Bone & Joint Research

Supplementary Material

10.1302/2046-3758.1111.BJR-2022-0101.R1

Supplementary Text

Materials used

Phosphate-buffered saline (PBS; pH 7.0 to 7.2) was supplied by Cytiva, USA. Sodium pyruvate was purchased from Thermo Fisher Scientific (USA). Heat-inactivated fetal bovine serum (FBS), trypsin-EDTA solution (0.25%), sodium pyruvate solution, and MTT reagent were purchased from MilliporeSigma (Germany). MC3T3-E1 subclone 4 cells were purchased from ATCC (USA). α-minimum essential medium (αMEM) without phenol red was purchased from Life Technologies Europe BV (Sweden).

Preparation and characterization of antibiotic loaded CaS/HA composites

Antibiotic-loaded CaS/HA composite synthesis

Table i. Details of the amount and concentration of materials used for the preparation of CaS/HA-antibiotic pellets.

Materials used	CaS/HA-GEN	CaS/HA-VAN	CaS/HA-RIF	CaS/HA-GEN + RIF	CaS/HA-VAN + RIF
CaS/HA*, mg	500	500	500	500	500
Gentamicin, mg	10.33 mg	N/A	N/A	10.33 mg	N/A
Vancomycin, mg	N/A	24.57	N/A	N/A	24.57
Rifampicin, mg	N/A	N/A	8.11	8.11	8.11
Mixing solution, µl†					
Normal saline	283.5	N/A	283.5	283.5‡	N/A
Iohexol	N/A	215	N/A	N/A	215

*For CaS/HA-GEN, CaS/HA-RIF, and CaS/HA-GEN + RIF groups, CaS/HA provided as a pre-mixed powder from Cerament G, Bonesupport AB (Sweden) was used, whereas for the CaS/HA-VAN and CaS/HA-VAN + RIF groups, CaS/HA from Cerament V, Bonesupport AB was used.
†Normal saline and iohexol used as mixing solutions for CaS/HA from Cerament G and Cerament V, respectively.
‡An intermittent mixing technique was used for CaS/HA-GEN + RIF. Briefly, 500 mg of CaS/HA powder was first hand-mixed with GEN (10.35 mg) dissolved in 200 µl normal saline and 30 seconds later, RIF (8.11 mg) dissolved in 83.5 µl normal saline was added and mixed again.
CaS/HA, calcium sulphate/hydroxyapatite; GEN, gentamicin; N/A, not applicable; RIF, rifampicin; VAN, vancomycin.

Fourier transform infrared spectroscopy

Calcium sulphate/hydroxyapatite-vancomycin (CaS/HA-VAN)/gentamicin (GEN) composites with or without rifampicin (RIF) were made in a clean and sterile hood and then crushed to form a fine powder, after which they were loaded on a Bruker Alpha FT-IR spectrometer (Bruker Optics, Germany). The % transmittance values were plotted against wavelength to identify vibrations from RIF.

Injectability and setting time

CaS/HA composites were prepared as mentioned in the main text. The injectability of the composite was then evaluated by extruding 250 µl of the paste manually through a graduated 1 ml syringe connected to an 18G needle (Ø = 1.2 mm, L = 4 cm) into a mould with hemispherical wells (Ø = 4.8

mm). The total weight of the syringe with paste before and after extrusion was measured. To normalize the amount of composite that remained in the needle and syringe tip after injection, the mean of the paste extruded from the syringe at the first timepoint (for GEN, four minutes; for VAN and RIF, three minutes) was calculated for the tested concentrations (normalized value). Injectability (%) was calculated by dividing the weight of the paste extruded from the syringe by a normalized value. Each test was repeated three times at various time points (GEN: four, six, and eight minutes; VAN and RIF: three, five, and seven minutes). The composite extruded into the mould wells was left to solidify as per the manufacturer's recommendations for 20 minutes. To confirm the setting of the tested composites, the consistency of the pellets was evaluated manually by pressing the pellets with a sterile spatula, and also the pellets were allowed to fall from a height of 50 cm to check whether they crumbled or not.

$$\text{Injectability (\%)} = \frac{\text{weight of the paste extruded from the syringe}}{\text{normalized value}} \times 100$$

Material degradation

The degradation profile of the preweighed pellets at each timepoint (day 1, 3, 7, 14, 21, 28, and 35) was analyzed by the protocol described by Liu et al.[1] Briefly, pellets were placed on a filter paper to soak excess PBS, and kept at 37°C for one hour before the pellets were weighed on an analytical scale. The loss of weight for the pellets was divided by the original weight of the pellets at the time of casting in order to compute the in vitro percentage of degradation.

Cell viability analysis via MTT assay

Antibiotic-containing CaS/HA pellets in triplets were placed in a 2 ml microcentrifuge tube containing 1 ml of αMEM (without FBS, sodium pyruvate, and antibiotics) and placed at 37°C. At different timepoints (day 1, 3, 7, 14, 21, 28, and 35), αMEM was collected and tubes were supplemented with fresh 1 ml αMEM. Under sterile conditions, MC3T3-e1 cells were cultured in αMEM with 1 mM sodium pyruvate, 10% FBS, and 1% antibiotics (penicillin-streptomycin) at 37°C and allowed to grow to 80%

to 90% confluence. Cells were then trypsinized and centrifuged at 400 × g for four minutes. A total of 5×10^4 cells were seeded on a 96-well plate and incubated for 24 hours. The media was then removed, and to the test wells 200 µl of αMEM containing antibiotic fractions from D-1, D-7, and D-28 either undiluted or diluted (×100) were added after they were supplemented with necessary media components. To the control wells, αMEM with 1 mM sodium pyruvate, 10% FBS, and 1% antibiotics (penicillin-streptomycin) were added and incubated for 24 hours. Following incubation, the medium was aspirated and 100 µl of MTT working solution (0.5 mg/ml) made in αMEM was added. After two hours of incubation, MTT reagent was replaced with 200 µl of dimethyl sulfoxide (DMSO) and incubated for ten minutes. The contents of each well were mixed with a pipette and the absorbance of the reaction products was measured at 600 nm (Labsystems Multiskan MULTISOFT; Labsystems, Finland).

Testing of antibacterial effects

Efficacy on planktonic bacteria by continuous Kirby-Bauer disk diffusion assay

The antimicrobial efficacy of composites on planktonic bacteria was tested by continuous Kirby-Bauer disk diffusion assay. For tested combinations of CaS/HA-antibiotics, pellets in triplets were used for each strain of *Staphylococcus aureus*. The bacteria were inoculated into sheep blood agar plates and incubated at 37°C for 24 hours. The bacterial suspensions were then prepared by dissolving the bacteria in sterile saline (NaCl 0.9%, B. Braun, Germany) to an optical density (OD) 600 = 0.1 ± 0.005 as measured by spectrophotometer (GENESYS 20, Thermo Fisher Scientific). By lawn culture, bacteria were inoculated on in-house made Mueller-Hinton agar (MHA) plates and using sterile forceps, pellets were transferred to the inoculated plates within three to 15 minutes of inoculation. The plates were then incubated at 37°C for 24 hours, and the diameter of the zone of inhibition (ZOI) was measured using a standard ruler. After measuring the ZOI, the pellets were transferred to a new set of plates inoculated with bacteria using sterile forceps, and the procedure was repeated until their ZOI became zero, or for a maximum of 28 days. At predetermined timepoints (day 1, 3, 7, 14, 21, and 28), MHA plates exhibiting ZOI were imaged using ChemiDoc MP imaging system (Bio-Rad Laboratories, USA). For GEN and RIF pellets, using CLSI guidelines, ZOIs of $\geq$ 15 and $\geq$ 20 mm, respectively, were taken as strong antibacterial effects, and ZOIs of less than these were represented as moderate antibacterial effects.[20] For VAN, ZOI of $\geq$ 17 mm was used as a surrogate for strong antibacterial effect.

Antibacterial effect of pellets after day-35

To mimic the in vivo conditions, after day 35 the antibacterial effect of the pellets that had been used for antibiotic release assay was tested in a similar way as mentioned above. Briefly, following the day 35 fraction collection for antibiotic release assay, the pellets were crushed to form a paste, and using a sterile spatula the paste was transferred to the MHA plates inoculated with *S. aureus* ATCC 25923.

After 18 h of incubation, the ZOIs were noted and plates were imaged using ChemiDoc MP imaging system (Bio-Rad Laboratories, Japan).

Efficacy on preformed biofilms

Using a modified biofilm quantification method,[21] the antibiotics released at different timepoints (day 1, 3, 7, 14, 21, 28, and 35) from the pellets of CaS/HA-antibiotic composites were tested for their ability to disrupt the preformed biofilm. *S. aureus* was grown in tryptic soy broth supplemented with 1.0% glucose (TSBG) in a rotary shaker (180 rpm) at 37°C for 16 to 18 hours. Then, the bacterial suspension was centrifuged at 4,000 rpm for ten minutes and immediately resuspended in fresh TSBG. The resuspended bacterial suspension was diluted to OD 0.17 measured at 600 nm (GENESYS 20, Thermo Fisher Scientific). From the diluted suspension, 11.4 µl was added to 138.6 µl of TSBG in the wells of 96-well flat-bottom tissue culture treated polystyrene microtitre plate (Costar, USA) coated with poly-L-lysine (0.2 mg/ml) (MilliporeSigma, Germany) and incubated at 37°C statically. Biofilms were allowed to form on the wells for 48 hours with a change of media after 24 hours of incubation. After incubation, biofilms were gently washed three times with sterile PBS to remove the planktonic bacteria. Thereafter, 200 µl of TSBG and TSBG-antibiotic fractions mixture (1:1) was added to the control and test wells, respectively, and incubated at 37°C for 24 hours. The wells were then gently washed two times with PBS and fixed with 150 µl of methanol for 20 minutes. After air-drying overnight, the remaining biofilms were stained with 150 µl of 1% (w/v) crystal violet solution (MilliporeSigma) for 15 minutes at room temperature. The wells were washed three times with PBS and air-dried before adding 95% ethanol to resolubilize the dye bound to cells. After 30 minutes, 100 µl from each well was transferred to a new 96-well plate and OD was measured at 590 nm using a microplate reader (iMark; Bio-Rad). The absorbance from the test wells, which correlates to the amount of biofilm that remained after exposure to the antibiotic fractions, was compared with the absorbance of untreated control wells. The efficacy of the antibiotic fractions was tested on both *S.*

aureus ATCC 25923 and *S. aureus* clinical strain P-3. For each tested group of antibiotic fractions, five separate wells were used (n = 5).

Scanning electron microscopy of biofilms

As mentioned in the main text, biofilms were first allowed to form on glass coverslips (Ø = 13 mm; Paul Marienfeld GmbH & Co. KG, Germany) coated with poly-L-lysine (0.2 mg/ml) for 48 hours in a 12-well plate (Costar) with a change of media after 24 hours of incubation. After incubation, biofilms were gently washed three times with sterile PBS to remove the planktonic bacteria. Thereafter, 700 µl of TSBG and TSBG-antibiotic fractions mixture (1:1) were added to the control and test wells respectively, and incubated at 37°C for 24 hours. The wells were then gently washed two times with PBS and fixed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4) overnight at room temperature. Fixed samples were washed with 0.15 M sodium cacodylate (pH 7.4) for ten minutes to a minimum of four cycles, and dehydrated with increasing concentrations of ethanol followed by critical point drying with liquid carbon dioxide in Critical point CPD30, (BalTec, Switzerland) using absolute ethanol as the intermediate solvent. Samples were then mounted onto aluminium holders and sputter coated with 20 nm gold/palladium in Leica EM ACE200 (Leica, Germany), and examined under a DELPHI microscope (Phenom-World, the Netherlands).

Viable bacteria after continuous treatment with antibiotic fractions, and development of resistance to VAN, GEN, and RIF

Viable bacteria after continuous treatment with antibiotic fractions, and the development of bacterial resistance to VAN, GEN, and RIF were determined by exposing the biofilm embedded bacteria continuously to released fractions of antibiotics from CaS/HA-VAN/GEN pellets with or without RIF from predetermined time points (day 1, 3, 7, 14, 21, 28, and 35). Briefly, 48 hour-old *S. aureus* biofilms were established on 96-well microtitre plates as mentioned previously. Biofilms were then gently washed twice with sterile PBS. The biofilms in the control and test wells were exposed to 200 µl of

plain TSBG and TSBG-antibiotic fractions mixture (1:1), respectively, for 16 hours, followed by eight-hour incubation in 150 µl of antibiotic-free TSBG. This cycle was repeated until antibiotic fractions from all timepoints were exposed: a total of seven days. Thereafter, each well was gently washed twice with sterile PBS. To remove the remaining biofilms from the wells, 200 µl sterile PBS was added, and wells were scraped with sterile plastic pipette tips followed by sonication of the microtitre plate for five minutes in an ultrasonic bath (Elmasonic S 30H; Elma Hans Schmidbauer GmbH & Co. KG, Germany). After serial dilution, the number of colony-forming units (CFUs) were determined by plating on both antibiotic-free tryptic soy agar (TSA) and TSA supplemented with varying concentrations of antibiotics (VAN/GEN: 4-24 µg/plate; RIF: 1-32 µg/plate). The development of resistance was tested on both *S. aureus* ATCC 25923 and *S. aureus* clinical strain P-3, whose minimum inhibitory concentration (MIC) values against VAN, GEN, and RIF were predetermined using MALDI-TOF MS system (Vitek MSTM; bioMérieux, France) (*S. aureus* ATCC 25923: VAN = 2 µg/ml, GEN = 0.064 µg/ml, RIF = 0.008 µg/ml; *S. aureus* clinical strain P-3: VAN = 1 µg/ml, GEN = 0.5 µg/ml, RIF = 0.008 µg/ml). Strains showing resistance to RIF were further confirmed using the MALDI-TOF MS system. For each tested group of antibiotic fractions, four separate wells were used (n = 4).

Table ii. Detailed statistical findings of the antibacterial efficacy of tested calcium sulphate/hydroxyapatite-antibiotics pellets on *Staphylococcus aureus* ATCC 25923 by continuous Kirby-Bauer disk diffusion assay.

Tukey's multiple comparison test group	**p-value**				
	Day 1	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Cas/HA-VAN vs CaS/HA-GEN	0.0077	0.9276	< 0.0001	< 0.0001	< 0.0001
Cas/HA-VAN vs CaS/HA-RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cas/HA-VAN vs CaS/HA-VAN+RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cas/HA-VAN vs CaS/HA-GEN+RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CaS/HA-GEN vs CaS/HA-RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CaS/HA-GEN vs CaS/HA-VAN+RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CaS/HA-GEN vs CaS/HA-GEN+RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CaS/HA-RIF vs CaS/HA-VAN+RIF	< 0.0001	0.0006	< 0.0001	< 0.0001	0.0180
CaS/HA-RIF vs CaS/HA-GEN+RIF	< 0.0001	0.0180	< 0.0001	< 0.0001	0.2002
CaS/HA-VAN+RIF vs CaS/HA-GEN+RIF	> 0.9999	< 0.0001	> 0.9999	0.0342	0.5393

CaS/HA, calcium sulphate/hydroxyapatite; GEN, gentamicin; RIF, rifampicin; VAN, vancomycin.

Table iii. Detailed statistical findings of the antibacterial efficacy of tested calcium sulphate/hydroxyapatite-antibiotics pellets on *Staphylococcus aureus* clinical strain P3 by continuous Kirby-Bauer disk diffusion assay.

Tukey's multiple comparison test group	**p-value**				
	Day 1	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Cas/HA-VAN vs CaS/HA-GEN	0.0018	0.0022	< 0.0001	< 0.0001	< 0.0001
Cas/HA-VAN vs CaS/HA-RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cas/HA-VAN vs CaS/HA-VAN+RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cas/HA-VAN vs CaS/HA-GEN+RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
CaS/HA-GEN vs CaS/HA-RIF	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0080
CaS/HA-GEN vs CaS/HA-VAN+RIF	< 0.0001	< 0.0001	< 0.0001	0.1168	0.5109
CaS/HA-GEN vs CaS/HA-GEN+RIF	< 0.0001	< 0.0001	< 0.0001	0.0019	0.0001
CaS/HA-RIF vs CaS/HA-VAN+RIF	0.0055	0.0126	0.0170	0.0004	0.0975
CaS/HA-RIF vs CaS/HA-GEN+RIF	0.0611	0.0052	0.9456	0.0179	0.0520
CaS/HA-VAN+RIF vs CaS/HA-GEN+RIF	0.5393	0.9723	0.0521	0.1168	0.0008

CaS/HA, calcium sulphate/hydroxyapatite; GEN, gentamicin; RIF, rifampicin; VAN, vancomycin.

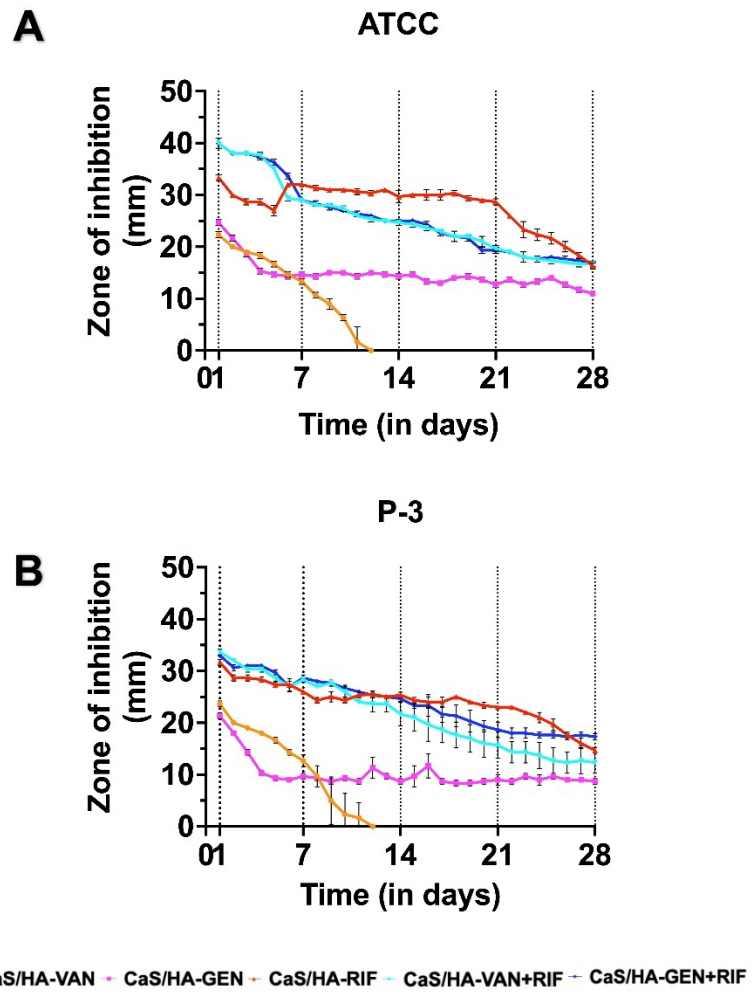

Fig a. Testing of antibacterial efficacy of calcium sulphate/hydroxyapatite (CaS/HA)-antibiotic pellets on planktonic bacteria by continuous Kirby-Bauer disk diffusion assay for 28 days. Graph data showing the zone of inhibition (ZOI) (in mm) of CaS/HA-antibiotic pellets against a) *Staphylococcus aureus* ATCC 25923, and b) *S. aureus* clinical strain P-3. GEN, gentamicin; RIF, rifampicin; VAN, vancomycin.

References

1. **Liu Y, Raina DB, Sebastian S, et al.** Sustained and controlled delivery of doxorubicin from an in-situ setting biphasic hydroxyapatite carrier for local treatment of a highly proliferative human osteosarcoma. *Acta Biomater*. 2021;131:555–571.