Title: Prevention of Propionibacterium acnes biofilm formation in prosthetic infections in vitro.

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**ABSTRACT**

**Background** The role of *Propionibacterium acnes* in shoulder arthroplasty and broadly in orthopaedic prosthetic infections (PIs) has historically been underestimated, with biofilm formation identified as a key virulence factor attributed to invasive isolates. With an often indolent clinical course, *P. acnes* infection can be difficult to detect and treat. The present study investigates absorbable cements loaded with a broad-spectrum antibiotic combination as an effective preventative strategy to combat *P. acnes* biofilms.

**Methods** *P. acnes* biofilm formation on an unloaded synthetic calcium sulfate (CaSO4) bone void filler cement bead was evaluated by scanning electron microscopy (SEM) over 14 days. Beads loaded with tobramycin alone, vancomycin alone (as comparative controls) and a vancomycin & tobramycin dual treatment were assessed for their ability to eradicate planktonic *P. acnes*,prevent biofilm formation and eradicate preformed biofilms was also evaluated using viable cell counts, confocal microscopy and SEM.

**Results** *P. acnes* surface colonisation and biofilm formation on unloaded CaSO4 beads was slow. Beads loaded with antibiotics were able to kill planktonic cultures of 106 CFU/ml, prevent bacterial colonisation and significantly reduce biofilm formation over periods of weeks. Complete eradication of established biofilms was achieved with a contact time of one week.

**Conclusions** This study demonstrates that antibiotic-loaded CaSO4 beads may represent an effective antibacterial and antibiofilm strategy to combat PIs in which *P. acnes* is involved.

Level of Evidence: Basic Science Study

Keywords: *Propionibacterium acnes*, Biofilm, Shoulder Arthroplasty, Prosthetic Infection, Calcium Sulfate, Antibiotics

**INTRODUCTION**

The number of prosthetic infections (PIs) continues to rise due to the ever increasing number of joint arthroplasties carried out world wide [10](#_ENREF_10). Joint remodelling or replacement has the capacity to improve function and mobility for the patient, but conversely the risk of infection has the potential to result in significant morbidity and cost to the healthcare service [27](#_ENREF_27); [34](#_ENREF_34).

Prosthetic infections are strongly linked to the formation of bacterial biofilms, initially seeded during the intraoperative process or later through haematogenous spread and intrusion through the scar [42](#_ENREF_42); [53](#_ENREF_53); [54](#_ENREF_54). Infiltrating bacteria are able to attach to and form a biofilm on a heterogeneous range of host and manufactured surfaces, such as prosthetic components, surrounding tissue and bone architecture [27](#_ENREF_27). Although physically distinct, these act as communicating niches where reseeding can occur from one area despite successful bacterial clearance from another, thus making complete eradication challenging. Moreover, the biofilm phenotype itself confers bacterial cells with an increased resistance to clearance by the host immune system and substantial tolerance to antimicrobials [14](#_ENREF_14). Consequently, the difficulty in clearing biofilm-mediated chronic infection means reinfection rates can rise up to 20 % after revision compared to 1-4 % of all primary procedures [7](#_ENREF_7); [24](#_ENREF_24); [25](#_ENREF_25).

*Staphylococcus aureus* and coagulase negative staphylococcus are considered the predominant causative pathogens in PI, based largely on culture-dependent methods [47](#_ENREF_47). However, gaining increasing prominence is the skin commensal and facultative anaerobe *Propionibacterium acnes* whose role inPI pathogenesis has historically been underestimated but whose impact in shoulder arthroplasty has been well documented [38](#_ENREF_38). A slow growing, aerotolerant anaerobe, *P. acnes* can be difficult to isolate using conventional methods, with incubation times of up to 3 weeks leading to false negative cultures [31](#_ENREF_31). Additionally, its relative low-virulence and abundance as part of the normal skin microbiota, coupled with an often indolent clinical course, make its clinical significance upon identification in PIs difficult to determine [8](#_ENREF_8); [13](#_ENREF_13); [52](#_ENREF_52). However, neurosurgical and orthopaedic procedures have been shown to be serious risk factors involved in *P. acnes* infection [45](#_ENREF_45). In particular, its common association with hair follicles and sebaceous glands whose numbers are elevated around the face, scalp, chest and back, means *P. acnes* is particularly numerous around the shoulder [17](#_ENREF_17). Consequently, it is the most predominant microorganism associated with shoulder infections and following revision shoulder arthroplasties [39](#_ENREF_39); [41](#_ENREF_41); [46](#_ENREF_46) . Moreover, its location deep within sebaceous glands means that instruments used around superficial tissues may be contaminated with *P. acnes* below the skin, making wound incision site sterilisation difficult and increasing the possibility of introduction into deep joint tissues [22](#_ENREF_22); [26](#_ENREF_26); [36](#_ENREF_36).

One approach in combatting PIs involves the use of antibiotic-loaded cement spacers or beads to provide high localised antibiotic concentrations at the surgical site, with the aim of device protection and dead space management [18](#_ENREF_18). Dissolvable cements, such as calcium sulfate, have the added benefit of sustained elution kinetics and complete reabsorption, therefore not requiring a further surgical procedure to remove [16](#_ENREF_16). This current study investigates the ability of sustained antibiotic elution of a broad spectrum combination from cement beads to prevent bacterial colonisation and biofilm formation, as well as their impact on established biofilms of *P. acnes*.

MATERIALS & METHODS

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

*Propionibacterium acnes* ATCC 11827 cultures were grown in brain heart infusion (BHI; Sigma-Aldrich) anaerobically (AnaeroGen; Oxoid) for 72 h at 37 °C. The strain was selected as it is one of the most commonly used *P. acnes* strains for biofilm studies[1](#_ENREF_1); [11](#_ENREF_11). Holmberg *et al*. 2009 demonstrated the importance of biofilm formation as a characteristic of invasive *P. acnes* clinical isolates[15](#_ENREF_15); consequently, the propensity for the strain to form biofilms was of key importance in this study in order to be able to evaluate the antibiofilm efficacy of PG-CSH beads. The cultures were then diluted in fresh BHI to an optical density (OD620nm) corresponding to 106 CFU/ml. As a result, this constitutes a modified MIC and MBC assay, performed so that starting cultures more closely matched the colony forming units (CFUs) recovered from biofilm MBCs which can not be readily controlled (planktonic control for MBC after 72 h growth: 2.1 x 107 CFU/ml, biofilm control for MBC after 7 days growth: 2.29 x 107 CFU/ml). Using the adjusted bacterial culture, a dilution series from 0.5 µg/ml to 15,000 µg/ml vancomycin (Hospira UK Ltd.) and 0.5 µg/ml to 5000 µg/ml tobramycin sulfate (Sigma-Aldrich) was performed and the cultures again incubated anaerobically for 72 h at 37 °C. Beyond 1024 µg/ml vancomycin was noted to be incompletely soluble in the media and as a result this was the maximum concentration of antibiotics evaluated. The antibiotic MIC was measured using a microplate reader (BMG Omega) at 680nm (OD680nm). The cultures were serial diluted in Hanks balanced salt solution (HBSS; Sigma-Aldrich), cultured onto tryptic soy agar (TSA; Sigma-Aldrich) containing 5 % defibrinated sheep blood (Fisher) and then incubated anaerobically for 72 h at 37 °C with the MBC considered as the concentration achieving a greater than 3 log reduction in CFUs.

Concurrently, biofilm MBCs were undertaken on pre-established biofilms grown anaerobically for 7 days at 37 °C in 6 well plates from a starting inoculum of 4 ml of 106 cells/ml with fresh media replacement performed every 72 h. Antibiotic treatment was performed anaerobically for 72 h at 37 °C. The wells were rinsed in HBSS and biofilms physically removed by scraping into 1 ml HBSS. The biofilm suspension was then vortexed for 20 secs to disrupt and homogenise the biofilm bacteria and then plated onto TSA + 5 % defibrinated sheep blood plates. Plates were incubated anaerobically for 72 h at 37 °C. The final surface concentration of biofilms immediately prior to antibiotic addition was 2.38 x 107 CFU/cm2.

Preparation of calcium sulfate alpha-hemihydrate and PMMA beads

Calcium sulfate alpha-hemihydrate and PMMA beads were made as previously described [16](#_ENREF_16). Briefly, 20 g of 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), 1000 mg vancomycin hydrochloride powder plus 6 ml sterile water (vancomycin-loaded beads) or 1000 mg vancomycin hydrochloride powder plus 6 ml of 40 mg/ml tobramycin solution (vancomycin & tobramycin in combination beads). The use of beads loaded with either tobramycin alone or vancomycin alone was as comparative controls for the dual antibiotic combination. In each case, the resultant paste was used to form 4.8 mm diameter hemispherical beads using a flexible mold where each bead weighed 0.108 g and contained either 4.13 mg of vancomycin, and/or 1.02 mg of tobramycin when set.

PMMA beads (TBCem 3, Tornier, France) were prepared by combining 40 g of cement powder with either 3.8 g vancomycin hydrochloride powder or 0.90 g tobramycin sulfate powder or both antibiotics in combination and 4.8 mm hemispherical beads formed as described above. Unloaded beads weighed 0.065 g and contained 3.4 mg of vancomycin and/or 0.9 mg of tobramycin, which was determined to be 18% less by weight per bead than the corresponding PG-CSH bead.

*P. acnes* biofilm formation on unloaded PG-CSH beads

To assess biofilm formation on a clinically relevant surface, unloaded PG-CSH beads were placed in 6 well plates and immersed in a 4 ml 106 CFU/ml culture of *P. acnes* ATCC 11827. Biofilms were grown anaerobically at 37 °C for 14 days with media replacement performed every 72 h. *P. acnes* surface colonisation and biofilm formation on the beads was assessed using scanning electron microscopy (SEM). Preparation of the beads for SEM was performed by initially immersing each bead in a fixative of 3 % gluteraldehyde, 0.1 M sodium cacodylate (pH 7.2) and 0.15 % Alcian blue for 24 h at 4 °C. Following aspiration of the fixative, a subsequent 1 h rinse in 0.1 M sodium cacodylate (pH 7.2) was performed followed by a secondary fixation in 0.1 M osmium tetroxide in 0.1 M sodium cacodylate (pH 7.2) for 1 h; both rinse and secondary fixation were performed at room temperature. Following another 1 h rinse with 0.1 M sodium cacodylate (pH 7.2), the beads were run through an ethanol series (30, 50, 70, 95 and x2 100 %), each for 10 mins. Each bead was then critical point dried and sputter coated in a gold/palladium alloy before mounting onto specimen stubs and imaged using a FEI Quanta 200 Scanning Electron Microscope. The bacteria, which were readily identified by their cell morphology, were false coloured using Photoshop CS6 so they could be clearly identified from the crystalline background of the bead surface.

Inhibition of planktonic growth by antibiotic eluting PG-CSH and PMMA beads

Agar diffusion assays were undertaken to assess the effective elution concentration of antibiotics from each bead type. A starting initial bacterial lawn was spread onto TSA + 5 % defibrinated sheep blood plates using 50 µL of a planktonic culture of *P. acnes* ATCC 11827. Using sterile forceps, a single bead was transferred onto individual plates and incubated anaerobically at 37 °C for 72 h. Zones of inhibition (ZOI) around the beads were photographed and the beads were then transferred onto a freshly prepared lawn of bacteria every 72 h until ZOI were lost. The area (cm2) of the ZOI was calculated using Image J (version 1.48) with the 90 mm diameter of the Petri dish used for spatial calibration.

Biofilm prevention using antibiotic eluting PG-CSH beads

Ten antibiotic-loaded and unloaded beads (per well or plate) were placed into polystyrene, poly-d-lysine coated 6 well plates for CFU enumeration and poly-d-lysine coated MatTek tissue culture plates (MatTek Corporation) for microscopic imaging on the glass coverslip surface. Both 6 well and MatTek culture plates were poly-d-lysine coated to enhance early bacterial colonisation *in vitro*. With the beads present upon addition, 4 ml of 106cells/ml *P. acnes* ATCC 11827 culture was added to the wells. Every 72 h, a media exchange was performed with fresh media containing 4 ml of 106 cells/ml as a bacterial challenge. At Days 1 (24 h post inoculation), 2, 3, 7 and 14, viable cell counts were performed as described above to determine the rate of surface colonisation and biofilm formation. At the same time points, confocal laser scanning microscopy (CSLM; inverted Leica DMI600 SP5) was performed on the residual biomass in the MatTek plates following fluorescent staining with Live/Dead BacLight (2 µL of Syto 9 and Propidium iodide per ml of HBSS; Invitrogen) for 20 mins.

Additionally, unloaded and vancomycin & tobramycin in combination loaded beads were processed for SEM as described previously to determine the extent of colonisation at Days 1, 3, 7 and 14.

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

Biofilms were cultured in 6 well plates for 7 days as described above and ten beads loaded with vancomycin & tobramycin in combination or unloaded beads as negative controls were placed the wells. The plates were incubated anaerobically for a further 24 h, 72 h or 7 days (with media changes every 72 h) at 37 °C and viable cells counts performed at each time point 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

*P. acnes* ATCC 11827 was susceptible to vancomycin (MIC: 1 µg/ml; MBC: 2 µg/ml) and intermediately susceptible to tobramycin (MIC and MBC: 64 µg/ml; Table I). Comparison of planktonic and biofilm MBC showed that the biofilms displayed a tolerance to both antibiotics greater than the concentration range evaluated (>1024 µg/ml). For tobramycin and vancomycin, respectively, residual bacteria following 1024 µg/ml antibiotic (0.426 mg/cm2) was 23593907.1 and 1132923.2 CFU/cm2, equating to a 0.003 (no statistical difference; P=0.909) and 1.3 (statistically significant difference; P=<0.001) log reduction relative to untreated control biofilm.

*P. acnes* biofilm formation

*P. acnes* biofilm formation on an unloaded PG-CSH bead was assessed using SEM. Surface colonisation was slow, even in an optimal growth media with media changes every 72 h, with single cell attachment not observed until Day 3 (before the first media exchange) (Fig. 1). At Day 7, deposition of extracellular polymeric substance (EPS) was observed on the bead surface, with bacterial surface colonisation more pronounced. At Day 14, extensive biofilm formation and EPS production could be seen across the bead surface.

Planktonic *P. acnes* inhibition by antibiotic eluting beads

Unloaded PG-CSH and PMMA beads did not elicit a ZOI (Fig. 2). Similar to the resistance profile of *P. acnes* ATCC 11827 established in Table I, tobramycin-loaded beads were less effective and elicited no ZOI with PMMA beads and a relatively small ZOI with PG-CSH beads which was lost rapidly by Day 6. Vancomycin and vancomycin & tobramycin in combination loaded PG-CSH beads produced a ZOI which was maintained until Day 60 and Day 27 respectively. The maintenance of the ZOI for longer with vancomycin-loaded beads relative to vancomycin & tobramycin in combination suggests an antagonistic effect of tobramycin on vancomycin efficacy when incorporated into PG-CSH beads, possibly due to direct interaction between the two antibiotics or to a change in porosity of the calcium sulfate. However, as demonstrated by the larger standard deviation from Day 30 in the data for vancomycin-loaded PG-CSH beads, this was a consequence of a single bead maintaining efficacy despite a loss of ZOI associated with the other two PG-CSH beads in the triplicate assay, suggesting possible variation in the PG-CSH-vancomycin mixing process. Importantly, loss of the ZOI at Day 60 corresponded to complete dissolution of the beads.

The ZOI associated with PMMA beads loaded with vancomycin and vancomycin & tobramycin in combination was maintained for 111 and 108 days respectively, corresponding to 37 and 36 different lawn challenges (Fig. 2).

Biofilm prevention using antibiotic eluting PG-CSH beads

The ability of antibiotic-loaded PG-CSH beads to prevent *P. acnes* surface colonisation (Days 1, 2 and 3) and biofilm formation (Days 7 and 14) in the presence of 106 CFU/ml bacterial challenges every 72 h was assessed (Fig. 3 & 4). Imaging by CLSM, demonstrated quicker surface colonisation in the poly-d-lysine coated plates (Fig. 3), relative to the unloaded beads as observed by SEM (Fig. 1).

Surface colonisation and biofilm formation in the presence of unloaded PG-CSH beads progressed similar to the control (no beads) with no statistical difference in CFU/cm2 at all but one time point (Fig. 4, Day 1: p=0.157, Day 2: p=0.371, Day 3: p=0.003, Day 7: p=0.851 and Day 14: p=0.072).

Despite an intermediate resistance to tobramycin (Table I), tobramycin loaded beads elicited a ~3 log reduction in CFU/cm2 at Day 1, which was maintained at Day 2. At Day 3, prior to a fresh bacterial challenge, recovery in CFU/cm2 was observed with an increase from 98.3 CFU/cm2 at Day 2 to 2917.1 CFU/cm2 at Day 3 suggesting a reduction in eluting tobramycin. Further recovery in CFU/cm2 was noted at Day 7 which remained statistically different to the unloaded treatment group (p=<0.001) until Day 14 when no statistical difference in CFU/cm2 was noted between tobramycin-loaded beads and unloaded beads (p=0.226). Importantly, CLSM imaging demonstrated that at all time points, biofilm formation in the presence of tobramycin-loaded PG-CSH beads was markedly reduced. Vancomycin and vancomycin & tobramycin in combination loaded PG-CSH beads were able to achieve a complete kill for 7 days (sensitivity cut off value for the assay: 6.92 CFU/cm2) despite two fresh bacterial challenges of 106 CFU/ml. Vancomycin & tobramycin in combination loaded beads also achieved a complete kill at Day 14 following 4 bacterial challenges during the assay. Conversely, at Day 14, CFU/cm2 in the presence of vancomycin-loaded beads had recovered by ~5 log to 88000.7 CFU/cm2 indicating a synergistic interaction between tobramycin and vancomycin in this assay. As with tobramycin-loaded beads, CLSM images demonstrate a marked reduction in biofilm formation in the presence of vancomycin and vancomycin & tobramycin in combination loaded beads, relative to unloaded beads.

SEM imaging of the surface of the vancomycin & tobramycin in combination loaded PG-CSH beads demonstrated a complete attenuation in bacterial surface attachment over 14 days, despite 4 bacterial challenges (Fig. 5).

Antibiotic-loaded PG-CSH beads to kill pre-established biofilms

*P. acnes* biofilms grown for 7 days prior to treatment with vancomycin & tobramycin in combination loaded PG-CSH beads were significantly more difficult to clear (Fig. 6). Bead contact times of 1 day with the established biofilms elicited a 1.5 log reduction in CFU/cm2 (96.95 % reduction) relative to the unloaded controls which increased to a 3.16 log reduction (99.93 %) after 3 days. A contact time of 7 days achieved an 8.24 log decrease in CFU/cm2 which corresponded to a 100 % reduction in *P. acnes* biofilm (sensitivity cut off value for the assay: 6.92 CFU/cm2).

**DISCUSSION**

*P. acnes* surface colonisation and biofilm formation was slow, despite optimised *in vitro* conditions with a rich media source. Studies have strongly linked biofilm formation to virulence, with genome mapping identifying several genes encoding adhesins and enzymes involved in extracellular polysaccharide synthesis used to form a biofilm [4](#_ENREF_4). Biofilm formation has been shown to be a characteristic of invasive isolates and the capacity of blood plasma to prevent biofilm formation may explain why *P. acnes* as a common skin commensal is more pathogenic around the plasma poor environment of an arthroplasty surgical site [5](#_ENREF_5); [15](#_ENREF_15). Nevertheless, slow biofilm growth kinetics would appear to link to the characteristic slow onset of infection, with symptoms presenting up to 36 months postoperatively and substantial cultures found during revision in apparently aseptic shoulders three years or more after the initial index arthroplasty [29](#_ENREF_29); [38](#_ENREF_38).

Initial colonisation and early biofilm formation was slower on unloaded PG-CSH beads than culture dishes used in subsequent assays. Preferential adherence to a culture dish is unsurprising. However, put in a clinical context, this again highlights the variability in attachment depending on surface composition [40](#_ENREF_40), with increased colonisation and infection rates observed with PMMA as a carrier surface relative to polyethylene, stainless steel and cobalt chromium alloys in canine studies [35](#_ENREF_35).

*P. acnes* is routinely reported as the predominant microorganism associated with shoulder PIs [39](#_ENREF_39); [41](#_ENREF_41); [46](#_ENREF_46). Treatment more commonly involves Penicillin G and ceftriaxone as first line antibiotics for severe infections with vancomycin and daptomycin as secondary alternatives in cases of β-lactam allergy or antimicrobial resistance [2](#_ENREF_2); [33](#_ENREF_33). Aminoglycosides generally have weak activity against *P. acnes*, and this was again demonstrated in this study with an elevated tobramycin MIC and MBC and reduced efficacy relative to vancomycin in subsequent assays. The combination of vancomycin and tobramycin is often applied in the treatment of hip and knee PIs [19](#_ENREF_19); [30](#_ENREF_30). Their suitability for incorporation into carrier substrates for localised release is well documented as, along with gentamicin, they meet many of the requirements for inclusion (solubility, chemical and thermal stability, broad-spectrum antibacterial activity, good elution properties), which other antibiotics may not, and their kinetics of release are well defined [3](#_ENREF_3); [6](#_ENREF_6); [21](#_ENREF_21). While infection rates vary from the hip and knee to the shoulder, specific to the shoulder and after *P. acnes*, coagulase negative staphylococci and *S. epidermidis* are secondary causative pathogens, with gram negative bacilli and polymicrobial infections, albeit relatively uncommon, still reported, with the latter consistently described as a worst-case scenarios broadly across prosthetic infection groups [39](#_ENREF_39); [41](#_ENREF_41); [44](#_ENREF_44); [46](#_ENREF_46); [50](#_ENREF_50); [51](#_ENREF_51). With an often indolent clinical course associated with *P. acnes*, with fewer clinical manifestations than observed with other bacterial species and a prolonged incubation time of more than a week in some cases for confirmation by culture, infection can be difficult to determine and therefore effectively treat[39](#_ENREF_39). Topolski *et al*. 2006 reported 75 patients who underwent revision shoulder arthroplasty without any signs of overt infection but whose intra-operative cultures returned positive, with *P. acnes* followed by *S. epidermidis* the cause [46](#_ENREF_46). Another study by Pottinger *et al.* 2012 demonstrated that 55 % of *P. acnes* cultures required more than one week to grow [39](#_ENREF_39). Consequently, the confirmation of the efficacy of a localised, broad spectrum antibiotic prophylactic, previously shown to be effective against *S. aureus* and *S. epidermidis* [16](#_ENREF_16), with demonstrable efficacy against *P. acnes,* may be beneficial approach in the management of shoulder PIs.

*P. acnes* was susceptible to vancomycin as shown by MIC and MBC evaluation. Importantly however, established biofilms were markedly more tolerant to both tobramycin and vancomycin than their planktonic counterparts, once again demonstrating the difficulty in clearing established biofilms. In prosthetic infections, this inherent phenotypic tolerance is compounded by the lack of blood supply to a foreign body and the build-up of hypovascular scar tissue thus restricting immune cell and antibody penetration [27](#_ENREF_27). As a result, early infection prevention measures are important in arthroplasty management and previous studies have shown the ability of antimicrobial-modified surfaces to retard bacterial colonisation and biofilm formation [9](#_ENREF_9); [20](#_ENREF_20). Antibiotic-loaded PG-CSH beads have also previously demonstrated antibacterial and antibiofilm efficacy *in vitro* [16](#_ENREF_16); [28](#_ENREF_28). In this study, agar diffusion assays also demonstrated long term antibacterial efficacy which was maintained for 60 days until complete reabsorption. As a comparison with the current standard of care, PMMA beads demonstrated elution for up to 111 days. The increased sensitivity of planktonic *P. acnes,* with its relatively low vancomycin MIC in this study, is likely to account for the prolonged efficacy of PMMA, even at low eluting concentrations. Previous studies have demonstrated efficacy of antibiotics released from PMMA to be reduced relative to PG-CSH beads, possibly owing to the inability of PMMA to completely elute loaded antibiotics, with some studies suggesting only about 10 % of the antibiotic is ever released from the cement [16](#_ENREF_16); [28](#_ENREF_28); [49](#_ENREF_49). To this end, dissolvable substrates may be more desirable as complete elution can be accomplished and no foreign body remains for subsequent colonization. Importantly however, PG-CSH would not be able to substitute for PMMA in procedures where mechanical strength and integrity is the primary requirement.eg. prosthesis fixation to bone in primary arthroplasties or as a spacer in staged revisions. Additionally, due to the absorbable nature of calcium sulphate, although with osteoconductive properties, the primary stability of the implant should rely on the integration of the press fit prosthesis into host bone directly, and not through an interface via PG-CSH beads.  It must be ensured that the beads do not interfere with the press fit due to the potential for loosening. Primary application would be with a view to device protection and soft tissue/dead space management around the implants during either one or two stage revision procedures or as an early preventative measure in the initial surgery, particularly in high risk groups such as young males. With the difficulty in detecting shoulder infections clear, the use of localised antibiotic-eluting absorbable carriers may be a beneficial anti-biofilm strategy, for example in cases of an apparent aseptic shoulder where post-operative deep cultures return positive, particularly with long culture time intervals. Additionally, with continuing work investigating the potential for incorporation of a wider range of antibiotics, the beads could also be employed as a targeted prophylactic approach in known infections, with the capability of achieving high, localised antibiotic concentrations not possible with systemic delivery.

In assays to observe the effect of antibiotic-loaded PG-CSH beads on *P. acnes* biofilm formation, the duration of bead efficacy was reduced relative to the agar diffusion assays due to the aqueous solution the beads are immersed in for assay duration and the media exchanges performed every 72 h. Further studies are needed to realistically reproduce the fluid exchange that a postoperative joint undergoes. However, antibiotic-loaded PG-CSH beads were capable of achieving reduced surface colonisation and exhibited prolonged biofilm prevention throughout the 14 day assay. Vancomycin-loaded PG-CSH was able to achieve a complete kill for 1 week in the presence 2 bacterial challenges of 106 CFU/ml. Interestingly, vancomycin & tobramycin in combination was able to extend the period for a complete kill to 14 days in the presence of a further 2 bacterial challenges, indicating possible synergy between these antibiotics in this experimental set up. Previous studies with different microorganisms but a similar experimental set-up have also indicated synergy between vancomycin and tobramycin [16](#_ENREF_16). Although this warrants further investigation, importantly vancomycin and an aminoglycoside such as tobramycin are often combined clinically for their potential synergistic effect in the treatment of MRSA infections [21](#_ENREF_21). Moreover, synergy between vancomycin and gentamicin [48](#_ENREF_48) and streptomycin and penicillin [37](#_ENREF_37) in the context of planktonic bacteria has been noted previously. Synergy in the context of antimicrobials targeted to biofilms has also been noted between rifampicin and cell-wall active antibiotics directed against *S. epidermidis* [12](#_ENREF_12).

Established biofilms were harder to eradicate. Increasing contact times of antibiotic-loaded PG-CSH beads to *P. acnes* biofilms previously grown for 1 week produced statistically significant increases in biofilm clearance with complete eradication observed at 7 days. This demonstrates that while difficult to eradicate once fully established, biofilm eradication, at least in some strains, can be achieved given optimal antibiotic choice, eluting concentrations and sufficient exposure time.

Several limitations exist with regards to this study. Firstly, *in vitro* assays can not replicate the complexity of the *in vivo* environment, bead packing density or the continuous rate of drainage in a joint that the beads would encounter clinically and, consequently, studies into the longevity of antibiotic elution from the beads and the time to complete reabsorption may vary from that reported *in vivo* (up to 3 weeks in soft tissue and 6 weeks in bone tissue) [23](#_ENREF_23); [32](#_ENREF_32). Further planned studies aim to address this deficit in experimental set up. Additionally, ZOI assays, whilst effectively assessing the spatial range of antibiotic elution, demonstrate elution in two-dimension, whereas *in vivo* elution would occur in three-dimension, possibly reducing the timeframe for elution described in this study. Conversely however, the concentration of *P. acnes* inoculum and the extent of biofilm coverage encountered *in vitro* in these assays is likely much greater than would be encountered *in vivo* and, as such, represents an extreme scenario, greater than would be encountered by the beads clinically. Future work also aims to investigate the capacity of other antibiotics to be incorporated into calcium sulfate carriers, such that a targeted prophylaxis in cases of known infection, rather than broad-spectrum preventative, may be applied.

**CONCLUSION**

Studies continue to demonstrate the importance of *P. acnes* in periprosthetic shoulder infections and in PIs in general, with *P. acnes* capable of forming biofilms which are strongly implicated as a key virulence factor. In orthopaedics, the use of antibiotic-loaded absorbable beads has the ability to achieve high, local concentrations at the surgical margin, with complete antibiotic elution and no residual unprotected foreign body surface [43](#_ENREF_43). Their ability to retain high bioactivity for periods of weeks *in situ* is crucial, particularly for slow growing pathogens such as *P. acnes*. The use of fully absorbable antibiotic-loaded PG-CSH may therefore represent an effective antibacterial and antibiofilm strategy to combat PIs in which *P. acnes* is involved.

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FIGURE AND TABLE LEGENDS

**Figure 1.** *P. acnes* ATCC 11827 biofilm formation on the surface of an unloaded PG-CSH bead over a period of 14 days. Surface colonisation was observed at Day 3 with evidence of EPS strands (indicated by red arrows), with small microcolonies noted at Day 7 with more extensive EPS deposition. At Day 14, biofilm was observed across the bead surface across a matted layer of EPS. Image scale bars: 10 μm.

**Figure 2.** Repeat agar diffusion assays to assess zones of inhibition (ZOI) of *P. acnes* ATCC 11827 over a period of up to 120 days. Graphs show the ZOI (cm2) surrounding unloaded, tobramycin-loaded, vancomycin-loaded and vancomycin & tobramycin in combination-loaded PG-CSH and PMMA beads. Data represents the mean of 3 experimental repeats with standard deviation bars.

**Figure 3.** *P. acnes* ATCC11827 biofilm formation on the glass surface of MatTek tissue culture plate *in vitro* as observed by Live/Dead BacLight fluorescent staining and confocal laser scanning microscopy (CLSM). Image scale bars: 25 µm.

**Figure 4.** *P. acnes* ATCC11827 biofilm formation over 14 days in the presence of antibiotic-loaded PG-CSH beads as determined by CLSM images and colony forming unit counts (CFU/cm2). Data represents the mean of 3 experimental repeats (15 data points, n=5 per per experimental repeat) with standard deviation bars. Arrows indicate a fresh bacterial challenge of 106 CFU/ml bacterial challenges every 72 h. Image scale bars = 25 µm.

**Figure 5.** Representative SEM image of the surface of an unloaded and a vancomycin & tobramycin in combination loaded PG-CSH bead incubated with *P. acnes* ATCC 11827 for 14 days with media exchange and a fresh bacterial challenge of 106 CFU/ml every 72 h. Image scale bar: 10 µm.

**Figure 6.** The effect of antibiotic-loaded PG-CSH beads with 1, 3 and 7 day contact times on established biofilms of *P. acnes* ATCC 11827. Data represent the mean log reduction in viable cells elicited by vancomycin & tobramycin in combination loaded beads relative to unloaded beads treatment from 10 data points (n=5 per experimental repeat). \* and \*\* indicated statistical difference (P=<0.001).

**Table I.** Data summary of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *P. acnes* ATCC 11827 in planktonic and biofilm phenotypes.