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Enzymes-enhanced antibiotic therapy reduces biofilms to undetectable levels in an implant-associated infection model

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Implant-associated infections caused by biofilm-forming bacteria, such as *Staphylococcus aureus*, remain a major clinical challenge due to their high tolerance to conventional antibiotic therapies. We report a dual-targeted therapeutic strategy that combines a tri-enzymatic cocktail designed to degrade key components of the biofilm matrix (TEC; comprising a DNA/RNA endonuclease, an endo-1,4-β-D-glucanase, and a β-*N*-acetylhexosaminidase), with vancomycin, both delivered via a thermosensitive poloxamer 407 hydrogel, for localized treatment of *S. aureus* biofilms. The formulation was evaluated both in vitro, on titanium-adherent biofilms, and in vivo, using a model of tissue cages containing titanium beads implanted in the back of guinea pigs. Animals additionally received intraperitoneal administration of vancomycin alone or combined with rifampicin. In vitro, this formulation enabled sequential drug release, with TEC delivered within the first 24 h and vancomycin for up to 96 h, and achieved >5 Log_{10} reductions in CFU counts after two applications at 48 h interval. In vivo, biofilm-associated bacterial counts reached the detection limit (100 CFU; >5 Log_{10} decrease from the initial inoculum) in 75% of implants 1 day post-treatment and remained undetectable in 37.5% of them 5 days post-treatment, with no emergence of resistance. Treatment efficacy was reduced if TEC or vancomycin were omitted in the hydrogel or if rifampicin was not included in the intraperitoneal treatment. Vancomycin in the hydrogel also prevented the emergence of rifampicin resistance. These findings underscore the therapeutic potential of a dual-targeted approach, combining biofilm disruption with local sustained antibiotic release, to improve the management of implant-associated infections.

Implant-associated infections (IAIs) remain significant and unresolved complications in modern medicine. Among these, prosthetic joint infections (PJIs) are particularly challenging due to their chronic nature[1], high rate of treatment failure[2,3], and substantial impact on both patients and healthcare systems[4–6]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is identified in 12.8–48.1% of the cases, with considerable geographical variations in prevalence[7]. MRSA is frequently associated with acute infections[8] and with an increased risk of treatment failure following debridement, antibiotic, and implant-retention (DAIR) procedures as compared to infections caused by Methicillin-susceptible *Staphylococcus aureus* (MSSA), likely reflecting the more limited range and lower efficacy of antibiotics available against MRSA[9]. Infections by *S. aureus* in general have been recognized as an independent risk factor of poor outcome in implant-retention strategies[10]. While DAIR offers the benefits of a less invasive approach and may preserve joint function[11], reported failure rates range from 16% to 57%[2,12,13]. Some of these failures might be attributed to the antibiotic tolerance of biofilm-forming bacteria[3,14].

The capacity of *S. aureus* to form biofilms on implant surfaces[15,16] represents a major obstacle to effective treatment. The biofilm matrix acts as a protective barrier, shielding bacteria from host immune responses and significantly impairing or delaying the ability of antibiotics to effectively reach and act upon embedded bacteria. In addition to this mechanical

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protection, biofilm-associated bacteria undergo profound phenotypic changes, including reduced growth rates and altered gene expression profiles, which further contribute to antibiotic tolerance. This protective effect leads to chronic and recurrent infections. Biofilm-associated bacteria can tolerate antibiotic concentrations up to 1000 times higher than those required to eradicate planktonic bacteria[17], highlighting the limitations of conventional antimicrobial therapies to treat IAIs. In response, innovative strategies considering the local administration of supratherapeutic concentrations of antibiotics, combined or not with adjuvants, are evaluated. Among them, bacteriophages[18,19] or the endolysins[20–22] they produce have regained interest, including in the clinics. Our team has instead focused on biofilm-disruptive enzymes as adjuvants[23,24]. These enzymes degrade components of the extracellular matrix to enhance antibiotic access to embedded bacterial populations[25].

In previous work, we demonstrated that a tri-enzymatic cocktail (TEC; comprising a DNA/RNA endonuclease [degrading extracellular DNA/RNA], an endo-1,4-β-D-glucanase, and a β-*N*-acetylhexosaminidase [endoglycosidases respectively targeting β(1–4)-O-glycosidic linkages and poly-β(1–6)-*N*-acetylglucosamine abundant in biofilms]) reduced DNA and polysaccharide contents in MRSA biofilms[23]. When combined with supratherapeutic concentrations of vancomycin, TEC exhibited potent antibiofilm activity against MRSA infections[23], both in vitro and in vivo.

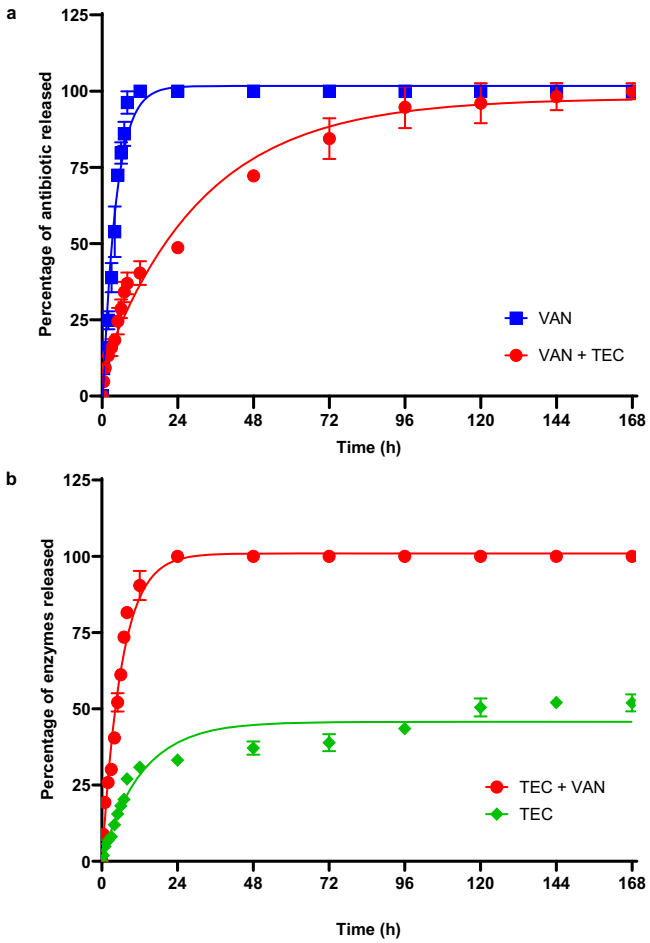


Fig. 1 | In vitro release kinetics of vancomycin and enzymes from 25% w/v poloxamer 407 hydrogel in a TRIS-HCl buffer 20 mM at pH 7.4. a Vancomycin release from P407 loaded with vancomycin alone (VAN; initial concentration: 20 mg/mL; blue square) or combined with TEC (VAN + TEC; red circles). **b** TEC release form P407 loaded with TEC alone (400 U/mL of aspecific DNA/RNA endonuclease, 50 U/mL of endo-1,4-β-D-glucanase, and 2.6 U/mL of β-*N*-acetylhexosaminidase; green diamonds), or combined with VAN (TEC + VAN; red circles). Values are means ± SD of triplicates ($n = 3$).

These agents were incorporated in a thermosensitive poloxamer P407 (P407) hydrogel that showed desirable properties for local antibiotic delivery: it is biodegradable, biocompatible, well-tolerated, and capable of sustaining drug release[26,27]. P407 is already widely used for biomedical applications[28], transitioning from a liquid at low temperatures to a gel at body temperature[29]. In in vitro assays using biofilms growing on titanium coupons, we showed that P407 containing vancomycin and TEC significantly reduced both CFU and biofilm biomass. In vivo, using a model of MRSA-infected tissue cages containing titanium beads implanted in the back of guinea pigs, a single local administration of the same formulation of vancomycin and TEC, coupled with intraperitoneal vancomycin, was also highly effective[23]. This treatment caused an additional reduction of bacteria adhering to the cages and the beads of 4.3 Log_{10} CFU as compared to intraperitoneal vancomycin alone. However, this effect was transient, with partial rebound of CFU observed following treatment cessation, emphasizing the resilience of biofilm-associated infections[30] and the challenge of killing bacteria in these structures.

The aim of the present study was to enhance the efficacy of this promising treatment by refining the local delivery system and optimizing systemic antibiotic therapy. To this end, the weight-to-volume ratio of poloxamer P407 was increased to modulate the release kinetics of the embedded active agents[31]. Moreover, to address the transient nature of the therapeutic effect observed in our previous study[23], we also repeated the local administration of the TEC-vancomycin formulation after 48 h. While repeated local injections are uncommon and remain poorly documented in the context of PJIs, a few recent clinical cases exploring this approach have been reported[19]. This strategy was inspired by routine orthopedic practices for degenerative joint diseases, which involve repeated intra-articular injections[32]. Similarly, local delivery protocols involving repeated or continuous intra-site administration of antibiotics have also been investigated in fracture-related infections, to maintain therapeutic concentrations[33]. We therefore aimed to transpose this established strategy to the IAI context, hypothesizing that prolonged local exposure to antimicrobial agents could improve treatment outcome. Lastly, to more closely replicate the clinical management of MRSA-mediated PJIs, we co-administered the drug-loaded hydrogel locally with a combination of vancomycin and rifampicin by intraperitoneal injection[1].

In line with our previous work, this new therapeutic scheme was evaluated in vitro, on biofilms grown on titanium coupons, and in vivo, in guinea pigs with implanted teflon-perforated cages containing titanium alloy beads. In brief, these adjustments allowed to achieve undetectable levels of adhering bacteria in a high proportion of the cages, including 5 days after the end of the therapy.

Results

Kinetics of release and stability of active agents from P407 hydrogel in vitro

As compared to our previous work[23], we increased poloxamer concentration in the hydrogel from 20 to 25% w/v and doubled vancomycin concentration. We therefore re-examined the kinetics of release of vancomycin (VAN) and TEC from this new formulation in vitro. VAN formulated alone was rapidly released from P407 (κ_{VAN}: 0.23 h^{-1} vs. 0.18 h^{-1} in the previous formulation), with 100% release achieved within 24 h When formulated in combination with TEC, VAN release was 7-fold slower ($\kappa_{VAN\text{-}TEC}$: 0.03 h^{-1} vs. 0.23 h^{-1} in the previous formulation), with total release occurring over 96 h (Fig. 1a; Table 1). The opposite behavior was observed for TEC (Fig. 1b; Table 1): TEC was released twice faster in combination with VAN than alone (κ_{TEC}: 0.08 h^{-1} vs. $\kappa_{TEC+VAN}$: 0.16 h^{-1}; $p < 0.001$), a rate similar to that observed with the previous formulation in both conditions (κ_{TEC}: 0.16 h^{-1} vs. $\kappa_{TEC+VAN}$: 0.15 h^{-1}). Thus, increasing P407 concentration to 25% allows releasing enzymes first, and VAN for a longer period, which is desirable based on the mode of action of enzymes degrading the biofilm matrix.

In parallel, we evaluated the stability of the active agents in this formulation over the time course of this experiment. For VAN, which was quantified using a microbiological assay, we can confirm full recovery (100%

of the added concentration) as long as release was complete, indicating that the drug retained its full activity within the hydrogel. For TEC, stability was evaluated by assessing the activity of the released enzymes on a fresh biofilm. TEC activity remained >80% of that of a freshly prepared tri-enzymatic cocktail during 24 h and declined to 35% after 48 h (Supplementary Fig. 1). For this reason, two successive administrations of the hydrogel separated by 48 h were used in further experiments.

Rheological characterization of P407-based formulations containing vancomycin, TEC, or their combination

Rheological analyses were conducted to determine whether the incorporation of active agents into P407 modified its sol-gel transition characteristics. The tested formulations included P407 alone, P407 with VAN, with TEC, or with their combination (VAN + TEC). As shown in Fig. 2, the sol-gel transition temperature (Tgel), defined as the crossover between the elastic (G′) and viscous (G″) moduli, was unaffected by the addition of VAN (22.2 ± 0.3 °C, ns), but significantly decreased with TEC (17.7 ± 0.6 °C) and VAN + TEC (17.0 ± 0.0 °C), compared to unloaded P407 (22.2 ± 1.4 °C; $p < 0.05$). This reduction in Tgel suggests an earlier gelation onset, which may limit injectability at room temperature due to faster transition to the gel state, thus requiring cold storage prior to administration. However, from a clinical perspective, a lower Tgel can be advantageous, as it promotes rapid gelation once injected into the joint, potentially enhancing local drug retention and minimizing leakage from the injection site.

The plateau elastic modulus (G′) recorded between 35.5 and 38.5 °C remained within the kilopascal range across all groups (2.3–3.2 kPa), with no statistically significant differences among formulations. This indicates a preservation of gel mechanical integrity at physiological temperatures.

Gelation kinetics, evaluated through the slope of the G′ rise (ΔG′/ΔT), were markedly altered by the presence of active agents. Compared to P407 (155 ± 2 Pa/°C), VAN only slightly increased this value (166 ± 2 Pa/°C, $p < 0.05$), and this effect was amplified with VAN + TEC (180 ± 2 Pa/°C). In sharp contrast, TEC caused a marked decrease in gelation rate (34 ± 4 Pa/°C; $p < 0.05$), indicating slower network formation. Overall, these findings demonstrate that TEC, alone or combined with VAN, significantly modulates the thermogelling behavior of P407 by lowering its transition temperature and altering gelation kinetics, while maintaining its final mechanical strength.

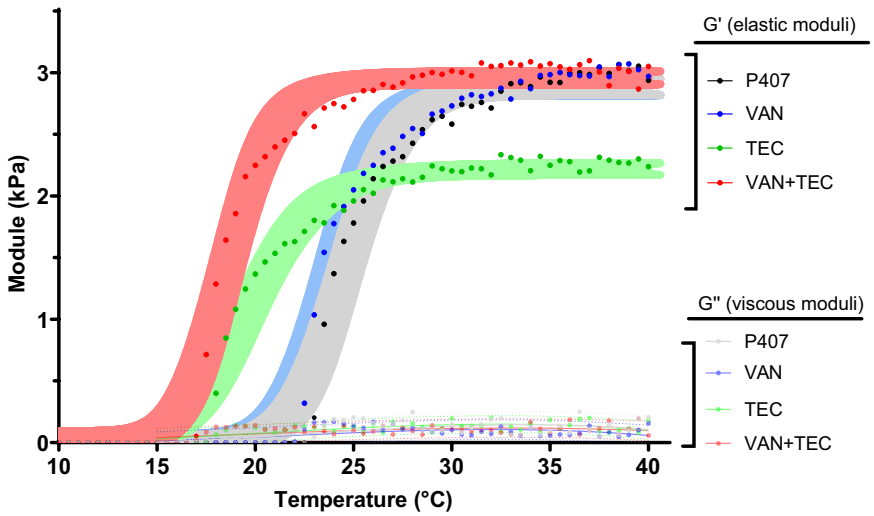
Table 1 | Parameters of release kinetics study

Active agents in P407	Parameters		Half-life (h) [95% CI]
	K (h^{-1})	*p*-value	
Vancomycin (VAN)[a]			
Alone	0.23	0.03	2.94 [2.64; 3.27]
With TEC	0.03		22.10 [17.19; 28.66]
TEC[a]			
Alone	0.08	<0.001	8.82 [6.87; 11.9]
With VAN	0.16		4.26 [3.93; 4.64]

Constant rate K (h^{-1}) and half-life $t_{1/2}$ (h).
[a] Concentrations in Poloxamer 407: vancomycin, 20 mg/mL; TEC, 400 U/mL of aspecific DNA/RNA endonuclease, 50 U/mL of endo-1,4-βD-glucanase and 2.6 U/mL of β-N-acetylhexosaminidase.

In vitro antibiofilm activity of antibiotics and TEC formulated in P407 25% w/v

The activity of vancomycin, TEC and their combination formulated in P407 hydrogel was evaluated on MRSA biofilms grown on titanium coupons. These biofilms were incubated with P407 loaded or not with VAN (20 mg/mL), TEC, or their combination for up to 48 h, with samples taken every 24 h to assess residual CFU counts and biomass. P407 alone had no effect (no difference with control conditions; not shown). The maximal reduction in CFU (2.6 Log_{10}) and biomass (79%) was reached after 24 h for P407 containing both VAN and TEC, with no gain in activity when the incubation was prolonged for an additional 24 h period (Fig. 3 and Supplementary Table 1 for detailed statistical analysis). As compared to P407 loaded with VAN alone, the VAN + TEC combination allowed to gain 1.4 Log_{10} reduction in CFU counts and a 16% reduction in biomass. As expected, TEC alone was ineffective on CFU and as effective as the combination on



Conditions	Tgel (°C)	G' plateau 35.5°C–38.5°C (kPa)	Slope ΔG'/ΔT (Pa/°C)
P407	22.2 ± 1.4	3.2 ± 0.5	155 ± 2
VAN	22.2 ± 0.3 (ns)	3.0 ± 0.6 (ns)	166 ± 2*
TEC	17.7 ± 0.6*	2.3 ± 0.9 (ns)	34 ± 4*
VAN+TEC	17.0 ± 0.0*	3.0 ± 0.4 (ns)	180 ± 2*

Statistical analysis: one-way ANOVA with Dunnett, comparing treatments versus P407 (*p < 0.05)

Fig. 2 | Rheological properties of poloxamer 407-based hydrogels with vancomycin (VAN, 20 mg/mL), tri-enzymatic cocktail (TEC), or both (VAN + TEC). Temperature-dependent evolution of viscoelastic moduli G′ (elastic modulus, solid lines) and G″ (viscous modulus, dotted lines) between 10 °C and 40 °C. Asymmetric sigmoidal regressions were fitted to the data, with 95% confidence prediction bands shown on the graph. The Table summarizes key rheological parameters, including the sol-gel transition Tgel (°C), the plateau modulus (kPa), and gelation slope (Pa/°C). Data are shown as mean ± SD ($n = 3$). Statistical significance was assessed using one-way ANOVA with Dunnett's post hoc test, comparing treatments versus P407 (*$p < 0.05$). Rheological measurements were performed using a stress-controlled rotational rheometer (1 Hz, 0.1% strain, 1 °C/min).

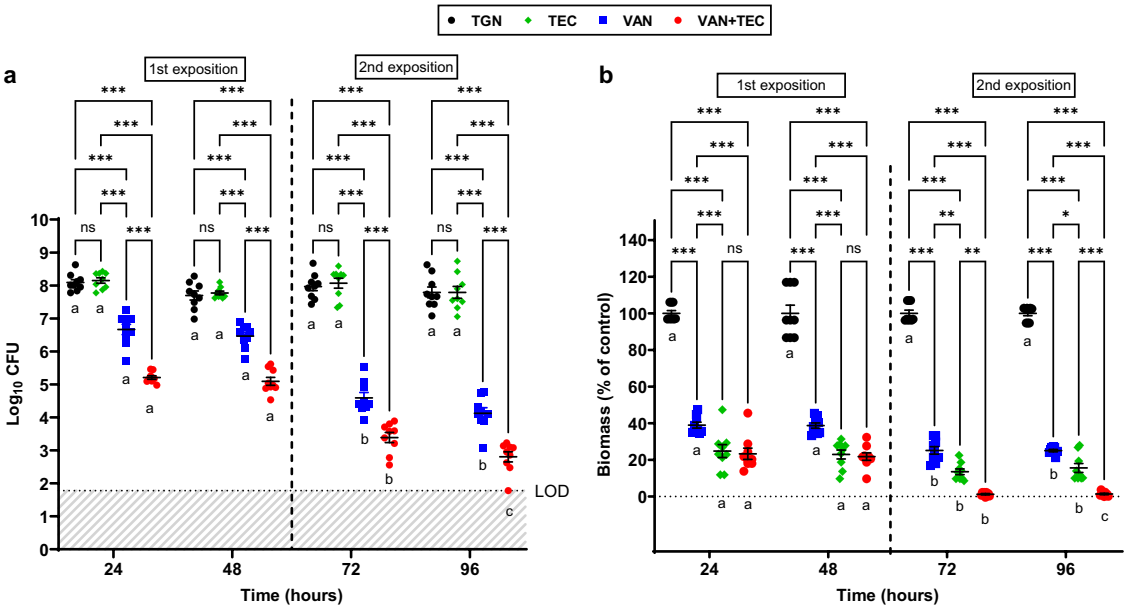


Fig. 3 | Activity of vancomycin and TEC in poloxamer P407 hydrogel when applied twice on biofilms in vitro. 24h-biofilms grown on titanium coupons were incubated with P407 hydrogel (25% w/v) containing TEC (400 U/mL of aspecific DNA/RNA endonuclease, 50 U/mL of endo-1,4-β-D-glucanase, and 2.6 U/mL of β-*N*-acetylhexosaminidase) and/or vancomycin (VAN; 20 mg/mL) for up to 48 h, rinced, and reincubated with fresh P407 containing the same active agents for an additional 48 h period. **a** CFU counts (Log_{10} per coupon). **b** Biomass (percentage of control value [TGN]). The horizontal lines are the mean and SEM of three independent experiments performed in triplicates ($N = 3$; $n = 3$), with individual values represented by colored dots. LOD limit of detection. Statistical analysis: two-way ANOVA with Tukey HSD. Comparison between different treatments at a given time point: ns, not significant, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Comparison of different time points for the same treatment: Different letters indicate a statistically significant difference ($p < 0.05$).

biomass. Of note, previous experiments demonstrated that TEC activity was not impaired in hydrogels as compared to solutions[23]. Since prolonging the time of exposure did not improve the efficacy of the treatment, probably due to TEC degradation (see Supplementary Fig. 1), we studied whether re-exposing biofilms at the end of this first 48 h period to fresh P407 hydrogels containing the same agents would bring some benefit. Following this second application of the formulation, VAN as well as the combination VAN + TEC led to an additional 2.4 or 2.2 Log_{10} reduction in CFU counts at 96 h compared to the effect reached at 48 h. Particularly, the combined treatment allowed to achieve more than 5 Log_{10} reduction compared to control samples. Regarding biomass, it was reduced to almost 0% of control values (P407 without active agent) already 24 h after the second exposure to the VAN + TEC combination, while TEC alone achieved more than 90% reduction.

Rifampicin is considered an antibiotic of choice for bone infections on one side[1], and biofilm infections on the other side[34], but is always used in combination to avoid selection of resistance. Later, VAN, combined or not with RIF, will be administered intraperitoneally in in vivo experiments. We therefore examined firstly the activity of VAN + RIF combinations in solution, at concentrations representative of those achieved in the bone or joint of treated patients[35], against 24-h biofilms grown on titanium coupons, in combination with TEC embedded in the hydrogel (Fig. 4). The antibiotic combination alone was active on biofilms, with significantly higher reductions in bacterial counts observed when RIF concentrations were ≥2.5 µg/mL ($p < 0.05$). The antibiotic combination also markedly reduced biomass, independently of the RIF concentration, as previously observed in a simpler model exposed to increasing concentrations of RIF alone[36]. TEC alone decreased biomass only in the absence of antibiotics but had no effect by itself on bacterial counts. The addition of TEC to the antibiotic combination significantly improved the activity on CFU counts, but not on biomass: the same level of activity was observed whatever the concentration of RIF used for both parameters. This suggests that an approx. 4 log_{10} CFU decrease is the maximal reachable effect in these conditions, probably due to antibiotic tolerance or persistence in the biofilm[30]. Of note, the most effective strategy to act upon persisters in biofilms (the ClpP protease activator ADEP4 combined with rifampicin) achieves full eradication after 3 days only[37], while the duration of incubation was limited to 24 h in this specific experiment.

Innocuity of the VAN + TEC hydrogel formulation for human cells and tissues

Prior to initiating in vivo studies, we evaluated the potential toxicity of the formulation containing both the antibiotic and the enzymes (VAN + TEC) by performing in vitro cytotoxicity assays on mouse fibroblasts (L929 cell line) and cutaneous irritation test on an in vitro 3D reconstructed human epidermis (RHE). Those tests were performed following established ISO 10993 and OECD guidelines, respectively[38,39]. They are commonly employed for assessing local toxicity of medical devices[40], and due to the anatomical proximity of the skin to the implanted tissue cages in our animal model.

Results of the in vitro cytotoxicity assay showed that the hydrogel formulation exhibited no cytotoxic potential toward fibroblasts, with cell viability consistently exceeding the 70% set threshold value[38] in all conditions tested. Likewise, results obtained in the RHE model showed that the formulation can be considered as non-irritant for human skin (Supplementary Fig. 2).

Rifampicin and vancomycin pharmacokinetic profile in tissue cage fluid of implanted animals

The different groups of animals included in this study are presented in Fig. 5, and details on sampling are provided in Supplementary Table 2.

Before assessing the activity of the formulation in vivo, we first determined the PK profile of antibiotics in the tissue cage fluid of implanted guinea pigs. Figure 6a presents the concentration-time profiles of RIF following the administration of a single intraperitoneal (i.p.) dose of 12.5 mg/kg, with the corresponding pharmacokinetic parameters summarized in Table 2. RIF reached its peak concentration (Cmax) in the tissue cage fluid at 3 h post-injection, after which the concentration declined rapidly to a residual value of 0.1 µg/mL by 12 h. Figure 6b shows the same

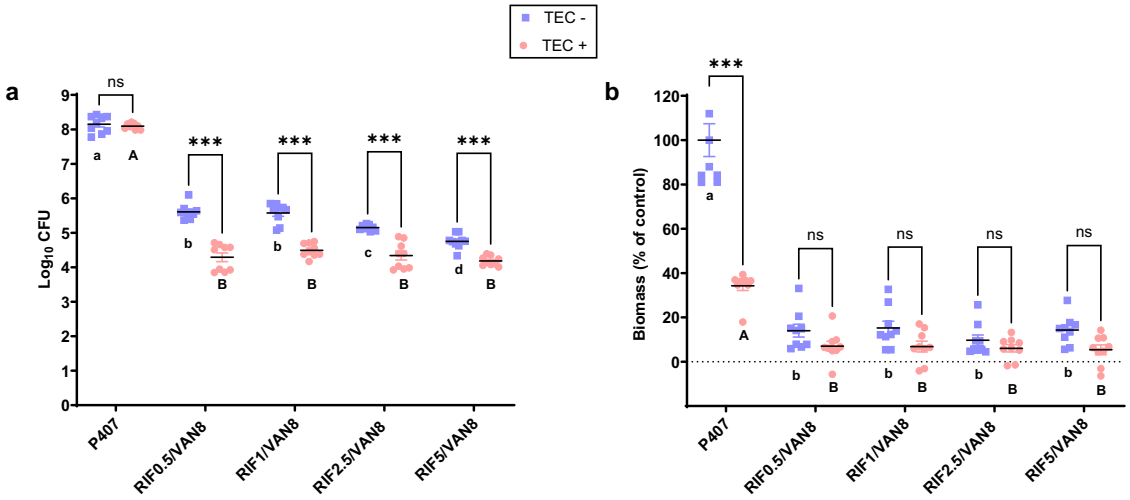


Fig. 4 | Activity on in vitro biofilms of dual antibiotic therapy by rifampicin and vancomycin in solution, combined with TEC in poloxamer P407 hydrogel. 24-h biofilms were exposed during 24 h to vancomycin (VAN) 8 µg/mL was combined with different clinically relevant concentrations of rifampicin (RIF), with or without 1 mL of P407 hydrogel (25% w/v) containing TEC (400 U/mL of aspecific DNA/RNA endonuclease, 50 U/mL of endo-1,4-β-D-glucanase, and 2.6 U/mL of β-*N*-acetylhexosaminidase). **a** CFU counts (Log_{10} per coupon). **b** Biomass (percentage of control (no antibiotic)). The horizontal lines are the mean and SEM from three independent experiments performed in triplicates ($N = 3$; $n = 3$), with individual values represented by colored dots. Statistical analysis: two-way ANOVA with Tukey HSD (ns, not significant; $^{***}p < 0.001$).

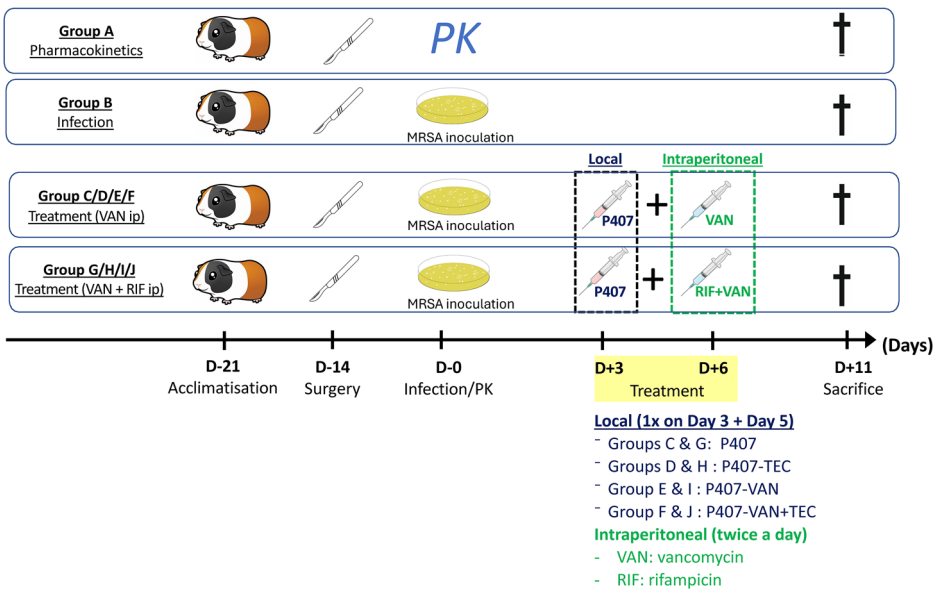


Fig. 5 | Study design. Animals were assigned to 10 groups: rifampicin or vancomycin pharmacokinetics (group A), infected/non-treated (group B), infected/treated locally in tissue cages with P407 (groups C and G), with P407 containing TEC (groups D and H), with P407 containing vancomycin at 20 mg/mL (groups E and I) and with P407 containing their combination (groups F and J). Groups C to F also received vancomycin i.p. at 15 mg/kg twice daily during 4 days, while groups G to J received a combination of vancomycin i.p. at 15 mg/kg twice daily and rifampicin i.p. at 12.5 mg/kg once a day, during 4 days. Local treatment was given once a day, on day 3 and on day 5.

PK profile, expressed as multiples of the RIF MIC (0.004 µg/mL)[41], and compared to the previously reported pharmacokinetic profile of intraperitoneal VAN in the tissue cage fluid[23], similarly expressed as multiples of its MIC (1 µg/mL). This normalization highlights a high local exposure to RIF, with C_{max}/MIC and AUC/MIC (Area Under the Curve/MIC) ratios of 128 and 862 h^{-1}, respectively. In contrast, local exposure to VAN after intraperitoneal administration was considerably lower, with C_{max}/MIC and AUC/MIC ratios of 7.5 and 61.2 h^{-1}, respectively. Figure 6c displays the local pharmacokinetic profile of VAN released in the tissue cage fluid from the P407 hydrogel formulation containing both VAN and TEC (VAN + TEC), with the corresponding pharmacokinetic parameters summarized in Table 3. Local VAN concentration peaked at 2000 µg/mL immediately after hydrogel administration, followed by a biphasic decline: an initial rapid decline to approx. 522.2 µg/mL [363.6 to 680.7 CI_{95}] within the first 12 h, and a subsequent sustained release phase that maintained the local concentration well above the MIC (22 x MIC) through the end of the measurements (120 h). The $AUC_{0\text{-}120h}$/MIC calculated for VAN following local administration of VAN + TEC hydrogel into the cage was 24,565 h^{-1}, i.e., a value 400-fold higher than that achieved by intraperitoneal injection.

Animal infection and assessment of the activity of different treatment modalities

Infection was first assessed in untreated animals by CFU counting of bacteria collected from the tissue cage fluid (at preset time points until the end of follow-up), or those adhering to the implanted material (at the end of the follow-up). The planktonic bacterial counts in tissue cage fluid remained stable over time (between 7.2 and 7.7 Log_{10} CFU/mL; Fig. 7a). No spontaneous recovery was observed during the follow-up. At day 11, the adherent bacteria count averaged 8.3 log_{10} CFU on removed material (Fig. 7b).

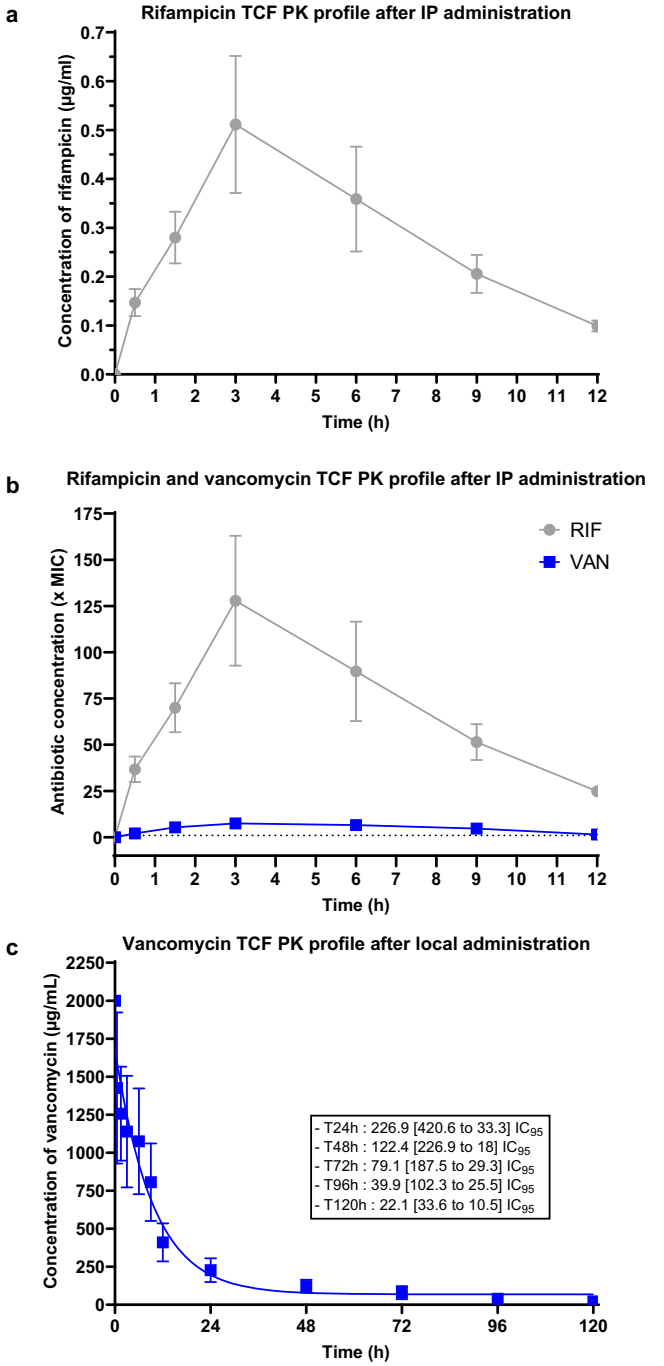


Fig. 6 | Pharmacokinetic profile of antibiotics in the tissue cage fluid (TCF). **a** Rifampicin after a single intraperitoneal dose of 12.5 mg/kg, concentrations expressed in µg/mL. **b** Same data for rifampicin and for vancomycin after a single intraperitoneal administration of 15 mg/kg[23], but expressed in multiples of their respective MIC (0.004 µg/mL for rifampicin; 1 µg/mL for vancomycin; dotted line). **c** Vancomycin in the tissue cage fluid following local administration of P407 containing vancomycin at 20 mg/mL. Residual concentrations with their 95% confidence interval are shown at specific time points in (**c**). **a**, **c** $n = 3$. **b** $n = 5$ for vancomycin. Values are means ± SD.

Intraperitoneal antibiotics (VAN 15 mg/kg twice daily alone [groups C–F] or VAN + RIF 12.5 mg/kg once daily [groups G–J]) were administered from day 3 post-infection till day 6 (4 days) while 2 local injections of P407 loaded or not with active agents (TEC and/or VAN 20 mg/mL) were performed on days 3 and 5.

Figure 7a shows the activity of the different treatment modalities against planktonic bacteria collected from tissue cage fluid over time. Prior to treatment initiation (day 3), bacterial counts in cage fluid ranged from 6.7 to 7.2 Log_{10} CFU/mL across all groups. By day 4, one day after treatment onset, a significant reduction in CFU counts was observed in all groups, except those having received a local injection of P407 without active agent (group C) or with TEC only (group D), combined with i.p. VAN. Addition of RIF to the i.p. treatment conferred significant activity: Animals receiving local treatment with P407 or P407 with TEC (groups G and H) and i.p. VAN + RIF showed additional reduction of 3.7 and 3.4 Log_{10} CFU/mL, respectively, compared to animals treated with i.p. VAN alone (Groups C and D, $p < 0.001$). The most active treatments were those combining local administration of P407 loaded with VAN (Group I) or with VAN + TEC (Group J) together with i.p. VAN + RIF. Specifically, Group I (local VAN combined with i.p. VAN + RIF) showed a 1.3 Log_{10} CFU/mL greater reduction than group E (local VAN combined with i.p. VAN; $p < 0.001$). Likewise, Group J (local VAN + TEC combined with i.p. VAN + RIF) showed a 1.1 Log_{10} CFU/mL greater reduction than Group F (local VAN + TEC combined with i.p. VAN; $p = 0.02$) at day 4.

CFU numbers decreased even further in all groups receiving active treatments (groups E to J) up to day 7 (1 day after the end of the therapy). Thereafter, a slight regrowth was noticed in all groups at day 11, though the increase remained below 1 Log_{10} CFU/mL. In the most effective treatment group (Group J; local VAN + TEC combined with i.p. VAN + RIF), the regrowth was limited to 0.5 Log_{10} CFU/mL and was not significant ($p = 0.57$).

Figure 7b shows the adherent bacterial counts on implanted material at specified time points. By day 5 (2 days after therapy initiation), animals receiving i.p. VAN and local VAN + TEC combination (Group F) exhibited an additional reduction of 1.5 Log_{10} CFU ($p < 0.0001$) in adherent bacteria compared to those treated by local TEC alone (Groups D) or local VAN alone (Group E). In animals treated with i.p. VAN + RIF, significant reductions in adherent bacteria were observed across all subgroups, including those receiving P407 hydrogel without active agents. Notably, greater reductions were achieved in animals treated locally with TEC alone (Group H) or combined with VAN (Group J), showing additional decreases of 1.0 Log_{10} ($p = 0.12$) and 1.3 Log_{10} ($p = 0.007$), respectively, compared to Group I receiving local VAN alone. At this time point, the most effective treatments were those combining local administration of TEC with i.p. VAN + RIF (group F) or local administration of VAN + TEC combined with either i.p. VAN or VAN + RIF (groups H and J).

At Day 7 (24 h post-treatment), differences in treatment efficacy among groups receiving i.p. VAN became more pronounced compared to day 5. Animals receiving local VAN + TEC (Group F) showed significantly greater reductions in adherent bacterial counts, with a difference of 3.2 log_{10} CFU with Group D (local TEC alone; $p < 0.0001$) and of 1.6 log_{10} CFU with Group E (local VAN alone; $p = 0.0005$). The addition of RIF to VAN by the i.p. route further enhanced efficacy across groups. The greatest effect was observed in Group J, which received i.p. VAN + RIF combined with local TEC + VAN: adherent bacterial counts were reduced by an additional 1.3 Log_{10} ($p = 0.007$) and 1.7 Log_{10} ($p < 0.0001$) compared to Group I (local VAN alone) and group H (local TEC alone), respectively. Notably, no adherent CFU could be detected in 6 of 8 cages (75%) in group J (limit of detection set at 100 CFU/mL).

By the end of follow-up (day 11), a slight regrowth of adherent bacteria counts (0.5–1.5 Log_{10} CFU) was noticed in all groups. However, the statistically significant differences between animals treated with i.p. VAN vs. i.p. VAN + RIF persisted. Group J, which received the most comprehensive treatment (i.p. VAN + RIF combined with local VAN + TEC), continued to show the highest efficacy. At this time point, no adherent CFU were detected in 6 of 16 cages.

Selection of resistance in vivo in adherent bacterial isolates

Resistance to RIF is known to easily develop during treatment. Therefore, its MIC was reassessed on *S. aureus* recovered from the adherent biofilms that

could be collected from infected tissue cages at the end of the follow-up (day 11). Only the cages with the highest CFU counts were used for this experiment, in order to maximize the chance of finding resistant colonies. A caveat of this approach, however, is that it may underestimate the risk, since rifampicin resistance can also develop at low inoculum, where detection is inherently difficult[42,43]. MICs were determined in cation-adjusted Mueller-Hinton broth (MHB-Ca; standard medium) and in TGN (medium used to grow biofilms in vitro). According to EUCAST (The European Committee on Antimicrobial Susceptibility Testing)[44], the susceptibility/resistance breakpoints for *S. aureus* are ≤0.06 mg/L/>0.06 mg/L in MHB-Ca. The MIC of the inoculated MRSA ATCC 33591 strain was 0.004 mg/L in MHB-Ca and in TGN. Despite all groups receiving i.p. VAN + RIF, the post-treatment MICs of RIF varied depending on the local treatment applied (Fig. 8a).

In group G (local P407 hydrogel only), MIC increased by 1 to 3 doubling dilutions in all animals, but remained within the susceptible range. In group H (local P407 hydrogel with TEC only), MIC reached 0.128 mg/L, a value higher than the EUCAST resistance threshold, in two animals, but remained in the susceptible range for the two other animals (≤0.008 mg/L). In Group I or J (local P407 hydrogel with vancomycin or with VAN + TEC), all isolates remained susceptible, with MIC of 0.004–0.008 mg/L, suggesting that high local concentration of vancomycin was sufficient to prevent resistance emergence.

Although the risk of resistance development to VAN is much lower[45], we also measured its MIC in the same samples and did not detect any significant change whatever the treatment group (median MIC increase of <1 doubling dilution as compared to the initial value (1 mg/L in MHB-Ca and 8 mg/L in TGN)) (Fig. 8b).

Discussion

Managing prosthetic joint infections, particularly those caused by MRSA, remains highly challenging, due to the persistence of biofilms on implanted materials and to their high antibiotic tolerance[9,46]. Building on our previous work evaluating a thermosensitive hydrogel containing vancomycin and TEC[23], we aimed here to improve therapeutic outcomes by addressing the issue of bacterial regrowth observed at the end of follow-up, despite significant reductions in both adherent and planktonic bacteria at the end of treatment. We optimized both the hydrogel formulation and the treatment strategies in order to achieve prolonged antibiotic release and enhanced and sustained biofilm disruption. We managed to achieve these goals, with no adherent bacteria detected in 75% of the implants 24 h after the end of the therapy, and still in 37.5% of them at the end of the follow-up.

A first improvement was achieved by reformulating the poloxamer 407 hydrogel. As anticipated[29], increasing the w/v percentage of poloxamer P407 from 20% to 25% prolonged the vancomycin release profile while preserving TEC activity. In contrast to the previous 20% formulation, which released nearly all vancomycin within 24 h in vitro[23], the optimized formulation enabled sustained release over 96 h. In vivo pharmacokinetic data confirmed prolonged antibiotic exposure, with local concentrations remaining above the MIC for more than 5 days, and an AUC approximately twice that measured in our previous study.

Particularly, the modifications of the formulation (increased percentage of P407, same TEC load and higher vancomycin load compared to our original hydrogel[23]) also improved its rheological properties. The new hydrogel exhibits a lower gelation temperature (17 °C vs. 22 °C), enabling an earlier sol-to-gel transition upon administration. It also displays a higher plateau elastic modulus (3.0 ± 0.4 kPa vs. 2.0 ± 0.7 kPa), suggesting enhanced mechanical stability at physiological temperature, closely matching the stiffness of unloaded P407. Most importantly, the gelation slope ($\Delta G'/\Delta T$) was steeper in the new formulation (180 Pa/°C vs. 97 Pa/°C), indicating a faster and more efficient micellar network assembly. Collectively, these rheological changes point to a structurally reinforced gel, better suited to maintain its integrity and localization following injection in vivo. Even higher P407 concentrations have been studied in the literature[47], but not tested here, as they would markedly modify rheological properties and induce faster gelation, which is undesirable for our purpose since the formulation must remain in liquid form during injection.

Unlike in the previous 20% P407 formulation[23], TEC was not fully released from the 25% P407 formulation. This incomplete release is likely due to the increased density and viscosity of the hydrogel, which forms a more compact micellar network with narrower aqueous channels compared to the 20% formulation. This denser structure may restrict enzyme diffusion and promote their retention via stronger hydrogen bonds and hydrophobic interactions. This phenomenon had been previously reported for poloxamer 407-based systems: Ricci et al.[48] demonstrated that higher P407 concentrations reduce drug release by forming tighter micellar networks[31]. Additionally, hydrogen bonds between the hydrophilic segments of P407 and encapsulated molecules can enhance their adsorption within the gel, further limiting release[49]. The co-inclusion of vancomycin in the formulation appeared to mitigate these effects. The high vancomycin concentration likely increased gel hydration and porosity, facilitating the full release of the enzymes.

Conversely, vancomycin release was slowed down in the presence of TEC. In P407 hydrogels, small hydrophilic drugs like vancomycin primarily diffuse through aqueous channels formed above the gelation temperature[50]. TEC proteins can interfere with this process through steric hindrance, macromolecular crowding, and specific interactions with poloxamer. These interactions comprise hydrophobic adsorption to polypropylene oxide (PPO) micellar cores and hydrogen bonding with polyethylene oxide (PEO) coronas, which can alter micelle packing and reduce channel accessibility[51]. As a result, the formulation can display (i) increased micellar spacing and reduced connectivity, impairing aqueous channel integrity[52]; (ii) partial physical obstruction of aqueous pores by proteins[27]; and (iii) decreased micellar mobility. Altogether, these effects result in a more rigid network[51], decrease gel porosity and increase tortuosity[31,53]. As a consequence, vancomycin becomes confined to narrower, less connected domains, slowing its release. This controlled kinetic profile is particularly relevant in the context of biofilm-associated infections, where prolonged local antibiotic exposure is therapeutically advantageous[49,54].

Table 2 | Pharmacokinetic parameters of rifampicin in tissue cage fluid after a single intraperitoneal injection of 12.5 mg/kg[a]

C_{max} (µg/mL)	C_{min} (µg/mL)	$AUC_{0\text{-}12h}$ (µg.h/mL)	$T_{1/2}$ (h)	T_{max} (h)
0.51 (±0.14)	0.1 (±0.01)	3.5 [2.8 to 4.1] 95% CI	3.4 [2.9 to 4.0] 95% CI	3

[a] Data are presented as mean ± SD ($n = 3$) for concentrations, and 95% confidence intervals for other parameters.

Table 3 | Pharmacokinetic parameters of vancomycin in tissue cage fluid after a single injection of P407 formulation containing 20 mg/mL of vancomycin and TEC[a]

C_{max} (µg/mL)	C_{min} (µg/mL)	$AUC_{0\text{-}120h}$ (µg.h/mL)	$T_{1/2}$ (h)	K (h^{-1})
2000 (±1.1)	22.1 (±4.7)	24,565 [20,487 to 28,642] 95% CI	6.6 [4.5 to 9.8] 95% CI	0.11 [0.07 to 0.16] 95% CI

[a] Data are presented as mean ± SD ($n = 3$) for concentrations, and 95% confidence intervals for other parameters.

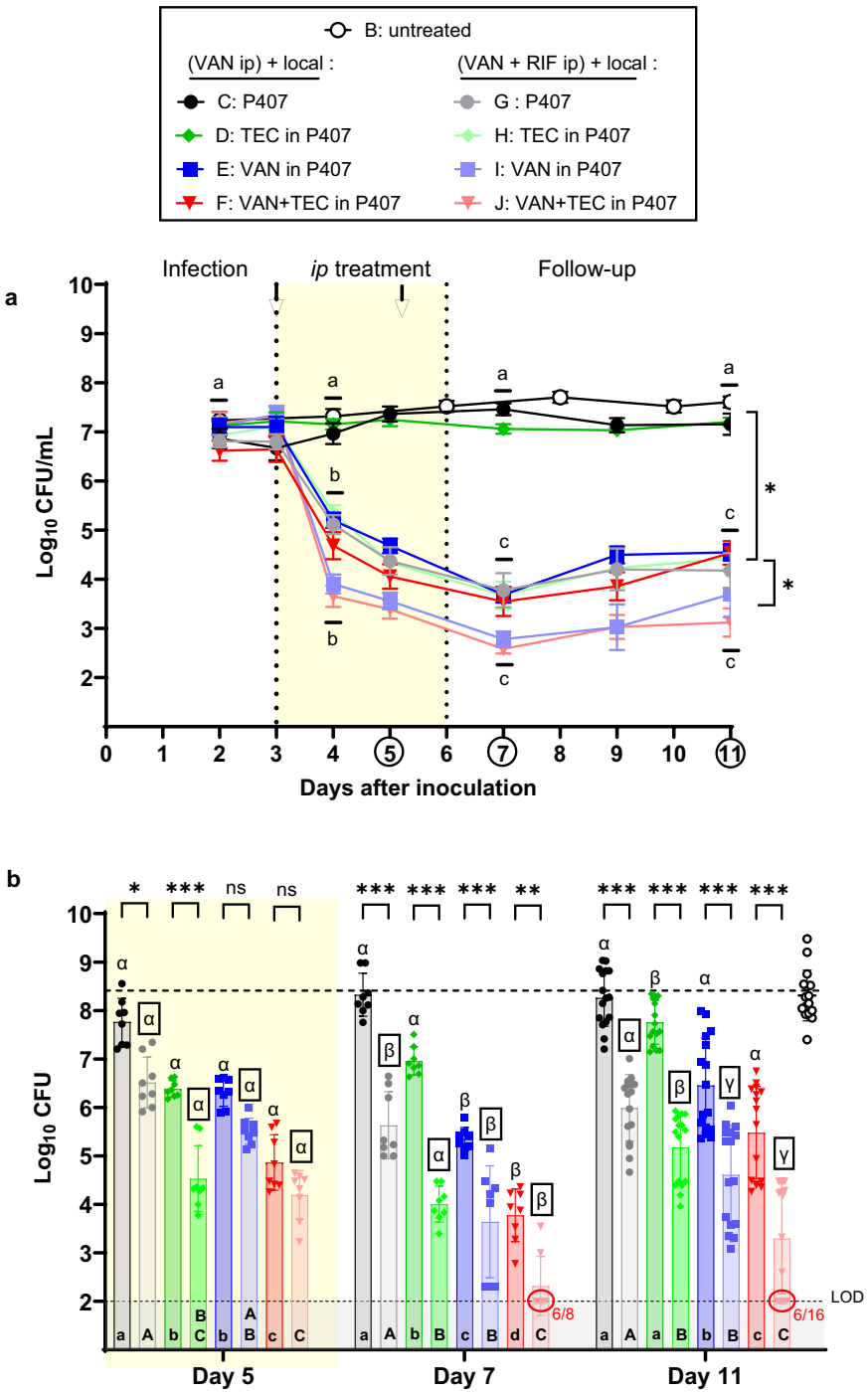


Fig. 7 | In vivo activity of vancomycin and TEC in poloxamer P407 hydrogel combined with intraperitoneal antibiotics. Evolution of planktonic bacteria in tissue cage fluid (**a**) and of bacteria adhering to the implanted material (**b**) over time. Two weeks after implantation, MRSA ATCC 33591 (10^7 CFU) was inoculated into tissue cages (day 0). Animals from group B were left untreated, and the number of CFU was determined in aspirated cage fluid at preset time points until the end of follow-up or on implanted material at day 11 after sacrifice ($n = 32$ from 8 animals). Animals from the other groups received two successive local injections at days 5 and 7 (indicated by the black arrows) of 1-mL of P407 (groups C, G) or P407 containing either TEC (groups D, H) or vancomycin (VAN) at 20 mg/mL (groups E, I) or their combination (VAN + TEC; groups F, J), as well as vancomycin at 15 mg/kg i.p. (twice daily) combined [Groups G–J] or not [Groups C–F] with rifampicin (RIF) at 12.5 mg/kg (once daily) for 4 days, indicated by the yellow area [days 3–6]. The number of CFU in aspirated cage fluid was determined regularly over time, and on material, in animals sacrificed at specified intervals, circled in (**a**) (day 5; 48 h after starting the treatment): $n = 8$ from 2 animals; day 7; 24 h after the end of the treatment: $n = 8$ from 2 animals; day 11; end of follow-up: $n = 16$ from 4 treated animals or 32 from 8 animals for untreated group. Results are presented as means ± SEM (**a**) or SD (**b**). Statistical analysis, two-way ANOVA followed by Tukey HSD multiple comparison. **a** $^*p < 0.05$ for comparisons between any animal groups. Different letters indicate statistically significant differences over time within the same treatment group (comparison of treatment effects across different days in animals receiving either intraperitoneal or local treatments); **b** Comparison of animals having received i.p. VAN vs. i.p. VAN + RIF and the same local treatment: ns: not significant; $^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$. Comparison of different local treatments on the same day: Different letters indicate a statistically significant difference in animals having received i.p. VAN [small letters]; or i.p. VAN + RIF [capital letters]. Comparison of the same local treatment over time: different Greek letters indicate statistically significant differences over time within the same local treatment group in animals having received i.p.VAN (Greek letters) or i.p. VAN + RIF (squared Greek letters). The proportion of harvested cages for which CFU counts were below the detection limit (100 CFU/mL) is indicated for group J at day 7 and 11. Note that CFU were evaluated only at Day 11 in the control group, and data are visible on the right of the other treatment groups.

A second major improvement in our treatment strategy consisted of repeating the local administration of the VAN + TEC formulation, in order to prolong the local exposure to the active agents and compensate for TEC limited stability over time. Local antibiotic delivery is crucial in PJI management, enabling high drug concentrations at the infection site[55] while minimizing the risk of systemic toxicity[33] and reducing the risk of resistance development[56]. The elevated vancomycin load in the hydrogel mirrors established clinical practices such as antibiotic-loaded cement spacers[57,58], bone cement[59], and intra-articular antibiotic injections[18,19,21,60], all designed to sustain local drug delivery and enhance bacterial clearance. Similarly, the dual application of the VAN + TEC hydrogel markedly reduced biofilm burden, both in vitro and in vivo. These results are consistent with previous studies showing that repeated exposure to biofilm-targeting therapies can substantially improve their efficacy[61–63].

Lastly, combining vancomycin with rifampicin further enhanced efficacy in vivo. Rifampicin is a cornerstone of systemic therapy for prosthetic joint infections, particularly those caused by MRSA, owing to its excellent bone penetration[35] and potent activity against biofilms[64]. However, its use as monotherapy is hampered by the rapid development of resistance[1,65], supporting its routine co-administration with other antibiotics. The high efficacy of the vancomycin-rifampicin combination is consistent with previous studies demonstrating their synergistic

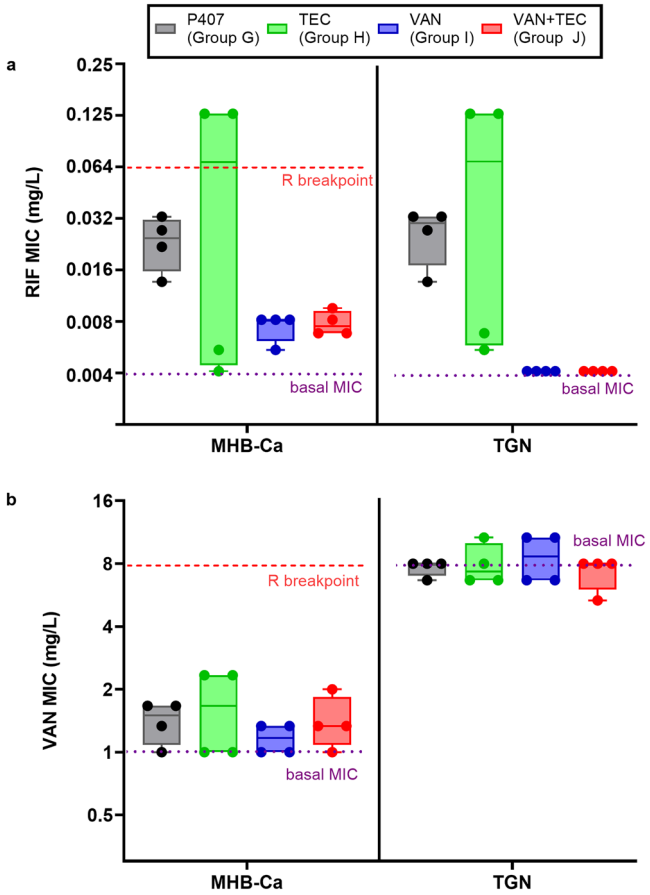


Fig. 8 | Post-treatment MIC of rifampicin and vancomycin for *S. aureus* isolates recovered from adherent biofilm, stratified by treatment group. Each point represents the mean MIC of rifampicin (**a**) and vancomycin (**b**) for one animal, measured in triplicate, in either MHB-Ca (cation-adjusted Mueller-Hinton broth) or TGN (Tryptic Soy Broth + 2% w/v NaCl + 1% w/v glucose) media. Isolates were recovered from the most infected tissue cage per animal at day 11. All groups received systemic rifampicin and vancomycin; local treatment varied as follows: Group G (P407 only), Group H (P407 loaded with TEC), Group I (P407 loaded with vancomycin [VAN]), and Group J (P407 loaded with VAN + TEC). The red dashed line indicates the EUCAST resistance breakpoint for rifampicin or vancomycin against *S. aureus* (>0.06 mg/L and >8 mg/L, respectively)[44] in MHB-Ca (no breakpoint set in TGN), and the violet dotted line, the basal MIC of rifampicin or vancomycin in both media.

activity against biofilms[66–68]. In vivo, adding rifampicin to vancomycin intraperitoneally improved treatment efficacy against planktonic bacteria in all study groups. The low MIC of rifampicin and its excellent diffusibility ensured more easy achievement of therapeutic concentrations within the tissue cages, reaching the pharmacodynamic targets for *S. aureus* infections (AUC/CMI ratio > 450 h^{-1}; Cmax/CMI ratio <12). This exposure surpassed thresholds for optimal bactericidal activity including against biofilms[69]. It explains why rifampicin, even when administered intraperitoneally, contributed to enhance vancomycin efficacy in vivo.

In addition, we demonstrate that the local administration of vancomycin seemed to prevent the emergence of rifampicin resistance in adherent bacteria. Rifampicin-resistant isolates were detected only in animals treated locally with TEC alone or unloaded P407, despite adequate rifampicin concentrations in the tissue cage fluid. Intraperitoneal vancomycin administration resulted in subtherapeutic local concentrations, which failed to prevent the selection of rifampicin-resistant bacteria that remained adherent to the implant surface after partial biofilm disruption by TEC. These findings emphasize the dual benefit of combining matrix-disrupting enzymes and bactericidal antibiotics at the infection site: enhanced efficacy and reduced risk of resistance development.

Consistent with our previous findings[23], TEC further enhanced the efficacy of antibiotics against adherent bacteria, indicating that enzymatic degradation of the biofilm matrix facilitates antibiotic access to encased bacteria and improves bactericidal activity. Quite remarkably, the most effective treatment regimen therapy (systemic antibiotic combination coupled with two successive administrations of VAN + TEC hydrogel) reduced adherent bacterial counts to the detection limit (100 CFU) in 75% of the cages 1 day after the end of the therapy, and still in 37.5% of the cages at the end of the follow-up. These results underscore the need for a combined local and systemic antibiotic strategy for substantial biofilm reduction in implant-associated infections. Sustained antibiotic delivery, controlled release, and repeated application appear critical for a more effective action on biofilms. Nevertheless, complete and durable eradication was not yet achieved, underscoring the high tolerance of biofilm-embedded bacteria and indicating that further optimization remains possible.

Importantly, no adverse effects were observed in treated animals, highlighting the safety and biocompatibility of the hydrogel formulation. This aligns with a recent study on hydrogel-based delivery systems of vancomycin and bacteriophages, which demonstrated both antimicrobial efficacy and a favorable biocompatibility profile[70]. The biocompatibility of our formulation was further supported by cytotoxicity and skin irritation assays. These two assays adequately address acute cytotoxicity and irritation potential of the tested formulations. This is particularly relevant for short-term local therapies (≤7 days), as planned for our hydrogel system. Chronic toxicity and systemic toxicity are not anticipated concerns given the localized application, the nature of the components (P407, vancomycin, TEC), and the controlled release profile. In accordance with ISO 10993-1, the need for additional testing depends on the type and duration of contact and must be determined through a comprehensive biological evaluation plan. The two tests performed here represent the basic assessments required for all medical devices[40]. However, the potential for sensitization needs to be addressed, particularly due to the presence of bacterial enzymes in the TEC cocktail, which may likely be immunogenic in humans.

This study has some limitations that warrant consideration. First, the treatment duration in our model was relatively short compared to clinical practice, where prolonged antibiotic therapy is often necessary for managing prosthetic joint infections. Exploring extended treatment protocols, including multiple hydrogel administrations, may help better approximate clinical scenarios; however, concerns regarding antibiotic-induced disruption of guinea pig gut microbiota justified the chosen setup. Second, the relatively brief follow-up period may limit assessment of long-term outcomes, including bacterial persistence or delayed infection recurrence. Yet, this is partly offset by the short treatment duration, which does not fully mimic chronic therapy effects. Third, while the tissue cage model enables controlled evaluation of biofilm-associated infections, it does not fully recapitulate the biomechanical stresses and immune environment of human prosthetic joints. Lastly, pharmacokinetic profiles have been determined only in non-infected animals, which may have underestimated exposure, as local inflammation may enhance drug permeability[71].

Future studies employing more clinically relevant models, such as large animal experiments or ex vivo human tissue systems, are needed to enhance the translational relevance of our findings. Lastly, although rifampicin resistance was assessed using standardized broth microdilution (CLSI) on isolates obtained from colonies growing on TSA, only the most heavily colonized implant was tested in each animal. This selective sampling approach may have underestimated the presence of low-frequency resistant subpopulations.

To conclude, this study highlights the potential of a VAN + TEC combination formulated in a poloxamer 407 hydrogel to improve the treatment of implant-associated infections by enabling sustained local antibiotic release and enhanced efficacy on biofilms. Our findings demonstrate a clear synergy between enzymatic degradation of the biofilm matrix

and high-dose local antibiotic therapy in vivo, resulting in bacterial counts below the limit of detection in the treatment group benefiting from optimized therapy. Furthermore, the combination of local treatment with systemic dual therapy (vancomycin and rifampicin) further improved efficacy and prevents resistance emergence, underscoring the importance of a multimodal approach in tackling biofilm-associated infections. Importantly, repeated administration of the VAN + TEC hydrogel provided an additional reduction in bacterial burden, suggesting that multiple local applications may further improve treatment outcome. However, the relatively short treatment and follow-up periods warrant further investigations to assess long-term efficacy, refine treatment strategies and strengthen the translation toward clinical applications.

Methods

Bacteria strain

The *Staphylococcus aureus* strain ATCC 33591, a methicillin-resistant *S. aureus* (MRSA) strain susceptible to vancomycin and rifampicin, was used for all in vitro and in vivo experiments. The minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) were 1 mg/L and >1024 mg/L for vancomycin, and 0.004 mg/L and 2 mg/L for rifampicin, respectively[24]. The strain was stored at −80 °C and cultured on tryptic soy agar (TSA; Sigma-Aldrich, Steinheim, Germany).

Antibiotics

Vancomycin powder for intraperitoneal (i.p.) injection (Mylan BVBA, Hoeilaart, Belgium) was reconstituted in 0.9% saline (Baxter SA, Lessines, Belgium) at a concentration of 50 mg/mL. When combined with rifampicin in the in vitro experiments, the concentration tested corresponded to a clinically relevant level typically found locally in bone and joint tissues after intravenous administration[35]. The vancomycin concentration formulated in poloxamer P407 was set at 20,000 × MIC against the *S. aureus* ATCC 33591 strain.

Rifampicin powder for i.p. injection (100% potency; Sanofi, Diegem, Belgium) was reconstituted in 0.9% saline at a concentration of 60 mg/mL. When combined with vancomycin in the in vitro experiments, rifampicin concentrations were in the range of those detected locally in bone and joint tissues after oral administration[35].

Tri-enzymatic cocktail

The tri-enzymatic cocktail solution, Orthenzy Qure (referred to as TEC in this paper), was specifically developed and provided by OneLife SA (Louvain-la-Neuve, Belgium) for this study. This sterile solution is buffered with Tris (20 mM; pH 7.4) and contains a combination of enzymes: 500 U/mL of a nonspecific DNA/RNA endonuclease derived from *Serratia marcescens* (c-LEcta GmbH, Leipzig, Germany), 50 U/mL of endo-1,4-β-D-glucanase from *Aspergillus niger* (Sigma), and 10 U/mL of β-*N*-acetylhexosaminidase from *Actinobacillus pleuropneumoniae* (Novozymes [now, Novonesis], Bagsværd, Denmark).

Preparation of Poloxamer 407 formulation. Poloxamer 407 (P407; Pluronic® F-127) was supplied by Merck (Steinheim am Albuch, Germany). Poloxamer solutions were prepared following a protocol adapted from the "cold method" originally described by Schmolka[72]. Briefly, a weighed amount of 2.5 g of poloxamer 407 was gradually dissolved in 10 mL of cold (4 °C) sterile Tris-buffered (20 mM; pH = 7.4) aqueous solution with gentle stirring. The container was left overnight in a cold room (2–4 °C) on a roller shaker running continuously at the fixed speed of 30 rpm to ensure complete dissolution, forming a clear, viscous solution. For gels containing active ingredients, the same procedure was applied. Vancomycin and/or TEC were added to a sterile Tris-buffered (20 mM; pH = 7.4) aqueous solution. The final concentration of vancomycin in the gel was 20 mg/mL, and that of enzymes, equivalent to the composition described above. All solutions were sterilized by filtration using 0.22 µm pore size filters prior to experimentation. All formulations were prepared extemporaneously.

In vitro release kinetic study of vancomycin or TEC from Poloxamer 407

The release profiles of vancomycin and/or TEC from poloxamer 407 hydrogels were determined according to the protocol previously established[23]. In short, 1 mL of the formulation was solidified at room temperature in 15 mL conical tubes, then overlaid with 9 mL of sterile Tris-HCl buffer and incubated at 37 °C. Aliquots (0.5 mL) were collected at predefined time points (up to 7 days) and replenished immediately. Vancomycin concentrations were determined using the disk diffusion method with *S. aureus* strain ATCC 25923 as the test organism. Enzyme concentrations were determined using the BCA (BiCinchoninic Acid assay) protein assay (ThermoFisher, Waltham, MA).

Stability of vancomycin and TEC in the Poloxamer P407 formulation

Stability of vancomycin was directly evaluable in samples collected from kinetics of release studies, as it was assayed by microbiological assay, detecting the active molecule. Stability of TEC was evaluated by measuring its activity on a freshly prepared 24-h biofilm grown on titanium coupons (see the paragraphs below on "Biofilm culture in vitro" and "Biofilm treatments in vitro" for more details on the methodology). To this effect, supernatants from kinetics of release studies were collected and aliquots diluted 10 times in Tris-buffered (20 mM; pH = 7.4) aqueous solution. Preformed biofilms were gently rinsed with PBS (Phosphate Buffer Saline at pH 7.4) and exposed for 24 h to 1 mL of diluted supernatants. Residual biomass was then measured by crystal violet staining using a previously established procedure[23] and compared to that of a freshly prepared TEC diluted the same way. Data were expressed as percentage of activity compared to that of the freshly prepared TEC.

Rheological characterization of P407-based formulations

The rheological behavior of the newly optimized P407-based formulations was evaluated to assess potential alterations in thermogelation properties following the incorporation of active agents. Rheological measurements were performed using a stress-controlled rotational rheometer (MCR102; Anton-Paar, Graz, Austria) operating in oscillatory mode with a plate–plate configuration (plate diameter: 40 mm; gap: 0.076 mm). The temperature was linearly increased from 10 °C to 40 °C at a rate of 1 °C/min, under conditions of 1 Hz frequency and 0.1% strain[23]. The following parameters were examined: (i) The gelation temperature (Tgel), defined as the temperature at which G′ (storage modulus) crosses G″ (loss modulus), indicates the thermosensitive transition point from liquid to gel. (ii) The plateau G′ value (35.5–38.5 °C) provides insight into gel consistency over physiological temperature ranges[73]. (iii) The gelation kinetics (ΔG′/ΔT), calculated a window of [T°gel +2°], quantifies the rate of network formation, which can influence in situ retention.

Biofilm culture in vitro

Biofilm formation on titanium coupons was performed following a previously established protocol[23]. In short, bacterial suspensions were standardized to OD_{620} = 0.5 (~8.5 $\log_{10}$ CFU/mL), diluted in TGN (Tryptic Soy Broth [VWR Chemicals, Leuven, Belgium] + 2% w/v NaCl + 1% w/v glucose) medium, and incubated on Ti-6Al-4V coupons for 24 h at 37 °C with gentle orbital shaking (50 rpm) to allow mature biofilm development.

Biofilm treatments in vitro

When studying the activity of a combination of vancomycin and TEC formulated in P407 hydrogel, coupons were transferred to 6-well plates containing 1 mL of P407 hydrogel containing no active agent, vancomycin, TEC, or their combination and 9 mL of TGN. Control wells contained 10 mL of TGN medium. All plates were incubated for 24 or 48 h with orbital shaking (at 50 rpm), then rinsed and re-exposed to a fresh hydrogel preparation containing the same concentrations of active agents as at time 0 h and reincubated for 24 or 48 h. Analyses of CFU and biomass were performed every 24 h.

When studying the activity of a combination of rifampicin and vancomycin in solution combined with P407 hydrogel loaded with TEC, coupons were transferred to 6-well plates containing 1 mL of P407 hydrogel loaded with TEC, and 9 mL of TGN containing vancomycin (final concentration 8 µg/mL) and rifampicin (final concentrations 0.5-, 1-, 2.5-, and 5 µg/mL). Control wells contained 10 mL of TGN medium. The plates were incubated for 24 h at 37 °C under constant orbital shaking at 50 rpm.

Quantification of biofilms in vitro

CFU enumeration and biomass assessment were conducted using the same standardized procedure reported in our previous study[23]. In brief, coupons were washed twice with sterile PBS (Phosphate buffer Saline), then immersed in 3 mL sterile PBS and subjected to a combined vortex-sonication-vortex treatment for biofilm detachment, followed by serial dilutions for CFU plating, or biomass evaluation via crystal violet staining and absorbance measurement.

In vitro cytotoxicity and skin irritation testing

The safety of the formulation was assessed using a combination of the ISO 10993-5 MTT assay[38] and the OECD TG 439 Reconstructed Human Epidermis (RHE) irritation assay[39]. Together, these tests address two critical biological endpoints for a locally applied biomedical hydrogel intended for short-term orthopedic use: cytotoxicity and local irritation.

The tested hydrogel is designed for application around orthopedic implants, where it will interface with connective and soft tissues. The L929 MTT assay is the international standard for screening potential cell toxicity of medical devices or extracts[38]. The use of a fibroblast cell line in this test is highly suitable to detect adverse cytotoxic effects on peri-implant cells (fibroblasts being abundant in bone, synovial, and subcutaneous tissues)[74].

The OECD TG 439 RHE model is an accepted surrogate for evaluating nonspecific local irritation[75], covering risks related to accidental peri-surgical exposure to skin, subcutaneous tissue, or exposed wounds during implantation. Although the model primarily evaluates epidermal irritation, its predictive value for general tissue irritation has been supported in recent adaptations of ISO 10993-23[75–77].

Both tests are widely recognized by regulatory agencies (EMA, FDA, MHRA) and constitute essential components of the biocompatibility assessment matrix defined by ISO 10993-1[40], especially for devices involving local application with tissue contact.

The cytotoxicity of poloxamer 407-based hydrogel formulations (25% w/v), with or without vancomycin (20 mg/mL) and/or a tri-enzymatic cocktail (TEC), was evaluated using the MTT assay in compliance with ISO 10993-5:2009 guidelines for medical device biocompatibility testing[38]. The study was performed under full Good Laboratory Practice (GLP) conditions by SenzaGen AB (Study n°1368-2406). Murine fibroblasts (L929 cell line, NCTC clone 929, Acc. No. 85011425), as recommended by ISO 10993-5[38], were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM GlutaMAX™. Cells were seeded at a density of 10^4 cells/well in 96-well plates and incubated for 24 h at 37 °C in a 5% CO_2 atmosphere to achieve a subconfluent monolayer. Controls included 0.9% NaCl (negative control) and TouchNTuff 92-605 Nitrile material (positive control known to be cytotoxic). After 24 h of exposure, MTT reagent (1 mg/mL; 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was added, and plates were incubated for 2 h. Formazan crystals were solubilized in isopropanol, and absorbance was measured at 570 nm, with 650 nm used as a reference wavelength to correct for nonspecific absorbance ($A_{570} - A_{650}$) such as tissue residues or optical background interferences[38]. Viability was calculated relative to the negative control, with viability values <70% considered cytotoxic, following ISO 10993-5 criteria[38].

The skin irritation potential of the formulations was assessed according to OECD Test Guideline No. 439 Skin irritation potential using reconstructed human epidermis (OECD TG 439)[39]. The study was performed by StratiCELL SA (Study n°CR24ONE01b) in a GLP-like environment. RHE tissues generated from normal human epidermal keratinocytes (three independent donors) were exposed to 20 µL of each hydrogel formulation (P407 + TEC or P407 + TEC + vancomycin) for 42 min at room temperature. Following exposure, tissues were thoroughly rinsed with PBS and incubated for 42 h in fresh maintenance medium at 37 °C under 5% CO_2. Tissue viability was assessed by MTT assay. Formazan production was quantified spectrophotometrically at 560 nm. A viability >50% classified the sample as non-irritant, while a viability ≤50% indicated irritancy, as per OECD TG 439 prediction model[39].

Establishment of the animal model for in vivo antibiofilm activity study

We used the so-called tissue cage guinea pig model established by Zimmerli et al.[78] and previously exploited in our laboratory[23]. All animal procedures were conducted in accordance with the Belgian Royal Decree of 29 May 2013 on the protection of experimental animals and the European Directive 2010/63/EU. The study was reviewed and approved by the UCLouvain Ethical Committee for Animal Welfare (2023/UCL/MD/52 and 2024/MD/42), which authorized the use of guinea pigs (moderate pain level). Animals were housed in pairs under controlled temperature and humidity with a 12-h light/dark cycle and had ad libitum access to food and water. After a 7-day acclimatization period, four sterile cylindrical polytetrafluoroethylene "Teflon" tissue cages (10 by 32 mm and perforated with 130 holes) (Europlex, Nivelles, Belgium) containing 10 titanium alloy beads (Ti-6Al-4V; 3 mm diameter) per cage, were aseptically implanted in the back of guinea pigs weighing 450–600 g. The animals were anesthetized with an intraperitoneal injection of ketamine 50 mg/kg (Nimatek®, Dechra Pharmaceuticals PLC, Norwich, United Kingdom) and xylazine 5 mg/kg (Rompun®, Bayer AG, Leverkusen, Germany). They were monitored daily for signs of pain, lameness, lethargy, or weight loss by trained personnel. Humane endpoints were predefined (weight loss >15%, persistent wound dehiscence, or severe behavioral changes). Analgesia (buprenorphine 0.05 mg/kg subcutaneously every 12 h for 48 h) was systematically administered post-operatively and prolonged if needed based on veterinary evaluation.

The experiments were started after complete wound healing (i.e., approximately 2 weeks after surgery). Before each experiment, the cages were checked for sterility by culturing the aspirated cage fluid (150–300 µL) for 7 days on TSA plates. A total of 70 female guinea pigs were randomly assigned to ten groups: vancomycin pharmacokinetics (A), infected/non-treated (B), infected/treated locally in cages with P407 (C and G: P407), with P407 containing TEC (D and H: P407-TEC), with P407 containing vancomycin at 20 mg/mL (E and I: P407-VAN) and with P407 containing their combination (F and J: P407-VAN + TEC). Groups C to F received 15 mg/kg vancomycin i.p. only, and groups G to J received dual therapy of 12.5 mg/kg rifampicin and 15 mg/kg vancomycin i.p. (Fig. 5). Local injections of hydrogel were performed with a 23-gauge needle (blue). The hydrogel formulation was prepared immediately before administration and injected directly into the tissue cage lumen. No trace of residual hydrogel was visible upon aspiration performed before the second injection, indicating that the first dose had completely disintegrated by 48 h. In tissue cages removed at day 5 (before the second administration), no visible hydrogel remnants were observed either.

Pharmacokinetic studies of antibiotics in tissue cage fluid

Rifampicin concentrations were measured at preset time points (30 min, 1, 3, 6, 9, and 12 h) in tissue cage fluid (TCF) of non-infected animals (group A) following a single intraperitoneal dose of rifampicin (12.5 mg/kg). Aliquots (150–300 µL) of all samples were transferred to Eppendorf tubes and centrifuged at 2500×*g* for 15 min, and supernatants were stored at −20 °C until further analysis. Rifampicin concentrations were determined using the disk diffusion method with *S. aureus* strain ATCC 25923 as the test organism.

Vancomycin concentrations were measured at 30 min, 1, 3, 6, 9, 12, 24, 96, and 120 h in TCF of non-infected animals (group A) that received 1 mL of P407 containing vancomycin (20 mg/mL) and TEC locally into the cages. Samples were processed as described for rifampicin, and vancomycin

concentrations were determined using disk diffusion method with *S. aureus* strain ATCC 25923 as the test organism.

Infection of animals, treatment and measure of treatment efficacy

Bacteria were taken from a frozen stock, streaked onto a TSA plate, and incubated overnight. A few colonies were then suspended in sterile PBS and adjusted to an OD_{620} of 0.5 (~8 Log_{10} CFU/mL). The suspension was diluted 1:10 in PBS and stored at 4 °C for 2 h before inoculation. Serial dilutions were plated on TSA to verify the bacterial inoculum.

Cages were infected by percutaneous injection of 200 µL bacterial suspension in each cage (day 0), allowing planktonic proliferation in the tissue cage fluid and biofilm development on both the cage and the beads. Infection was confirmed by quantitative culture of TCF at day 3. Animals in group B were not treated. Animals in group C to J received two local injections (into cages; day 3 and 5) of 1 mL of P407, with or without active agents, followed by intraperitoneal injection of vancomycin at 15 mg/kg (group C to F) or dual therapy of rifampicin 12.5 mg/kg and vancomycin 15 mg/kg (group G to J) every 12 h for 4 days (day 3 to 6). All four tissue cages within a single animal received the same local treatment. For ethical reasons, this design was chosen to limit the number of animals while providing several replicates per condition. To assess the efficacy of the treatment against planktonic bacteria, TCF samples were taken at preset times (day 3, 4, 5, 7, 9 and 11) from all animals. Samples were placed in 500-µL conical Eppendorf tubes, vortexed for at maximum intensity 30 s, serially diluted in PBS if needed to ensure accurate counting, and then plated on TSA for bacterial quantification. The efficacy of the treatment against adherent bacteria was assessed using implanted materials removed from euthanized animals 48 h after the start of treatment, 24 h after the end of treatment, and at the end of the follow-up period.

After collection, each tissue cage was first flushed with 10 mL of sterile PBS using a 10 mL syringe to remove planktonic material and any potential antibiotic residues. The cages were then washed twice more by immersion in 10 mL of PBS to eliminate remaining tissue debris or fibrinous material, since subcutaneous implantation induces fibrous encapsulation around the titanium beads. After washing, the ten titanium beads were removed from each cage and transferred together with the corresponding cage in 15-mL conical tubes containing 5 mL of sterile PBS, vortexed at maximum intensity for 30 s, sonicated for 5 min (Branson 5510 Ultrasonic Bath, Emerson Electric, Saint Louis, MO), and vortexed again for 30 s to dislodge adhering bacteria, and aliquot plated on TSA for colony counting (after appropriate serial dilution if needed). CFU data were calculated for each individual cage, as commonly done in tissue cage studies, since all cages in the same animal are physically independent compartments. This approach is considered an ethically acceptable compromise to limit the number of animals when intra-animal variability is low[79,80].

MIC determination of post-treatment adherent isolates collected from tissue cages

At the end of the follow-up period (day 11), the MIC of rifampicin and vancomycin was reassessed on *S. aureus* isolates recovered from biofilms adherent to the implanted titanium beads and tissue cages. For each animal, among the four implanted tissue cages, only the cage exhibiting the highest bacterial burden (based on CFU quantification) was selected for MIC testing, as it was considered the most critical ecological niche where resistant subpopulations are most likely to emerge. Implants were treated as described above to dislodge adherent bacteria, and aliquots were spread on TSA for quantification. A few colonies were then homogenized in buffer, adjusted to 0.5 McFarland, and this suspension was used to determine MIC according to the CLSI guidelines.

Data transformation and statistical analysis

The release kinetics data were analyzed using nonlinear regression. The combined effect of TEC and antibiotics was assessed separately for in vitro and in vivo models. In the in vitro model, a one-way ANOVA was used after checking for normality with QQ plots and variances with the Levene test, while in the in vivo model, a two-way ANOVA was performed. Pairwise comparisons were made using the Tukey or Dunnett Honestly Significant Difference (HSD) test. Vancomycin serum AUCs were calculated using the trapezoid rule. Interaction between treatments was assessed through ANOVA[81]. The threshold for alpha errors was set at 0.05. Data are reported as means with 95% confidence intervals (CI) or medians with 95% CI obtained through bootstrapping. All statistical analyses were conducted using GraphPad 9.4.1 (GraphPad Software Inc., San Diego, CA, USA).

Data availability

No datasets were generated or analyzed during the current study.

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Author contributions

R.B.M.: methodology, conceptualization, investigation, formal analysis, writing—original draft. H.P.: conceptualization, writing—review & editing. L.M.: investigation, writing—review & editing. R.V.: methodology, formal analysis, writing—review & editing. F.V.B.: conceptualization, formal analysis, funding acquisition, writing—review & editing. O.C.: conceptualization, formal analysis, funding acquisition, writing—review & editing.

Competing interests

Onelife provided the enzymatic cocktail used in this study and contributed to cover the functioning costs of this study, without intervening in the design of the study, the interpretation of the data or the writing of the paper. Prof. Van Bambeke has been named among the inventors of the international Patent Application WO2022079315, assigned to OneLife and UCL. The authors declare no other competing interests.

Additional information

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Enzymes-enhanced antibiotic therapy reduces biofilms to undetectable levels in an implant-associated infection model

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Activity of vancomycin and TEC in poloxamer P407 hydrogel when applied twice on biofilms *in vitro* : multiple comparisons between TGN and P407 over time using Tukey's test (α = 0.05) (analysis of data from Figure 3)

(a) Tukey's multiple comparisons test results for CFU

Time point	Comparison	Mean difference	95% CI of difference	Adjusted P value	Significance
24 h	TGN vs. P407	-0.10	-0.58 to 0.38	0.9775	ns
48 h	TGN vs. P407	-0.05	-0.53 to 0.43	0.9984	ns
72 h	TGN vs. P407	0.01	-0.47 to 0.49	>0.9999	ns
96 h	TGN vs. P407	0.17	-0.31 to 0.65	0.8709	ns

(B) Descriptive statistics and test details for CFU

Time point	Comparison	Mean (TGN)	Mean (P407)	SE of difference	Sample size (per group)
24 h	TGN vs. P407	8.09	8.19	0.17	9
48 h	TGN vs. P407	7.70	7.75	0.17	9
72 h	TGN vs. P407	7.97	7.96	0.17	9
96 h	TGN vs. P407	7.79	7.62	0.17	9

(c) Tukey's multiple comparisons test results for biomass

Time point	Comparison	Mean difference	95% CI of difference	Adjusted P value	Significance
24 h	TGN vs. P25	2.39	-8.47 to 13.25	>0.9999	ns
48 h	TGN vs. P25	2.29	-8.57 to 13.15	>0.9999	ns
72 h	TGN vs. P25	2.58	-8.28 to 13.44	>0.9999	ns
96 h	TGN vs. P25	3.01	-7.85 to 13.87	>0.9999	ns

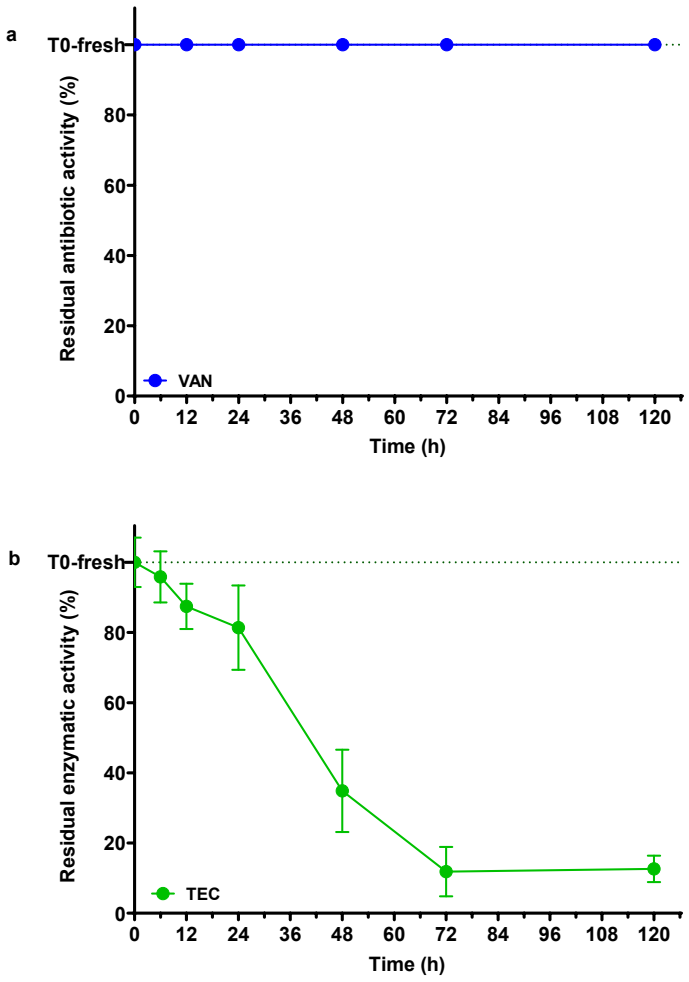
(d) Descriptive statistics and test details for CFU

Time point	Comparison	Mean (TGN)	Mean (P407)	SE of difference	Sample size (per group)
24 h	TGN vs. P25	100.0	97.61	3.26	9
48 h	TGN vs. P25	100.0	97.71	3.26	9
72 h	TGN vs. P25	100.0	97.42	3.26	9
96 h	TGN vs. P25	100.0	96.99	3.26	9

Supplementary Table 2 : Treatment groups and sample size in the *in vivo* study

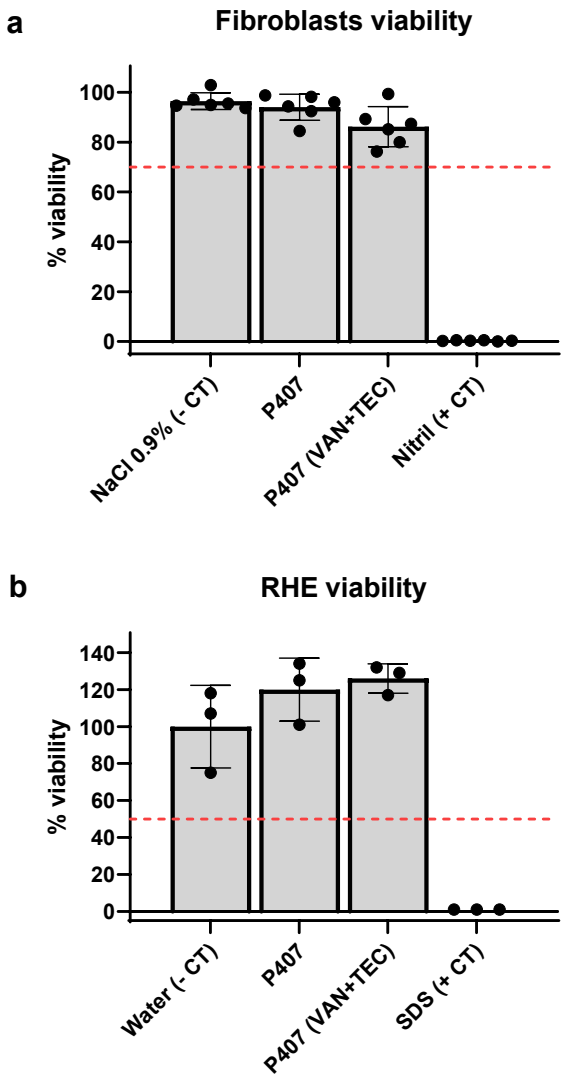
		Number of samples (number of animals) for adhering bacteria									
Day / **Group**		**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Group B	**untreated**	32 (8)		32 (8)		32 (8)		32 (8)		32 (8)	32 (8)
Group C	**VAN ip + P407**	32 (8)	32 (8)	32 (8)	32 (8)		24 (6)		16 (4)		16 (4)
Group D	**VAN ip + P407 TEC**	32 (8)	32 (8)	32 (8)	32 (8)		24 (6)		16 (4)		16 (4)
Group E	**VAN ip + P407 VAN**	32 (8)	32 (8)	32 (8)	32 (8)		24 (6)		16 (4)		16 (4)
Group F	**VAN ip + P407 VAN+TEC**	32 (8)	32 (8)	32 (8)	32 (8)		24 (6)		16 (4)		16 (4)
Group G	**(VAN+RIF) ip + P407**	32 (8)	32 (8)	32 (8)	32 (8)		24 (6)		16 (4)		16 (4)
Group H	**(VAN+RIF) ip + P407 TEC**	32 (8)	32 (8)	32 (8)	32 (8)		24 (6)		16 (4)		16 (4)
Group I	**(VAN+RIF) ip + P407 VAN**	32 (8)	32 (8)	32 (8)	32 (8)		24 (6)		16 (4)		16 (4)
Group J	**(VAN+RIF) ip + P407 VAN+TEC**	32 (8)	32 (8)	32 (8)	32 (8)		24 (6)		16 (4)		16 (4)
		Number of samples (number of animals) for adhering bacteria[a]									
Day / **Group**		**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Group B	**untreated**										16 (4)
Group C	**VAN ip + P407**				8 (2)		8 (2)				16 (4)
Group D	**VAN ip + P407 TEC**				8 (2)		8 (2)				16 (4)
Group E	**VAN ip + P407 VAN**				8 (2)		8 (2)				16 (4)
Group F	**VAN ip + P407 VAN+TEC**				8 (2)		8 (2)				16 (4)
Group G	**(VAN+RIF) ip + P407**				8 (2)		8 (2)				16 (4)
Group H	**(VAN+RIF) ip + P407 TEC**				8 (2)		8 (2)				16 (4)
Group I	**(VAN+RIF) ip + P407 VAN**				8 (2)		8 (2)				16 (4)
Group J	**(VAN+RIF) ip + P407 VAN+TEC**				8 (2)		8 (2)				16 (4)

[a] each cage considered as an independent replicate, based on the low inter-animal variation (DOI: 10.1089/ten.TEB.2012.0059; 10.1042/ETLS20190061)



Supplementary Figure 1: Stability of vancomycin and TEC in the 25 % w/v poloxamer 407 hydrogel formulation.

Samples collected during the experiments presented in Figure 1 (kinetics of release) for the VAN+TEC formulation were used for assessing the residual activity of the active components in the buffer. (**a**) VAN was measured using a microbiological assay. TEC activity was evaluated by measured by its capacity to reduce biomass in a fresh biofilm. Data are expressed as percentage of the activity of a freshly prepared solution of vancomycin or of TEC. Values are means ± SD of triplicates (n=3).



Supplementary Figure 2: Evaluation of the innocuity of the formulation.

(a): Percentage of viability of fibroblasts exposed to P407 alone, or containing vancomycin and TEC (VAN-TEC) during 24 h, as assessed using MTT assay. Negative control: NaCl 0.9%; positive control: TouchNTuff 92-605 Nitrile material. The accepted limit is a viability ≥ 70% (highlighted by the red dotted line). Data are means ± SD of 2 independent experiments performed in triplicates (N=2, n=3). **(b):** viability of reconstituted human epidermis exposed to P407 alone, or containing vancomycin and TEC (VAN+TEC) for 42 minutes at room temperature, rinsed with PBS and incubated for 42 hours in fresh medium. Viability was thereafter assessed by MTT assay. Negative control: water; Positive control: SDS 5%. The accepted limit is a viability ≥ 50% (highlighted by the red dotted line). Data are mean ± SD of 3 independent experiments.