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Injectable gentamicin-loaded thermo-responsive hyaluronic acid derivative prevents infection in a rabbit model

Gert-Jan A. ter Boo [a,b], Daniel Arens [a], Willem-Jan Metsemakers [a], Stephan Zeiter [a], R. Geoff Richards [a], Dirk W. Grijpma [b], David Eglin [a], T. Fintan Moriarty [a,*]

[a] AO Research Institute Davos, Davos, Switzerland
[b] MIRA Institute for Biomedical Technology and Technical Medicine, Department of Biomaterials Science and Technology, Faculty of Science and Technology, University of Twente, Enschede, The Netherlands

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ABSTRACT

Despite the use of systemic antibiotic prophylaxis, the surgical fixation of open fractures with osteosynthesis implants is associated with high infection rates. Antibiotic-loaded biomaterials (ALBs) are increasingly used in implant surgeries across medical specialties to deliver high concentrations of antibiotics to the surgical site and reduce the risk of implant-associated infection. ALBs which are either less or not restricted in terms of spatial distribution and which may be applied throughout complex wounds could offer improved protection against infection in open fracture care.

A thermo-responsive hyaluronic acid derivative (hyaluronic acid-poly(*N*-isopropylacrylamide) (HApN)) was prepared by a direct amidation reaction between the tetrabutyl ammonium (TBA) salt of hyaluronic acid and amine-terminated poly(*N*-isopropylacrylamide) (pN). The degree of grafting, and gelation properties of this gel were characterized, and the composition was loaded with gentamicin. The rheological- and release properties of this gentamicin-loaded HApN composition were tested *in vitro* and its efficacy in preventing infection was tested in a rabbit model of osteosynthesis contaminated with *Staphylococcus aureus*. The gentamicin-loaded HApN composition was able to prevent bacterial colonization of the implant site as shown by quantitative bacteriology. This finding was supported by histopathological evaluation of the humeri samples where no bacteria were found in the stained sections.

In conclusion, this gentamicin-loaded HApN hydrogel effectively prevents infection in a complex wound, simulating a contaminated fracture treated with plating osteosynthesis.

Statement of Significance

Fracture fixation after trauma is associated with high infection rates. Antibiotic loaded biomaterials (ALBs) can provide high local concentrations without systemic side effects. However, the currently available ALBs have limited accessibility to contaminated tissues in open fractures because of predetermined shape. Thus, a novel thermo-responsive hyaluronan based hydrogel with control over gelation temperature is reported. The efficacy of this gentamicin loaded hyaluronan derivative is demonstrated in an *in vivo* fracture model in the presence of fracture fixation hardware. The bacterial burden is cleared in all of the inoculated rabbits in the presence of the ALB. Thus, the proposed injectable thermo-responsive hyaluronan presents an effective ALB for the prevention of infection.

1. Introduction

Implant-associated infection remains a challenging complication in surgically fixed fractures. On average, 5% of the patients receiving internal fracture fixation implants develop an infection [1]. In closed fractures, where the protective barrier of the skin is intact, the infection rate is between 0.5% and 2%. However, the infection rate can exceed 30% in patients with severe open fractures [2]. Contamination of the fracture site prior to or during the surgical intervention can lead to bacterial adhesion and biofilm formation on tissue and implant surfaces [3,4]. Once developed, the treatment of implant-associated infection is challenging and expensive, and leads to delayed fracture healing and potential loss of limb function [5,6].

* Corresponding author at: AO Research Institute Davos, AO Foundation, Clavadelerstrasse 8, Davos-Platz CH-7270, Switzerland.
E-mail address: fintan.moriarty@aofoundation.org (T.F. Moriarty).

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In order to reduce infection rates in operative fracture treatment, systemic antibiotic prophylaxis is typically administered pre-operatively and may be continued for a number of days depending upon injury severity. Although appropriate antibiotic prophylaxis reduces infection rates, systemic administration may not be able to provide sufficiently high antibiotic concentrations to locations where distribution is limited by vascular damage or edema [7,8]. This may be the case in many fracture sites or contused soft tissues.

Antibiotic-loaded biomaterials (ALBs) offer the prospect of delivering high local concentrations of antibiotics directly to the site of interest, without requiring an intact vasculature. ALBs, such as antibiotic-loaded poly(methylmethacrylate) (PMMA) cement and beads [9,10], collagen fleece [11] and PDLLA coatings [12–14] with gentamicin have shown their potential in orthopedic and trauma surgery. Nevertheless, currently available ALBs possess certain suboptimal properties. PMMA cement/beads lack degradability often requiring a removal surgery, have poor release kinetics of antibiotics [10,15,16] and their setting can cause heat damage to various antibiotics [10,17]; collagen fleeces have undesirable handling properties, immunogenic potential [18,19]; and coatings of poly(α-hydroxyacid)s form acidic degradation products and have to be approved separately for each type of implant [6]. The complex nature of trauma wounds, *i.e.* the presence of contamination, inaccessible sites, poorly definable wound margins, and involvement of multiple tissues, demands alternative ALBs that are suited for use in these circumstances. The previously described ALBs have confined dimensions at the time of insertion and may have limited distribution in a complex wound.

Hyaluronan (HA) is a biocompatible and degradable polymer with many biomedical applications, notably as vehicle for drug delivery systems [20]. HA and HA derivative-based aqueous formulations can be injectable and subsequently gelate *in situ*, and are hence good material candidates for delivery of hydrosoluble antibiotics throughout complex wounds [21,22]. In this study, a direct amidation reaction was performed to graft amine-terminated thermo-responsive poly(*N*-isopropylacrylamide) (pN) to HA [23]. The thermo-responsive hyaluronic acid-poly(*N*-isopropylacrylamide) (HApN) hydrogel was loaded with gentamicin and designed to have a low modulus at room temperature (RT) and easy applicability at the surgical site. Once in contact with tissue and body fluids, the HApN solution shifts from a sol state to a gel state as it heats up to temperatures higher than its lower critical solution temperature (LCST). This property of the hydrogel improves the versatility of its application, since it can be applied at any stage before, during or at completion of the surgery and all potentially contaminated surrounding and overlying tissues, implant surfaces and implant cavities can be covered or filled.

In this preclinical study, a gentamicin-loaded thermo-responsive HApN hydrogel was prepared and characterized *in vitro* and *in vivo*. A rabbit humerus model has been established in the past [24], and this model mimics, as close as possible, a real trauma case including an osteotomy and a fracture fixation implant. Additionally, by choosing the humerus the animal's burden can be minimized since rabbits have the opportunity to reduce weight-bearing on the operated limb which is not feasible in their hind limbs. In this study the model was used to assess the efficacy of prophylactic antibiotic delivery.

2. Materials and methods

2.1. Materials

HA sodium salt from *Streptococcus equi* (HANa) was purchased from Contipro Biotech s.r.o. (Czech Republic), number average molecular weight (M_n) = 170.6 kDa and polydispersity (M_w/M_n) = 1.73. Tetrabutylammonium hydroxide solution (TBAOH) (~40% in H_2O, ~1.5 M); 2,2′-azobis(2-methylpropionitrile) (AIBN) (⩾98.0% GC); methanesulfonic acid (MSA) (⩾99.5%), methanol (analytical grade (99.8%)), ethanol (⩾99.8% puriss) and bromophenol blue were purchased from Fluka (Buchs, Switzerland). *N*-isopropylacrylamide (nIPAm) (⩾99%); cysteamine hydrochloride (AESH) (⩾98% (titration)), *N*,*N*-dimethylformamide (DMF) anhydrous (99,8%), 1,4-dioxane (⩾99.5%, p.a., ACS); Dowex M-31 cation exchange resin, 1,1′-carbonyldiimidazole (CDI) (reagent grade), dimethyl sulfoxide (DMSO) (⩾99.5% for synthesis), sodium carbonate (Na_2CO_3) (puriss., anhydrous, 99.5–100.5% calc. on the dried substance), potassium sulfate (K_2SO_4) (⩾99.0%), gentamicin sulfate salt (GEN) (potency ⩾590 μg gentamicin base per mg); boric acid (BAc) (⩾99.5%), phthaldialdehyde (⩾99.0 by high-performance liquid chromatography) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethyl ether (⩾99.5%, p.a.), 2-mercaptoethanol (99%), 2-propanol (⩾99.8%), glycerol (⩾99%) and sodium bromide (NaBr) (⩾99% p.a., ACS) were purchased from Carl Roth (Karlsruhe, Germany). Blood Agar (BA), Mueller-Hinton broth (MHB), Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were purchased from Oxoid AG (Basel, Switzerland). Gentamicin sulfate loaded-collagen fleece, Gentafleece®, was purchased from Baxter AG (Volketswil, Switzerland). Blank disks (BBL™ Sensi-disc™ blank disk) and disks with gentamicin (BBL™ Sensi-disc™ gentamicin 10 μg) were purchased from BD Diagnostics (Sparks, MD, USA).

2.2. Thermo-responsive hyaluronan (HApN) synthesis

2.2.1. Amine-terminated poly(N-isopropylacrylamide) synthesis

DMF was degassed by N_2 bubbling for 15 min. Ten grams (88.4 mmol) of nIPAm monomer was then dissolved in 20 ml DMF. N_2 bubbling at RT was performed for 2 h to remove all oxygen from the solution. Subsequently, 100.2 mg (8.8×10^{-1} mmol) AESH and 492.8 mg (3.0 mmol) AIBN were added to the solution. The system was then heated to 70 °C and the N_2 bubbling was continued during the radical polymerization reaction, which proceeded for 6 h. Then the system was cooled down to RT. Hereafter, the poly(*N*-isopropylacrylamide) amine (pN-NH_2) solution was precipitated in diethyl ether at RT.

After precipitation, the product was redissolved in 35 ml of 1,4-dioxane overnight. Then the pN-NH_2 solution was precipitated again in diethyl ether. The redissolution and precipitation steps were repeated 2–3 times until a fine white precipitate was obtained. The precipitate was collected and dried in a vacuum oven at 32 °C until all residual solvent was removed. The product was characterized by proton nuclear magnetic resonance (^{1}H NMR) on a Bruker Avance AV 500 NMR spectrometer using deuterium oxide (D_2O) as solvent with 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (TMS) as a standard to set the zero-shift. The NMR spectra were processed and analysed using Mestrenova™ software. The molecular weight of the amine-terminated pNs was determined by multi-detector size exclusion chromatography (SEC). The polymers M_n and polydispersity were determined by a modular multi-detector SEC system with an Alliance 2695 separation module from Waters (Milford, USA) with two on-line detectors: a MALS Dawn DSP-F photometer from Wyatt (Santa Barbara, USA) and a 2414 differential refractometer from Waters (Milford, USA) used as a concentration detector. The different signals from the two detectors were aligned to compensate for their intrinsic temporal delay. Chromatographic column specifications were as follows: Column: 2PL gel Mixed C from Polymer Laboratories (UK); Mobile phase: DMF + 0.05 M LiCl; Flow rate: 0.8 ml/min; Temperature: 50 °C; Injection volume: 218.5 μl; Sample concentration: 2 mg/ml.

2.2.2. HApN synthesis

A cationic exchange process was performed to obtain the tetrabutyl ammonium (TBA) salt of HA (HATBA) from HANa [25]. At RT, 1.0 g of HATBA was dissolved in 80 ml of dry DMSO under mild stirring. For the synthesis of HApN, 2.63 g of pN-NH_2 was dissolved in 20 ml of DMSO at 25 °C overnight. HATBA solutions were heated to 42 °C and 225 µl of MSA and 270 mg of CDI were added to the reaction vessel. After 1 h of stirring at 42 °C the pN-NH_2 solution was added to the reaction vessel and the constituents were allowed to cool down to 25 °C and react for 48 h. A saturated solution of NaBr (aq) was then added to quench the reaction and the solution was stirred at 25 °C for 2 h. At this point, the solution containing the HApN was transferred to cellulose dialysis tubing with a molecular weight cut-off (MWCO) of 50 kDa (Spectrapor no 6, 34 mm flat width). The products were dialyzed against demineralized water for 5 days. The HApN solutions were frozen and lyophilized. The samples were finally kept under vacuum for 3 days and sterilized using a cold ethylene oxide cycle and further degassed for 5 days. The degree of substitution of the HApN was then determined by ^{1}H NMR [23]. Residual TBA content in the final HApN samples was quantified by a Bromophenol Blue Assay [26].

2.3. Rheological behavior of gentamicin-loaded HApN formulations

The temperature-induced sol-gel transition of the HApN was assessed by the vial inversion method. HApN was dissolved in phosphate buffer saline (PBS; pH 7.4) at 13% w/w in hermetically closed vials. The flowability of the solutions was assessed visually using a vial inversion test at 22 ± 2 °C and after 5 min incubation at 37 °C.

Rheological measurements were performed on an Anton Paar Physica MCR 302 rheometer with a Peltier controller with plate-plate geometry (Ø 25 mm). HApN samples were dissolved at 13% w/w in PBS or in PBS containing 1% w/w gentamicin sulfate. The shear moduli (G') and loss moduli (G'') were recorded as a function of the temperature for these HApN polymers. Solutions were then subjected to 0.5% oscillatory strain at 1 Hz while heating from 20 °C to 40 °C at a rate of 1 °C/min. In order to avoid evaporation of water from the hydrogel composition at the solution-atmosphere interface, low viscosity silicone oil was applied along the border of the plates after sample placement. Measurements were assured to be run within the viscoelastic linear region.

The effect of the sulfate salt of gentamicin on the LCST of HApN was investigated for HApN (derived from high MW HANa; M_n = 952.5 kDa, M_w/M_n = 1.65) solutions with increasing concentrations of sulfate from gentamicin sulfate dissolved in PBS (0, 26, 52 and 104 mM sulfate). In order to verify that the shift could be attributed to the sulfate salt in the gentamicin sulfate molecule, HApN solutions dissolved in PBS with increasing concentrations of potassium sulfate (K_2SO_4) were also screened. In order to make a valid comparison, the amount of K_2SO_4 that needed to be dissolved to obtain a stoichiometric equivalent of sulfate ions in the final solution was calculated as compared to the HApN solutions with gentamicin sulfate. Shear moduli and viscous moduli were again recorded as a function of the temperature, as described above.

2.4. Gentamicin release in vitro

Release of gentamicin from the HApN hydrogel was assessed *in vitro*. For the preparation of the gentamicin-loaded HApN, either a 1% w/w or a 2% w/w gentamicin solution in PBS was used to dissolve HApN at 13% w/w. To keep the total gentamicin payload equal (8 mg gentamicin sulfate in total), 800 µl of the 1% w/w gentamicin-loaded HApN or 400 µl of the 2% w/w gentamicin-loaded HApN were injected into 8 ml of pre-warmed PBS (37 °C) to form single hydrogel spheres. Vials were then incubated at 37 °C in an incubator, while shaking at 20 rotations/min on a shaker plate (New Brunswick Scientific co. Inc. Classic C1, Edison, New Jersey, US). Supernatants (1 ml) were collected after 1 h, 3 h, 6 h, 1 day, 2 days, 4 days and 7 days, and 1 ml of fresh PBS was added to the vials. Each condition was performed in triplicate. The amount of gentamicin released was quantified by a modified absorbance assay measured at λ = 332 nm on a spectrophotometer (Multiskan GO, Thermo Scientific) as reported by Sampath and Robinson, after derivatization with *o*-phthaldialdehyde (OPDA) reagent [27]. The detection limit was calculated to be 94 µg/ml for this assay [28].

Gentamicin-loaded collagen fleece (4 cm × 1 cm × 0.5 cm), containing 8 mg gentamicin sulfate, was used as a clinically available comparator in the *in vitro* release study. The release was expressed as cumulative percentage (%) of the total payload, the removal of 1 ml of supernatant and dilution with 1 ml of fresh PBS was accounted for in the calculation.

2.5. In vivo assessment of a gentamicin-loaded HApN formulation in a contaminated plated fracture model in rabbits

2.5.1. Study design

An overview is provided with a description of the 3 study groups in Table 1. Bacteriological evaluations were performed on 8 animals of each group, and the remaining 2 animals per group were used for histological evaluation.

2.5.2. Institutional animal care and use committee approvals

The *in vivo* study was approved by the Ethical Committee of the Canton of Grisons, Switzerland (TVB number 28/2014). All procedures were performed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International approved facility and according to Swiss animal protection law and regulations. Thirty skeletally mature, specific pathogen free (SPF) female New Zealand white rabbits (Charles River, Suzfeld, Germany) older than 24 weeks of age and a mean body weight of 3.6 kg ± 0.2 kg were included in this study. All rabbits were assessed by a veterinarian and determined to be healthy prior to inclusion in this study and were allowed to acclimatize to their surroundings for two weeks prior to the start of the study. During the acclimatization period, they were group-housed with a 12 h dark/12 h light cycle, fed with hay, lettuce and supplemental feed for rabbits (Biomill, Switzerland). After the surgical intervention, the animals were single-housed until euthanasia.

For the first five post-operative days, each animal was checked using a score sheet at least twice daily by a veterinarian or an experienced animal caretaker, thereafter the rabbits were checked at least once a day for the rest of the study duration.

2.5.3. Bacteria

A gentamicin sensitive *Staphylococcus aureus* strain (JAR 060131), isolated from a male patient with a knee prosthesis infection was used in the present study [29]. Bacteria were stored in MHB supplemented with 20% of glycerol for long-term preservation at −20 °C.

Table 1
In vivo study plan.

Group name	ALB	Gentamicin sulfate[a]
1. Untreated control	–	–
2. Collagen Gen	Collagen fleece	+
3. HApN Gen	HApN	+

[a] Amount of gentamicin sulfate in ALB is 8 mg, n = 10 per study group.

Bacterial inocula were prepared in PBS as previously described [30], with a target inoculum of 2.0×10^6 colony-forming units (CFU) of *S. aureus*. Quantitative culture of each inoculum was performed immediately after preparation to assure the accuracy of the used inocula.

2.5.4. Antibiotic-loaded biomaterials

Gentamicin sulfate loaded collagen fleece was cut into 4 cm × 1 cm pieces using a sterile custom-made steel template. These dimensions were selected in order to allow the fleece to completely overlay the implants used in the *in vivo* study. All the sterile HApN samples were reconstituted at 13% w/w overnight in 1% w/w gentamicin sulfate in sterile PBS at 4 °C, 1 day prior to surgery. The HApN hydrogel samples with gentamicin were chilled on ice prior to administration. During the surgery, 800 µl of the gentamicin-loaded HApN hydrogel was injected over the implant. In order to speed up the gelation process, an infrared lamp was used to pre-heat the surgical site. This lamp was removed prior to injection of the hydrogel. The total payload was 8 mg gentamicin sulfate in both the hydrogel and the fleece groups.

2.5.5. Operative procedure

The surgical anesthesia, plating procedure and pre-operative and post-operative analgesia regimens have been described previously [24].

Inoculation of the rabbits was performed after creation of the osteotomy with a 0.45 mm Gigly saw, as three separate 34 µl injections into the central screw hole overlying the osteotomy and onto the adjacent proximal and distal screw holes. Subsequently, the wounds were closed without any local antibiotic prophylaxis (group 1), or after gentamicin-loaded collagen fleece was placed (group 2) or HApN hydrogel with gentamicin (group 3) was injected over the 7-hole locking plate (Fig. 1).

2.6. Evaluation of the in vivo study

2.6.1. Post-operative care and radiological analyses

Weight was measured at surgery, and 3 and 7 days post-operatively. Radiographs of the operated limb were taken in two planes (lateromedial and caudocranial) after surgery and post-mortem to assess proper implant positioning. Animal exclusion criteria were set at a weight loss exceeding 15% of the initial body weight, local infection with severe lameness, persistent swelling and discharge, or signs of systemic infection such as fever, depression and anorexia. One of the rabbits of study group 2, which received the gentamicin-loaded collagen fleece, died during anesthesia due to cardiac arrest and was excluded from the study. The rabbit was intended to be used for histology and was not replaced. Upon completion of the observation period (7 days), all animals were euthanized using intravenously administered Pentobarbital (200 mg/kg, Esconarkon®, Streuli Pharma AG, Switzerland).

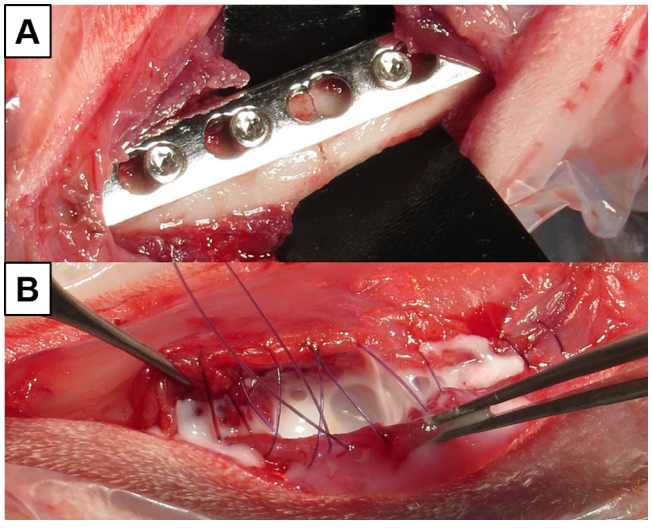

Fig. 1. Intra-operative image before (A) and after (B) application of the gentamicin-loaded HApN hydrogel within the surgical field. The HApN hydrogel (white color) fills the surgical field and turns from a sol to a gel state upon contact with the tissue.

2.6.2. Blood analyses

Blood samples were drawn from all included rabbits pre-operatively, 3 and 6 h, 1 day post-operatively and then every day until the end of the study. White blood cell (WBC) count was measured at each time point (Vet ABC, Scil animal care, Germany). C-reactive protein (Rabbit CRP Elisa Kit, ICL Inc. Portland, OR, USA) and gentamicin concentrations were measured in serum samples at the same time points. Serum gentamicin levels were quantified by a fluorescence polarization immunoassay (FPIA, lower limit of detection, 0.5 µg/ml) on a Cobas Integra® 400 plus analyzer (Roche Diagnostics AG, Switzerland) using reagents from Roche Diagnostics. Test solutions and calibration standards were diluted with human plasma.

2.6.3. Bacteriology

The soft tissue covering the locking plate and any abscess material were removed using a scalpel, weighed, and then placed into a sterile receptacle containing 10 ml of PBS. The soft tissue samples were then homogenized using an Omni-TH hand-held homogenizer (LabForce AG, Switzerland) with sterile Omni-tip plastic probes. All hardware (plates and screws) were transferred to sterile glass test tubes containing 10 ml of PBS. Plates and screws were sonicated (Bandelin Ultrasonic waterbath RK 510 H, Bandelin, Germany) for 3 min, thereafter they were vortexed for 10 s. Humerus bone samples were crushed into small fragments with a sterile luer and immediately homogenized in 40 ml of PBS using a Polytron PT3100 homogenizer (Kinematica AG, Switzerland).

All tissue and implant samples were serially diluted (10-fold steps) and plated on BA plates. Agar plates were incubated at 37 °C and colonies were counted after 24 h and 48 h. The limit of detection of *S. aureus* colonies was 5×10^1 CFU for soft tissue and hardware samples and 2×10^2 CFU for bone samples. All *S. aureus* bacterial growth was tested by a latex agglutination test (Staphaurex™ Plus Latex Agglutination Test, Remel, UK). In case of a negative test result (*i.e.* infection with organism other than *S. aureus*), isolates were identified using a Vitek2 machine (bioMérieux Vitek Inc., Hazelwood, MO, USA). The susceptibility of the *S. aureus* strain used for inoculation and the susceptibility of other isolated organisms to gentamicin were assessed by the disk diffusion method. For *S. aureus* the disk diffusion test was performed on Mueller-Hinton agar (MHA), using *S. aureus* ATCC® 25923 as quality control strain. All zone of inhibition (ZOI) tests were performed in triplicate for each bacterial species. Bacterial suspensions were adjusted to a 0.5 McFarland standard before use. Susceptibility to gentamicin was tested by using disks with 10 µg gentamicin. The bacterial strain was determined to be susceptible, intermediate or resistant according to the clinical and laboratory standards institute (CLSI) guidelines M100-S25 (2015) [31].

2.6.4. Histology

Rabbit humeri, including implant and adjacent soft tissue, were fixed in 70% (v/v) methanol for a minimum of 2 weeks with fresh methanol changes weekly. Contact radiographs (full thickness) were taken using high resolution technical film (D4 Structurix DW ETE, Agfa, Belgium) and a cabinet X-ray system (Model No.

4385A, Faxitron X-ray Corporation, AZ, USA). After fixation, samples were dehydrated by an ascending series of ethanol and were transferred to xylene. Finally, they were infiltrated and embedded in methylmethacrylate (MMA). The polymerized MMA blocks were trimmed using a butcher saw (Bizerba FK 22, Bizerba AG, Switzerland) prior to cutting with an annular diamond saw (Leitz 1600 saw microtome, Leica AG, Switzerland). The samples were glued with cyanoacrylate onto Beracryl holders for sectioning. Two sections of each sample were selected, which were glued onto opaque Plexiglass® slides, ground and fine polished. Sections were stained with Giemsa-Eosin and histopathological analysis of the slides was performed using a transmission light microscope (BX40, Olympus, Switzerland). Histological findings were described, wherever possible, according to distribution (focal, multifocal, diffuse), morphological character and to severity by a veterinary pathologist.

3. Results

3.1. Preparation and characterization of gentamicin-loaded HApN formulations

The M_n of the pN-NH_2 was determined to be 11.2 kDa with a polydispersity index of 3.85, and a recovered mass of 95.2%. The average degree of substitution of pN for the carboxylate group on hyaluronic acid was 15% as verified by integration of the HA proton NMR signals between δ = 3.00 ppm and 3.77 ppm (equivalent to 9 HA protons) and integration of the pN signal at δ = 1.14 ppm (6 protons, $-CH_3$) [23]. Residual TBA content in the HApN polymer was low and quantified to be 0.1%.

A vial inversion test of the HApN composition in PBS showed that at concentrations above 9% w/w the composition flowed easily at RT, upon heating to 37 °C the composition did not flow anymore. A 13% w/w concentration was selected as it has the required temperature dependent gelation for this study. Rheology was performed on the 13% w/w HApN, dissolved in PBS alone or in PBS with 1% w/w gentamicin sulfate (Fig. 2A).

The 13% w/w HApN composition in PBS (Fig. 2A, black circles) was found to be a flowing sol at RT, with moderate shear and viscous moduli below 1 Pa. Upon heating above the lower critical solution temperature where the shear modulus G′ over take the viscous modulus G″, the shear moduli of all the HApN compositions (with and without gentamicin) increased more than 3 orders of magnitude up to 10 kPa at 37 °C. The onset of gelation for the HApN without gentamicin added, was approximately 28 °C. Upon addition of 1% w/w gentamicin sulfate the LCST decreases to 25 °C.

As can be seen from Fig. 2B and C, the shift in LCST is proportional to the gentamicin sulfate concentration in the PBS, with the largest shift observed for the highest concentration. The same effect is observed when HApN is dissolved in K_2SO_4 at stoichiometric equal concentrations. However, the effect is stronger for K_2SO_4 than for gentamicin sulfate.

3.2. In vitro gentamicin release

The cumulative release of gentamicin sulfate from collagen fleece and HApN hydrogels with 1% and 2% w/w gentamicin over a 1 week period were measured *in vitro* at 37 °C (Fig. 3).

An initial burst release of gentamicin was observed for both of the tested ALBs (Fig. 3). After 1 h, 67% of the gentamicin

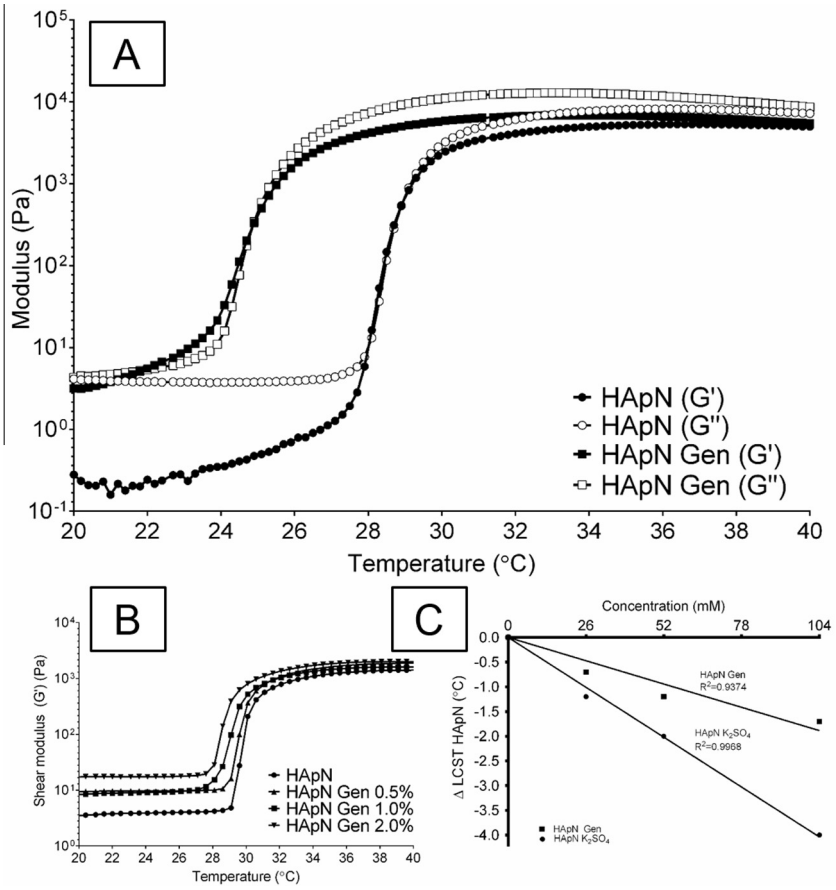

Fig. 2. Viscoelastic shear moduli of 13% w/w HApN solution in PBS, without or with addition of 1% w/w of gentamicin sulfate (A). Shear moduli of a 13% w/w HApN solution in PBS with increasing gentamicin sulfate content (B). Concentration dependent LCST shift (ΔT) for HApN (13% w/w) solutions in PBS with increasing sulfate content from gentamicin sulfate (0–104 mM sulfate) or increasing stoichiometric equal concentration of sulfate ions from potassium sulfate (K_2SO_4) (C).

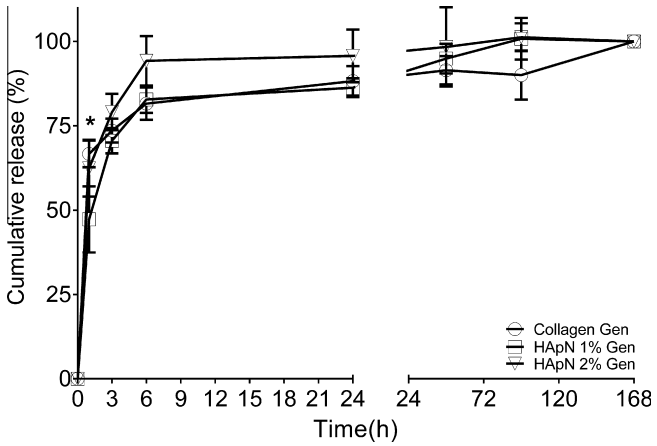

Fig. 3. Gentamicin cumulative release profile of collagen-gentamicin fleece and HApN hydrogels loaded with 1% w/w and 2% w/w gentamicin incubated at 37 °C for 1 week in PBS. n = 3, error bars represent standard deviation.

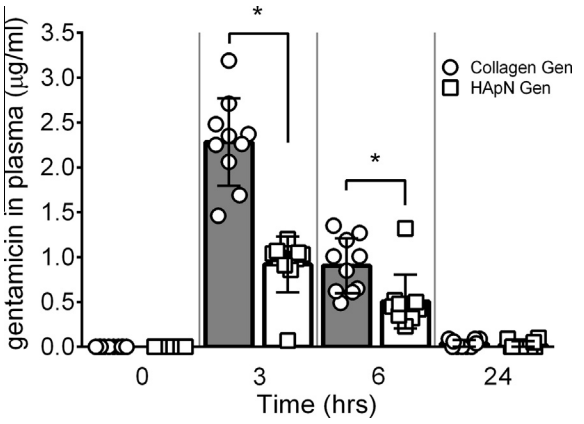

Fig. 5. Plasma concentrations of gentamicin after application of antibiotic loaded ALBs *in vivo*: Collagen Gen and HApN Gen at 0, 3, 6, 24 h. Values for the plasma concentrations of gentamicin are expressed as the mean value per group (n = 10) ± standard deviations. Statistical analysis on the gentamicin concentrations was performed by Mann-Whitney test (*, $P \leqslant 0.05$).

incorporated in the collagen fleece was released, whereas for the HApN hydrogel with 1% w/w gentamicin and 2% w/w, 47% and 62% of the total gentamicin payload was released, respectively. After 24 h, 88% of the total amount of gentamicin was released from the collagen fleece, and 86% and 96% respectively for the 1% w/w and 2% w/w gentamicin containing HApN hydrogels. After 7 days incubation in PBS, all ALBs had released their entire gentamicin payload. The HApN hydrogel (800 µl) with 1% w/w gentamicin sulfate was chosen for the *in vivo* study as it matches more closely the volume of the applied collagen fleece in the *in vivo* study (2 cm^3) and because of its slightly slower initial release of gentamicin as compared to the 2% w/w gentamicin sulfate containing HApN hydrogel.

3.3. In vivo observations

Between the rabbits from the 3 study groups no difference in weight development was observed over the course of the observation period. Rabbit weights were 3.4 kg (±0.2 kg) (untreated control group rabbits), 3.5 kg (±0.2 kg) (gentamicin loaded collagen fleece treated rabbits) and 3.4 kg (±0.3 kg) (gentamicin loaded HApN hydrogel treated rabbits) after 1 wk, respectively.

3.3.1. C-reactive protein & white blood cell count

After surgery the C-reactive protein (CRP) levels in plasma were elevated in all 3 study groups (Fig. 4).

The peak in CRP production is found 2 days after surgery, after which CRP levels started to decrease for all 3 study groups. For the 2 groups treated with either the Collagen Gen or the HApN Gen, CRP levels returned to base line values. However, after day 3 the CRP levels of the untreated group stayed significantly elevated until the end of the study (Fig. 4). A similar trend is observed for the white blood cell (WBC) counts in the rabbit plasma, where 7 days after surgery rabbits of the untreated group have 12.3×10^6 WBCs/ml as opposed to 8.9×10^6 (Collagen Gen) and 9.3×10^6 (HApN Gen) WBCs/ml in the treated groups (Fig. 4).

3.3.2. In vivo systemic gentamicin concentrations

The plasma concentration of gentamicin sulfate released from both HApN and Collagen fleece was measured over a 1 week period (Fig. 5).

Systemic gentamicin concentrations at 3 h upon administration were found to be 2.3 µg/ml for Collagen Gen, and 0.9 µg/ml for the HApN Gen, respectively. After 24 h no gentamicin could be traced in the plasma from both ALBs (Fig. 5).

3.4. Bacteriology

Quantitative culture of each inoculum, showed that CFU counts ranged from 1.2×10^6 CFU – 3.5×10^6 CFU for the inocula

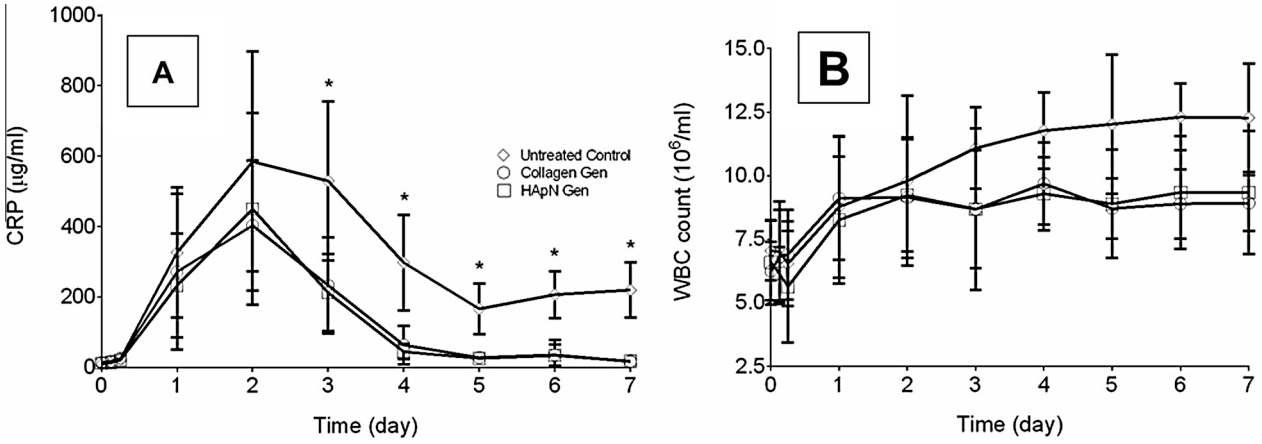

Fig. 4. Plasma C-reactive protein (CRP) levels (A) and white blood cell (WBC) values (B) for the untreated group, gentamicin-loaded collagen fleece (Collagen Gen) treated group and the gentamicin-loaded HApN hydrogel (HApN Gen) treated group. Values are expressed as mean value per group (n = 10) ± standard deviations. Statistical analysis on the CRP levels was performed by Mann-Whitney test in a pairwise comparison with untreated control (*, $P \leqslant 0.05$).

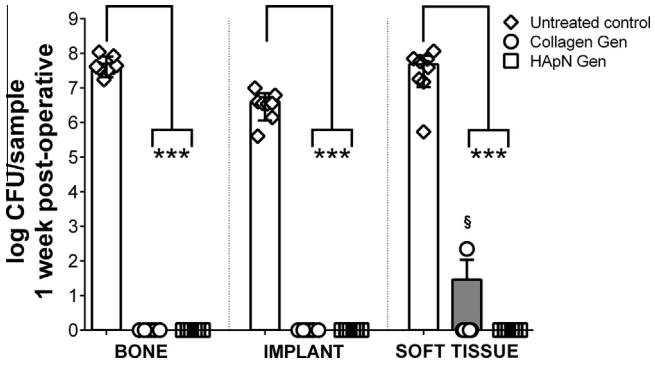

Fig. 6. Bacteriological quantification of harvested tissues and implant 1 week post-operatively from: Untreated control group (Untreated Control); Collagen fleece with gentamicin (Collagen Gen); HApN hydrogel with gentamicin (HApN Gen). § indicates causative organism other than *S. aureus*. n = 8, error bars represent standard deviation. Statistical analysis on the number of CFUs was performed by Mann-Whitney test in a pairwise comparison with untreated control (***, $P \leqslant 0.001$).

administered to the rabbits. Bacteriological quantification for harvested tissues and implant is shown in Fig. 6.

All rabbits (8/8) that did not receive any antibiotic treatment (untreated control) developed an infection (Fig. 6). Bone, soft tissues and implant material of these rabbits were all colonized by *S. aureus*, as proven by a positive latex agglutination test for *S. aureus*. The *S. aureus* strain used in this *in vivo* study was found to be susceptible to gentamicin (average zone diameter for *S. aureus* JAR 060131 was 20 mm and for the quality control strain, the average zone diameter for *S. aureus* ATCC® 25923 was 22 mm indicating the test was performed correctly). In the group of rabbits treated with the collagen fleece with gentamicin (Collagen Gen), all tissues and implant material, except for the soft tissue of one rabbit (1/8), stayed free from bacterial colonization. The soft tissue sample of the single infected rabbit was negative for the *S. aureus* latex agglutination test and was identified as *Streptococcus salivarius* (*S. salivarius*). This strain was at least intermediately susceptible to gentamicin as determined by the disk diffusion method (average zone diameter was 19.5 mm ± 0.9 mm). None of the rabbits (0/8) of the group that received the HApN hydrogel with gentamicin (HApN Gen) developed an infection. Bone, soft tissue and implant were free of bacteria.

3.5. Histological evaluation

Images of Giemsa Eosin stained sections of the operated humerus, including implants and surrounding soft tissues, are shown in Fig. 7. All three groups displayed signs of surgery-induced damage at the 7-day euthanasia time point, such as fibrin formation and muscle necrosis. However, some distinctions between treated and untreated groups were evident. In the untreated control group (Fig. 7, left column), obvious suppurative inflammation was observed surrounding the implant. At high magnification, Giemsa-positive coccoid bacterial micro-colonies were identified in the untreated control group, and seen to be embedded in fibrin and surrounded by polymorphonuclear granulocytes at the periphery of a micro-abscess (Fig. 7D, inset).

In comparison, the Collagen Gen and HApN groups (center and right columns, respectively) displayed a more physiological response at the implant interface and there were early signs of muscle regeneration (Fig. 7E and F).

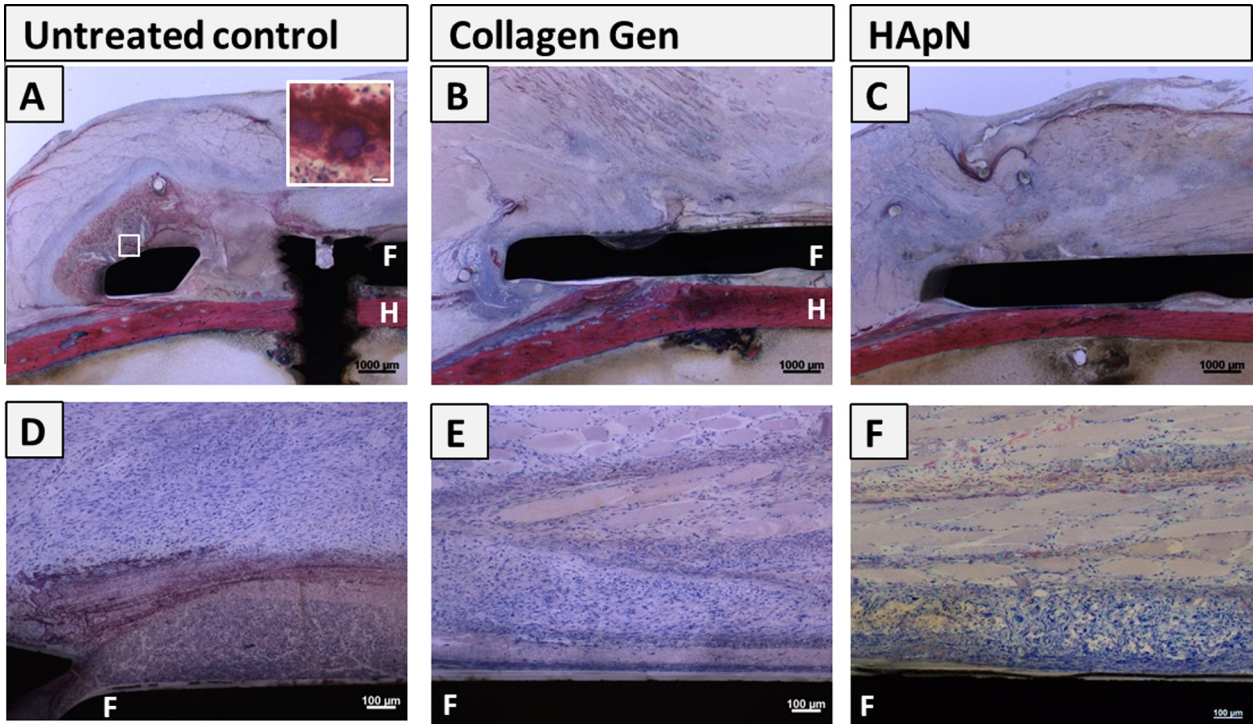

Fig. 7. Microphotographs of histological humerus samples (incl. stainless steel implant and soft tissue (A, B, C)) at day 7 after inoculation with *S. aureus* (Giemsa-Eosin stained thick-sections; objective lens [A, B, C]: 1.25×; [D, E, F]: 10×; [inset Figure A]: 100× oil immersion). Fracture fixation implant (F) and the cortex of the humerus (H) are indicated in the image. The inset in figure A represents a magnification of the area within the white bordered box. The scale bar in the inset in figure A represents 10 µm. Histological changes: see text.

4. Discussion

Open fracture treatment has been shown to benefit from the local application of ALBs [12,32]. However, improvements in the development of ALBs may offer the possibility of further reducing the infection rates associated with operative fracture care [2,33]. Hydrogels are attractive ALBs as they offer the prospect of a less invasive application than non-injectable materials such as PMMA or collagen fleece. Hydrogels also offer versatility in that they can be applied as an anti-infective adjunct to any fracture fixation system and can protect the entire surgical site regardless of the size or extent of tissue damage and regardless of the 3D geometry of the repaired fracture [34–36]. Furthermore, hydrogels based on biodegrabable polymers do not require removal. Coated implants or bone cement beads may not achieve these particular performance targets, especially in cases where tissue damage and contamination extends beyond the immediate vicinity of the implant/ALB.

Previously described hydrogel systems that were based on hydrated polymer matrices with already formed networks, are applied as highly viscous compositions prior to surgery to coat the implant [37]. Therefore, there is a risk of retaining only a low percentage of the starting amount of gel on the implant during the insertion procedure. This high viscosity may also result in a suboptimal dispersion within the surgical field. The gentamicin-loaded HApN formulation characterized in this study showed liquid-like behavior at RT, enabling distribution throughout the contaminated surgical site. Once placed within the wound, the gentamicin-loaded HApN dispersed throughout the wound, gelated soon thereafter, and effectively prevented bacterial infection. The treatment was also shown to prevent infection-induced CRP increases over time in all rabbits and prevented the appearance of suppurative abscess formation adjacent to the implant.

The addition of gentamicin sulfate to the HApN resulted in a reduction in LCST (from 28 °C to 25 °C). The temperature-induced phase transition of thermo-responsive polymers is influenced by the presence of co-solutes, such as salts [38], and their position in the Hoffmeister series [39]. Of all anions, sulfate has the strongest salting-out potential [38]. In addition, anions like SO_4^{2-} have shown to increase the viscosity of polysaccharide solutions, as observed as an increase in G″ at temperatures below the LCST for HApN [40]. As the temperature of tissues in the extremities of the body is ordinarily much lower than 37 °C, the shift in LCST is beneficial for their application to obtain fast gelation in traumatic wounds.

The HApN Gen showed an initial and rapid burst release of antibiotic, followed soon thereafter by complete exhaustion of the antibiotic from the material. This release profile is favorable in terms of reducing toxicity concerns, but also in terms of optimal pharmacodynamics of gentamicin, which is a concentration dependent antibiotic. *In vitro*, the gentamicin released at 1 h was significantly lower for the HApN Gen hydrogel than the collagen fleece, and this was also seen in the plasma measurements of rabbits three and six hours after surgery. This may indicate a slightly lower burst release of gentamicin upon application of the thermo-responsive HApN Gen than for the Collagen Gen. This is, however, difficult to verify as the half-life of gentamicin in the blood circulation of the rabbit is very short and accurate assessment of the pharmacokinetics with such few time-points is therefore a challenge [41,42]. Nevertheless, the peak plasma gentamicin levels measured in both groups are much lower than the commonly observed maximum concentration (C_{max}) of gentamicin in the human serum (18 μg/ml) upon intravenous administration of a conventional dose of 6 mg gentamicin/ kg body weight [43], indicating systemic toxicity is not a concern after application of either ALB. Clearly, both the thermo-responsive HApN hydrogel and collagen fleece with gentamicin provided adequate protection against infection, despite the low total amount of gentamicin applied, highlighting the fact that local delivery is a highly effective prophylactic strategy.

Although numerous publications have described hydrogels as antibiotic or antifungal delivery systems, few hydrogels have been tested for their efficacy in *in vivo* models [36,37,44–51], and those that have done so present varied success in their primary goal of infection prevention. Giavaresi *et al.* tested a non-responsive HA hydrogel with poly-D,L-lactic acid grafted as side groups, and loaded with vancomycin in an *in vivo* model with methicillin-resistant *S. aureus* (MRSA) in the rabbit femur. The vancomycin loaded hydrogel reduced, but was not able to clear the MRSA infection [37], possibly due to the high bacterial load of MRSA (10^4 CFU and 10^6 CFU) used in the study. Jia *et al.* tested a platelet-rich plasma derived leucocyte gel in a rabbit tibia model with *S. aureus* inoculation without the presence of a fracture fixation implant. Again, however, 4 out of 9 treated rabbits became infected [47], although as a non-conventional antibacterial agent, this result is nevertheless of interest. Taken together, none of these *in vivo* studies showed complete clearance of the bacterial burden, as opposed to the present study, in which the bacterial burden is completely cleared. Soon after inoculation of bacteria into the surgical site, the innate immune defenses will be activated and begin to combat the contaminating bacteria. Although the amount of bacteria killed by the innate defense system relative to those killed by the antibiotic cannot be estimated, in the absence of antibiotic, this dose of bacteria will consistently achieve a longstanding infection for up to at least ten weeks in this model [24]. This indicates that the antibiotic delivery is a critical feature in the eradication of infection by the hydrogel. In using the HApN hydrogel for prophylaxis, we prove that a flowable degradable thermo-responsive hydrogel loaded with gentamicin can eliminate a single species *S. aureus* contamination. As such, the efficacy against other pathogens such as Gram-negative or resistant bacteria is not shown. Furthermore, the bacteria in this model are planktonic and so are more easily targeted than for example bacteria within a biofilm. However, gentamicin does retain activity against many Gram-negative pathogens, and gentamicin has even been selected as the antibiotic of choice for the only commercially available antibiotic coated trauma implant on the market, which is used prophylactically in the same clinical context as the intended use for the HApN.

Should the HApN hydrogel be required to treat an infection, it would present a much more formidable challenge, considering that the bacteria will likely have formed biofilm on implanted hardware rendering them highly resistant to antibiotics. Additionally, the local tissues will likely be inflamed, with granulation tissue and abscess formation possible. The antibiotic distribution and concentrations required to achieve eradication in this situation may well require greater control over, or greater amounts of release from, the hydrogel than is achieved with the tested formulation. Therefore, the data presented should not be extrapolated to any claim of efficacy in the treatment of established infection. Overstreet *et al.* investigated the efficacy of a gentamicin loaded poly(*N*-isopropylacrylamide-co-dimethyl-γ-butyrolactone acrylate-co-Jeffamine® M-1000 acrylamide) (G-PNDJ) viscous hydrogel as treatment of established infection in a rabbit model [36]. The G-PNDJ hydrogel, with an average 30 mg gentamicin, was able to treat the infection in combination with debridement. Future work with the HApN hydrogel described here may involve evaluating the efficacy as a treatment option in a similar *in vivo* model.

Collagen fleece is a benchmark delivery product used for the local delivery of gentamicin in infection prophylaxis and was chosen as an ideal comparison for the hydrogel in the absence of

any commercially available, gentamicin loaded hydrogels. The systemic gentamicin concentration observed in the collagen fleece group is in accordance with previous published work by Mehta *et al.* [52], where collagen fleece resulted in an average systemic gentamicin concentration of 3.2 μg/ml. The single rabbit in the collagen gen group that was found to have a superinfection with a streptococcus (of intermediate gentamicin resistance [53,54]), is believed to be due to the rabbit licking the wound and delivery of oral microflora after the initial gentamicin is released from the collagen. This highlights the importance of proper post-surgical wound care and the need to protect the surgical field until wound closure, rather than as a particular failing of the collagen fleece. Several clinical cases have been published where collagen fleece with gentamicin was applied to prevent infection: e.g. after major or minor amputations in patients with diabetes or diabetic complications; before insertion of plate osteosynthetic hardware; and at the site of the removed disk after discectomy in spinal surgery [55]. In a case series report of 35 patients with open fractures, the efficacy of applying the gentamicin-loaded collagen fleece during the plating procedure was investigated. The majority of the fractures were considered to have united 40 weeks post-surgery. Local wound complications were observed in 10% of the patients, and 7% of the patients had deep tissue infections following the procedure [55]. In general, the wound healing time was shorter after application of the gentamicin-loaded collagen fleece and no patient progressed to non-union or implant failure during long-term follow-up.

Although similarly effective in preventing infection, the collagen fleece lacks many of the handling features of HApN. HApN may therefore be expected to be equally effective as collagen fleece, however, with greater ease of use and greater distribution throughout even complex wounds. Furthermore, there is the possibility to reconstitute the HApN hydrogel with different antibiotics for loading. This may eventually result in greater clinical uptake, should such a material become clinically available. Prior to clinical implementation however, the effect of the HApN hydrogel with gentamicin on the bone healing process should be investigated.

5. Conclusions

The described gentamicin-loaded HApN hydrogel is designed for complex trauma cases, where contamination may have spread throughout complex wound sites. The gentamicin-loaded HApN hydrogel was effective in preventing *S. aureus* infection in a rabbit prophylaxis model in the presence of osteosynthesis material. The HApN hydrogel offers comparable prophylaxis against a current clinical product, yet promises greater versatility in terms of application.

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