

## Accepted Manuscript

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PII: S0927-7765(17)30700-2  
DOI: <https://doi.org/10.1016/j.colsurfb.2017.10.050>  
Reference: COLSUB 8931

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received date: 18-7-2017  
Revised date: 7-10-2017  
Accepted date: 17-10-2017

Please cite this article as: Maria Eugenia Butini, Sabrina Cabric, Andrej Trampuz, Mariagrazia Di Luca, *In vitro* anti-biofilm activity of a biphasic gentamicin-loaded calcium sulfate/hydroxyapatite bone graft substitute, *Colloids and Surfaces B: Biointerfaces* <https://doi.org/10.1016/j.colsurfb.2017.10.050>

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***In vitro* anti-biofilm activity of a biphasic gentamicin-loaded calcium sulfate/hydroxyapatite bone graft substitute**

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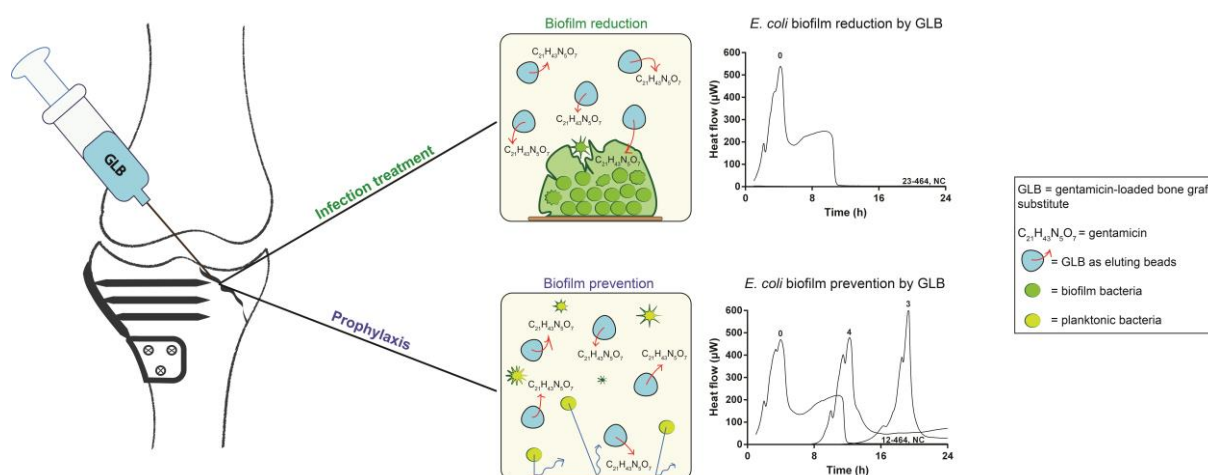
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**Total number of words: 4639**

**Number of figures/tables: 8**

## Graphical Abstract



## HIGHLIGHTS:

- $CaSO_4/HA$  bone graft substitute loaded with gentamicin (GLB) was tested.
- An accurate analysis of GLB antibiofilm activity was performed by microcalorimetry.
- High local gentamicin concentration was released within the first 3 hours.
- GLB reduced viability of sessile bacteria and prevented biofilm formation.
- GLB is promising biomaterial for local anti-biofilm treatment.

## ABSTRACT

Bone and implant-associated infections caused by microorganisms that grow in biofilms are difficult to treat because of persistence and recurrence of infection. Along with surgical debridement, the combination of systemic and controlled local administration of antimicrobials represents the background for an efficient treatment strategy. Gentamicin is one of most used antibiotics for the local treatment of bone-related infections, alone or in combination, due to its bactericidal and broad-range activity. Gentamicin-loaded beads (GLBs), composed of calcium sulfate/hydroxyapatite, were assessed for their *in vitro* antimicrobial activity against planktonic and biofilm *S. agalactiae*, *S. aureus*, *S. epidermidis*, *E. faecalis* and *E. coli*, using standard

methods and ultra-sensitive isothermal microcalorimetry. Gentamicin released from GLBs to clinically relevant concentrations (200 - 2500 µg/mL) within 1 h was able to kill planktonic *S. agalactiae*, *S. epidermidis* and *E. coli* at lower concentrations (MIC: ≤4 µg/mL). Moreover, 12 and 23 µg/mL of released gentamicin were able to prevent bacterial adhesion and suppress a 24 h-old biofilm of *E. coli*, respectively. Conversely, higher amounts of antibiotic, ranging from 171 to 1260 µg/mL, were needed to prevent and eradicate biofilms of gram-positive bacteria. Likewise, the emergence of resistance to GLBs *in vitro* and the bacterial attachment on the bone graft substitute, when the amount of gentamicin in the material is reduced, was also reported. This study provides further information regarding the *in vitro* anti-biofilm activity of the biphasic gentamicin-loaded bone graft substitutes, suggesting the validity of this antibiotic-loaded material for the prophylaxis and treatment of bone and implant-associated infections.

**Keywords:** Antibiotic-loaded biomaterials, local antibiotic release, biofilm, bone infection, implant-associated infection, bacterial resistance, isothermal microcalorimetry.

## INTRODUCTION

Osteomyelitis is an inflammatory process caused by microorganisms involving bone and bone marrow [1] that causes bone disruption and formation of necrotic tissue [2]. The onset of bone infections might be secondary to the spreading of bacteria from either a contiguous infected focus or blood circulation [3]. Microorganisms induce acute inflammation in bone tissue causing necrosis. These avascular areas are unreachable for systemic antibiotics and therefore represent an optimal environment for bacterial adhesion, persistence and biofilm development [4]. Similar to dead bone and soft tissue, fixation devices used to stabilize bones fractures constitute an additional attractive surface for microbial colonization and biofilm formation [4].

Biofilms consist of communities of sessile bacteria embedded in a self-produced matrix, which evade host immunity and antimicrobials [4, 5]. Indeed, biofilm-embedded bacteria exhibit considerably greater resistance to antimicrobial killing in comparison to planktonic counterparts. The most common pathogen isolated in bone and implant-associated infections is *Staphylococcus aureus*, responsible for more than 50% of infections. Other microorganisms include coagulase-negative staphylococci, streptococci, enterococci and gram-negative rods such as *Escherichia coli*, *Pseudomonas* spp. and *Enterobacter* spp. [2].

The treatment consists of surgical debridement and pharmacological therapy [6, 7], which combines long course systemic antibiotics with the local administration thereof released from drug-delivery systems [8-10]. Major advantages of the local release are represented by the high antibiotic concentration *in situ*, typically exceeding the minimum inhibitory concentrations (MICs) for most of bacteria [11], together with a minimal systemic toxicity [7, 12, 13]. Moreover, it is suggested that extremely high concentrations of antimicrobials may eradicate biofilms [14], improving the therapeutic efficacy and contributing to bone healing after debridement.

Poly(methyl methacrylate) (PMMA) [14, 15], a non-resorbable biomaterial, was used since decades as local antibiotic delivery carrier. However, it requires surgical removal after

completion of drug release when employed as temporary spacer or PMMA chains. Hence, extensive research has targeted fully degradable biomaterials as drug releasing vehicles [16-18], which do not require follow-up surgeries. Calcium sulfate bone graft substitutes play a major role in the biomedical field thanks to their biocompatibility, porosity and biodegradability [19]. Many resorbable scaffolds result from the combination of calcium sulfate and hydroxyapatite, in which the former typically ensures a fast resorption, whereas the latter resembles the biological composition of the bone. Several studies have been performed aiming to incorporate antibiotics into hydroxyapatite/calcium sulfate composites [20, 21]. Recently, a gentamicin-loaded bone graft substitute (175 mg gentamicin/10 mL), consisting of 40% hydroxyapatite particles within a 60% calcium sulfate matrix, became commercially available. The biphasic ceramic allows for the achievement of high gentamicin concentrations locally and it safely prevents bone infections in different clinical cases [22]. Other biodegradable materials which are clinically used for the local treatment of bone infections include calcium phosphates [23, 24], allograft bones [25], collagen implants [26] and bioactive glasses [27].

The aim of this study was to investigate the *in vitro* anti-biofilm activity of gentamicin-eluting bone graft substitute against selected biofilm-forming bacteria that are most frequently isolated in bone and implant-associated infections. In particular, we studied the ability of gentamicin-eluting bone graft substitute to prevent the bacterial colonization of an abiotic surface and to eradicate a 24 h-old biofilm of *Streptococcus agalactiae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Escherichia coli*.

## MATERIALS AND METHODS

### Tested strains.

*S. agalactiae* ATCC 13813, methicillin-resistant *S. aureus* (MRSA) ATCC 43300, methicillin-susceptible *S. aureus* (MSSA) ATCC 29213, *S. epidermidis* ATCC 12228, *E. faecalis* ATCC 19433 and *E. coli* Bj HDE-1 were used in this study. Bacteria were stored in cryovial bead preservation system (Microbank; PRO-LAB DIAGNOSTICS, Richmond Hill, ON, Canada) at -80 °C. All bacterial strains were cultured on Columbia Blood Agar (VWR Chemicals, Leuven, Belgium) for 24 h at 37 °C in an ambient air incubator, except for *S. agalactiae*, which was cultivated under 5% CO<sub>2</sub> atmosphere. Inocula were prepared according to a McFarland standard turbidity of 0.5 and determined by Colony Forming Units (CFUs) counting.

#### **Antimicrobial agent.**

Gentamicin sulfate (CERAMENT™|GENTAMICIN A0421) was supplied as a powder by BONESUPPORT AB (Ideon Science Park, Scheelevägen 19, SE-223 70 Lund, Sweden) for the determination of the *in vitro* susceptibility of the strains used in the study.

#### **Gentamicin-eluting bone graft substitute.**

Sterile CERAMENT™|G beads containing 1.75% w/v gentamicin were supplied by BONESUPPORT AB. Four different types of beads labeled as FB1, SB1, SB2 and SB3, containing 101, 258, 947 and 1625 µg of gentamicin, respectively, were prepared using bead molds (EQ299 and EQ300) in clean room facilities (Class A) at Mikrolab Stockholm AB (Kung Hans Väg 3, 192 68, Sollentuna, Sweden). Briefly, a kit of CERAMENT™|G (CERAMENT™|GENTAMICIN gentamicin paste + CERAMENT™|MIXING LIQUID 9 mg/mL saline) was mixed according to the Instruction for Use (IFU).

#### **Bone void filler.**

CERAMENT™|BONE VOID FILLER beads were supplied by BONESUPPORT AB. Four different sizes of beads were prepared using bead molds (EQ299 and EQ300) in clean room facilities (Class A) at Mikrolab Stockholm AB. A kit of CERAMENT™|BVF (40% hydroxyl

apatite + 60% calcium sulfate) was mixed according to the IFU and beads were prepared following the previously reported procedure.

### **Drug release from gentamicin-loaded beads (GLBs), agar well diffusion assay and calculation of active gentamicin concentration.**

Different combinations of GLBs, corresponding to distinct theoretical gentamicin concentrations (Table 1), were incubated under static conditions in Cation Adjusted Müller Hinton broth (CAMHB) for 0, 1, 3, 6, 12 and 24 h at 37 °C. After incubation, the solutions containing the released drug were used for agar well-diffusion assay against MSSA, slightly modifying the procedure reported by Bonev et al. [28]. Briefly, a bacterial inoculum was prepared according to a McFarland standard turbidity of 0.5 ( $\approx 10^8$  CFUs/mL) and a sterile cotton swab was dipped into the inoculum suspension. The entire surface of a Cation Adjusted Müller Hinton agar plate (CAMHA) was carefully streaked evenly in three directions and the excess of moisture was allowed to be fully absorbed. A 6 mm hole was punched on inoculated agar plates and filled with 60  $\mu$ l of the solution containing the released drug. Upon each sampling, the same amount of medium was replenished to accurately simulate *in vivo* conditions of continuous liquid flow and gentamicin diffusion, as previously reported [22]. After 24 h-incubation at 37 °C, the inhibition halos were measured and the concentration of active gentamicin was obtained according to the logarithmic equation reported in Figure S 1. Experiments were performed in duplicates.

For the calibration curve, 60  $\mu$ l of two folds serial dilutions of gentamicin ranging from 5000 to 2.4  $\mu$ g/mL were dropped on CAMHA inoculated with MSSA. After 24 h-incubation at 37 °C, the inhibition halos were measured and diffusion distances were calculated as the half of the inhibition halo diameter, after subtracting the diameter of the well. Diffusion distance (mm) was plotted versus gentamicin concentration ( $\mu$ g/mL) on logarithmic scale.



**Table 1 Combinations of GLBs (FB1, SB1-3) and related CAMHB volumes (mL) used for gentamicin release experiments.**

GLBs combination	FB1 <sup>a</sup>	SB1 <sup>a</sup>	SB2 <sup>a</sup>	SB3 <sup>a</sup>	CAMHB (mL)
<b>a</b>	1				42
<b>b</b>	1				21
<b>c</b>	1				10,5
<b>d</b>	1				5,25
<b>e</b>	1				2,6
<b>f</b>		1			3
<b>g</b>	2	1			3
<b>h</b>			1		3
<b>i</b>			2		3
<b>j</b>	2	1		2	3
<b>k</b>	1		1	4	3

<sup>a</sup> Number of GLBs used for each combination.

GLBs: gentamicin-loaded beads. CAMHB: Cation Adjusted Müller Hinton Broth.

### **Antimicrobial activity of GLBs against planktonic bacteria.**

The antimicrobial activity versus planktonic bacteria was determined by broth macrodilution method, slightly modifying the CLSI guidelines [29]. Briefly, a standard inoculum of  $1-5 \times 10^5$  CFUs/mL was prepared in CAMHB and different combinations of GLBs (Table 1) were added. Subsequently, the macrodilution tubes were incubated at 37 °C for 20 h. For the antimicrobial susceptibility of *S. agalactiae*, CAMHB supplemented with 2.5% lysate horse blood (CAMHB-2.5% LHB) was used and tubes were incubated under 5% CO<sub>2</sub> atmosphere. The MIC was defined as the lowest gentamicin concentration that completely inhibited the growth of the organism in the tubes, as detected by unaided eye. Experiments were performed in triplicates.

### **Evaluation of the *in vitro* bacterial resistance to GLBs releasing sub-MIC concentrations of gentamicin.**

The development of resistance to gentamicin was determined by exposing bacteria to sub-MIC concentrations of antibiotic released from GLBs. Briefly, GLBs combinations corresponding to 1x MIC were incubated in medium for 24 h at 37 °C to induce the gentamicin release. Then,  $1 \times 10^6$  CFUs/mL bacterial cells were incubated in CAMHB in the presence of the partially unloaded

beads (still releasing a sub-MIC concentration of gentamicin) at 37 °C. After 24 h, the optical density of the bacterial culture was measured and, upon dilution in fresh medium to a  $1 \times 10^6$  CFUs/mL final inoculum, bacteria were incubated with a second combination of unloaded beads (treated as previously described) for 24 h at 37 °C. This cycle was repeated every 24 h for 5 days. After 120 h, bacteria were finally diluted in fresh medium to a  $1 \times 10^6$  CFUs/mL inoculum. Different combinations of GLBs corresponding to 1x, 2x, 4x and 8x MIC for *S. agalactiae*, MSSA, *S. epidermidis*, *E. faecalis* and *E. coli* were then incubated with a  $1 \times 10^5$  CFUs/mL final inoculum in CAMHB. The CLSI broth macrodilution procedure [29] was slightly modified to evaluate the emergence of resistance of all bacterial strains. The MIC<sub>II</sub> was defined as the lowest gentamicin concentration that completely inhibited the bacterial growth, as detected by unaided eye, after the exposure to sub-MIC concentrations. Each experiment was performed in triplicate.

#### **Anti-biofilm activity of GLBs.**

The analysis was performed using a 48-channel isothermal microcalorimeter (TAM III; TA Instruments, New Castle, DE, USA), as previously described [30-32]. To investigate the anti-biofilm activity of GLBs,  $1-5 \times 10^6$  CFUs/mL of bacterial cells were inoculated in Tryptic Soy Broth and incubated in the presence of porous glass beads (VitraPor; ROBU, Germany) (diameter 2-3 mm, pore size 60 µm) for 24 h at 37 °C. For *S. agalactiae*, 1.0 McFarland bacterial suspension was diluted 1:10 in CAMHB-2.5% LHB and incubated with porous glass beads for 24 h at 37 °C under 5% CO<sub>2</sub> atmosphere. The ratio between glass beads and bacterial suspension was 1 bead : 1 mL. Then, glass beads were gently washed three times with 0.9% sterile saline and co-incubated with different combinations of GLBs in CAMHB. A positive control, in which the biofilm was incubated in the absence of GLBs, and a sterile bead as negative control were also included. After 24 h co-incubation, glass beads were rinsed and placed in air-tight sealed microcalorimetric ampoules containing 3 mL of fresh medium, to monitor the heat produced by the biofilm cells still viable after the treatment. The heat production was continuously measured at 37 °C for 24 h

and the results were plotted as heat flow (microwatts) versus time (hours). The minimum biofilm bactericidal concentration (MBBC) measured by microcalorimetry was defined as the lowest gentamicin concentration that strongly reduced the number of viable bacterial cells within the biofilm, therefore leading to the lack of heat production related to bacterial regrowth during 24 h-incubation in fresh medium in the microcalorimeter. All experiments were performed in triplicate.

### **Biofilm prevention activity of GLBs.**

For biofilm prevention studies,  $1-5 \times 10^6$  CFUs/mL bacterial cells were co-incubated with porous glass beads and different combinations of GLBs for 24 h at 37 °C in CAMHB. After incubation, glass beads were gently washed three times with 0.9% sterile saline and placed in air-tight sealed ampoules containing 3 mL of fresh medium. The heat production was continuously measured at 37 °C for 24 h and the results were plotted as heat flow (microwatts) versus time (hours). The minimum biofilm preventing concentration (MBPC) was defined as the lowest gentamicin concentration that prevented the formation of biofilm on the glass beads, thus leading to the lack of heat production related to bacterial metabolism during 24 h-incubation in fresh medium in the microcalorimeter. All experiments were performed in triplicate.

### **Biofilm formation on gentamicin-unloaded GLBs (GUB).**

A combination of GLBs corresponding to the MBPC for each bacterial species was incubated in CAMHB for 7 days. Medium was refreshed every 24 h. After 7 days, GUB were incubated for 24 h at 37 °C under static conditions in CAMHB inoculated with  $\sim 10^6$  CFUs/mL bacterial cells. Then, GUB were rinsed three times and placed in air-tight ampoules containing 3 mL of CAMHB. The heat production was continuously measured at 37 °C for 48 h and the results were plotted as heat flow (microwatts) versus time (hours). Combinations of bone void filler beads (BVF) corresponding to each MBPC were also included as controls. Each experiment was

performed in triplicate.

### **Data analysis.**

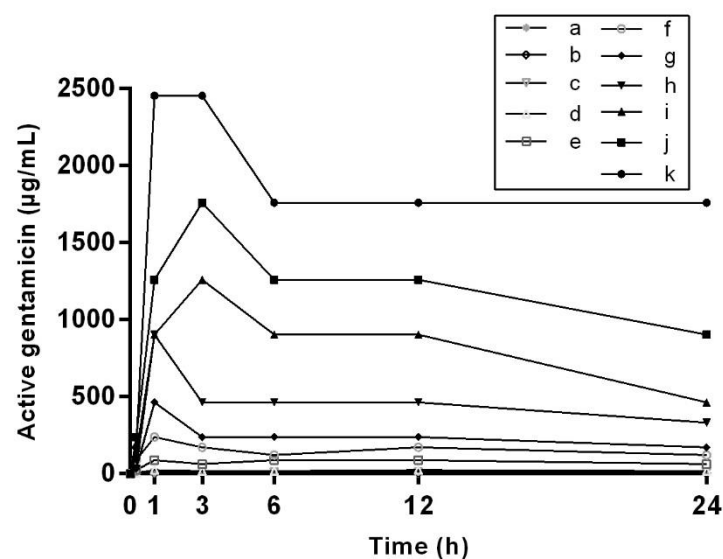
Microcalorimetric data analysis was accomplished using the manufacturer's software (TAM Assistant; TA Instruments, New Castle, DE, USA). Figures were plotted using GraphPad Prism 7.03 (GraphPad Software, La Jolla, CA, USA).

## **RESULTS AND DISCUSSION**

### **Release kinetic of active gentamicin from GLBs evaluated by agar well diffusion assay.**

Elution of the active antibiotic from different combinations of GLBs containing 1.75% w/v gentamicin was monitored over 24 h by agar diffusion assays. This assay estimates the antibiotic efficiency in inhibiting the bacterial growth, based on the assumption that drugs can diffuse freely in the solid medium [28]. The inhibition halo obtained from the solution containing active gentamicin released at each time point was compared to a standard curve of known gentamicin concentrations.

As shown in Figure 1, an initial burst release of gentamicin was detected within the first 3 hours of incubation, whereas the concentration of active drug slightly decreased during the following 3 hours, remaining then approximately constant up to 24 hours. These data are in agreement with those previously reported in an *in vitro* elution study [22], in which an initial high amount of gentamicin ( $2420 \pm 45$  µg/mL) was released in a Ringer's solution from the biphasic material.



**Figure 1 Release kinetics of active gentamicin from different combinations of GLBs (a-k) during 24 h-incubation at 37 °C under static conditions.** Aliquots of release medium were sampled after 0, 1, 3, 6, 12 and 24 h-incubation. The concentration of active eluted gentamicin was obtained by agar diffusion assay. Active gentamicin concentration ( $\mu\text{g/mL}$ ) is plotted versus time (h).

The final active gentamicin concentrations, obtained for each GLBs combination after 24 h drug release in broth, are reported in Table 2. These GLBs combinations were used to evaluate the antimicrobial and anti-biofilm activity of GLBs in the following experiments, as they ensure a sufficient released gentamicin amount to treat most bacteria causing bone infections.

**Table 2 Concentrations of active gentamicin ( $\mu\text{g/mL}$ ) released from GLBs (FB1, SB1-3) after 24-h incubation in CAMHB (mL).**

GLBs combination	FB1 <sup>a</sup>	SB1 <sup>a</sup>	SB2 <sup>a</sup>	SB3 <sup>a</sup>	CAMHB (mL)	Active gentamicin ( $\mu\text{g/mL}$ )
<b>a</b>	1				42	3
<b>b</b>	1				21	4
<b>c</b>	1				10,5	12
<b>d</b>	1				5,25	23
<b>e</b>	1				2,6	75
<b>f</b>		1			3	171
<b>g</b>	2	1			3	204
<b>h</b>			1		3	464
<b>i</b>			2		3	903
<b>j</b>	2	1		2	3	1260
<b>k</b>	1		1	4	3	2106

<sup>a</sup> Number of GLBs used for each combination.

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GLBs: gentamicin-loaded beads. CAMHB: Cation-Adjusted Müller Hinton Broth

### Antimicrobial activity of GLBs against planktonic bacteria.

The activity of GLBs against planktonic bacteria belonging to five of the most representative species isolated in bone and implant-associated infections was assessed using the conventional broth macrodilution assay [29]. The GLBs MICs for planktonic bacteria are shown in Table 3. Most of the strains tested in their planktonic form were susceptible to GLBs with MIC values similar to those observed when gentamicin was tested as free drug in the same conditions (Table S 1). The MIC value for MRSA was >2106 µg/mL, confirming the resistance of this strain to gentamicin observed when a routine antibiogram was performed. In addition, MSSA and *E. faecalis* exhibited about 37 and 10 times higher MIC values, respectively, when treated with GLBs.

**Table 3 Antimicrobial susceptibility test of gentamicin released from GLBs against planktonic bacteria by broth macrodilution assay.**

Strain	MIC (µg/mL)
<i>S. agalactiae</i>	≤4
MSSA	75
<i>S. epidermidis</i>	≤3
<i>E. faecalis</i>	171
<i>E. coli</i>	≤3
MRSA	>2106

The Food and Drug Administration (FDA) defines the bioavailability of a drug as “the rate and extent to which the active moiety becomes available at the site of action” (21 CFR 320.1(a)). The pharmaceutical formulation can affect the bioavailability of a given drug [33]. The results

obtained suggest that the drug formulation has an influence on the pharmacokinetic profile of gentamicin when tested against MSSA and *E. faecalis* bacterial cells in a specie-specific manner. Although enterococci are intrinsically resistant to aminoglycosides and, therefore, aminoglycoside monotherapy is ineffective, we included *E. faecalis* strain in this study because gentamicin shows a synergistic activity against enterococci when tested in combination with penicillin derivatives or glycopeptides [34] and it is generally suggested for the therapy of implant-associated osteomyelitis of long bones [4].

### **Evaluation of *in vitro* microbial resistance to gentamicin released from GLBs.**

As was shown by the kinetics, the initial burst (within 24 h) of gentamicin release from GLBs is followed by a plateau in which lower concentrations of the antibiotic are released over a longer period. For this reason, we evaluated whether the exposure of different bacterial species to sub-inhibitory gentamicin concentrations could select resistant cells. To this aim, microorganisms were repeatedly exposed to GLBs that were previously partially unloaded by a 24-h gentamicin elution. After the treatment, the MIC of gentamicin released by GLBs was calculated again and referred to as MIC<sub>II</sub>. Whenever resistance emerged, the resulting MIC<sub>II</sub> was higher than the MIC previously obtained for the susceptible strains.

Table 4 reports the MIC<sub>II</sub>, which ranged from  $\leq 3$  to  $>903$   $\mu\text{g/mL}$ . When *S. epidermidis* was exposed to sub-MIC gentamicin concentration, the MIC<sub>II</sub> remained  $\leq 3$   $\mu\text{g/mL}$ , indicating that no selection of resistant cells occurred. On the contrary, the MIC<sub>II</sub> for *S. agalatiæ* was at least 6-fold higher than the MIC determined before the resistance-inducing treatment. Similar results were observed for *E. coli*, in which prolonged exposure to sub-inhibitory concentrations of gentamicin triggered the ability to grow at concentrations about 8-times higher than the initial MIC. In addition, the MIC<sub>II</sub> obtained after MSSA and *E. faecalis* exposure to low gentamicin concentrations also showed at least a 5-fold increase as compared to the MIC (from 75 to  $>464$   $\mu\text{g/mL}$  and from 171 to  $>903$   $\mu\text{g/mL}$ , respectively). Indeed, both strains developed resistance, to

a different extent, to residual concentrations of active gentamicin.

**Table 4 MIC<sub>II</sub> values of gentamicin released from GLBs obtained after exposure of bacteria to GLBs partially unloaded after 24 h-gentamicin elution.**

Strain	MIC <sub>II</sub> (µg/mL)
<i>S. agalactiae</i>	23
MSSA	>464
<i>S. epidermidis</i>	≤3
<i>E. faecalis</i>	>903
<i>E. coli</i>	>23

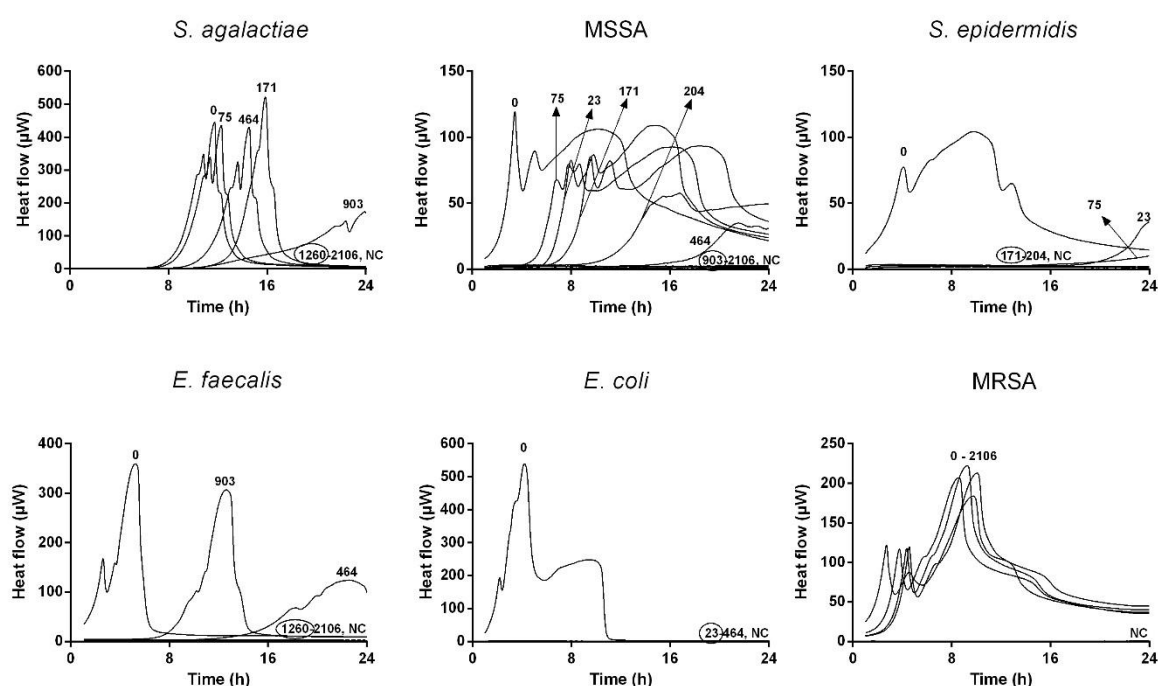
Our *in vitro* results suggest that prolonged exposure of bacteria to sub-MIC concentrations of the antibiotic, rather than the presence of the carrier, facilitated the emergence of resistance. These data might be in agreement with the observations reported by other authors on gentamicin-loaded PMMA beads [35, 36]. In fact, we observed the development of reduced susceptibility for most of the treated strains, as compared to the susceptible untreated controls. However, it is unclear whether the GLBs affect the *in vivo* bacterial resistance or tolerance to the locally released gentamicin, delivered to the interface between resorbable carrier and bone. Due to the extremely high local gentamicin concentrations and the dose-dependent antimicrobial mode of action of gentamicin, it is very unlikely that resistant bacterial strains will emerge during the treatment of bone and implant-associated infections.

#### **Anti-biofilm activity of GLBs.**

The bactericidal activity of GLBs against bacterial biofilms formed on porous glass beads, used as abiotic scaffolds, was investigated by isothermal microcalorimetry. The development of a metabolism-related heat during the microbial growth process is the key feature that supported the



application of this technique in the microbiology research field for the quali-quantitative analysis of microbial growth and as a non-conventional tool for antimicrobial susceptibility tests [37-40]. When investigating the biofilm bactericidal activity of GLBs, the heat production detected is related to the regrowth of viable bacteria survived the anti-biofilm treatment. Figure 2 shows the heat flow generated by the tested bacterial strains after a 24 h-treatment with GLBs. As shown by the thermogenic curves, the MBBC values of GLBs obtained for all of the tested bacterial biofilms were higher than the MICs observed when GLBs were tested against their planktonic counterparts.



**Figure 2 Microcalorimetric evaluation of the anti-biofilm activity of GLBs.** Heat flow (μW), related to the metabolic activity of viable sessile cells of *S. agalactiae*, MSSA, *S. epidermidis*, *E. faecalis*, *E. coli* and MRSA after 24 h-treatment with GLBs, is plotted versus time (h). Numbers indicate gentamicin concentration (μg/mL). NC: negative control. Circled value represents the MBBC: minimum biofilm bactericidal concentration.

*E. coli* biofilm-embedded cells showed a more pronounced susceptibility to the treatment with GLBs, since a concentration around 20 μg/mL of released gentamicin was sufficient to inhibit any heat production. Conversely, the MBBC value of released gentamicin against *S. agalactiae*

biofilm was 1260 µg/mL, suggesting that a bactericidal effect could be achieved only in the presence of high local concentrations of active drug.

Nevertheless, a delay in detection time of the streptococcal metabolic activity, in comparison to the heat produced by untreated sample, was observed after the incubation with 464 µg/mL of gentamicin, thus indicating that the treatment could give a reduction in the number of viable biofilm cells, although a complete eradication is not achievable *in vitro*. Similar to that observed for *S. agalactiae*, the MBBC of released gentamicin against enterococcal biofilm was exceeding 1000 µg/mL (1260 µg/mL). The initial classification of *E. faecalis* as part of *Streptococcus* group D might explain the comparable resistant response of the two strains to the anti-biofilm treatment based on GLBs.

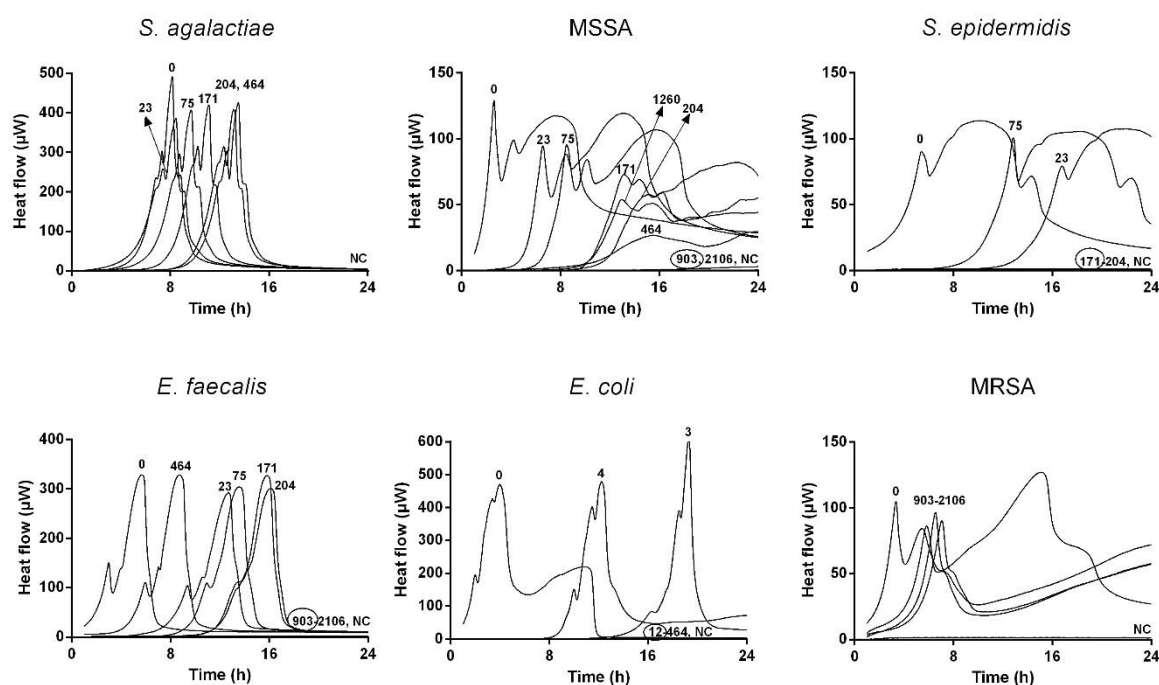
The metabolic activity of MSSA and *S. epidermidis* biofilm-embedded cells have been inhibited by 903 and 171 µg/mL of gentamicin released by GLBs, respectively. When GLBs were tested against MSSA, a concentration-dependent activity inhibiting bacterial heat production was observed (Figure 2), in which a decrease in the heat flow peak and a change in the curve shape were associated to a delay in the heat development in the presence of increasing concentrations of gentamicin in the GLBs. As we expected, the heat production related to the regrowth of MRSA biofilm cells was still detectable after the treatment with 2106 µg/mL of gentamicin released from GLBs.

In addition, an increase in MBBC values of GLBs, with respect to those reported for the anti-biofilm treatment with free gentamicin (Table S 1), was also observed. Similarly to what obtained in the antimicrobial assays against planktonic MSSA and *E. faecalis*, the presence of the material seems to reduce the anti-biofilm activity of the eluted gentamicin against all of the tested strains, except for *E. coli* biofilm. Interestingly, MBBCs of GLBs against *S. epidermidis* and *S. agalactiae* biofilms were more than 10 and 100-folds higher, respectively, in comparison to those obtained in the presence of free gentamicin tested under the same conditions. However, this difference was not visible when GLBs and free gentamicin were tested against planktonic strains.

This might suggest that the presence of the material interferes with the diffusion or penetration of gentamicin in the biofilms of these two bacterial strains.

### Biofilm prevention activity of GLBs.

Isothermal microcalorimetry was also used to investigate the activity of GLBs in preventing biofilm formation on glass beads. Figure 3 shows the microcalorimetric results obtained monitoring for 24 h the heat flow generated by viable cells surviving the treatment. Indeed, the heat production detected is related to the metabolic activity of viable sessile cells within the newly-formed biofilm. On the contrary, the lack of heat production is correlated to the inhibition of biofilm formation. GLBs MBPCs obtained were >464, 903, 171, 903 and 12  $\mu\text{g/mL}$  for *S. agalactiae*, MSSA, *S. epidermidis*, *E. faecalis* and *E. coli*, respectively. The GLBs combination releasing the highest tested concentration of active drug (>2106  $\mu\text{g/mL}$  gentamicin) was not able to prevent MRSA biofilm formation.



**Figure 3 Microcalorimetric evaluation of the biofilm prevention activity of GLBs.** Heat flow ( $\mu\text{W}$ ), generated by *S. agalactiae*, MSSA, *S. epidermidis*, *E. faecalis*, *E. coli* and MRSA biofilms grew on glass beads during the 24 h co-incubation with GLBs in inoculated medium, is plotted

versus time (h). Numbers indicate gentamicin concentration ( $\mu\text{g/mL}$ ). NC: negative control. Circled value represents the MBPC: minimum biofilm preventing concentration.

Gentamicin concentrations lower than  $464 \mu\text{g/mL}$  did not prevent the development of the streptococcal biofilm, whereas the formation of both staphylococcal biofilms was prevented by the same concentrations that also exerted a bactericidal activity against them ( $171$  and  $903 \mu\text{g/mL}$  against *S. epidermidis* and against MSSA, respectively). Similar response was observed for *E. faecalis* biofilm, which demonstrated to be prevented during the incubation with  $903 \mu\text{g/mL}$  of gentamicin. The colonization and attachment of *E. coli* to the glass beads was prevented by lower concentrations (MBPC =  $12 \mu\text{g/mL}$ ), demonstrating to be the most susceptible strain among the tested ones. These gentamicin concentrations are achievable in bone and soft tissues in the clinical setting for local treatment.

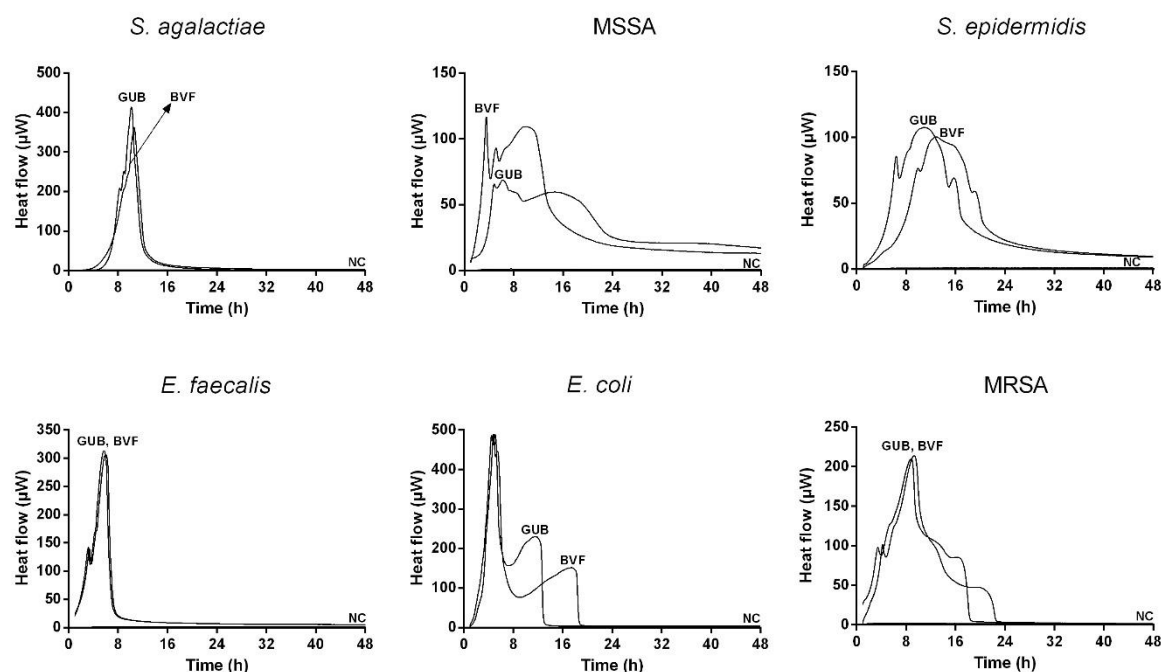
Attention should be drawn to the fact that all MBPC values resulted higher than the MIC values obtained for planktonic bacteria. These findings might suggest that, in the presence of a substrate, gentamicin MIC is able to inhibit bacterial replication but not the bacterial adhesion on an abiotic surface.

However, the impossibility of *in vitro* tests to reproduce accurately the physiological conditions of an organism or a real clinical situation represents one of the shortcomings of this kind of experiments. In our case, the relatively high concentrations needed to prevent the glass beads colonization might also reflect the stringent experimental conditions applied for these *in vitro* experiments. Indeed, the high bacterial inoculum ( $\sim 10^6$ ), the absence of supportive surgical and pharmacological systemic treatment and the presence of an abiotic surface (foreign body) mimic the worst scenario that could be experienced in the management of bone and implant-associated infections. Moreover, the lack of vascularization strongly facilitates the bacterial colonization, as the host immune activity is completely missing. It is then unsurprising that local high

concentrations are needed *in vitro* to observe the prevention of a biofilm development and the strong reduction of biofilm-embedded sessile cells.

### Biofilm formation on GUB.

As the unloaded biomaterial is not resorbed immediately after the total release of gentamicin, we investigated the possibility that bacteria can colonize the remaining unloaded scaffold, simulating a preventive use of the material in an arthroplasty surgery. Combinations of GLBs that determined the MBPC were co-incubated, after unloading cycles for 7 days, with bacteria for 24 h and the presence of biofilm was then evaluated by microcalorimetry. As shown in Figure 4, high values of heat flow were detected at early times for all the strains tested in presence of GUB, similarly to that observed when BVF (without any gentamicin) was used as control substrate. The detection of heat production indicates that all of the tested bacterial strains were able to form biofilm on both GUB and BVF.



**Figure 4 Microcalorimetric evaluation of biofilm formation on gentamicin-unloaded beads (GUB).** Heat flow (μW), generated by *S. agalactiae*, MSSA, *S. epidermidis*, *E. faecalis*, *E. coli* and MRSA biofilms on gentamicin-unloaded beads (GUB) and bone void filler beads (BVF) after 24 h-incubation in inoculated medium, is plotted versus time (h). NC, negative control.

In contrast to data reported by Lindberg [41] and Stravinskas et al. [22], who observed sustained levels of gentamicin above the MIC breakpoint up to 28 days for most of the gentamicin-susceptible strains, our observations could suggest the possibility of an initial gentamicin burst release which does not support the retention of sufficiently high concentrations after 7 days of incubation in release medium. Nevertheless, it is noteworthy the evidence of a slight temporal shift and a decreased amount of heat produced by MSSA biofilm when grown on GUB. This behavior could suggest either a retained susceptibility to some residual amounts of gentamicin or a minor capability of the strain to adhere to GUB within 24 h-incubation. Although also *E. coli* formed biofilm on the unloaded biomaterial, the analysis of the heat flow suggests that the metabolic activity of bacteria stops several hours earlier than that observed for the biofilm grown on BVF. Differently, *E. faecalis* and *S. agalactiae* biofilms reported no marked differences in growth profile when formed either on GUB or on control material. Interestingly, *S. epidermidis* biofilm revealed an unpredictable growth behavior: unlike for all of the other tested strains, the adherence and development of a microbial aggregate appears to be enhanced by the presence of GUB after 24 h-incubation. As compared to the control, the heat production started to be detected earlier in time and it reached higher values (maximal peak  $>100\mu\text{W}$ ) approximately 10 hours after the experiment started.

The different conditions lying between *in vitro* and *in vivo* experimental settings could understandably validate our data. As an example, the static incubation of the beads and the low-frequency of medium exchange are two experimental conditions that may significantly vary the resorption rate of the biomaterial, as compared to *in vivo* conditions. Indeed, besides the continuous exchange of body fluids, also cellular resorption and macrophage activity in the surroundings of the bone defect or implant are factors that play a fundamental role in shortening the residence time of the biomaterial *in vivo* and in reducing the number of bacteria on the site of a potential infection.

## CONCLUSIONS

Microorganisms growing as sessile communities acquire high resistance to host defenses, as well as to the antimicrobial treatment, and therefore biofilm-related infections are difficult to treat. Invasive surgical procedures and long-lasting pharmacological treatments represent a clinical challenge; hence non-invasive and highly effective therapeutic strategies are needed. The use of resorbable biomaterials as reservoir for the local release of antimicrobials is considered a valid option as supportive treatment to achieve high local concentrations of drug, therefore avoiding infections relapses and microbial resistance. We found that the biphasic gentamicin-loaded calcium sulfate/hydroxyapatite bone graft substitute, which protects bone healing from microbial colonization and promotes the regenerative process [42, 43], also possess a preventive and bactericidal anti-biofilm activity *in vitro* against some selected bacterial strains that are responsible for bone infections. Additionally, we believe that the high concentrations of active drug achieved through the initial burst release within 3 hours, together with the retention of a sustained level of antibiotic for at least 24 hours, could effectively prevent an early infection in the first stages of bacterial replication. Our results also suggest that prolonged exposure of most of bacteria to sub-MIC gentamicin concentrations facilitates the emergence of resistance. However, the clinical relevance of this *in vitro* observation is unknown since GLBs induce new bone formation, which enhances the local immunity and may clear bacteria, including resistant ones.

In conclusion, this *in vitro* study demonstrates the potential of the gentamicin-loaded bone graft substitute to prevent and treat biofilm-related bone and implant infections. Further *in vivo* studies are required to deeply investigate the anti-biofilm activity using a foreign-body infection model.

## AUTHORS CONTRIBUTION

MDL and AT conceived and designed the experiments; MEB and MDL performed the experiments, with the contribution of SC; MEB and MDL analysed the data; MEB and MDL drafted the manuscript, with the contribution of AT. All authors revised and approved the final manuscript draft.

## **ACKNOWLEDGMENTS**

An educational grant was provided by the PRO-IMPLANT Foundation ([www.pro-implant-foundation.org](http://www.pro-implant-foundation.org)) and BONESUPPORT AB (Lund, Sweden). The gentamicin sulfate and gentamicin-loaded beads were kindly provided by BONESUPPORT AB.

## **APPENDIX A**

Supporting information.



## REFERENCES

- [1] M.C. Birt, D.W. Anderson, E. Bruce Toby, J. Wang, Osteomyelitis: Recent advances in pathophysiology and therapeutic strategies, *J Orthop*, 14 (2017) 45-52. DOI: 10.1016/j.jor.2016.10.004
- [2] S. Chihara, J. Segreti, Osteomyelitis, *Dis. Mon.*, 56 (2010) 5-31. DOI: 10.1016/j.disamonth.2009.07.001
- [3] D.P. Lew, F.A. Waldvogel, Osteomyelitis, *The Lancet*, 364 (2004) 369-379. DOI: 10.1016/s0140-6736(04)16727-5
- [4] W. Zimmerli, P. Sendi, Orthopaedic biofilm infections, *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica*, 125 (2017) 353-364. DOI: 10.1111/apm.12687
- [5] T. Bjarnsholt, O. Ciofu, S. Molin, M. Givskov, N. Høiby, Applying insights from biofilm biology to drug development [mdash] can a new approach be developed?, *Nature Reviews Drug Discovery*, 12 (2013) 791-808. DOI: 10.1038/nrd4000
- [6] A. Trampuz, W. Zimmerli, Diagnosis and treatment of implant-associated septic arthritis and osteomyelitis, *Curr. Infect. Dis. Rep.*, 10 (2008) 394-403. DOI: 10.1007/s11908-008-0064-1
- [7] H. Winkler, P. Haiden, Treatment of Chronic Bone Infection, *Oper. Tech. Orthop.*, 26 (2016) 2-11. DOI: <http://dx.doi.org/10.1053/j.oto.2016.01.002>
- [8] J.C. Wenke, S.A. Guelcher, Dual delivery of an antibiotic and a growth factor addresses both the microbiological and biological challenges of contaminated bone fractures, *Expert Opin Drug Deliv*, 8 (2011) 1555-1569. DOI: 10.1517/17425247.2011.628655
- [9] A.W. Smith, Biofilms and antibiotic therapy: is there a role for combating bacterial resistance by the use of novel drug delivery systems?, *Adv Drug Deliv Rev*, 57 (2005) 1539-1550. DOI: 10.1016/j.addr.2005.04.007
- [10] M.P. Ginebra, C. Canal, M. Espanol, D. Pastorino, E.B. Montufar, Calcium phosphate cements as drug delivery materials, *Adv Drug Deliv Rev*, 64 (2012) 1090-1110. DOI: 10.1016/j.addr.2012.01.008
- [11] K. Kanellakopoulou, I. Galanopoulos, V. Soranoglou, T. Tsaganos, V. Tziortzioti, I. Maris, A. Papalois, H. Giamarellou, E.J. Giamarellos-Bourboulis, Treatment of experimental osteomyelitis caused by methicillin-resistant *Staphylococcus aureus* with a synthetic carrier of calcium sulphate (Stimulan) releasing moxifloxacin, *Int. J. Antimicrob. Agents*, 33 (2009) 354-359. DOI: 10.1016/j.ijantimicag.2008.09.008
- [12] S.S. Aiken, J.J. Cooper, H. Florance, M.T. Robinson, S. Michell, Local Release of Antibiotics for Surgical Site Infection Management Using High-Purity Calcium Sulfate: An In Vitro Elution Study, *Surg. Infect. (Larchmt.)*, 16 (2015) 54-61. DOI: 10.1089/sur.2013.162
- [13] P. Wahl, F. Livio, M. Jacobi, E. Gautier, T. Buclin, Systemic exposure to tobramycin after local antibiotic treatment with calcium sulphate as carrier material, *Arch. Orthop. Trauma Surg.*, 131 (2011) 657-662. DOI: 10.1007/s00402-010-1192-2
- [14] H. Winkler, Rationale for one stage exchange of infected hip replacement using uncemented implants and antibiotic impregnated bone graft, *Int. J. Med. Sci.*, 6 (2009) 247-252. DOI: 10.7150/ijms.6.247
- [15] Z. Wentao, G. Lei, Y. Liu, W. Wang, T. Song, J. Fan, Approach to osteomyelitis treatment with antibiotic loaded PMMA, *Microb. Pathog.*, 102 (2017) 42-44. DOI: <http://dx.doi.org/10.1016/j.micpath.2016.11.016>
- [16] M. El-Husseiny, S. Patel, R.J. MacFarlane, F.S. Haddad, Biodegradable antibiotic delivery systems, *J. Bone Joint Surg. Br.*, 93 (2011) 151-157. DOI: 10.1302/0301-620x.93b2.24933
- [17] J.W. Kuiper, R.M. Brohet, S. Wassink, M.P. van den Bekerom, P.A. Nolte, D.A. Vergroesen, Implantation of resorbable gentamicin sponges in addition to irrigation and debridement in 34 patients with infection complicating total hip arthroplasty, *Hip Int.*, 23 (2013) 173-180. DOI: 10.5301/hip.2013.10612
- [18] R. Howlin, M. Brayford, J. Webb, J. Cooper, S. Aiken, P. Stoodley, Antibiotic-loaded synthetic calcium sulfate beads for prevention of bacterial colonization and biofilm formation in periprosthetic infections, *Antimicrob. Agents Chemother.*, 59 (2015) 111-120. DOI: 10.1128/AAC.03676-14

- [19] M. Nilsson, J.-S. Wang, L. Wielanek, K. Tanner, L. Lidgren, Biodegradation and biocompatibility of a calcium sulphate-hydroxyapatite bone substitute, *Bone & Joint Journal*, 86 (2004) 120-125. DOI: 10.1302/0301-620X.86B1.14040
- [20] M.A. Rauschmann, T.A. Wichelhaus, V. Stirnal, E. Dingeldein, L. Zichner, R. Schnettler, V. Alt, Nanocrystalline hydroxyapatite and calcium sulphate as biodegradable composite carrier material for local delivery of antibiotics in bone infections, *Biomaterials*, 26 (2005) 2677-2684. DOI: 10.1016/j.biomaterials.2004.06.045
- [21] J.C. Karr, J. Laurretta, G. Keriazes, In vitro antimicrobial activity of calcium sulfate and hydroxyapatite (Cerament Bone Void Filler) discs using heat-sensitive and non-heat-sensitive antibiotics against methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*, *J. Am. Podiatr. Med. Assoc.*, 101 (2011) 146-152.
- [22] M. Stravinskas, P. Horstmann, J. Ferguson, W. Hettwer, M. Nilsson, S. Tarasevicius, M.M. Petersen, M.A. McNally, L. Lidgren, Pharmacokinetics of gentamicin eluted from a regenerating bone graft substitute: In vitro and clinical release studies, *Bone Joint Res*, 5 (2016) 427-435. DOI: 10.1302/2046-3758.59.bjr-2016-0108.r1
- [23] C.L. Romano, N. Logoluso, E. Meani, D. Romano, E. De Vecchi, C. Vassena, L. Drago, A comparative study of the use of bioactive glass S53P4 and antibiotic-loaded calcium-based bone substitutes in the treatment of chronic osteomyelitis: a retrospective comparative study, *The bone & joint journal*, 96-b (2014) 845-850. DOI: 10.1302/0301-620x.96b6.33014
- [24] I. Takigami, Y. Ito, D. Ishimaru, H. Ogawa, N. Mori, T. Shimizu, N. Terabayashi, K. Shimizu, Two-stage revision surgery for hip prosthesis infection using antibiotic-loaded porous hydroxyapatite blocks, *Arch. Orthop. Trauma Surg.*, 130 (2010) 1221-1226. DOI: 10.1007/s00402-009-0991-9
- [25] H. Winkler, A. Stoiber, K. Kaudela, F. Winter, F. Menschik, One stage uncemented revision of infected total hip replacement using cancellous allograft bone impregnated with antibiotics, *J. Bone Joint Surg. Br.*, 90 (2008) 1580-1584. DOI: 10.1302/0301-620x.90b12.20742
- [26] H. Knaepler, Local application of gentamicin-containing collagen implant in the prophylaxis and treatment of surgical site infection in orthopaedic surgery, *Int. J. Surg.*, 10 Suppl 1 (2012) S15-20. DOI: 10.1016/j.ijssu.2012.05.020
- [27] N. Lindfors, J. Geurts, L. Drago, J.J. Arts, V. Juutilainen, P. Hyvonen, A.J. Suda, A. Domenico, S. Artiaco, C. Alizadeh, A. Brychcy, J. Bialecki, C.L. Romano, Antibacterial Bioactive Glass, S53P4, for Chronic Bone Infections - A Multinational Study, *Adv. Exp. Med. Biol.*, 971 (2017) 81-92. DOI: 10.1007/5584\_2016\_156
- [28] B. Bonev, J. Hooper, J. Parisot, Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method, *J. Antimicrob. Chemother.*, 61 (2008) 1295-1301. DOI: 10.1093/jac/dkn090
- [29] CLSI, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, Approved Standard—Ninth Edition, CLSI document M07-A9, (2012).
- [30] U. von Ah, D. Wirz, A. Daniels, Isothermal micro calorimetry – a new method for MIC determinations: results for 12 antibiotics and reference strains of *E. coli* and *S. aureus*, *BMC Microbiol.*, 9 (2009) 1-14. DOI: 10.1186/1471-2180-9-106
- [31] M. Di Luca, E. Navari, S. Esin, M. Menichini, S. Barnini, A. Trampuz, A. Casani, G. Batoni, Detection of Biofilms in Biopsies from Chronic Rhinosinusitis Patients: In Vitro Biofilm Forming Ability and Antimicrobial Susceptibility Testing in Biofilm Mode of Growth of Isolated Bacteria, *Adv. Exp. Med. Biol.*, 2017.10.1007/5584\_2017\_34
- [32] M. Gonzalez Moreno, A. Trampuz, M. Di Luca, Synergistic antibiotic activity against planktonic and biofilm-embedded *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Streptococcus oralis*, *J. Antimicrob. Chemother.*, (2017). DOI: 10.1093/jac/dkx265
- [33] B.G. Katzung, S.B. Masters, A.J. Trevor, *Farmacologia generale e clinica*, 2012.
- [34] C.J. Kristich, L.B. Rice, C.A. Arias, Enterococcal infection—treatment and antibiotic resistance, (2014).
- [35] D. Neut, H. van de Belt, I. Stokroos, J.R. van Horn, H.C. van der Mei, H.J. Busscher, Biomaterial-associated infection of gentamicin-loaded PMMA beads in orthopaedic revision surgery, *J. Antimicrob. Chemother.*, 47 (2001) 885-891. DOI: <https://doi.org/10.1093/jac/47.6.885>

- [36] J.G. Hendriks, J.R. van Horn, H.C. van der Mei, H.J. Busscher, Backgrounds of antibiotic-loaded bone cement and prosthesis-related infection, *Biomaterials*, 25 (2004) 545-556. DOI: [https://doi.org/10.1016/S0142-9612\(03\)00554-4](https://doi.org/10.1016/S0142-9612(03)00554-4)
- [37] O. Braissant, D. Wirz, B. Göpfert, A.U. Daniels, Use of isothermal microcalorimetry to monitor microbial activities, *FEMS Microbiol. Lett.*, 303 (2010) 1-8. DOI: 10.1111/j.1574-6968.2009.01819.x
- [38] I. Wadsö, Isothermal Microcalorimetry. Current problems and prospects, *J. Therm. Anal. Calorim.*, 64 (2001) 75-84. DOI: 10.1023/a:1011576710913
- [39] U. Furustrand Tafin, J.F. Meis, A. Trampuz, Isothermal microcalorimetry for antifungal susceptibility testing of *Mucorales*, *Fusarium* spp., and *Scedosporium* spp, *Diagn. Microbiol. Infect. Dis.*, 73 (2012) 330-337. DOI: 10.1016/j.diagmicrobio.2012.05.009
- [40] G. Buckton, S.J. Russell, A.E. Beezer, Pharmaceutical calorimetry: a selective review, *Thermochim. Acta*, 193 (1991) 195-214. DOI: [http://dx.doi.org/10.1016/0040-6031\(91\)80184-K](http://dx.doi.org/10.1016/0040-6031(91)80184-K)
- [41] B.F. Lindberg, EBJIS Conference, 2012.
- [42] A. Dvorzhinskiy, G. Perino, R. Chojnowski, M. Van Der Meulen, F. Ross, M. Bostrom, X. Yang, CERAMENT BONE VOID FILLER WITH GENTAMICIN INCREASES BONE FORMATION AND DECREASES DETECTABLE INFECTION IN A RAT MODEL OF DEBRIDED OSTEOMYELITIS, *Bone & Joint Journal Orthopaedic Proceedings Supplement*, 97-B (2015) 9-9.
- [43] D.B. Raina, A. Gupta, M.M. Petersen, W. Hettwer, M. McNally, M. Tägil, M.H. Zheng, A. Kumar, L. Lidgren, Muscle as an osteoinductive niche for local bone formation with the use of a biphasic calcium sulphate/hydroxyapatite biomaterial, *Bone & Joint Research*, 5 (2016) 500-511. DOI: 10.1302/2046-3758.510.BJR-2016-0133.R1