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Antibiotic-Loaded Synthetic Calcium Sulfate Beads for Prevention of Bacterial Colonization and Biofilm Formation in Periprosthetic Infections

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Periprosthetic infection (PI) causes significant morbidity and mortality after fixation and joint arthroplasty and has been extensively linked to the formation of bacterial biofilms. Poly(methyl methacrylate) (PMMA), as a cement or as beads, is commonly used for antibiotic release to the site of infection but displays variable elution kinetics and also represents a potential nidus for infection, therefore requiring surgical removal once antibiotics have eluted. Absorbable cements have shown improved elution of a wider range of antibiotics and, crucially, complete biodegradation, but limited data exist as to their antimicrobial and antibiofilm efficacy. Synthetic calcium sulfate beads loaded with tobramycin, vancomycin, or vancomycin-tobramycin dual treatment (in a 1:0.24 [wt/wt] ratio) were assessed for their abilities to eradicate planktonic methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* relative to that of PMMA beads. The ability of the calcium sulfate beads to prevent biofilm formation over multiple days and to eradicate preformed biofilms was studied using a combination of viable cell counts, confocal microscopy, and scanning electron microscopy of the bead surface. Biofilm bacteria displayed a greater tolerance to the antibiotics than their planktonic counterparts. Antibiotic-loaded beads were able to kill planktonic cultures of 10^6 CFU/ml, prevent bacterial colonization, and significantly reduce biofilm formation over multiple days. However, established biofilms were harder to eradicate. These data further demonstrate the difficulty in clearing established biofilms; therefore, early preventive measures are key to reducing the risk of PI. Synthetic calcium sulfate loaded with antibiotics has the potential to reduce or eliminate biofilm formation on adjacent periprosthetic tissue and prosthesis material and, thus, to reduce the rates of periprosthetic infection.

Periprosthetic infection (PI) is a serious complication of total joint arthroplasty with high rates of associated morbidity (1, 2), and a growing body of data suggests that bacterial biofilms are the underlying cause (3–9). Within a biofilm, bacteria display a ≥1,000-fold tolerance to antibiotics than their planktonic counterparts (10) and significant resistance to innate and adaptive host immunity (11). Moreover, biofilms associated with orthopedic hardware are typically difficult to culture using conventional clinical microbiological methods, and the lack of a definitive diagnosis may result in an underestimate of infection rates (12, 13). Consequently, the underlying infection is difficult to diagnose and treat (6, 7), and often the only effective intervention is the twin strategy of thorough debridement and prostheses removal (14).

Existing prevention strategies include the use of antibiotic-loaded poly(methyl methacrylate) (PMMA) cement spacers or beads to elevate local antibiotic levels at the surgical site. Studies have demonstrated a significant reduction in infection rates using antibiotic-impregnated cement in total hip arthroplasty and total knee arthroplasty patient populations (15, 16). Conversely, other studies indicated limited clinical benefit, albeit with various antibiotics and concentrations (17, 18) and poor descriptions of elution kinetics (19). Additionally, once the antibiotics have eluted from a nonabsorbable cement, the surface becomes a foreign body that is subject to bacterial colonization and biofilm formation (20, 21).

Absorbable mineral-based bone cements are not as mechanically strong as acrylic cements, but they provide some advantages for antibiotic delivery and infection control. First, they do not require removal, since they are naturally absorbed. Second, there is little temperature increase during setting, so they have the capacity to accommodate a wider range of antibiotics. Last, as they slowly dissolve, there is a sustained release of antibiotics over the cement's lifetime. Studies using carriers constructed from calcium sulfate have shown improved antibiotic release relative to that of PMMA beads and, importantly, complete biodegradation (22–24). The efficacy of antibiotic-loaded cements has been corroborated in animal models in which data demonstrated the effective treatment of experimental osteomyelitis in rabbits (25, 26) and of contaminated fractures in a goat model (27). These data support the rationale for the more widespread clinical use of antibiotic-

Received 19 June 2014 **Returned for modification** 26 September 2014
Accepted 8 October 2014

Accepted manuscript posted online 13 October 2014

Citation Howlin RP, Brayford MJ, Webb JS, Cooper JJ, Aiken SS, Stoodley P. 2015. Antibiotic-loaded synthetic calcium sulfate beads for prevention of bacterial colonization and biofilm formation in periprosthetic infections. Antimicrob Agents Chemother 59:111–120. doi:10.1128/AAC.03676-14.

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doi:10.1128/AAC.03676-14

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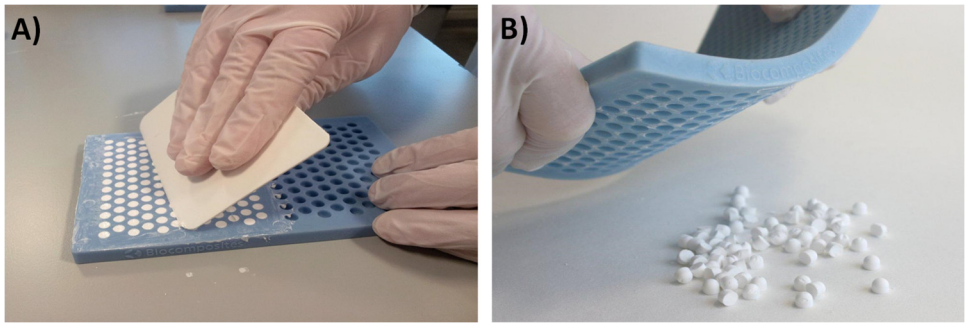

FIG 1 Preparation of PG-CSH beads: production of the beads from a smooth paste (A) and removal of the set beads from the mold (B).

loaded cements; however, a better understanding of their antibacterial and antibiofilm efficacies is required. The goals of the present study were to quantify the long-term bioactivities of antibiotics released from synthetic calcium sulfate beads, their ability to prevent biofilm formation on adjacent surfaces and on the beads themselves, and their ability to kill preformed biofilms.

MATERIALS AND METHODS

Bacterial strains and growth media. Epidemic methicillin-resistant *Staphylococcus aureus* 16 (EMRSA-16) NCTC 13143 and *Staphylococcus epidermidis* ATCC 35984 were each cultured in tryptic soy broth (TSB) (Sigma-Aldrich). EMRSA-16 is one of the two main epidemic bacteremia MRSA strains in the United Kingdom (28) and has been shown to form biofilms (29). *S. epidermidis* ATCC 35984 is an intercellular adhesion (*ica*)-positive strain and is a strong biofilm former (30).

Antibiotic MIC and MBC and biofilm MBC assays. Overnight cultures of each strain were diluted in fresh TSB to an optical density (OD) of 10^6 cells/ml. In 10 ml of bacterial culture, dilution series of 0.5 μg/ml to 15,000 μg/ml vancomycin and 0.5 μg/ml to 5,000 μg/ml tobramycin were prepared by using tobramycin sulfate alone (Sigma-Aldrich), vancomycin hydrochloride alone (Hospira UK Ltd.), or a vancomycin-tobramycin combination treatment (in a 1:0.24 [wt/wt] ratio). The cultures were incubated for 15 h at 37°C. The ratio of vancomycin to tobramycin was selected based on previous clinical use (31). The MIC of each antibiotic was determined by measuring the cultures' OD at 680 nm (OD_{680}) with a microplate reader (BMG Omega). The cultures were serially diluted in Hanks buffered salt solution (HBSS) (Sigma-Aldrich) and plated onto tryptic soy agar (TSA) (Sigma-Aldrich); then, they were incubated for 24 h at 37°C to determine the minimum bactericidal concentration (MBC) that elicited a >3-log reduction in planktonic cells.

Additionally, biofilm MBCs were determined following growth in 6-well plates for 72 h at 37°C from a starting inoculum of 4 ml of 10^6 cells/ml, with nutrient exchanges every 24 h. Antibiotic treatment was performed for 15 h at 37°C. The wells were rinsed in HBSS, and the surface was scraped to remove the attached biofilms into 1 ml HBSS. Following vortexing for 20 s to homogenize the biofilm bacteria, serial dilutions were performed in HBSS and plated onto TSA solid agar plates.

Preparation of calcium sulfate alpha-hemihydrate and PMMA beads. A 10-ml kit containing 20 g pharmaceutical-grade calcium sulfate alpha-hemihydrate powder (PG-CSH) (Stimulan; Biocomposites Ltd., United Kingdom) was mixed with 6 ml of sterile water (unloaded beads), 6 ml of a 40-mg/ml tobramycin sulfate solution (tobramycin-loaded beads), or 1,000 mg of vancomycin hydrochloride powder plus the 6 ml sterile water required to make the paste and cure the cement (vancomycin-loaded beads). For the beads containing vancomycin and tobramycin, a 10-ml kit of PG-CSH was mixed with 1,000 mg vancomycin hydrochloride powder plus 6 ml of a 40-mg/ml tobramycin solution. In each case, all the components were mixed for 30 to 60 s to form a smooth paste, which was pressed into 4.8-mm-diameter hemispherical cavities in a flexible mold (Fig. 1A). The beads were left undisturbed for 30 to 60 min to set. When set, the beads were removed by flexing the mold (Fig. 1B). The unloaded beads with diameters of 4.8 mm weighed 0.108 g each. When loaded with antibiotics, each bead contained 4.13 mg of vancomycin and/or 1.02 mg of tobramycin.

To compare release from the calcium sulfate bioabsorbable beads with the current standard of care, we prepared nonabsorbable PMMA beads (TBCem 3; Tornier, France) per standard operating room protocols (32, 33). A 40-g pack of powder was combined with either 3.8 g vancomycin or 0.90 g tobramycin or with the two antibiotics in combination. This concentration is higher than recommended when structural support is required, but it is in the (high-end) range used for antibiotic-eluting beads (32). The PMMA paste was spread into a 4.8-mm bead mold identical to that used for the pharmaceutical-grade calcium sulfate beads (Fig. 1A), and the cavities were completely filled. The unloaded 4.8-mm-diameter beads weighed 0.065 g each. When loaded with antibiotics, each bead contained 3.4 mg of vancomycin and/or 0.9 mg of tobramycin, which was 18% less by weight per bead than a PG-CSH bead.

Antibiotic-eluting PG-CSH and PMMA beads for inhibiting planktonic bacterial growth. Modified Kirby-Bauer assays were used to determine the release and potency of antibiotics from the beads over time. First, a lawn of bacteria was spread onto TSA plates using 50 μl of an overnight culture. Beads were placed onto the agar plate with sterile forceps and incubated at 37°C for 24 h. Zones of inhibition (ZOI) were assessed and photographed, and the beads were transferred onto a freshly prepared lawn of bacteria. This process was repeated each day until the ZOI were lost. The areas (in cm^2) of the ZOI were calculated using ImageJ (version 1.48) (34), and the 90-mm diameter of the petri dish in each image was used for spatial calibration. Analyses were performed on days 1, 2, 3, and 4 and every 2 days thereafter. The areas, rather than the diameters, of the ZOI were calculated to account for irregularities in the shape of the ZOI.

Antibiotic-eluting PG-CSH beads for preventing biofilm formation. Antibiotic-loaded and unloaded beads (10 beads per well or plate) were placed into 6-well plates for CFU enumeration and into MatTek tissue culture plates to allow for optimal sample imaging (MatTek Corporation). The number of beads was chosen to compromise between higher bead numbers, which may physically inhibit substratum colonization, and too few beads, which would limit clinical relevance. A total of 4 ml of a culture of 10^6 cells/ml was added to the wells, with the beads present upon addition. Every 24 h, the medium was replaced and the beads were subjected to a fresh bacterial challenge of 4 ml of 10^6 cells/ml. At days 1 (24 h postinoculation), 2, 3, 7, and 14, CFU counts of the surface-attached bacterial populations in the 6-well plates were performed as described above. Concurrently, at days 1, 2, 3, 7, and 14, the fluorescent stain Syto 9 was used to microscopically assess the surface-attached biomass in the MatTek plates. Plates were rinsed with HBSS and stained (with 2 μl of Syto 9 per ml of HBSS) for 20 min. The plates were gently rinsed and analyzed using an inverted Leica DMI600 SP5 confocal laser scanning microscope (CLSM).

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TABLE 1 MIC and MBC data for EMRSA-16 NCTC 13143 and *S. epidermidis* ATCC 35984 in planktonic and biofilm growth[a]

Strain and antibiotics	Planktonic growth data (μg/ml)		Biofilm growth data (MBC [μg/ml])
	MIC	MBC	
EMRSA NCTC 13143			
Tobramycin	>5,000	>5,000	>5,000
Vancomycin	2	2	>15,000
Vancomycin-tobramycin	2/0.48	2/0.48	>15,000/3,600
S. epidermidis ATCC 35984			
Tobramycin	64	256	>5,000
Vancomycin	2	4	>15,000
Vancomycin-tobramycin	2/0.48	4/0.96	>15,000/3,600

[a] Planktonic assays were performed in triplicate, and the data are expressed as the means of 15 data points (5 points per experimental repeat). Biofilm MBCs were determined in duplicate, and the data are expressed as the means of 10 data points (5 points per experimental repeat).

Additionally, beads were visualized by scanning electron microscopy (SEM) to determine the extent of colonization. Beads were transferred to an initial fixative of 3% glutaraldehyde, 0.1 M sodium cacodylate (pH 7.2), and 0.15% Alcian blue for 24 h at 4°C. The fixative was replaced with 0.1 M sodium cacodylate (pH 7.2) and incubated for 1 h at room temperature followed by secondary fixation in 0.1 M osmium tetroxide in 0.1 M sodium cacodylate (pH 7.2) for 1 h. Following a further incubation with 0.1 M sodium cacodylate (pH 7.2) for 1 h, the beads were placed through an ethanol series (at 30, 50, 70, and 95% and twice at 100%), each for a 10-min incubation period. The beads were then critical-point dried and sputter coated in a gold-palladium alloy. Imaging was carried out using an FEI Quanta 200 scanning electron microscope.

Antibiotic-eluting PG-CSH beads for killing preexisting biofilms. Biofilms were cultured as described, and beads (10 per well) were placed onto 6-well plates and incubated for a further 24 h or 72 h (with medium changes every 24 h) at 37°C. CFU counts were performed on the residual surface biomass as described above.

Statistics. Data were compared using a Mann-Whitney rank sum test for nonnormally distributed data, and a difference was considered significant when the P value was <0.05.

RESULTS

Planktonic and biofilm MICs/MBCs. Data to determine the planktonic MICs and MBCs (Table 1) showed that EMRSA-16 NCTC 13143 was resistant to tobramycin but sensitive to vancomycin (tobramycin MBC, >5,000 μg/ml; vancomycin MBC, 2 μg/ml). The MIC and MBC of the vancomycin-tobramycin dual treatment were equal to those of vancomycin alone. A similar profile was observed for *S. epidermidis* ATCC 35984, although it had intermediate sensitivity to tobramycin. A comparison of the planktonic and biofilm MBCs showed that the biofilms displayed ≥100-fold antimicrobial resistances.

Antibiotic-loaded PG-CSH and PMMA beads for inhibiting planktonic bacteria. Throughout the duration of the assay (40 days), unloaded PG-CSH and PMMA beads did not elicit a zone of inhibition (ZOI) with either bacterial strain (Fig. 2). With the PG-CSH beads, the MRSA strain was resistant to tobramycin, as shown by a small ZOI (0.39 cm^2 at day 1), which was not present at day 2 (Fig. 2). The vancomycin-loaded and vancomycin-tobramycin-loaded beads produced strong ZOI, which were maintained until approximately day 26, when a gradual decrease in the sizes of the ZOI was observed, with complete loss by day 40. *S. epidermidis* was susceptible to vancomycin, tobramycin, and the antibiotic combination, with large ZOI observed in all cases at day 1 (Fig. 2). A rapid decline in the size of the tobramycin ZOI was observed between days 1 (2.78 cm^2) and 4 (0.62 cm^2), and the loss of the ZOI was observed at day 5. As with MRSA, vancomycin-loaded and vancomycin-tobramycin-loaded beads persistently elicited strong ZOI until approximately day 34, when a steady decline in the sizes of the ZOI was noted, with complete loss at day 40.

With the PMMA beads, similar resistance profiles of each strain to the antibiotics was observed. Notably, however, the size of the ZOI decreased more rapidly than with the PG-CSH beads. The ZOI of the vancomycin-loaded and vancomycin-tobramycin-loaded beads were completely lost by day 12 for the MRSA and *S. epidermidis* strains.

Antibiotic-loaded PG-CSH beads for preventing biofilm formation. The vancomycin and antibiotic combination beads significantly reduced MRSA surface colonization, with an initial ~3-log reduction in the number of CFU/cm^2 at day 1. Despite fresh bacterial challenges every 24 h, the vancomycin-loaded and the vancomycin-tobramycin-loaded beads further reduced the number of viable surface-bound cells, with a maximal reduction to 7.6×10 CFU/cm^2 with the antibiotic combination beads at day 3 relative to 1.0×10^6 CFU/cm^2 with unloaded beads. Furthermore, at day 14, the vancomycin beads and the antibiotic combination beads still displayed significantly reduced numbers of CFU/cm^2 relative to those with the unloaded beads ($P \leq 0.001$). Confocal microscopy corroborated the CFU measurements but gave the additional benefit of allowing direct observation of biofilm formation (Fig. 3). Importantly, even at day 14, when the CFU data indicated a large increase in the number of CFU/cm^2 relative to that on day 7 with the vancomycin-loaded and vancomycin-tobramycin-loaded beads, CLSM indicated that biofilm formation was markedly reduced relative to that with the unloaded beads.

In agreement with previous data (Table 1 and Fig. 2), the tobramycin-loaded beads had a limited effect on MRSA, although a statistically significant reduction in the number of CFU/cm^2 was noted relative to that of the unloaded-bead treatment groups at days 1, 2, and 3 ($P \leq 0.001$ for each day). After 3 days, biofilm formation progressed similar to that of the controls.

SEM showed that the dual-loaded vancomycin-tobramycin beads were the most effective at preventing biofilm formation (Fig. 4). Unloaded beads demonstrated early bacterial colonization on day 2 and extensive biofilm formation by day 7. Conversely, there was no detectable MRSA colonization up to or on day 7 of using the vancomycin-tobramycin-loaded beads; bacterial colonization and biofilm formation were observed only at day 14.

The tobramycin-loaded and vancomycin-tobramycin-loaded beads resulted in complete killing of *S. epidermidis* at day 1 (Fig. 5). With respect to tobramycin-loaded beads at day 2, the number of surface-associated *S. epidermidis* cells increased rapidly until days 7 and 14, when no further difference was observed in the number of CFU/cm^2 relative to that of the unloaded beads ($P > 0.05$).

Conversely, in the presence of vancomycin-loaded beads, colonization of the substratum was markedly less at days 2, 3, and 7 than for controls. Crucially, the combination of vancomycin and tobramycin loaded in the beads achieved complete killing at days 2 and 3, suggesting that concentrations of eluted antibiotics remained higher than the MBCs for *S. epidermidis* for at least 72 h.

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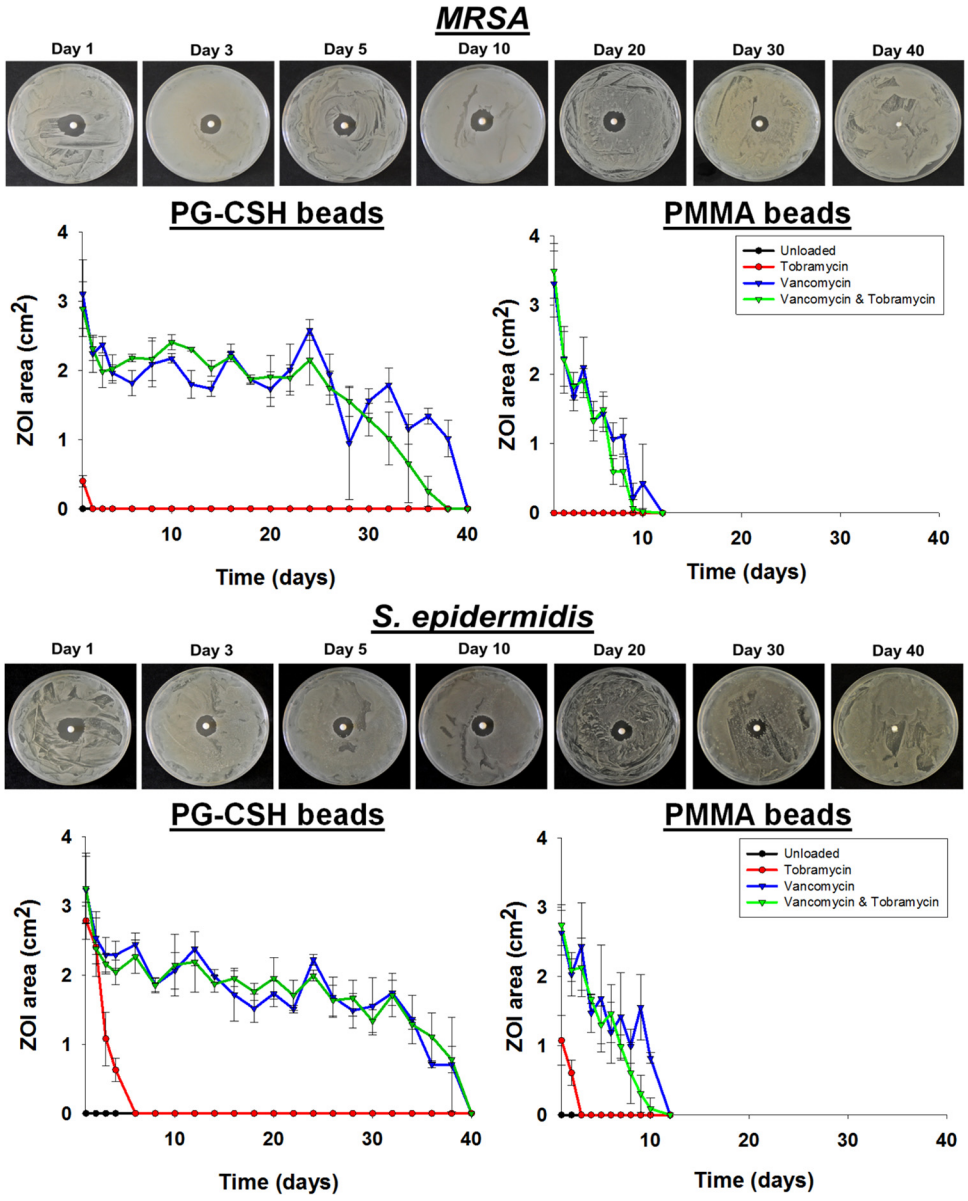

FIG 2 Repeat modified Kirby-Bauer assays for assessing zones of inhibition (ZOI) of EMRSA-16 NCTC 13143 and *S. epidermidis* ATCC 35984 over a period of 40 days. Images are representative photographs of the ZOI of the two bacterial strains observed on agar plates at days 1, 3, 5, 10, 20, 30, and 40 of vancomycin-tobramycin-loaded PG-CSH beads. Graphs show the sizes of the ZOI (in cm^2) over time, as calculated using ImageJ. Assays were performed in triplicate, and data are expressed as the means of 3 data points with standard error bars.

Even at day 7, the bacterial concentrations remained low (2.1×10^2 CFU/cm^2).

Confocal analysis (Fig. 5) corroborated the CFU data. The vancomycin-loaded and vancomycin-tobramycin-loaded beads suppressed bacterial colonization up to day 7. Importantly, by day 14, while the CFU/cm^2 data showed surface colonization similar to that of the control, CLSM images highlighted that this existed only as a thin monolayer, with biofilm formation markedly suppressed.

Visualization of the unloaded bead surfaces demonstrated rapid *S. epidermidis* colonization and biofilm formation (Fig. 6). However, the vancomycin-tobramycin-loaded beads demonstrated markedly delayed surface colonization, with no visible bacteria on the bead surface at day 2. Through day 14 of the experiment, biofilm formation was significantly reduced relative to that of the unloaded beads, with limited bacterial colonization and extracellular polymeric substance deposition.

Antibiotic-loaded PG-CSH for killing preexisting biofilms. To assess the ability of the antibiotic-loaded beads to eradicate established biofilms, 10 beads were incubated with biofilms for 24 and 72 h. The data are expressed as log reductions relative to that for the unloaded treatment groups to account for possible physical abrasion and disruption of the biofilm during growth through the presence of the bead itself. After 24 h, the greatest effect observed was an ~1-log reduction in the numbers of viable MRSA and *S. epidermidis* cells observed with combination vancomycin-

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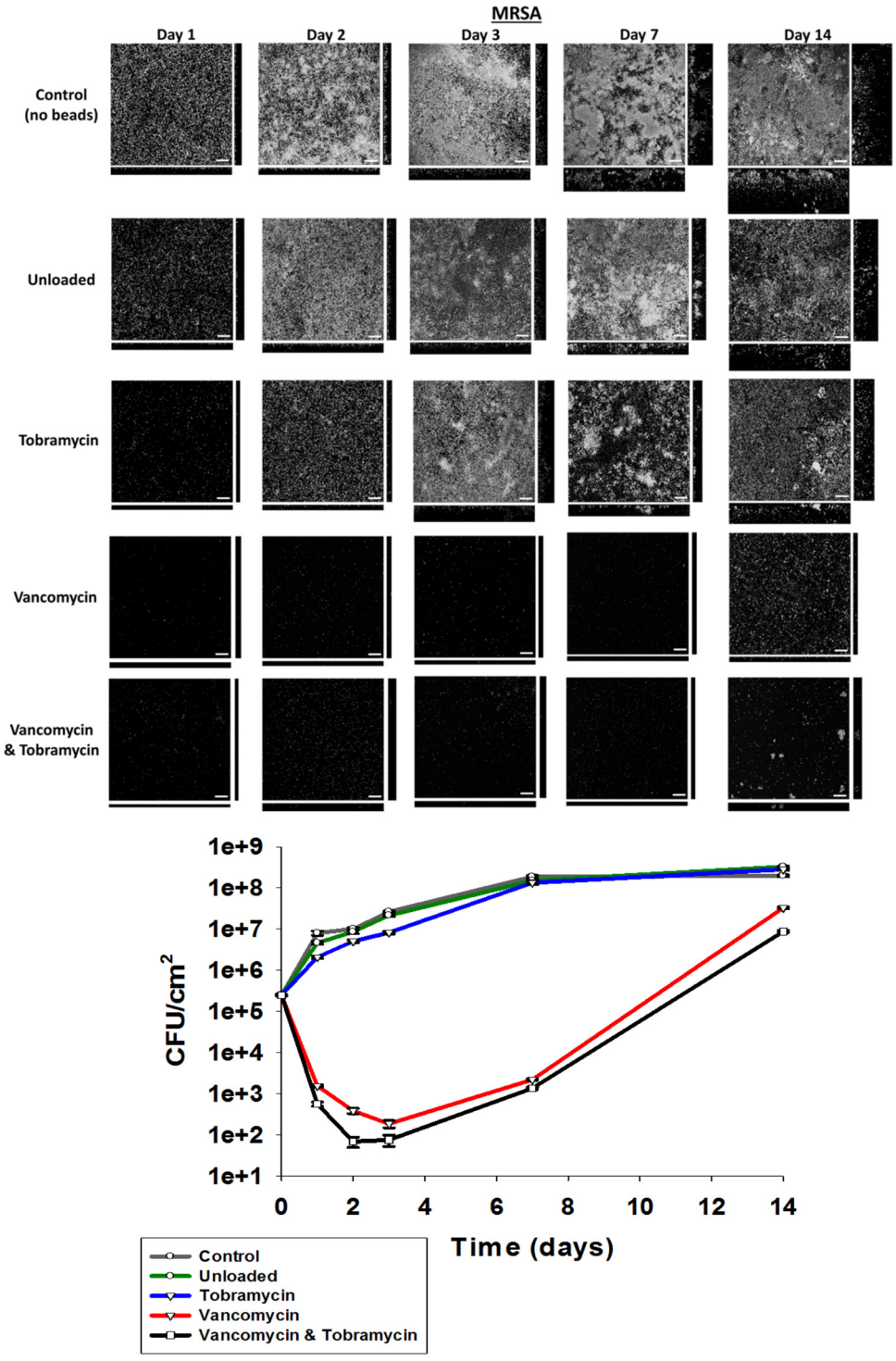

FIG 3 EMRSA-16 NCTC 13143 biofilm formation over time in the presence of antibiotic-loaded synthetic calcium sulfate beads as determined by CLSM images and CFU counts (per cm^2). Data are expressed as the means of 15 data points (5 data points per experimental repeat) with standard error bars. Bars, 25 μm.

tobramycin-loaded beads (Fig. 7), which represents 88% and 90% biofilm reductions, respectively. The efficacy of antibiotic-loaded beads toward preexisting MRSA biofilms was increased by extending the contact time to 72 h, when a 4-log (99.999%) reduction in the number of CFU/cm^2 was obtained with the antibiotic combination beads relative to that of the control. However, in the case of *S. epidermidis*, increasing the bead contact time to 72 h did not greatly increase biofilm killing (24-h vancomycin-tobramycin reduction, 0.98 log [89.67%], versus 72-h vancomycin-tobramycin reduction, 1.01 log [90.43%]).

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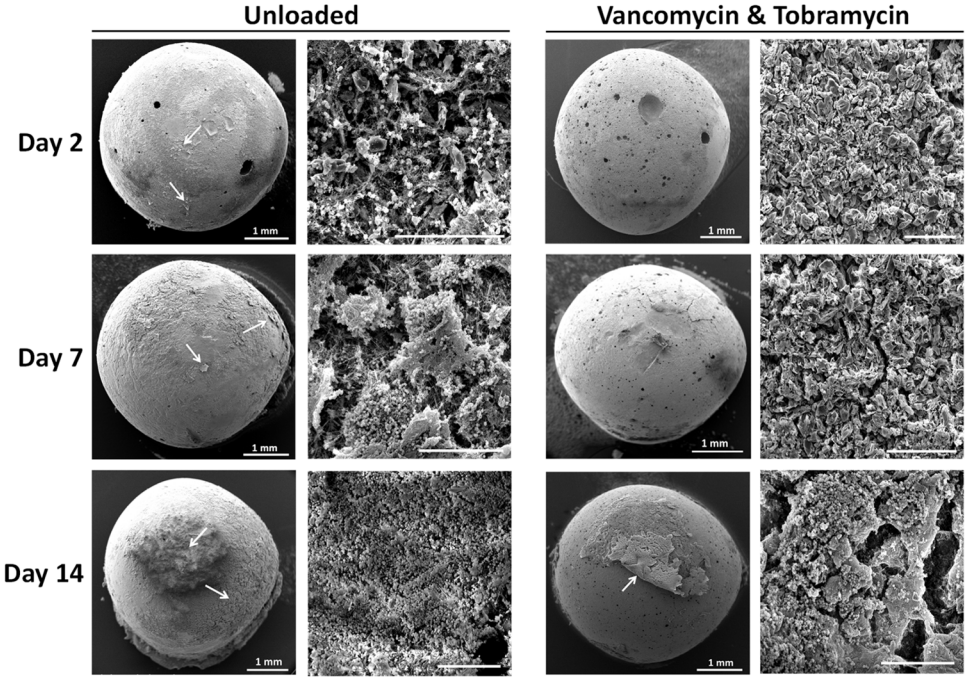

FIG 4 Biofilm formation of EMRSA-16 NCTC 13143 on vancomycin- and tobramycin-loaded beads relative to that on unloaded beads over time as determined by SEM. White arrows indicate bacterial colonization and biofilm formation. Bars, 25 μm unless otherwise stated.

DISCUSSION

In this study, we evaluated antibiotic release from a synthetic calcium sulfate for its long-term capacity to kill planktonic MRSA and *S. epidermidis* strains and, for the first time, to address the issue of biofilm prevention and eradication. The beads were loaded with vancomycin, tobramycin, or a combination of vancomycin and tobramycin. Our initial aim was to characterize the strains using assays to determine the planktonic MICs and MBCs, demonstrating that *Staphylococcus aureus* 16 NCTC 13143, a MRSA strain, was susceptible to vancomycin but resistant to tobramycin. *S. epidermidis* ATCC 35984 was susceptible to vancomycin and intermediately sensitive to tobramycin. Similar to previous studies, a comparison of the planktonic MBCs with those of biofilm cells showed, in all cases where planktonic bacteria were initially sensitive, that biofilms were ≥100 times more resistant (10), further demonstrating the importance of preventing robust biofilm formation in PIs.

This was highlighted further when PG-CSH beads loaded with a combination of vancomycin and tobramycin were able to achieve only a 1-log reduction in viable bacteria after a contact time of 24 h. Increasing the contact time to 72 h resulted in a further 3-log reduction in the number of MRSA cells, demonstrating the importance of maintaining locally high concentrations for as long as possible. While an overall 4-log reduction can be considered highly effective *in vivo*, complete MRSA biofilm eradication was not achieved. However, the *S. epidermidis* biofilm was more difficult to clear, and although the combination beads reduced almost 90% of bacterial coverage over a 24-h period, there was no further killing up to 72 h.

A Kirby-Bauer-type diffusion test showed that antibiotics loaded into PG-CSH were potent against planktonic bacteria for multiple days. When the bacteria were sensitive to the antibiotic, zones of clearing were observed and maintained for 39 days, indicating that eluted antibiotics (in this case, vancomycin) from PG-CSH remained at concentrations higher than the MIC throughout this time. This observation is in agreement with that of a 2014 study by Roberts et al., who showed similar elution rates of vancomycin from calcium sulfate beads (35). Importantly, in the present study, we also demonstrated that the antibiotics have antimicrobial potency against staphylococcal species over the same time period. However, PMMA, the current standard of care, demonstrated a much shorter elution profile, with the ZOI lost by day 12. Previous studies have shown comparable efficacies of PG-CSH and PMMA; however, this is the first study to compare antimicrobial activity over a long duration (33). While the amount of antibiotic per PMMA bead is 18% less than in PG-CSH, this difference would not be expected to account for the difference in the length of bioactivity. Moreover, the amount of antibiotic added to the PMMA is in the upper limit of what can be practically added to PMMA cement (32). Part of this difference in elution rates may be explained by variations in the porosity of PMMA relative to that of PG-CSH. However, since the initial ZOI were similar in size, it is more likely that the extended period of bioactivity of the PG-CSH beads can be explained primarily by the gradual absorption of this material, resulting in the sustained release of a larger amount of antibiotics over time. Increasing the number of PG-CSH beads to fill dead space would effectively increase the total antibiotic reservoir and ensure maximal contact with potentially infected tissue at the surgical margin. The elution kinetics from the present study suggest that this strategy would ensure high localized antibiotic concentrations to counteract potential infection at the surgical site through intraoperative contamination and also infection acquired later through hematogenous spread almost over the entire 6-week period recommended for treating orthopedic staphylococcal infections. However, it is important to note that, despite a longer

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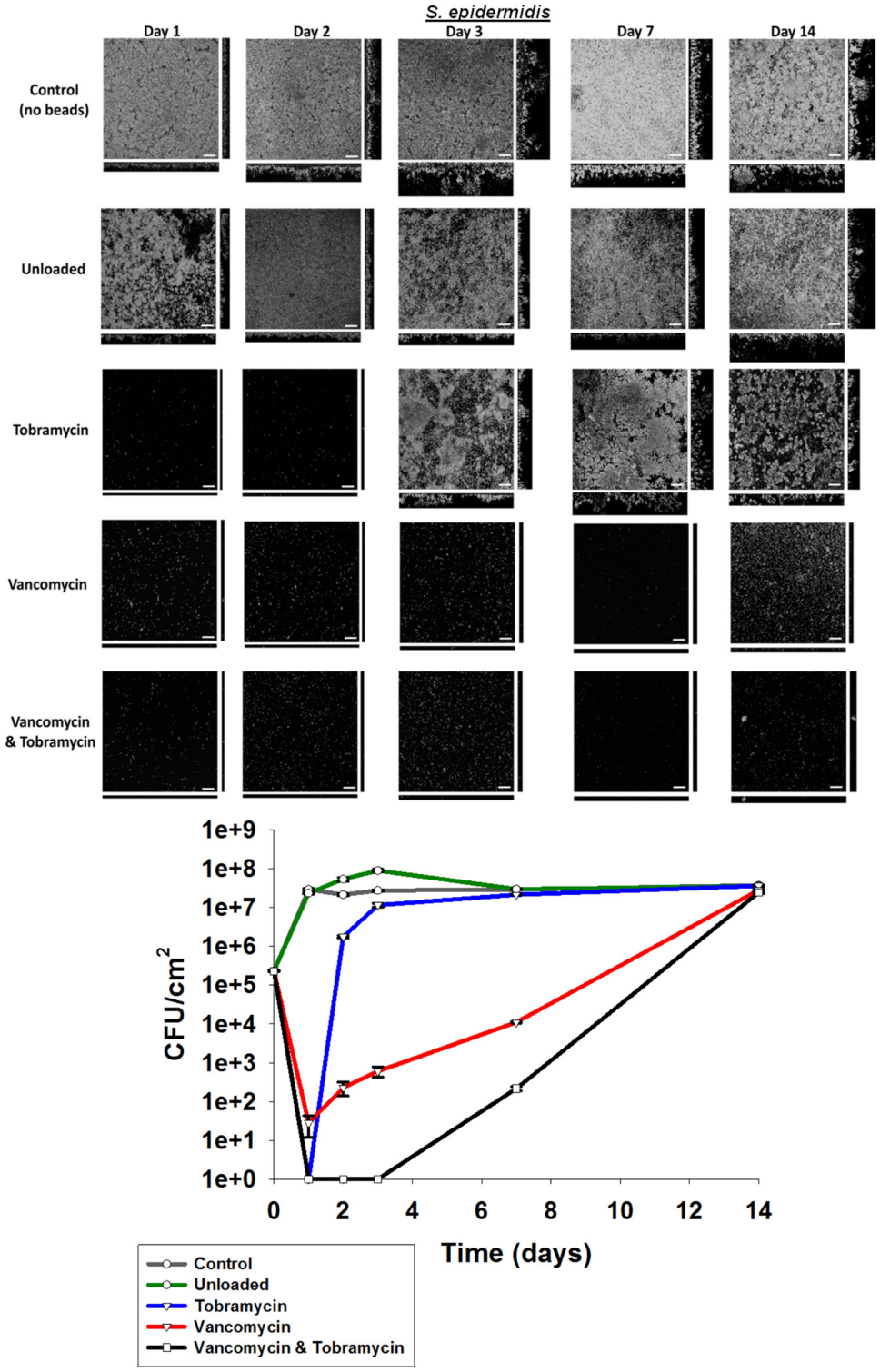

FIG 5 *S. epidermidis* ATCC 35984 biofilm formation over time in the presence of antibiotic-loaded synthetic calcium sulfate beads, as determined by CLSM images and CFU counts (per cm^2). Data are expressed as the means of 15 data points (5 data points per experimental repeat) with standard error bars. Bars, 25 μm.

duration of antimicrobial efficacy in this study, PG-CSH cannot be expected to replace PMMA in situations where mechanical strength and integrity are of paramount importance to the procedure (e.g., fixing a prosthesis to the bone in primary arthroplasty or use as a spacer in a staged revision). However, it is envisioned that PG-CSH beads can be used in conjunction with PMMA, with PG-CSH beads used for the management of the dead space and PMMA providing structural integrity.

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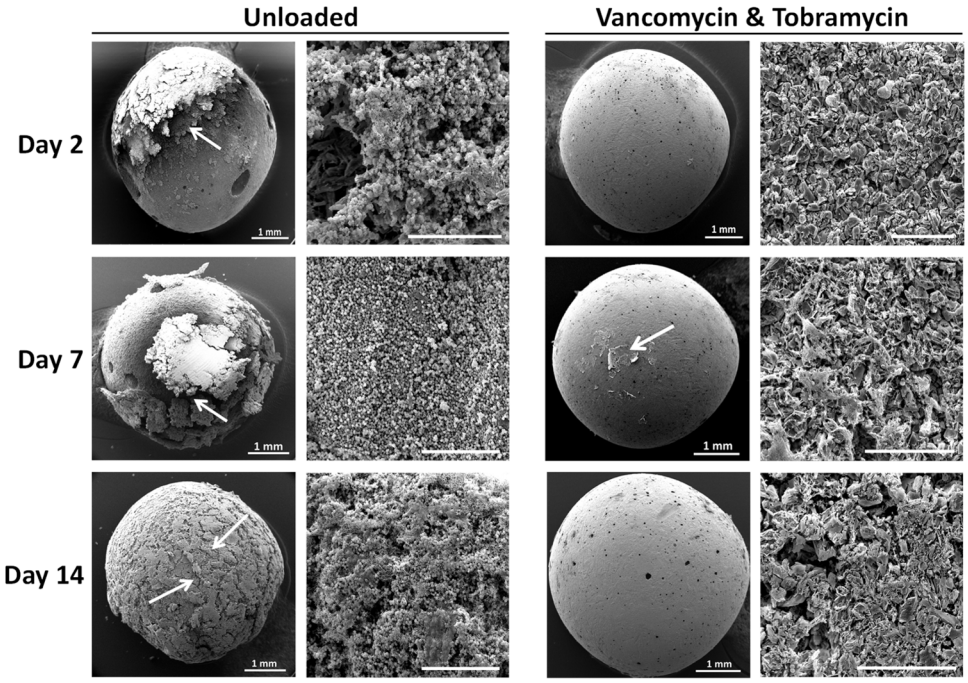

FIG 6 Biofilm formation of *S. epidermidis* ATCC 35984 on vancomycin- and tobramycin-loaded beads relative to unloaded beads over time as determined by SEM. White arrows indicate bacterial colonization and biofilm formation. Scale bars equal 25 μm unless otherwise stated.

Due to the link between the biofilm phenotype and the establishment of periprosthetic infection, additional studies were undertaken to determine how effective antibiotic-loaded PG-CSH beads are at preventing surface colonization and biofilm formation when the beads remain *in situ* and are subjected to daily bacterial challenges. Media containing 10^6 CFU/ml, much greater than would be expected in a single infective dose *in vivo*, were delivered daily for up to 14 days after the initial inoculation. The concentration was chosen to provide a vigorous evaluation of the antibacterial capacities of the beads over time.

In the case of MRSA, growth was attenuated in the presence of vancomycin-loaded and vancomycin-tobramycin-loaded beads for up to 1 week, despite daily bacterial challenges of 10^6 cells/ml, as observed by the numbers of CFU, confocal microscopy, and SEM of the bead surface. With *S. epidermidis*, tobramycin-loaded, vancomycin-loaded, and vancomycin- tobramycin-loaded beads produced significant reductions in the numbers of *S. epidermidis* cells on day 1 as observed by CFU counts, with both the tobramycin and combination antibiotic beads achieving complete killing for 1 and 3 days, respectively. Importantly, markedly reduced

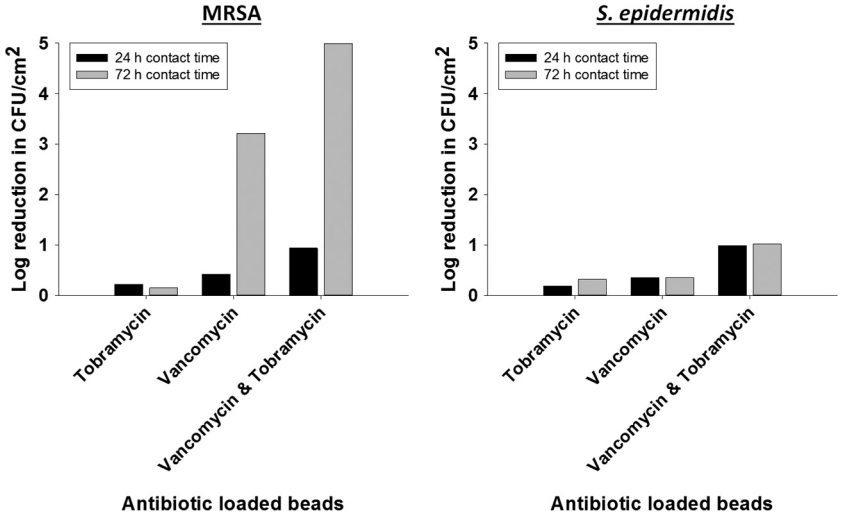

FIG 7 Effects of antibiotic-loaded synthetic calcium sulfate beads with 24-h and 72-h contact times on preexisting biofilms of EMRSA-16 NCTC 13143 and *S. epidermidis* ATCC 35984. Data are expressed as log reductions of the means of 15 data points (5 points per experimental repeat, with each assay performed in triplicate) relative to those of the unloaded treatment group.

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MRSA and *S. epidermidis* biofilm formation during all 14 days was noted with the vancomycin-tobramycin-loaded beads which, importantly, would enable the host immune system to cope more effectively *in vivo* (36). While this duration of efficacy is reduced relative to the 39 days observed in the repeated ZOI study (Fig. 2), this can be explained by the change in focus of the assay outcome, from the bacterial inhibition of rapidly respiring cells on a removable substratum, as in the ZOI assay, to the bactericidal efficacy and biofilm formation on a static substratum in the biofilm prevention assays. Crucially, this again highlights the difficulty in clearing a biofilm once colonization of a substratum and biofilm formation begin. Conversely, however, beads were immersed in medium for the duration of the biofilm prevention study, which is in stark contrast to the experimental setup of the ZOI assay in which the beads are placed on agar. With daily medium changes, antibiotics can be expected to elute from porous PG-CSH more rapidly than on an agar plate, so combined with daily high-dose bacterial challenges, the duration of antibiotic efficacy in the biofilm prevention study can be considered a worst-case scenario.

Importantly, while *in vitro* studies do not account for the flow of body fluids, limb motility, host immune processes, and antibiotic stability *in vivo*, the 7- to 14-day antibacterial activity observed with PG-CSH can be put into the context of similar studies. PMMA, the current standard of care, loaded with vancomycin showed a retention of bioactivity for 48 h (37). Recently, a novel SiO_2-TiO_2-ZnO-CaO-SrO-based glass polyalkenoate cement loaded with vancomycin was also shown to be effective against *S. aureus* for 48 h (38). Studies into the release kinetics of tobramycin and vancomycin from a demineralized bone matrix showed complete elution within periods of 3 and 14 days, respectively (39). Our data indicate bactericidal efficacy of PG-CSH within a time frame similar to that of the release kinetics study and highlight the importance of sustained antibiotic release from these materials for many days after surgical intervention. Also, the SEM data demonstrate the potential for eluting materials to be a nidus for infection and biofilm formation once antibiotic concentrations fall below bacterial MBCs and, therefore, emphasize the benefit of absorbable substrates. Studies have shown complete absorption of high-purity calcium sulfate within 4 to 6 weeks when combined with effective management of penile implant infection and of osteomyelitis (25, 40–42), indicating potentially wide-ranging benefits in the management of PIs.

In conclusion, the use of antibiotic-loaded beads at the site of infection is becoming the standard of care, as the beads enable localized supra-MIC levels which would be difficult to achieve by other means (43). In this study, antibiotic-impregnated PG-CSH demonstrated high bioactivity in preventing early bacterial colonization and biofilm formation by MRSA and *S. epidermidis* strains of bacteria *in vitro*, with long periods of sustained efficacy. This suggests that the use of fully absorbable antibiotic-loaded PG-CSH has the potential to be a highly effective strategy for preventing biofilm formation and therefore reducing the risk of the establishment of chronic infection in total joint arthroplasty and other surgical procedures.

ACKNOWLEDGMENTS

This work was funded by Biocomposites Ltd. and was supported by the NIHR Southampton Respiratory Biomedical Research Unit.

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