Materialia 24 (2022) 101473

Contents lists available at ScienceDirect

Materialia

journal homepage: www.elsevier.com/locate/mtla

Coating of bone implants with silica, hyperbranched polyethyleneimine, and gentamicin prevents development of osteomyelitis in a porcine model

Louise Kruse Jensen[a,*], Henrik Elvang Jensen[a], Sophie Amalie Blirup-Plum[a], Mats Bue[b], Pelle Hanberg[b], Lasse Kvich[c], Bent Aalbæk[a], Yuly López[d], Sara M. Soto[d], Marilina Douloudi[e], Michaela Papageorgiou[e], Eleni Nikoli[e], Michael Arkas[e], Ignacio Gutiérrez-del-Río[f], Sara López-Ibáñez[f], Claudio J. Villar[f], Felipe Lombó[f], Paraskevi Gkomoza[g,h], Ioanna Kitsou[h], Athina Tsetsekou[h], Michalis Vardavoulias[g]

[a] Department of Veterinary and Animal Sciences, University of Copenhagen, Ridebanevej 3, Frederiksberg 1870, Denmark
[b] Department of Orthopaedic Surgery, Aarhus University Hospital, Denmark
[c] Department of Immunology and Microbiology, Costerton Biofilm Center, University of Copenhagen, Denmark
[d] The Barcelona Institute for Global Health (ISGlobal), University of Barcelona, Spain
[e] Institute of Nanoscience Nanotechnology, National Center for Scientific Research- Demokritos, Athens, Greece
[f] Department of Functional Biology, Biotechnology in Nutraceuticals and Bioactive Compounds-BIONUC, University of Oviedo, Spain
[g] PyroGenesis SA, Lavrion, Greece
[h] Laboratory of Metallurgy, School of Mining & Metallurgical Engineering, National Technical University of Athens, Athens, Greece

ARTICLE INFO

Keywords:
Osteomyelitis
Antibacterial coating
Bone implants
Porcine model

ABSTRACT

The use of bone implants and prostheses has contributed to a revolution in modern medicine; however, in the beginning, not much was asked from the implant and prosthetic materials *per se*. Therefore, the next game-changer in orthopedic research will come from new material designs which for instance can aid in prevention of implant-associated bone infections. Here, we describe the development of a new sol-gel coating technique that can deliver an efficient antimicrobial surface coating on orthopedic implants. Gentamicin was stocked in a novel nanocomposite xerogel made from silica and hyperbranched polyethyleneimine. The xerogel was anchored inside a porous surface made by coating of bone implants with titanium microspheres. Thereby, only the small water-soluble gentamicin molecules diffused in an aqueous environment, i.e., just after surgical insertion and leaving behind a titanium scaffold for osseointegration. The novel xerogel coating prevented development of severe *Staphylococcus aureus* induced osteomyelitis in a porcine model, which untreated, replicated the pathology seen in stage 3A on the Cierny–Mader classification system for osteomyelitis in adults.

1. Introduction

Orthopedic implants, such as fracture fixation devices and total joint prostheses, have significantly improved the quality of life for many patients [1]. However, despite their essential benefits, insertion of bone implants exhibits a risk for development of deep biofilm infections like prosthetic joint infection (PJI) and implant-associated osteomyelitis (IAO) [1,2]. Often PJI and IAO show an exogenous pathogenesis due to bacterial contamination at surgery [2,3]. Hence, new antimicrobial technologies aiming to prevent PJI and IAO should focus on creating, at surgery, a sufficient local antimicrobial environment on both the implant surface and within the surrounding bone tissue [4]. Current prophylactic strategies involve preoperative systemic antimicrobial therapy, but increasing evidence suggest local adjuvant therapy in, e.g., fracture care and primary and revision arthroplasty [1]. Therefore, preclinical studies have for decades focused on to develop new and innovative antimicrobial coating solutions for orthopedic implants; however, not many have made it to the clinical market [1,5,6]. Although no prospective randomized control trial has evaluated post-operative infection rates of available gentamicin-coated tibial nails vs. uncoated control implants, it was recently concluded that there is an overall good risk-benefit ratio for patients treated with gentamicin-coated implants [1,5,6]. Therefore, the present study aimed to develop a new coating material for orthopedic implants and prostheses with the ability to encapsulate and release gentamicin immediately after insertion. The new coating is a xerogel made from silica and the dendritic polymer hyperbranched polyethyleneimine (PEI). In brief, a xerogel is a hydrogel that in a sol-gel process dries into a solid composite. The novel gentamicin-loaded xerogel coating showed extraordinarily good results, in comparison to many existing preclinical coating

* Corresponding author.
E-mail address: Louise-k@sund.ku.dk (L.K. Jensen).

https://doi.org/10.1016/j.mtla.2022.101473
Received 14 March 2022; Accepted 6 June 2022
Available online 7 June 2022

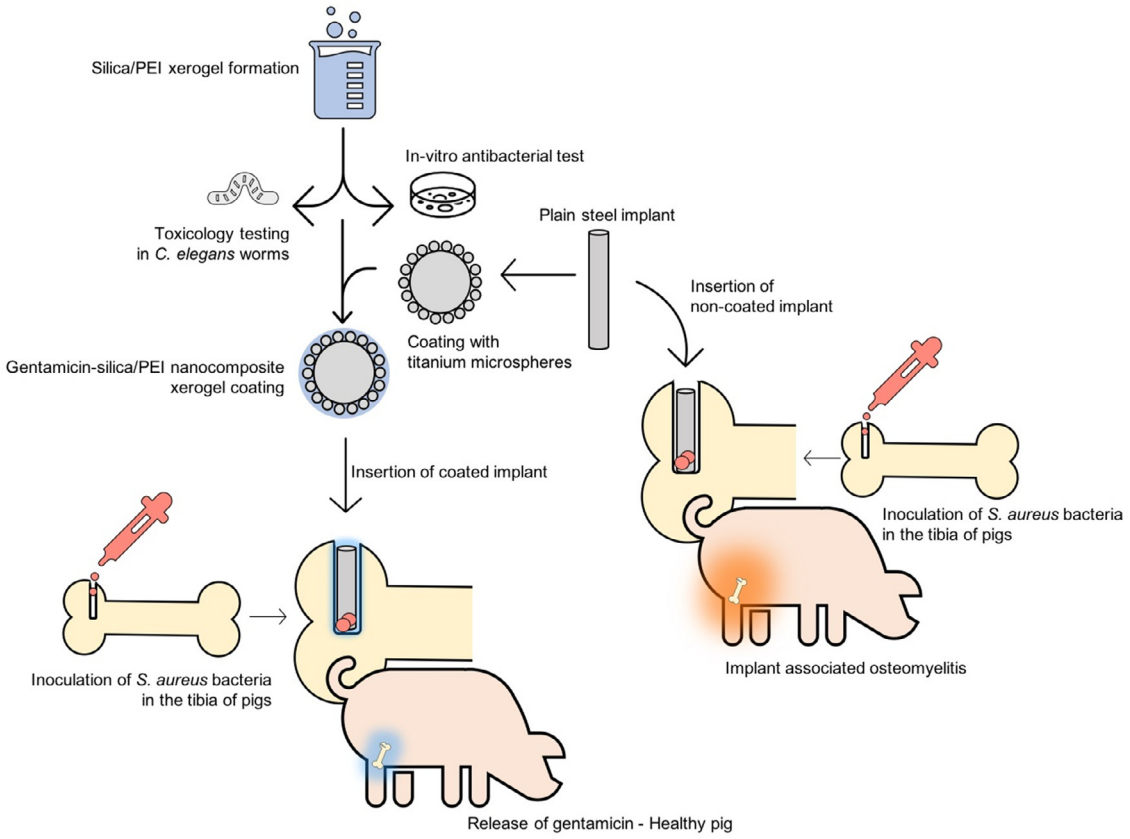

Fig. 1. Study overview. A novel gentamicin+silica/PEI xerogel was produced and tested for toxicity in invertebrtes and antibacterial activity *in-vitro*. Plain steel implants were surface coated with titanium microspheres in order to increase the surface area and create a structure into which the xerogel could be anchored. The gentamicin+silica/PEI xerogel coated and uncoated implants were applied in a clinicaly relevant porcine model of implant associated osteomyelitis.

studies, as it completely prevented IAO (both implant and surrounding tissue were found sterile) in a porcine osteomyelitis model challenged with an extremely high virulent *S. aureus* strain (Fig. 1) [7,8]. Many orthopedic antibacterial-coating studies exists and, therefore, it is relevant to sum up the novelty of present study: The coating is dual as it has an inorganic (titanium microspheres) and organic part (silica and PEI). The desired antibiotic release profile is pathogenesis based, as the new coating was designed to release antibiotics directly after primary insertion in order to eradicate perioperative contamination. It is the first time; an orthopedic antibacterial coating has been evaluated *in-vivo* by using infected pigs and with inclusion of *in-vivo* microdialysis pharmacokinetic.

2. Material and methods

2.1. Development of silica/PEI xerogel and incorporation of gentamicin

Acid hydrolysis of 5 mL tetraethoxysilane solution 1 M (Sigma-Aldrich, Steinheim, Germany) with 25μL HCl 1 M under stirring for 15 min resulted in 1 M orthosilicic acid. Afterwards 2.5 mL of PEI 0.1 mM (BASF, Ludwigshafen, Germany) were added, and the pH adjusted to 7.5 with Trizma (Research Organics, Cleveland, OH, USA). Following 2 h a hydrogel was formed and submitted to drying overnight under vacuum and over phosphorus pentoxide (P_2O_5) (Sigma-Aldrich, Steinheim, Germany) in order to form a silica/PEI xerogel. Subsequently, 100 μl 10% w/w gentamicin sulphate solution (Sigma-Aldrich, Steinheim, Germany) were drop wise added to the silica/PEI xerogel layer and the resulting composite hydrogel was again dried overnight under vacuum and over P_2O_5 in order to re-form the xerogel. An identical second gentamicin addition step followed.

2.2. Antibacterial test of gentamicin-silica/PEI xerogel in-vitro

In order to investigate if the antibacterial activity was maintained after encapsulation of gentamicin into the silica/PEI xerogel, a MIC (Minimum Inhibitory Concentration) assay was carried out following the guidelines of two established organizations and committees, the Clinical and Laboratory standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) [9,10]. A glycerol stock of the *Staphylococcus aureus* to be used *in-vivo* was used. The xerogel were dispersed in 14% MeOH in water (v/v) up to a standardised stock concentration of 4096 mg/L and serial two-fold dilutions ranging from 1024 to 64 mg/L were carried out in a 96-well U-bottom microtiter plate with 50 μL of pre-added sterile Milli-Q water. 50 μL of bacterial suspension were added to all wells up to the desired concentration (5×10^5 CFU/mL). Growth, sterility and solvent controls were included. MIC values were defined as the lowest concentration that inhibited visible growth. The experiment was carried out by triplicate.

2.3. Toxicity assessment of gentamicin-silica/PEI xerogel using Caenorhabditis elegans model

Caenorhabditis elegans worms were grown in Nematode Growth Media (NGM). *Escherichia coli* (strain OP50) was grown overnight in Luria Bertani culture. The number of worms was adjusted to 15–20 worms/50μL in a final volume of 5.5 mL using M9 buffer. A total of 600 μL of *E. coli* was added to the worm solution. A black flat bottom 96-well plate was filled with 50 μL of worm solution+ *E. coli* (about 15 worms/well). The last well contained the positive growth control.

Table 1
Overview of *in-vivo* study groups.

Group	Inoculum	Implant	*n*	Time of euthanasia
In-vivo study of efficacy				
A*	saline	Plain steel	10	5 days after inoculation
B*	*S. aureus*10^4CFU	Plain steel	10	5 days after inoculation
C	*S. aureus*10^4CFU	Titanium microspheres+ Gentamicin-silica/PEI xerogel	10	5 days after inoculation
D	*S. aureus*10^4CFU	Titanium microspheres+ Silica/PEI xerogel	3	5 days after inoculation
In-vivo pharmacokinetic study of gentamicin release				
E	*S. aureus*10^4CFU	Titanium microspheres+ Gentamicin-silica/PEI xerogel	2	24 h after inoculation

* 6 animals in group A and 1 animal in group B were historic controls (3R compliance). Group D was included in order to observe if there were signs of positive or negative effects of the coting in itself, i.e., without antibiotics.

A death-control was included using acetonitrile as toxic reagent. A flat bottom 96-well plate was filled with 50 μL of M9 buffer. Afterwards, 50 μL of gentamicin-silica/PEI xerogel solution (32 mg/L) were added to the first well and 2x serial dilutions were made. 50 μL of each well of the xerogel plate were transferred to the correspondent well of the worm plate and incubated at 25 ºC. Using a stereomicroscope worm activity/movement was registered by visual observation following 1, 2, 24 and 48 h of inoculation, respectively. In addition, the self-fertilization behavior was also registered following 48 h.

2.4. *Coating of implants with gentamicin-silica/PEI xerogel for* in-vivo *studies*

Implants for the *in-vivo* study were made of stainless steel (K-wire, 2 × 15 mm). A porous layer of metallic titanium microspheres was deposited on the implant surface using Cold High Velocity Oxygen Fuel (Cold HVOF) thermal spray. The aim was to obtain optimal surface roughness and porosity characteristics, i.e., a larger surface area before application of gentamicin-silica/PEI xerogel. The titanium microsphere layer was fabricated with a METCO DJ 2700 HVOF gun. The delivered spherical titanium particles were 15–45 microns sprayed with a pressure of 30 bar at 600 °C. Nitrogen was used as spraying gas. The spray distance was 40 mm and the spraying velocity 250 m/se. The porosity was evaluated with scanning electron microscopy (SEM) (EDS, JEOL, JSM-6390, Tokyo, Japan). Titanium microspheres covered implants were immersed into the silica/PEI hydrogel forming solution as described in the beginning. After drying, the procedure was repeated once in order to add a second xerogel layer. Gentamicin was added dropwise in two single steps as described in the beginning. SEM and Fourier-transform infrared spectroscopy (FTIR) using a Nicolet Magna-IR Spectrometer 550 (Thermo Nicolet Co., Madison, WI, USA) were used to verify the morphology and molecular interactions of coated implants. Finally, thermogravimetry experiments were used to assess the gentamicin loading efficiency of the wetting procedure using a Setaram SETSYS Evolution 17 instrument 8, (SETARAM, Caluire, France) with a 5 °C/min heating rate under oxygenic atmosphere.

2.5. In-vivo *study of efficacy of coated implants*

In total, 35 female specific pathogens free (SPF) pigs were included in the study. The body weight of the pig was 25–35 kilogram. The pigs were housed in pens with 2–4 animals in each. They were feed a normal standard pig diet and had free access to tap water. The pigs were inspected by authorized personal several times a day during the study period. The Danish Animal Experiments Inspectorate approved the experimental protocol (license No. 2013/15-2934-00946). Table 1 gives, an overview of the animals used. The pigs were randomized into the study groups A-D.

2.5.1. *Inoculation procedure*

Anaesthetised pigs were placed in right lateral recumbency exposing the medial side of the right tibia. An incision through skin, subcutis and periosteum was made over the drilling point of the implant cavity located parallel and distal (10 mm) to the proximal tibial growth plate. A 20 mm deep implant cavity was drilled with a 4 mm K-wire. The inoculum (or sterile saline) was injected into the implant cavity followed by insertion of a small steel implant with or without coating (2 × 15 mm), see Table 1. Once the implant was inserted, the periosteum, subcutaneous tissue and skin were sutured. All animals received daily analgesic treatment with non-steroidal anti-inflammatory drugs (NSAIDs) throughout the study period. Impaired ability to stand, anorexia and systemic signs of sepsis, e.g., depressed respiration and fever, were set as human endpoints. Animals were euthanised by an intravenous overdose of pentobarbital. All drugs and doses used for anesthesia, analgesia and euthanasia has previously been reported in detail [8]. No systemic antibiotics was used at all.

2.5.2. *Inoculum*

Group B-D animals were inoculated with *S. aureus* S54F9 10^4 CFU in 10 μL sterile saline. The dose of 10^4 CFU/kg BW was estimated to be among the lowest infective dose, based on a former dose-response study using the same model and *S. aureus* strain [7,8]. The *S. aureus* strain was originally isolated from a porcine lung abscess and has previously been used to model human osteomyelitis, endocarditis and sepsis in pigs [11]. The strain has been whole genome sequenced and belong to SPA-type t1333 and multi-locus sequence type (MLST) ST433 [9]. Recently, it was demonstrated that the strain produces biofilm [12]. The strain is highly virulent and has genes encoding several toxins, including phage-associated enterotoxin, exotoxins and superantigen [11].

2.5.3. *Computed tomography*

Following euthanasia, the right hindlimb was scanned with a single slide computed tomography (CT) scanner (Siemens Somatom Emotion, Erlangen, Germany). The tibia was scanned in the craniocaudal direction with a slide thickness of 2 mm (kV = 130 and mAs = 55). The scans were reconstructed using a standard soft tissue algorithm (B80s). CT volumetry (cm^3) of the implant cavity and associated osteomyelitis, if present, was estimated using the software system Osirix MD (Pixmeo, Geneva, Switzerland) [7,13]. The evaluation of CT scans was blinded.

2.5.4. *Macroscopic pathology*

Abdomen and thorax were cut open and all organs were inspected and palpated. A small sample of the lung, kidney and liver were collected for histology. The surgical wound was inspected and cut open. The subcutaneous tissue was inspected and dissected in order to expose the periosteal sutures. The implant was removed, and the tibial bone was sagittal sectioned through the implant cavity. During necropsy of the right tibial bone the following findings were registered; (1) wound infection, (2) subcutaneous inflammation (oedema, hemorrhage, and swelling), (3) subcutaneous abscess formation, (4) exudate in implant cavity, and (5) signs of bone infection (including intra osseous pus, sequestrum formation, and osteolysis) [14]. Registration with a “yes” resulted in one point and, thereby, a total of 5 points could be given in macroscopic pathology score.

2.5.5. Microbiology

During necropsy, a small piece of lung tissue was collected for microbiological examination in order to disclose systemic spread of the inoculated bacteria. In relation to the operated bone, a swab was taken from the subcutaneous tissue and the implant cavity, respectively. Swabs were inoculated on blood agar supplied with 5% sterile bovine blood and incubated at 37 °C for 24 h. Isolates were identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Vitek MS RUO, bioMérieux, France). SPA-typing was used to disclose the genetic identity of isolated *S. aureus* strains [15]. The bone implants were analyzed for biofilm attachment using sonication as previously described [16]. The detection limit for sonication was 50 CFU/mL. Evaluation of swabs and sonication results were blinded.

2.5.6. Histology and immunohistochemistry

The right tibial bone was placed in 10% neutral buffered formalin for 1 week followed by 4 weeks of decalcification in formic acid (22%). Following decalcification, the bone was cut into representative pieces containing the implant cavity and processed through graded concentrations of alcohol and embedded in paraffin wax. Tissue sections (4–5 μm) were stained with haematoxylin and eosin (HE). The peri-implanted pathological bone area (PIBA) was described and measured. PIBA was defined as the maximal perpendicular distance from the border of the implant cavity until normal pattern of trabecular bone and bone marrow appeared. The number of neutrophil granulocytes (NGs) inside PIBA was counted by the method developed by Morawietz et al. [17]. Briefly, potential 'hot spots' rich in NG were identified at low magnification. These areas were then evaluated under high power (400 x magnification) and all cells clearly identifiable as NG were counted. In each high-power field (HPF), a maximum of 10 NG was counted. In this way 10 HPFs were examined, resulting in a maximum count per pig of 100 NG [17]. Immunohistochemistry (IHC) with a primary antibody against *S. aureus* (Monoclonal, mouse, Clone 704, Abcam, ab37644, Cambridge, UK) was performed in order to visualize bacteria inside PIBA. The protocol has previously been described [18]. Positive bacteria were red, and the number of positive colonies were registered, although a maximum of 100 colonies were counted. All PIBA measurements, NG and bacterial counts were obtained blinded.

2.6. *Pharmacokinetic* in-vivo *study of gentamicin release from coated implants*

Two pigs (Group E) were anaesthetized [19] and an implant cavity was drilled as previously described. Microdialysis (MD) catheters (membrane length 20 mm, molecular cut-of 20 kDa) were placed in the adjacent subcutaneous tissue, within the implant cavity, and within the bone tissue 10 mm next to the implant cavity. All MD catheters were connected to separate 107 MD precisions pumps producing flowrates of 1 μL/min. MD pumps and catheters were obtained from M Dialysis AB, Stockholm, Sweden. Briefly, microdialysis is a minimally invasive catheter-based technique that allows for continuous sampling of solutes in the interstitial space (extracellular fluid) [20]. Calibration of each separate catheter is imperative if total tissue concentrations are to be determined. In the present study, the MD catheters were calibrated before introduction of the gentamicin-silica/PEI xerogel coated implant with 0.9% NaCl containing gentamicin at a concentration of 2.5 μg/mL. After a 30 min tissue equilibration period, all catheters were individually calibrated by collecting a 60-min sample. Following calibration, the perfusate was changed to blank isotonic saline, and a 180-min washout period was allowed for. Prior to sampling start, the implant cavity was inoculated with *S. aureus* S54F9 (10^4 CFU in 10 μL sterile saline) and a gentamicin-silica/PEI xerogels coated implant was inserted. Afterwards, dialysates from the MD catheters and venous blood samples were collected regularly for 24 h. The animals were euthanized after the last sample by administration of intravenous pentobarbital. The free gentamicin concentration in plasma and dialysates was measured by homogenous enzyme immunoassay technique and liquid chromatography-tandem mass spectrometry, respectively, as previously described [19]. A swab was taken for microbiological examination (see *Microbiology* for procedure) from the implant cavity after 12 h and just before euthanasia. Furthermore, the implants were sampled for sonication (see *Microbiology* for procedure).

2.7. *Statistic*

Primary endpoint; gentamicin-silica/PEI coated implants + bacterial inoculation should result in the same quantity of pathology as inoculation with saline only. A power analysis showed that a sample size of 9 had an 80% power to detect an effect size of 2 mm PIBA assuming a 5% significance level and a one-sided test. A one-way ANOVA analysis followed by post hoc comparison was used to look for differences in macroscopic pathology score, CT bone lesion volume, sonication results, NG count, and PIBA size between Groups A, B and C. Statistical significance was assigned to differences having *P-value* ≤ 0.05. All calculations were performed using Prism version 7 (Graphpad Software Inc., San Diego, CA, USA). All analysis was performed blinded, i.e., the animal ID could not be identified during either preparation or evaluation.

3. Results

3.1. *Development of xerogel and incorporation of gentamicin*

The produced silica/PEI hydrogel consisted of a hydrogen bond network between PEI and orthosilicic acid with subsequent polymerization of silanol groups to siloxane bridges in neutral pH (Fig. 2).

3.2. *Antimicrobial test* in-vitro

Gentamicin-silica/PEI xerogel inhibited the growth of *S. aureus* with an estimated MIC value of 128 μg/mL. All controls performed as expected.

3.3. *Toxicity assessment using* Caenorhabditis elegans *model*

The gentamicin-silica/PEI xerogel showed no toxicological or lethal effects to any of the examined time points, i.e., all worms had normal behavior. Furthermore, the self-fertilization behavior was not affected. Positive and death controls performed as expected.

3.4. *Coating of bone implants for* in-vivo *study*

Cold HVOF coating with titanium microspheres increased the surface area and porosity of the bone implants (Fig. 3). FTIR revealed that titanium microsphere covered bone implants successfully were coated by silica/PEI xerogel and that gentamicin was incorporated (Fig. 4). Thermogravimetry was used to assess the gentamicin loading efficiency (Fig. 4.) An initial weight loss was observed up to 150 °C due to intrinsic moisture, i.e., water molecules loosely hydrogen bonded to PEI and evaporation of silanol groups (7.4% of the initial silica/PEI xerogel, 5.1% after the first and 4.25% after the second gentamicin wetting step.) At higher temperatures, silica/PEI xerogel lost 17.85% of its initial weight up to 700 °C. Thus, the rest of the decomposition percentages (33.9% and 37.8%) were due to gentamicin, indicating an 18.5% incorporation of the drug after the first treatment and a 24.3% in total. A simple comparison of the weights of uncoated and coated implants reveals that each pin was loaded with about 50 μg gentamicin.

3.5. In-vivo *study of efficacy of coated implants*

All animals eat, drink and demonstrated normal behavior during the 5-day study period. One Group C animal was excluded from the study due to inoculation procedure failure, i.e., non-correct drilled implant cavity.

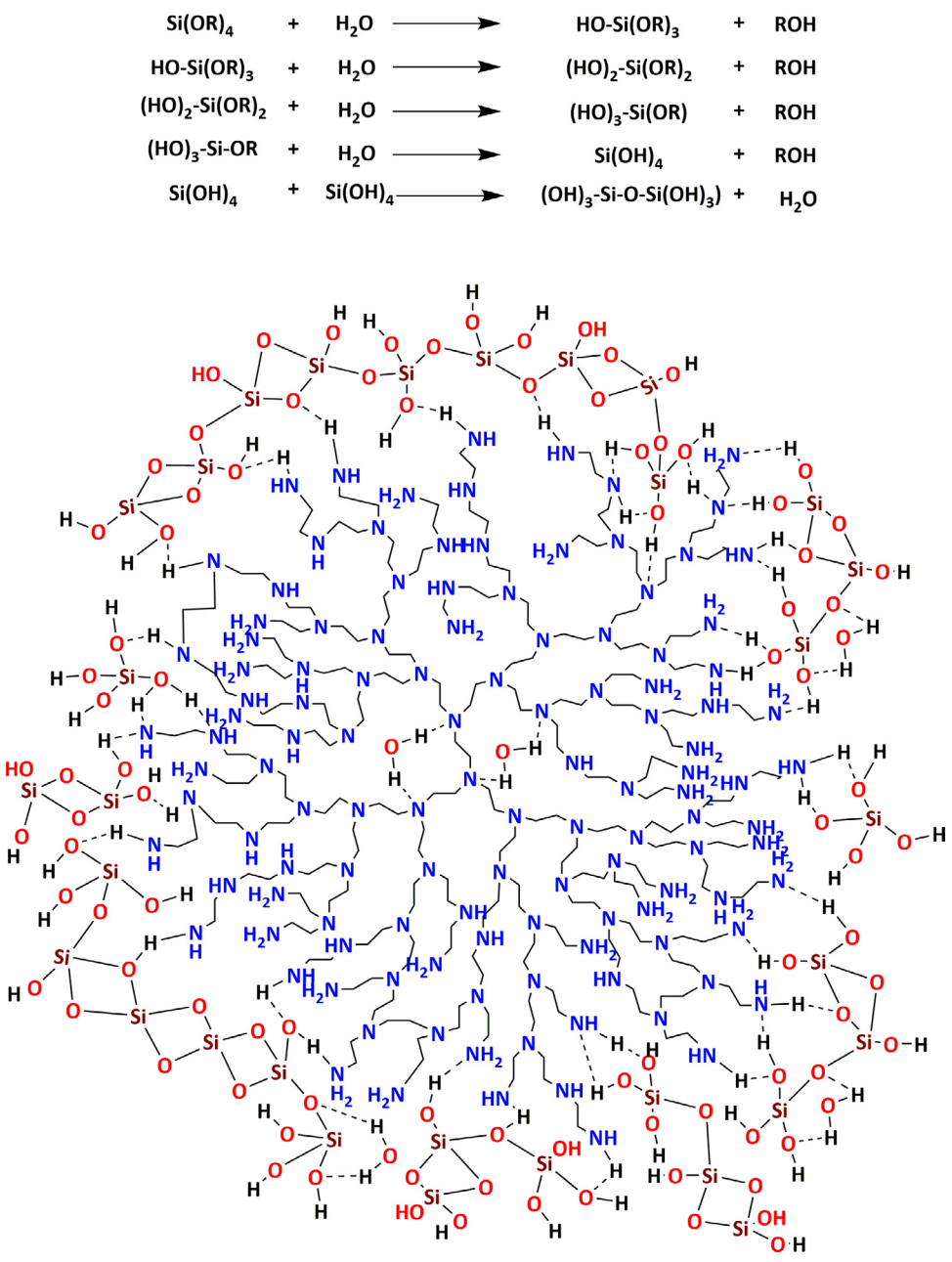

Fig. 2. Chemical reactions for formation of the silica/PEI hydrogel and structure depicting the hydrogen bonds network before drying of the hydrogel into xerogel.

3.5.1. Computer tomography

The 3D estimated volumes of implant cavity plus bone lesions were significantly (*p-value* = 0.016) increased in Group B in comparison to Group A. (Figs. 5 and 6).

3.5.2. Macroscopic pathology

Lesions were not observed in any abdominal or thoracal organs. The common macroscopic finding in tibial bones with plain steel implants and saline inoculation (Group A) was minor subcutaneous inflammation due to surgery *per se*. In contrast, all animals with plain steel implants and *S. aureus* inoculation (Group B) demonstrated several confirmatory diagnostic criteria for IAO and PJI in humans like wound swelling, soft tissue abscesses, and pus surrounding the implant [3,20]. Furthermore, osteolysis and necrosis were seen in the osseous tissue around the implant cavity. All animals with gentamicin-silica/PEI xerogel coated implants were comparable to Group A. Coating with silica/PEI xerogel alone (Group D) resulted in severe pathology as seen in Group B. The macroscopic pathology score of bone lesions was significantly reduced (*p-value* > 0.000001) between Group B and C, but not between Group A and C (Fig. 5).

3.6. Microbiology

Groups C and A animals were *S. aureus* free in both subcutis and implant cavity. In contrast, all group B animals had *S. aureus* bacteria in subcutis and within the implant cavity. The *S. aureus* isolates were found identic to the strain used for inoculation (SPA-type t1333). Sonication showed that one control animal (Group A) had a few bacteria on the implant (the implant cavity was found positive for a porcine *Streptococcus)*. For the gentamicin-silica/PEI xerogel coated group (Group C) bacteria were not identified on any implants. Thus, CFU load on implants were significantly (*p-value* > 0.000001) reduced between Group B and C, but not between Group A and C (Fig. 7). All lung samples were found sterile.

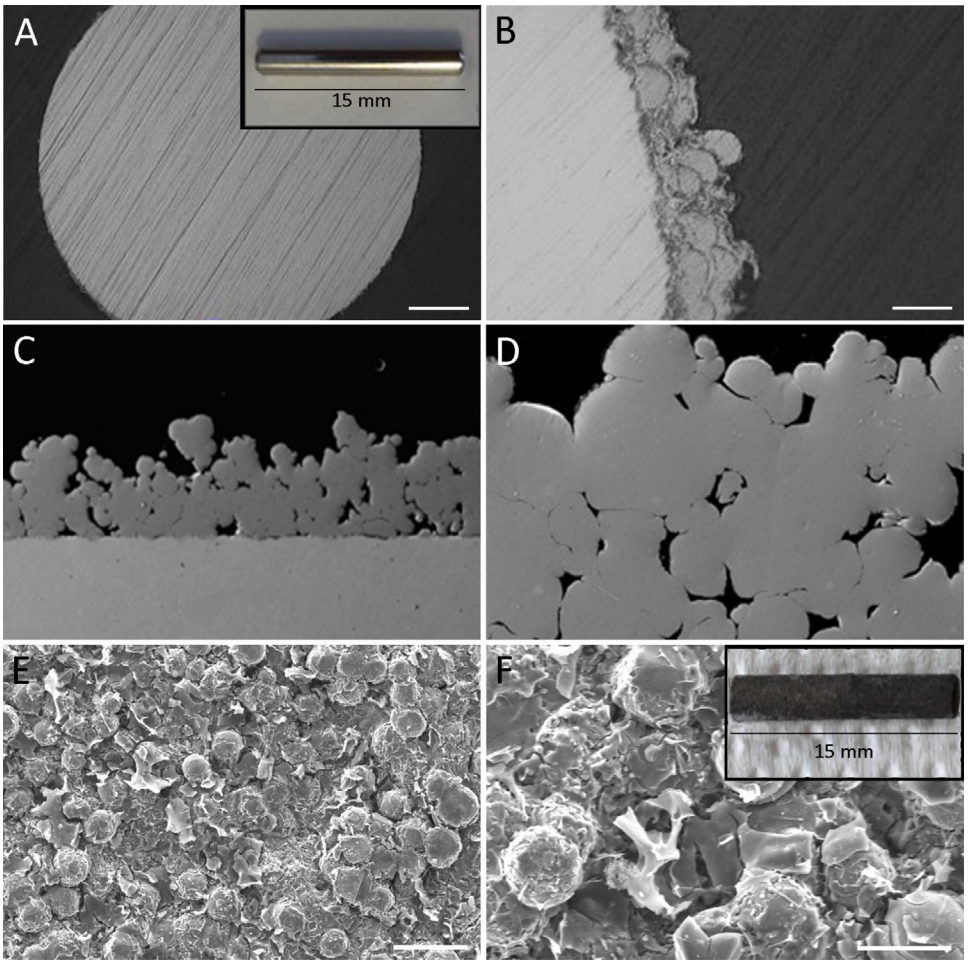

Fig. 3. A: Cross-sectional scanning electron microscopy (SEM) of an uncoated implant. Bar = 100 μm. Insert shows the uncoated implant. B: Cross-sectional microstructure of an implant coated with titanium microspheres using cold high velocity oxygen fuel (HVOF) thermal spray. Unmolten larger titanium powder particles are observable in layers, while smaller powder particles have melted and formed splats acting as binding material throughout the coating. SEM, Bar = 200 μm. C+D: Cross-sectional micrographs of titanium microsphere coating showing the increased porosity and surface area. E+F: SEM of implant coated with both titanium microspheres and silica/PEI xerogel. The xerogel follows and covers the profile of the microspheres. Bar = 100 and 50 μm, respectively. Insert in F shows a coated implant ready for *in-vivo* application.

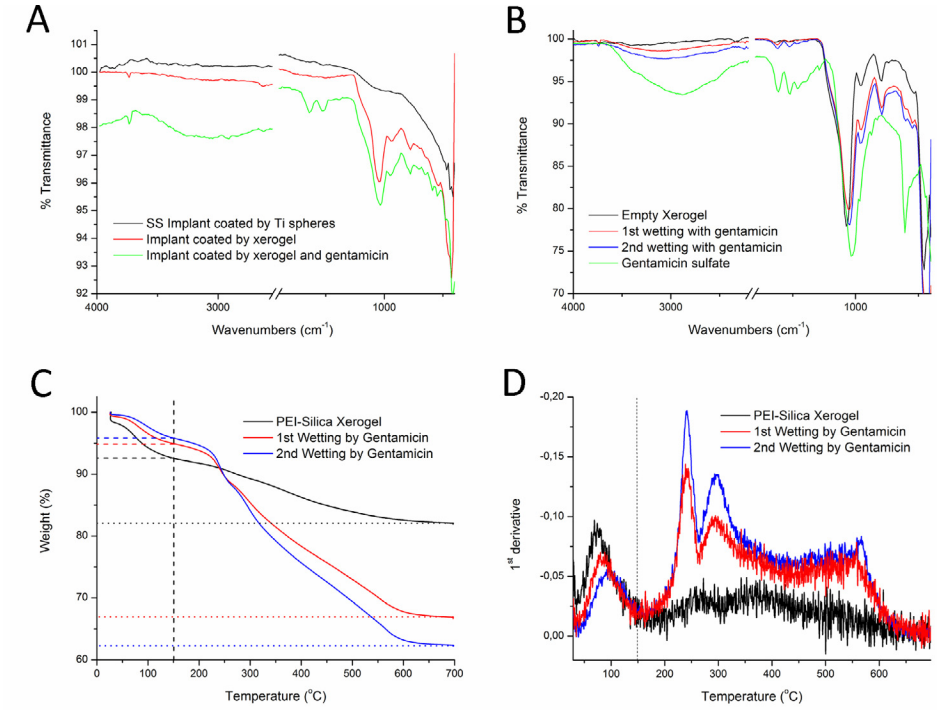

Fig. 4. Analysis of gentamicin-silica/PEI xerogel coating. A: Fourier-transform infrared (FTIR) spectra depicting the various processing stages of the stainless-steel (SS) bone implants; Treatment with titanium microspheres (black), two stages of coating by silica/PEI xerogels (red), two stages of wetting by gentamicin sulfate solution (green). B: FTIR spectra of pure silica/PEI xerogel (black), gentamicin sulfate (green) and xerogel after the first (red) and second (blue) treatment with gentamicin sulphate solution. C+D: Thermogravimetric analysis profile of silica/PEI xerogel before and after 2 gentamicin wetting steps (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.).

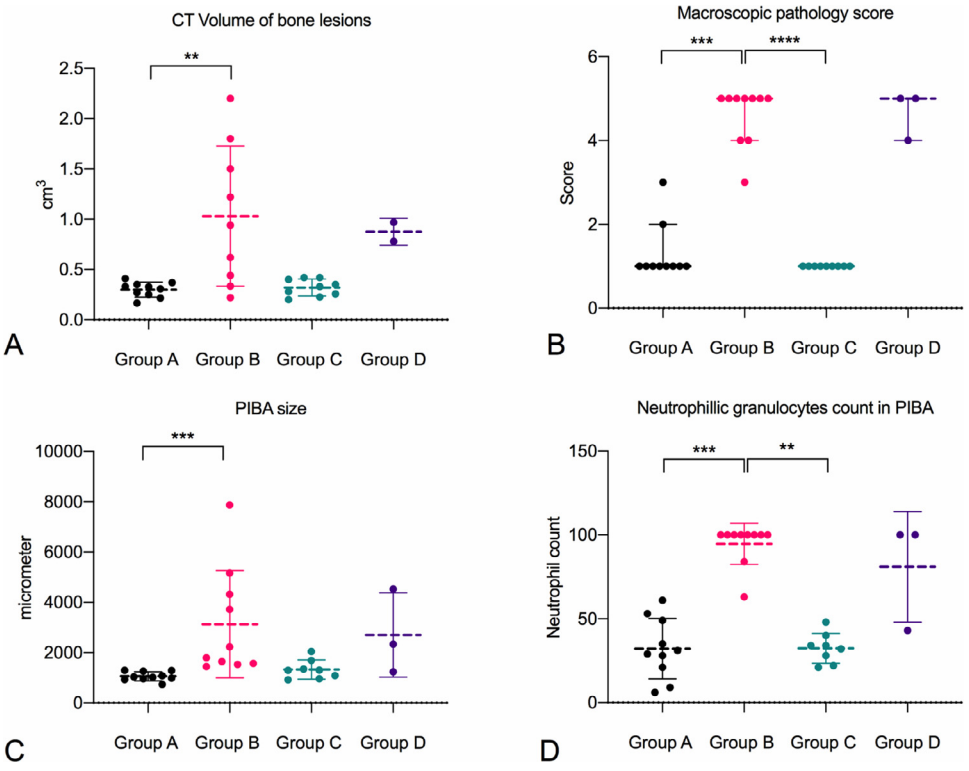

Fig. 5. Pathology results in a porcine model of implant associated osteomyelitis euthanised 5 days after inoculation. Group A; Pigs inoculated with saline and plain implants, Group B; Pigs inoculated with *S. aureus* and plain implants, Group C; Pigs inoculated with *S. aureus* and gentamicin- silica/PEI xerogel coated implants, Group D; Pigs inoculated with *S. aureus* and silica/PEI xerogel coated implants. A, C, D; Mean ± SD. B; Median with 95 confidence intervals. PIBA; Peri-implant pathological bone area. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.7. Histology and immunohistochemistry

In saline inoculated animals (Group A) and animals inoculated with bacteria and gentamicin-silica/PEI xerogel coated implants (Group C), the surgical inoculation procedure *per se* resulted in thermal bone necrosis, debris, and a small amount of inflammatory cellular infiltration, which resulted in PIBA values of approximately 1.3 mm (Fig. 5). A quite different PIBA pattern was observed in the infected animals of Group B and D. In these animals, a layer of cells that included fibroblasts, neutrophils (NG) (sometimes arranged as micro-abscesses), macrophages, giant cells, and bone debris was observed around the implant cavity (Fig. 8). A significant reduction (*p-value* > 0.000001) was seen in NG count between Group B and C, but not between Group A and C (Fig. 5). Immunohistochemistry visualized bacterial localization within PIBA in 9 out of 10 Group B animals and in 7 pigs, more than 100 colonies (maximum count) were reported (Figs. 7 and 8). Bacteria were not observed in the peri-implant tissue of any Group A or C animals. Histologically, both lung, liver, kidney and spleen within all animals were found free for inflammation and lesions like sublethal cellular damage and necrosis.

3.8. Pharmacokinetic study – release behavior of gentamicin

Gentamicin could only be detected within the implant cavity. The gentamicin content peaked immediately after insertion (Fig. 9). The gentamicin concentration within the implant cavity was above the MIC value for gentamicin against *S. aureus* for several hours. Microbiological swab analyses taken 12 h into the sampling procedure were found sterile. Following euthanasia, after the 24 h sampling period, the implants were also found sterile by sonication.

4. Discussion

A new gentamicin-silica/PEI xerogel coating of bone implants successfully prevented infection in a well-characterized porcine IAO model [7]. The porcine IAO model corresponded to stage 3A on the Cierny–Mader classification system for osteomyelitis in adults [21]. This stage describes localised osteomyelitis, usually involving cortical and medullary bone in a patient without systemic or local compromising factors [21,22]. Previously, and in several studies, the porcine IAO model has been reported to be 100% effective, i.e., all animals inoculated with bacteria develop osteomyelitis [7]. This is central and emphasizes the reliability of the present results as the results of prophylactic antimicrobial interventions only can be completely trusted, if the applied animal model has an absolute infection rate [23]. The absence of osteomyelitis in pigs with coated implants was due to a complete bacterial elimination and thus prevention of the cardinal step of biofilm formation, bacterial adherence [24]. Complete eradication of infection has previously also been shown in other preclinical coating studies by Diefenbeck et al. [25] and Metsemarkers et al. [26]. However, most preclinical studies aiming to investigate the prophylactic effect of antimicrobial surface coatings for orthopedic devices, have only been able to demonstrate a reduction in the bacterial load [24,27–32]. It is difficult to evaluate the effect of a bacterial reduction in terms of clinical significance. An intervention which almost eliminates the bacterial load may have an impact, but a re-established biofilm infection is likely [23]. A recent review of animal models of bone infection by Moriarty et al. suggested that clinically relevant efficacy of anti-infective interventions should be assessed in terms of complete eradication of infection [23,33].

Polymers possessing cavities with groups capable of intermolecular interactions have attracted scientific interest, as they have emerged in the frontier for local controlled drug release [34]. One of the most promising categories is dendritic polymers due to their tree-like structure, which derive from radial instead of conventional linear polymerization [35]. The main classification of dendritic polymers is the symmetric monodisperse dendrimers and the non-symmetric analogues, the hyperbranched dendritic polymers. PEI, the basis for the developed silica/PEI xerogel coating, is classified as a cationic hyperbranched dendritic polymer. In the present study, a hydrogel solution based on PEI was transformed into a solid xerogel in a sol-gel process. Normally, the drying (or aging process) from hydrogel to xerogel is slow, and if high temperatures are used to speed up the process, internal stresses are exercised during shrinking [36]. For this reason, drying under vacuum and

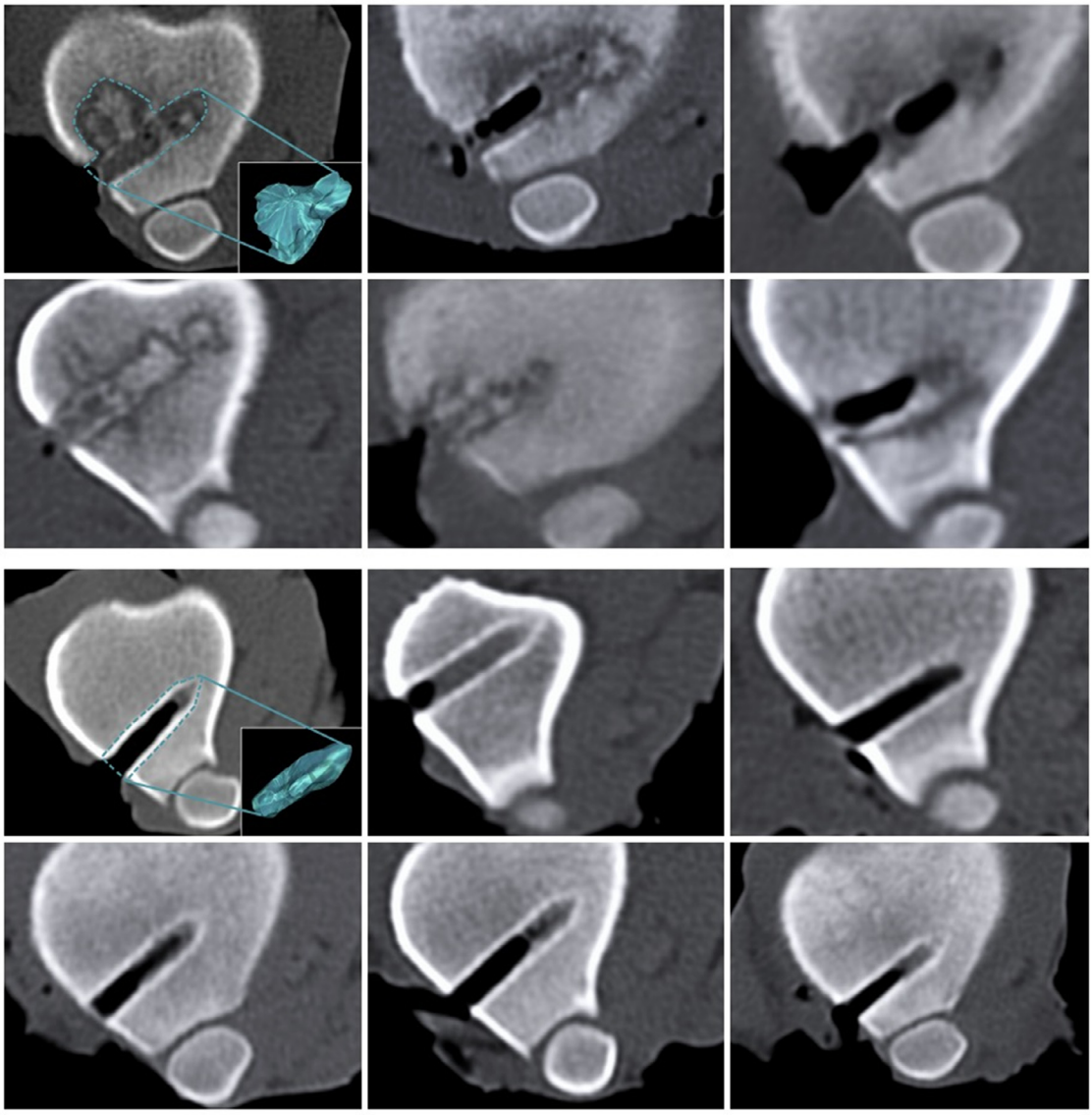

Fig. 6. CT scans in a porcine model of implant associated osteomyelitis euthanized 5 days after inoculation. Upper panel; inoculation with *S. aureus* and no coating of the implant in 6 pigs. The contour of the implant cavity is lost due to surrounding osteolysis and trabecular sequesters are seen. Lower panel; inoculation with *S. aureus* and gentamicin-silica/PEI coated implants in 6 pigs. The contour of the implant cavity is clear and sclerotic with no signs of surrounding osteolysis. The inserts show an example of the estimated 3D volume of the lesions.

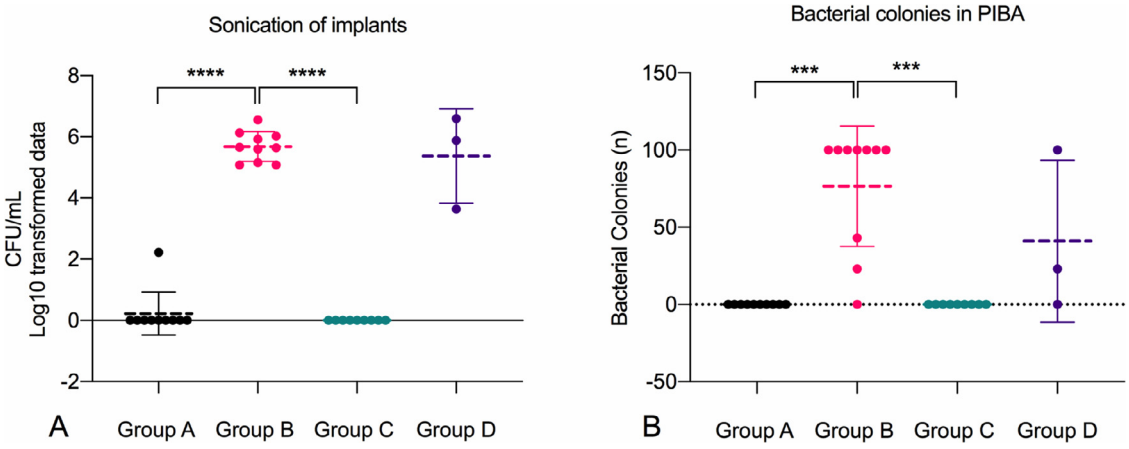

Fig. 7. Microbiology results in a porcine model of implant associated osteomyelitis. A: Identification of bacteria on implants. B: Identification of *S. aureus* within peri-implant pathological bone area (PIBA). Group A; saline and plain implants, Group B; *S. aureus* and plain implants, Group C; *S. aureus* and gentamicin- silica/PEI xerogel coated implants, Group D; *S. aureus* and silica/PEI xerogel coated implants. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

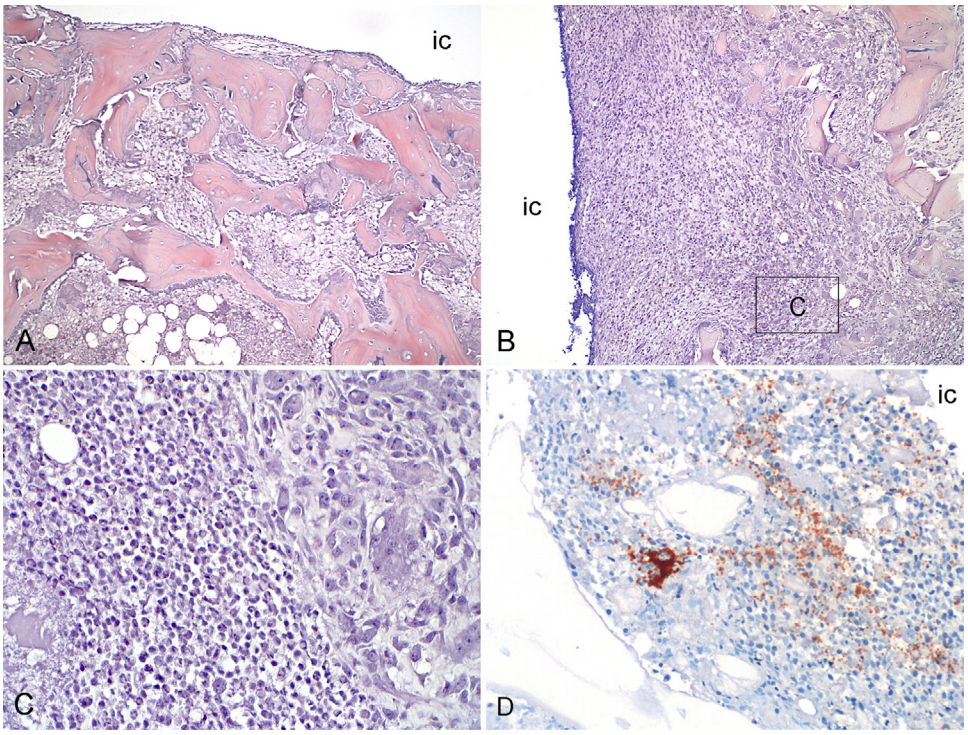

Fig. 8. Histology in a porcine model of implant associated osteomyelitis euthanized 5 days after inoculation with *S. aureus*. The pictures represent the bone tissue surrounding the implant which was located in the implant cavity (ic). A: Pig with a gentamicin silica/PEI xerogel coated implant. Next to the implant cavity minor fibroplasia (only a few cell leayer) is present. No signs of infection is seen. HE x 40. B+C+D: From *S. aureus* inoculated pig with a non-coated implant. B+C: The peri-implant tissue demonstrates a massive inflammatory response dominated by fibroplasia, neutrophils and macrophages. HE x 100 and 200, respectively. D: Bacteria (in red) are seen inside the peri-implant bone tissue. IHC towards *S. aureus* x 150 (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.).

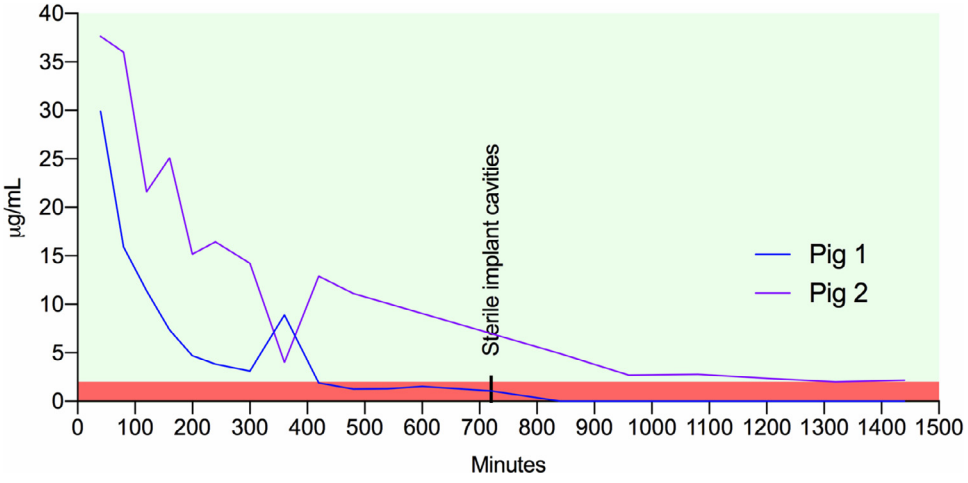

Fig. 9. Pharmacocenteic study of gentamicin release from a gentamicin-silica/PEI xerogel coated implant. Results of 24 h microdialysis study in two anesthetized pigs. Prior to microdialysis *S. aureus* bacteria was inoculated into the drilled implant cavity and a coated implant was inserted. Microdialysis catheters were placed in the implant cavity and extracellular fluid collected continuasly and measured for gentamicin content. The pink area shows the common range for gentamicin MIC values against *S. aureus*.

over phosphorus pentoxide (P_2O_5) was developed in order to maintain the structure of the hydrogel within the xerogel. FTIR analysis indicated that gentamicin sulphate was successfully encapsulated in the silica/PEI matrix and that no significant chemical or physic interaction, that could have changed the gentamicin molecule, occurred. The fast and efficient gentamicin immersion from the xerogel seen *in-vivo*, together with the very high *in-vitro* gentamicin-silica/PEI xerogel MIC value of 128 mg/L, support that the gentamicin was encapsulated in the xerogel and not chemically bonded to it. Due to stereochemical limitations the silica/PEI xerogel was anchored inside the pores of the titanium microspheres and only the small water-soluble gentamicin molecules diffused within the aqueous environment of the implant cavity, i.e., just after surgical insertion.

The present pharmacokinetic study confirmed a fast burst release profile for the new coating. Of note, the pharmacokinetic study represents the first microdialysis study to investigate antimicrobial release from a surface coating within a bone infection model. The new coating released gentamicin concentrations above the reported clinical breakpoint, MIC value of 2 mg/L (reported aggressive target for gentamicin against *S. aureus*), already from the first measurements of the MD study and until 8 h [37]. This means that the critical time point of most relevant surgical procedures potentially could be protected by the novel coating [38,39]. The PROtect tibial nail (Synthesis GmbH, Oberdorf, Switzerland) is a commercially available gentamicin-coated tibial nail that shows good clinical results in the prevention of infection following correction of fractures [6]. The PROtect tibial nail system uses a biodegradable poly(lactic acid) (PLA) coating for the release of gentamicin. The gentamicin release profile of the PROtect coating has been examined *in-vivo* using a rat model (uninfected model with 1 × 20 mm coated implant, total gentamicin load pr implant = 64 µg) [38]. In that study, the gentamicin release profile from the PROtect coating was found completely identic to the present silica/PEI xerogel coating (infected model with 2 × 15 mm coated implant, total gentamicin load pr implant = 50 µg), i.e., the main release from both coatings occurred within the first hour [38].

The novel gentamicin-silica/PEI xerogel coating shows clinical properties for local gentamicin prophylaxis in cementless arthroplasty or osteosynthesis. During arthroplasty, local antimicrobials are normally pro-

vided using antimicrobial-loaded bone cement. However, there is established a trend for application of cementless components at both primary and revision surgeries, and currently, there are only limited antimicrobial delivery systems for these cementless procedures [39,40]. The gentamicin-silica/PEI xerogel coating is designed to prevent infection, i.e., by eradicating perioperative contamination and, therefore, there is a particular perspective for application in patients at high risk of infection [41]. Antimicrobial coating of implants, like the present, primarily reduces the patient's susceptibility to infection [38]. In the present study, no signs of acute toxicity could be observed, and serum concentrations of gentamicin could not be measured. However, this might change when significantly larger amounts of gentamicin-silica/PEI coating are applied on full-scale implants or prostheses. In the future, we aim to investigate the gentamicin-silica/PEI xerogel coating *in-vivo* by using porcine models with larger size implants and longer study periods in order to examine pharmacokinetics including modulating of time with maximum release (days) and combination with a second long-term release profile (weeks), osseointegration, mechanical stability and bioavailability in more details. The present study was designed to show antibacterial proof of concept for the new coating and this was successfully achieved, although, limited by the fact that only one single bacterial strain and species were used.

Author contribution

LKJ, HEJ, YL, SS, MD, MP, EN, MA, IGR, SLI, CJV, FL, PG, IK, AT and MV were all partners of the EU Horizon 2020 project called NOMORFILM. The present study was designed as a part of the NOMORFILM project. MD, MP, EN and MA developed and characterized the gentamicin-silica/PEI xerogel and coated the implants for *in-vivo* testing. YL and SS performed toxicity testing in the *C. elegans* model. IGR, SLI, CJV and FL performed *in-vitro* microbiology. PG, IK, AT and MV developed the protocol for titanium microsphere coating of implants and prepared the implants for *in-vivo* testing. LKJ, HEJ, ABP, LK and BA performed the porcine *in-vivo* efficacy study including postmortem pathology and microbiology. LKJ, ABP, BA, LK, MB and PP designed and performed the microdialysis study. LKJ drafted the manuscript. All authors read and commented on the manuscript.

Declaration of Competing Interest

There are no conflicts of interest.

Acknowledgments

We would like to thank Betina Andersen and Elizabeth Petersen for excellent laboratory assistance with histology and immunohistochemistry. This study was financed by the European Union's Horizon 2020 research and innovation program under NOMORFILM project grant agreement No 634588 and grant no. R-345-2020-1674 from the Lundbeck Foundation to LKJ. All financial aspects have been covered by the two grants.

References

[1] V. Alt, Antimicrobial coated implants in trauma and orthopaedics-a clinical review and risk-benefit analysis, Injury 48 (3) (2017) 599–607.
[2] W. Zimmerli, W. Zimmerli, Periprosthetic joint infection: general aspects, in: Bone and Joint Infections from Microbiology to Diagnosis and Treatment, 1st ed., Jhon Wiley&Sons Ltd., New York, 2015, pp. 113–130. p.
[3] M. McNally, S. Parham, W. Zimmerli, Implant-associated osteomyelitis of long bones, in: Bone and Joint Infections from Microbiology to Diagnosis and Treatment, 1st ed., Jhon Wiley&Sons Ltd., New York, 2015, pp. 303–323. p..
[4] C.L. Romano, H. Tsuchiya, I. Morelli, et al., Antibacterial coating of implants: are we missing something? Bone Jt. Res. 8 (5) (2019) 199–206.
[5] T. Fuchs, R. Stange, G. Schmidmaier, M.J. Raschke, The use of gentamicin-coated nails in the tibia: preliminary results of a prospective study, Arch. Orthop. Trauma Surg. 131 (10) (2011) 1419–1425.
[6] W.J. Metsemakers, M. Reul, S. Nijs, The use of gentamicin-coated nails in complex open tibia fracture and revision cases: a retrospective analysis of a single centre case series and review of the literature, Injury 46 (12) (2015) 2433–2437.
[7] L.K. Jensen, Implant-associated osteomyelitis: development, characterisation, and application of a porcine model, APMIS 129 (2021) 1–44 Suppl 141.
[8] L.K. Jensen, J. Koch, K. Dich-Jorgensen, B. Aalbaek, et al., Novel porcine model of implant-associated osteomyelitis: a comprehensive analysis of local, regional, and systemic response, J. Orthop. Res. 35 (10) (2017) 2211–2221.
[9] European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology, Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution, Clin. Microbiol. Infect. 9 (8) (2003) 1–7.
[10] Clinical and Laboratory Standards Institute, An informational supplement for global application developed through the clinical and laboratory standards institute consensus processPerformance Standards for Antimicrobial Susceptibility Testing, Clinical and Laboratory Standards Institute, 2016.
[11] B. Aalbaek, L.K. Jensen, H.E. Jensen, et al., Whole-genome sequence of staphylococcus aureus S54F9 isolated from a chronic disseminated porcine lung abscess and used in human infection models, Genome Announc. 3 (2015) 5.
[12] L.K. Jensen, N.L. Henriksen, T. Bjarnsholt, et al., Combined staining techniques for demonstration of staphylococcus aureus biofilm in routine histopathology, J. Bone Jt. Infect. 3 (1) (2018) 27–36.
[13] J.R. van der Vorst, R.M. van Dam, R.S. van Stiphout, et al., Virtual liver resection and volumetric analysis of the future liver remnant using open source image processing software, World J. Surg. 34 (10) (2010) 2426–2433.
[14] M.S. Smeltzer, J.R. Thomas, S.G. Hickmon, et al., Characterization of a rabbit model of staphylococcal osteomyelitis, J. Orthop. Res. 15 (3) (1997) 414–421.
[15] M. Stegger, P.S. Andersen, A. Kearns, et al., Rapid detection, differentiation and typing of methicillin-resistant Staphylococcus aureus harbouring either mecA or the new mecA homologue mecA(LGA251), Clin. Microbiol. Infect. 18 (4) (2012) 395–400.
[16] L.K. Jensen, T. Bjarnsholt, K.N. Kragh, et al., *In vivo* gentamicin susceptibility test for prevention of bacterial biofilms in bone tissue and on implants, Antimicrob. Agents Chemother. 63 (2) (2019) 1–10.
[17] L. Morawietz, O. Tiddens, M. Mueller, et al., Twenty-three neutrophil granulocytes in 10 high-power fields is the best histopathological threshold to differentiate between aseptic and septic endoprosthesis loosening, Histopathology 54 (7) (2009) 847–853.
[18] F.L. Luthje, S.A. Blirup-Plum, N.S. Moller, et al., The host response to bacterial bone infection involves a local upregulation of several acute phase proteins, Immunobiology 225 (3) (2020) 151914, doi:10.1016/j.imbio.2020.151914.
[19] M.B. Thomassen, P. Hanberg, M. Stilling, et al., Local concentrations of gentamicin obtained by microdialysis after a controlled application of a GentaColl sponge in a porcine model, J. Orthop. Res. 38 (2020) 1793–1799.
[20] C.S. Chaurasia, M. Muller, E.D. Bashaw, et al., AAPS-FDA workshop white paper: microdialysis principles, application, and regulatory perspectives, J. Clin. Pharmacol. 47 (5) (2007) 589–603.
[21] G. Cierny, J.T. Mader, J.J. Penninck, A clinical staging system for adult osteomyelitis, Clin. Orthop. Relat. Res. 414 (2003) 7–24.
[22] D.R. Osmon, E.F. Berbari, A.R. Berendt, et al., Executive summary: diagnosis and management of prosthetic joint infection: clinical practice guidelines by the infectious diseases society of America, Clin. Infect. Dis. 56 (1) (2013) 1–10.
[23] T.F. Moriarty, L.G. Harris, R.A. Mooney, et al., Recommendations for design and conduct of preclinical *in vivo* studies of orthopedic device-related infection, J. Orthop. Res. 37 (2) (2019) 271–287.
[24] E.A. Masters, R.P. Trombetta, Mesy de, K.L. Bentley, et al., Evolving concepts in bone infection: redefining "biofilm", "acute vs. chronic osteomyelitis", "the immune proteome" and "local antibiotic therapy", Bone Res. 7 (20) (2019), doi:10.1038/s41413-019-0061-z.
[25] M. Diefenbeck, C. Schrader, F. Gras, et al., Gentamicin coating of plasma chemical oxidized titanium alloy prevents implant-related osteomyelitis in rats, Biomaterials 101 (2016) 156–164.
[26] W.J. Metsemakers, N. Emanuel, O. Cohen, et al., A doxycycline-loaded polymer-lipid encapsulation matrix coating for the prevention of implant-related osteomyelitis due to doxycycline-resistant methicillin-resistant Staphylococcus aureus, J. Control. Release 209 (2015) 47–56.
[27] A. Gulcu, A. Akman, A.F. Demirkan, et al., Fosfomycin addition to poly(D,L-Lactide) coating does not affect prophylaxis efficacy in rat implant-related infection model, but that of gentamicin does, PLoS One 11 (11) (2016) e0165544, doi:10.1371/journal.pone.0165544.
[28] M. Lucke, G. Schmidmaier, S. Sadoni, et al., Gentamicin coating of metallic implants reduces implant-related osteomyelitis in rats, Bone 32 (5) (2003) 521–531.
[29] D. Liu, C. He, Z. Liu, W. Xu, Gentamicin coating of nanotubular anodized titanium implant reduces implant-related osteomyelitis and enhances bone biocompatibility in rabbits, Int. J. Nanomed. 12 (2017) 5461–5471.
[30] C. Folsch, M. Federmann, K.D. Kuehn, et al., Coating with a novel gentamicin-palmitate formulation prevents implant-associated osteomyelitis induced by methicillin-susceptible staphylococcus aureus in a rat model, Int. Orthop. 39 (5) (2015) 981–988.
[31] J.S. Moskowitz, M.R. Blaisse, R.E. Samuel, et al., The effectiveness of the controlled release of gentamicin from polyelectrolyte multilayers in the treatment of Staphylococcus aureus infection in a rabbit bone model, Biomaterials 31 (23) (2013) 6019–6030.
[32] J.A. Jennings, K.E. Beenken, R.A. Skinner, et al., Antibiotic-loaded phosphatidylcholine inhibits staphylococcal bone infection, World J. Orthop. 7 (8) (2016) 467–474.

[33] L.K. Jensen, N.L. Henriksen, H.E. Jensen, Guidelines for porcine models of human bacterial infections, Lab. Anim. 53 (2) (2019) 125–136.
[34] W.B. Liechty, D.R. Kryscio, B.V. Slaughter, N.A. Peppas, Polymers for drug delivery systems, Annu. Rev. Chem. Biomol. Eng. 1 (2010) 149–173.
[35] E.R. Gillies, J.M. Frechet, Dendrimers and dendritic polymers in drug delivery, Drug Discov. Today 10 (1) (2005) 35–43.
[36] G. Pajonk, Drying methods preserving the textual properties of gels, J. Phys. Coll. 50 (1989) C4-13-C4-22.
[37] European Commitee on Antimicrobial Succeptibility Testing. Data from the EUCAST MIC distribution website 2021 [cited 2021 13/10]. Available from: http://www.eucast.org.
[38] S. Nast, M. Fassbender, N. Bormann, et al., *In vivo* quantification of gentamicin released from an implant coating, J. Biomater. Appl. 31 (1) (2016) 45–54.
[39] T. Nichol, J. Callaghan, R. Townsend, et al., The antimicrobial activity and biocompatibility of a controlled gentamicin-releasing single-layer sol-gel coating on hydroxyapatite-coated titanium, Bone Jt. J. 103-B (3) (2021) 522–529.
[40] C.L. Romano, K. Malizos, N. Capuano, et al., Does an antibiotic-loaded hydrogel coating reduce early post-surgical infection after joint arthroplasty? J. Bone Jt. Infect. 1 (2016) 34–41.
[41] P.S. Stewart, T. Bjarnsholt, Risk factors for chronic biofilm-related infection associated with implanted medical devices, Clin. Microbiol. Infect. 26 (8) (2020) 1034–1038.