



## ■ BIOMATERIALS

# Extended local release and improved bacterial eradication by adding rifampicin to a biphasic ceramic carrier containing gentamicin or vancomycin

**S. Sebastian,  
F. Tandberg,  
Y. Liu,  
D. B. Raina,  
M. Tägil,  
M. Collin,  
L. Lidgren**

From Lund University,  
Lund, Sweden

### Aims

There is a lack of biomaterial-based carriers for the local delivery of rifampicin (RIF), one of the cornerstone second defence antibiotics for bone infections. RIF is also known for causing rapid development of antibiotic resistance when given as monotherapy. This in vitro study evaluated a clinically used biphasic calcium sulphate/hydroxyapatite (CaS/HA) biomaterial as a carrier for dual delivery of RIF with vancomycin (VAN) or gentamicin (GEN).

### Methods

The CaS/HA composites containing RIF/GEN/VAN, either alone or in combination, were first prepared and their injectability, setting time, and antibiotic elution profiles were assessed. Using a continuous disk diffusion assay, the antibacterial behaviour of the material was tested on both planktonic and biofilm-embedded forms of standard and clinical strains of *Staphylococcus aureus* for 28 days. Development of bacterial resistance to RIF was determined by exposing the biofilm-embedded bacteria continuously to released fractions of antibiotics from CaS/HA-antibiotic composites.

### Results

Following the addition of RIF to CaS/HA-VAN/GEN, adequate injectability and setting of the CaS/HA composites were noted. Sustained release of RIF above the minimum inhibitory concentrations of *S. aureus* was observed until study endpoint (day 35). Only combinations of CaS/HA-VAN/GEN + RIF exhibited antibacterial and antibiofilm effects yielding no viable bacteria at study endpoint. The *S. aureus* strains developed resistance to RIF when biofilms were subjected to CaS/HA-RIF alone but not with CaS/HA-VAN/GEN + RIF.

### Conclusion

Our in vitro results indicate that biphasic CaS/HA loaded with VAN or GEN could be used as a carrier for RIF for local delivery in clinically demanding bone infections.

**Cite this article:** *Bone Joint Res* 2022;11(11):787–802.

**Keywords:** Antimicrobial resistance, Biofilm, Biomaterial, Bone infections, Calcium sulphate, Hydroxyapatite, Rifampicin

### Article focus

- We evaluated a clinically used, biphasic bioresorbable calcium sulphate/hydroxyapatite (CaS/HA) biomaterial containing vancomycin (VAN)/gentamicin (GEN) as a carrier for the local delivery of rifampicin (RIF).
- In vitro experiments focused on the antibiotic release profile, antibacterial and antibiofilm characteristics, and development of RIF resistance.

### Key messages

- CaS/HA biomaterial containing VAN or GEN could be used as a carrier for RIF.
- A sustained release of RIF above the minimum inhibitory concentration of *Staphylococcus aureus* was observed until day 35.
- CaS/HA-VAN/GEN combined with RIF showed excellent antibacterial and antibiofilm effects, and did not cause resistance development.

Correspondence should be sent to  
Sujeesh Sebastian; email:  
sujeeshsebastian@gmail.com

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

*Bone Joint Res* 2022;11(11):787–  
802.

## Strengths and limitations

- CaS/HA biomaterial used in the present study is a clinically proven and approved material, which potentially makes it easy for future clinical studies and clinical translation of CaS/HA as a carrier of RIF.
- Dual antibiotic delivery using CaS/HA biomaterial provides extended antibiotic release and improved bacterial eradication without development of RIF resistance.
- The *in vitro* findings need to be validated in more mature biofilms and relevant animal models. However, being a proof-of-concept study, we believe our findings are valuable and aid the ongoing *in vivo* experiments.

## Introduction

Bone infections are some of the most serious complications in orthopaedic surgery. In the poorly vascularized infected bone tissue, achieving an effective and sustained local concentration of systemically administered antibiotics is a challenge.<sup>1,2</sup> The current treatment methods involving long-term systemic antibiotics, following local debridement and filling of the remaining dead space, may also lead to unwanted toxicities and selection of antibiotic-resistant bacteria. In addition, multiple bacterial species associated with bone infections use biofilms as a defence mechanism, which further limits conventional treatment efficacy.<sup>2,3</sup>

To achieve high local antibiotic levels, exceeding minimum inhibitory concentration (MIC) or minimum biofilm eradication concentration (MBEC) in bone infections, locally implanted polymethylmethacrylate (PMMA) containing antibiotics have been used clinically for the last few decades. However, the mechanism of antibiotic release from PMMA is a passive surface diffusion phenomenon. Hence, the material as a carrier does not provide sustained and optimal antibiotic release.<sup>4</sup> Furthermore, PMMA lacks bone-regenerating properties and is non-biodegradable. There are recently clinically approved biphasic ceramic carriers that have been shown to provide high local release of gentamicin (GEN) or vancomycin (VAN) reporting good outcomes in debrided bone infections.<sup>5-7</sup> However, no osteoconductive biomaterial currently contains rifampicin (RIF), considered to be a cornerstone second defence antibiotic in the treatment of severe longstanding bone infections.<sup>8,9</sup> Since PMMA combined with RIF results in incomplete setting and altered mechanical properties, PMMA is not suitable as a carrier for local delivery of RIF.<sup>10,11</sup>

Commercially available, biphasic, bioresorbable calcium sulphate/hydroxyapatite (CaS/HA) bone void filler, composed of 60 wt%  $\alpha$ -CaS hemihydrate and 40 wt% HA, has the ability to deliver biologically active molecules, hormones, and anti-cancer drugs.<sup>12-15</sup> Additionally, various clinical studies have reported its potential as a carrier for antibiotics such as GEN and VAN for the treatment of bone infections, with fewer side effects as compared to the commonly used systemic treatment

mode.<sup>5,16</sup> Previously, Sebastian et al<sup>17</sup> showed that CaS/HA is a potential carrier for local delivery of RIF and reported that a maximum of 300 mg RIF was possible to mix with 10 ml CaS/HA composite, eluting concentrations several times higher than the MIC of most common pathogens of bone infections.<sup>18</sup> However, RIF monotherapy is not recommended due to rapid development of bacterial resistance.<sup>19</sup>

In order to overcome the limitations stated above, we addressed the following research question in the present study: can a biphasic CaS/HA biomaterial be used as a carrier for combined local delivery of a HA-binding antibiotic (RIF) and CaS-mediated passively diffusing antibiotics (VAN/GEN) to exert an extended antibacterial effect?

Composites containing RIF/GEN/VAN, either alone or in combination, were first prepared and physiochemically characterized. To test the feasibility of using the biomaterial carrier delivering the antibiotic combinations in a clinical setting, important material characteristics such as setting time, material degradation, and the *in vitro* antibiotic elution profile were studied in detail. Finally, the antibacterial effect was tested on both planktonic bacteria and biofilm-embedded bacteria aged 48 hours.

## Methods

**Materials used.** Medical-grade RIF (Rifampicin Ebb, 600 mg; Sanofi, Italy) was purchased from the local pharmacy (Apoteket AB, Sweden). CaS/HA composites containing GEN (Cerament G) or VAN (Cerament V) were purchased from Bonesupport AB (Sweden).

**Antibiotic loaded CaS/HA composite synthesis.** The following combinations of CaS/HA and antibiotics were prepared — CaS/HA-VAN, CaS/HA-VAN + RIF, CaS/HA-GEN, CaS/HA-GEN + RIF, and CaS/HA-RIF. For the CaS/HA-VAN and CaS/HA-VAN + RIF composites, 500 mg of CaS/HA powder (Cerament V; Bonesupport AB) was hand-mixed in a 48-well plate with either only VAN (24.57 mg) or in combination with RIF (8.11 mg) dissolved in 215  $\mu$ l iodine-based mixing solution (Iohexol, C-TRU; Bonesupport AB) using a sterile wooden spatula for 30 seconds. The paste was transferred to a 1 ml syringe connected to an 18 G needle ( $\varnothing = 1.2$  mm) and extruded into a mould with hemispherical wells ( $\varnothing = 4.8$  mm) to obtain uniform-sized pellets for evaluating antibiotic release profile, material degradation, and continuous Kirby-Bauer disk diffusion assay. CaS/HA-GEN or CaS/HA-RIF composites were prepared by mixing 500 mg of CaS/HA powder (Cerament G; Bonesupport AB) and GEN (10.35 mg) or RIF (8.11 mg) dissolved in 283.5  $\mu$ l of saline (Bonesupport AB). For the CaS/HA-GEN + RIF composites, preparation was the same but with the difference of intermittent mixing technique. Briefly, 500 mg of CaS/HA powder (Cerament G) was first hand-mixed with GEN (10.35 mg) dissolved in 200  $\mu$ l mixing solution (Bonesupport AB) and 30 seconds later, RIF (8.11 mg) dissolved in 83.5  $\mu$ l mixing solution (Bonesupport AB) was added and mixed again. This intermittent mixing technique was adopted to prevent the rapid setting of the composite when GEN and

RIF were mixed simultaneously. The details of the material and their concentrations used for pellet preparation are given in Supplementary Table i.

**Scanning electron microscopy analysis.** To compare the structure and surface of the CaS/HA-VAN/GEN with or without RIF, hemispherical discs of the composites were sputter-coated and analyzed using a scanning electron microscope (SEM) (JSM-7800F; JEOL, Sweden) at an operating voltage of 10 kV.

**Fourier transform infrared spectroscopy.** Fourier transform infrared spectroscopy (FTIR) was used to verify the presence of RIF in CaS/HA-VAN/GEN composites. A fine powder of the composites was made, 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 previously. The injectability of the composite was then evaluated by extruding 250  $\mu$ l of the paste manually through a graduated 1 ml syringe connected to an 18 G needle into a mould with hemispherical wells ( $\varnothing = 4.8$  mm). The total weight of the syringe with paste before and after extrusion was measured. Each test was repeated three times at various timepoints (GEN: four, six, and eight minutes; VAN and RIF: three, five, and seven minutes). To confirm the setting of the tested composites, after 20 minutes of extrusion 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.

**Antibiotic release profile and material degradation.** For each combination, pellets in triplets were placed in 2 ml microcentrifuge tubes containing 1 ml of sterile phosphate-buffered saline (PBS) and placed at 37°C. At different timepoints (day 1, 3, 7, 14, 21, 28, and 35), PBS in the test tube was collected and tubes were supplemented with fresh 1 ml PBS. The amount of VAN and RIF released from the pellets was quantified using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Thermo Fisher Scientific, USA), and GEN quantification was performed by cloned enzyme donor immunoassay (CEDIA) on Roche Cobas, C501 (Roche Diagnostics, Switzerland). 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.<sup>15</sup>

**Cell viability analysis via MTT assay.** The effect of released antibiotic fractions in the cell viability of MC3T3-e1 cells was tested via MTT assay.

**Testing of 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 using *Staphylococcus aureus* standard strain obtained from American Type Culture Collection (*S. aureus* ATCC 25923), and *S. aureus* clinical strain P3, isolated from a case of periprosthetic joint infection (PJI) at our hospital.

For tested combinations of CaS/HA-antibiotics, pellets in triplets were used for each strain of *S. aureus*. For GEN and RIF pellets, using Clinical and Laboratory Standards Institute (CLSI) guidelines, zone of inhibitions (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.<sup>20</sup> For VAN, ZOI of  $\geq 17$  mm was used as a surrogate for strong antibacterial effect.<sup>20</sup>

**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 by continuous Kirby-Bauer disk diffusion assay.

**Efficacy on preformed biofilms.** Using a modified biofilm quantification method,<sup>21</sup> 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. Briefly, *S. aureus* biofilms were allowed to form on 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) for 48 hours with a change of media after 24 hours of incubation. Thereafter, 200  $\mu$ l of tryptic soy broth supplemented with 1.0% glucose (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 stained with 1% (w/v) crystal violet solution (MilliporeSigma) and their optical density was measured at 590 nm using a microplate reader (iMark; Bio-Rad Laboratories, Japan). 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 ( $n = 5$ ).

**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) in 96-well microtitre plate. The development of resistance was tested on both *S. aureus* ATCC 25923 and *S. aureus* clinical strain P-3 whose MIC values against VAN, GEN, and RIF were predetermined using MALDI-TOF MS system (Vitek MSTM; bioMérieux, France). Strains showing resistance to RIF were further confirmed using MALDI-TOF MS system. For each tested group of antibiotic fractions, four separate wells were used ( $n = 4$ ).

**Statistical analysis.** All data were presented as mean and standard deviation (SD). An independent-samples *t*-test was used to analyze injectability of CaS/HA-VAN/GEN

with or without RIF, material degradation of the pellets, MTT assay, and to compare the differences in biofilm destruction. An independent-samples *t*-test or one-way analysis of variance (ANOVA) with Dunn's multiple comparisons test was used to analyze the cumulative release profile of VAN, GEN, or RIF. For comparison of the difference in ZOI between different groups, ANOVA with Tukey's multiple comparison test was used. A *p*-value of < 0.05 was considered statistically significant. Details of statistical analysis are indicated in each figure legend. All data processing was carried out on GraphPad Prism version 9.1.2 for MacOS (GraphPad Software, USA). For detailed information about the methods used in the study, please refer to the Supplementary Material.

## Results

**Preparation and characterization of antibiotic-loaded CaS/HA composites.** Pellets of all tested combinations of antibiotic-loaded CaS/HA composites were characterized regarding the surface morphology and physicochemical properties. CaS/HA pellets with either GEN or VAN were white in colour while combinations of RIF had dark orange colour (Figures 1a and 1b). SEM revealed that the addition of RIF to CaS/HA-VAN/GEN composites appeared to have no effect either on the surface morphology or the structure (Figures 1a and 1b). FTIR characterization further confirmed that the RIF was loaded onto CaS/HA-VAN/GEN composites. CaS/HA-VAN + RIF showed the same transmittance peaks at 1,584  $\text{cm}^{-1}$ , 1,559  $\text{cm}^{-1}$ , 1,430  $\text{cm}^{-1}$ , 1,225.3  $\text{cm}^{-1}$ , and 889  $\text{cm}^{-1}$  as RIF, whereas CaS/HA-GEN + RIF showed the same transmittance peaks as those of RIF at 1,558  $\text{cm}^{-1}$ , 1,433.4  $\text{cm}^{-1}$ , 1,372.2  $\text{cm}^{-1}$ , 1,225.3  $\text{cm}^{-1}$ , and 891  $\text{cm}^{-1}$ , which could not be seen in only CaS/HA-VAN/GEN composites (Figures 1c and 1d).

**Injectability and setting time.** CaS/HA-VAN composites could be completely extruded from the syringes at all three tested timepoints (three, five, and seven minutes) whereas addition of RIF to CaS/HA-VAN significantly reduced the injectability, and by five minutes the composite became uninjectable (Figure 1e). The CaS/HA-GEN composites could be completely extruded from the syringes at four minutes without blocking the syringe outlet or causing obvious pressure filtration effects, and by eight minutes it dropped to almost 47% (Figure 1f). By dropping the injectability to almost 12% by six minutes, a significant injectability reduction trend was observed with the addition of RIF to CaS/HA-GEN as well. At both three and five minutes, CaS/HA-RIF composites maintained the injectability and by seven minutes it reduced to almost 11%. All the CaS/HA-antibiotic composites were completely set within 15 minutes of injection, and they subjectively seemed durable and did not crumble while handled.

**Antibiotic release profile and material degradation.** In the tested CaS/HA pellets of both VAN or a combination of VAN + RIF, at days 1, 3, 7, and 21 there was a significant

difference in the amount of VAN released per day. The overall cumulative release profile of VAN remained almost unchanged (58% and 57%, respectively) (Figures 2a and 2b). A similar cumulative release trend was observed with GEN as well (CaS/HA-GEN, 59% and CaS/HA-GEN + RIF, 63%) (Figures 2c and 2d). From CaS/HA-VAN and CaS/HA-VAN + RIF, VAN concentrations above the MIC levels of *S. aureus* were obtained until day 14 (3.08  $\mu\text{g/ml}$ ) and day 21 (2.1  $\mu\text{g/ml}$ ), respectively. Until study endpoint at day 35, CaS/HA-GEN and CaS/HA-GEN + RIF pellets eluted GEN of concentrations, well above the MIC levels of *S. aureus*; 11.8  $\mu\text{g/ml}$  and 9.5  $\mu\text{g/ml}$ , respectively. For RIF, an increased cumulative release profile was obtained when it was combined with CaS/HA-VAN (56%) or GEN (61%), compared to CaS/HA-RIF alone (46%) (Figures 2e and 2f). On day 35, concentrations of RIF from CaS/HA-RIF (1.94  $\mu\text{g/ml}$ ), CaS/HA-VAN + RIF (1.87  $\mu\text{g/ml}$ ), and CaS/HA-GEN + RIF (1.87  $\mu\text{g/ml}$ ) were all found to be above the MIC levels of *S. aureus*.

During the first week, almost 30% of CaS/HA-VAN, CaS/HA-VAN + RIF, and CaS/HA-RIF pellets degraded, whereas it was approximately 36% for CaS/HA-GEN and CaS/HA-GEN + RIF pellets (Figures 2g and 2h). Until day 35, no statistically significant difference in the material degradation was observed for any of the CaS/HA-antibiotic combinations.

**Cell viability via MTT assay.** MTT assay of both CaS/HA-VAN/GEN undiluted fractions from all tested timepoints exhibited no significant toxic effects on the MC3T3-e1 cell lines (Figures 3a and 3b). Undiluted fractions from CaS/HA-VAN/GEN + RIF or CaS/HA-RIF did not achieve  $\geq 80\%$  cell viability (Figures 3c to 3e). However, with 100% cell viability, all diluted fractions of CaS/HA-antibiotic combinations showed a significant difference in the cell viability with that of undiluted fractions.

**Efficacy on planktonic bacteria by continuous Kirby-Bauer disk diffusion assay.** There was a general trend that ZOI of all different composite pellets decreased over time (Figures 4a to 4d; Supplementary Figure a). Out of all the tested CaS/HA pellets, antibacterial effect of CaS/HA-VAN against both *S. aureus* strains declined sharply and showed no effect by day 12 (Supplementary Figure a). CaS/HA-GEN pellets exhibited a strong antibacterial effect until day 15 and day 3 against *S. aureus* ATCC 25923 and clinical strain P-3, respectively (Supplementary Figure a). Thereafter it maintained a low but almost steady antibacterial effect until day 28. Against *S. aureus* ATCC 25923, both CaS/HA-VAN/GEN + RIF combinations showed a strong antibacterial effect until day 20 but in case of clinical strain P-3, it was day 16 for CaS/HA-VAN + RIF and day 20 for CaS/HA-GEN + RIF. Compared to pellets of all combinations, the antibacterial effect persisted for a longer time for CaS/HA-RIF pellets against both *S. aureus* ATCC 25923 (until day 26) and clinical strain P-3 (until day 25) (Supplementary Figure a). Overall, against both tested bacterial strains, CaS/HA-VAN/GEN + RIF combinations

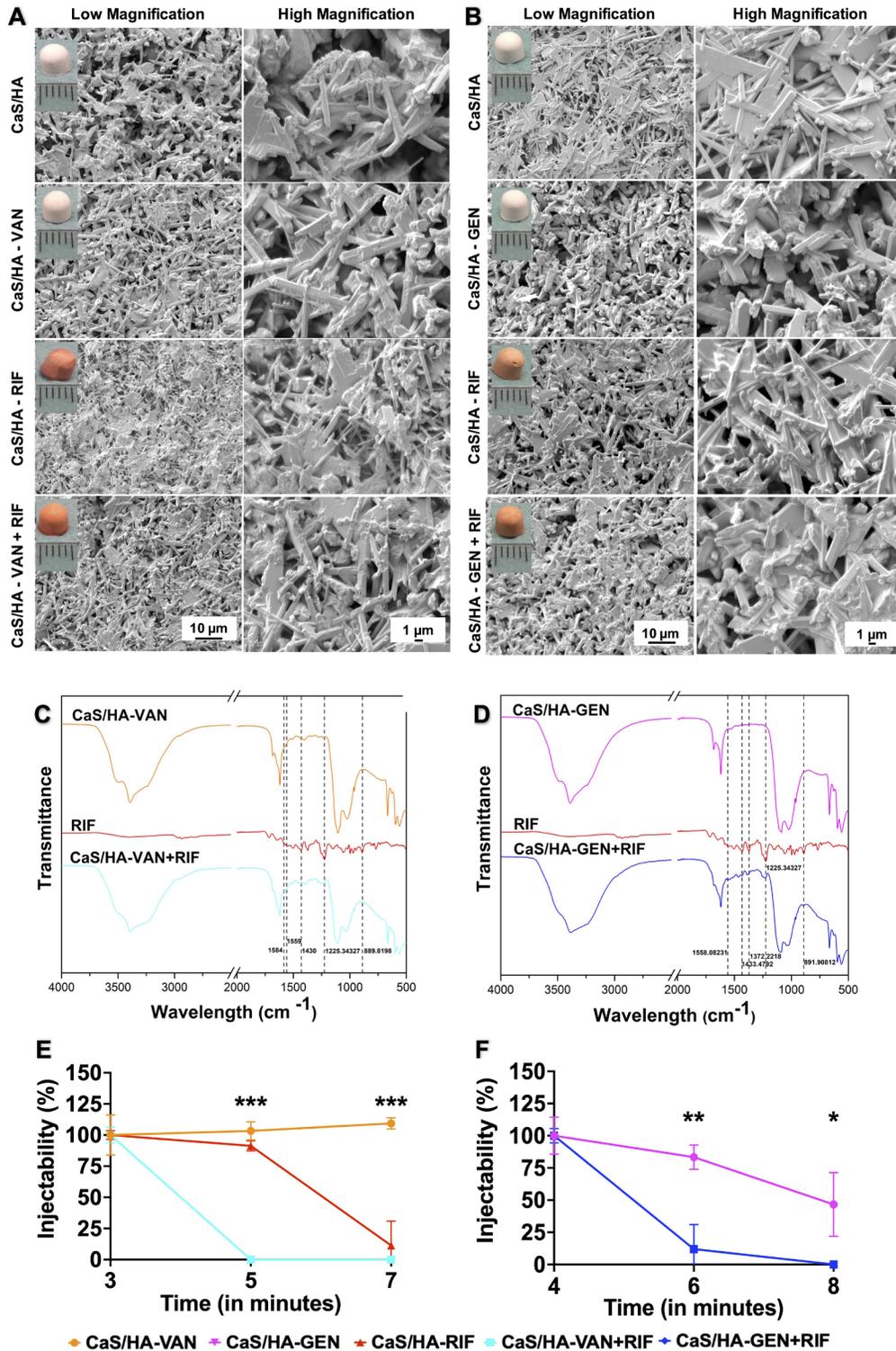


Fig. 1

Morphological and physicochemical characterization of calcium sulphate/hydroxyapatite-vancomycin/gentamicin (CaS/HA-VAN/GEN) with or without rifampicin (RIF). a) and b) Scanning electron microscopy (SEM) images showing surface characteristics and pore distribution of CaS/HA-VAN/GEN pellets with or without RIF. Hemispherical pellets casted using an elastic mould of 4.8 mm diameter are shown in inset of a) and b). c) and d) Fourier transform infrared spectroscopy (FTIR) spectra from: c) CaS/HA-VAN, pure RIF, or CaS/HA-VAN + RIF; and d) CaS/HA-GEN, pure RIF, or CaS/HA-GEN + RIF. e) and f) Injectability of tested CaS/HA-VAN/GEN composites with or without RIF. An independent-samples *t*-test was used to compare the injectability of CaS/HA having VAN/GEN alone or in combination with RIF. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

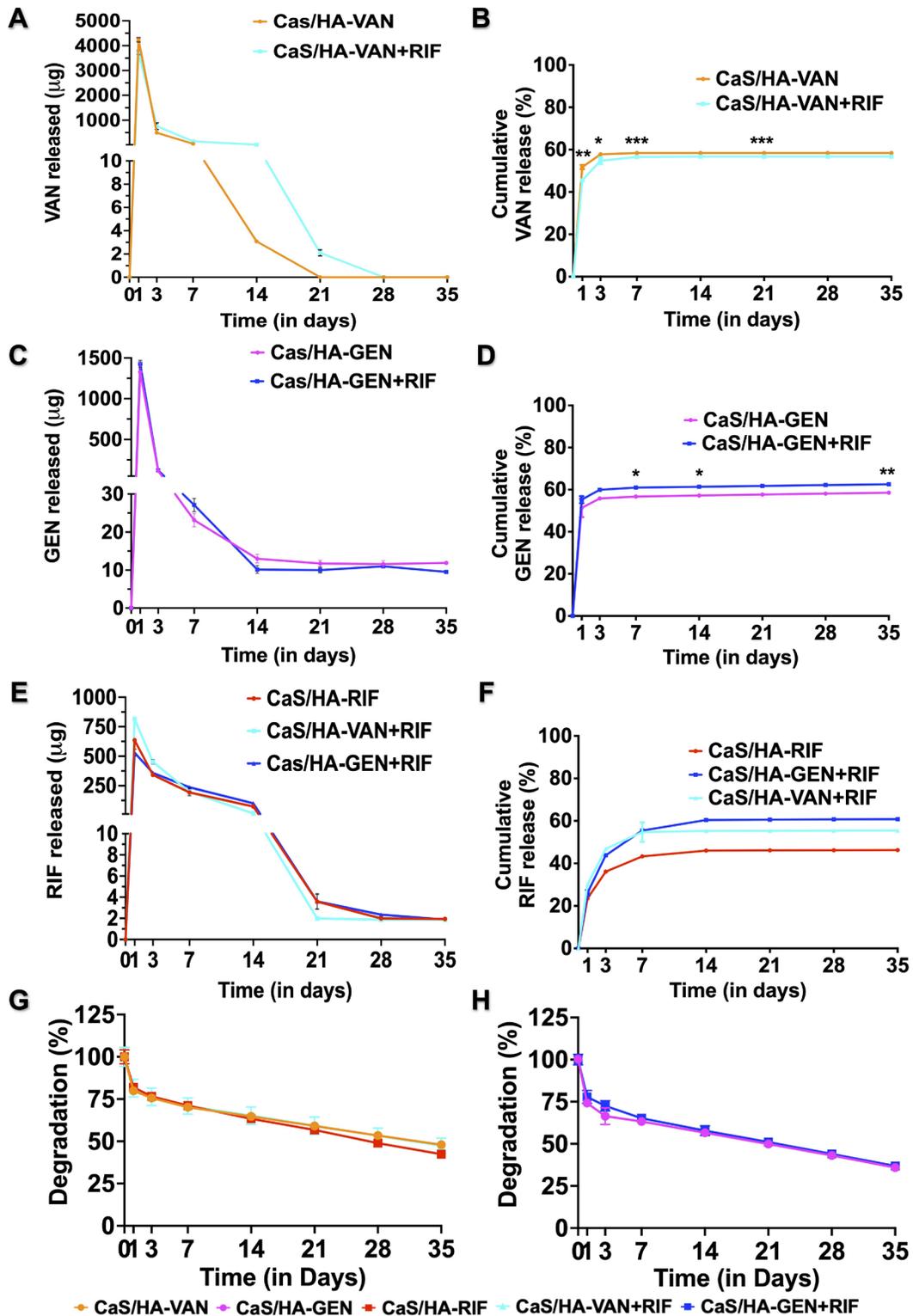


Fig. 2

In vitro antibiotic release profile and material degradation. a) to f) Release per day and cumulative release profile of vancomycin (VAN), gentamicin (GEN), and rifampicin (RIF) from calcium sulphate/hydroxyapatite (CaS/HA)-VAN/GEN with or without RIF at days 1, 3, 7, 14, 21, 28, and 35. g) and h) In vitro material degradation profile of tested CaS/HA-antibiotic composites in phosphate-buffered saline. An independent-samples *t*-test was used to compare the in vitro material degradation profile and cumulative release profile CaS/HA-VAN/GEN alone or in combination with RIF. For comparison of cumulative release profile of RIF, one-way analysis of variance with Dunn's multiple comparison test was used. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

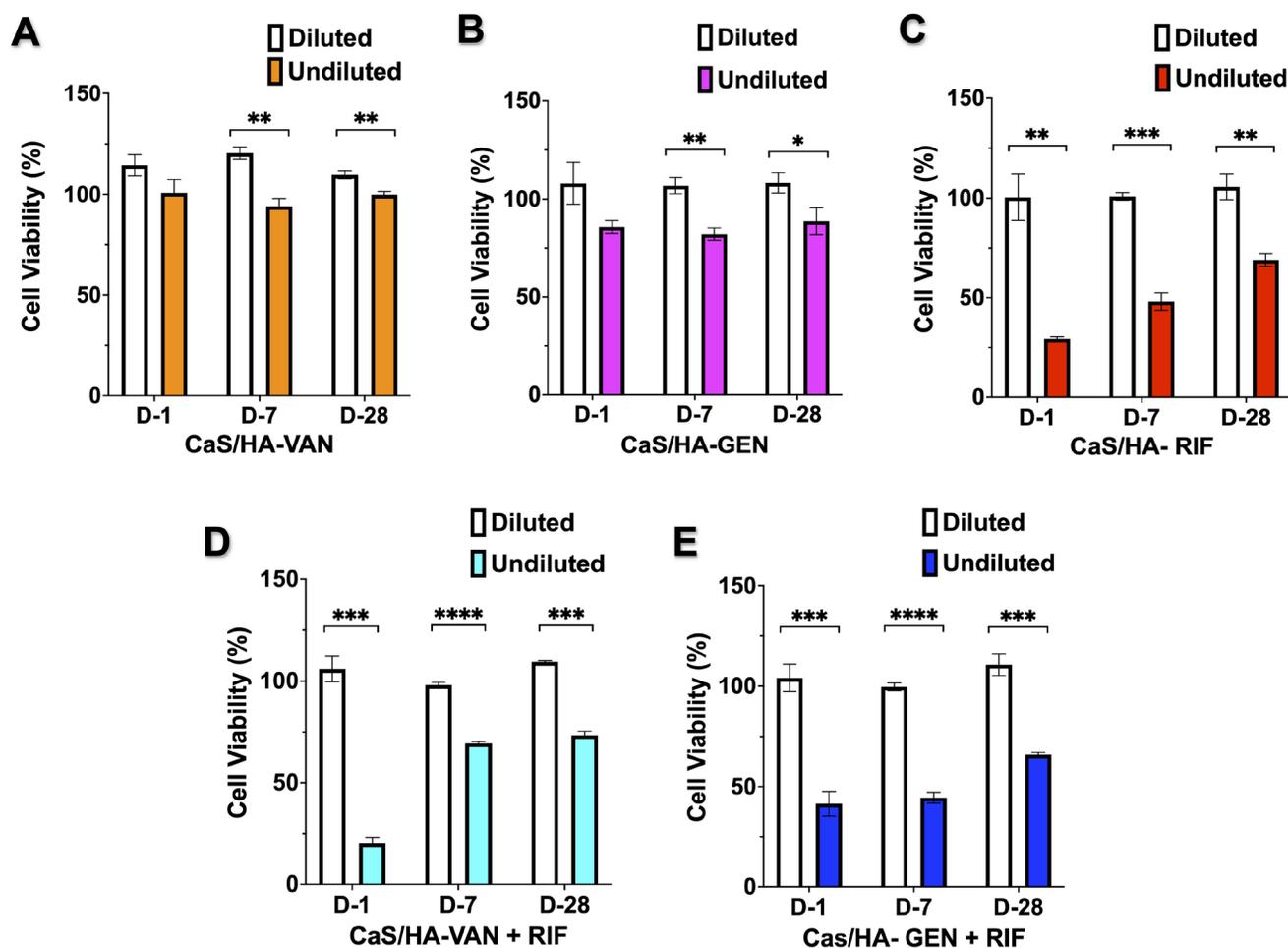


Fig. 3

Cell viability via MTT assay. a) to e) Evaluation of in vitro cytotoxicity on MC3T3-E1 cell lines by MTT assay of both diluted and undiluted antibiotic fractions from day 1, 7, and 28. An independent-samples *t*-test was used to compare the difference in cytotoxicity between diluted and undiluted antibiotic fractions. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001. CaS/HA, calcium sulphate/hydroxyapatite; GEN, gentamicin; RIF, rifampicin; VAN, vancomycin.

were found to be significantly more effective than CaS/HA-VAN/GEN alone composites (Supplementary Tables ii and iii).

**Antibacterial effect of pellets after day 35.** Except for CaS/HA-VAN, all other tested CaS/HA-antibiotics groups showed strong antibacterial effects at day 35 (Figures 5a and 5b). By exhibiting larger ZOI of all groups, CaS/HA-GEN + RIF appears to have the strongest antibacterial effects.

**Efficacy on preformed biofilms.** Overall, there was an excellent biofilm destruction trend observed with CaS/HA-VAN/GEN + RIF combinations compared with CaS/HA-VAN/GEN alone (Figures 6a to 6f). In comparison to CaS/HA-VAN, all tested fractions from CaS/HA-VAN + RIF showed significant destruction of *S. aureus* ATCC biofilms, but against clinical strain P-3 biofilms only day 14 and day 35 achieved significant difference. Almost all tested fractions of CaS/HA-GEN with RIF showed significant biofilm destruction against clinical strain P-3 biofilms. Compared

to CaS/HA-VAN/GEN alone, CaS/HA-RIF fractions exhibited strong antibiofilm properties against both *S. aureus* ATCC and P-3 biofilms (Figure 6). SEM analysis of the preformed biofilm destruction also showed a comparable trend with that of crystal violet staining results (Figure 7).

**Testing for development of bacterial resistance to VAN, GEN, and RIF.** Following continuous treatment of *S. aureus* ATCC 25923 and clinical strain P-3 biofilms with CaS/HA-VAN/GEN/RIF alone antibiotic fractions, the number of viable colony-forming units (CFUs) obtained on antibiotic-free TSA ranged from 0 to  $6.5 \times 10^7$  CFU and  $1.4$  to  $3.1 \times 10^8$  CFU, respectively (Table I). However with complete destruction of biofilm-embedded bacteria, no viable CFUs were obtained for CaS/HA-VAN/GEN + RIF combinations, which were clearly more potent in biofilm eradication than CaS/HA-VAN/GEN/RIF alone groups.

*S. aureus* ATCC and clinical strain P-3 biofilms that had been subjected to CaS/HA-RIF alone developed resistance to RIF, and were grown on plates with 32 mg/l of

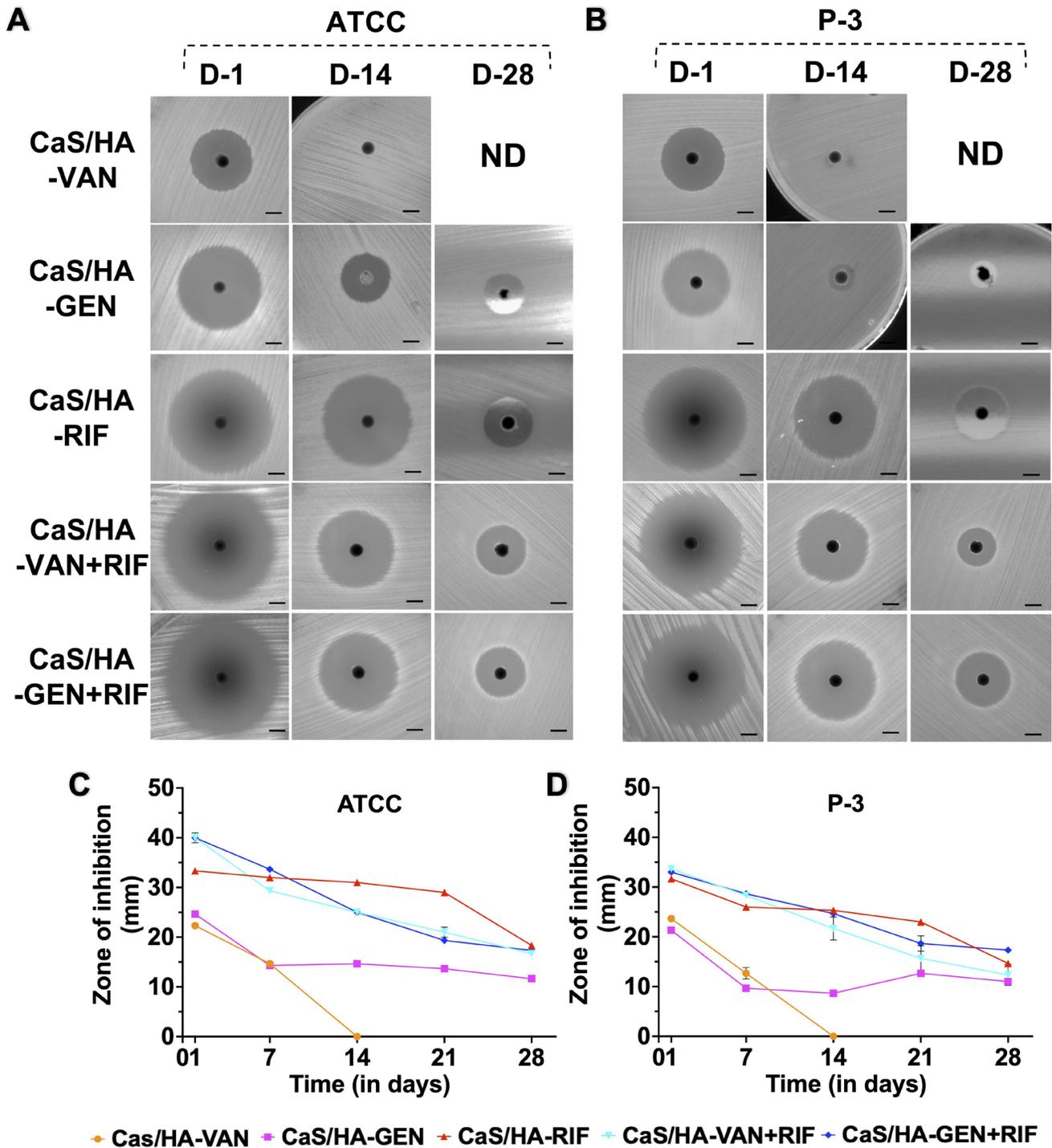


Fig. 4

Testing of antibacterial efficacy of calcium sulphate/hydroxyapatite (CaS/HA)-antibiotic pellets on planktonic bacteria by continuous Kirby-Bauer disk diffusion assay. a) and b) Mueller–Hinton agar plates inoculated with *Staphylococcus aureus* ATCC 25923 and clinical strain P3 showing zone of inhibition (ZOI) obtained on days 1, 14, and 28 (scale bar = 1.6 cm). c) and d) Graph data showing the ZOI (in mm) of CaS/HA-antibiotic pellets against c) *S. aureus* ATCC 25923 and d) *S. aureus* clinical strain P3. For comparison of difference in ZOI between different groups, one-way analysis of variance with Tukey's multiple comparison test was used. Against both *S. aureus* ATCC 25923 and clinical strain P3, CaS/HA-vancomycin (VAN)/gentamicin (GEN) + rifampicin (RIF) combinations were found to be significantly more effective than CaS/HA-VAN/GEN alone composites. Detailed statistical findings are provided in Supplementary Tables ii and iii. ND, not done.

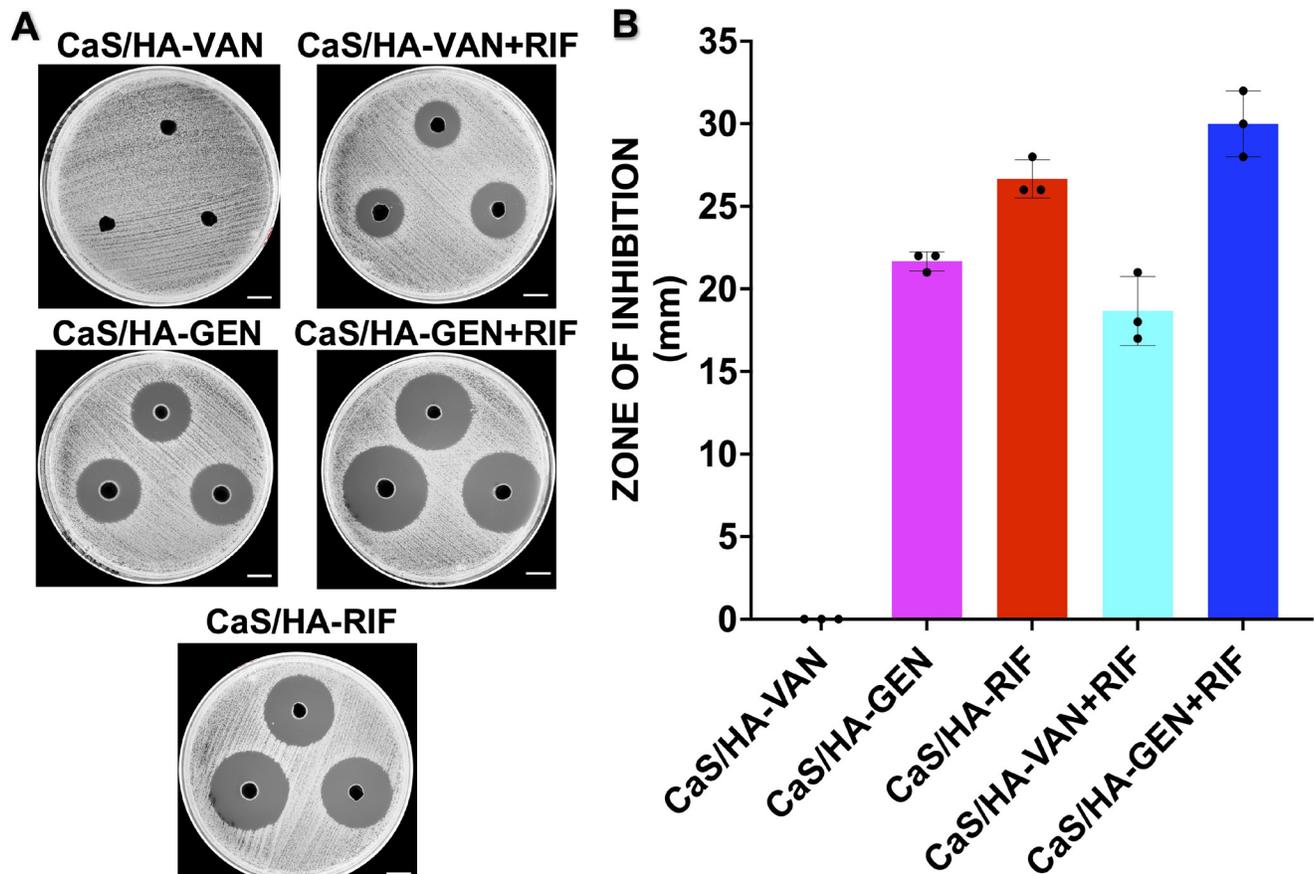


Fig. 5

Antibacterial effect of pellets after day 35. a) Mueller-Hinton agar plates inoculated with *Staphylococcus aureus* ATCC 25923 showing zone of inhibition (scale bar = 2.6 cm). b) Graph data showing the antibacterial effect of the pellets placed in phosphate-buffered saline for 35 days. CaS/HA, calcium sulphate/hydroxyapatite; GEN, gentamicin; RIF, rifampicin; VAN, vancomycin.

RIF indicating high level of resistance. This was further confirmed using MALDI-TOF. However, none of the tested biofilms exposed to RIF in combination with VAN/GEN developed resistance to RIF. Also, no resistance to VAN or GEN was noted for any of the CaS/HA-antibiotic combinations. Bacteria in control wells were sensitive to all tested antibiotics.

### Discussion

Herein, we show that RIF could be combined with, and delivered locally via, the clinically approved biphasic ceramic carrier CaS/HA containing GEN or VAN. There are a few published studies that have reported carriers capable of RIF delivery for the treatment of bone infections.<sup>18,22-24</sup> In an in vitro study, Ahola et al<sup>23</sup> reported the potential of a  $\beta$ -tri-calcium phosphate-based material to deliver RIF or ciprofloxacin, but no combination studies were performed. In two separate studies, Qayoom et al<sup>18,25</sup> reported on a biphasic ceramic material as a carrier for RIF. In their first in vitro study, via local delivery, RIF and isoniazid showed potential for the treatment of bone tuberculosis. In a subsequent paper, the carrier delivered RIF alone for in vivo osteomyelitis treatment without

focusing on the risk of resistance development with RIF monotherapy. Recently, a silorane-based biomaterial made up of two silicon-containing oxiranes combined with  $\text{SiO}_3$  fillers and Lamoreaux's catalyst initiators was able to deliver RIF locally but lacked the biodegradable properties of the CaS/HA material.<sup>22</sup> In comparison to the materials used in these studies, the CaS/HA material used in the present study is a clinically proven and approved material, which potentially makes it easy for future clinical studies and clinical translation of CaS/HA as a carrier of RIF. One of the main indications for local delivery with an antibiotic containing CaS/HA is cavity filling of eradicated osteomyelitis. There may be a potential risk of abrasive HA wear in component-retaining treatment of prosthetic joint infections.

Using our experimental setup, combinations of CaS/HA-GEN/VAN + RIF were injectable at the manufacturer's recommended starting injection time of three or four minutes. However, compared to the CaS/HA-GEN/VAN composites, a significant reduction in injectability was noticed at later timepoints with the addition of RIF. With the use of a commercial mixing system, which is recommended by the manufacturer for mixing CaS/

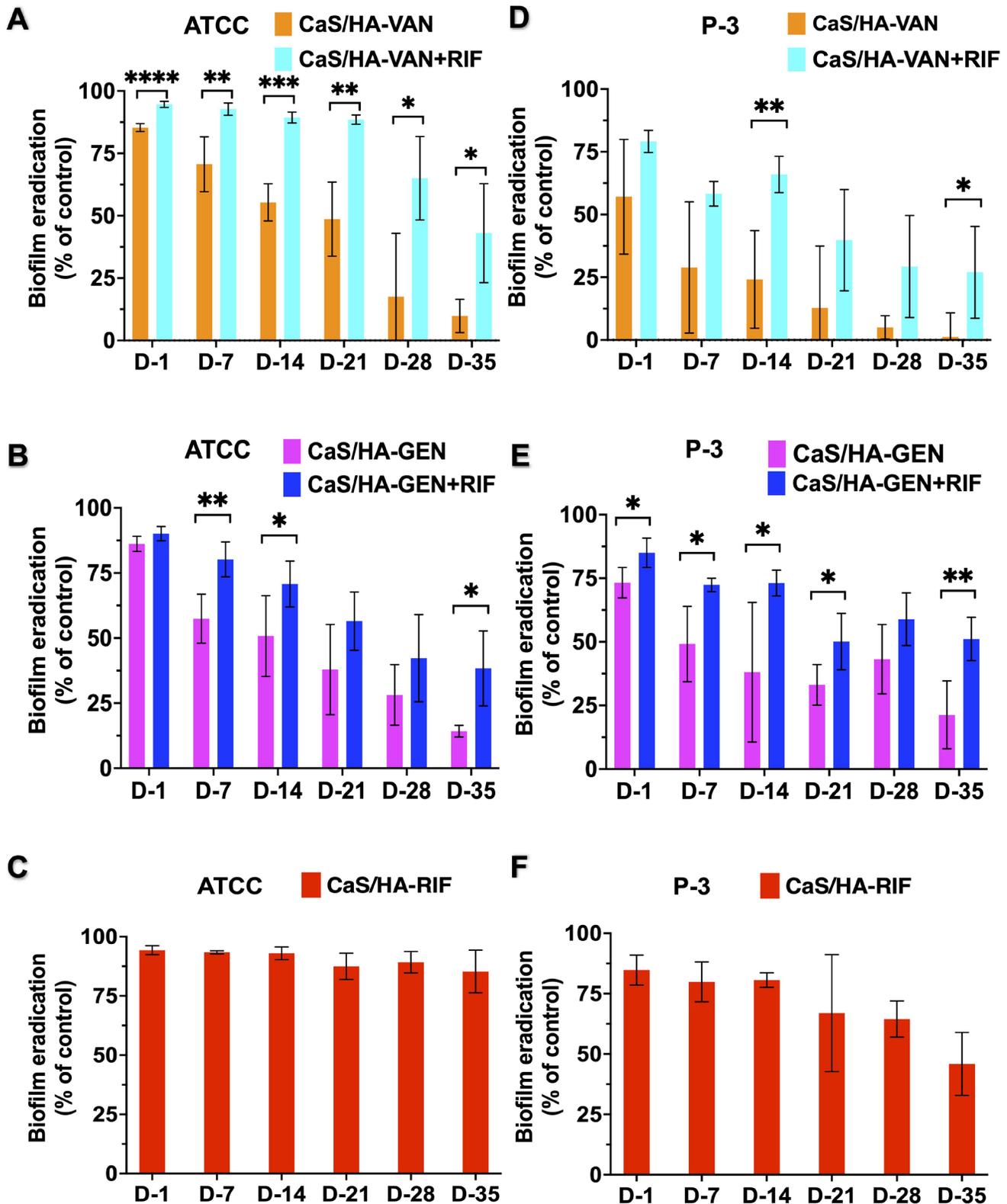


Fig. 6

Efficacy of antibiotic fractions from days 1, 7, 14, 21, 28, and 35 on preformed biofilms. a) to f) Graphical representation of the *Staphylococcus aureus* ATCC 25923 and clinical strain P3 biofilm destruction obtained by crystal violet staining. An independent-samples *t*-test was used to compare the differences in biofilm destruction between calcium sulphate hydroxyapatite (CaS/HA)-vancomycin (VAN)/gentamicin (GEN) with or without RIF. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . RIF, rifampicin.

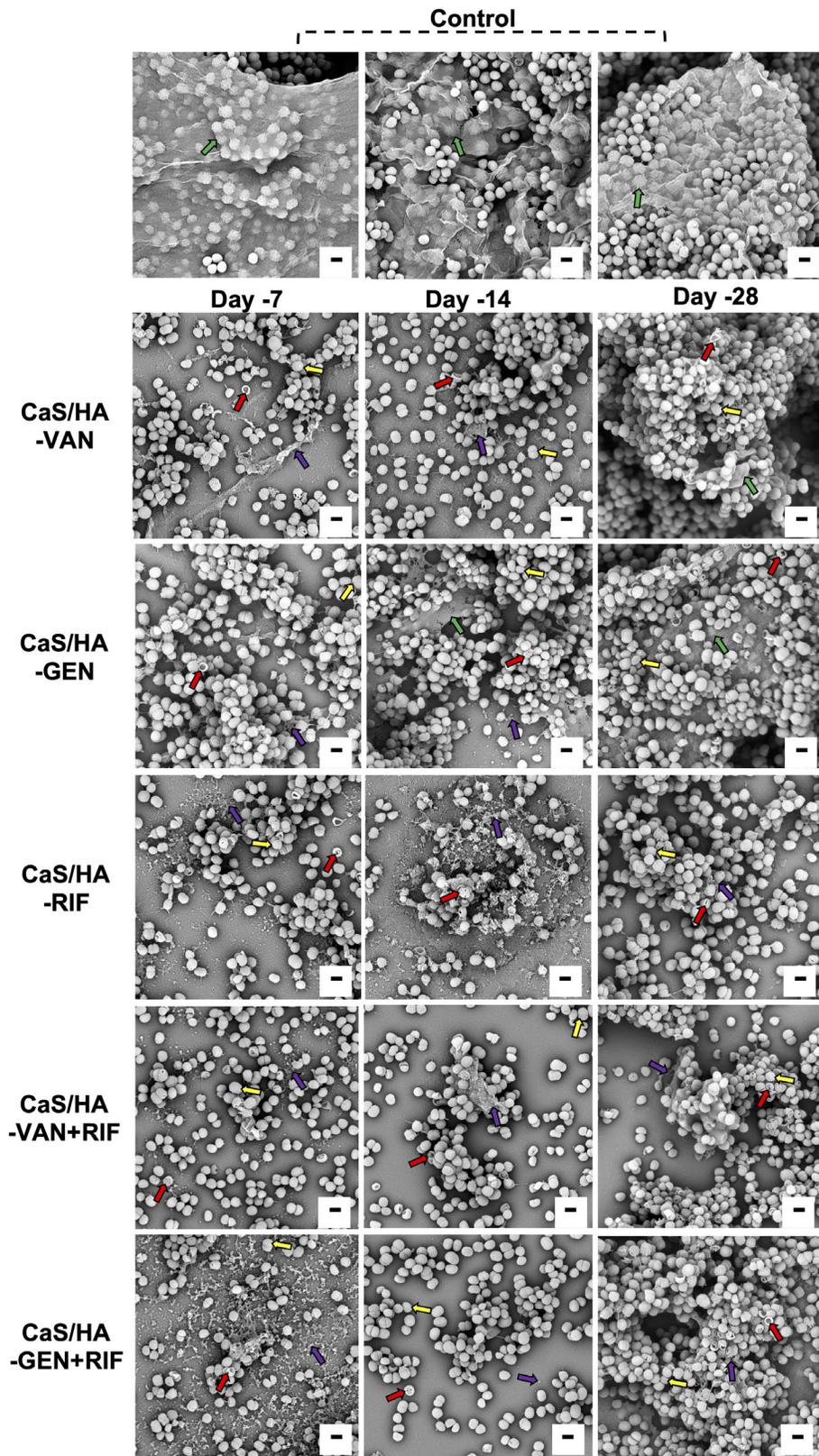


Fig. 7

Scanning electron microscopy images of *Staphylococcus aureus* ATCC 25923 biofilm destruction. For all tested CaS/HA-antibiotic groups, biofilm destruction and antibacterial effects were evident on day 7 and day 14. At day 28, number of planktonic bacteria were increased and biofilm were visible. Green arrow indicates biofilm while violet arrow indicates destroyed biofilm. Red arrow indicates dead bacteria. Yellow arrow indicates membrane disruption and extravasation of cytoplasmic components of bacteria. Scale bar = 1  $\mu\text{m}$ . CaS/HA, calcium sulphate/hydroxyapatite; GEN, gentamicin; RIF, rifampicin; VAN, vancomycin.

**Table 1.** Viable colony-forming units after continuous treatment with antibiotic fractions, and development of rifampicin-resistant CFUs.

Antibiotic fractions from CaS/HA-antibiotics pellets	Bacteria tested, and total CFUs before treatment	Mean remaining viable CFUs (range)	Mean rifampicin-resistant CFUs (range)
CaS/HA-VAN		$5.9 \times 10^7$ ( $5.6 \times 10^7$ to $6.5 \times 10^7$ )	0
CaS/HA-GEN	<i>S. aureus</i>	$2.4 \times 10^7$ ( $1.4 \times 10^7$ to $3.7 \times 10^7$ )	0
CaS/HA-RIF	ATCC 25923,	$1.1 \times 10^7$ (0 to $4.5 \times 10^7$ )	$1.8 \times 10^5$ (0 to $7.5 \times 10^5$ )
CaS/HA-VAN+RIF	$6.5 \times 10^7$	0	0
CaS/HA-GEN+RIF		0	0
CaS/HA-VAN		$1.73 \times 10^8$ ( $1.4 \times 10^8$ to $1.92 \times 10^8$ )	0
CaS/HA-GEN	<i>S. aureus</i>	$2.54 \times 10^8$ ( $2.2 \times 10^8$ to $3.1 \times 10^8$ )	0
CaS/HA-RIF	clinical strain P-3,	$1.05 \times 10^8$ ( $2.5 \times 10^2$ to $2.8 \times 10^8$ )	$9.2 \times 10^7$ (0 to $1.9 \times 10^8$ )
CaS/HA-VAN+RIF	$2.8 \times 10^8$	0	0
CaS/HA-GEN+RIF		0	0

CaS/HA, calcium sulphate/hydroxyapatite; CFU, colony-forming unit; GEN, gentamicin; RIF, rifampicin; VAN, vancomycin.

HA materials, we believe that it would be easier to mix and deliver these composites. However, during surgery clinicians should aim to start the injection immediately after mixing rather than waiting for the recommended injection time, which needs to be revised for the new combinations of antibiotics containing RIF. Although the possibility of increasing the volume of mixing solutions to be used for combinations of RIF was not in the scope of the present study, future studies are planned to explore this possibility as well.

Adding RIF substantially prolongs the setting time of PMMA to more than 30 minutes, preventing it from being used clinically as a RIF carrier.<sup>10</sup> To circumvent this, Sanz-Ruiz et al<sup>26</sup> used microencapsulation of RIF to introduce it in PMMA, while Cyphert et al<sup>27</sup> added RIF to insoluble cyclodextrin. However, none of these methods of RIF incorporation in PMMA are in clinical use. In the present study, all tested combinations of RIF showed a recommended setting time of 15 minutes when combined with either GEN or VAN containing CaS/HA material.

The elution patterns of all tested antibiotics from CaS/HA had an initial burst release, which is congruent with previous reports on CaS/HA-VAN or GEN composites.<sup>28,29</sup> Since the MBEC levels of many antibiotics are reported to be 1,000-fold higher than the MIC levels, high levels of antibiotics delivered by burst release are indeed necessary to penetrate the biofilms and achieve bactericidal activity.<sup>30,31</sup> In agreement with the previous reports, following the initial high concentration, both GEN and RIF, either alone or in combination, maintained a sustained low concentration, still well above the MIC levels of most common pathogens such as staphylococci.<sup>18,29</sup> Similarly, as reported by Stravinskas et al,<sup>28</sup> from the CaS/HA-VAN + RIF composites, VAN with concentrations above the MIC levels was eluted for three weeks but from CaS/HA-VAN it was only eluted for two weeks. This could be attributable to the differences in the experimental setup with previous in vitro and clinical studies. Although the mixing of RIF did not affect the cumulative release profile of both VAN and GEN, it significantly changed the release profile of RIF itself. Since both GEN and VAN have no accretion to particulate HA, their fast elution from the CaS-based material might have influenced the RIF as well.<sup>28,29</sup>

Most of the current guidelines for the treatment of bone- and implant-associated infections recommend the use of longer duration of systemic treatment of RIF in combination, one to two weeks post-surgery.<sup>32,33</sup> However, its timing of introduction in treatment, length of use, dose, and efficacy, especially in acute *Staphylococcus* or *Cutibacterium* infections, is repeatedly debated.<sup>34</sup> A recent multicentre observational study reported the efficacy of RIF in acute staphylococcal PJI treated with debridement, and found no association with the dose of RIF but its early start within the first five days after debridement was associated with treatment failure.<sup>9</sup> The explanation pointed to a possible drug-drug interaction when the bacteria are still in planktonic phase, but the authors fell short of any definitive conclusions. In contrast, Becker et al<sup>35</sup> reported no association on the timing of RIF introduction in treatment, but rather a minimum RIF treatment duration of 14 days or more is recommended. In a cohort of acute staphylococcal PJI treated with debridement, implant retention, and a short course of RIF which the authors started intraoperatively, Schepers et al<sup>36</sup> reported good clinical outcomes without any resistance development to RIF. So the available literature is inconclusive on the immediate starting of RIF following surgery due to issues with development of resistance. Moreover, it is well known that long-term systemic or oral treatment of RIF is associated with systemic toxicities and often patients find it difficult to comply with the treatment regimen. In such cases, local application of RIF via a controlled delivery system could definitely circumvent such problems. Since there is no current recommendation on the duration of local antibiotic delivery, and recommended systemic treatment duration of bone infections is six to 12 weeks, we believe that the RIF release profile observed in the current study may be valid.

Due to the low dose of RIF added to the material, the possible systemic effects should be less than those of systemic administration of the same drug. We used a concentration (300 mg) that corresponds to half of one clinically used dose (600 mg). Based on the in vitro data, it would not be possible for us to comment on the potential development of resistance, particularly in the gut microbiome, but in an in vivo model Kim et al<sup>37</sup> reported

no RIF resistance development following treatment with rifaximin, a drug belonging to the same class. We hope that future in vivo data will give us more detailed information on low level of RIF in systemic circulation, and how it may affect the patient's normal bacterial flora.

Although local delivery via carriers results in high initial level of antibiotics in bone infections, it should not negatively affect either osteoblasts or their ability to induce bone formation. Previous studies using different antibiotic classes reported their toxic effects in high concentrations in vitro on osteoblast cells.<sup>38-41</sup> Isefuku et al<sup>40</sup> reported that RIF at concentrations of 3 µg/ml can decrease the proliferation of osteoblast-like cells in vitro. However, using a 3D-printed calcium phosphate scaffold containing RIF (~33 µg/scaffold) and sitafloxacin in a murine model of femoral implant-associated osteomyelitis, Trombetta et al<sup>42</sup> conversely reported increase in bone formation at three and ten weeks.<sup>42</sup> In an in vivo osteomyelitis rat model, Qayoom et al<sup>25</sup> implanted RIF 3 mg/animal locally with nano-HA, which corresponds to clinically used RIF dose, 600 mg. The locally applied nano-HA-RIF combination completely eradicated the infection and increased bone formation, and the defect was completely healed. In a recent study, single and multiple doses of RIF < 16 µg/ml had no impairment on osteoblast proliferation or metabolism, but results differed when RIF concentrations were ≥ 50 µg/ml.<sup>43</sup> Interestingly, when the same authors monitored the effect of multiple doses of RIF 8 µg/ml between *S. aureus*-infected osteoblasts and uninfected control groups during 21 days of osteogenesis, a lower amount of mineralized extracellular organic matrix was observed in *S. aureus*-infected osteoblasts on day 7 but this significantly increased on day 21. These mentioned reports indicate that even at minimal concentrations RIF is toxic to normal uninfected osteoblast cells, but in bone infections where bacteria such as *S. aureus* are hiding within the osteoblasts, minimal to high RIF concentrations seem to improve the osteoblasts' viability in the long term. In the present study, in vitro RIF elution shows concentrations > 16 µg/ml for at least two weeks, which clearly had an effect on the osteoblasts. However, we would also like to highlight the fact that bone is a tightly organized matrix of collagen and HA with embedded osteocytes, osteoblasts, and osteoclasts, therefore perfusion of drugs within this tight matrix is somewhat difficult, which should potentially help in alleviating the cytotoxic effect of antibiotics on bone and its cells. Previously, our group has shown that the carrier CaS/HA also plays a role in reducing the toxic effects of the drugs which arise when they are administered alone in the targeted site,<sup>15</sup> but conclusions on the actual biocompatibility and osteogenic properties of CaS/HA-GEN/VAN + RIF combinations could only be drawn from an experimental in vivo study designed to answer those particular questions.

While testing the antimicrobial efficacy, we used entire antibiotic-loaded biomaterial pellets for disk diffusion assay. In a previous study, Wahl et al<sup>44</sup> raised the concerns of this method over variation of antibiotic quantity in

each pellet and its contact with agar surface. During the entire study period, we did not observe any notable variations in ZOI within each group, which means that the concentration of antibiotics present in each pellet was nearly equal. Although the contact surface of the pellet with the agar is not standardized and varies over time, it could be managed to be within an acceptable limit by standardizing the placement of CaS/HA pellets on the agar. The antibacterial effect of CaS/HA-VAN pellets decreased sharply by day 12 but correlated well with the VAN elution profile at day 14, when the VAN concentrations were below the MIC. Also, this validates our results obtained from the antibiotic release experiment. A moderate antibacterial effect of GEN was seen until day 28, and a strong antibacterial effect of RIF was seen for almost four weeks. These results were also in line with the release profile of both GEN and RIF. Using CaS beads, Aiken et al<sup>24</sup> reported a similar trend of comparable release profile and antimicrobial efficacy obtained with modified Kirby-Bauer assay of RIF. CaS/HA-RIF exhibited strong antibacterial effects for four weeks, but in the case of CaS/HA-VAN/GEN + RIF this was three weeks. As mentioned previously, the release trend of RIF when combined with VAN/GEN might be the reason. However, as shown repeatedly in previous studies, to prevent the development of RIF resistant bacterial populations, combinations of RIF with other antibiotics exert better antibiotic effects on biofilms than non-RIF combinations.<sup>31,45</sup> Moreover, in clinical conditions the initial burst release of antibiotics could potentially kill the majority of bacteria, and thereafter the residual bacterial populations could be eradicated by the sustained MIC levels released from the carriers.

Although the antibacterial effects of the CaS/HA-antibiotic pellets were tested by continuous disk diffusion assay, we also evaluated the antibacterial effects of the pellets used for the antibiotic elution assay, since they had been placed in PBS which was replenished at predetermined timepoints, thereby closely mimicking in vivo conditions. No extended antibacterial effect exhibited by CaS/HA-VAN was expected since its elution profile showed no VAN release by day 21. In parallel to the consistent ZOI observed during continuous disk diffusion assay, CaS/HA-GEN showed antibacterial effects at day 35. This persistent antibacterial effect of CaS/HA-GEN points to the possibly limited interaction between GEN and HA particles or the slower resorption of CaS. However, this needs to be validated in future studies. The strong antibacterial effects exhibited by CaS/HA-VAN/GEN + RIF combinations after 35 days confirm RIF accretion to microparticulate HA.<sup>46</sup> This implies that these composites have the potential to elute antibiotics that could exert an antibacterial effect for at least a month even under actual in vivo conditions through either passive diffusion via material fluid interaction, material degradation, or osteoclast scavenging of antibiotic-bound HA particles.

Although biofilm-embedded bacteria are characterized by their resistance to antibacterial agents, our results

showed that most of the tested antibiotic fractions of CaS/HA-VAN/GEN + RIF combinations showed excellent antibiofilm effects against 48-hour-old biofilms. It is important to note the significant difference in biofilm destruction effects between fractions of CaS/HA-VAN/GEN with or without RIF, within two weeks and at day 35. This highlights the potential of the initial high concentrations of combinations of the selected antibiotics to eradicate the biofilm. Using a porcine model of osteomyelitis, Blirup-Plum et al<sup>47</sup> reported that following limited or extended debridement, CaS/HA-GEN alone cannot eradicate infection without the addition of systemic antibiotics. Drawing similarity from their findings, our results show that even after continuous treatment of biofilms, CaS/HA-VAN/GEN fractions could not effectively eradicate established biofilms. Further, a recent study by Goetz et al<sup>48</sup> showed the significant effect of RIF combinations in reducing the bacterial counts on bone and implants compared with antibiotic monotherapy, and highlights the crucial therapeutic role of RIF combinations in bone infections.

Various studies have reported that RIF monotherapy leads to rapid development of resistance.<sup>49,50</sup> In agreement, we also noted the emergence of RIF resistance following continuous treatment of all tested *S. aureus* strains with CaS/HA-RIF fractions. Interestingly, when RIF was combined with VAN or GEN, resistance to RIF was not observed. This is not in line with a previous study by Holmberg et al,<sup>50</sup> where low rates of resistance to RIF were noted even when combined with other antibiotics. As observed, the delivery mode with an initial high concentration of antibiotics, and their excellent antibacterial and antibiofilm properties, could be the possible reasons for not developing RIF resistance. In agreement with a study by Bidossi et al,<sup>51</sup> no resistance to VAN or GEN was detected in the present study. In another study, Butini et al<sup>52</sup> reported the emergence of resistance to GEN following exposure to sub-MIC concentrations of GEN eluted from beads of CaS/HA-GEN. By employing the continuous exposure of sub-MIC concentrations of GEN, their experimental methodology differed from the present study where we exposed fractions from different timepoints including the initial high concentration of GEN from day 1 to above MIC at day 35.

This study has a few limitations. First, the in vitro findings need to be validated in relevant animal models. However, being a proof-of-concept study, we believe that our findings are valuable and aid the ongoing in vivo experiments. Second, although the use of current release profile setup using PBS is a well-established system to study the release of various biomolecules added to different biomaterial carriers, we acknowledge that an elution test using plasma or serum as exchange fluid could have added values to our observations.<sup>29,44</sup> For biofilm eradication we used a 48-hour-old immature biofilm rather than a 14-day-old biofilm, which is the reported time for biofilm maturation post-infection.<sup>53</sup> However, in a recent study Okae et al<sup>54</sup> reported that the

MBEC of GEN and cefazolin obtained on 72-hour-old biofilms was similar to or even twice that of 14-day-old biofilms. VAN also showed a similar trend when tested using a methicillin-resistant *S. aureus* strain.<sup>54</sup> Taking these findings into consideration, we believe that the preliminary data obtained using a 48-hour-old biofilm provide useful insights about the biofilm destructive action of RIF combinations eluted via CaS/HA carrier. Finally, an analysis of the mechanical properties of CaS/HA alone and in combination with antibiotics was lacking in this study. However, these osteoconductive biomaterials are used as bone void fillers, and in weightbearing applications need additional mechanical support.

In conclusion, in this study we demonstrated a biphasic ceramic platform for the local and sustained delivery of RIF along with GEN or VAN. With a favourable injectability and sustained release of antibiotics, this bioresorbable carrier could avoid the off-target toxic effects of systemically administered RIF, and has the potential to be used as a novel treatment regime for bone infections. Compared to the VAN or GEN alone composites, RIF and VAN or GEN combinations did yield convincing antibacterial and antibiofilm effects without the development of resistance to RIF. Since CaS/HA is a clinically approved biomaterial for bone applications, translation of this as an off-the-shelf RIF carrier would be easier. However, for further validation of our in vitro findings, in vivo models are necessary and currently ongoing.

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### Supplementary material



Details of the following: materials used in the study, amount and concentration of materials and antibiotics used for pellet preparation, scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy protocol for analysis of calcium sulphate/hydroxyapatite composites, testing of injectability and setting time, degradation assay, MTT assay, efficacy on planktonic bacteria by continuous Kirby-Bauer disk diffusion assay, antibacterial effects of the pellets after day-35, efficacy on preformed biofilms, biofilm SEM protocol and viable bacteria after continuous treatment with antibiotic fractions, and development of resistance to vancomycin, gentamicin, and rifampicin. Figure showing the continuous Kirby-Bauer disk diffusion assay of pellets, and tables showing their detailed statistical findings.

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#### Author information:

- S. Sebastian, PhD, Research Engineer, Postdoctoral Fellow
- F. Tandberg, MD, Medical Student
- Y. Liu, MD, Doctoral Student
- D. B. Raina, PhD, Associate Researcher, Postdoctoral Fellow
- M. Tägil, MD, PhD, Professor
- L. Lidgren, MD, PhD, Emeritus Professor  
Department of Clinical Sciences, Orthopedics, Lund University Faculty of Medicine, Lund, Sweden.
- M. Collin, PhD, Professor, Division of Infection Medicine, Department of Clinical Sciences, Lund University Faculty of Medicine, Lund, Sweden.

#### Author contributions:

- S. Sebastian: Investigation, Methodology, Data curation, Formal analysis, Funding acquisition, Writing – original draft.

- F. Tandberg: Investigation, Data curation, Writing – review & editing.
- Y. Liu: Investigation, Methodology, Data curation, Writing – review & editing.
- D. B. Raina: Conceptualization, Project administration, Supervision, Resources, Validation, Writing – review & editing.
- M. Tägil: Supervision, Resources, Validation, Writing – review & editing.
- M. Collin: Supervision, Resources, Validation, Writing – review & editing.
- L. Lidgren: Conceptualization, Supervision, Funding acquisition, Resources, Validation, Writing – review & editing.

#### Funding statement:

- The authors disclose receipt of the following financial or material support for the research, authorship, and/or publication of this article: funding from the Royal Physiographic Society of Lund (grant number 142843-2020) and Landshövding Per Westlings Minnesfond (grant number RMv 2021-0022).

#### ICMJE COI statement:

- Y. Liu, D. B. Raina, M. Tägil, and L. Lidgren are stockholders in Moroxite AB, Sweden.
- L. Lidgren is a board member of Bonesupport AB, Sweden and Orthocell, Australia.
- All other authors have nothing to declare.

#### Data sharing:

- Data are available upon reasonable request to the corresponding author.

#### Acknowledgements:

- The authors would like to thank Anna Stefansdottir and Linnéa Polland for providing the clinical strains of *Staphylococcus aureus*. The authors deeply acknowledge the excellent technical assistance provided by Maria Baumgarten in performing the biofilm scanning electron microscopy imaging and its analysis. Ann-Charlotte Strömdahl, Ganna Petruk, Ariane Neumann, and Anna Dahlman are acknowledged for all their help during the bacteriology laboratory work. Xiaoya Li is greatly acknowledged for her help during the Fourier transform infrared spectroscopy experiment.

#### Open access funding

- The authors report that they received open access funding for their manuscript from Lund University.

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