# An *in vitro* biofilm model of *Staphylococcus aureus* infection of bone

Running head: Bone biofilms

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## Significance and Impact of the Study

The majority of studies of antibiotic efficacy in the treatment of chronic osteomyelitis are carried out in animals. We developed an *in vitro* model of *Staphylococcus aureus* infection of bone to evaluate the ability of antibiotics to eradicate mature biofilms on surfaces analogous to necrotic bone. The results demonstrated the difficulties which occur in osteomyelitis treatment, with only very high concentrations of antibiotic able to penetrate the bone sufficiently to reduce bacterial survival whilst still failing to eradicate biofilms. This model could be of use in initial screening of novel compounds intended for use in the treatment of osteomyelitis.


## Abstract

Chronic osteomyelitis is difficult to treat, with biofilm growth and the diffusion barrier to antibiotics presented by bone contributory factors. The aim of this study was to develop and evaluate an *in vitro* model of osteomyelitis. A bioluminescent strain of *Staphylococcus aureus* was grown in bone blocks made from bovine femur. Light output was insufficient for detection of bacterial cells within bone by 24 h and viable counting of crushed bone blocks was used to determine bacterial survival. Challenge of 72 h biofilms with gentamicin and daptomycin for 24 h demonstrated that only concentrations of ten times the clinical peak serum target levels (100 mg $l^{-1}$ gentamicin and 1000 mg $l^{-1}$ daptomycin) resulted in significant reductions in cell viability compared to controls. Once daily dosing over seven days resulted in ≥3 log reductions in cell numbers by 48 h. Thereafter no significant reduction was achieved, although emergence of resistance was suppressed. Determination of antibiotic concentration in bone blocks over seven days indicated that neither agent was able

to consistently reach levels in bone of greater than 10% of the original dose. The model was, therefore, able to demonstrate the challenges posed by biofilm growth on and within bone.

**Keywords**: biofilm, *Staphylococcus aureus*, osteomyelitis, *in vitro* model

## Introduction

Osteomyelitis, microbial infection of cortical and cancellous bone, can occur in any bone in the human body (Beck-Broichsitter *et al*. 2015). It may be acquired exogenously via direct inoculation of microorganisms through trauma or surgery, or endogenously via haematogenous spread of microorganisms (Walter *et al*. 2012) or contiguous spread from, for example, diabetic foot ulcers (Malhotra *et al*. 2014). With population aging and concomitant increases in orthopaedic intervention and prosthesis implantation, as well as an increasing diabetic population (WHO 2016), the incidence of osteomyelitis is likely to increase. Osteomyelitis is inherently difficult to diagnose and treat. In its chronic form, bone necrosis and microbial biofilm formation limit delivery of antibiotics to, and within, the infection site (Cook *et al*. 2015). Relapse of infection is common and surgical debridement of the infected site (Tuchscherr *et al*. 2016) and prolonged antimicrobial chemotherapy add to the already considerable economic burden of this disease (Birt *et al*. 2017).

*Staphylococcus aureus* is the bacterium most commonly associated with chronic osteomyelitis, responsible for up to 90% of cases (Olson and Horswill 2013).

It expresses a number of adhesins for bone extracellular matrix components and, once chronic osteomyelitis is established, it likely resides within the fluid spaces of de-vascularised, dead bone, known as sequestra (Ciampolini and Harding, 2000). However, *S. aureus* also persists within live cortical bone cells, leading to biofilm formation in osteocyte lacunae (de Mesy Bentley *et al*. 2017).

If diagnosed early, acute and haematogenous osteomyelitis caused by methicillin sensitive *S. aureus* can be treated by six weeks of antibiotic therapy, whilst chronic infection treatment may span from eight weeks to lifelong suppression. Gentamicin, an aminoglycoside, has been widely used in orthopaedic surgery, intravenously, impregnated into bone cement or implanted in the form of beads in order to deliver high concentrations at the site of device implantation (McHugh *et al*. 2011). Daptomycin, a cyclic lipopeptide, has been used experimentally and clinically in the treatment of osteomyelitis and device-related infections caused by *S. aureus*, often in combination with rifampicin. Although often used successfully, there are reports of relapse of infection and emergence of resistance (Falagas *et al*. 2007) particularly with monotherapy, and optimal dosing regimes are still being investigated (Senneville *et al*. 2016).

Clearly, further study and new, novel agents are required. However, with limited new approaches available, it is pertinent to investigate current drugs with new models. Although reports in the literature of *in vitro* biofilm models of osteomyelitis have increased in recent years (Tuchscherr *et al*. 2016; Junk *et al*. 2017; Melicherčík *et al.* 2018; Raic *et al*. 2018), most pharmacodynamic studies of antibiotic efficacy are carried out in animals. The aim of this work was to use a novel *in vitro* pharmacodynamic model of biofilm in bone in order to study gentamicin and daptomycin efficacy against a bioluminescent *S. aureus* reporter strain.

## Results and discussion

### The bone model

The work described here highlights the challenges involved in *in vitro* modelling of osteomyelitis. Bovine bone was chosen for several reasons; its availability, its similarities in chemical composition and architectural geometry to human bone (Stravropoulos 2008) and also its reported similar antibiotic elution properties to human bone (Winkler *et al*. 2000). The size of bovine femur enabled a large number of blocks to be cut from the cortical region of an individual bone, however, natural variation in the bone marrow lumen diameter and bone density, precluded the production of identical bone blocks, as encountered elsewhere (Melicherčík *et al,* 2018). To minimise variation, only blocks between 1 and 2 g were used. Changes to the bone that may have resulted from autoclaving, such as reduction in mechanical strength (Köhler *et al*. 1986) were deemed less important than the requirement to sterilise the bone and, as the model aimed to simulate *in vivo* necrotic bone devoid of blood supply, some denaturation of the organic matrix was acceptable.

### Visualisation of *S. aureus* 8325p1 biofilm on bovine femur bone substratum

Scanning electron microscopy of 72 h biofilms confirmed that *S. aureus* 8325p1 was adhering to bovine femur bone (see supporting information). As this strain is a dual reporter (expressing *gfp* and *lux* genes), it was hoped that information on its location and metabolic activity within bone blocks could be acquired via confocal laser scanning microscopy and bioluminescence imaging, respectively. However, preliminary work (data not shown) demonstrated that bone autofluorescence

interfered with detection of *gfp*-expressing bacterial cells and, although it may be possible to mitigate this (Zimmermann *et al*. 2014; Capasso *et al*. 2017), only bioluminescence imaging was further investigated.

During low light level imaging of *S. aureus* 8325p1 cultures in bone blocks over 24 h (Fig. 1), bioluminescence initially increased, indicating that cells were metabolically active and primarily maintained within the bone blocks. Thereafter, light intensity decreased within the bone, but increased in the broth surrounding the bone, as the bacterial reporter spread outwards due to the vascularised nature of bone. At 24 h, there was an obvious darker region where the bone was present in each of the samples (represented by arrows in Fig. 1). This reduction in bioluminescence was not due to an absence of cells, as viable counts performed by crushing the bone blocks and counting cells washed from the bone fragments all yielded approximately $4 \times 10^6$ cfu $ml^{-1}$ (ca. $8.0 \times 10^7$ cfu per bone block). One possible reason was that the number of reporter organisms was too low for bioluminescence to be detected through the bone. An improved *lux* gene construct generating increased light output, recently described for use in *Escherichia coli* by Gregor *et al*. (2018), may address this problem. It is, however, more likely that cells adherent to the bone were metabolically inactive or growing slowly and, therefore, not emitting sufficient detectable light. The likelihood of slow growing or persister cells within biofilms (Conlon 2014) will limit the utility of reporters reliant on active metabolism and a combination of bioluminescence imaging and conventional viable counting will likely be required for the study of mature biofilms (Ogunniyi *et al*. 2018) and chronic infection.

142

143 **Time/kill studies of gentamicin and daptomycin against biofilms of *S. aureus***
144 **8325p1**

145 Gentamicin and daptomycin were chosen as relevant antimicrobials with which to
146 investigate antibiotic penetration into and efficacy within the bone model. *S. aureus*
147 8325p1 was susceptible to both antibiotics, with MICs of 0.25 mg $l^{-1}$ and 1.0 mg $l^{-1}$ for
148 gentamicin and daptomycin, respectively.

149 For gentamicin dosing of 72 h biofilms, at concentrations of 10, 30 and 100 mg $l^{-1}$
150 for 24 h, only 100 mg $l^{-1}$, corresponding to approximately ten times the target peak
151 concentration in plasma ($C_{max}$), resulted in a significant difference in viable cell counts
152 compared to the growth control (p=0.04) (Fig. 2), with a >2 $log_{10}$ reduction. Overall
153 drug effect (i.e. bacterial survival) was determined from the area under the bacterial
154 kill curve (AUBKC). Gentamicin at 10 and 30 mg $l^{-1}$ had minimal effect, however, at
155 100 mg $l^{-1}$ the mean AUBKC was 70.46 ±0.6 log cfu $ml^{-1}$ h, corresponding to a 38.9%
156 reduction in the bacterial population.

157 Exposure to daptomycin for 24 h at concentrations of 100 and 300 mg $l^{-1}$ did not
158 result in a reduction in viable cells from 72 h biofilms compared to controls (Fig. 3). As
159 a result, biofilms were challenged with daptomycin at 1000 mg $l^{-1}$ thereafter. The effect
160 of exposing 72 h biofilms to once daily dosing of daptomycin at 1000 mg $l^{-1}$ and
161 gentamicin at 100 mg $l^{-1}$, over 7 days, was investigated (Fig. 4). A significant reduction
162 in the recovery of *S. aureus* 8325p1 from biofilms was achieved over 7 days with both
163 antibiotics when compared to recovery of cells from the growth control (p = 0.002 for
164 daptomycin and p = 0.0005 for gentamicin), with gentamicin superior to daptomycin
165 (p=0.0005). AUBKC for each antibiotic (186.80±71 log cfu $ml^{-1}$ h for gentamicin and

549.42±24 log cfu $ml^{-1}$ h for daptomycin) represented 11.9% predicted survival with gentamicin treatment and 35% survival with daptomycin when compared to the AUC of the untreated control. A ≥3 $log_{10}$ cfu $ml^{-1}$ reduction was achieved for both drugs by 24 h and, for gentamicin, maintained over the remaining experimental period. However, despite both drug concentrations being approximately ten times the target $C_{max}$ for effective plasma concentrations *in vivo*, viable populations remained. These results reflect those of other *in vitro* studies (Parra-Ruiz *et al*. 2010; Molina-Manso *et al*. 2013) where high dose antibiotic levels were unable to eradicate *S. aureus* biofilms. Although the concentrations used in this current study are unlikely to be achieved or tolerated with parenteral administration, the continued improvement of targeted antimicrobial delivery systems should allow high local antibiotic doses whilst minimising systemic toxicity (Birt *et al*. 2017). It is likely that maintaining high concentrations over a prolonged period will be crucial.

**Diffusion of antibiotic into the bone model**

Reduced antibiotic activity against bacterial biofilms embedded in bone may be, in part, due to a lack of diffusion into bone or variability of diffusion rates, due to different bone densities. To evaluate diffusion, the concentrations of both gentamicin and daptomycin were measured in bone blocks over time. Once daily dosing over 7 days resulted in both drugs accumulating within the bone blocks (Fig. 5), with the relationship between dose and drug accumulation stronger for daptomycin. The reason for this is unclear, although may be as a result of daptomycin's charge and the fact that it readily binds to calcium ions (Humphries *et al*. 2013). However, neither drug consistently reached levels that were greater than 10% of the dosing concentration,

highlighting the difficulties that are encountered when attempting to achieve clinically useful levels in necrotic bone *in vivo*.

The gentamicin levels achieved within the bone should, in theory, be sufficient to reduce bacterial load as they exceeded the plasma concentration generally regarded as therapeutically effective (5-10 mg $l^{-1}$ for multiple daily dosing (Joint Formulary Committee 2018)). Comparison of the results of daily dosing on bacterial survival (Fig. 4) and drug concentration within bone (Fig. 5A), suggests that, although the sustained antibiotic concentration was unable to further reduce the biofilm population, it was still sufficient to prevent regrowth over the time period studied.

Reports of the concentration of daptomycin in bone vary, with concern about its bioavailability in different tissues due to its high plasma protein binding (>90%). Nevertheless, it has been shown to equilibrate with bone *in vivo* within 3 h of infusion (Traunmüller *et al*. 2010) and reach a sufficient free concentration for clinical efficacy against *S. aureus* infection (Montange *et al*. 2014). In this current study, daptomycin lacked efficacy at clinically acceptable concentrations and a significant reduction in bacterial numbers was only seen at tenfold the target level. This was sufficient only to prevent re-growth of cells and maintain bacteriostasis. It should be noted, however, that there was considerable variation in drug concentration within replicate bone blocks, likely attributable to the inherent variability in bone density of the blocks. Although there have been varied reports for daptomycin efficacy against staphylococci in biofilms both *in vitro* and *in vivo* (Hurdle *et al*. 2011), a review of clinical outcomes for treatment of osteomyelitis and orthopaedic device infection (EU-CORE$^{SM}$) reported efficacy in 82.7% of *S. aureus* infections (Malizos *et al*. 2016). Work to further improve and expand its use continues, particularly for high dose delivery via polymethyl methacrylate (PMMA) bone cement (Eick *et al*. 2017), or in the form of daptomycin-

loaded PMMA microparticles to enhance delivery to *S. aureus* within osteoblasts (Woischnig *et al.* 2018) or biofilms (Santos Ferreira *et al*. 2018).

The results of this current work suggest that, without a dynamic blood supply, bone presents a barrier to the diffusion of even small molecules. Local antibiotic delivery methods *in vivo*, such as antibiotic-loaded beads, antibiotic-coated intramedullary rods or antibiotic-containing cement, expose the infecting microorganisms to high doses without causing toxic levels systemically (Hake *et al*. 2015). However, sustaining the required effective concentration for a sufficient period is not without potential problems. *S. aureus* small colony variants (SCVs) are isolated from osteomyelitis infections (Kahl *et al.* 2016) and were noted when *S. aureus* 8325p1 was exposed to gentamicin. It is likely that treatment failure and relapse of chronic osteomyelitis are, at least in part, attributable to this antibiotic tolerant phenotype. Nonstable SCVs with long lag phases originate from persister cells and their delayed growth is advantageous in the presence of antibiotics (Vulin *et al*. 2018). Under the stressful conditions that likely exist in necrotic bone during (inadequate) antibiotic treatment, such SCVs would be at a selective advantage. Furthermore, known to survive in osteoblasts and osteocytes (Yang *et al*. 2018), SCVs have the potential to persist even when bone is being remodelled and reformed. Three-dimensional scaffolds which allow bone generation alongside sustained release of antibiotic are under development (Cicuéndez *et al*. 2018) and it will be critical to their success that sufficient antibiotic can be delivered in order to suppress the development of SCVs.

It is clear from this work that *in vitro* modelling of bone infection is challenging, both in terms of consistency of model construction and the non-destructive monitoring

of the infecting organisms. Nevertheless, the model has the potential to generate information on bacterial survival over sustained time periods which can be related to the ability of antibiotic to penetrate into the bone. Most *in vitro* pharmacokinetic and pharmacodynamic models only investigate short term drug efficacy which is not appropriate when modelling chronic osteomyelitis. Given that the levels required for measurable reduction in our study were 400 x MIC for gentamicin and 1000 x MIC for daptomycin, the model was able to reflect some of the challenges faced with *in vivo* control of *S. aureus* on and within bone.

## Material and methods

### Bacterial strain

The bacterial strain used was *S. aureus* ATCC 8325, transformed (Sweeney 2010) with a recombinant plasmid containing the *luxABCDE* gene cassette of *Photorhabdus luminescens* (pSB3007; a 3 fragment expression cassette in the shuttle vector pUNK1 $Em^r$ : $P_{xylA}$:*gfp*:*luxABCDE*:*rrnBT1T2* [4 *attB*]) (Perehinec *et al*., 2007). The transformant, *S. aureus* 8325p1, was maintained on nutrient agar (NA) (Oxoid, Basingstoke, UK) supplemented with 10 mg $l^{-1}$ erythromycin (Sigma-Aldrich Company Ltd., Poole, UK) for plasmid selection and overnight cultures were grown in nutrient broth (NB) (Oxoid) supplemented with 10 mg $l^{-1}$ erythromycin (Sigma-Aldrich).

### Bone model preparation

Bovine femur, obtained from an abattoir (Bristol University Veterinary School, Langford, UK) or a local butcher, was sliced into approximately 4 cm sections. Bone marrow and debris were removed and bone slices washed in 10% Hycolin™. Bone blocks of 1 $cm^3$ were cut from each section and those weighing between 1 and 2 g were selected. A hole (2 mm diameter x 5 mm depth) was drilled into each block and the blocks were sterilised by autoclaving (121°C, 15 lb $inch^{-2}$, for 15 min).

**Bone model set up and confirmation of biofilm formation**

The well of each bone block was inoculated with 10 µl of an overnight culture of *S. aureus* 8325p1 incubated in NB containing 10 mg $l^{-1}$ erythromycin and diluted to a final concentration of $10^6$ cfu $ml^{-1}$. Each bone block was then immersed in 5 ml of pre-warmed NB, in universal bottles and incubated, without agitation, at 37°C in air for 72 h to allow biofilm development. Bones were transferred to fresh NB every 24 h.

**Bioluminescence imaging of *S. aureus* 8325p1 in bone blocks**

Bone blocks were prepared and inoculated with *S. aureus* 8325p1, as described previously, placed in a tissue culture dish (Nunclon® 60 x 15 mm) containing 3 ml NB and light output monitored over 24 h, at 37°C, using a High Resolution Photon Counting system (HRPCS-3; Photek, St. Leonards-on-Sea, UK).

**Recovery of viable bacterial cells from bone blocks**

To recover *S. aureus* 8325p1 from bone blocks, they were crushed in an iron bore mortar. The fragments were given four successive 1 min vortex-washes in 5 ml

sterile PBS. The 20 ml washings were pooled, serially diluted in saline, plated onto NA containing 10 mg $l^{-1}$ erythromycin and incubated overnight at 37˚C for bacterial enumeration.

**Antimicrobial susceptibility testing**

Minimum inhibitory concentrations (MICs) of gentamicin and daptomycin were determined for *S. aureus* 8325p1 using the BSAC standard broth macrodilution method (Andrews 2001), modified for daptomycin by using Mueller Hinton broth (MHB) (Oxoid) supplemented with 50 mg $l^{-1}$ calcium ions.

**Time/kill studies of antibiotics against bone biofilms**

The activity of gentamicin and daptomyin (both Sigma-Aldrich) against biofilms within bone blocks was determined. Bone blocks were inoculated, as described previously, and incubated for 72 h at 37°C, with NB changes every 24 h. Bones were then transferred to tissue culture plates containing 3 ml sterile NB, NB containing gentamicin (10, 30 or 100 mg $l^{-1}$) or MHB containing daptomycin (100, 300 or 1000 mg $l^{-1}$) plus 50 mg $l^{-1}$ $Ca^{2+}$ in the form of $CaCl_2$. At 0, 1, 2, 4, 6, 9, 12 and 24 h, three bone blocks for each treatment were removed, briefly washed in fresh PBS and blotted dry to remove adherent bacteria/antibiotic and the cells recovered for viable counting. To monitor the effect of once-daily dosing, bone biofilms were developed and monitored in the same way and maintained for 7 days. Every 24 h, growth controls were transferred to fresh NB or MHB+$Ca^{2+}$ and test samples were transferred to the relevant broth containing the appropriate antibiotic dose. Three bone blocks were sampled for viable counting at each 24 h interval.

## Determination of antibiotic diffusion into bone by FPIA and HPLC

Sterile bone blocks were weighed and placed into universals containing 5 ml of aqueous solutions of either 100 mg $l^{-1}$ gentamicin or 1000 mg $l^{-1}$ daptomycin. At intervals of 0, 1, 2, 3, 4, 6, 12 and 24 h bone blocks were crushed and re-weighed. Following transfer to universal bottles, PBS was added in the ratio 1:2 w/v and the samples left at room temperature for four hours to allow the drug to elute into the solution. Gentamicin concentrations were determined by Fluorescence Polarisation Immunoassay (FPIA) (Abbott TDxFLx®, Abbott Laboratories, Abbott Park, IL, USA) and daptomycin was determined by High Performance Liquid Chromatography (HPLC). In brief, FPIA samples were prepared by 1:1 dilution with TDx buffer (Abbott Laboratories) and then assayed following the manufacturer's instruction. HPLC samples were diluted 1:1 with acetonitrile (Sigma-Aldrich), allowed to stand for 5 min then centrifuged for 5 min (2,500 *g*) to remove any cell protein and then assayed as previously described (Tobin *et al*. 2008). Chromatography was performed on a Hypersil 5ODS column using a mobile phase composed of 0.2 M phosphate buffer (pH 5.5) and acetonitrile (70:30). The pump flow rate was 1.5 ml $min^{-1}$ and detection was by UV absorbance at 223 nm (Thermo-Sep Electron Corp., Waltham, MA, USA; Dionex Chromeleon Analyser, Dionex Corp, Sunnyvale, CA, USA). All assays were calibrated against appropriate standards using the external standard method.

## Statistical analysis

All data were analysed using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, USA). All assays were performed in triplicate. Two way ANOVA was used to compare antibiotic-treated biofilms with controls. *P* values of $< 0.05$ were considered statistically significant.


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## Conflict of interest

The authors have no conflicts of interest to declare.

## References


Andrews, J.M. (2001). Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* **48** (Suppl 1), 5-16.

Beck-Broichsitter, B.E., Smeets, R. and Heiland, M. (2015) Current concepts in pathogenesis of acute and chronic osteomyelitis. *Curr Opin Infect Dis* **28**, 240-245.

Birt, M.C., Anderson, D.W., Toby, E.B. and Wang, J. (2017) Osteomyelitis: recent advances in pathophysiology and therapeutic strategies. *J Orthop* **14**, 45-52.

Capasso, L., D'Anastasio, R., Guarnieri, S., Viciano, J. and Mariggiò, M. (2017) Bone natural autofluorescence and confocal laser scanning microscopy: Preliminary results of a novel useful tool to distinguish between forensic and ancient human skeletal remains. *Forensic Sci Int* **272**, 87-96.

Ciampolini, J. and Harding, K.G. (2000) Pathophysiology of chronic bacterial osteomyelitis. Why do antibiotics fail so often? *Postgrad Med J* **76**, 479-483.

Cicuéndez, M., Doadrio, J.C., Hernández, A., Portolés, M.T., Izquierdo-Barba, I. and Vallet-Regí, M. (2018) Multifunctional pH sensitive 3D scaffolds for treatment and prevention of bone infection. *Acta Biomater* **65**, 450-461.

Conlon, B. (2014) *Staphylococcus aureus* chronic and relapsing infections: Evidence of a role for persister cells: an investigation of persister cells, their formation and their role in *S. aureus* disease. *Bioessays* **36**, 991-996.

Cook, G.E., Markel, D.C., Ren, W., Webb, L.X., McKee, M.D. and Schemitsch, E.H. (2015) Infection in orthopaedics. *J Orthop Trauma* **29**, S19-S23.

Eick, S., Hofpeter, K., Sculean, A., Ender, C., Klimas, S., Vogt, S. and Nietzsche, S. (2017) Activity of fosfomycin- and daptomycin-containing bone cement on selected bacterial species being associated with orthopaedic infections. *Biomed Res Int* **2017**, 2318174.

Falagas, M.E., Giannopoulou, K.P., Ntziora, F. and Papagelopoulos, P.J. (2007) Daptomycin for treatment of patients with bone and joint infections: a systematic review of the clinical evidence. *Int J Antimicrob Agents* **30**, 202-209.

373 Gregor, C., Gwosch, K.C., Sahl, S.J. and Hell, S.W. (2018) Strongly enhanced
374 bacterial bioluminescence with the *ilux* operon for single-cell imaging. *Proc Natl Acad*
375 *Sci USA* **115**, 962-967.

376 Hake, M.E., Young, H., Hak, D.J., Stahel, P.F. and Hammerberg, E.M. (2015) Local
377 antibiotic therapy strategies in orthopaedic trauma: Practical tips and tricks and review
378 of the literature. *Injury* **46**, 1447-1456.

379 Humphries, R.M., Pollett, S. and Sakoulaas, G. (2013) A current perspective on
380 daptomycin for the clinical microbiologist. *Clin Microbiol Rev* **26**, 759-780.

381 Hurdle, J.G., O'Neill, A.J., Chopra, I. and Lee, R.E. (2011) Targeting bacterial
382 membrane function: an underexploited mechanism for treating persistent infections.
383 *Nat Rev Microbiol* **9**, 62-75.

384 Joint Formulary Committee. *British National Formulary* (online) London: BMJ Group
385 and Pharmaceutical Press <http://www.medicinescomplete.com> [Accessed on
386 [5/10/18]

387 Junka, A., Szymczyk, P., Ziółkowski, G., Karuga-Kuzniewska, E., Smutnicka, D., Bil-
388 Lula, I., Bartoszewicz, M., Mahabady, S. and Sedghizadeh, P.P. (2017) Bad to the
389 one: on *in vitro* and *ex vivo* microbial biofilm ability to directly destroy colonized bone
390 surfaces without participation of host immunity or osteoclastogenesis. *PLoS One* **11**,
391 e0169565.

392 Kahl, B.C., Becker, K and Löffler, B. (2016) Clinical significance and pathogenesis of
393 staphylococcal small colony variants in persistent infections. *Clinical Microbiology*
394 *Reviews* **29**, 401-427.

Köhler, P., Kreicbergs, A. and Strömberg, L. (1986) Physical properties of autoclaved bone. Torsion test of rabbit diaphyseal bone. *Acta Orthop Scand* **57**,141-145.

Malhotra, R., Chan, C.S.Y and Nather, A. (2014) Osteomyelitis in the diabetic foot. *Diabet Foot Ankle* **5**, 10.3402/dfa.v5.24445.

Malizos, K., Sarma, J., Seaton, R.A., Militz, M., Menichetti, F., Riccio, G., Gaudias, J., Trostmann, U. *et al*. (2016) Daptomycin for the real world treatment of osteomyelitis and orthopaedic device infections: real-world clinical experience from a European registry. *Eur J Clin Microbiol Infect Dis* **35**, 111-118.

McHugh, S.M., Collins, C.J., Corrigan, M.A., Hill, A.D.K. and Humphreys, H. (2011) The role of topical antibiotics used as prophylaxis in surgical site infection prevention. *J Antimicrob Chemother* **66**, 693-701.

Melicherčík, P., Nešuta, O and Čeřovský, V. (2018) Antimcrobial peptides for topical treatment of osteomyelitis and implant-related infections: study in the spongy bone. *Pharmaceuticals (Basel)* **11**, E20.

de Mesy Bentley, K.L., Trombetta, R., Nishitani, K., Bello-Irizarry, S.N., Ninomiya, M., Zhang, L., Chung., HL., McGrath, J.L. *et al*. (2017) Evidence of *Staphylococcus aureus* deformation, proliferation, and migration in canaliculi of live cortical bone in murine models of osteomyelitis. *J Bone Miner Res* **32**, 985-990.

Molina-Manso, D., del Prado, G., Ortiz-Pérez, A., Manrubia-Cobo, M., Gómez-Barrena, E., Cordero-Ampuero, J. and Esteban, J. (2013) *In vitro* susceptibility to antibiotics of staphylococci in biofilms isolated from orthopaedic infections. *Int J Antimicrob Agents* **41**, 521-523.

417 Montange, D., Berthier, F., Leclerc, G., Serre, A., Jeunet, L., Berard, M., Muret, P.,
418 Vettoretti, L. *et al*. (2014). Penetration of daptomycin into bone and synovial fluid in
419 joint replacement. *Antimicrob Agents Chemother* **58**, 3991-3996.

420 Ogunniyi, A.D., Kopecki, Z., Hickey, E.E., Khazandi, M., Peel, E., Belov, K., Boileau,
421 A., Garg, S. *et al*. (2018) Bioluminescent murine models of bacterial sepsis and scald
422 wound infections for antimicrobial efficacy testing. PLoS One **13**, e0200195.

423 Olson, M.E. and Horswill, A.R. (2013) *Staphylococcus aureus* osteomyelitis: bad to
424 the bone. *Cell Host Microbe* **13**, 629-631.

425 Parra-Ruiz, J., Vidaillac, C., Rose, W.E. and Ryback, M.J. (2010) Activities of high-
426 dose daptomycin, vancomycin, and moxifloxacin alone or in combination with
427 clarithromycin or rifampin in a novel *in vitro* model of *Staphylococcus aureus* biofilm.
428 *Antimicrob Agents Chemother* **54**, 4329-4334.

429 Perehinec, T.M., Qazi, S.N., Gaddipati, S.R., Salisbury, V., Rees, C.E. and Hill, P.J.
430 (2007) Construction and evaluation of multisite recombinatorial (Gateway) cloning
431 vectors for Gram-positive bacteria. *BMC Mol Biol* **8**, 80.

432 Raic, A., Riedel, S., Kemmling, E., Bieback, K., Overhage, J., Lee-Thedieck, C.
433 (2018) Biomimetic 3D *in vitro* model of biofilm triggered osteomyelitis for
434 investigating hematopoiesis during bone marrow infections. *Acta Biomater* **73**, 250-
435 262.

436 Santos Ferreira, I., Kikhney, J., Kursawe, L., Kasper, S., Gonçalves, L.M.D.,
437 Trampuz, A., Moter, A., Bettencourt, A.F. and Almeida, A.J. (2018) Encapsulation in
438 polymeric microparticles improves daptomycin activity against mature staphylococci
439 biofilms – a thermal imaging study. *AAPS PharmSciTech* **19**, 1625-1636.

440 Senneville, E., Caillon, J., Calvet, B. and Jehl, F. (2015) Towards a definition of
441 daptomycin optimal dose: Lessons learned from experimental and clinical data. *Int J*
442 *Antimicrob Agents* **47**, 12-19.

443 Stravopoulos, A. (2008) Deproteinized Bovine Bone Xenograft, in Pietrzak, W.S. (ed.)
444 (2008) *Musculoskeletal Tissue Regeneration.* New Jersey: Humana Press, 119-151.

445 Sweeney, E. (2010) The development and evaluation of novel *in vitro* model of
446 osteomyelitis. PhD, University of the West of England, Bristol.

447 Tobin, C.M., Darville, J.M., Lovering, A.M. and MacGowan, A.P. (2008) An HPLC
448 assay for daptomycin in serum. *J Antimicrob Chemother* **62**, 1462-1476.

449 Traunmüller, F., Schintler, M.V., Metzler, J., Spendel, S., Mauric, O., Popovic, M.,
450 Konz, K.H., Scharnagl, E. and Joukhadar, C. (2010) Soft tissue and bone penetration
451 abilities of daptomycin in diabetic patients with bacterial foot infections. *J Antimicrob*
452 *Chemother* **65**, 1252-1257.

453 Tuchscherr, L., Kreis, C.A., Hoerr, V., Flint, L., Hachmeister, M., Geraci, J., Bremer-
454 Streck, S., Kiehntopf, M. *et al*. (2016) *Staphylococcus aureus* develops increased
455 resistance to antibiotics by forming dynamic small colony variants during chronic
456 osteomyelitis. *J Antimicrob Chemother* **71**, 438-448.

457 Vulin, C., Leimer, N., Huemer, M., Ackermann, M. and Zinkernagel, A.S. (2018)
458 Prolonged bacterial lag time results in small colony variants that represent a sub-
459 population of persisters. *Nat Commun* **9**, 4074.

460 Walter, G., Kemmerer, M., Kappler, C. and Hoffmann, R. (2012) Treatment algorithms
461 for chronic osteomyelitis. *Dtsch Arztebl Int* **109**, 257-264.

462 Winkler H., Janata, O., Berger, C., Wein, W. and Georgopoulos, A. (2000). *In vitro*
463 release of vancomycin and tobramycin from impregnated human and bovine bone
464 grafts. *J Antimicrob Chemother* **46**, 423-428.

465 Woischnig, A.K., Gonçalves, L.M., Ferreira, M., Kuehl, R., Kikhney, J., Moter, A.,
466 Ribeiro, I.A.C., Almeida, A.J. *et al*. (2018) Acrylic microparticles increase daptomycin
467 intracellular and *in vivo* anti-biofilm activity against *Staphylococcus aureus*. *Int J*
468 *Pharm* **550**, 372-379.

469 World Health Organization (2016) *Global Report on Diabetes*. Geneva: World Health
470 Organization.

471 Yang, D., Wijenayaka, A.R., Solomon, L.B., Pederson, S.M., Findlay, D.M., Kidd, S.P.
472 and Atkins, G.J. (2018) Novel insights into *Staphylococcus aureus* deep bone
473 infections: the involvement of osteocytes. *MBio* **9**, e00415-18.

474 Zimmermann, T., Marrison, J., Hogg, K. and O'Toole, P. (2014) Clearing up the signal:
475 spectral imaging and linear unmixing in fluorescence microscopy. *Methods Mol Biol*
476 **1075**, 129-48.

477

478

479 **Figure legends**

480

481 **Figure 1** Bioluminescence imaging of cultures of *S. aureus* 8325p1, growing within 1
482 $cm^3$ sterile bovine bone blocks (indicated by superimposed square), surrounded by 3
483 mL NB in tissue culture plates (indicated by superimposed circle), over 24 h. Arrows

484 at time 24 h indicate dark areas where bioluminescence is no longer detected. A-C
485 represent 3 replicates.

486

487 **Figure 2** Effect of varying concentration on the antimicrobial activity of single dose gentamicin
488 against 72 h biofilms of *S. aureus* 8325p1 in bone, monitored over 24 h. Growth Control (■),
489 gentamicin at 10 mg $l^{-1}$ (□), 30 mg $l^{-1}$ (▼) and 100 mg $l^{-1}$ (●). (n = 3, error bars indicate SD).

490

491 **Figure 3** Effect of varying concentration on the antimicrobial activity of single dose daptomycin
492 + 50 mg $l^{-1}$ $Ca^{2+}$ against 72 h biofilms of *S. aureus* 8325p1 in bone, monitored over 24 h.
493 Growth Control (■), daptomycin at 100 mg $l^{-1}$ (□) and 300 mg $l^{-1}$ (▼). (n = 3, error bars indicate
494 SD).

495

496 **Figure 4** Antimicrobial activity of once daily doses of 100 mg $l^{-1}$ gentamicin and 1000 mg $l^{-1}$
497 daptomycin +50 mg $l^{-1}$ $Ca^{2+}$ against 72 h biofilms of *S. aureus* 8325p1 in bone, monitored over
498 7 days. Untreated control (■, n=1), gentamicin 100 mg $l^{-1}$ (●, n=3), daptomycin 1000 mg $l^{-1}$
499 +50 mg $l^{-1}$ $Ca^{2+}$ (○, n=3). (Error bars indicate SD).

500

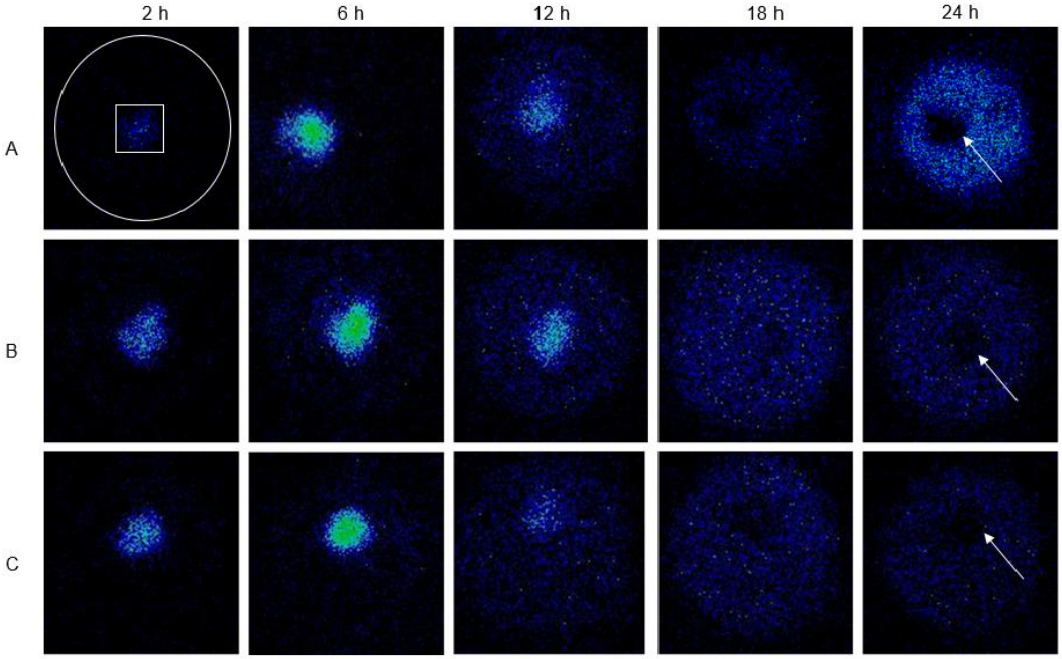
501 **Figure 5** Measurement of antibiotic diffusion into bone over 7 days with once daily dosing of
502 100 mg $l^{-1}$ gentamicin (■) and 1000 mg $l^{-1}$ daptomycin (▼). Gentamicin concentration
503 determined by FPIA and daptomycin concentration by HPLC. (n=3, error bars indicate SD).

504

505 **Supporting information legend**

ESEM images of 100 nm bone slices with biofilm (a) SEM image of Haversian canal in bone, after 72 h biofilm development. (b) Magnification of area indicated by arrow in (a) shows densely packed cocci cells. Scale bar 1 µm.

512 **Figure 1**

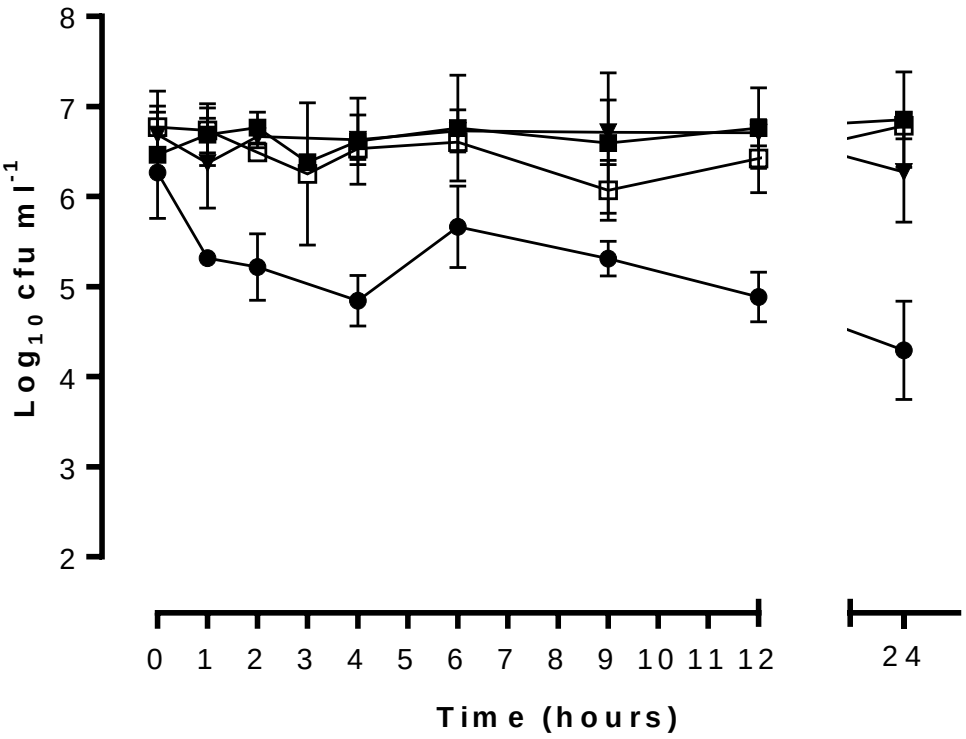



513

514 **Figure 2**

515

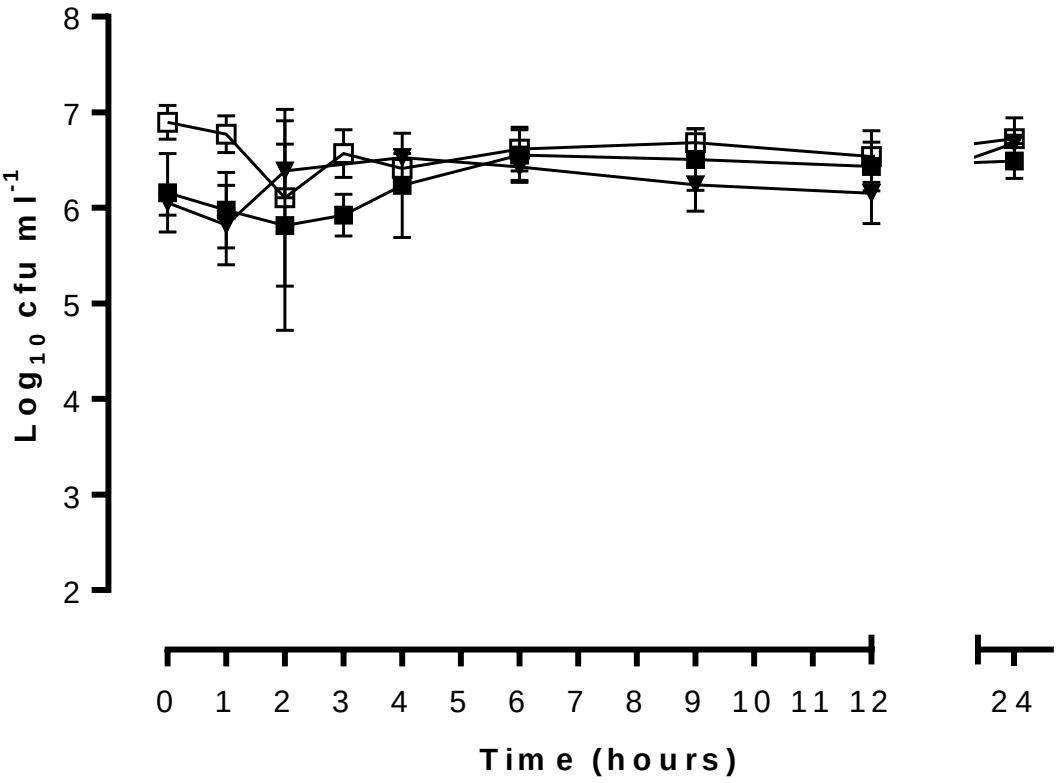
516



517

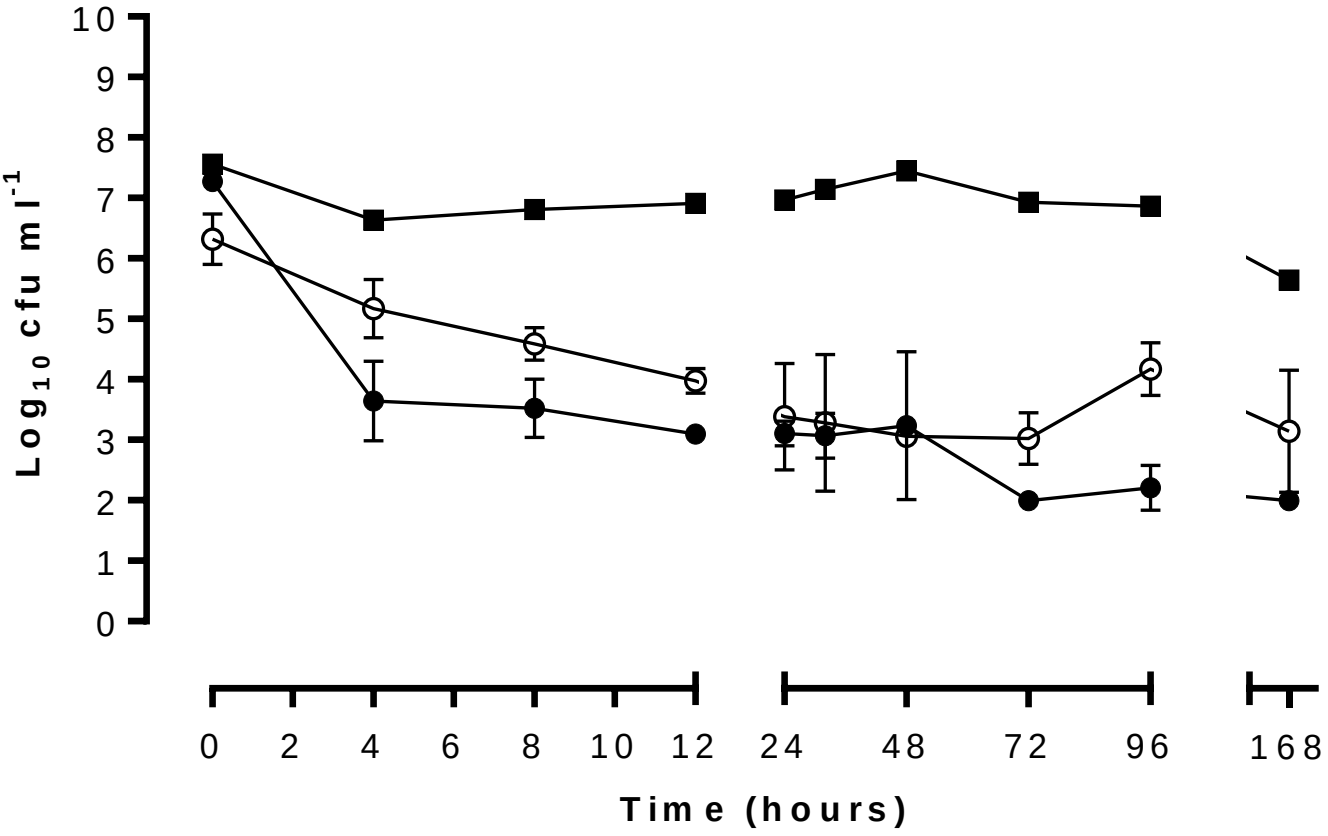
518 **Figure 3**

519



520 **Figure 4**

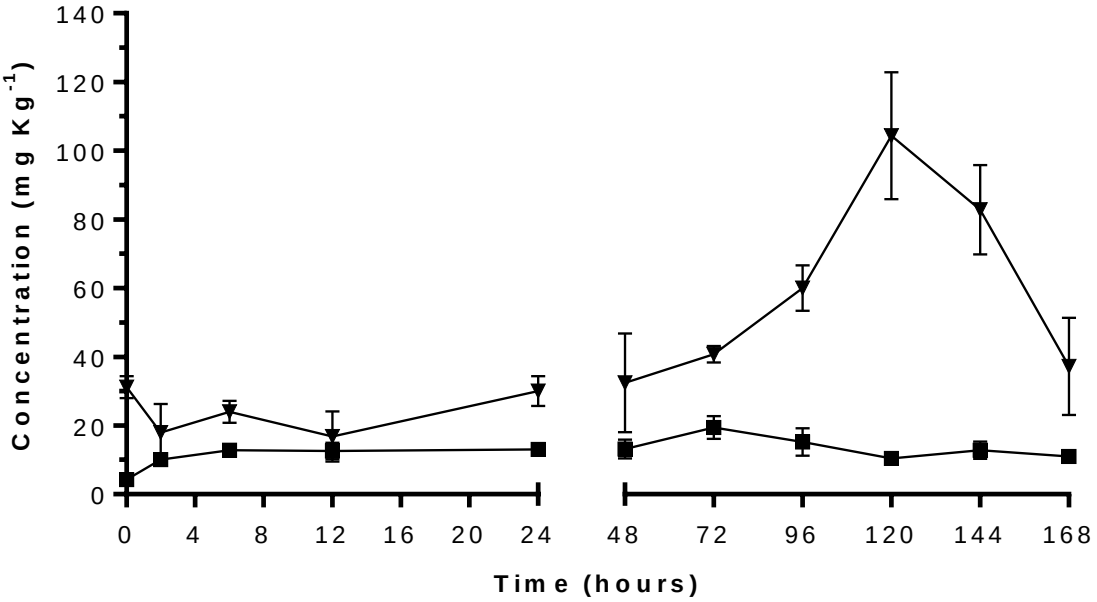
521



522

523 **Figure 5**

524

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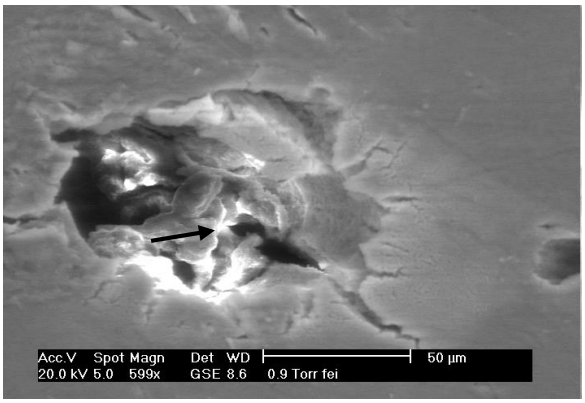
526

**Supporting information:**

(a)




(b)