved in EtOH at room temperature, while stirring. For the unloaded control coatings, no chlorhexidine was added to solution B. Just prior to the coating procedures, solutions A and B were mixed. The weights of the solids used for the solutions A and B are listed in Table 1. In this report, the

<table>
<thead>
<tr>
<th>Coating</th>
<th>Batch</th>
<th>Sol. A (g)</th>
<th>Sol. B (g)</th>
<th>Epoxy (g)</th>
<th>IPD (g)</th>
<th>Dopamine (g)</th>
<th>CHX (g)</th>
<th>NH/epoxy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHX_3</td>
<td>1</td>
<td>5.00</td>
<td>10.00</td>
<td>4.993 (64.9%)</td>
<td>1.731 (22.5%)</td>
<td>0.069 (12.6%)</td>
<td>-</td>
<td>1.04</td>
</tr>
<tr>
<td>CHX_4</td>
<td>1</td>
<td>5.00</td>
<td>10.00</td>
<td>4.992 (61.8%)</td>
<td>1.720 (21.3%)</td>
<td>0.069 (12.0%)</td>
<td>0.389 (4.9%)</td>
<td>1.03</td>
</tr>
<tr>
<td>CHX_10</td>
<td>1</td>
<td>5.00</td>
<td>10.00</td>
<td>5.021 (59.0%)</td>
<td>1.719 (20.2%)</td>
<td>0.082 (11.3%)</td>
<td>0.809 (9.5%)</td>
<td>1.03</td>
</tr>
<tr>
<td>CHX_3</td>
<td>2</td>
<td>10.00</td>
<td>15.00</td>
<td>5.012 (65.0%)</td>
<td>1.740 (22.8%)</td>
<td>0.062 (12.5%)</td>
<td>-</td>
<td>1.07</td>
</tr>
<tr>
<td>CHX_10</td>
<td>2</td>
<td>10.00</td>
<td>15.00</td>
<td>5.012 (58.5%)</td>
<td>1.740 (20.3%)</td>
<td>0.062 (11.2%)</td>
<td>0.858 (10.0%)</td>
<td>1.07</td>
</tr>
</tbody>
</table>

Table 1. Coating formulation compositions. Batch 1 was used for spin-coating of the aluminium sheets and Q-panels and titanium disks, batch 2 for dip-coating of the \textit{in vivo} titanium implants. To obtain a similar coating thickness in both coating procedures, the viscosity of the formulation had to be lower in case of the dip-coating procedure and therefore a lower solids in EtOH content was used in batch 2.
coating formulations were designated CHX₀ (no chlorhexidine), CHX₅ (5 wt% chlorhexidine) and CHX₁₀ (10 wt% chlorhexidine). Two batches of the CHX₀ and CHX₁₀ coating formulations were prepared. The chemical composition was kept maximally constant in both batches with particular attention to the ratio between the amino groups and the epoxy groups determining the crosslink density. The coating formulations of CHX₀ (1st batch), CHX₅ and CHX₁₀ (1st batch) were used to coat aluminium sheets and titanium disks for the *in vitro* assays. The second batch CHX₀ and CHX₁₀ formulations were used to coat the titanium implants for the *in vivo* assays. Formulations were prepared fresh prior to application to the different materials.

**APPLICATION OF COATING TO ALUMINIUM SHEETS AND TITANIUM DISCS VIA SPIN-COATING**

For the *in vitro* experiments, coatings were applied on aluminium sheets (5 × 5 cm) and Q-panels (15.3 × 10.1 cm), and medical grade titanium (Ti 6Al-4V ELI, surface finish 2B (ASTM A480)) discs (diameter 20 mm, height 1 mm), which had been cleaned by submerging in ethanol and acetone and subsequent treatment with ultrasound for 5 min. Aliquots of approximately 1 ml and 0.1 – 0.2 ml of the coating formulation were applied to the surface of aluminium sheets and titanium disks, respectively, and the samples were spin-coated at a rotation speed of 1,000 rpm for 30 sec. The samples were air-dried for 0.5 – 2 h and subsequently placed in an oven at ~90 °C for 3 h.

**APPLICATION OF COATINGS TO TITANIUM IMPLANTS VIA DIPPING**

For the *in vivo* experiments, medical-grade titanium implants (10 × 4 × 1 mm, with a slit to allow cutting of the implant after explantation, see below) were manufactured by Flowcut (Nederweert, The Netherlands) and coated using the following procedure. First, the implants were cleaned by boiling them for 1 h in 10 wt% aqueous NaOH, followed by washing by ultrasonic treatment in ethanol and acetone for 5 min. The implants were dip-coated to allow complete covering of the surface in spite of their complex shape. The implants were connected to a paperclip and submerged completely in the dipping solution using a dip coater (Specialty Coating Systems, type PL-3201, SCS, Indianapolis, IN, USA) with a dip speed of 2 mm/s. The dipped implants were dried for 0.5 – 1 h, placed in a convection oven at 90 °C for 3 h for curing. The paperclip was removed, and the coated implants sterilized by autoclaving at 121 °C for 20 min.
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IN VITRO CHLORHEXIDINE RELEASE ASSAY

Samples of 1 × 5 cm were cut from the coated aluminium sheets and incubated with 4 ml of phosphate buffered saline (PBS; pH = 7.0) at 37 °C under shaking at 100 rpm. At different time points (0, 1, 2, 3 and 4 d) the medium was exchanged for fresh PBS, and the chlorhexidine concentration in the removed medium was determined by ultraviolet-visible spectroscopy (UV-Vis). The absorption values at λ = 255 nm were converted to mass concentrations of chlorhexidine via a calibration curve (m = 22.427 * A + 0.2474; with m: mass of chlorhexidine, A: measured absorbance at λ = 255 nm), and these values were plotted against time in a cumulative way. The lower limit of detection for the absorption was 0.005 AU. In case of an absorption above 1.5 AU, the sample was diluted and measured again. The amount of chlorhexidine released at 96 h was defined as the total releasable amount. Percentual release at other time points was calculated based on this amount. The results of one representative experiment are shown.

MECHANICAL STABILITY ASSAYS

SOLVENT RESISTANT ASSAY

A double rub test was used to assess the mechanical stability of the coatings under dry conditions. A number of the coated aluminium Q-panels were washed for 48 h in PBS at 37 °C to assess the mechanical stability of the coatings under wet conditions. The double rub test was performed essentially as described in the solvent resistance standard test method (ASTM D5402), with some modifications. In short, a piece of felt (diameter 2 cm), soaked in EtOH (more appropriate solvent than methyl ethyl ketone (MEK) used in ASTM D5402), was moved back and forth (1 double rub) over a distance of 12 cm (in 1 s) using an automated rubbing apparatus (DSM, in house equipment) on a defined area of the coated substrate with a constant load of 400 g. When macroscopic damage to the coating was observed, the test was stopped and the number of rubs were noted. A maximum of 200 double-rubs was applied and the resistance was rated by eye on a 0 – 5 scale, with 0 meaning penetration to the substrate in 200 double rubs or less and 5 meaning no effect on surface after 200 double rubs.

TAPE ADHESION TEST

The adhesion of the coating to the substrate was tested essentially as described in the tape standard test method (ASTM D3359). Due to the coating hardness, it was not possible to make cuts as described in ASTM D3359 prior to the procedure. In short, 25 mm wide semi-
transparent pressure-sensitive tape was pressed firmly onto a defined area of the coating and pulled off rapidly under an angle close to 180°. The test was repeated on two other sites of the coated aluminium Q-panels, and the level of adhesion was rated by eye on 0 – 5 scale, with 0 meaning full removal of the coating and 5 meaning no peeling or removal of the coating.

SCANNING ELECTRON MICROSCOPY
A JEOL JSM-IT100 scanning electron microscope (SEM; JEOL, Tokyo, Japan) was used to examine non-coated and CHX10-coated titanium implants. Before imaging, specimens were sputter-coated with carbon layer of ~6 nm thickness. The images were collected at an acceleration voltage of 5 – 15 kV.

ATOMIC FORCE MICROSCOPY
Atomic force microscopy (AFM) was performed with an AFM Dimension Edge (Bruker, Camarillo, CA, USA). Non-coated and CHX10-coated titanium implants were scanned in tapping mode with a standard silicon cantilever at a resonance frequency of 370 kHz and a scan rate of 0.5 Hz using a tip with a radius of 10 nm and a height of 10 – 15 µm. The average surface roughness (Ra) was quantified on three different locations of each sample using images of 100 × 100 µm², and presented as average with standard deviation.

BACTERIA
S. aureus ATCC 49230 is a biofilm-producing strain originally isolated from a patient with osteomyelitis. In order to verify the identity of the challenge strain after re-isolation in the in vivo studies, we constructed a green fluorescent S. aureus ATCC 49230 strain by introducing plasmid WVW189, essentially as described earlier. This plasmid carries the gfpuvr gene under control of a constitutive S. aureus promoter, and a chloramphenicol resistance gene for selection. The strain, S. aureus ATCC 49230 GFP, was designated S. aureus GFP in this paper, for brevity. The plasmid was stably maintained by the S. aureus GFP bacteria even in absence of chloramphenicol during growth. Chloramphenicol (10 µg/ml) was however always added to cultures of the strain used to prepare inocula, in order to avoid any potential loss of the plasmid. The susceptibility to chlorhexidine and the growth rate in tryptic soy broth (TSB; BD Difco) of S. aureus GFP and of the wild type S. aureus ATCC 49230 were equal (data not shown). In previous studies we showed that the presence of
plasmid WVW189 did not influence the virulence of *S. epidermidis* in mice\(^{30}\).

**SURFACE BACTERICIDAL ACTIVITY ASSAY**

A modified version\(^{32}\) of the Japanese Industrial Standard test for surface microbicidal activity (JIS Z 2801:2000\(^{33}\)) was used to evaluate the bactericidal activity of quadruplicate coated titanium disks against *S. aureus*. In short, *S. aureus* was cultured in TSB with 10 µg/ml chloramphenicol to the logarithmic growth phase at 37 °C, shaking. Subsequently, the bacteria were diluted with TSB to 10\(^7\) colony forming units (CFU)/ml, based on the optical density at 620 nm (OD\(_{620}\)). UV-sterilized nitrocellulose filter disks (diameter 13 mm) were placed on a Colombia sheep blood agar plate and 20 µl of the diluted bacterial culture, containing 2 × 10\(^5\) CFU, was pipetted onto the filters. The medium was absorbed by the agar while the bacteria were retained on the filter. Twenty µl of 1% TSB in 10 mM phosphate buffer was pipetted centrally on the surface of each titanium disk and an inoculated filter disk was carefully placed on top, with the bacteria contacting the coated surface. All disks with bacterial filters were placed individually in petri dishes and incubated at 37 °C for 24 h in a humid atmosphere. After incubation, each disk and the corresponding filter were together placed in 5 ml of TSB, sonicated for 30 sec in a sonicator water bath (Elma Transsonic T460, 35 kHz; Elma Schmidbauer GmbH, Singen, Germany) and vortexed for 1 min to dislodge adherent bacteria. This procedure does not affect bacterial viability\(^{34}\). Per sample, two independent ten-fold serial dilutions were made in a microtiter plate and duplicate 10 µl aliquots of the undiluted suspension and of the dilutions were pipetted onto blood agar plates. The blood agar plates were incubated overnight at 37 °C and the numbers of colonies were counted the following day.

**MOUSE EXPERIMENTAL BIOMATERIAL-ASSOCIATED INFECTION MODEL**

An established subcutaneous biomaterial-associated infection model to assess prevention of infection was used\(^{30,35–37}\). In this model, planktonic bacteria are injected along the implant, as opposed to biofilm treatment models, where bacterial biofilm if pre-grown on the implant surface prior to implantation\(^{30}\). The mouse study was approved by the Animal Ethical Committee of the Academic Medical Center at the University of Amsterdam, the Netherlands. Specific pathogen-free C57BL/6J OlaHsd immune competent female mice (Envigo, Horst, The Netherlands), aged 7 to 9 weeks old and weighing 17 to 20 g, were used.

Mice were anesthetized with 2% isoflurane (Pharmachemie) in oxygen in a laminar flow cabinet, followed by a subcutaneous injection of buprenorphine (Temgesic, RB Pharmaceuticals
Limited; 0.05 mg/kg) 15 min prior to the surgical procedure for pain control. The backs of the mice were shaved and disinfected with 70% ethanol. On each side, an incision of 0.5 cm was made 1 cm lateral to the spine. Subsequently, the (coated) titanium biomaterials were implanted subcutaneously with minimal tissue damage using a transponder, specifically designed for these implants. The incisions were closed with a single 0/6 vicryl stitch (Vicryl, Ethicon).

*S. aureus* GFP was cultured in TSB with 10 µg/ml chloramphenicol to the mid-logarithmic growth phase. The bacteria were washed in 0.9% NaCl (saline) and resuspended in saline, based on the OD<sub>620</sub> to 4 × 10<sup>6</sup>, this inoculum size was based on previous experience with *S. aureus* in the mouse BAI model, or 4 × 10<sup>7</sup> CFU/ml. Immediately following implantation, 25 µl of either of the *S. aureus* GFP inoculum suspensions, containing 10<sup>5</sup> or 10<sup>6</sup> CFU, respectively, were injected along the (coated) implants using a repetitive injector (Stepper model 4001-025; Dymax Corporation, Torrington, CT, USA). Groups of 9 mice with 2 implants each were used in the experiments. Mice were housed singly in individually ventilated cages (IVCs) and were provided with sterile food and water ad libitum.

One or 4 days after implantation, mice were anesthetized with 2% isoflurane in oxygen and buprenorphine was administered for pain control 15 min before biopsies were taken. Standardized biopsies (Ø 12 mm) were taken from the implantation sites as described previously and subsequently mice were euthanized by cervical dislocation. Each biopsy included skin, subcutaneous tissue and the implant. The implant was separated from the tissue and both implant and tissue were used for quantitative culture of bacteria. The implants were vortexed briefly in 0.5 ml of PBS to remove non-adherent bacteria, and then sonicated in saline for 5 min in a water bath sonicator to dislodge adherent bacteria. The tissue samples were homogenized in 0.5 ml of PBS using 5 zirconia beads (2 mm diameter, BioSpec Products, Bartlesville, OK, USA) and the MagnaLyser System (Roche), with 3 cycles of 30 sec at 7000 rpm, with 30 sec cooling on ice between cycles. Like the sonication procedure of the implants, this homogenization procedure does not affect the viability of staphylococci. The sonicates and homogenates were 10-fold serially diluted and plated on blood agar plates. In addition, the sonicated implants and 50 µl of each homogenate were cultured in 5 ml TSB containing 0.5% (v/v) Tween 80, for 48 h at 37 °C, shaking. The cultured *S. aureus* GFP bacteria were distinguished from mouse flora by inspection under exposure to UV light in a G:box (Syngene). The numbers of cultured bacteria were expressed as log CFU per implant or per homogenized biopsy. The lower limit of detection was 10 CFU. This value was assigned to samples positive in broth and negative in blood agar plate culture. For statistical analysis and to visualize the data on a logarithmic scale, a value of 1 CFU per sample was assigned when no growth had occurred in either culture system.
HISTOLOGY

Tissue biopsies were embedded in plastic (methyl methacrylate/butyl methacrylate (MMA/BMA); Merck Schuchart), and three-µm sections were cut from these samples. Per experimental group, 4 biopsies were randomly selected for histological examination and one or two microscopic sections per biopsy were immohistochemically stained. The sections were deplastified by incubating twice in 100% acetone for 6 min, washed in demineralized water for 5 min, and incubated in methanol with 0.3% H₂O₂ for 20 min at RT in the dark to block endogenous peroxidase activity. The sections were subjected to heat-induced epitope retrieval (HIER)³⁹, performed in a PreTreatment Module (Lab Vision) by incubation in citrate buffer pH 6.0 (Thermo Fisher Scientific, TA-250-PM1X) for 20 min at 98 °C, rinsed with tap water and incubated with Superblock (Klinipath, AAA999) for 10 min at RT. Sections were incubated with rat anti-mouse monoclonal F4/80 (macrophages; Serotec, MCA497GA, clone: Cl:A3-1) diluted 1:1000 in Tris-HCl-buffered saline (TBS; 50 mM Tris, 0.9% NaCl) overnight at 4 °C, subsequently with rabbit anti-rat IgG (fab2; SBA/ITK, 6130-01) diluted 1:3000 in TBS with 20% normal mouse serum for 30 min at RT, followed by undiluted BrightVision anti-rabbit AP (Immunologic, DPVB-55AP) for 30 min at RT, and finally with Vector Blue (Vector Labs, SK-5300) for 10 min at RT, with three TBS washes between all incubations. After rinsing with tap water, a HIER-citrate antigen retrieval step of 10 min at 98 °C, and a Superblock step were performed as described above. Next, sections were incubated with 1:10000 diluted rabbit anti-\textit{S. aureus} polyclonal IgG (Abcam, 20920) for 1 h at RT, with undiluted BrightVision anti-rabbit AP for 30 min at RT, and with Vector Red (Vector Labs, SK-5100) for 10 min at RT, again with three TBS washes between all incubations. After another HIER-citrate (10 min at 98 °C) and Superblock incubation, sections were incubated with 1:1000 diluted rat anti-mouse Ly-6G-FITC (neutrophils; BD, 553126) in TBS for 2 h at RT, with 1:1000 diluted rabbit anti-FITC (Nuclilab, 4510-7804) in the presence of 5% normal mouse serum in TBS for 15 min at RT, with undiluted BrightVision anti-rabbit IgG-HRP (Immunologic, DPVR-110HRP) for 30 min at RT, and finally stained for ~8 min with DAB (Immunologic, BS04-110) until they turned yellow/brownish. Sections were then washed with tap water, stained with 10-fold diluted haematoxylin (Klinipath, 4085-9002) for 2 min, washed with tap water and dried on a heat block at 50 °C. A drop of VectaMount (Vector Labs, H-5000) was applied and the sections were covered with a coverslip.

Between 2 and 5 multispectral imaging data sets were acquired of the immunohistochemically stained sections using the Nuance Imaging System (PerkinElmer/Caliper Life Science) at 420 to 720 nm with intervals of 20 nm. The data sets were spectrally
unmixed to identify the different cell types (macrophages, neutrophils and bacteria). In the negative controls without primary antibodies no staining was observed.

STATISTICAL ANALYSIS
All statistical analyses were performed in Graphpad Prism. For CFU counts, two-sample comparisons were made using a two-tailed Mann-Whitney rank sum test. The significance of differences between the frequencies of categorical variables was determined using Fisher’s exact test. For all tests, p-values of ≤0.05 were considered significant.

RESULTS

COATING SYNTHESIS AND CHARACTERIZATION
A procedure for the preparation of the coating formulation was established utilizing the diglycidyl ether of glycerol as the epoxy resin, IPD for curing and dopamine to optimize the adhesion to titanium (Figure 1a). Ensuring a close to equimolar ratio of NH/epoxy in the coating formulations (Table 1) resulted in a highly cross-linked network (Figure 1c) when the procedure was applied to aluminium sheets and Q-panels as well as to titanium disks. The coatings showed excellent resistance to rubbing, with no visible damage after 200 double-rubs (classification: 5). Moreover, no visual damage was observed upon tape stripping (classification: 5), proving a good adhesion of the coating to the substrate. The mechanical properties of the coatings did not change when the coated substrates had been incubated in PBS at 37 °C for 48 h prior to the mechanical testing. Next, we prepared the same coating formulations with chlorhexidine diacetate added at approximately 5 and 10 wt% of the total solid content, or without this agent (Figure 1b). Separate batches of coating formulations were prepared for the in vitro studies (CHX_0, CHX_5 and CHX_10) and for the in vivo studies (CHX_0 and CHX_10) (Table 1). No differences were observed between the mechanical properties of the separate batches of identical formulations.

The SEM analyses of the non-coated surfaces showed a rough topography with visible grooves (Figure 2a) and enhanced local roughness (Figure 2c), which was also affirmed by the 3D morphology as assessed by AFM with an estimated average surface roughness (Ra) of 861.9 ± 27 nm (Figure 2e). In contrast, the CHX_10-coated samples showed a smooth (Figure 2f; Ra of 530.5 ± 15 nm), compact and non-porous layer completely covering the original titanium topography (Figure 2b and 2d). In addition, white structures protruding from the
surface are visible on the coated samples both in SEM (Figure 2b and 2d) and AFM (Figure 2f). The nature of these protrusions remains elusive.

**IN VITRO CHLORHEXIDINE RELEASE**

The release curves show that the total amount of released chlorhexidine can be adjusted by changing the concentration of chlorhexidine in the formulation, i.e. 5 versus 10 wt% chlorhexidine (CHX$_5$ and CHX$_{10}$ in Figure 3). Chlorhexidine was released from the CHX$_5$- and CHX$_{10}$-coated aluminium sheets in a burst-release fashion with >80% of the release, 19 and 41 µg/cm$^2$ chlorhexidine, respectively, occurring in the first 24 h. After 4 days 23 and 47 µg/cm$^2$ chlorhexidine was released from the CHX$_5$- and CHX$_{10}$-coated sheets, respectively.

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**Figure 1.** Overview of the coating components (a) and architecture (b and c). a, The coating components: epoxy resin (i.e. the diglycidyl ether of glycerol), dopamine, chlorhexidine diacetate (CHX) and isophorone diamine (IPD). b, Schematic representation of the coating architecture, including chlorhexidine diacetate (CHX; diamonds) release from the epoxy-based coating on a substrate (///). c, Representation of a (possible) crosslink within the network.
Figure 2. Surface morphology of non-coated (left panels) and CHX\textsubscript{10}-coated titanium implants (right panels), shown by SEM recordings at 50x (a and b) and 500x magnification (c and d), and the 3D morphology by AFM analysis (e and f).

Figure 3. Cumulative release of chlorhexidine (µg/cm\textsuperscript{2}) from aluminium sheets coated with CHX\textsubscript{0} (no CHX; squares), CHX\textsubscript{5} (5 wt% CHX; triangle) or CHX\textsubscript{10} (10 wt% CHX; circles) over time. Results of one representative experiment.
IN VITRO SURFACE BACTERICIDAL ACTIVITY

The CHX$_{5}$ and CHX$_{10}$ coatings applied on titanium disks completely killed the S. aureus inoculum after 24 h of incubation (Figure 4). After washing with PBS for 24 h, both the CHX$_{5}$ and CHX$_{10}$ coatings remained fully bactericidal. The coating without chlorhexidine (CHX$_{0}$) did not reduce survival of S. aureus, showing that the bactericidal activity of the CHX$_{5}$ and CHX$_{10}$ coatings was due to the release of chlorhexidine.

IN VIVO ANTIBACTERIAL ACTIVITY OF CHX$_{10}$-COATED TITANIUM IMPLANTS IN MOUSE EXPERIMENTAL BAI

In a first experiment, mice were either sham-operated or they received non-coated, CHX$_{0}$-, or CHX$_{10}$-coated titanium subcutaneous implants. An S. aureus GFP inoculum of $10^5$ CFU was subsequently injected at the site of sham surgery in the sham-operated mice, or along the implants in the other mice. A potentially protective effect of the CHX$_{10}$ coating against colonization of the implants could not be assessed, since the level of implant colonization in the animals receiving non-coated or CHX$_{0}$-coated implants appeared to be unexpectedly low (Figure 5). The level of tissue colonization did however allow evaluation. At 1 day after challenge, tissues from sham-operated mice and from mice receiving non-coated implants did not differ in frequency of culture positive tissues, nor in numbers of CFU cultured from these tissues. In the group of mice with CHX$_{10}$-coated implants the frequency of culture-positive tissues was lower than in the mice with non-coated implants ($p = 0.0016$), and the numbers of CFU retrieved were lower than from tissues of mice with CHX$_{0}$-coated ($p = 0.029$) or non-coated implants ($p = 0.0022$) (Figure 5). No apparent macroscopic signs of toxicity or inflammation were observed in any of the mice.

In order to allow evaluation of an effect of the CHX$_{10}$ coating on implant colonization, we repeated the experiment with an S. aureus GFP inoculum of $10^6$ CFU with expected higher levels of in vivo colonization of non-coated implants. We used the coating with the highest chlorhexidine content (CHX$_{10}$) and non-coated implants for comparison. With this increased inoculum size, both the implant surfaces and the peri-implant tissues were highly colonized in the group of mice with non-coated implants (Figure 6). At 1 day after challenge 100% of the tissues and 89% of the implants, and at 4 days 83% of the tissues and 28% of the implants were culture positive in this group, and numbers of CFU retrieved from the implants and the surrounding tissue were higher with this increased inoculum size than with an inoculum of $10^5$ CFU used in the previous experiment (compare Figure 6 to Figure 5).

At 1 day after challenge, the CHX$_{10}$ coating significantly reduced the number
**Figure 4.** Surface bactericidal activity of chlorhexidine-releasing epoxy-based coating on titanium disks against $2 \times 10^5$ CFU *S. aureus*. The survival of the bacteria 24 h after application on unwashed (white bars) and washed (24 h in PBS; black bars) disks is shown. Non-coated, titanium disks without coating; CHX$_0$, titanium disks with coating not containing chlorhexidine; CHX$_5$ and CHX$_{10}$, titanium disks with coating containing 5 or 10 wt% chlorhexidine, respectively. The detection limit was log 2.7 CFU of *S. aureus*.

**Figure 5.** Bactericidal activity of chlorhexidine-releasing epoxy-based coatings applied on titanium implants against $1 \times 10^5$ CFU *S. aureus* GFP in mouse experimental biomaterial-associated infection at 1 day after implantation. Sham, surgery but no implant; non-coated, titanium implant without coating; CHX$_0$, titanium implant with coating not containing chlorhexidine; CHX$_5$, titanium implant with coating containing 5 wt% chlorhexidine. Results are expressed as the frequencies and percentages of culture-positive samples, and as the log of the numbers of CFU retrieved. The horizontal line represents the median log CFU. Frequencies of culture-positive samples and log numbers of CFU were analysed by Fisher’s exact and Mann-Whitney tests, respectively. *, $p \leq 0.05$ and **, $p \leq 0.01$. 
of culture-positive implants \((p < 0.001)\) as well as the numbers of CFU in the tissue \((p = 0.0015)\) and on the implants \((p < 0.001)\). At 4 days after challenge, the group with CHX\(_{10}\)-coated implants had significantly lower numbers of culture-positive tissues \((p = 0.0153)\) and culture-positive implants \((p = 0.0455)\) than mice with non-coated implants. At 1 day after challenge, only 4 of the CHX\(_{10}\)-coated implants were culture positive, yielding low numbers of CFU, and the surfaces of all CHX\(_{10}\)-coated implants were culture negative at 4 days after challenge. No apparent macroscopic signs of toxicity or inflammation were observed in the peri-implant tissue of any of the mice. Thus, the release of chlorhexidine proved to be effective to prevent \textit{S. aureus} colonization of the implant and the surrounding tissue \textit{in vivo} with no visible adverse effects.
Figure 7. Histological sections of biopsies of mice with non-coated titanium implants (Non-coated; a and c) or with \( \text{CHX}_{10} \)-coated implants (b and d), challenged with \( 1 \times 10^6 \) CFU \( \text{S. aureus} \) GFP and sacrificed after 1 day (a and b) or 4 days (c and d). High magnification images recorded from boxed area are shown below the respective images. Macrophages immunostained with F4/80, neutrophils with Ly-6 and bacteria with anti-\( \text{S. aureus} \) antibodies are shown in multispectral images as blue, green and red, respectively. Co-localization of macrophages and bacteria are shown in pink, co-localization of macrophages and neutrophils in light-blue and co-localization of bacteria and neutrophils in yellow. The images are representative for sections from different levels of the biopsies examined.
HISTOLOGY
To assess the influence of the coatings on the local immune response, we simultaneously stained polymorphonuclear neutrophils (PMNs), macrophages and bacteria in single histological slides of tissue sections, and visualized the cells using multispectral imaging. At 1 day after challenge, a strong infiltration of PMNs as well as of macrophages around the non-coated titanium samples was observed (Figure 7a). Around the CHX\textsubscript{10}-coated implants, clearly less PMNs were present at this early time point. At 4 days after challenge the foreign body response had progressed from neutrophil to macrophage (F4/80 positive cells) predominance, around both the non-coated and the CHX\textsubscript{10}-coated titanium implants (Figure 7c and 7d). So, in line with our macroscopic observations, there were no microscopic signs of \textit{in vivo} adverse effects such as excessively increased or protracted neutrophil-dominated inflammatory responses nor necrosis which might have been caused by the coating components or the chlorhexidine released.

At 1 day after challenge, many bacteria were observed in the tissue around the non-coated implant (Figure 7a) and only few bacteria around the CHX\textsubscript{10}-coated implant (Figure 7b), which is in accordance with the quantitative culture results (Figure 6). The bacteria were mainly co-localized with the PMNs. Although the number of culture-positive tissues around CHX\textsubscript{10}-coated implants at 4 days after challenge was lower than of the tissues around non-coated implants, the numbers of CFUs retrieved were similar. This was also true for the number of bacteria observed in histology in tissue surrounding the non-coated (Figure 7c) and CHX\textsubscript{10}-coated implants (Figure 7d). At 4 days after challenge the bacteria were mainly co-localized with macrophages.

DISCUSSION
We have developed an epoxy-based mechanically stable coating for titanium implants providing a predetermined release of chlorhexidine to prevent biomaterial-associated infection (BAI). The epoxy-based coatings containing 5 or 10 wt\% chlorhexidine, CHX\textsubscript{5} and CHX\textsubscript{10}, displayed an initial burst release in the first 24 h and the residual release occurring within 4 days. Additional \textit{in vitro} studies showed that the coating completely eradicated the \textit{S. aureus} inoculum even after washing for 24 h in PBS. The CHX\textsubscript{10} coating prevented mouse subcutaneous biomaterial-associated \textit{S. aureus} infection and did not show macroscopic nor microscopic signs of adverse effects.

Coatings and materials derived from epoxy resins have found widespread use
in numerous applications. Because of their good adhesion properties and excellent mechanical stability, epoxy-based coatings are highly suited for metallic orthopaedic implants, which are often inserted under mechanical stress. These coatings are particularly suited for non-cemented implants which in contrast to cemented implants cannot be protected by e.g. antibiotic releasing bone cement\textsuperscript{40,41} or other antimicrobial approaches such as the use of bioactive glass\textsuperscript{42,43}, and are more prone to recurrent infection\textsuperscript{44,45}.

Perhaps the most well-known epoxy resin imparting good mechanical properties to its derived coatings is the diglycidyl ether of bisphenol-A (DGEBA)\textsuperscript{20}. Cured networks of DGEBA and isophorone diamine (IPD) for biomedical applications indeed showed respectable mechanical properties and good biocompatibility \textit{in vitro}\textsuperscript{20}. However, bisphenol A derivatives are under continuous debate for their safety\textsuperscript{46}. Therefore, we selected the diglycidyl ether glycerol as the epoxy resin, to be cured with IPD, a system shown to be hemocompatible\textsuperscript{47}, and not toxic in a rabbit bone implant model\textsuperscript{48}. Ensuring a nearly stoichiometric ratio of NH to epoxy groups resulted in a highly crosslinked network. To further enhance adhesion of the coating to the titanium, dopamine was added to the formulation. The coating did not show any damage after extensive testing in the double rub as well as tape adhesion assays, illustrating its excellent mechanical properties. The coating therefore is a promising candidate for application to titanium implants in orthopaedics and dentistry.

Although antibiotic releasing coatings are widely used for different medical applications, e.g. in sutures and central venous and urinary tract catheters, antibiotic release coatings for orthopaedic devices remain mainly experimental. A study in rabbits with titanium fixation plates coated with poly-L-lactide (PLLA) containing a combination of rifampicin and fusidic acid placed on the tibia showed a significant reduction in \textit{S. aureus} infection after 28 d\textsuperscript{49}. Direct application of rifampicin and minocycline on titanium significantly reduced \textit{S. aureus} colonization of femoral medullary nails and device-related osteomyelitis in rabbits after 1 week\textsuperscript{50}. In a rabbit tibia \textit{S. aureus} infection model, the application of tobramycin to hydroxyapatite-coated titanium implants reduced infection rates and improved osseointegration after 28 days\textsuperscript{51}. In another rabbit study, the combination of hydroxyapatite with gentamicin also showed significant reduction in \textit{S. aureus} infection rates, good biocompatibility and bone integration as observed with pure hydroxyapatite\textsuperscript{52}. A gentamicin-loaded biodegradable poly(\textit{D,L}-lactide) (PDLLA) coating on titanium Kirschner wires showed a significant reduction in implant-related infection after 6 weeks of implantation in a rat tibia infection model\textsuperscript{53}. A recent prospective study of the
first commercially available antibiotic-releasing intramedullary tibia nail, with a coating of PDLLA containing gentamicin, has shown promising results, with no deep surgical wound infections recorded within the first six months after implantation of the coated nails\textsuperscript{54}. However, antibiotic-releasing coatings have two major disadvantages. Due to the worldwide rapid increase in antibiotic resistance there is a risk that a patient will have an infection with a bacterium which is resistant to the released antibiotic. Conversely, due to the local release, graded concentrations of antibiotic will be present near the implant, increasing the risk of selection of resistant bacteria and thus contributing to resistance development and ensuing implant infection.

In order to avoid antibiotics, we sought to use an antiseptic as the antimicrobial agent for our coating. One of the most frequently used antiseptics is chlorhexidine, with a broad spectrum of activity against Gram-positive and Gram-negative bacteria and yeasts, and with acceptable tolerability and a good safety record\textsuperscript{55}. Chlorhexidine has been approved by the American Food and Drug Administration (FDA) for application to intracorporally used medical devices, such as surgical meshes\textsuperscript{56,57} and intravenous catheters\textsuperscript{57}, and has a history of successful clinical\textsuperscript{58} and experimental use in preventing biomaterial-associated and other infections\textsuperscript{59,60}. Chlorhexidine combined with chloroxylenol in a dip-coating on rabbit tibia intramedullary nails prevented experimental S. aureus osteomyelitis\textsuperscript{18,19}.

Chlorhexidine is an important agent in the prevention of the spread of antibiotic resistant organisms and hospital-acquired infections\textsuperscript{61}. The effectiveness and widespread use of chlorhexidine has led to some concerns regarding the emergence of bacterial resistance\textsuperscript{62,63}, including MRSA resistance\textsuperscript{61}. However, reported levels of susceptibility of the bacterial isolates are still several orders of magnitude below the clinically applied dosages\textsuperscript{63}.

We tailored our coatings to release most of the chlorhexidine within the first 24 h. As proof of concept for prevention of infection, we studied efficacy in our mouse subcutaneous implant infection model. The CHX\textsubscript{10} coating proved to be highly efficacious in preventing S. aureus infection and did not cause any signs of adverse effects. Interestingly, at 1 day post challenge only few bacteria were observed in histology, and there was only a relatively low level of neutrophil influx in the tissue surrounding the CHX\textsubscript{10}-coated implants. This low influx likely is due to the efficacy of the coating to kill the S. aureus bacteria rapidly, thereby reducing e.g. local complement activation and consequent neutrophil attraction.

Studies in a rabbit tibia intramedullary nail infection model with titanium implants
with the same epoxy-based chlorhexidine-releasing coatings as used in our study, showed no signs of local nor of systemic toxicity during the 6 week experiment\textsuperscript{48}. Moreover, significantly higher bone apposition on the implant surface of both the CHX\textsubscript{0} (98.8\% coverage) and CHX\textsubscript{10} (81.3\% coverage) than on non-coated titanium nails (71.5\% coverage) was observed after 6 weeks. However, in contrast to our mouse results, no protection against \textit{S. aureus} infection was observed. This may have been due to the prolonged time period of the rabbit experiments. In the first post-operative week the rabbits showed lower \textsuperscript{18}F-FDG tracer uptake suggesting lower levels of inflammation. This might indicate that a partial suppression of the \textit{S. aureus} infection might have occurred in the first days of the experiment. Apparently, the chlorhexidine reservoir and its release profiles would need to be optimized for the rabbit model, which certainly is possible with this coating technology.

Bacteria colonizing the surface of a biomaterial not only are a focus of a localized biofilm infection, but can also be the source of tissue colonization\textsuperscript{30}. Conversely, bacteria residing in the tissue can be a cause of infection after re-implantation, in experimental infection\textsuperscript{11} as well as in patients. Tissue-residing bacteria can be hard to eradicate by antibiotic treatment\textsuperscript{10,64}, and in patients may necessitate a prolonged regimen of systemic and local antibiotic treatment prior to re-implantation\textsuperscript{65}. Thus, prevention of bacterial colonization of tissue surrounding implants is of vital importance. In our study, large numbers of bacteria were cultured from the tissues around the non-coated implants. Particularly at 4 days after challenge, these bacteria were co-localized with macrophages, suggesting that the macrophages were not effectively killing the bacteria. This phenomenon has been observed in many earlier studies\textsuperscript{34,66,67}. From the tissue around the CHX\textsubscript{10}-coated implants however, only few bacteria were cultured, and in histology few bacteria were seen. Apparently, the rapid initial release of chlorhexidine killed the vast majority of the infecting bacteria, preventing formation of a biofilm on the implant surface as well as colonization of the tissue, thus protecting both these sites against infection.

\textbf{CONCLUSION}

Taken together, our mechanically stable chlorhexidine-releasing epoxy-based coating on titanium holds promise for further development towards clinical application. Because of its mechanical stability it is suited for all types of bone implants, including but not limited to non-cemented orthopaedic implants, and possibly dental implants as well. Our coating
provides a good alternative for coatings releasing conventional antibiotics and therefore has the potential not only to prevent implant infection but also contribute to reduction of antibiotic resistance development.

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