



Research Article

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Influence of bioactive glass S53P4 granules and putty on osteomyelitis associated bacteria *in vitro*

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Abstract: Bacterial infection of bone tissue and bone marrow, referred to as osteomyelitis, is a challenging clinical problem. In this study we analysed the influence of the granule size of the bone substitute bioactive glass (BAG) S53P4 and the novel putty material containing BAG S53P4 on four clinically important bacteria associated with osteomyelitis; *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Reference materials were the frequently used biomaterial in surgical bone grafting procedures; tricalcium phosphate and an inert glass. Powder of BAG S53P4 was used as a positive control. The materials were incubated with bacterial suspension and the viability of the microbes was determined as colony forming units after cultivation on agar plates.

All pathogens lost their viability in contact with the BAG S53P4 granules and the powder of the BAG S53P4. The reference materials tricalcium phosphate and the inert glass had no effect on the viability of the bacteria. The BAG S53P4 putty containing 0.5–0.8 mm granules did not show any antibacterial effect on any of the tested bacteria. New putty compositions need to be investigated to obtain antibacterial properties for this novel bone regeneration biomaterial.

Keywords: antibacterial effect, bioactive glass S53P4 putty, granule size, osteomyelitis associated pathogens

1 Introduction

Bacterial infection of bone tissue and bone marrow, *i.e.* osteomyelitis, is a challenging clinical problem [1]. The infection is often related to severe trauma of bone or/and the soft tissue or can be a result of a haematogenous infection. According to the literature, the incidence of posttraumatic osteomyelitis and non-union of the long bones occurs in average in 4.4% of the patients [2, 3]. In open fractures, however, the incidence of postoperative complications is even higher. The pathogen related to osteomyelitis is most often *Staphylococcus aureus*, however, the infection can be caused by a variety of other different species. *Staphylococcus aureus* is a gram-positive coccus causing a range of illnesses from minor skin infections to life-threatening diseases such as pneumonia, meningitis and sepsis. It is also one of the five most common species causing postoperative wound infections [4]. Methicillin resistant *Staphylococcus aureus*, (MRSA), is due to its resistance to beta-lactam antibiotics, one of the most feared strains of *Staphylococcus aureus* [5]. *Staphylococcus epidermidis* is a gram-positive coccus, typically found on the skin and mucosa. Due to its ability to colonize surfaces and its known ability to form thick, multilayered biofilms, it is often related to infections on catheters and implants [6]. *Pseudomonas aeruginosa* is a gram negative rod-shaped γ -Proteobacteria found in soil, water and human skin flora. It typically infects the pulmonary and urinary tract, wounds and also causes other blood infections. As it preferably grows on surfaces, this bacterium is frequently associated with medical device infections, including catheters [7]. Debridement combined with local and systemic broad-spectrum antibiotics, such as gentamycin, vancomycin and amphotericin B, is the gold standard treatment-method of osteomyelitis [8]. Typically, local administration of antimicrobial agents is performed after surgical debridement by putting chains of polymethylmethacrylate (PMMA) beads, collagen fleeces or calcium sulphate pellets impregnated with antibiotics into the bone defect [9–11]. One disadvantage of the treatment-method with antibiotic loaded beads is the second surgical intervention needed for removing the beads and filling the defect with autograft bone or a

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bone substitute. To avoid this two-stage procedure, antibiotics have also been incorporated into bone substitutes, such as hydroxyapatite (HA), tricalcium phosphate (TCP) and calcium sulphates (CaS) [12, 13]. Another well known method to manage infected bone is a two-stage treatment, by the name of Masquelet technique, where an antibiotic-containing PMMA spacer is implanted in the first stage and the induced membrane compartment is grafted with autograft in the second stage after removal of the spacer [14, 15].

Resistant bacterial strains, such as, *MRSA* are, however, a growing problem. In the treatment of severe bone infections, a bone substitute with intrinsic antibacterial properties that would not need to rely on added antimicrobial drugs and therefore not induce microbial resistance would be very valuable.

BAG, S53P4 is a clinically used bone substitute with a composition of 53% SiO_2 , 23% Na_2O , 20% CaO , 4% P_2O_5 . In addition to its osteoconductive and osteostimulative properties, BAG S53P4 has also shown excellent antibacterial properties on both aerobic and anaerobic species *in vitro* [16–22]. BAG S53P4 is also the first known biomaterial that has been shown to decrease biofilm burden, is effective towards multi-resistant “super bugs” and does not induce resistance in bacteria [23–25]. The antibacterial properties of the glass are related to a high pH and an osmotic pressure in the vicinity of the glass, caused by the dissolution process at the glass surface [26]. BAG S53P4 has been studied clinically in randomised prospective trials in bone tumour [27, 28], trauma [29] and spine surgery [30, 31]. Due to its antibacterial properties it has also been used in the treatment of severe chronic osteomyelitis, mastoiditis, spine infections and frontal sinus infections [21, 32–38].

The primary purpose of the study was to evaluate *in vitro*, if different granule sizes of BAG S53P4 affect the known antibacterial property of this biomaterial, by testing its antibacterial capacity on four clinically important bacterial species related to osteomyelitis: *Staphylococcus aureus*, *MRSA*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. The secondary purpose was to investigate the unknown antibacterial property of a S53P4-based new putty material used as biomaterial for bone regeneration. Tricalcium phosphate, which is a frequently used biomaterial in surgical bone grafting procedures, and inert glass were used as reference materials. Powder of BAG S53P4 was used as a positive control.

2 Materials and methods

Bacterial strains of *Staphylococcus aureus*, ATCC 29213, *Staphylococcus aureus* MetR, ATCC 43300, *Staphylococcus epidermidis*, ATCC 14990 and *Pseudomonas aeruginosa*, ATCC 27853 were used. Bacteria cultured in sterile Tryptone Soy Broth (TSB) without additives were defined as the negative control.

BAG S53P4 (BonAlive® granules, BonAlive Biomaterials Ltd, Turku, Finland) of different granule sizes (0.5–0.8mm, 1.0–2.0mm and 2.0–3.15mm), and a putty of the BAG S53P4 (BonAlive® putty, BonAlive Biomaterials Ltd, Turku, Finland) were investigated. Powder of the BAG S53P4 (<45 μm) was used as a positive control, as earlier studies have shown that a concentration of 100 mg/ml of powder of BAG-S53P4 can effectively inhibit bacterial growth [18]. β -tricalcium phosphate (TCP) (ChronOS granules, DePuy-Synthes GmbH, Oberdorf, Switzerland) and inert glass (Iittala clear glass, Iittala, Finland) were used as references. The putty polymer binder was also tested (Table 1). All materials tested were gamma sterilized except for the inert glass, which was sterilized in hot air.

The procedures were performed as previously described by Munukka and co-workers [18, 19]. The bacteria were incubated in sterile TSB, in 5 ml sterile test tubes (Becton Dickinson) together with the materials. The materials were weighted (Mettler AE 50) and mixed properly with two ml of TSB. Three replicates with the exception for the putty-product of six replicates were performed. Three replicates of the putty-product were incubated for two hours at room temperature where after the TSB-solution with the dissolved polymer was replaced by a new two ml batch of TSB. This product is later referred to as washed granules of the putty-product. Finally the amount of 1×10^5 bacteria inoculums determined by optical densitometry (Thermo GeneSys 20) was added to the mixture. Bacterial cultures without added materials and pure TSB served as controls. The concentrations of the tested materials incubated with the bacterial solution are shown in Table 1.

The viability of the bacterial suspension incubated with the material was assessed by using commercial solid blood agar plates (Trypticase Soy Agar II with 5% Sheep Blood, Becton Dickinson). At consecutive 24 h cultivation points, 10 μL samples taken directly from the suspensions were plated as described by Vuorenoja and co-workers [39]. In addition, one replicate of 1:10 000 dilutions of the samples and bacterial controls were plated to assure the quantifiable, single colony formation.

After agar plate cultivation (+37 °C) for 16 hours, the growth of bacteria was evaluated by comparing the results

Table 1: Tested materials, controls and reference materials.

Product	Description	Concentration (mg/ml)
BAG S53P4	0.5-0.8mm	1000
BAG S53P4	1.0-2.0mm	1000
BAG S53P4	2.0-3.15mm	1000
BAG S53P4 Putty	Paste like binder with S53P4 granules (granule size 0.5-0.8 mm + spheres 0.09-0.425 mm)	1400
Putty binder	Pure polymer (PEG-glycerol) material without granules	560
Tri-calcium phosphate (TCP)	Reference material, granule size not defined	100
BAG S53P4 powder (positive control)	<45 µm	600
Inert glass (non-bioactive reference)	littala clear glass, recipe 1350, granule size not defined	600

obtained for the materials, to the results of the control. Absence of growth on the plates was an indicator of the antibacterial ability of a given product. The cultivation period was seven days.

The pH of the samples was estimated from the test tubes by using pH paper (pH range 7.5 - 14 Merck Alkalit 81.09532 and range 6.4 - 8.0 Nacherey-Nagel, REF 90210) by dipping it into the broth. pH was measured after eight days of cultivation for *Staphylococcus aureus* and *Staphylococcus epidermidis*, after seven days for *MRSA* and at 5 days for *Pseudomonas aeruginosa*.

The study was performed in the laboratory facilities of the Department of Medical Microbiology and Immunology (Finas approved, ISO SFS-EN ISO/IEC17025), University of Turku, Finland.

3 Results

The antibacterial effect of the different sizes of the BAG S53P4 granules on the four osteomyelitis related bacterial species is represented in Figure 1A-1D.

All bacteria tested lost their viability during incubation with granules of the BAG S53P4 (Figure 1a-1d). No clear difference in the antibacterial effect on the osteomyelitis related bacteria could be seen between the different granule sizes tested.

The effect of the BAG S53P4 with a granule size of 1.0-2.0 mm on all the four strains tested (Figure 2) showed total loss of viability in 6 days for *P.aeruginosa* and *MRSA* and in 7 days for *S.aureus* ATCC 29213. The viable counts of

S.epidermidis decreased from 10^5 to 10^2 , indicating 99,9% loss of viability in 7 days.

In contact with the powder of the BAG S53P4 (<45µm), (positive control), all bacteria also lost their viability 99-100% within 2-3 days, except for *S. aureus* ATCC 29213 in 6 days. (Figure 1a-1d).

No clear antibacterial effect was observed for the BAG S53P4 putty (Figure 3), the PEG-glycerol polymer nor for the washed BAG S53P4 granules (Table 2). The negative controls gave consistently a result of too numerous bacteria to count after plating. This was seen with all strains and at all time points showing that the bacteria were viable throughout the study period. After the 5th day of incubation the CFU's of *P. aeruginosa* were not countable due to slime formation (Table 2).

Granules of the TCP had no antibacterial effect on *P. aeruginosa* or *S. aureus* ATCC 29213 at the end of the test period. A slight antibacterial effect on *MRSA* and *S. epidermidis* was, however, observed. For this phenomenon we found no explanation (Figure 4).

Pure TSB without an inoculant was used as a control to demonstrate aseptical conditions. There was no bacterial growth in pure TSB at any of the time points.

A clear increase in pH up to 9.5 was observed for all sizes of BAG-S53P4 granules at the end of the incubation period. The highest pH change was seen for the positive control, the BAG S53P4 powder (<45 µm), up to 10. No clear pH changes over 9.0 were seen for the other materials tested (Table 3).

Table 2: Amount of osteomyelitis associated bacteria as colony forming units (Log 10) indicating no significant changes of the viability during incubation with the BAG S53P4 putty, washed granules from the putty and the putty-matrix on *Staphylococcus epidermidis* and *Staphylococcus aureus*, Methicillin resistant (MetR) *Staphylococcus aureus* and *Pseudomonas aeruginosa*. U= colonies unable to count due to agglutination.

Bacteria species	1 day			2 day			3 day			4 day		
	Putty	Washed granules	Matrix	Putty	Washed granules	Matrix	Putty	Washed granules	Matrix	Putty	Washed granules	Matrix
<i>S. epidermidis</i>	5	5	5	5	5	5	4	5	5	4	5	5
<i>S. aureus</i>	5	5	5	5	5	5	5	5	5	5	4	5
<i>S. aureus</i> MetR	5	5	5	5	5	5	5	5	5	4	4	4
<i>P. aeruginosa</i>	5	5	5	5	5	5	4	4	5	5	5	5
Bacteria species	5 day			6 day			7 day					
	Putty	Washed granules	Matrix	Putty	Washed granules	Matrix	Putty	Washed granules	Matrix	Putty	Washed granules	Matrix
<i>S. epidermidis</i>	4	5	4	5	5	4	5	5	4	5	4	
<i>S. aureus</i>	5	4	5	5	4	5	5	4	5			
<i>S. aureus</i> MetR	5	4	5	5	4	5	U	U	U			
<i>P. aeruginosa</i>	5	5	5	U	U	U	U	U	U			

Table 3: pH at the end of the incubation period: *Staphylococcus epidermidis* and *Staphylococcus aureus* at 8 days, Methicillin resistant (MetR) *Staphylococcus aureus* at 7 days, *Pseudomonas aeruginosa* at 5 days.

	<i>S. aureus</i>	<i>S. epidermidis</i>	MetR	<i>P. aeruginosa</i>
Granules 0.5 – 0.8	9.5	9.5	9.5	9.5
Granules 1.0 – 2.0	9.5	9.5	9.5	9.5
Granules 2.0 – 3.15	9.5	9.5	9.5	9.5
BonAlive® Putty	6.8	8.5	7.8	6.8
Putty binder	6.4	6.4	6.4	6.4
Washed granules	6.6	6.4	6.8	8.5
Tricalcium phosphate	8.0	7.4	8.0	8.0
S53P4 glass powder	10.0	10.0	9.5	10.0
TSB + bacteria	8.5	8.0	7.4	8.5

4 Discussion

In this *in-vitro* study, granules of BAG S53P4 were able to prevent colonization of all the four clinically important pathogens tested. All granule sizes of BAG S53P4 glass provided bacterial inhibition at the end of the study period. This is in accordance with the *in-vitro* bactericidal capacity recently observed for BAG S53P4 of 500-800 μm size on *S. aureus* and *epidermidis*, *P. aeruginosa* and *Acinetobacter baumannii* by Drago and co-workers [21], as well as for powder and granules of 2.0-3.0 mm size on *S. aureus*, *S. epidermidis*, *E. coli* and *K. pneumoniae* [7].

Earlier findings on the efficacy of BAG S53P4 powder to prevent bacterial growth *in-vitro* were also confirmed in this study; the powder was the most effective material against all the pathogens tested [10, 18, 19, 26]. This also

shows that regardless of the sterilization method results can be compared, as previous materials have been hot air sterilized whereas the materials in this study were gamma sterilized.

According to Gergely and co-workers, the antibacterial effect of BAG S53P4 is highly influenced by the granule size and the contact time. Comparing the effect of BAG S53P4 of the large 2.0-3.15 mm granule size and gentamicin impregnated polymethylmethacrylate (PMMA) beads, the antibacterial effect of BAG S53P4 on *S. aureus* was found to be as effective as gentamicin impregnated PMMA beads, with no statistically significant difference. In contrast, PMMA beads showed a superior antibacterial effect on *S. epidermidis* and *K. pneumoniae* [10]. The materials tested in this study showed a similar clear rise of the pH value, up to 9.5, measured at the end of the incubation period correlating to the similar antibacterial effects they gave rise to.

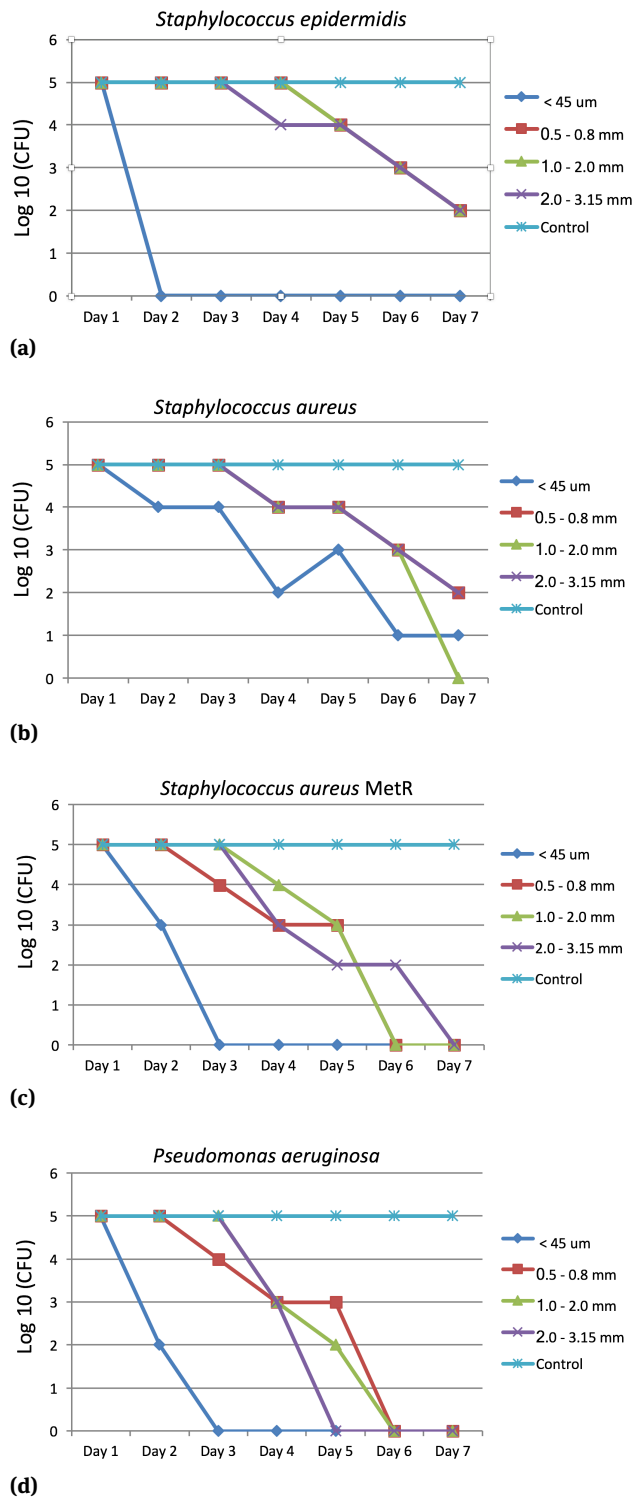


Figure 1: The effect of the different granule size of the bioactive glass S53P4 tested on viable counts of A) *Staphylococcus epidermidis*, B) *Staphylococcus aureus*, C) Methicillin resistant *Staphylococcus aureus* and D) *Pseudomonas aeruginosa* in suspension. CFU= Colony forming units.

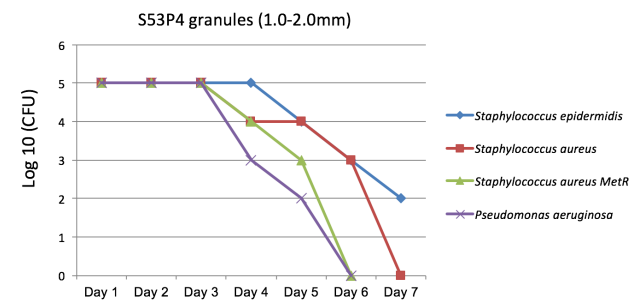


Figure 2: The effect of the 1.0-2.0mm bioactive glass S53P4 granules on viable counts of the different osteomyelitis associated pathogens in suspensions. CFU= Colony forming units.

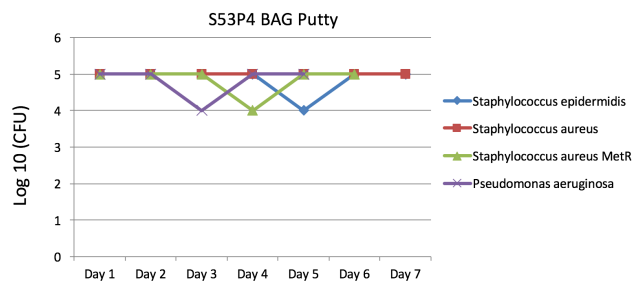


Figure 3: The effect of the putty containing BAG S53P4 granules (0.5-0.8mm) on viable counts of the different osteomyelitis associated pathogens in suspensions. CFU= Colony forming units.

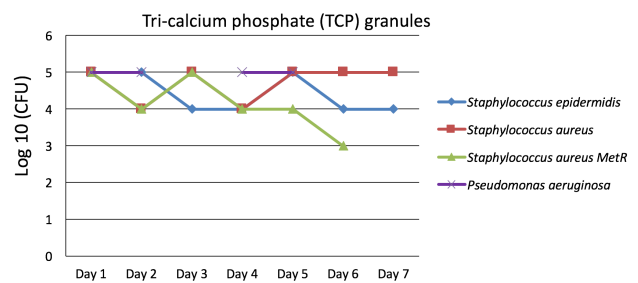


Figure 4: The effect of the reference material β -tricalcium phosphate on viable counts of the different osteomyelitis associated pathogens in suspensions. CFU= Colony forming units.

Our results did, however, not show any clear differences in antibacterial effects with regard to the granule size. All granule sizes showed clear efficacy, but the contact time was relevant.

According to our results the BAG S53P4 putty was not able to prevent the colonization of the four pathogens tested. The granules in the putty are embedded in a matrix which is a water-soluble synthetic binder made of a blend of polyethylene glycols (PEGs) and glycerol. For this reason the granules in the putty will not immediately be exposed to the aqueous environment, and there will, therefore, be a delay in the surface reactions of the BAG in the putty. This

was seen also in the only mild rise of the pH, varying from 6.8 to 8.5, at the end of the incubation. Another detail is that there will be a sequential reaction cascade with granules reacting first on the surface of the putty mass in the defect and the granules in the middle of the putty mass will only react after a given time point when the body fluids have reached the middle. Hence, with BAG granules all the granules will immediately react and this will effectively increase the pH and osmotic pressure.

In the treatment of osteomyelitis, a high local antibiotic concentration is needed. A concentration, as high as, 100 times of the minimal inhibitory concentration (MIC) is desirable during the first 1-2 days [40]. Systemic administration of these antibiotics would cause high systemic side effects and due to the compromised local vascularity, the concentrations and antimicrobial effects at the infection site are low [41].

Our results show that the 0.5-0.8 mm and 1.0-2.0 mm sized BAG S53P4 granules show a similar capacity to inhibit bacterial growth. BAG S53P4 has been used as bone graft substitute in the treatment of chronic osteomyelitis, according to a one-stage procedure with very promising results [21]. The obvious antibacterial effect of the BAG S53P4 granules contributes to the beneficial outcome of the treatment of this severe chronic infection [32, 33, 38]. Without the need of local antibiotics the potential risk of developing bacterial resistance is also decreased.

Our *in vitro* results thus further support the use of BAG S53P4 as bone substitute in potentially contaminated areas. However, the BAG S53P4 putty containing PEG-glycerol polymer and BAG S53P4 granules of the size 0.5-0.8 mm does not show antibacterial properties.

In conclusion, BAG S53P4 has proven antibacterial properties. However, as a putty, containing BAG S53P4 granules with the size 0.5-0.8 mm, our *in vitro* results confirm no antibacterial effects on osteomyelitis associated pathogens. New formulations of putty need to be investigated to obtain antibacterial properties for this novel bone regeneration biomaterial.

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