Antibiotic-loaded synthetic calcium sulfate beads for the prevention of bacterial colonisation and biofilm formation in periprosthetic infections

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ABSTRACT

Periprosthetic infection (PI) causes significant morbidity and mortality in fixation and joint arthroplasty and has been extensively linked to the formation of bacterial biofilms. Poly(methyl methacrylate) (PMMA) cement or beads are commonly used for antibiotic release to the site of infection but display variable elution kinetics and also represent a potential nidus for infection, therefore requiring surgical removal. 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 and a vancomycin & tobramycin dual treatment (1:0.24 w/w ratio) were assessed for their ability to eradicate planktonic methicillin resistant *S. aureus* (MRSA) and *S. epidermidis,* relative to PMMA beads. The ability of the beads to prevent biofilm formation over multiple days and 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 106 CFU/mL, prevent bacterial colonisation and significantly reduce biofilm formation over multiple days. However, established biofilms were harder to eradicate.

This data further demonstrates the difficulty in clearing established biofilms and therefore early preventative 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 reduce rates of periprosthetic infection.

INTRODUCTION

Periprosthetic infection (PI) is a serious complication of total joint arthroplasty (TJA) with high associated morbidity ([1](#_ENREF_1), [2](#_ENREF_2)) and a growing body of data suggests that bacterial biofilms are the underlying cause ([3-9](#_ENREF_3)). Within a biofilm, bacteria display a 1,000-fold or higher tolerance to antibiotics than their planktonic counterparts ([10](#_ENREF_10)) with a significant resistance to innate and adaptive host immunity ([11](#_ENREF_11)). Moreover, biofilms associated with orthopaedic hardware are typically difficult to culture using conventional clinical microbiological methods and lack of a definitive diagnosis may underestimate infection rates ([12](#_ENREF_12), [13](#_ENREF_13)). Consequently, the underlying infection is both difficult to diagnose and treat ([6](#_ENREF_6), [7](#_ENREF_7)) with often the only effective intervention being the twin strategies of thorough debridement and prostheses removal ([14](#_ENREF_14)).

Existing prevention strategies include the use of antibiotic-loaded poly (methyl methacrylate) (PMMA) cement spacers and beads to elevate local antibiotic levels at the surgical site. Studies have demonstrated a significant reduction in infection rates using antibiotic-impregnated cement in THA and TKA patient populations ([15](#_ENREF_15), [16](#_ENREF_16)). Conversely, other studies indicate limited clinical benefit, albeit with varying antibiotics and concentrations ([17](#_ENREF_17), [18](#_ENREF_18)) and poor description of elution kinetics ([19](#_ENREF_19)). Additionally, once the antibiotics have eluted from a non-absorbable cement the surface becomes a foreign body, subject to bacterial colonisation and biofilm formation ([20](#_ENREF_20), [21](#_ENREF_21)).

Absorbable mineral based bone cements are not as mechanically strong as acrylic cements but have some advantages for antibiotic delivery and infection control. Firstly, they do not require removal since they naturally absorb. Secondly, there is little temperature rise during setting so they have the capacity to accommodate a wider range of antibiotics and lastly, as they slowly dissolve there is a sustained release of antibiotics over their lifetime. Studies using carriers constructed from calcium sulfate have shown improved antibiotic release relative to PMMA beads and, importantly, complete biodegradation ([22-24](#_ENREF_22)).Their efficacy has been been corroborated in animal models with data demonstrating the effective treatment of experimental osteomyletis in rabbits ([25](#_ENREF_25), [26](#_ENREF_26)) and contaminated fractures in a goat model ([27](#_ENREF_27)). These data support the rationale for a more widespread clinical use, however a better understanding of their antibacterial and antibiofilm efficacy is required. It was the goal of the present study to quantify 1) the long term bioactivity of antibiotics released from synthetic calcium sulfate beads, 2) their ability to prevent biofilm formation on adjacent surfaces, as well as on the beads themselves and 3) their ability to kill preformed biofilms.

MATERIALS AND METHODS

Bacterial strains & growth media

*Staphylococcus aureus* EMRSA-16 NCTC 13143 and *Staphylococcus epidermidis* ATCC 35984 were each cultured in tryptic soy broth (TSB; Sigma-Aldrich). EMRSA-16 is one of two main epidemic bacteraemia MRSA strains in the UK ([28](#_ENREF_28)) and has been shown to form biofilms ([29](#_ENREF_29)). *S. epidermidis* ATCC 35984 is an intercellular adhesion (ica) positive strain and is a strong biofilm former ([30](#_ENREF_30)).

Antibiotic minimum inhibitory and bactericidal concentration (MIC & MBC) and biofilm MBC assays

Overnight cultures of each strain were diluted in fresh TSB to an optical density (OD) corresponding to 106 cells/mL. In 10 mL of bacterial culture, a dilution series from 0.5 µg/mL to 15,000 µg/mL vancomycin and 0.5 µg/mL to 5000 µg/mL tobramycin was performed using either tobramycin sulfate alone (Sigma-Aldrich), vancomycin hydrochloride alone (Hospira UK Ltd.), or a vancomycin & tobramycin combination treatment (1:0.24 w/w ratio). Cultures were incubated for 15 h at 37 °C. The ratio of vancomycin to tobramycin was selected based on previous clinical use ([31](#_ENREF_31)). The MIC of each antibiotic was determined by measuring the culture OD using a microplate reader (BMG Omega) at 680nm (OD680nm). The cultures were serial diluted in Hanks buffered salt solution (HBSS; Sigma-Aldrich) and plated onto tryptic soy agar (TSA; Sigma-Aldrich), incubated for 24 h at 37 °C, to determine the MBC eliciting a greater than 3 log reduction in planktonic cells.

Additionally, biofilm MBCs were performed following growth in 6 well plates for 72 h at 37 °C from a starting inoculum of 4 mL of 106 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 scraped to remove attached biofilms into 1 mL HBSS. Following vortexing for 20 secs to homogenise 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 cc kit containing 20 g pharmaceutical grade calcium sulfate alpha-hemihydrate powder (PG-CSH; Stimulan®, Biocomposites Ltd, U.K.) was mixed with either 6 mL of sterile water (unloaded beads), 6 mL of a 40 mg/mL tobramycin sulfate solution (tobramycin-loaded beads) or 1000 mg 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 both vancomycin and tobramycin, a 10 cc kit of PG-CSH was mixed with 1000 mg vancomycin hydrochloride powder plus 6 mL of 40 mg/mL tobramycin solution. In each case, all components were mixed for 30 to 60 secs to form a smooth paste which was pressed into 4.8 mm diameter hemispherical cavities in the flexible mold (Figure 1A). The beads were left undisturbed for 30-60 mins to set. When set, beads were removed from the mold by flexing (Figure 1B). Unloaded 4.8 mm diameter beads weigh 0.108 g. When loaded with antibiotics, each bead would contain 4.13 mg of vancomycin, and/or 1.02 mg of tobramycin.

So as to compare release from the calcium sulfate bioabsorbable beads with current standard of care we prepared nonabsorbable PMMA beads (TBCem 3, Tornier, France) as per standard operating room protocols ([32](#_ENREF_32), [33](#_ENREF_33)). A 40 g pack of powder was combined with either 3.8 g vancomycin or 0.90 g tobramycin or both antibiotics in combination. This concentration is higher than is recommended if structural support is required, but is in the (high end) range used for antibiotic eluting beads ([32](#_ENREF_32)). The PMMA paste was spread into an identical 4.8 mm bead mold as used for the pharmaceutical grade calcium sulfate beads (Figure 1A), taking care to completely fill the cavities. Unloaded 4.8mm diameter beads weigh 0.065 g. When loaded with antibiotics, each bead would contain 3.4 mg of vancomycin, and/or 0.9 mg of tobramycin, which is 18% less by weight per bead than a PG-CSH bead.

Antibiotic eluting PG-CSH and PMMA beads to inhibit planktonic bacteria 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 on the agar plate using 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 ZOI were lost. The area (cm2) of the ZOI was calculated using Image J (version 1.48) ([34](#_ENREF_34)) using the 90 mm diameter of the petri dish in each image for spatial calibration, with analysis performed on Days 1, 2, 3 and 4 and then every subsequent 2 days. Area, rather than diameter, of the ZOI was calculated to account for irregularities in the shape of the ZOI.

Antibiotic eluting PG-CSH beads to prevent biofilm formation

Antibiotic-loaded and unloaded beads (10 beads per well or plate) were placed into 6 well plates for CFU enumeration and 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 colonisation and too few beads which would limit clinical relevance. 4 mL of 106cells/mL culture was added to the wells with the beads present upon addition. Every 24 h, media was replaced and the beads were subjected to a fresh bacterial challenge of 4 mL of 106 cells/mL. At Days 1 (24 h post inoculation), 2, 3, 7 and 14, CFU counts of the surface-attached bacterial population in the 6 well plates were performed as described above. Concurrently, at Days 1, 2, 3, 7 & 14, the fluorescent stain Syto 9 was used to microscopically assess surface attached biomass in the MatTek plates. Plates were rinsed with HBSS and stained (2 µL of Syto 9 per mL of HBSS) for 20 mins. The plates were gently rinsed and analysed using an inverted Leica DMI600 SP5 confocal laser scanning microscope (CLSM).

Additionally, beads were visualized by scanning electron microscopy (SEM) to determine the extent of colonisation. Beads were transferred to an initial fixative of 3 % gluteraldehyde, 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 (30, 50, 70, 95 and x2 100 %), each for 10 mins. The beads were then critical point dried and sputter coated in a gold/palladium alloy. Imaging was carried out using a FEI Quanta 200 Scanning Electron Microscope.

Antibiotic eluting PG-CSH beads to kill pre-existing biofilms

Biofilms were cultured as described and beads (10 per well) placed into 6 well plates and incubated for a further 24 h or 72 h (with media changes every 24 h) at 37 °C. CFU counts were performed on the residual surface biomass as described above.

Statistics

Data was compared using a Mann-Whitney Rank Sum test for non-normally distributed data and difference considered significant where P<0.05.

RESULTS

Planktonic and biofilm MIC/MBC

Data to determine the planktonic MIC and MBC (Table 1) showed that *S. aureus* EMRSA-16 NCTC 13143 was resistant to tobramycin but sensitive to vancomycin (tobramycin MBC: >5000 µg/mL, vancomycin MBC 2 µg/mL). The MIC/MBC of the vancomycin and tobramycin dual treatment was equal to that of vancomycin alone. A similar profile was observed for *S. epidermidis* ATCC 35984, although with intermediate sensitivity to tobramycin. Comparison of planktonic and biofilm MBC showed that the biofilms displayed a resistance to the antimicrobial of 100 fold or greater.

Antibiotic-loaded PG-CSH and PMMA beads to inhibit planktonic bacteria

Unloaded beads of both PG-CSH and PMMA beads did not elicit a zone of inhibition (ZOI) with either bacterial strain (Figure 2) throughout the duration of the assay (40 days). With respect to the PG-CSH beads, the MRSA strain was resistant to tobramycin as shown by small ZOI (0.39 cm2 at Day 1) which were not present at Day 2 (Figure 2). Both vancomycin and vancomycin & tobramycin-loaded beads produced strong ZOI which were maintained until approximately Day 26 when a gradual decrease in size 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 (Figure 2). A rapid decline in the size of the tobramycin ZOI was observed between Days 1 (2.78 cm2) and 4 (0.62 cm2), with loss of the ZOI observed at Day 5. As with MRSA, vancomycin and vancomycin & tobramycin-loaded beads persistently elicited strong ZOI, until approximately Day 34 when a steady decline in the size of the ZOI was noted, with complete loss at Day 40.

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

Antibiotic-loaded PG-CSH beads to prevent biofilm formation.

Vancomycin and the antibiotic combination beads significantly reduced MRSA surface colonisation with an initial ~3 log reduction in CFU/cm2 at Day 1. Despite fresh bacterial challenges every 24 h, both vancomycin and vancomycin & tobramycin combination-loaded beads further reduced surface bound viable cells with a maximal reduction to 7.6x101 CFU/cm2 with the antibiotic combination beads at Day 3, relative to 1.0x106 CFU/cm2 with unloaded beads. Furthermore, at Day 14 both vancomycin and the antibiotic combination beads still displayed a significantly reduced CFU/cm2 relative to the unloaded beads (P=<0.001). Confocal microscopy corroborated the CFU measurements but gave an additional benefit of allowing direct observation of biofilm formation (Figure 3). Importantly, even at Day 14, where CFU data indicated a large increase in CFU/cm2 relative to Day 7 with vancomycin and vancomycin & tobramycin-loaded beads, CLSM indicated that biofilm formation was markedly reduced relative to unloaded beads.

In agreement with previous data (Table 1 & Figure 2), tobramycin-loaded beads had limited effect on MRSA, although a statistically significant reduction in CFU/cm2 was noted, relative to unloaded bead treatment groups, at Days 1, 2 and 3 (P=<0.001 at Days 1, 2 & 3). After 3 days, biofilm formation progressed similar to controls.

SEM showed that the dual-loaded vancomycin & tobramycin beads were the most effective at preventing biofilm formation (Figure 4). Unloaded beads demonstrated early bacterial colonisation at Day 2 and extensive biofilm formation by Day 7. Conversely, there was no detectable MRSA colonisation up to and including Day 7 of vancomycin & tobramycin-loaded beads, with bacterial colonisation and biofilm formation only observed at Day 14.

Tobramycin and vancomycin & tobramycin-loaded beads resulted in a complete kill of *S. epidermidis* at Day 1 (Figure 5). With respect to tobramycin-loaded beads at Day 2, surface-associated *S. epidermidis* increased rapidly, until Days 7 and 14 where no further difference was observed in CFU/cm2 relative to unloaded beads (P>0.05).

Conversely, in the presence of vancomycin-loaded beads, colonisation of the substratum was markedly less at Days 2, 3 and 7 than for controls. Crucially, the combination of vancomycin & tobramycin-loaded beads achieved a complete kill at Day 2 and 3, suggesting concentrations of eluted antibiotics remained higher than the MBC for *S. epidermidis* for at least 72 h. Even at Day 7, bacteria concentrations remained low (2.1x102 CFU/cm2).

Confocal analysis (Figure 5) corroborated the CFU data. Vancomycin and vancomycin & tobramycin-loaded beads suppressed bacterial colonisation up to Day 7. Importantly, by Day 14, whilst CFU/cm2 data show surface colonisation similar to the control, CLSM images highlight that this exists only as a thin monolayer, with biofilm formation markedly suppressed.

Visualization of the bead surface demonstrated rapid *S. epidermidis* colonisation and biofilm formation of unloaded beads (Figure 6). However, vancomycin & tobramycin-loaded beads demonstrated markedly delayed surface colonisation, with no visible bacteria on the bead surface at Day 2. Up to and including Day 14 of the experiment, biofilm formation was significantly reduced relative to unloaded beads with limited bacterial colonisation and EPS deposition.

Antibiotic-loaded PG-CSH to kill pre-existing biofilms

To assess the ability of antibiotic-loaded beads to eradicate established biofilms, ten beads were incubated with biofilms for 24 and 72 h. The data was expressed as log reductions relative to unloaded treatment groups to account for possible physical abrasion and disruption of the biofilm during growth through the presence of the bead itself. With 24 h, the greatest effect observed was ~1 log reduction in viable MRSA and *S. epidermidis* observed with combination vancomycin & tobramycin-loaded beads (Figure 7A & B) which represents 88 and 90 % biofilm reduction, respectively. The efficacy of antibiotic-loaded beads toward pre-existing MRSA biofilms was increased by extending the contact time to 72 h where a 4 log (99.999 %) reduction in CFU/cm2 was obtained with the antibiotic combination beads relative to 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: 0.98 log (89.67 %) reduction vs 72 h vancomycin & tobramycin: 1.01 log (90.43 %)].

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, as well as, for the first time, addressing the issue of biofilm prevention and eradication. The beads were loaded with vancomycin, tobramycin and a combination of vancomycin & tobramycin. Initial data aimed to characterise the strains with assays to determine the planktonic MIC and MBC, demonstrating that *Staphylococcus aureus* 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, comparison of planktonic MBCs with that of biofilm cells showed, in all cases where planktonic bacteria were initially sensitive, biofilms were 100x or greater more resistant ([10](#_ENREF_10)) further demonstrating the importance in preventing robust biofilm formation in PIs.

This was highlighted further with PG-CSH beads loaded with a combination of vancomycin & tobramycin only able to achieve a 1 log reduction in viable bacteria after a contact time of 24h. Increasing the contact time to 72 h resulted in a further 3 log reduction in MRSA demonstrating the importance of maintaining locally high concentrations for as long as possible. Whilst a total 4 log reduction can be considered highly effective *in vivo*, complete MRSA biofilm eradication was not achieved providing further evidence of the difficulty in removing robust, established biofilms. 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. Where bacteria were sensitive to the antibiotic, zones of clearing were observed and maintained for 39 days indicating that eluted antibiotics from PG-CSH (in this case, vancomycin) remain at concentrations higher than the minimum inhibitory concentration throughout this time. This observation is in agreement with a previous study from Roberts *et al*. 2014 who showed similar elution rates of vancomycin from calcium sulfate beads ([35](#_ENREF_35)). Importantly in the present study we additionally demonstrate 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 ZOI lost by Day 12. Previous studies have shown comparable efficacy between the two compounds however this is the first to compare antimicrobial activity over a long duration ([33](#_ENREF_33)). Whilst the amount of antibiotic per PMMA bead is 18 % less than PG-CSH, this 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](#_ENREF_32)). Part of this difference in elution rate may be explained by variations in porosity of PMMA relative to 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 primarily explained by the gradual absorption of this material resulting in sustained release of higher amount of antibiotics over time. Increasing the number of PG-CSH beads to fill dead space would effectively increase the total antibiotic reservoir as well as ensuring maximal contact with potentially infected tissue at the surgical margin. The elution kinetics from the present study suggest that this strategy would ensure localised high antibiotic concentrations to counteract potential infection at the surgical site through intra-operative contamination and also that acquired later through haematogenous spread almost over the entire 6 week period recommended to treat orthopaedic staphylococcus infections. Crucially however, it is important to note that despite a longer duration of antimicrobial efficacy in this study, PG-CSH can not be expected to replace PMMA in situations where mechanical strength and integrity is of paramount importance to the procedure.eg. fixing prosthesis to bone in primary arthroplasties or use as a spacer in staged revisions. However it could be envisioned that PG-CSH beads could be used in conjunction with PMMA, with the former used for the management of the dead space and the latter providing structural integrity.

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 were at preventing surface colonisation and biofilm formation, where the beads remained *in situ* and were subjected to daily bacterial challenges. Media containing 106 CFU/mL, much greater than would be expected in a single infective dose *in vivo*, were delivered daily for up to 14 days post initial inoculation. The concentration was chosen as a vigorous evaluation of the antibacterial capacity of the beads over time.

In the case of MRSA, growth was attenuated in the presence of vancomycin and vancomycin & tobramycin-loaded beads for up to 1 week, despite daily bacterial challenges of 106 cells/mL, as observed by CFUs, confocal microscopy and SEM of the bead surface. With respect to *S. epidermidis*, tobramycin, vancomycin, and vancomycin & tobramycin-loaded beads produced a significant reduction of *S. epidermidis* on Day 1 as observed by CFU counts, with both the tobramycin and combination antibiotic beads able to achieve a complete kill for 1 and 3 days respectively. Importantly, a markedly reduced MRSA and *S. epidermidis* biofilm formation during all 14 days was noted with the vancomycin & tobramycin-loaded beads which, importantly *in vivo*, would enable the host’s immune system to cope more effectively ([36](#_ENREF_36)). Whilst this duration of efficacy is reduced relative to the 39 days observed in the repeated ZOI study (Figure 2), this can be explained by the change in focus of the assay outcome from bacterial inhibition of rapidly respiring cells on a removable substratum as in the ZOI assay, to 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 colonisation of a substratum and biofilm formation begins. Conversely however, beads were immersed in media for the duration of the biofilm prevention study, which is in stark contrast to the experimental set-up of the ZOI assay with beads placed on agar. With daily media changes, antibiotics could be expected to elute from porous PG-CSH more rapidly than on an agar plate, meaning that, combined with daily, high-dose bacterial challenges, the duration of antibiotic efficacy in the biofilm prevention study could be considered a worse-case scenario.

Importantly, whilst *in vitro* studies do not account for the flow of body fluids, limb motility, host immune processes and antibiotic stability *in vivo*, the antibacterial activity observed with PG-CSH of 7-14 days 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](#_ENREF_37)). Recently, a novel SiO2-TiO2-ZnO-CaO-SrO-based glass polyalkenoate cement also loaded with vancomycin was also shown to be effective against *S. aureus* for 48 h ([38](#_ENREF_38)). Studies into the release kinetics of tobramycin and vancomycin from a demineralised bone matrix showed complete elution within a period of 3 and 14 days, respectively ([39](#_ENREF_39)). Our data indicates bactericidal efficacy from PG-CSH within a similar time frame as the latter study and highlights, firstly, the importance of sustained antibiotic release from these materials for many days post-surgical intervention. Secondly, the SEM data demonstrates the potential for eluting materials to be a nidus for infection and biofilm formation once antibiotic concentrations fall below bacterial MBCs and therefore emphasises the benefit of absorbable substrates. Studies have shown complete absorption of high purity calcium sulfate within 4-6 weeks combined with effective management of penile implant infection as well as osteomyelitis ([40-43](#_ENREF_40)), indicating potentially wide ranging benefits in the management of PIs.

CONCLUSIONS

The use of antibiotic loaded beads at the site of infection is becoming the standard of care as it enables localised, supra-MIC levels which would be difficult to achieve by other means ([44](#_ENREF_44)). In this study, antibiotic impregnated PG-CSH has demonstrated high bioactivity in preventing early bacterial colonisation 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 to prevent biofilm formation, therefore reducing the risk of the establishment of chronic infection in total joint arthroplasty and other surgical procedures.

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FIGURE LEGENDS

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

Figure 2. Repeat Modified Kirby Bauer assays to assess zones of inhibition (ZOI) of *S. aureus* EMRSA-16 NCTC 13143 and *S. epidermidis* ATCC 35984 over a period of 40 days. Images show 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 combination PG-CSH beads. Graphs show the size of the ZOI (cm2) over time, as calculated using Image J. Assays were performed in triplicate and data expressed as the mean of 3 data points with standard error bars.

Figure 3. *S. aureus* EMRSA-16 NCTC 13143 biofilm formation over time in the presence of antibiotic-loaded synthetic calcium sulfate beads as determined by CLSM images and colony forming unit counts (CFU/cm2). Data expressed as the mean of 15 data points (5 data points per experimental repeat) with standard error bars. Scale bars = 25 µm.

Figure 4. Biofilm formation of *S. aureus* EMRSA-16 NCTC 13143 on vancomycin & tobramycin loaded relative to unloaded beads over time as determined by SEM. White arrows indicate bacterial colonisation and biofilm formation. Scale bars = 25 µm unless otherwise stated.

Figure 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 colony forming unit counts (CFU/cm2). Data expressed as the mean of 15 data points (5 data points per experimental repeat) with standard error bars. Scale bars = 25 µm.

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

Figure 7. The effect of antibiotic-loaded synthetic calcium sulfate beads with 24 h and 72 h contact times on pre-existing biofilms of *S. aureus* EMRSA-16 NCTC 13143 and *S. epidermidis* ATCC 35984. Data were expressed as log reduction relative to the unloaded treatment group of the mean of 15 data points (5 per experimental repeat with each assay performed in triplicate).