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A doxycycline-loaded polymer-lipid encapsulation matrix coating for the prevention of implant-related osteomyelitis due to doxycycline-resistant methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Implant-associated bone infections caused by antibiotic-resistant pathogens pose significant clinical challenges to treating physicians. Prophylactic strategies that act against resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA), are urgently required. In the present study, we investigated the efficacy of a biodegradable Polymer-Lipid Encapsulation MatriX (PLEX) loaded with the antibiotic doxycycline as a local prophylactic strategy against implant-associated osteomyelitis. Activity was tested against both a doxycycline-susceptible (doxy^S) methicillin-susceptible *S. aureus* (MSSA) as well as a doxycycline-resistant (doxy^R) methicillin-resistant *S. aureus* (MRSA).

In vitro elution studies revealed that 25% of the doxycycline was released from the PLEX-coated implants within the first day, followed by a 3% release per day up to day 28. The released doxycycline was highly effective against doxy^S MSSA for at least 14 days in vitro. A bolus injection of doxycycline mimicking a one day release from the PLEX-coating reduced, but did not eliminate, mouse subcutaneous implant-associated infection (doxy^S MSSA). In a rabbit intramedullary nail-related infection model, all rabbits receiving a PLEX-doxycycline-coated nail were culture negative in the doxy^S MSSA-group and the surrounding bone displayed a normal physiological appearance in both histological sections and radiographs. In the doxy^R MRSA inoculated rabbits, a statistically significant reduction in the number of culture-positive samples was observed for the PLEX-doxycycline-coated group when compared to the animals that had received an uncoated nail, although the reduction in bacterial burden did not reach statistical significance.

In conclusion, the PLEX-doxycycline coating on titanium alloy implants provided complete protection against implant-associated MSSA osteomyelitis, and resulted in a significant reduction in the number of culture positive samples when challenged with a doxycycline-resistant MRSA.

1. Introduction

One of the most challenging complications in orthopedic and trauma surgery is the development of implant-associated osteomyelitis. The incidence of osteomyelitis following elective orthopedic surgery ranges from 0.7 to 4.2% [1–4]. In trauma patients, the incidence of infection can be much higher and ranges from approximately 1% after operative fixation of closed low-energy fractures to more than 30% in complex open tibia fractures [5,6]. Infection after fracture fixation delays fracture healing and usually necessitates a prolonged and expensive treatment [7,8]. The emergence of antibiotic-resistant bacteria, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), has become a significant global healthcare issue [9,10] that has also influenced trauma medicine: between 25 and 32% of infections after fracture fixation are caused by MRSA [11,12].

Appropriate antibiotic prophylaxis can reduce infection rates in operative fracture treatment, but the distribution of systemically applied antibiotics to the fracture site and the tissue-implant interface may be limited due to localized vascular damage, tissue destruction and edema [5,13]. Improved prophylaxis against infection may be achieved

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by providing local delivery of potent antimicrobials directly to the tissue–implant interface. Numerous biomaterials have been investigated as potential local delivery vehicles, with antibiotic-loaded bone cement (ALBC) beads, collagen fleeces, and implant coatings being amongst the most prominent and clinically adopted approaches in fracture fixation [14–16]. Gentamicin-loaded ALBC beads have been shown to reduce acute and chronic infections even in the most severe fractures, although the beads require a second surgery for removal or, in the case of the bead pouch technique, delay wound closure significantly [14,17]. Antibiotic-coated implants on the other hand do not necessitate additional removal surgeries or delay wound closure. A gentamicin-coated tibial intramedullary (IM) nail has already proven to be successful in preclinical and clinical studies [16,18].

Currently available antibiotic releasing materials used in orthopedic medicine most often incorporate gentamicin, although it remains to be seen whether these gentamicin-releasing technologies will remain efficacious in an environment of increasing gentamicin-resistant bacteria [19–22]. Gentamicin also displays limited intracellular penetration and therefore lacks significant antibacterial activity against intracellular-residing bacteria, which have emerged as an important feature of implant related infection [23,24]. In the present study, we selected doxycycline, an antibiotic displaying very low resistance amongst staphylococci [25] yet with good intracellular penetration [26], and incorporated it in a Polymer-Lipid Encapsulation MatriX technology (PLEX) [27] coating on titanium alloy implants.

We assessed the antimicrobial activity of the PLEX-doxycycline-coated implants in vitro and in mouse and rabbit models as a prophylactic against implant-related osteomyelitis caused by a doxycycline-sensitive *S. aureus* (doxy^S MSSA), and against a doxycycline-resistant MRSA (doxy^R MRSA).

2. Methods

2.1. Bacteria

S. aureus strain JAR060131 (hereafter referred to as doxy^S MSSA), isolated from a patient with an infected hip prosthesis [23,26], and MRSA strain LUH15101 (hereafter referred to as doxy^R MRSA), isolated from a patient with constitutional eczema, were used in the present study. Bacteria were preserved for prolonged periods at −80 °C. Antibiotic susceptibility profiles were determined using Vitek and/or E-test (BioMerieux). The doxy^S MSSA was susceptible to all antibiotics, except penicillin (Minimum Inhibitory Concentration, MIC 33 μg/ml) [28]; the doxy^R MRSA was resistant to penicillin (MIC 33 μg/ml), oxacillin (MIC 32 μg/ml), tetracycline (MIC ≥ 16 μg/ml), doxycycline (MIC 32 μg/ml), and cotrimoxazol (MIC ≥ 326 μg/ml).

2.2. 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. Eighteen specific pathogen-free C57BL/6 OlaHsd immune competent female mice (Harlan), aged 7 to 9 weeks old and weighing 17 to 20 g, were used. The rabbit study was approved by the Ethical Committee of the canton Grisons, Switzerland. Twenty-eight skeletally mature, female New Zealand White rabbits (Charles River) between 24 and 36 weeks of age and a mean body weight of 4.3 ± 0.4 kg were included in this study. All experiments were performed according to the animal protection law and regulations of the country where the experiments were performed.

2.3. Implant manufacture

Several types of metal implants have been used in this study. For the in vitro antibacterial activity experiments, solid medical grade (ISO 5832/11) titanium–6% aluminum–7% niobium (TAN) circular discs (5 mm in diameter) with a handle allowing for retrieval of the discs from the wells were used. For the murine infection model, rectangular implants (10 × 4 × 1 mm) composed of medical grade titanium were used. For the rabbit study, IM nails custom designed to fit the intramedullary cavity of the adult rabbit humerus were manufactured from medical grade TAN (55 mm long, 3 mm diameter along the implant shaft). All discs and implants were machined at the AO Research Institute and anodized in the final processing step (KKS Ultraschall AG). The surface topography of the uncoated IM nail was observed by scanning electron microscopy (SEM) using a Hitachi S4700 (Hitachi) and was quantitatively measured by non-contact, white light profilometry (FRT MicroProf 200 Profilometer, Fries Research & Technology) [29].

2.4. Coating procedure

The TAN discs and IM nails were coated with a previously described biodegradable Polymer-Lipid Encapsulation MatriX (PLEX) coating containing polylactic-co-glycolic acid (PLGA), dipalmitoyl phosphatidyl choline (DPPC) and distearoyl phosphatidyl choline (DSPC) (Lipoid), cholesterol with doxycycline hyclate (Yangzhou Pharmaceuticals) [27]. The initial antibiotic loading can be adjusted both by the % drug loading and/or by the number of the applied layers [27]. In this study, we identified an initial burst of 25% followed by four weeks of steady release as our target release profile. The appropriate formulation conditions were thus applied, as described below.

For the in vitro studies, TAN discs were dip-coated by dipping implants in the polyester solution containing doxycycline. Control discs were treated identically, but the solution did not contain doxycycline. For the in vivo rabbit study, the implants were spray-coated whereby the formulation ingredients were dissolved in an organic solvent mixture and subsequently sprayed on the IM nail surface. The residual solvents were evaporated in moderate conditions, i.e. 45 °C for 2 h followed by overnight vacuum of 1 Pa at room temperature. A second coating layer was applied over the first layer in the same manner in order to achieve our target total coating and doxycycline weight. Coated implants were imaged using a Magellan 400L scanning electron microscope (FEI, Oregon, USA). The final weight of the coating applied to discs and nails was determined after subtracting the naked implant weight from the coated implant weight (individually for IM nails and from a combined total of 30 discs). The coating thickness (l) was calculated from the implant surface area (in mm^2) and the coating weight (in mg) and density (d ~0.7 mg/mm^3) using the following formula; $l = (m / V) / d$. The amount of doxycycline was then calculated from the total coating weight.

2.5. Doxycycline release from the PLEX-coating in vitro

Doxycycline release from the coated IM nails was measured by immersing the implants in 5% fetal bovine serum (FBS) in water. The medium was completely replaced after the first hour (hr) followed by medium replacements daily (working days) for 4 weeks. At each time point, the eluted doxycycline was analyzed by high-pressure liquid chromatography (HPLC), according to US pharmacopeia protocols, which has a lower limit of quantification of 2 μg/ml. This method quantifies the released doxycycline and any degradation products (4-epidoxycycline, 6-epidoxycycline, and methacycline). The measurements were performed in duplicate.

2.6. Antibacterial activity of doxycycline released from the PLEX-coating in vitro

TAN discs with an empty or a doxycycline-loaded PLEX coating were placed in wells of a 96-well polypropylene flat-bottom plate (Greiner Bio-One). Then, 100 μl of phosphate buffered saline (PBS; pH 7.4) was added to each well. Plates were sealed and incubated in a humidified