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Prevention of *Staphylococcus aureus* biomaterial-associated infections using a polymer-lipid coating containing the antimicrobial peptide OP-145

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Abstract

The scarcity of current antibiotic-based strategies to prevent biomaterial-associated infections (BAI) and their risk of resistance development prompted us to develop a novel antimicrobial implant-coating to prevent *Staphylococcus aureus*-induced BAI. We incorporated the antimicrobial peptide OP-145 into a Polymer-Lipid Encapsulation MatriX (PLEX)-coating to obtain high peptide levels for prolonged periods at the implant-tissue interphase. We first confirmed that OP-145 was highly effective in killing *S. aureus* and inhibiting biofilm formation *in vitro*. OP-145 injected along *S. aureus*-inoculated implants in mice significantly reduced the number of culture-positive implants. OP-145 was released from the PLEX coating in a controlled zero-order kinetic rate after an initial 55%-burst release and displayed bactericidal activity *in vitro*. In a rabbit intramedullary nail-related infection model, 67% of rabbits with PLEX-OP-145-coated nails had culture-negative nails after 28 days compared to 29% of rabbits with uncoated nails. In rabbits with PLEX-OP-145-coated nails, bone and soft tissue samples were culture-negative in 67% and 80%, respectively, whereas all bone samples and 71% of the soft tissue samples of rabbits with uncoated nails were infected. Together, PLEX-OP-145 coatings, of which both compounds have already been found safe in man, can prevent implant colonization and *S. aureus*-induced BAIs.

Keywords (6): antimicrobial peptides, OP-145, *Staphylococcus aureus*, antimicrobial coating, orthopaedic implant, rabbit

Introduction

Biomaterial-associated infections (BAI) are dreaded complications in surgery and may result in serious disabilities [1]. BAI are caused predominantly by *Staphylococcus aureus* and *Staphylococcus epidermidis*, bacterial species able to form biofilms, i.e. aggregates of bacteria, often adherent to implant surfaces and encased in an extracellular matrix [1]. Due to their low metabolic activity and the protective matrix, biofilm-encased bacteria are less susceptible to the actions of conventional antibiotics [2], resulting in hard-to-treat chronic infections that ultimately may require implant removal. In some cases, the infection persists even after removal or replacement of the implant [3; 4]. This can be due to impaired clearance of bacteria residing in the tissue or within phagocytes [5; 6]. Treatment of the infection is compromised due to local vascular and tissue destruction and inflammation, which limit local concentrations of systemically applied antibiotics. Prevention of BAI by controlled release of the antibiotic at the tissue-implant interphase for prolonged periods is therefore a more promising strategy. Indeed, orthopaedic implants with controlled release coatings containing antibiotics like gentamicin [7; 8] and doxycycline [9] have been successful in preventing bone and tissue infections in (pre)clinical studies. However, in light of the increasing antibiotic resistance [10], including resistance to gentamicin and doxycycline [9; 11-13], the future efficacy of antibiotic coatings is uncertain and alternatives to prevent BAI should be developed.

Antimicrobial peptides (AMPs) like human defensins and cathelicin LL-37 can overcome the shortcomings of conventional antibiotics in multidrug-resistant and biofilm infections [14; 15]. Such peptides are generally small, cationic and amphipathic and typically exert their antimicrobial activity by interacting with bacterial cell membranes. They cause depolarization, destabilization and permeabilization of the bacterial membrane within minutes and subsequent lysis of the bacteria [16], irrespective of their metabolic state [17].

This fast, membrane-directed mechanism of action is considered one of the major reasons why development of resistance to AMPs is less likely to occur [18]. In addition to their antimicrobial activities, AMPs often display immunomodulatory activities, including regulation of inflammatory responses, chemo-attraction of leukocytes, enhancement of bacterial killing by human phagocytes [19], and wound healing [20]. Earlier, we developed the LL-37-derived synthetic peptide OP-145, previously named P60.4Ac [21]. OP-145 was more effective than LL-37 in eradicating *S. aureus* in wound infection models *in vitro* [22], while both neutralize the bacterial toxins lipopolysaccharide (LPS) and lipoteichoic acid (LTA) [21]. Moreover, OP-145 proved to be safe and successful as treatment for patients with chronic suppurative otitis media [23].

Based on the above considerations, the aim of the present study was to develop a novel antimicrobial coating for bone implants that allows the controlled release of OP-145 at the tissue-implant interphase for prolonged periods. For the coating, we chose the so-called Polymer Lipid Encapsulation Matrix (PLEX) technology, which we used successfully for the controlled release of doxycycline [9]. The ability of this PLEX-OP-145 coating to prevent bacterial colonization of the implant and implant-associated osteomyelitis was assessed in a rabbit intramedullary nail *S. aureus* infection model.

Materials and Methods

Bacteria

S. aureus strain JAR060131, isolated from a patient with an infected hip prosthesis [24] was used in the present study. Prior to each experiment, inocula from frozen stocks in nutrient broth supplemented with 20% (v/v) glycerol were grown overnight at 37°C on sheep blood agar plates (BioMerieux).

Peptide

OP-145 (acetyl-IGKEFKRIVERIKRFLRELVRPLR-amide) was prepared by normal 9H-fluorenylmethyloxycarbonyl (Fmoc)-chemistry using preloaded tentagel resin, benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop)/N-methylmorpholin (NMM) for *in situ* activation and 20% piperidine in NMP for Fmoc removal [25]. After final Fmoc removal, acetylation was performed by PyBop/NMM-mediated coupling using acetic acid. Couplings were performed for 60 min with 6-fold acylating species. After final Fmoc removal, OP-145 was cleaved with trifluoroacetic acid/H_2O 19/1 (v/v). The peptide was isolated by ether/pentane 1/1 (v/v) precipitation and centrifugation. After air-drying at 40°C, the peptide was dissolved in acetic acid/water 1/10 (v/v), lyophilized and HPLC-purified. Purity of the final product was >95% (RP-HPLC, detection at 214 nm). Integrity of the peptide was determined with Maldi-Tof (Microflex, Bruker). MH^+(calc) = 3094.81 MH^+(meas) =3095.06.

For peptide release experiments, the OP-145 amino acid sequence was not acetylated, but instead N-terminally labeled with the fluorophore nitrobenzoxadiazole (NBD) by coupling NBD-glycine as the final residue.

Antimicrobial activity *in vitro*

Bacteria were cultured to mid-logarithmic growth phase in tryptic soy broth (TSB; Oxoid) at 37°C and 200 rpm, pelleted, washed once with phosphate-buffered saline (PBS; pH 7.4), resuspended and diluted in PBS to $5x10^6$ colony forming units (CFU)/ml, as calculated from the absorbance of the suspension at 600 nm. In wells of a 96-wells polypropylene flat bottom plate (Greiner), 20 µl of the diluted bacterial suspension were added to 80 µl of peptide solution (final concentrations of 0.4-204.8 µM) in either PBS, PBS with pooled human plasma (Sanquin, final concentrations of 10-50% (v/v)) or HEPES-buffered saline with $MgCl_2$ or $CaCl_2$ (final concentrations of 0.625-10 mM). As untreated control, bacteria were incubated in the respective solutions without peptides. After 2 hrs of incubation at 37°C and 200 rpm, the number of viable bacteria was determined by plating samples and 10-fold serial dilutions on diagnostic sensitivity agar (DST, Oxoid). The lower limit of detection was 50 CFU. To visualize the data on a logarithmic scale, a value of 1 CFU per sample was assigned when no growth had occurred.

Prevention of biofilm formation *in vitro*

Bacteria were cultured to mid-logarithmic growth phase in TSB at 37°C and 200 rpm, pelleted, washed once with PBS and resuspended and diluted in biofilm-adjusted BM2 medium (62 mM potassium phosphate buffer (pH 7), 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 µM $FeSO_4$, 0.4% (wt/v) glucose and 0.5% (wt/v) casamino acids [26]) to $2x10^8$ CFU/ml, as calculated from the absorbance of the suspension at 600 nm. Fifty microliters of the bacterial suspension were added to 50 µl of peptide solution (final peptide concentrations 1.6-12.8 µM) in BM2 medium in wells of a 96-wells polypropylene flat-bottom plate (Greiner). As untreated control, bacteria were incubated in BM2 medium without peptides. After 24 hrs of stationary incubation at 37°C, planktonic bacteria were removed by four washes with PBS and biofilms were stained with 1% (w/v) crystal violet (Sigma-Aldrich) for 15 min. After

four washes with water and solubilization of the remaining crystal violet with 150 µl of 96% ethanol, 100 µl were transferred to a polystyrene 96-wells flat-bottom plate and the optical density at 595 nm (OD_{595nm}) was determined as a measure of biofilm mass.

To assess the effect of plasma on anti-biofilm activity of the peptides, bacteria and peptide solutions were added to plasma-coated microtiter plates as described by others [27]. This method was chosen since culture of the bacteria in BM2 medium with 50% (v/v) human plasma resulted in formation of thick pellicles at the bottom of the wells that were easily disrupted during washing, leading to inconsistent results. For coating, 96-wells plates were incubated overnight with 20% (v/v) pooled human plasma in PBS at 4°C, and washed with sterile water prior to adding the bacteria and peptide solutions. Biofilm mass after 24 hrs incubation was determined as described above.

Implant manufacture and coating procedure

A previously described biodegradable Polymer-Lipid Encapsulation MatriX (PLEX) coating consisting of poly lactic-co-glycolic acid (PLGA), dipalmitoyl phosphatidyl choline (DPPC), distearoyl phosphatidyl choline (DSPC) and cholesterol [28] was mixed with 10% (w/w) OP-145 or no peptide for the coating of the implants. The latter coating is further referred to as "empty coating". For *in vitro* experiments, solid medical grade TAN (titanium 7%-aluminium 6%-niobium; ISO 5832/11) discs (Ø 5 mm) with a handle allowing gentle removal of the discs from the wells, were submerged in the coating solution. Final weight of the coatings was determined by subtracting the weight of the implant prior to coating from its weight after coating. As the peptide concentration in the coating formulation was 10% (w/w), we could calculate that the average coating contained 109 µg of OP-145 per TAN disc.

For *in vivo* experiments, solid medical grade TAN intramedullary nails with a diameter of 2.5 mm and a length of 85 mm were coated by spraying the coating solution on

the nail surface to create a thin coating. Residual solvent was evaporated during 2 hrs of incubation at 45°C at 600 mm Hg, followed by overnight incubation at room temperature under vacuum. A second coating layer was applied in the same manner and final weight of the coating was calculated as described above. The average PLEX-OP-145 coating contained 6 ± 0.7 mg of OP-145 per TAN nail. All discs and implants were machined at the AO Research Institute (Davos, Switzerland), anodized in the final processing step at KKS Ultraschall AG (Steinen, Switzerland), and PLEX-coated at PolyPid (St. Petach-Tikva, Israel).

OP-145 release from the PLEX coating *in vitro*

For release experiments, a PLEX-OP-145 coating, containing 2% (w/v) NBD-labelled OP-145, was applied to tri-calcium phosphate (TCP) granules as described previously [28]. The average coating of the TCP granules contained 8.6 ± 0.5 µg of OP-145 per mg of TCP. In order to quantify the amount of OP-145 released from the coating, 100 mg of PLEX-OP-145-coated TCP granules were incubated in 1 ml of distilled water at 37°C. The supernatants were collected and replaced with fresh water after the first hour of incubation and daily thereafter for a period of 30 days. At each time-point, the amount of released peptide in the water was quantified by measuring the level of NBD-fluorescence using a spectrophotometer (BMG LABTECH). Based on previous experience, the release of the peptide from coated TCP granules was assumed to be similar to its release from coated metallic implants.

Antimicrobial activity of OP-145 released from the PLEX coating *in vitro*

TAN discs with the PLEX-OP-145 coating or with an empty coating were placed in wells of a 96-wells polypropylene flat-bottom plate and 100 µl of PBS were added to each well. Plates were sealed and incubated in a humidified atmosphere at 37°C. To assess the total amount of

OP-145 released over time, the PBS volumes of individual TAN disks were collected as samples at 1, 4, 6 hrs or 1, 2, 3, 7 or 14 days of incubation at 37°C. For each time point, three wells were harvested and the samples were stored at -20°C until further analysis. Of note, incubation periods of 21 and 28 days resulted in liquid loss due to evaporation and were therefore not included.

To determine the activity of OP-145 released from the coatings, the collected samples from the empty coatings were diluted 5-fold (to have sufficient volume) and those from the OP-145-containing coatings 5- to 625-fold in PBS. To 80 µl of the diluted samples, 20 µl of $5x10^6$ CFU/ml of *S. aureus* from a mid-logarithmic growth phase culture were added. As non-treatment control, bacteria were incubated in PBS without peptides. After 2 hrs of incubation at 37°C and 200 rpm, the number of viable cells was determined by plating 10-fold serial dilutions on DST agar. The lower limit of detection was 50 CFU. To visualize the data on a logarithmic scale, a value of 1 CFU per sample was assigned when no growth had occurred.

Institutional animal care and use committee approvals

The mouse study was approved by the Animal Ethical Committee of the Academic Medical Center at the University of Amsterdam, the Netherlands. Twenty-seven specific pathogen-free C57BL/6J OlaHsd immune competent female mice (Harlan), aged 7 to 9 weeks and weighing 17 to 20 g, were used. The rabbit study was approved by the Ethical Committee of the canton Grisons, Switzerland. Seventeen healthy, skeletally mature, age-matched, specific pathogen-free female New Zealand White rabbits (Charles River) were used in this study. All experiments were performed according to the animal protection law and regulations of the country where the experiments were performed.

Mouse subcutaneous implant-associated infection model

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 0.4 cm was made 1 cm lateral to the spine. Subsequently, 1 cm long silicone elastomer catheter segments (Medtronic Medical) were implanted subcutaneously with minimal tissue damage using a transponder, specifically designed for these implants [6]. The incisions were closed with a single 6-0 vicryl stitch (Vicryl, Ethicon).

S. aureus was cultured in TSB (BD Difco) to the mid-logarithmic growth phase. The bacteria were washed in 0.9% NaCl (saline) and resuspended in saline to $4x10^6$ CFU/ml, based on the optical density at 620 nm. Immediately following implantation, a 25 µl *S. aureus* inoculum containing $1x10^5$ CFU, was injected along each silicone elastomer implant. One hour later, mice were anesthetized again, and 50 µl of PBS (negative control) or 50 µl of PBS supplemented with 135 µg or 675 µg of OP-145 were injected along the implants. These were equimolar and 5-fold higher amounts to those of antimicrobial peptide BP2, which yielded protection in the same mouse model [29]. All injections were performed with a repetitive injector (Stepper model 4001-025; Dymax). Groups of 9 mice with 2 implants each were used in the experiments. Due to an error with the anesthesia during the implantation procedure 2 mice were excluded, resulting in 18 implants in the PBS group and 16 in the OP-145 groups. Mice were housed in individually ventilated cages (IVCs) and were provided with sterile food and water *ad libitum*.

One day 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 [6; 30] 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 the 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 fresh PBS for 5 min in a water bath sonicator (Elma Transsonic T460, 35 kHz; Elma) to dislodge adherent bacteria. The tissue samples were homogenized in 0.5 ml of PBS using 5 zirconia beads (Ø 2 mm, BioSpec Products) and the MagnaLyser System (Roche), with 3 cycles of 30 sec at 7,000 rpm, with 30 sec cooling on ice between cycles. Neither the sonication procedure of the implants [30], nor this homogenization procedure [6] affects the viability of staphylococci. The sonicates and homogenates were 10-fold serially diluted and plated on agar. In addition, the sonicated implants and 50 µl of each homogenate were cultured in 5 ml of TSB containing 0.5% (v/v) Tween 80, for 48 hrs at 37°C. Up to 5 10-fold serial dilutions of the samples were plated. In case the highest dilution yielded non-countable numbers of colonies, the log number of CFU was displayed as >6. For statistical analysis, a value of 6.5 was given to these samples. 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.

Rabbit intramedullary nail infection model

Premedication, anaesthesia and intra- and postoperative pain management were performed as described previously [31]. The medullary cavity of the right humerus of each rabbit was entered with a 2 mm centering drill bit. The medullary cavity was then reamed with a 2.5 mm reamer to the length of the nail, which was followed by lavage with lactated Ringer's Solution and finally all liquid was suctioned out. *S. aureus* was cultured as described previously [31].

Approximately $6x10^4$ CFU of *S. aureus* in 100 µl of saline were immediately dispensed into the medullary cavity with a pipette. Next, the (coated) nail was inserted and immediately screwed into the bone until it became flush with the bone, thus preventing loss of the inoculum or blood from the intramedullary cavity. A total of 17 rabbits received the *S. aureus* inoculum. Due to an error in inoculation and to unexpected fractures following implantation, four rabbits were excluded, resulting in 7 rabbits in the uncoated group and 6 in the PLEX-OP-145-coated group.

Rabbits were checked regularly by a veterinarian or an experienced animal caretaker. Blood samples for white blood cell counts and C-reactive protein (CRP) measurements were taken pre-operatively, three days post-operative and weekly thereafter until the end of the observation period. Weights were determined on the days of blood sampling. Radiographs of the operated limb were taken in two planes after surgery and once a week thereafter. Criteria for premature discontinuation of the study were set at weight loss exceeding 10% of the initial body weight within two weeks, local infection with severe lameness, persistent swelling and discharge, or signs of systemic infection such as fever, depression and anorexia. Four weeks after inoculation, all rabbits were euthanized by an overdose of pentobarbital (Esconarkon, Streuli Pharma). After euthanasia, a contact radiograph of the operated humerus was taken using high resolution technical film (D4 Structurix DW ETE, Agfa) and a cabinet X-ray system (Model No. 43855A, Faxitron X-Ray Corporation). Thereafter, the nail was removed and the newly formed soft tissue overlying the nail head was removed with a sterile scalpel and homogenized using an Omni-TH hand-held homogenizer (LabForce AG) with a sterile Omni-tip plastic probe. The nail was submerged in PBS, vortexed for 30 sec and subsequently placed in a sonicating water bath (Bandelin Sonorex Super 10P, Bandelin) operating at a frequency of 40 kHz for 3 min before a final vortex for 30 sec. The humerus was homogenized in PBS using a Polytron PT3100 homogenizer (Kinematica). The sampling

procedures did not affect viability of the bacteria. The number of viable bacteria were determined by plating 10-fold serial dilutions of the nail sonicate and the soft tissue and bone homogenates on blood agar plates (Oxoid). The lower limit of detection was 50 CFU/nail, 50 CFU/soft tissue and 100 CFU/bone. 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. All bacterial growth was evaluated by latex agglutination test (Staphaurex Plus, Oxoid AG).

Statistics

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 release kinetics, curves were fitted using linear regression analysis. For all tests, p values of ≤ 0.05 were considered significant.

Results

In vitro antimicrobial and anti-biofilm activities of OP-145 against *S. aureus*

We have previously shown that OP-145 displays bactericidal and anti-biofilm activity against clinical MRSA isolates *in vitro* [22]. Here, we assessed whether OP-145 is also effective against *S. aureus* JAR060131, a clinical isolate collected from a BAI. At concentrations of 1.6 μM and higher, OP-145 reduced the number of viable bacteria to undetectable levels in the presence of physiological salt concentrations (Figure 1A). As the presence of divalent cations and plasma proteins inhibits the antimicrobial action of many AMPs [32-34], we assessed the activity of OP-145 in PBS with increasing concentrations of $MgCl_2$, $CaCl_2$ and plasma. Up to 10 mM of $MgCl_2$ and $CaCl_2$ - which are up to 5- to 10-fold higher concentrations than in blood - did not reduce the efficacy of OP-145 against *S. aureus* (LC99.9 of 1.6-3.2 μM). Plasma, however, dose-dependently reduced the efficacy of OP-145 (Figure 1A).

After 24 hrs, *S. aureus* JAR060131 formed a moderate biofilm on uncoated and plasma-coated polypropylene microtiter plates (OD_{595nm} of 1.1-1.8 and 0.6-0.9, respectively). OP-145 inhibited this biofilm formation in a dose-dependent fashion with maximal inhibition of 77% seen at 6.4 μM in uncoated plates (Figure 1B). In plasma-precoated microtiter plates, the maximal inhibition was 87% (at 6.4 μM, Figure 1B). Of note, at the concentrations tested, OP-145 did not reduce the number of viable bacteria in biofilm-adjusted BM2 medium after 24 hrs ($1.5x10^8$-$1.4x10^9$ CFU compared to $1.3x10^8$ CFU in the absence of peptide), indicating that the observed inhibition of biofilm formation in this medium was not due to killing of the bacteria by the peptide.

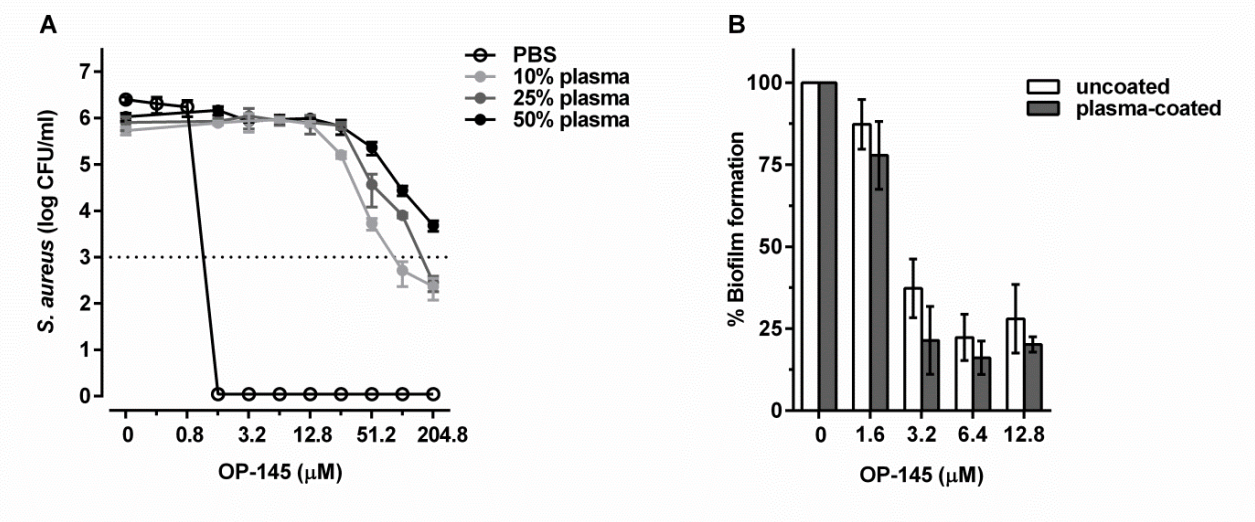

Figure 1. Antimicrobial (A) and anti-biofilm (B) activity of OP-145 against *S. aureus*.

A, $1x10^6$ CFU/ml of *S. aureus* JAR060131 were exposed to 0-204.8 μM of OP-145 in the absence (open circles) or presence (closed circles) of 10% (light gray), 25% (dark gray) and 50% (black) human plasma. After 2 hrs of incubation, the numbers of viable bacteria were determined microbiologically. Values are means and standard deviations of three independent experiments. Dotted line represents cut-off for 99.9% lethal concentration (LC99.9) using an inoculum of $1x10^6$ CFU/ml. **B**, $1x10^8$ CFU/ml of *S. aureus* JAR060131 were incubated with 0-12.8 μM of OP-145 in uncoated (white bars) or plasma-coated (gray bars) wells. After 24 hrs, the biofilm mass was determined using crystal violet staining. Results are percentage biofilm formation relative to the control, which was set to 100%. Values are means and standard deviations of three independent experiments.

Effects of OP-145 on implant-associated *S. aureus* colonization in mice

Next, we investigated the *in vivo* activity of OP-145 against *S. aureus* JAR060131 using an established mouse subcutaneous implant infection model [5; 29]. We assessed colonization of silicone elastomer implants and surrounding tissues by *S. aureus* one day after injection of OP-145 or PBS. Injection of 135 μg and 675 μg of OP-145 resulted in a significantly lower percentage of culture-positive implants (69%; $p \leq 0.05$ and 63%; $p \leq 0.01$, respectively) than injection of PBS (100%) (Figure 2A). Moreover, the numbers of bacteria on the implants were significantly ($p \leq 0.05$) lower in mice that had received 675 μg of OP-145 than in mice

that had received PBS injections. Tissue colonization by the bacteria was not reduced by OP-145 (Figure 2B).

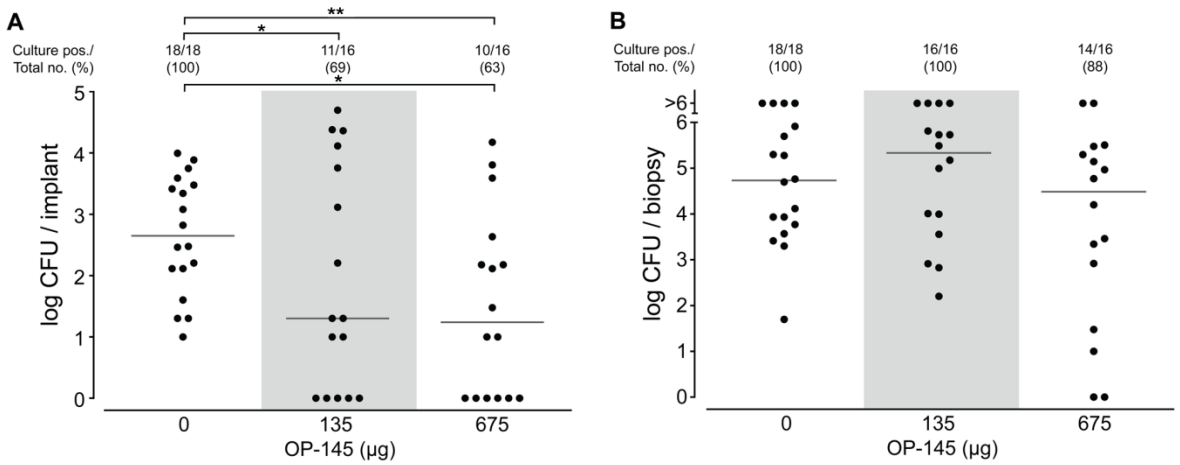

Figure 2. Effects of OP-145 on implant colonization by *S. aureus* in mice.

Immediately following subcutaneous implantation of a silicone elastomere implant, $1x10^5$ CFU of *S. aureus* JAR060131 were injected along the implants. One hour later, 50 µl of PBS containing 135 µg or 675 µg of OP-145 or as control no peptide were injected along the implants. Results are expressed as the proportion (and percentage) of culture-positive implants and the numbers of viable bacteria per implant (**A**), and peri-implant tissue (**B**), one day after treatment. The horizontal lines represent the median values. *p≤0.05, **p≤0.01, different from the PBS-injected control group as calculated using the Mann-Whitney rank sum test for the log CFU values, and the Fisher's exact test for the numbers of culture-positive samples.

Release of OP-145 by the PLEX coating

Next, we incorporated OP-145 in a PLEX coating on TCP granules and assessed its release. The results showed a burst release of approximately 55% of the peptide by the coating during the first 48 hrs followed by a daily release of about 1% for 30 days (R^2=0.94, Figure 3A). In addition, OP-145 released by the PLEX-OP-145 coating collected daily up to 14 days completely killed $1x10^6$ CFU of *S. aureus* (Figure 3B), showing that OP-145 released by the coating during this interval was biologically active.

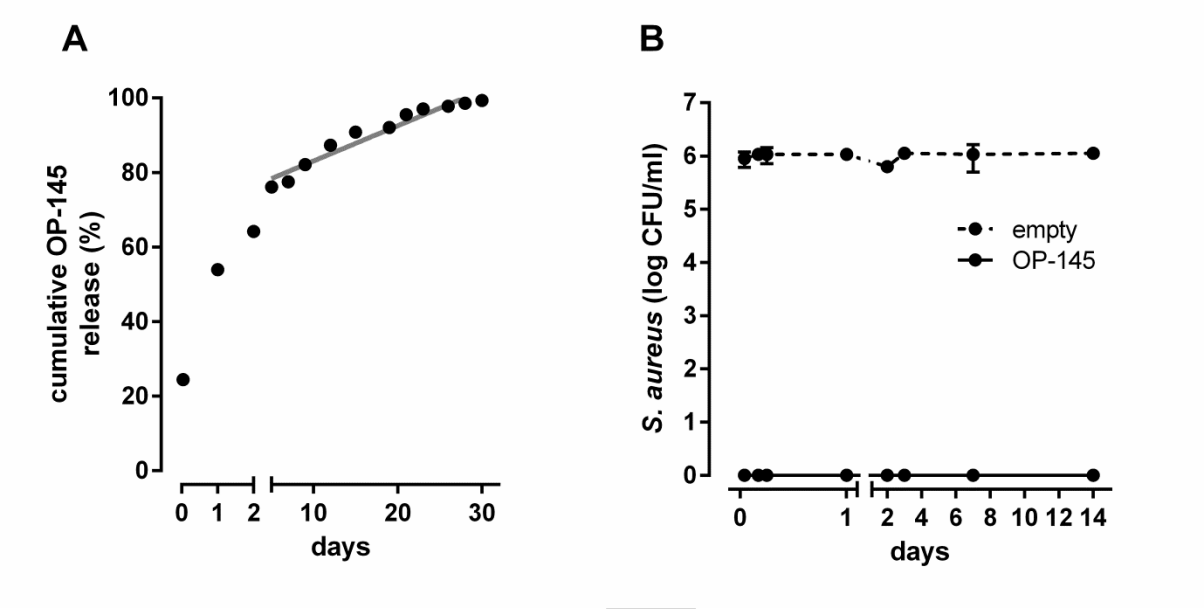

Figure 3. Release (A) and bactericidal activity (B) of OP-145 released from the PLEX-OP-145 coating.

A, TCP granules coated with PLEX-OP-145 coating were incubated in distilled water for 1 h. Water was collected as samples and refreshed daily thereafter for a period of 30 days. The amount of fluorescently-labelled OP-145 in all samples was measured spectrophotometrically. Results are expressed as percentage cumulative release (relative to the total amount of OP-145 incorporated into the coating) of OP-145 during 30 days. The linear fit of the percentage cumulative release from day 2-30 is shown as a gray line. Results of one representative experiment. **B**, TAN discs coated with PLEX-OP-145 or empty coating were incubated in PBS and samples were collected after 1, 4, 6 hrs and 1, 2, 3, 7, 14 days. Approximately $1x10^6$ CFU/ml of *S. aureus* JAR060131 were exposed to diluted samples from PLEX-OP-145-coated TAN discs (solid line) or from TAN discs with empty coating (dotted line). The numbers of viable bacteria were determined after 2 hrs of incubation in the samples. Values are mean numbers of viable bacteria (in log CFU/ml) and standard deviations of at least three independent experiments.

Effect of PLEX-OP-145 coating on implant colonization and osteomyelitis by *S. aureus* in rabbits

To investigate the capacity of the PLEX-OP-145 coating to prevent implant colonization and implant-associated osteomyelitis caused by *S. aureus*, we used an intramedullary nail infection model in rabbits. At sacrifice after 28 days, the nails and soft tissue biopsies of five out of seven (71%) rabbits in the uncoated group were culture-positive with a median number

of $2.9x10^4$ CFU per nail and $1.3x10^5$ CFU per soft tissue biopsy, respectively (Figure 4A). All rabbits (100%) from this group had positive bone cultures with a median number of $2.5x10^4$ CFU per bone biopsy. In the PLEX-OP-145-coated group, 33% of nails and 20% of soft tissue biopsies were culture-positive (Figure 4A), and the number of culture-positive bones in the PLEX-OP-145 group (33%) was significantly lower ($p \leq 0.05$) than the uncoated group (100%). The rabbits with PLEX-OP-145-coated nails had a 4.5-, 4.4- and 5.1-log lower median number of bacteria on the implants, in the bones and in the soft tissues, respectively, than rabbits with uncoated nails (Figure 4A). Three out of six rabbits (50%) in the PLEX-OP-145-coated group were completely infection-free, i.e. had negative implant-, bone- and soft tissue cultures, after 28 days.

In agreement with the quantitative bacteriology data, post mortem contact macro-radiographs showed signs of osteomyelitis in the rabbits receiving an uncoated nail (Figure 4B left, white arrow). Some rabbits receiving an uncoated nail displayed a peri-implant and periosteal reaction with joint involvement. Rabbits receiving the PLEX-OP-145-coated nails that had a culture-positive nail or bone showed only minor signs of infection on the radiographs, such as a mild periosteal reaction or slightly irregular bone surface (Figure 4B middle, white arrow). In agreement, rabbits that received an OP-145-PLEX-coated nail and that were completely infection-free after the study period did not show any signs of bone or joint infection in the conventional radiographs at any time-point, which was also confirmed in the post mortem contact macro-radiographs (Figure 4B right).

Rabbits with an uncoated nail displayed significantly ($p \leq 0.01$) more weight loss from day 7 onward (0.3-0.4 kg weight loss) than rabbits with a PLEX-OP-145-coated nail (0.04 kg weight loss at day 7 and 0.1-0.2 kg weight gain at day 14-28; Figure 4C). Furthermore, compared to the count at the start of the experiment, an elevated white blood cell count was

seen at day 7 in rabbits with uncoated nails and at day 7 ($p \leq 0.01$), 14 and 21 ($p \leq 0.05$) in rabbits with PLEX-OP-145-coated nails.

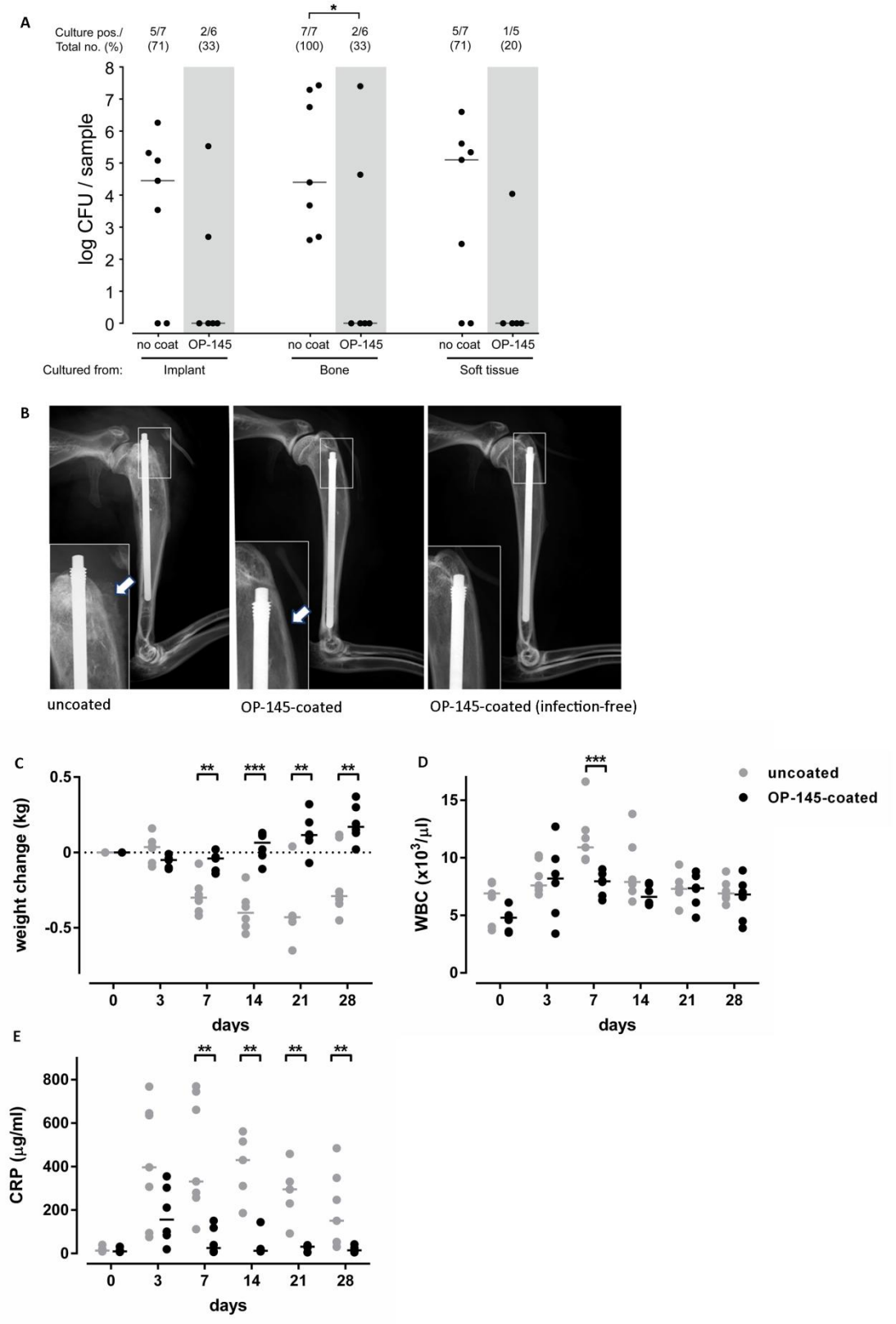

Figure 4. Effects of PLEX-OP-145 on implant-associated *S. aureus* osteomyelitis in rabbits.

Rabbits were infected with $6x10^4$ CFU of *S. aureus* JAR060131 in the intramedullary cavity and subsequently received an uncoated or a PLEX-OP-145-coated nail. After 28 days, the numbers of viable bacteria on the nail, in the bone cavity and in the soft tissue surrounding the bone were determined. **A**, Results are expressed as the proportion (and percentage) of culture-positive implants, bones and soft tissues, and as the numbers of viable bacteria retrieved from these materials after 28 days. The horizontal lines represent median values. Of note, the soft tissue sample of one rabbit in the PLEX-OP-145-coated group was not available. *$p \leq 0.05$, different from the uncoated group as calculated using the Fisher's exact test for the numbers of culture-positive samples. **B**, Contact radiographs of uncoated and PLEX-OP-145-coated nails in rabbits after 28 days. White arrows indicate irregular bone surface on the cranial side of the proximal part of the humerus, and periosteal reaction, which are signs of infection. The middle radiograph is taken from a rabbit of the PLEX-OP-145-coated group that was not completely culture-negative after 28 days and had minor signs of infection (irregular bone surface at arrow), whereas the right radiograph is from a completely infection-free animal. **C**, Change in body weight (kg) **D**, number of white blood cells (WBC, $x10^3$/µl) and **E**, level of C-reactive protein (CRP, µg/ml) for rabbits from uncoated (gray dots) and PLEX-OP-145-coated (black dots) group. The horizontal lines represent median values. **$p \leq 0.01$, ***$p \leq 0.001$, different from the uncoated group as calculated using the Mann-Whitney rank sum test.

At day 7, the peak in the number of white blood cells was lower ($p \leq 0.001$) for the rabbits with a PLEX-OP-145-coated nail ($8.0x10^3$/µl) than for the rabbits with an uncoated nail ($10.9x10^3$/µl). Rabbits from the uncoated group displayed an elevated ($p \leq 0.01$) CRP level during the entire study period compared to the levels at the start of the experiment. Rabbits with a PLEX-OP-145 coated nail only had an elevated (156 µg/ml, $p \leq 0.01$) CRP level at day 3 when compared to the level at the start of the experiment. From day 7 onward the CRP levels were 9.5-35-fold lower ($p \leq 0.01$) in rabbits with a PLEX-OP-145-coated nail than in those with an uncoated nail (Figure 4E).

Discussion

Current strategies to prevent BAI have mainly focused on the development of anti-adhesive surfaces, immobilization of antimicrobial compounds on the implant surface, and antibiotic-eluting implant coatings [35]. These approaches all suffer from considerable drawbacks. The first two approaches mainly prevent colonization of the implant, but have little or no effect on bacteria in the peri-implant tissues. The main drawback of antibiotic-eluting coatings is that at a certain point in time they will release sub-inhibitory concentrations of the antibiotic that promote the selection of resistant strains [36]. As an example, gentamicin-resistant bacteria have been isolated from orthopaedic implants in patients receiving gentamicin-loaded polymer beads [37]. Obviously, coatings releasing agents that are less likely to induce resistance, such as antimicrobial peptides [18], may circumvent this drawback.

In this connection, we selected the antimicrobial peptide OP-145, which has proven efficacy as treatment for patients with therapy-resistant chronic otitis media [23], to incorporate in such a coating. In this study, we first confirmed that the OP-145 peptide has potent *in vitro* bactericidal and anti-biofilm activities, essential traits in view of the application for biomedical devices, against the *S. aureus* JAR060131, an isolate collected from a BAI [24]. OP-145 was highly effective in killing this *S. aureus* strain in the presence of physiological concentrations of salt and divalent cations, but not human plasma. This reduced bactericidal activity in plasma may be explained by the binding of OP-145 to plasma components, such as lipoproteins [38], thus interfering with the peptide's ability to interact with the phospholipid bilayer [39]. Nevertheless, the peptide proved to be effective in preventing colonization of subcutaeneous implants in mice, showing that *in vitro* activities under defined conditions do not necessarily predict *in vivo* potency. Injection of OP-145, however, did not reduce the numbers of *S. aureus* in the tissue surrounding the implants, possibly because the peptide did not effectively penetrate the tissue, or was not taken up by

the host cells and thereby not capable of killing internalized bacteria. It has been reported that in the direct vicinity of biomaterials, staphylococci may survive inside host immune cells [6; 30] and may adapt to this micro-environment by the formation of small colony variants, which are in general more resistant to antimicrobial compounds [40]. Our observation that in the BM2 biofilm medium, OP-145 at the concentrations tested did not display bactericidal activity indicates that mechanisms other than killing, such as inhibition of bacterial adherence and inhibition of expression of genes essential for biofilm formation, as was shown for LL-37 [26], might be involved.

We developed a PLEX coating for the present study based on the results of a previous study in which the PLEX coating (i) allowed for the release of doxycycline in a controlled fashion, and (ii), prevented experimental implant-associated *S. aureus* infection in rabbits [9]. This coating provides an anhydrous environment for the antimicrobial agent incorporated and allows tailoring of the coating composition for optimized release over time [28]. In agreement, we found that the PLEX-OP-145 coating is characterized by a high initial release rate -which is necessary because the local immune defences may be compromised after implantation, leaving the implant susceptible to bacterial colonization [41]-, followed by a continuous slow release to maintain protective levels of this antimicrobial agent [42]. The coating provided effective antimicrobial activity against *S. aureus in vitro* for at least 14 days.

In the subsequent rabbit intramedullary nail infection study, the bones of rabbits with PLEX-OP-145-coated implants were more frequently culture-negative than those of rabbits with uncoated implants. Moreover, the numbers of CFU of *S. aureus* cultured from the implant, bone and soft-tissue were considerably lower in rabbits with the PLEX-OP-145-coated implants than those with the uncoated implants. Rabbits with the PLEX-OP-145-coated implants also displayed less signs of infection, i.e. less weight loss and lower levels of white blood cells and CRP, than rabbits with an uncoated nail.

Together, we conclude that the PLEX-OP-145 coating holds promise for further (pre)clinical development as an alternative for coatings releasing conventional antibiotics associated with resistance development. In addition, the present coating has the potential for rapid translation to man particularly since all compounds comprising the coating, including OP-145, have already been approved for human use.

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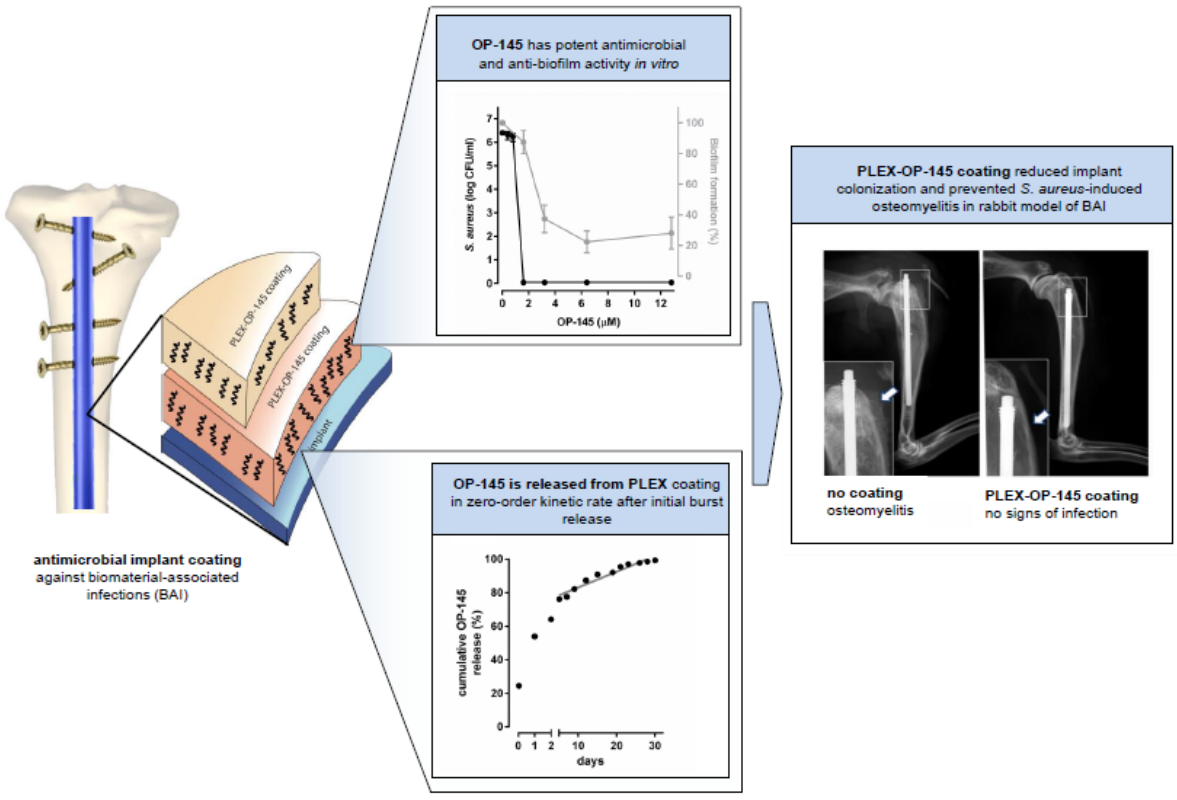

Graphical abstract