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## A BIODEGRADABLE GENTAMICIN-HYDROXYAPATITE-COATING FOR INFECTION PROPHYLAXIS IN CEMENTLESS HIP PROSTHESES

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### Abstract

A degradable, poly (lactic-co-glycolic acid) (PLGA), gentamicin-loaded prophylactic coating for hydroxyapatite (HA)-coated cementless hip prostheses is developed with similar antibacterial efficacy as offered by gentamicin-loaded cements for fixing traditional, cemented prostheses in bone. We describe the development pathway, from *in vitro* investigation of antibiotic release and antibacterial properties of this PLGA-gentamicin-HA-coating in different *in vitro* models to an evaluation of its efficacy in preventing implant-related infection in rabbits. Bone in-growth in the absence and presence of the coating was investigated in a canine model. The PLGA-gentamicin-HA-coating showed high-burst release, with antibacterial efficacy in agar-assays completely disappearing after 4 days, minimising risk of inducing antibiotic resistance. Gentamicin-sensitive and gentamicin-resistant staphylococci were killed by the antibiotic-loaded coating, in a simulated prosthesis-related interfacial gap. PLGA-gentamicin-HA-coatings prevented growth of bioluminescent staphylococci around a miniature-stem mounted in bacterially contaminated agar, as observed using bio-optical imaging. PLGA-gentamicin-HA-coated pins inserted in bacterially contaminated medullary canals in rabbits caused a statistically significant reduction in infection rates compared to HA-coated pins without gentamicin. Bone ingrowth to PLGA-gentamicin-HA-coated pins, in condylar defects of Beagle dogs was not impaired by the presence of the degradable, gentamicin-loaded coating. In conclusion, the PLGA-gentamicin-HA-coating constitutes an effective strategy for infection prophylaxis in cementless prostheses.

**Keywords:** cementless prosthesis, gentamicin-releasing coating, hydroxyapatite, antibiotic release, antibacterial efficacy, bone ingrowth.

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### Introduction

Total hip replacements have been performed since the early 1960s on patients suffering from hip joint arthrosis. At present, total hip replacement has an incidence higher than 150 procedures per 100,000 habitants in most industrialised countries, and accordingly about one million hips are replaced by prostheses worldwide every year (Web ref. 1). On average, the incidence rate of this procedure has increased by 25 % between 2000 and 2009 and this trend is predicted to continue due to ageing populations and growing prevalence of obesity (Web ref. 1). Projection models claim that the incidence of total hip replacement is expected to increase at least until 2030 in the United States (Kurtz *et al.*, 2014; Nemes *et al.*, 2014), which is likely a worldwide valid prediction. From the onset, high infection rates have plagued patients and treating orthopaedic surgeons. Nevertheless, total hip replacement is, at the same time, amongst the clinically most successful procedures, providing almost immediate relief of pain and restoration of function. Along with the projected increase in incidence of total hip replacement, is an increase in the numbers of patients suffering from infection. Although infections rates may be considered low at around 1-2 % (Hamilton and Jamieson, 2008), the economical downside of infection makes 1-2 % unacceptably high, due to the enormous number of implant recipients (Kurtz *et al.*, 2014). Moreover, many patients have a severely reduced quality of life after an episode of infection (Helwig *et al.*, 2014).

Despite the use of local and systemic antibiotic prophylaxis, strict hygiene protocols, and special sterile enclosures with laminar flow during surgery, the deep infection rate of primary hip replacements remains between 1 % and 2 % (Hamilton and Jamieson, 2008). Based on past experiences (Knobben *et al.*, 2006), it seems likely that improving hygiene and surgical discipline are insufficient countermeasures to prevent peri-operative contamination of implant surfaces. This is especially true for hip and knee replacements, often still requiring long incisions and surgical times of 1-2 h. When peri-operatively introduced bacteria are able to grow into a biofilm that protects the colonising organisms against the host immune system and antibiotics, the fate of the implant is a race for the surface between host tissue integration and bacterial colonisation (Gristina, 1987). This frequently results in surgical removal and subsequent replacement of the implant, at considerable costs (Vanhegan *et al.*, 2012). *In vitro* (Subbiahdoss *et al.*, 2010) as well as clinical evaluations (Deacon *et al.*, 1996) have demonstrated that tissue integration provides protection against infection.

This is especially important for the long-term protection of an implant against post-operative spreading of bacteria disseminated from infections elsewhere in the body or entering the bloodstream after routine dental treatments (Walman *et al.*, 1997).

Polymethylmethacrylate bone cement has traditionally been used to bond prosthetic replacements into bone. The use of antibiotic-loaded bone cements has been demonstrated to yield a lower risk of both septic (Engesaeter *et al.*, 2003; Wang *et al.*, 2013) and aseptic, or possibly more accurately “presumed aseptic” loosening than unloaded cements (Parvizi *et al.*, 2011) in primary prostheses as well as in revision surgery, despite administration of systemic antibiotic prophylaxis. In addition, local antibiotic delivery from bone cements has been proven to yield prophylactic effects against post-operative infections both in experimental and clinical studies (Malchau *et al.*, 2002; Tunney *et al.*, 2007). The Norwegian Arthroplasty Register (containing a total of 97,344 total hip replacements) indicated that there was a broad and consistent use of cemented prostheses over the period 1987-2007, representing about 85 % of all replacements (Dale *et al.*, 2009). Register data also indicate a continuous increase in the use of antibiotic-loaded cements, from 45 % in the period 1987-1992 up to 99 % in the period 2003-2007 (Dale *et al.*, 2009). Consequently, orthopaedic surgeons all over Europe now routinely use antibiotic-loaded bone cements in all cemented primary hip replacements and revision surgeries, and cemented fixation remains a common clinical solution.

Lately, due to progressive improvements in prosthesis design and changes in prosthetic material, providing better osseointegration, cementless prostheses have experienced increasing popularity and growth in use especially in younger and more active patients. Important in the increasing popularity of cementless prostheses is the development of grit-blasted, porous or hydroxyapatite (HA) coatings to stimulate osseointegration. Grit-blasting is known to stimulate osseointegration, and coating of prostheses with calcium phosphate ceramics, such as HA (Oosterbos *et al.*, 2004), further enhance bone bonding in a relatively short period of time (Geesink *et al.*, 1987; Søballe *et al.*, 1993) under load-bearing conditions (Søballe *et al.*, 1990). Clinically, osseointegration allowing full weight-bearing occurs within 4 to 12 weeks (Markmiller *et al.*, 2011). As a result of these developments, currently in the United States, about 90 % of all femoral stems implanted use cementless procedures (Web ref. 2). However, as a result of the use of cementless prostheses, antibiotic-loaded bone cements will no longer be available for prophylaxis to reduce risks of peri-operative bacterial contamination. Presently, no effective strategies are clinically available for local antibiotic prophylaxis in the case of cementless prostheses. A recent report shows that while infection rates associated with total hip replacements are increasing in general, the increase is most marked in the case of cementless hip replacements (Dale *et al.*, 2009).

*In vitro* experiments indicated that gentamicin-releasing coatings on grit-blasted titanium surfaces for use as cementless prostheses, with 1.0 mg gentamicin/cm<sup>2</sup> of the antibiotic sandwiched between the surface and

a biodegradable overlayer, possess a similar antibacterial efficacy as clinically employed gentamicin-loaded bone cements (Neut *et al.*, 2011). However, no such antibiotic-releasing coating has been described for cementless HA-coated prostheses.

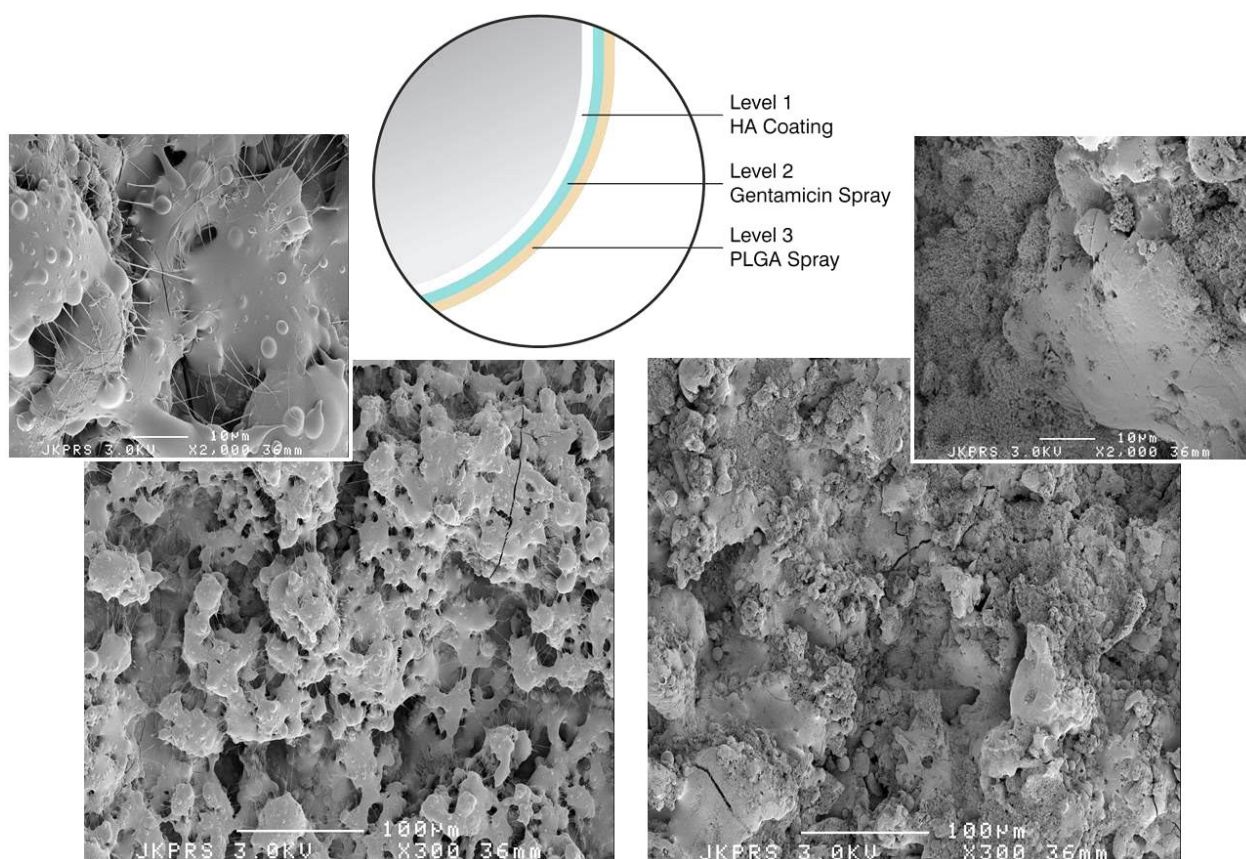
This study describes the development of a new, robust HA-coating containing gentamicin on titanium alloy (Ti-6Al-4V) covered with a protective, biodegradable poly (lactic-co-glycolic acid) (PLGA)-overlayer, that prevents colonisation of cementless orthopaedic prostheses by peri-operatively introduced bacteria. Initial *in vitro* experiments describe evaluation of the gentamicin release characteristics and antibacterial efficacies of the PLGA-gentamicin-HA-coatings against a number of staphylococcal strains, among which two were gentamicin-resistant. Staphylococcal strains were used since staphylococci constitute the number one causative strain for orthopaedic implant infection (Zimmerli *et al.*, 2004). Antibacterial efficacy was determined using traditional zone of inhibition measurements on agar plates, but also using models to analyse antibacterial efficacies in confined spaces and in a geometrically relevant set-up with miniature stems, using bio-optical imaging. *In vivo* evaluation of the antibacterial efficacy of the PLGA-gentamicin-HA-coating was carried out in a contaminated prosthesis model in rabbits, while effects of the coating on bone fixation and osseointegration were assessed in a canine condylar defect model, to demonstrate technology potential for clinical translation.

## Materials and methods

### PLGA-gentamicin-HA-coated titanium coupons, miniature stems, and pins

The Ti-6Al-4V surface was grit-blasted with alumina grit. Grit-blasting is used to roughen the surface to obtain better mechanical adhesion of the HA-coating. After cleaning ultrasonically in purified water and passivating, HA was spray-coated on the grit-blasted Ti-6Al-4V, according to DePuy's standard specification for the clinically established Corail® total hip system (plasma sprayed HA with a thickness of 155 µm, as per the detailed specification described by Reignier *et al.* (2011)). Gentamicin sulphate (Lek Pharmaceuticals, Slovenia) was applied on HA-coated, grit-blasted Ti-6Al-4V coupons (diameter 2.5 cm; height 0.4 cm; surface area 4.9 cm<sup>2</sup>) using a proprietary spraying technique (Web ref. 3) and its surface concentration was adjusted to 1 mg/cm<sup>2</sup> bioactive gentamicin. This concentration was chosen, because on grit-blasted Ti-6Al-4V surfaces, this gentamicin surface concentration yielded a similar antibacterial efficacy to that of gentamicin-loaded bone cement (Neut *et al.*, 2011). The gentamicin surface concentration of 1 mg/cm<sup>2</sup> is controlled through the process by spray time and the gentamicin content is checked by an HPLC method. Finally, a PLGA layer (PURAC® 5002, Purac Biochem, Gorinchem, The Netherlands) with a methyl ester as the terminal functional group was applied over the HA-containing gentamicin coating using an ultrasonic coating process. The PLGA used had an inherent viscosity of 0.2 (molecular weight of 17 kDa) and a 50:50 molar ratio of PLA: PGA. This





**Fig. 1.** Scanning electron micrographs of a PLGA-gentamicin-HA-coating prior to (left) and after gentamicin-release during 2 weeks in PBS (right), together with a schematic presentation of the built-up of the coating, including the protective PLGA-overlayer. The bar equals 100  $\mu\text{m}$  for low magnification micrograph, and 10  $\mu\text{m}$  for the insert.

thin PLGA-overlayer serves as a biodegradable layer to protect the gentamicin layer during transit and surgical implantation and not *a priori* to control antibiotic release. It quickly degrades *in vivo* to ensure normal osseointegration.

In addition, PLGA-gentamicin-HA-coated, grit-blasted Ti-6Al-4V miniature stems (diameter 1.2 cm and length 10.0 cm) and pins (diameter of 0.25 cm, length of 4.5 cm for the infection prophylaxis model, and diameter of 0.5 cm, length of 1.5 cm for the bone ingrowth studies) with a protective PLGA-overlayer were made. Fig. 1 presents a schematic overview of the different coating layers.

All coupons (HA- and PLGA-gentamicin-HA-coated), miniature stems, and pins were manufactured (DePuy Ireland, Cork, Ireland) under conditions employed for manufacturing implants and were supplied in individual 25 kGy gamma-sterilised surgical packs.

#### Gentamicin release

In order to establish the longevity of the gentamicin release from the PLGA-gentamicin-HA-coating, coupons were immersed in 20 mL of sterile phosphate buffered saline (PBS; NaCl 8.76 g/L,  $\text{K}_2\text{HPO}_4$  43.5 g/L,  $\text{KH}_2\text{PO}_4$  34.5 g/L, pH 7.4) and incubated at 37  $^\circ\text{C}$ , as described before in a study on gentamicin-coatings in absence of HA (Neut *et al.*, 2011). At designated time intervals (1, 6, 24, 30, 48, 72, 168 and 336 h), 500  $\mu\text{L}$  aliquots of the gentamicin-PBS solution were removed and stored

at 4  $^\circ\text{C}$  for subsequent determination of the gentamicin concentration. Gentamicin concentrations were determined by a fluorescence polarisation immuno-assay, having a sensitivity of 0.30  $\mu\text{g}/\text{mL}$  and an assay sample range of 0.44-13.80  $\mu\text{g}/\text{mL}$  (AxSYM, Abbott Laboratories, USA). Samples with gentamicin concentrations reported as greater than 10.00  $\mu\text{g}/\text{mL}$  were diluted.

#### Scanning electron microscopy (SEM)

The surfaces of the PLGA-gentamicin-HA-coated coupons before and after complete gentamicin release were investigated using SEM, as described before in a study on gentamicin-coatings in absence of HA (Neut *et al.*, 2011). Examination was done at 2.0 kV in a JEOL field emission scanning electron microscope type 6301F. Coupons were sputter-coated with a 3 nm thick conductive layer of gold/palladium (80/20).

#### Bacterial strains for *in vitro* studies

Six staphylococcal strains were used in the *in vitro* parts of this study, including two gentamicin-resistant strains, with minimal inhibitory concentration (MIC) > 4  $\mu\text{g}/\text{mL}$ . *S. aureus* 5298 (MIC gentamicin = 0.2  $\mu\text{g}/\text{mL}$ ), *S. aureus* 7323 (MIC gentamicin = 1.5  $\mu\text{g}/\text{mL}$ ), *S. epidermidis* 7388 (MIC gentamicin > 256  $\mu\text{g}/\text{mL}$ ), and *S. epidermidis* 7391 (MIC gentamicin = 24  $\mu\text{g}/\text{mL}$ ) were obtained from patients with infected joint arthroplasties and retrieved

during revision surgery (Department of Orthopaedic Surgery at the University Medical Center Groningen, The Netherlands). In addition, we used one ATCC strain *S. aureus* ATCC 12600 (MIC gentamicin = 1 µg/mL) and one bioluminescent strain *S. aureus* Xen36 (MIC gentamicin = 0.75 µg/mL). The bioluminescent strain produces luciferase and its substrate constitutively, resulting in a photon-emitting state when metabolically active. This strain was obtained commercially from Perkin Elmer (Massachusetts, USA). The MIC of gentamicin for all strains was determined using the E-test (Biomérieux, Marcy l'Etoile, France).

Staphylococci were cultured from cryopreservative beads (Protect Technical Service Consultants Ltd., United Kingdom) onto blood agar plates at 37 °C in ambient air for 24 h. For the preparation of experimental cultures, one colony was inoculated into a 10 mL batch culture of Tryptone Soya Broth (TSB: Oxoid, UK), cultured for 18 h at 37 °C, and this overnight culture was diluted with TSB to an optical density (at 600 nm) of 0.1 which equals approximately 10<sup>8</sup> bacteria/mL.

### Antibacterial efficacy of the PLGA-gentamicin-HA-coating

#### *Longevity of the antibacterial efficacy*

To determine the 24 h bacterial growth inhibition of the coating and its longevity, staphylococcal cultures were used to inoculate TSA plates with a sterile cotton swab, according to methodological details described before (Neut *et al.*, 2011). Ten minutes after inoculation, the PLGA-gentamicin-HA-coated coupons were placed in the centre of each plate, with little or no additional pressure. Agar plates were incubated for 24 h at 37 °C in ambient air, after which the width of the inhibition zones for each sample were measured. The inhibition zone was calculated by subtracting the diameter of the coupon from the inhibition zone diameter and dividing the difference by two. The experiments were done in triplicate with separately grown bacterial cultures and on separate agar plates. Immediately following the first zone of inhibition measurement, coupons were transferred to a freshly inoculated agar plate every 24 h to determine the longevity of the gentamicin release. This was repeated on a daily basis up to 4 days.

#### *Bacterial killing in an interfacial gap*

To determine the bacterial killing of PLGA-gentamicin-HA-coating in a confined spacing, the 3M Petrifilm Aerobic Count (AC) medium system was used, as described before from a methodological perspective (Neut *et al.*, 2011). A Petrifilm AC plate (3M Microbiology, St. Paul, USA) consists of two films, a bottom film containing standard nutrients, a cold-water gelling agent, and an indicator dye that facilitates colony counting, and a top film enclosing the sample within the Petrifilm system. The bottom film containing the gelling-agent can be inoculated with sterile demineralised water after which both films are pressed together and gelling occurs after 30 min. For the current experiments, 50 µL aliquots of bacteria suspended in fresh TSB to different concentrations between 10<sup>3</sup> and 10<sup>9</sup> bacteria/mL, as determined in a Bürker-Türk counting chamber, was placed on top of a PLGA-gentamicin-

HA-coated coupon after which the Petrifilm was folded together. Petrifilm plates were incubated at 37 °C for 48 h and colony forming units (CFU) counts determined. On average, the bacterial suspension spread to a diameter of 2.5 cm, implying a confined spacing with a thickness of 100 µm. Multiplication of the suspension volume (50 µL) with the bacterial concentration yields the total bacterial challenge per coupon (4.9 cm<sup>2</sup>). All experiments were done in triplicate with separately prepared bacterial suspensions.

#### *Bioluminescence imaging*

Potential suitability of the PLGA-gentamicin-HA-coating for use in total hip replacement is further assessed by determining its ability to prevent bacterial growth in a biofilm assay. Longitudinal bacterial growth prevention of a gentamicin-coating in absence of HA was demonstrated using a recent geometrically-relevant model in which a gentamicin-coated miniature stem was mounted in an aqueous agar surrounding into which the gentamicin released could diffuse, as described in detail before (Neut *et al.*, 2012). In short, the stem-holder allowed placement of a stem in its centre through a slot over half the depth of the bottom plate. The sterilised stem-holder was filled with liquid TSA with a sterile uncoated miniature stem in place. After cooling and solidification of the agar, the stem was removed, creating a perfect mould of the stem in the centre of the agar.

A culture of bioluminescent *S. aureus* Xen36 in TSB was then diluted and suspended to a concentration of 1 × 10<sup>4</sup> bacteria/ml in fresh TSB. These suspensions were used to contaminate the inside of the mould using a cotton swab. To this end, a sterile cotton swap was dipped 3 times in the bacterial suspension and gently moved over the inner mould surface. This procedure yielded transfer of approximately 150 bacteria from the swab to the mould surface (Neut *et al.*, 2012), corresponding with 4 bacteria/cm<sup>2</sup> mould surface. This represents a clinically realistic bacterial challenge based on available data on peri-operative contamination during a 1 h surgery in operating theatres with conventional ventilation systems (Sossai *et al.*, 2011; Verkkala *et al.*, 1998; Fitzgerald, 1979). After 10 min of drying, a sterile HA-coated or PLGA-gentamicin-HA-coated miniature stem was inserted and the entire system was incubated for 60 h at 37 °C in a bio-optical imaging system.

Bioluminescence was imaged with a CCD camera (IVIS® Lumina II, Caliper Life Sciences, Hopkinton, MA, USA) directly following insertion of the stem on an hourly basis throughout the experimental period up to 60 h. A bioluminescent image was obtained with 5 min exposure time using 7.5 cm field of view and 5 cm sample height, binning of 4, 1/*f* stop and open filter. Bioluminescence imaging was performed with the top cover of the stem holder removed, thus focusing on the top of the agar and imaging bioluminescence along the length of the stem. As a region of interest (ROI), a circular shell was taken around the stem. To this end, two circular ROIs were first defined: one with a diameter coinciding with the diameter of the miniature stem and one with a larger diameter of 3.0 cm around the centre (insert Fig. 4). Subsequently, the bioluminescences from both ROIs were subtracted

to yield the bioluminescence arising from the colonised zone around the stem. Bioluminescence was quantified as total bioluminescent flux (p/s) within the circular shell by using Living Image® 3.0 software (Xenogen, Alameda, CA, USA). The experiments were done in triplicate with separately cultured bacteria.

### Animal experiments – infection prophylaxis model

#### Study design

The study was carried out in four groups with a total of 14 female New Zealand White albino rabbits. Two groups (HA- and PLGA-gentamicin-HA-coated pins) were sacrificed at day 2 post-operatively (3 animals/group) and two similar groups were sacrificed at day 7 post-operatively (4 animals/group). The group sizes were determined by the following power calculation: at day 2, we anticipated a mean CFU count of 6 log ( $SD = 3$ ) in the HA-coated group and in the PLGA-gentamicin-HA-coated group  $\leq 1$  log ( $SD = 1$ ). With an alpha level of 0.05 and a power of 80 %, sample size needed in each group was 3 animals. At day 7, we anticipated a mean CFU count of 7 log ( $SD = 4$ ) in the HA-coated group and again  $\leq 1$  log ( $SD = 1$ ) in the PLGA-gentamicin-HA-coated group. With an alpha level of 0.05 and a power of 80 %, sample size needed in each group was 4 animals.

All experiments were carried out at a contract research facility (MPI Research, Mattawan, MI, USA). The study design was approved by MPI Research in compliance with all applicable regulations governing care and use of laboratory animals in the USA.

#### Surgical procedure

Surgery was carried out under aseptic conditions, after performing anaesthesia and starting pain management. Skin incision was made over the greater trochanter of the right limb and the gluteal muscles were retracted to allow access to the piriformis fossa. A hole with the final size of 2.5 mm was created by inserting a 1.0 mm Kirschner wire (K-wire) into the femur/medullary canal and subsequently the hole was gradually enlarged, to prevent fracture of the cortical bone, by drilling over the K-wire with a 2.0 mm and a 2.5 mm drill bit. After the hole was created, the medullary canal was flushed with sterile saline to remove most of the marrow.

The medullary canal was inoculated with gentamicin-sensitive *S. aureus* ATCC 25923 (MIC gentamicin = 1 µg/mL) (Hess *et al.*, 2011) using a micropipette. Each animal received 100 µL of a bacterial suspension ( $1 \times 10^6$  CFU/mL) to a final dose of  $1 \times 10^5$  CFU per site. This inoculation dose was chosen due to results of a pilot study, showing induction of infection without causing death of the animals. An HA- or PLGA-gentamicin-HA-coated pin was inserted into the canal using an insertion tool to avoid direct handling of the implant surface. Once the pin was placed, the defect opening was filled with bone wax, muscle and subcutaneous layers were sutured in a routine fashion, and the skin was closed with skin glue.

The animals were euthanised at day 2 or 7 and the right femur was harvested under sterile conditions. Macroscopic observations of the implant sites (e.g. abscess formation or cortical lysis) were recorded. The distal third of the

femur was removed using surgical rongeurs and the pin was explanted from the medullary canal. The distal and proximal femur segments and the pin itself were collected in separate sterile containers.

#### Microbiological evaluation

Samples of bone adjacent to the pins were weighed on receipt and placed in 50 mL tubes. If the samples weighted over 2 g, they were cut up using sterile instruments. Five mL of sterile saline per g bone was added to each tube and bone samples were homogenised for 1 min using a Polytron homogeniser PT 1200 E (Kinematica AG). The pins were transferred to a sterile 15 mL tube and 5.5 mL of TSB medium was added to each tube. Pins were sonicated for 7 min at 40 kHz and 150 W using a Crest Ultrasonics Sonicator and then vortexed for 3 min. All samples were kept on ice water in order to avoid bacterial growth.

Standard 96-well microdilution plates (Costar 3795; Fisher Scientific, Pittsburgh, US) were filled with 270 µL sterile saline or TSB medium, except for column 1. Three hundred µL of sample was loaded into column 1 and the samples were serially diluted 10-fold across the plate. From columns 1-8 (undiluted sample through the  $10^{-7}$  dilution), 10 µL aliquots were spotted in duplicate across the top of a square agar plate. The agar plates were then tilted at a 45-90° angle to allow the 10 µL aliquot to track across the agar surface to the opposite side of the plate. The plates were laid flat, allowed to dry at room temperature, inverted, and incubated at 35 °C for 24-48 h. Colonies were manually counted and counts were converted to log-units CFU per gram bone or per pin.

#### Haematological evaluation

Haematological evaluations were conducted pre-operatively for all animals, on days 1 and 2 post-operatively for animals in the day-2 sacrifice groups, and on days 4 and 7 post-operatively for the animals in the day-7 sacrifice groups. Blood samples were taken from the jugular vein. Samples were placed in tubes containing ethylenediaminetetraacetic acid for evaluation of haematology parameters (leukocyte count -total and absolute differential-, erythrocyte count, haemoglobin, haematocrit, absolute reticulocytes, platelet count, and blood cell morphology) using an automated haematological analyser ABX Micros ES 60 (HORIBA Medical, Kyoto, Japan).

### Animal experiments – bone ingrowth model

#### Study design

The study was carried out in four groups with a total of 12 male Beagles at a contract research facility (MPI Research, Mattawan, MI, USA). The study design was approved by MPI Research in compliance with all applicable regulations governing care and use of laboratory animals in the USA. Beagles were selected because of their wide-spread use for evaluating implants in bone (Turner, 2001). Additionally, the Beagle distal femoral condyle is sufficiently large to hold two implants per knee, reducing the number of animals required for the study. Two groups (HA- and PLGA-gentamicin-HA-coated pins) were sacrificed at week 4 post-operatively (5 animals), and two similar groups were sacrificed at week 12 post-operatively (7 animals).



The group sizes were determined by the following power calculation: at week 4, we anticipated a mean bone-implant contact of 40 % ( $SD = 6$  %) in the HA-coated group and 30 % ( $SD = 5$  %) in the PLGA-gentamicin-HA-coated group. With an alpha level of 0.05 and a power of 80 %, sample size needed in each group was 5 animals. At week 12, we anticipated a mean bone-implant contact of 60 % ( $SD = 7$  %) in the HA-coated group and 50 % ( $SD = 6$  %) in the PLGA-gentamicin-HA-coated group. With an alpha level of 0.05 and a power of 80 %, sample size needed in each group was 7 animals.

The 4-week period is regarded as the time required for initial bone ingrowth and fixation of the pin, while the time point of 12 weeks was added as further data on medium-term bone integration (Alt *et al.*, 2011; Bobyen *et al.*, 2009; Moojen *et al.*, 2009).

#### *Surgical procedure*

After induction of anaesthesia, each animal was placed on its side on the surgical table and a skin incision was made over the distal femur. The skin was retracted and the muscle and soft tissue over the femur were reflected to expose the lateral condyle. Two circular defects, approximately 4.8 to 5.4 mm in diameter, were placed 12 to 17 mm apart (from centre of defect to centre of defect) parallel to the distal femur condylar plateau. Each defect was drilled perpendicular to the lateral condyle surface, through the cortex, until the medullary cavity was breached. The first defect was created in the approximate centre of the lateral condyle and the second defect positioned relative to the first.

All pins were handled using a specifically designed insertion tool and they were placed so that the pin was surrounded by cancellous bone and not in contact with the opposing cortical bone. Once each pin had been placed, the drilled bone defect was filled with bone wax, the muscle and subcutaneous layers were sutured in a routine fashion, and the skin was closed with skin glue. The animal was repositioned on its opposite side and the procedure was repeated on the contralateral femur.

At the end of the 4 and 12 week recovery periods, the animals were euthanised by an intravenous overdose of sodium pentobarbital solution followed by exsanguination by severing of the femoral or axillary vessels. The left and right distal femora were removed.

#### *Biomechanical testing*

Each condyle was potted in dental cement making sure the cement did not come into contact with the pin directly or through the cancellous bone bed. The potted specimen was mounted directly under the pull-out grip and positioned so that the axis of the pin was aligned in parallel with the direction of the pull-out force. The pull-out grip was centred over the specimen and raised at a rate of 1 mm/min. Ultimate pull-out force was recorded for each pin and fixation strength was calculated (pull-out force / surface area of the pin).

#### *Histomorphometry*

Pins for histomorphometry were excised leaving a sufficient bone collar (5 mm minimum) around the pin,

placed in 10 % neutral buffered formalin, and shipped for histology slide preparation. Histology samples were embedded in an acrylic resin (Technovit 7200), sectioned and stained with Sanderson's Rapid Bone Stain. Digital photos of the slides were taken and image analysis was carried out using the colour recognition software in BioQuant. The bone-pin contact was measured and the scores were reported as a percentage of the pin surface area. Region of Interest encompassed the contact area along the full length of the interface between the bone defect surface and the pin surface.

#### **Statistical analysis**

Influence of the gentamicin-releasing coating on bacterial growth around the miniature stems was determined through comparison of the integrated photon flux around miniature stems with HA- or PLGA-gentamicin-HA-coatings using the unpaired Student's *t*-test, accepting  $p \leq 0.05$  as statistically significant. Likewise, for the animal experiments the influence of the PLGA-gentamicin-HA-coated pins on bone fixation, osseointegration, and infection resistance was determined through comparison of the fixation strength, percentage of bone-implant contact, and bacterial and neutrophil count in the animals groups with an HA-coated pin using the unpaired Student's *t*-test, accepting  $p \leq 0.05$  as statistically significant.

## **Results**

#### **Gentamicin release**

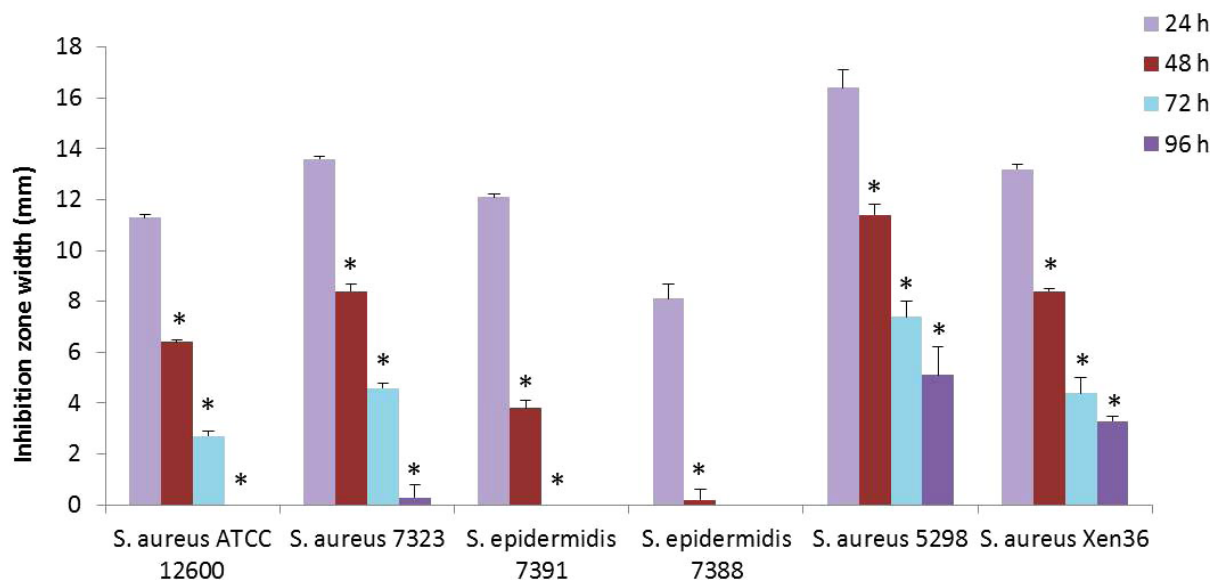
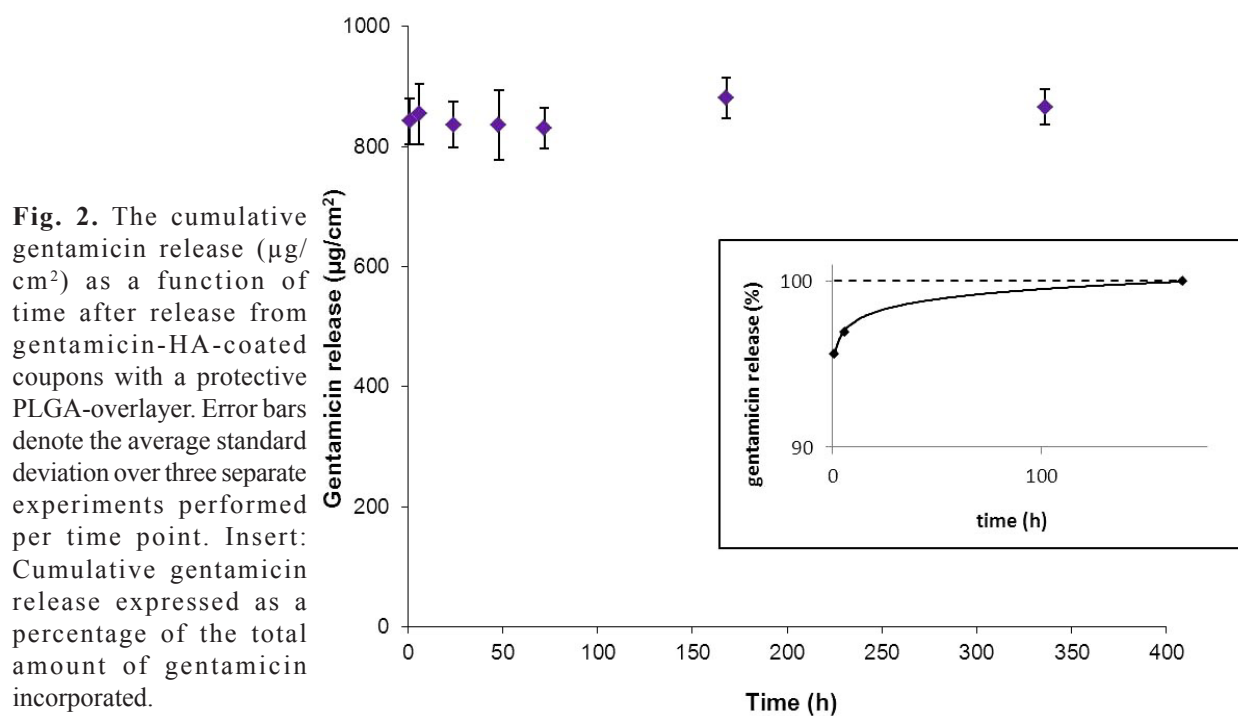
PLGA-gentamicin-HA-coatings demonstrate an initial burst release of around 95 % of the gentamicin incorporated within the first 1-2 h, followed by slow release of an additional 2 % up to 6 h. After about 168 h, 100 % of the gentamicin was released from the PLGA-gentamicin-HA-coating (Fig. 2). SEM micrographs (Fig. 1) indicate that the PLGA-overlayer had disappeared within 336 h, leaving the surface structure of the HA-coating intact.

#### **Antibacterial efficacy**

All staphylococcal strains, including gentamicin-resistant *S. epidermidis* 7388 and *S. epidermidis* 7391 are inhibited in their growth on agar during the first 24 h of gentamicin release from the coating (Fig. 3). The growth inhibition observed towards resistant strains can be attributed to the high initial burst release of the gentamicin from the coating. The antibacterial efficacy of the PLGA-gentamicin-HA-coating gradually decreases with time. Antibacterial efficacy disappears most readily for the gentamicin-resistant strains while some residual antibacterial activity is seen for the most sensitive strains after 96 h (Fig. 3). No zones of inhibition were observed around HA-coated coupons without gentamicin.

#### **Bacterial killing in interfacial gap model**

At a bacterial challenge of 10 bacteria/cm<sup>2</sup> substratum surface, HA-coated coupons allowed growth of around 27 CFUs per coupon in the confined spacing between coupons and the Petrifilm, regardless of the staphylococcal strain involved, whereas no CFUs or in a few occasions



**Fig. 3.** Width of the zones of inhibition (mm) around gentamicin-HA-coated titanium coupons with a protective PLGA-overlayer on TSA plates as a function of time for six different staphylococcal strains. Growth inhibition zones were measured 24 h after inoculation, after which coupons were transferred to a freshly inoculated agar plate and incubated for another 24 h. This procedure was repeated every 24 h to determine the longevity of the antibiotic release up to 4 days. Data represent averages over three separate experiments with error bars indicating the standard deviation. For each strain, significant differences ( $p < 0.05$ ) within a time series with respect to the day before is indicated by \*.

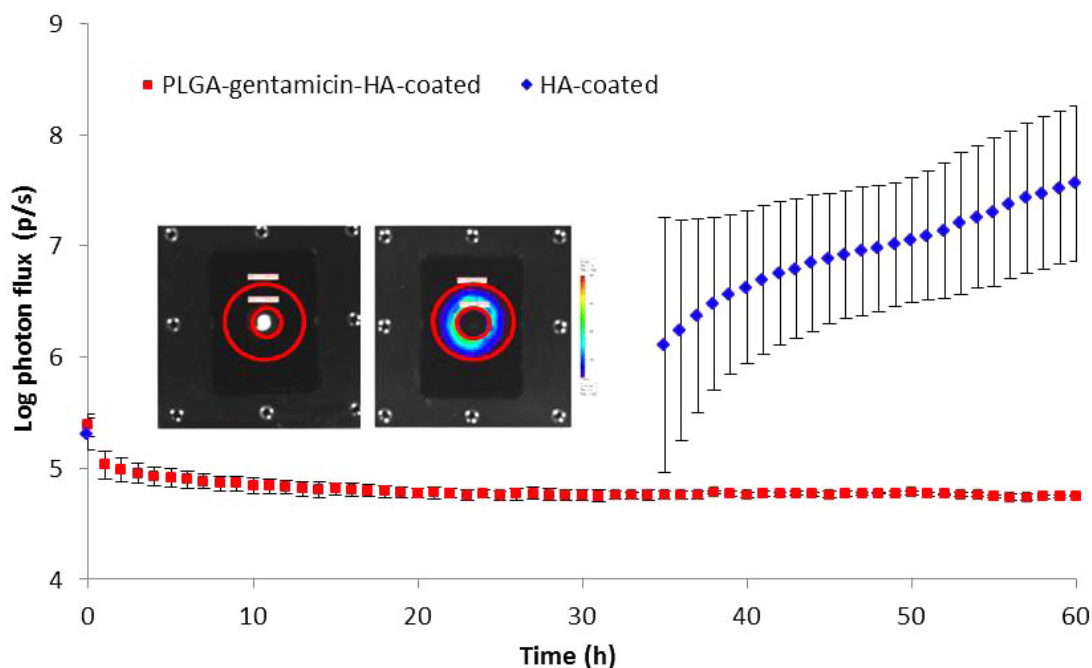
1 or 2 CFUs, were seen in the confined space between PLGA-gentamicin-HA-coated coupons and the Petrifilm until a bacterial challenge of  $10^7$  bacteria/ $\text{cm}^2$  was reached. Note that at a challenge of  $10^7$  bacteria/ $\text{cm}^2$ , representing a clinically unlikely high total challenge, no CFUs were seen, not even for the gentamicin-resistant strains included.

#### Longitudinal antibacterial efficacy in geometrically-relevant model

Our data indicate that the gentamicin-susceptibility of bioluminescent *S. aureus* Xen36 is within the range of the other staphylococcal strains used (Fig. 3).

Photon fluxes arising from bioluminescent *S. aureus* Xen36 colonising the miniature stem and its surrounding become measurable after 30-40 h of growth around HA-coated stems (Fig. 4) and increase more than two log-units with time up to 60 h (statistically significant at  $p = 0.005$ ) after which the agar started to dry out, in a fashion resembling a normal bacterial growth curve. No measurable photon fluxes arise from colonising staphylococci when a PLGA-gentamicin-HA-coated stem is inserted in bacterially contaminated agar, indicating the antibacterial efficacy of the coating.





**Fig. 4.** Bioluminescence imaging of staphylococcal growth around miniature stems. Photon fluxes arising from a circular shell around miniature stems mounted in an agar mould contaminated with bioluminescent *S. aureus* Xen36 for HA-coated and PLGA-gentamicin-HA-coated stems as a function of time. All experiments were done in triplicate and data represent averages  $\pm$  SDs. Insert: top-view of the bioluminescence signal around a miniature stem with (left) and without (right) a PLGA-gentamicin-releasing coating (indicated by the smallest red circle) in an agar mould, contaminated with bioluminescent *S. aureus* Xen36 after 31 h of incubation at 37 °C. Bioluminescence is displayed on a pseudo-colour scale, high numbers of photons per second (p/s) appear red, while low numbers appear blue. The circular shell in between the red circles indicates the ROI for quantitative analysis of the bioluminescence.

#### Animal experiments – infection prophylaxis model

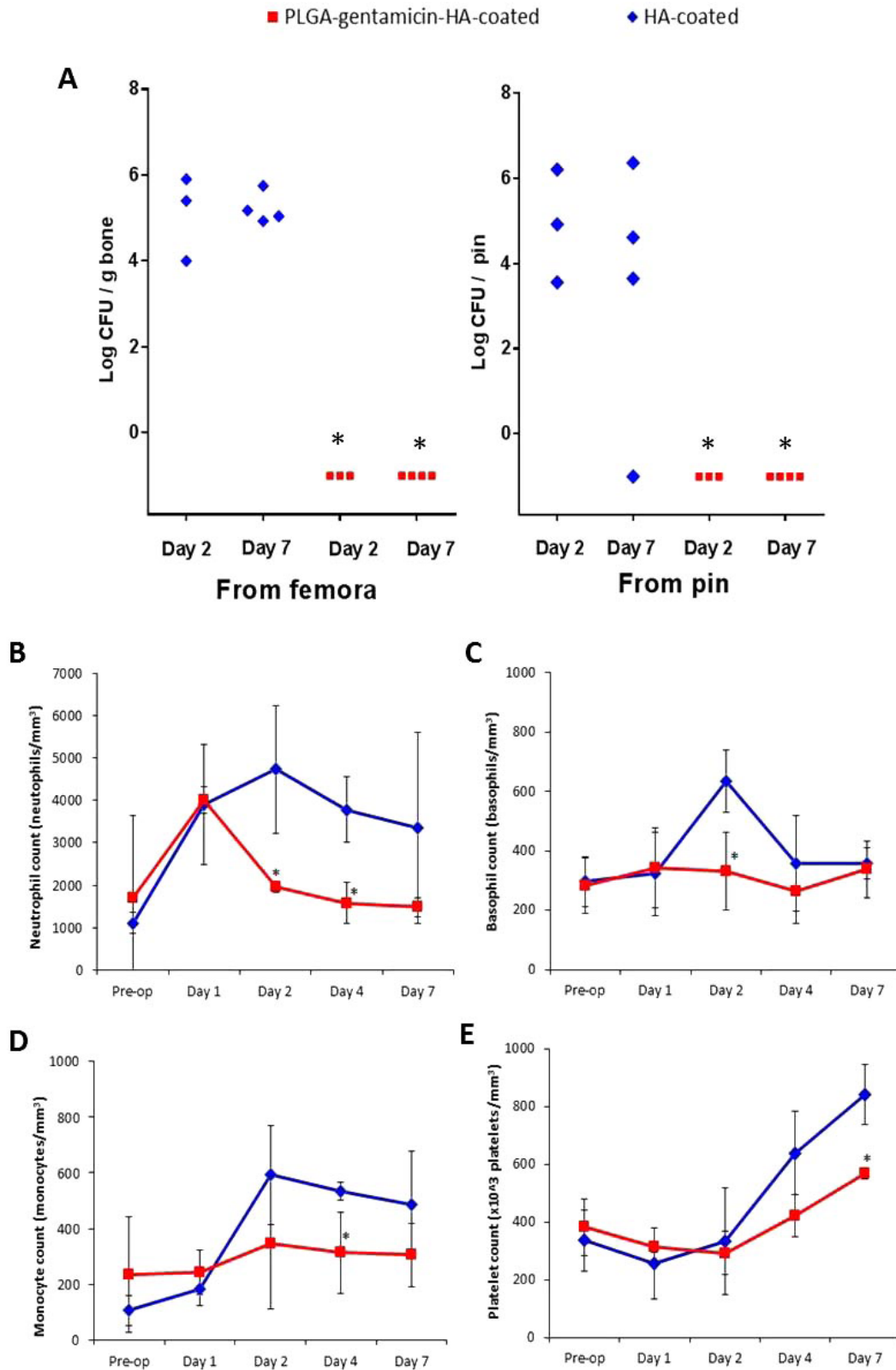
HA- and PLGA-gentamicin-HA-coated pins were inserted in bacterially contaminated medullary canals in rabbits, defining “infection” as staphylococcal presence at the bone-implant interface, *i.e.* colonisation of the pin surface and in surrounding tissue. Accordingly, infection rates were 100 % (7 out of 7 animals) for the rabbits with HA-coated pins and 0 % (0 out of 7 animals) for the rabbits with PLGA-gentamicin-HA-coated pins. High levels of staphylococci were recovered from the femora of rabbits implanted with an HA-coated pin, while all rabbits implanted with a PLGA-gentamicin-HA-coated pin failed to produce bacterial colonies from either the femur or pin samples (Fig. 5a), yielding a statistically significant reduction ( $p = 0.001$ ).

The infected animals did not show any signs of systemic septic disease during the entire observation period. Furthermore, body temperatures recorded during the study were comparable to pre-operative values for both groups. Noteworthy clinical findings (dehiscence or/and erythema) were limited to some animals implanted with an HA-coated pin. Mean body weight loss was slightly larger (not statistically significant) for animals implanted with the HA-coated pin (10 % weight loss) compared to animals with a PLGA-gentamicin-HA-coated pin (5 % weight loss). Haematological analyses in animals implanted with

HA-coated or PLGA-gentamicin-HA-coated pins showed a significant reduction in neutrophil ( $p = 0.003$ ; Fig. 5b), basophil ( $p = 0.035$ ; Fig. 5c), and monocyte ( $p = 0.027$ ; Fig. 5d) counts for the PLGA-gentamicin-HA group after implantation compared to the HA group. Accordingly total leukocyte counts were significantly ( $p = 0.023$ ) higher in animals implanted with HA-coated ( $9.85 \times 10^3/\mu\text{L}$ ) than in animals with PLGA-gentamicin-HA-coated pins ( $6.85 \times 10^3/\mu\text{L}$ ). In addition, platelet counts in animals implanted with PLGA-gentamicin-HA-coated pins showed a significant reduction ( $p = 0.002$ ; Fig. 5e) after implantation compared to the HA group. No significant differences were observed between both groups in eosinophil, lymphocyte, erythrocyte, and absolute reticulocyte counts, and in haemoglobin and haematocrit values.

#### Animal experiments – bone ingrowth model

Noteworthy clinical findings were observed in two animals, but resolved prior to euthanasia. During week 2 and 3, one animal had impaired hind limb function on the right side, which was implanted with PLGA-gentamicin-HA-coated pins, while another animal (implanted with HA-coated pins) had swelling on the left hind limb at week 3. Incidentally, the average amount of PLGA on two pins decreased with 61 % and 96 % after 1 and 4 weeks, as



**Fig. 5.** Infection prevention with PLGA-gentamicin-HA-coated pins in a rabbit model. (A) Number of staphylococci recovered from femora and the pin itself at day 2 and 7 after insertion of HA-coated or PLGA-gentamicin-HA-coated pins in the medullary canal of rabbits. The medullary canal was contaminated with  $1 \times 10^5$  CFU of *S. aureus* ATCC 25923 prior to insertion of the pin. \*denotes statistical significance at  $p < 0.05$  between PLGA-gentamicin-HA-coated and HA-coated. (B) Neutrophil counts (cells/mm<sup>3</sup>) for HA-coated and PLGA-gentamicin-HA-coated pins in the medullary canal of rabbits. The medullary canal was contaminated with  $1 \times 10^5$  CFU/site of *S. aureus* ATCC 25923 prior to insertion of the pin. Haematological evaluations were done pre-operative and 1, 2, 4 and 7 days post-operatively. Data represent mean  $\pm$  standard deviations. \*denotes statistical significance at  $p < 0.05$  between PLGA-gentamicin-HA-coated and HA-coated. (C) As in Fig. 5b, for basophil counts. (D) As in Fig. 5b, for monocyte counts. (E) As in Fig 5b, for platelet counts.

**Table 1.** A comparison of the pre- and post-exposure antibacterial efficacy of gentamicin-HA-coatings in absence and presence of a protective PLGA-overlayer against gentamicin-resistant *S. epidermidis* 7388, obtained prior to and after exposure of samples for 2 h in sterile PBS at 37 °C. Data represent averages over three separate experiments with  $\pm$  indicating the standard deviation.

Pre-exposure efficacy	
Coating	Number of CFU per coupon
HA-coated	28 $\pm$ 3 <sup>a</sup>
PLGA-gentamicin-HA-coated	0 $\pm$ 0 <sup>b</sup>
Post-exposure efficacy	
Gentamicin-HA-coated (no PLGA-overlayer)	21 $\pm$ 14 <sup>c</sup>
PLGA-gentamicin-HA-coated	9 $\pm$ 9 <sup>a,b,c</sup>

<sup>a</sup> significantly different at  $p = 0.026$

<sup>b</sup> no significant difference at  $p = 0.158$

<sup>c</sup> no significant difference at  $p = 0.280$

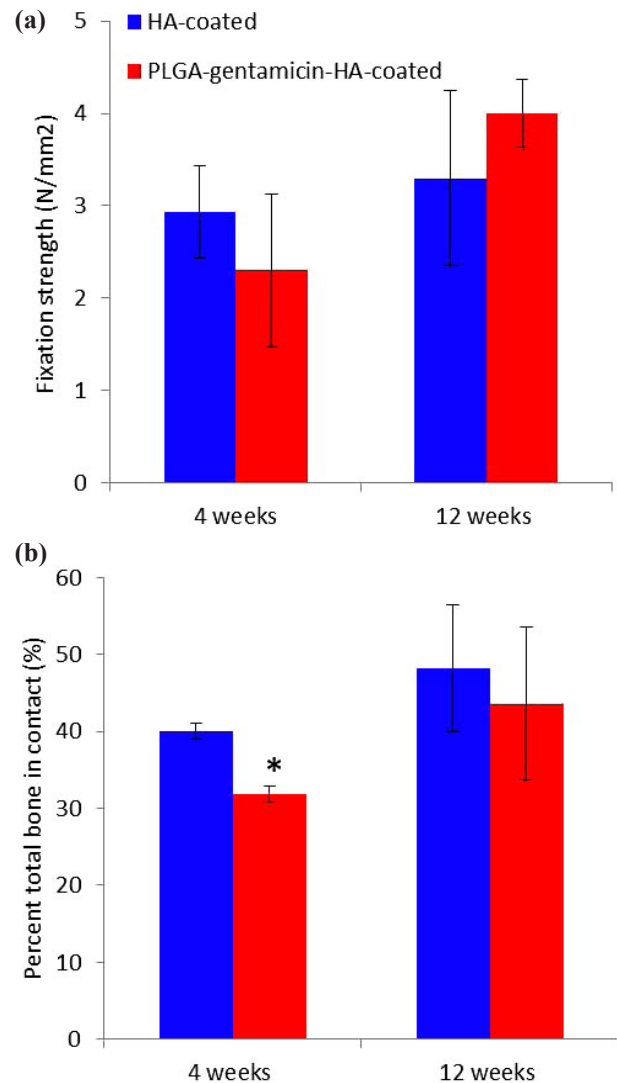
determined by Cordis Corporation/Conor Medsystems, LLC, Fremont, US). No PLGA was detected on explanted pins after 12 weeks.

At 4 weeks, PLGA-gentamicin-HA-coated pins had lower fixation strength than HA-coated pins (Fig. 6a;  $p = 0.184$ ). Nevertheless, the mechanical strength of the interface after 12 weeks was higher ( $p = 0.094$ ) for the PLGA-gentamicin-HA-coated pins (Fig. 6a), ensuring better bone fixation in time.

Image analysis showed that mean overall percentages of bone-implant contact after 4 weeks were 40.0 % for the HA-coated pins and 31.8 % for the PLGA-gentamicin-HA-coated pins (Fig. 6b;  $p = 0.0001$ ). After 12 weeks, HA-coated pins still showed slightly higher percentages of bone-implant contact (Fig. 6b), but this difference did not reach statistical significance ( $p = 0.365$ ).

## Discussion

The peri-prosthetic region in total joint arthroplasties is highly sensitive for bacterial biofilm formation (Campoccia *et al.*, 2006). Fast tissue integration of HA-coatings is considered to reduce the risk of biofilm formation after peri-operative bacterial contamination of the implant and its surrounding (Geesink *et al.*, 1987; Søballe *et al.*, 1990; Dhert *et al.*, 1993). However, Vogely *et al.* showed in a rabbit infection model that HA-coated implants had a higher infection susceptibility compared to uncoated titanium implants (Vogely *et al.*, 2000) which negates the advantage of better bone integration and implant fixation (Oosterbos *et al.*, 2002) and underscores the need for infection prophylaxis in cementless applications. Currently, there are no strategies available for local antibiotic delivery from cementless prostheses. HA is mostly applied to



**Fig. 6.** Bone fixation and osseointegration with PLGA-gentamicin-HA-coated pins in a canine condylar defect model. Data represent mean  $\pm$  standard deviations and \*denotes statistical significance at  $p < 0.05$  between PLGA-gentamicin-HA-coated and HA-coated. (a) Fixation strength (N/mm<sup>2</sup>) for HA-coated and PLGA-gentamicin-HA-coated pins in the distal femoral condyle of beagles. Pull-out testing was done 4 and 12 weeks post-operatively. (b) Osseointegration, expressed by amount of bone-implant contact at week 4 and 12 after insertion of HA-coated or PLGA-gentamicin-HA-coated pins in the distal femoral condyle of beagles. Results are reported as a percentage of the implant surface area.

prosthetic surfaces by plasma spraying and the high processing temperatures associated with plasma spraying make incorporation of antibiotics impossible (Stigter *et al.*, 2002). Incorporation of antibiotics in HA-coatings on titanium prostheses has been established using a “biomimetic” coating technology at 37 °C with deposition of amorphous calcium phosphate on titanium followed by immersion of the prostheses in a supersaturated calcium phosphate solution containing the antibiotics (Stigter *et al.*, 2004). However, the dose of antibiotics that could be



incorporated this way is very limited and about 300 times lower than the gentamicin dose applied in our current PLGA-gentamicin-HA-coating, making this an unsuitable way for effective prophylaxis. Moreover, these coatings suffer from a rapid wash-out by body fluids (Radin *et al.*, 1997): almost 100 % of incorporated gentamicin was released within 1 h (Stigter *et al.*, 2004). The coating investigated here protects the antibiotic sprayed onto the HA-coating through a PLGA-overlayer to yield an antibacterially effective release of gentamicin over clinically relevant time scales of up to 4 days, which is in fact longer than currently achieved using pre-operative antibiotics, usually given as a single dose 60 min before the surgical procedure (Bratzler *et al.*, 2013). Although the feature of burst release within the first hours of exposure of the coating to a fluid may seem disappointing, this high burst release is important in order to kill bacteria introduced during the surgery (Knobben *et al.*, 2006). At the same time, gentamicin release is fully confined to about 7 days which presents a clear advantage over the use of antibiotic-loaded bone cements in cemented applications, demonstrating a relatively low concentration tail-release of antibiotics, thereby limiting the risk of development of antibiotic-resistant strains (Thomes *et al.*, 2002).

One of the critically important considerations when evaluating new antimicrobial technologies is the selection of appropriate and relevant *in vitro* models. The antibacterial efficacy of our PLGA-gentamicin-HA-coating was evaluated in three different *in vitro* systems and in an *in vivo* animal model. While zones of inhibition are well-accepted and effective for comparing antibacterial efficacies of an antibiotic, they are not necessarily representative of the clinical situation. In orthopaedic prostheses, the clinical situation is characterised by both close proximity between the prosthesis and the bone surface, and limited fluid volumes and flow. Therefore, we studied staphylococcal survival in a confined space using a Petrifilm assay, as previously developed and published (Neut *et al.*, 2011). Appropriate and clinically relevant *in vitro* systems and *in vivo* animal models are especially important in the evaluation of coatings in orthopaedics intended to reduce infection rates, because clinical studies in humans to demonstrate statistically significant reductions in infection rates are practically impossible due to the high number of patients required for such studies. In the hypothetical case, where the efficacy of a coating in reducing the incidence of infection has to be demonstrated in a clinical trial, a simple power calculation shows that studies aiming to demonstrate a 50 % reduction – in an existing infection incidence of 2 % at a conservative statistical significance of  $p < 0.05$  – requires the inclusion of around 5,000 patients, who must be followed longitudinally over several years (Darouiche *et al.*, 1999). Such statistical considerations place a heavy burden on the selection of proper *in vitro* and *in vivo* animal experiments, although demonstration of the incidence of infection, as opposed to a reduction in biofilm formation, also requires large animal cohorts. All three *in vitro* systems applied here demonstrate antibacterial efficacy of our PLGA-gentamicin-HA-coatings, most notably so against gentamicin-resistant strains at very high bacterial challenge concentrations of up to  $1 \times 10^7$  bacteria/cm<sup>2</sup>. Importantly,

these *in vitro* results are confirmed in an animal experiment. The animal study had a limited, but sufficient power to yield statistically significant conclusions and support the findings of our *in vitro* methods, as currently the use of large numbers of animals *in vivo* is becoming increasingly troublesome (Richmond, 2002).

Although there are several published studies that investigate the *in vivo* efficacy of antibiotic releasing coatings for the prevention of infection (Giavaresi *et al.*, 2014), relatively fewer studies employ HA-coatings to do so. Alt *et al.* (2006) reported on the efficacy of two different gentamicin-HA-coatings on steel K-wires in the prevention of infection. They showed good results, as infection rates decreased from 88 % for HA-coated K-wires to 0 % for both types of gentamicin-HA-coated K-wires. More recently, Moojen *et al.* (2009) investigated the efficacy of tobramycin-HA coated implants in preventing infection. For a *S. aureus* challenge in a rabbit model 6 % of the tobramycin-HA-coated titanium foam rods were infected after 28 days, compared to 53 % and 67 % for the HA-coated titanium and uncoated titanium rods, respectively. Both studies, however, have no protective PLGA-overlayer, unlike our current PLGA-gentamicin-HA-coating. This thin biodegradable overlayer serves to protect the gentamicin layer during transit and surgical implantation, even when the prosthesis is hammered into a tight bone junction and the coating becomes disrupted, gentamicin release will occur at the implant-bone junction. The protective effects of the PLGA-overlayer are demonstrated in Table 1, showing antibacterial efficacy of the coatings after 2 h of exposure to a fluid (PBS in the present example). The PLGA-overlayer clearly slows down the release of gentamicin due to a longer diffusion trajectory, which is of clinical importance when the prosthesis might become in contact with body fluids prior to final insertion in the bone. The gentamicin-coated tibial nail that has been recently approved for clinical application in Europe and Canada, is equipped with a poly-DL-lactide (PDLLA)-layer in which gentamicin is dispersed (Schmidmaier *et al.*, 2006), but its degradation is considerably slower than of our coating through the use of higher-molecular weight PDLLA (30 kDa *versus* 17 kDa in our coating). This makes the gentamicin tibial nail with PDLLA-layer well suited for its application in trauma, where high antibiotic levels are needed during longer periods than in our application, which is aimed to prevent the development of infection due to peri-operative bacterial contamination of an implant and its surrounding tissue.

The prophylactic gentamicin-HA-coating with a protective PLGA-overlayer developed here has the ability to resist local infection; all animals in the PLGA-gentamicin-HA-coated group were free of infection despite an inoculation dose of  $10^5$  CFU/site of *S. aureus*. Infiltration of granulocytes is one of the first steps of an inflammatory process and generally encompasses neutrophils but also, to a much smaller, extent basophils (Schutte *et al.*, 2009). Yet, significant reductions in both neutrophil and basophil counts in animals implanted with PLGA-gentamicin-HA-coated pins were noticed. As neutrophils are the primary white blood cells that respond

to invading *S. aureus* in order to bind and ingest them (Rigby and DeLeo, 2012), this is an important finding. It is widely recognised that bacteria causing prosthetic hip infections grow in biofilms. Therefore, any antibiotic coating for cementless hip prostheses should be capable of preventing bacterial biofilm formation. Our animal model showed complete bacterial growth inhibition on all PLGA-gentamicin-HA pins implanted, both 2 and 7 days after placement. It seems presumable that “sealing” the gentamicin-HA-coating with a PLGA-overlayer bears the risk of impairing bone ingrowth, which determines the long-term survival of cementless prostheses in total joint replacements. However, 12 weeks after implantation, PLGA-gentamicin-HA-coated pins performed slightly better in a pull-out test than did HA-coated pins and this effect on bone fixation strength of a gentamicin coating has been noticed before (Fassbender *et al.*, 2013). Neither mechanically nor histomorphometrically were there any statistically significant differences, suggesting that our biodegradable PLGA-gentamicin-HA-coating does not impair long-term bone ingrowth.

### Conclusions

The gentamicin-HA-coating with a protective PLGA-overlayer shows good antibacterial efficacy *in vitro*, killing even gentamicin-resistant staphylococcal strains in the confined space of a simulated interfacial gap, as initially existing between a cementless prosthesis and cancellous bone. Moreover, we were able to longitudinally demonstrate the absence of bacterial growth of contaminating staphylococci along the PLGA-gentamicin-HA-coated stems for at least 60 h. These *in vitro* results were all in line with results obtained in a rabbit model, demonstrating infection prophylaxis for at least 7 days, while not impairing bone fixation and osseointegration 12 weeks after implantation in a non-rodent model. Our biodegradable PLGA-gentamicin-HA-coating is a so-called combination device: clinically approved components (HA, a drug and a biodegradable polymer overlayer) are combined into a single platform. Combination devices are offered a unique regulatory review process to facilitate translation to clinical application (Wu and Grainger, 2006), although they still undergo lengthy approval processes that include *in vitro* testing, animal studies and clinical trials. Importantly, and in line with what is needed for the approval of a new drug (Web ref. 4), animal studies have been carried out in rodent-like and non-rodent species. Inclusion of a non-rodent species is required in the safety assessment of pharmaceuticals to limit the uncertainty in the extrapolation process from animal toxicity data to the human situation (Web ref. 5). Based on these corroborative results, our PLGA-gentamicin-HA-coating has a high potential, as an extremely appreciated clinical option for infection prophylaxis in cementless total joint replacement. Regulatory agencies, especially in the USA have been critical toward antibiotic-releasing coatings, fearing that long-term tail release of the antibiotic, as from gentamicin-loaded bone cements, stimulates the development of resistance among bacterial strains.

Importantly, we demonstrate that the current coating effectively overcomes this critique, as antibiotic release is completed after dissolution of the PLGA-overcoat. Moreover, inclusion of other antibiotics in the coating will not affect the release mechanism.

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## Discussion with Reviewers

**Reviewer III:** If the polymer coating allows most fractional drug release within a few hours, why use PLGA at all? Why not use a slowly soluble calcium phosphate coating?

**Authors:** The thin PLGA-overlayer serves as a biodegradable layer to protect the gentamicin layer during transit and surgical implantation and not *a priori* to control antibiotic release. A calcium-phosphate coating was not used because calcium-phosphate is not strong enough during surgical implantation.

**Reviewer III:** What antibiotic dosing requirements are considered for this possible clinical application?

**Authors:** The gentamicin surface concentration was chosen, because its antibacterial efficacy matches with the antibacterial efficacy of gentamicin-loaded bone cement (Neut *et al.*, 2011, text reference).

**Reviewer III:** Is local cell toxicity from rapid drug dose dumping an issue for these closed compartment models?

**Authors:** Our Beagle dog study shows (Figure 6) that the local gentamicin application does not negatively interfere with the long-term healing process, but even shows a small stimulation of the fixation strength. Furthermore, *in vitro* studies with osteosarcoma cells did not show any cytotoxic effects of the coating (Web ref. 3, text reference).

**Reviewer III:** The future of gentamicin releasing technologies may be influenced by increasing gentamicin resistance amongst staphylococci. This is known to be a problem in gentamicin releasing bone cements, as the authors are well aware. How do the authors see this issue in terms of the performance of the described gentamicin releasing technology?

**Authors:** We are indeed aware of the increasing gentamicin resistance in staphylococci causing prosthetic joint infections. We chose gentamicin because of its widespread clinical use, but our coating may as well be used in combination with other antibiotics.

**Reviewer V:** Insertion of an artificial joint is a vigorous act using a hammer forcing the implant in a press-fit fixation in a pre-reamed bone bed. Is the coating strong enough to withstand these forces? What would be the effect if the coating is disrupted in parts? Will the implant than be contaminated only on these specific points or will the burst release of gentamicin kind of cover these areas?

**Authors:** We have not performed any stability testing of the coating. However, since this is a release coating and not a (mono-) molecular coating, hammer blows may disrupt the integrity of the coating, but this will yield an increased burst-release utmost, since the antibiotic is in all cases made locally available.

**Reviewer V:** Around  $\frac{2}{3}$  of blood loss in THR occur after surgery has finished with blood flowing from the medullary canal along the implant to the joint. How much wash out of antibiotics will be possible without dropping the antibiotic concentration below the MIC?

**Authors:** When the released antibiotic falls below the MIC levels *in vivo* cannot be answered in any *in vitro* study. The answer critically depends on the volume used to release the antibiotics in and the turn-over of the fluid in that volume. Gentamicin is highly hydrophilic and spontaneous release will occur when left unprotected on a surface. The protective effect of the PLGA- overlayer in a moist environment clearly follows from the data in Table 1.