Efficacy of Antibacterial Bioactive Glass S53P4 Against *S. aureus* Biofilms Grown on Titanium Discs In Vitro

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ABSTRACT: We evaluated the effectiveness of different sizes of bioactive glass S53P4 against *Staphylococcus aureus* biofilms grown on metal discs in vitro. *S. aureus* biofilms were cultivated on titanium discs. BAG-S53P4 (0.5–0.8 mm and <45 μm) were placed in contact with the discs containing biofilms. Glass beads (0.5 mm) were used as a control. After each interval, the pH from each sample was measured. Colony forming units were counted for the biofilm recovery verification. In parallel, we tested the activity of bioactive glass against *S. aureus* planktonic cells. We found that BAG-S53P4 can suppress *S. aureus* biofilm formation on titanium discs in vitro. The suppression rate of biofilm cells by BAG-S53P4 <45 μm was significantly higher than by BAG-S53P4 0.5–0.8 mm. BAG-S53P4 has a clear growth-inhibitory effect on *S. aureus* biofilms. BAG-S53P4 <45 μm is more efficient against biofilm growth in vitro comparing with BAG-S53P4 0.5–0.8 mm. Bioactive glass S53P4 has potential to be used as bone substitute for the resolution of infection complications in joint replacement surgeries and treatment of chronic osteomyelitis. © 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:175–177, 2014.

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The treatments of post-traumatic and implant-related skeletal complications are challenging. Bone grafting is usually required to stimulate bone healing. Several methods of reconstructing bone defects are available using autograft, allograft, demineralized bone matrix, hydroxyapatite, calcium phosphate, autologous bone marrow aspirates, bone morphogenetic proteins, and other related growth factors.[1] In treatment of bone infections, adequate debridement is mandatory. Unfortunately, this treatment often results in a poorly vascularized large bone defect.[2] Many methods have been used to treat infected bone defects, including free vascularized bone grafts, local muscle flaps, and antibiotic-impregnated polymers[3,4] and bone grafts.[5]

The bacterial colonization of host tissue or implanted materials is promoted by the ability of the bacteria to produce protein-specific adhesions on their surfaces, which is followed by interactions with host protein components, such as fibrinogen, fibronectin, and collagen. Bacteria in biofilms evade host defenses and are more resistant to antibiotics.[6] Staphylococci and Gram-negative bacilli are the pathogens most commonly involved in these cases.

Bioactive glass S53P4 (BAG-S53P4; 53% silicon dioxide, 23% sodium oxide, 20% calcium oxide, and 4% phosphorus pentoxide) is a biocompatible, osteoconductive bone substitute, with bone bonding capacity and antibacterial properties.[3,7,8] BAG-S53P4 does not favor adhesion or colonization of several pathogens on its surface.[9,10] Also, promising results were obtained in dead space management in chronic osteomyelitis.[3,9,11,12] The ability of BAG-S53P4 to inhibit bacterial growth is based on simultaneous processes that occur when the bioactive glass reacts with body fluids. First, sodium is released from the surface of the glass, inducing elevation of the pH that is unfavorable for bacteria. Further, the ions (sodium, calcium, phosphorus, and silicium) released from the surface increase the osmotic pressure, creating an environment where the bacteria cannot grow.[7,8]

No studies, however, show the effect of BAG-S53P4 against bacterial biofilms. The aim of this study was to evaluate the effectiveness of two different dimensions of BAG-S53P4 against *Staphylococcus aureus* biofilms grown on metal discs in vitro.

METHODS

Bioactive Glass

Bone substitute made by bioactive glass (BAG-S53P4) as granules sizing 0.5–0.8 mm and <45 μm (BonAlive Biomaterials Ltd., Turku, Finland) were used.

Bacterial Strains

American Type Culture Collection *S. aureus* (ATCC 29213) was used. For the preparation of the inoculums, the lyophilized strains were freshly grown overnight on Müller-Hinton (MH) agar plates (Sigma–Aldrich, Hamburg, Germany). Discrete colonies were obtained from MH agar plates and resuspended in MH broth to a McFarland turbidity of 0.5.

Substrate for Biofilm Formation

Discs with an area of 157 mm^2 and made from TMZF® alloy (TiMo12Zr6Fe2), usually employed for joint replacement implants confection, were purchased from Stryker GmbH & Co KG (Duisburg, Germany).[13]

Activity of BAG-S53P4 Against *S. aureus* Biofilm

Discs were washed, autoclaved, and immersed in 48-well plates containing 1 ml of a 2×10^5 *S. aureus*/ml stock solution. The plates containing the discs were incubated at 37°C for 24 h on a rocking table (12 cycles/min) for the attachment of planktonic cells on the disc surfaces and biofilm formation. After 24 h, the discs containing biofilms were washed three times with distilled water to remove the planktonic cells and

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transferred to a new 48-well plate. The following samples were placed in wells in contact with the discs: 500 mg of BAG-S53P4 (0.5–0.8 mm), 500 mg of BAG-S53P4 (<45 μm), and 500 mg of glass beads (0.5 mm) used as control. Five hundred microliters of fresh MH bullion was added in each well. The plates were incubated at 37°C for 24 and 48 h and 4 and 5 days. After each interval, the pH from each well was measured, and the discs were removed, washed three times in PBS and transferred to a 15 ml Falcon tube containing 2 ml of MHB. The tubes were sonicated for 1 min at 100% intensity for the disruption of the biofilms. After sonication, 20 μl of the sonication fluid were added to a MH agar plate and incubated at 37°C for 24 h. After 24 h, the colony forming units (CFUs) were manually counted for the biofilm recovery verification. All experiments were carried out in triplicate.

In parallel, we tested the activity of bioactive glass against *S. aureus* as planktonic cells. For that we added 500 mg of BAG-S53P4 (0.5–0.8 mm), 500 mg of BAG-S53P4 (<45 μm), and 500 mg of glass beads (0.5 mm) in tubes containing 2 ml of a 2×10^5 *S. aureus*/ml stock solution and incubated at 37°C for 24 h. After 24 h, 20 μl of the incubated fluid was added to a MH agar plate and incubated at 37°C for 24 h. After 24 h, the CFUs were counted. The experiments were carried out in duplicate.

Statistical Analysis

The difference in the biofilm recovery after treatment with bioactive glass between time steps was analyzed with Freedman tests. The difference among all samples was calculated using ANOVA and Games-Howell as post hoc tests. Statistical analyses were carried out using SPSS 20 (IBM, Armonk, NY). Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA) was used to create the graphs.

RESULTS

Activity of BAG-S53P4 Against *S. aureus* Biofilm

The discs treated with BAG-S53P4 (0.5–0.8 mm) and BAG-S53P4 (<45 μm) had less CFU in comparison with the control samples. A significant difference was observed between the time steps and also between the substrates ($p < 0.0001$) where after 24 h a mean of 6 CFU $\log_{10}$ (was counted for the BAG-S53P4 (0.5–0.8 mm) plates, a mean of 2 CFU $\log_{10}$ from BAG-S53P4 (<45 μm), and a mean of 8 CFU $\log_{10}$ for control group (Fig. 1).

The BAG-S53P4 (0.5–0.8 mm) started with a pH of 8, which increased to 11 by 5 days. The BAG-S53P4 (<45 μm) showed a pH of around 10 and 11 from time 0, while the glass beads kept the pH at 8 during all intervals (Fig. 2).

The activity of bioactive glass against *S. aureus* planktonic cells showed 8 CFU $\log_{10}$ for BAG-S53P4 (0.5–0.8 mm) and 8 CFU $\log_{10}$ for glass beads, while the CFU counting for BAG-S53P4 (<45 μm) was near 0 (Fig. 3).

DISCUSSION

We examined the anti-biofilm properties of BAG-S53P4 <45 μm and BAG-S53P4 0.5–0.8 mm. Smaller granules size is recommended for smaller bone defects and bigger granules for larger bone defects. As small and larger bone defects can be infected with biofilm formation, we chose to investigate both granules sizes.

A clinically important bacterial species was cultivated in broth on metal discs together with two different BAGs. We showed that BAG-S53P4 can suppress *S. aureus* biofilm formation on titanium alloy discs in vitro. However, the dimensions of the bioactive glass granules are an important factor for the effectiveness of this bone substitute against biofilm growth. The suppression rate of biofilm cells by BAG-S53P4 <45 μm was significantly higher than by BAG-S53P4 0.5–0.8 mm. These results also correlate with tests made with BAG-S53P4 <45 μm and BAG-S53P4 0.5–0.8 mm in contact with *S. aureus* planktonic cells, where just a few CFU could be counted from the samples treated by BAG-S53P4 <45 μm. Our results correlate with other studies carried out with BAGs.[3,9,10]

The exact mechanisms of the antibacterial action of BAGs are unknown. High pH and osmotic effects caused by the nonphysiological concentration of ions dissolved from the glass have been suggested.[10] We suggest that the better activity of smaller granules can be related to the increased surface area. Increasing surface area increases the contact of the bioactive glass with the aqueous environment that increases the release of ions from BAG, raising the local pH and osmotic pressure.[8,14] High pH is unfavorable to bacterial growth.[3,9] Another factor may be the high concentrations of calcium and alkalis likely to be released from BAG that could cause perturbations of the membrane potential of bacteria.[9] Our results agree with Zhang et al.[15] who affirmed that the largest particles did not markedly prevent diffusion between the ions inside the particle bed and simulated

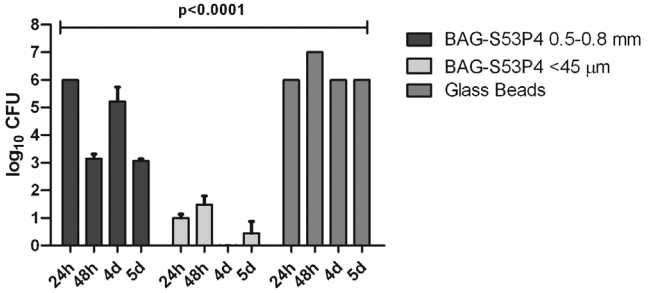

Figure 1. Biofilm recovery on titanium alloy surfaces after treatment with BAG 0.5–0.8 mm, BAG <45 μm, and glass beads as control. Threshold = 8 $\log_{10}$.

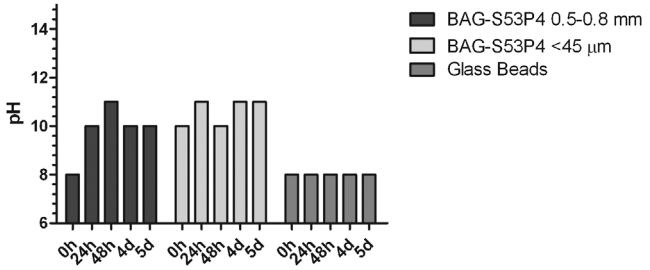

Figure 2. pH of the wells containing biofilm grown on the metallic surfaces and treated with BAG 0.5–0.8 mm, BAG <45 μm, and glass beads as control after 0, 24, and 48 h and for 4 and 5 days.

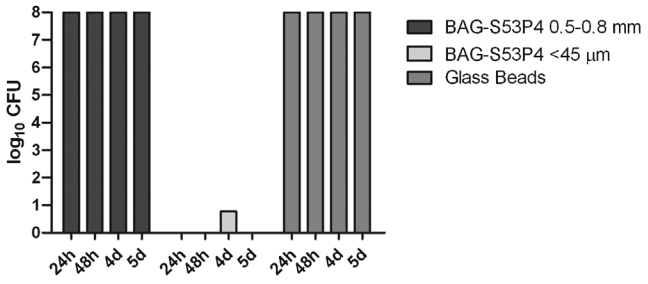

Figure 3. Quantification of planktonic cells after treatment with BAG 0.5–0.8 mm, BAG <45 μm, and glass beads as control after intervals of 0, 24, and 48 h and of 4 and 5 days.

body fluid. The higher pH values obtained with the smaller particles depended on their large surface area. The bigger the particles, the smaller the differences between the pH values.[15]

The stability of the calcium phosphate precipitation increased with pH. According to Lu and Leng,[16] the nucleation rate of calcium phosphate precipitation in simulated body fluid was significantly affected by pH, resulting in a different crystallized structure of the precipitate. A high pH environment was favorable for hydroxyapatite nucleation, and the hydroxyapatite nucleation rate approached that of octacalcium phosphate at pH 10.[16] The bone-like layer formed on the surface of BAG-S53P4 once in contact with corporal fluids is responsible for the osteointegration of the material, improving the bone remodeling and therefore, the resistance of the tissue against infections.

The possibility of bacterial colonization is a remarkable problem in the use of prostheses and other medical devices BAGs have bactericidal properties on both aerobic and anaerobic bacteria.[3,10,17] Promising results with BAG-S53P4 used in dead space management in chronic osteomyelitis has been shown.[3,12] Modifications of the surfaces of devices, for example, by coating them with a suitable BAG, or use of BAGs as bone substitute in joint replacement revisions may prevent bacterial adhesion and thus prevent the tissues around them from becoming infected.[9,17]

In conclusion, BAG S53P4 has a clear growth-inhibitory effect on *S. aureus* biofilms. BAG-S53P4 <45 μm is more efficient against biofilm growth in vitro comparing with BAG-S53P4 0.5–0.8 mm. Bioactive glass S53P4 has potential to be used as bone substitute in joint replacement surgeries aiming prevention and treatment of periprosthetic infections.

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