DOI: 10.1002/adem.200900081

In vitro Study of the Antibacterial Activity of Bioactive Glass-ceramic Scaffolds**

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Bioactive glasses (BGs) have been the subject of intensive research in view of their stimulatory effect on bone tissue formation. The bioactivity of these silicate and phosphate systems has been attributed to both surface reactions taking place at the material–tissue interface and to the direct effect of glass dissolution products on osteogenesis.[1,2] It has also been shown that several melt- and sol–gel-derived BGs have antimicrobial properties against different bacteria.[3–21] This antibacterial activity was very dependent on glass composition, glass concentration, particle size, and on the microorganisms tested.[3–21] This large body of positive results has led to the development of a series of foam-like scaffolds for bone tissue engineering based on BGs and glass-ceramics.[22–24] One group of highly porous scaffolds recently produced is based on the original 45S5 Bioglass® composition (45 SiO_2, 24.5 CaO, 24.5 Na_2O, and 6 P_2O_5 in wt%).[22,23] The microstructure, mechanical properties, surface reactivity and the in vitro and in vivo biocompatibility of the scaffolds have been investigated;[22,23,25,26] however, their antibacterial efficacy has not been evaluated to date. Therefore, the aim of this study was to evaluate the potential in vitro antibacterial effects of bioactive glass-ceramic scaffolds made from both 45S5 Bioglass and from boron containing bioactive glass (45S5.2B). The rationale for incorporating boron to BGs as scaffolds for bone tissue-engineering is the emerging scientific evidence that boron has beneficial effects on bone modeling, remodeling and repair.[27–30] In addition, there is some evidence that boron-containing BGs (MBG0118 and MBG0123) exert antibacterial effects against *Staphylococcus aureus*.[13] Thus, the incorporation of B_2O_3 into the BG matrix is aimed at minimizing the risk of microbial contamination through the potential antimicrobial activity of the leaching boron ions.

S. aureus was chosen for the preliminary investigation in view of its significance for the pathogenesis of infections associated to orthopedic implants.[31–33]

Materials and Methods

45S5 Bioglass® -derived glass-ceramic scaffolds ($5 \times 5 \times 2\,mm^3$) (labeled 45S5) with porosity in the range 90–95% were fabricated using the foam replica technique and sintering at 1100 °C for 1 h, as described elsewhere.[22,23] Scaffolds were also prepared from 45S5 Bioglass containing B_2O_3 (2 wt%) (labeled 45S5.2B).[27]

The antagonistic effect of the ionic dissolution products from glass-ceramic scaffolds 45S5 and 45S5.2B on bacteria was evaluated by incubating 1% w/v scaffolds in Hanks' balanced salt solution (HBSS) containing glucose ($1\,g\,L^{-1}$) at pH 7.4 and 37 °C for 7, 14, and 28 day. The ionic composition of the solution was analyzed pre- and post-incubation by inductively coupled plasma spectroscopy (ICP). *Staphylococcus aureus* strains (ATCC 25923; ATCC 29213 and ATCC 6538P) were activated in Mueller Hinton broth (Britania, Argentina) at 37 °C for 24 h. Bacterial cells were then harvested by centrifugation ($10\,000 \times g$ for 5 min at 4 °C), resuspended in 0.1% w/v buffered peptone water and the concentration adjusted close to $10^6\,CFU\,mL^{-1}$. These cell suspensions were put in contact with the dissolution products in a ratio 1:1 at 37 °C. At 0, 1, 2, and 24 h of incubation, cell viability was determined by plate count in agar Mueller Hinton.

The initial surface attachment of *S. aureus* (ATCC 29213) on sintered scaffolds was evaluated by incubating 45S5 and

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[**] *Acknowledgments, The authors gratefully acknowledge the expert assistance of Dr. Forrest H. Nielsen (USDA ARS, Grand Forks Human Nutrition Research Center) with ICP analysis, and Pedro Villagrán, Ch.E., and Silvia Blanco, Ch.E. with SEM, LASEM – ANPCyT -UNSa -CONICET, Salta, Argentina.*

45S5.2B scaffolds in the bacterial suspension for 1 and 24 h. Subsequently, the scaffolds were gently rinsed with physiological solution (NaCl 0.9%). The samples were then placed in 20 mL of physiological solution and vortexed for 1 min to remove nonattached bacteria and to disperse them into the suspension. Serial dilutions of the suspensions were carried out in buffered peptone water and spread onto agar Mueller Hinton plates. The plates were then incubated aerobically at 37 °C and viable counts (the number of CFU) were determined after 24 h.

The scaffolds were placed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) to fix the cells and were stored at 4 °C for 24 h. The samples were sequentially dehydrated for 10 min in 50, 70, 90, and 96% ethanol solutions and then twice for 20 min in absolute ethanol as described by Kockro *et al.*[34] After critical-point drying, mounting on stubs and gold sputtering, the samples were examined with a scanning electron microscope (JEOL JSM 6480 LV, Japan).

Assays were carried out in duplicate. The statistical significance of the data was determined using the analysis of variance test (ANOVA). Data are presented as means ± SD.

Results and Discussion

Antagonistic effect of the ionic dissolution products from glass-ceramic scaffolds.

Despite the high pH value (9.5–10) and/or the concentration of free boron ($120 \pm 2\ \mu g\ L^{-1}$) in the dissolution products of scaffolds 45S5 and 45S5.2B, respectively, no significant inhibition was observed on the different *S. aureus* analyzed. In particular, the results obtained with *S. aureus* ATCC 29213 are shown in Figure 1.

Our results were consistent with those of Bellantone *et al.*[8] who reported that after a 20 h incubation period, 45S5 BG particles (90–710 μm) had no effect on the viability of 5×10^7 CFU mL^{-1} culture of *S. aureus* (NCIMB 11852) even at a 10 mg mL^{-1} concentration. Recently, Xie *et al.*[19,20] showed that 300 mg particulate 45S5 BG did not reduce the rate of infection with *S. aureus* (ATCC 25923) after the fixation of open tibial fractures in rabbits.

However, Hu *et al.*[9] showed that 45S5 BG particles (<50 μm) exhibited after a 1 h incubation period, strong antibacterial activity against several pathogenic bacteria ($0.5–2 \times 10^8$ CFU mL^{-1}) in vitro. The bactericidal percent for *S. aureus* (ATCC 25923) was 55% at the concentration of 10 mg mL^{-1}. At concentration over 50 mg mL^{-1}, the bactericidal percentages increased up to 98%.

Munukka *et al.*[13] evidenced that particles (<45 μm) of 2 wt% of boron oxide-containing BGs (MBG0118 and MBG0123) had a growth-inhibitory effect against 10^6 *S. aureus* (ATCC 25923) cells after 24 h cultivation at a concentration of 100 mg mL^{-1}. This is in contrast with our results, probably due to the concentration of BGs in their study was significantly higher than the used in the present work. It has been described that for both melt- and sol–gel-derived bioactive glasses the dose-dependency effect on dissolution and bioactivity is an important issue when carrying out in vitro cell-culture studies to simulate conditions in vivo.[35,36] At high concentrations, when there are excessive calcium ions in solution causing an increase in solution pH, calcium carbonate (calcite) forms on the surface of the glasses at the expense of a biologically active hydroxycarbonate apatite layer. For 45S5 BG this was verified when the glass concentration increased above a value of 0.002 g mL^{-1}.[35]

Attachment of *S. Aureus* (ATCC 29213) on sintered scaffolds.

SEM analysis of the samples from 24 h postincubation showed only bacterial adhesion on 45S5.2B scaffolds (Fig. 2). Moreover, neither 45S5 nor 45S5.2B scaffolds were seen to affect significantly the viability of the bacterial strain under investigation (Fig. 3). In addition, no relation could be found

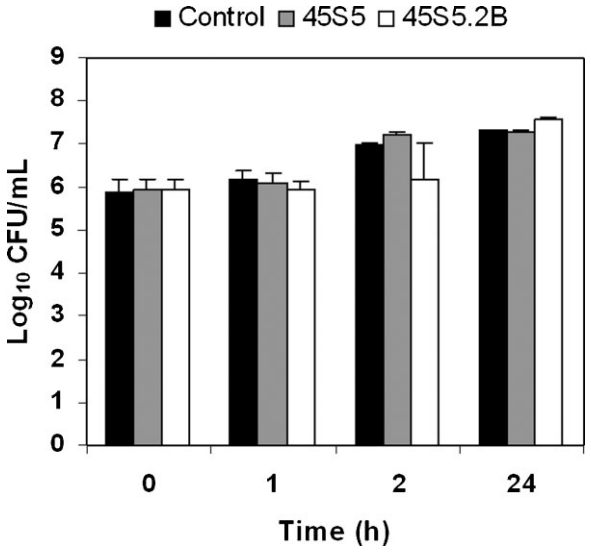

Fig. 1. Viable log_{10} numbers of CFU/mL of S. aureus (ATCC 29213) recovered post-incubation with the ionic dissolution products obtained from scaffolds after being immersed in HBSS for 28 day. Bacterial cultures without added ionic dissolution products served as controls.

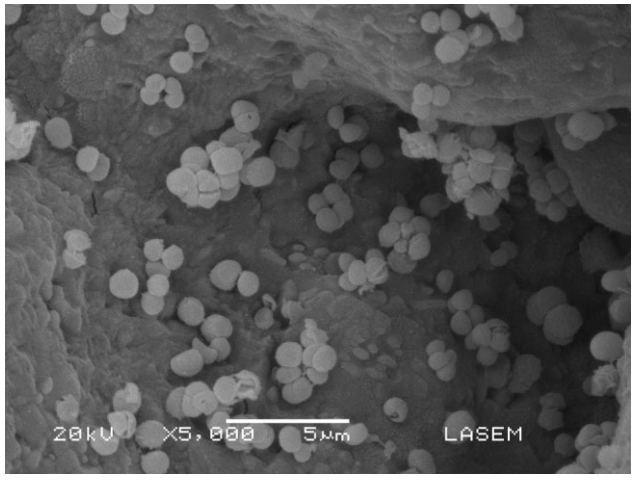

Fig. 2. SEM micrograph of a scaffold 45S5.2B exposed in vitro to S. aureus (ATCC 29213) for 24 h.

ADVANCED ENGINEERING MATERIALS

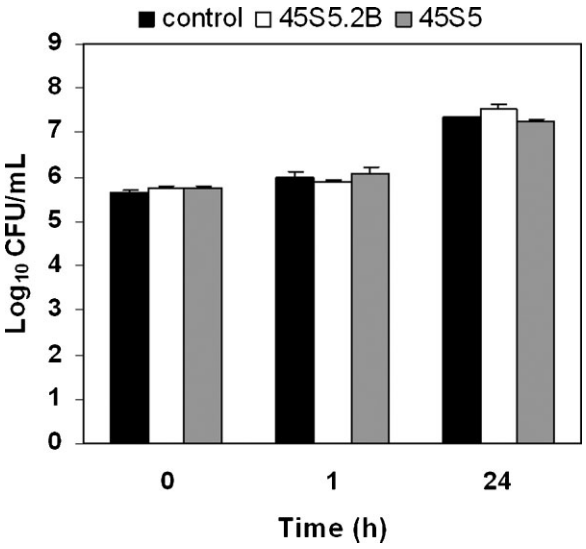

Fig. 3. Viable $\log_{10}$ numbers of CFU/mL of S. aureus (ATCC 29213) bacteria from the scaffolds after 1 and 24 h incubation in HBSS. Bacterial cultures without added BGs scaffolds served as controls.

between bacterial adhesion and scaffold's surface reactivity. After being immersed for 24 h, no statistically significant differences were observed in the pH values (6.51 ± 0.04) nor the ion concentrations of the major glass network former Si, as well as the glass modifiers Na, Ca, and P in solution (data not shown). It is noticeable that in the case of 45S5.2B scaffolds, the B content in the solution, 38 ± 1 and $101 \pm 5\ \mu g\ L^{-1}$ after 1 and 24 h post-incubation, respectively, did not exhibit antibacterial effect against *S. aureus*.

The adherence of *S. aureus* (15981) onto different multifunctional silica-based bioceramics has been evaluated by Kinnari *et al.*[37] It was found that bacterial adherence was higher on mesoporous bioceramics, although this higher microbial attachment was mainly due to the intergranular porosity and grain size/morphology rather than to the mesoporous structure. Additional studies are therefore necessary to clearly identify these different contributions to preferential adhesion of *S. aureus* to surfaces of boron-containing BG scaffolds, which are however beyond the scope of the present investigation at this stage.

The reports that analyzed the antibacterial effects of 45S5 Bioglass against several strains of *S. aureus* are controversial. Data were extremely variable, conceivably due to the fact that the success of *S. aureus* as an opportunistic pathogen is typically ascribed to its versatility via an inherent capacity to modulate behavior in response to changes in its environment.[38] Finally, the antibacterial activity of 45S5 Bioglass based glass-ceramic scaffolds on *S. aureus* was not supported by the present findings.

Future development of biodegradable tissue engineering scaffolds with tailored ion release and/or specific surface chemistry/topography, as well as functionalized surfaces through the use of biomolecules or antimicrobial agents may certainly prevent the initial bacterial adhesion and add knowledge to what is known about biomaterial-related infections representing an alternative to achieve better clinical results.

Conclusion

This experimental study provides the first evidence in vitro that bioactive glass-ceramic scaffolds made from both 45S5 Bioglass and from boron containing bioactive glass (45S5.2B) as well as their ionic dissolution products do not exhibit antibacterial effect against several strains of *S. aureus*.

Received: March 9, 2009
Final Version: April 3, 2009
Published online: June 18, 2009

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