J Mater Sci: Mater Med (2008) 19:547–551
DOI 10.1007/s10856-007-3018-5

Antibacterial effect of bioactive glasses on clinically important anaerobic bacteria in vitro

Outi Leppäranta · Minna Vaahtio · Timo Peltola · Di Zhang · Leena Hupa · Mikko Hupa · Heimo Ylänen · Jukka I. Salonen · Matti K. Viljanen · Erkki Eerola

Received: 18 October 2006 / Accepted: 27 March 2007 / Published online: 10 July 2007

Abstract Bioactive glasses (BAGs) of different compositions have been studied for decades for clinical use and they have found many dental and orthopaedic applications. Particulate BAGs have also been shown to have antibacterial properties. This large-scale study shows that two bioactive glass powders (S53P4 and 13–93) and a sol–gel derived material (CaPSiO II) have an antibacterial effect on 17 clinically important anaerobic bacterial species. All the materials tested demonstrated growth inhibition, although the concentration and time needed for the effect varied depending on the BAG. Glass S53P4 had a strong growth-inhibitory effect on all pathogens tested. Glass 13–93 and sol–gel derived material CaPSiO II showed moderate antibacterial properties.

O. Leppäranta · M. K. Viljanen · E. Eerola
Department of Medical Microbiology, University of Turku, Kiinamyllynkatu 13, Turku 20520, Finland

O. Leppäranta (✉)
Finnish Food Safety Authority Evira, Mustialankatu 3, Helsinki 00790, Finland
e-mail: oupale@utu.fi

M. Vaahtio · T. Peltola · H. Ylänen · J. I. Salonen
Turku Biomaterials Centre, University of Turku, Itäinen Pitkäkatu 4 B, Turku 20520, Finland

Present Address:
M. Vaahtio*Present Address:*
T. Peltola
Vivoxid Ltd., Tykistökatu 4 A, Turku 20520, Finland

D. Zhang · L. Hupa · M. Hupa · H. Ylänen
Process Chemistry Centre, Åbo Akademi University, Piispankatu 8, Turku 20500, Finland

Introduction

Bioactive materials are defined as materials eliciting a specific biological response at the interface of the material and tissue, resulting in the formation of a bond between them [1]. The bonding reflects the ability of these materials to react in body fluid and form a bone mineral-like calcium phosphate layer on their surfaces. Biocompatible, tissue-bonding bioactive glasses (BAGs) were first introduced in the early 1970s [2].

The base components in most bioactive glasses are SiO_2, Na_2O, CaO, and P_2O_5, and the weight percentages of these oxides vary in different glasses [1]. Since the invention of 45S5 Bioglass®, numerous glasses and glass ceramics with different compositions have been extensively studied for clinical use. Some compositions have also been successfully applied as solids and particulates to provide treatment for many disparate clinical conditions [3]. Nowadays BAGs are gaining use in both dental and orthopaedic applications.

BAG S53P4 has previously been shown to have an antibacterial effect on some oral microorganisms [4, 5]. Recently, the glass S53P4 and some other BAGs have shown similar properties on a variety of clinically important, aerobic pathogens [6]. The antibacterial action of BAGs has been suggested to be based on several factors, including high pH and osmotic effects caused by the nonphysiological concentration of ions dissolved from the glass [4], i.e. the antibacterial action of a BAG is influenced by its chemical composition and the dissolution conditions in its surroundings.

In this extensive work we evaluate the antibacterial efficacy of two conventional bioactive glass powders and a sol–gel derived material on 17 clinically important, anaerobic bacteria. For reliable results, the amount of live

bacteria exposed to BAGs at each run was standardized by analyzing the viability of the bacteria with a rapid flow cytometric method.

Materials and methods

Materials

The fine powders of bioactive glasses S53P4 and 13–93 were produced by Process Chemistry Centre, Åbo Akademi University, Turku [7]. The sol–gel derived material CaPSiO II was produced by the Turku Biomaterials Centre, Turku, according to a method reported previously [8]. E-glass (an inert reference glass) was obtained from Ahlström Glassfibre Oy (Karhula, Finland). Table 1 shows the compositions of the glasses. The glass powders were sieved to a particle size of ≤45 μm.

Microorganisms and culture conditions

The microorganisms used were *Bacteroides fragilis* (UK NEQAS, Sheffield, UK), *Bacteroides thetaiotaomicron* ATCC 29741 (American Type Culture Collection, Rockville, MD), *Bifidobacterium adolescentis* ATCC 15704, *Clostridium difficile* (a Finnish clinical isolate), *Clostridium perfringens* (a Finnish clinical isolate), *Clostridium septicum* 105020 (Hungary), *Eubacterium lentum* AHP 6425 (culture collection of the National Public Health Institute, Finland), *Fusobacterium necrophorum* CCUG 17326 (Culture Collection, University of Gothenburg, Sweden), *Fusobacterium nucleatum* NCTC 10562 (National Collection of Type Cultures, London, UK), *Peptostreptococcus anaerobius* AHC 5044 (culture collection of the National Public Health Institute, Finland), *Porphyromonas gingivalis* ATCC 35277, *Prevotella intermedia* ATCC 66563, *Prevotella melaninogenica* ATCC 25845, *Propionibacterium acnes* (a Finnish clinical isolate), *Propionibacterium propionicus* ATCC 14157, *Staphylococcus epidermidis* ATCC 14990, *Veillonella parvula* ATCC 10790.

All strains were grown anaerobically, i.e. in an anaerobic jar (Oxoid, Basingstoke, UK) filled with mixed gas (80% N_2, 10% H_2 and 10% CO_2). *S. epidermidis* was grown in Tryptone Soy Broth (LAB M, Bury, UK). All the other strains were cultivated in Fastidious Anaerobe Broth (LAB M). Prior to use, the broths were deoxidized in an anaerobic jar for at least 24 h.

The growth of *S. epidermidis* was evaluated on blood agar plates (Blood agar base (Pronadisa, Madrid, Spain) supplemented with 7.5% defibrinated sheep blood). The growth of other strains was evaluated on menadione-cysteine plates (Blood agar base (Pronadisa) supplemented with 2 g/L glucose, 5 g/L yeast extract (LAB M), 5% defibrinated sheep blood, 0.05% L-cysteine HCl (Merck, Darmstadt, Germany) and 0.5 ppm menadione (Merck)).

Bacterial viability testing

The bacteria were precultured in deoxidized broth at 37 °C for 20–24 h. The viability of the bacteria was monitored with a flow cytometric method by staining with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Eugene, OR) [6]. Briefly, a sample of the precultured bacteria was first diluted 100-fold in 0.9% NaCl. Then the cells were stained simultaneously with a green-fluorescent nucleic acid stain SYTO 9, which labels all bacteria in a population, and a red-fluorescent nucleic acid stain propidium iodide (PI), which penetrates only bacteria with compromised membranes. As a result, live bacteria stain green and dead ones red. For exact counting of bacteria in the samples, TruCount® tubes (Becton Dickinson, San Jose, CA) containing a known number of fluorescing microbeads were used.

Antimicrobial activity testing

The bacteria were cultured together with the BAG powders to evaluate their antibacterial activity. The dilution series of each glass was prepared in deoxidized broth in test tubes. The final glass concentrations tested were 400, 200, 100, and 50 mg/mL of broth. Powders of BAGs were first mixed and vortexed with the broth and the mixtures were deoxidized in an anaerobic jar for 2 h. Then 10^5–10^7 live bacteria were added to each tube.

The viability of the bacterial suspensions incubated with different concentrations of BAGs was assessed using solid agar plates. After 24 h cultivation in broth containing

Table 1 The chemical composition of the glasses in wt%

Glass	Na_2O	K_2O	MgO	CaO	B_2O_3	P_2O_5	Al_2O_3	SiO_2
S53P4	23.00	0.00	0.00	20.00	0.00	4.00	0.00	53.00
13–93	6.00	12.00	5.00	20.00	0.00	4.00	0.00	53.00
CaPSiO II	0.00	0.00	0.00	42.30	0.00	21.40	0.00	36.30
E-glass	0.13	0.70	0.66	23.50	6.40	0.00	14.10	53.90

BAG, 10 μL samples from the suspensions were plated. The growth of bacteria was evaluated after cultivation on agar plates at +37 °C for 2–3 days. Absence of growth on the plates was an indicator of bactericidal i.e. killing effect of a given BAG. A culture of the organism without added powder and a culture with inert E-glass (400 mg/mL) were included in each series as positive controls.

Results

All three BAGs tested inhibited bacterial growth at concentrations of 400, 200, 100, and 50 mg/mL. The effect of BAGs (400 mg/mL) on the growth of bacteria is presented in Table 2, and the average effect of a lower concentration of BAGs (200 mg/mL) on the growth of bacteria is shown in Fig. 1. The viability of bacteria in cultivations with BAGs was assessed by spreading samples to solid agar plates (see materials and methods). Absence of growth on the plate (–) was an indicator of bactericidal i.e. killing effect of a given BAG. Sparse and moderate growth, (+) and (++), respectively, indicate that the BAG has a growth-inhibitory effect. Good growth (+++) on a plate indicates no effect. The time needed for the effect varied depending on the BAG. The effect also varied between bacterial species, but no significant difference was seen between grampositive and gramnegative bacteria.

Table 2 The effect of BAGs (400 mg/mL) on the growth of bacteria at time points of 1 d/3 d

	S53P4	13–93	CaPSiO II
B. fragilis	nd/–	nd/+++	nd/+++
B. thetaiotaomicron	+/–	+++/nd	++/+++
B. adolescentis	–/–	+/–	+/–
C. difficile	–/–	+++/++	++/++
C. perfringens	+/–	+++/+++	++/+++
C. septicum	++/+	+++/++	+/++
*E. lentum**	–/–	+++/+++	–/–
F. necrophorum	++/–	+++/++	–/+++
F. nucleatum	–/–	++/–	+++/+++
P. anaerobius	–/–	+++/+++	++/++
P. gingivalis	–/–	–/–	+/–
P. intermedia	–/–	–/–	–/–
P. melaninogenica	–/–	–/–	–/–
P. acnes	–/–	+/–	–/–
P. propionicus	+/–	+/+++	+/+++
*S. epidermidis***	+/–	nd	+/+
V. parvula	+/–	+/–	+/–

–, No growth; +, Sparse growth; ++, Moderate growth; +++, Good growth; nd = not done

*2 d/7 d

**1 d/4 d (100 mg/mL)

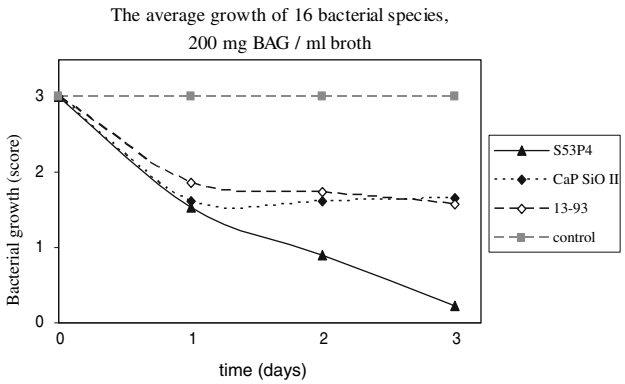

Fig. 1 The effect of BAGs on the growth of 16 pathogens. 3 = good growth (positive control), 2 = moderate growth, 1 = weak growth, 0 = no growth

The glass S53P4 was the most effective BAG since it had a clear growth-inhibitory effect even on the most resistant pathogens (Table 2). As seen in Table 2, BAG S53P4 generally had the fastest killing or growth-inhibitory effect. It also had antibacterial properties in lower concentrations than the other BAGs (*data not shown*). Table 2 also shows that while BAG S53P4 had an effect on the growth of all bacteria tested, BAGs 13–93 and CaPSiO II could inhibit the growth of only some species. Thus, BAGs 13–93 and CaPSiO II showed moderate antibacterial properties. E-glass (positive control) had no effect on the growth of bacteria.

In the presence of any BAG, even at the lowest concentrations, *P. intermedia* and *P. melaninogenica* were killed after 24 h of incubation and *V. parvula* after 72 h of incubation. *B. adolescentis* lost its viability in the presence of the BAGs at a concentration of 100 mg/mL (Fig. 2a).

All three BAGs killed *P. acnes* even at the lowest concentrations. During a seven-day experiment, *P. propionicus* lost its viability when exposed to 400 mg/mL S53P4 or 13–93. At the same concentration, CaPSiO II had a bacteriostatic effect on this bacterium.

Clostridium sp. forms a group of very resistant bacteria. Glass S53P4 had a bactericidal effect on *C. perfringens* while the other BAGs had no effect on its growth. All BAGs had a growth-inhibitory effect on *C. difficile* and *C. septicum*.

CaPSiO II had a bacteriostatic effect on *Fusobacterium* sp. *F. nucleatum* was killed when incubated with 100 mg/mL of either S53P4 or 13–93 (Fig. 2b). *F. necrophorum* was killed after 7 days of incubation in the presence of 100 mg/mL BAG S53P4 or 400 mg/mL BAG 13–93.

Bacteroides fragilis lost its viability in the presence of S53P4 while the other BAGs had only a minor bacteriostatic effect. *B. thetaiotaomicron* was killed by all three BAGs after 7 days of incubation.

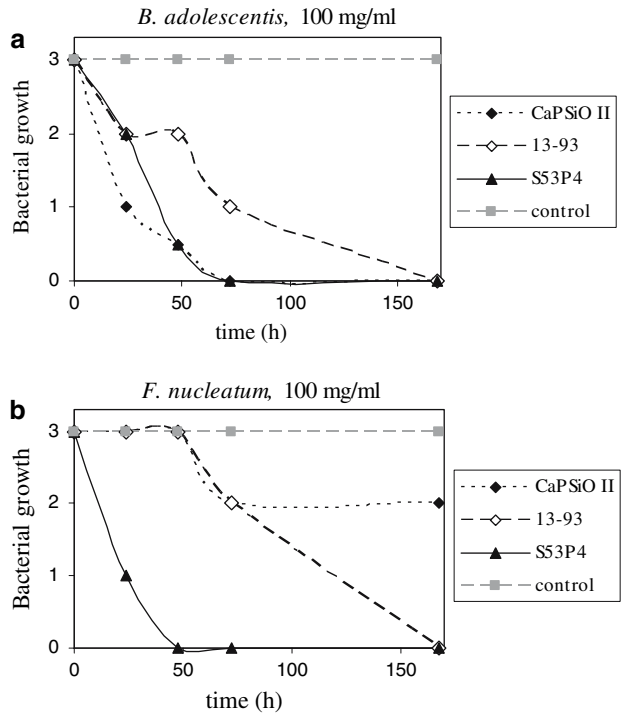

Fig. 2 (**a**) The effect of 100 mg BAG/mL on *Bifidobacterium adolescentis*. (**b**) The effect of 100 mg BAG/mL on *Fusobacterium nucleatum*

Glass 13–93 had no effect on the growth of *P. anaerobius* or *E. lentum*. CaPSiO II had a bacteriostatic effect and S53P4 a bactericidal effect on *P. anaerobius*. At a concentration of 400 mg/mL, the growth of *E. lentum* was totally inhibited by both S53P4 and CaPSiO II after 48 h of incubation.

Exposure to 100 mg/mL S53P4 killed *S. epidermidis* while at the same concentration CaPSiO II only reduced the number of CFUs. *Porphyromonas gingivalis* was killed after 24 h when exposed to 50 mg/mL S53P4 or 13–93, and after 48 h when incubated in the presence of 400 mg/mL CaPSiO II.

Discussion

We examined the antibacterial properties of two conventional bioactive glasses and one sol–gel derived material. Seventeen clinically important anaerobic bacterial species were cultivated in broth together with four concentrations of the BAGs. All materials tested inhibited the growth of bacteria. The anaerobic strains selected for the study contained the most common important anaerobic pathogens. In addition, some apathogenic (*B. adolescentis*, *E. lentum*, *P. acnes*, and *V. parvula*) common habitants of mouth, skin, and intestine normal flora were selected. These species were the most obvious anaerobic bacteria which could be involved if clinical applications of the glasses were designed.

The final glass concentrations in this study were chosen based on previous results [6]. In clinical applications, varying concentrations of BAGs can be used. For example, Stoor et al. [4] simulated a composition used for treatment of hypersensitive teeth with 1.67 g BAG S53P4/mL liquid and even higher concentrations of 45S5 Bioglass® were used in the experiments of Allan et al. [9]. This study shows that BAGs have antibacterial properties also at lower concentrations.

In aqueous solutions, dissolution of the glass network is rapid until the solution becomes saturated with respect to silica [10]. The release of network-modifying ions leads to an increase in interfacial pH [1]. The dissolution also creates increased osmotic pressure in the vicinity of a BAG [4]. The dissolution of the glass network is influenced by its chemical composition and the conditions in its surroundings. This might explain at least some of the differences in the antibacterial action of BAGs with varying chemical compositions. The dissolution behaviour of BAGs S53P4 and 13–93 has been studied by Zhang et al. [11, 12]. Ion release of sol–gel derived material CaPSiO II has also been published previously [13].

BAG S53P4 had a clear growth-inhibitory effect on all pathogens tested. This corroborates the findings from previous studies, which showed that particulate S53P4 has antibacterial properties on some oral bacteria [4, 5] as well as on many other clinically important aerobic bacteria [6].

In our experimental set-up, the mixtures of BAGs and broths were deoxidized prior to the antibacterial tests. This step removes all oxygen bound in the materials and these altered conditions may affect the solubility of a BAG. Sol–gel derived materials are very porous [8]. Due to the porous structure, there is more bound oxygen in sol–gel derived materials than in the other BAGs. In anaerobic conditions, CaPSiO II has only a minor increasing effect on the pH of the solution (M. Vaahtio, unpublished data).

The exact mechanisms of the antibacterial action of BAGs are unknown. It has been suggested to be based on several influences, including high pH and osmotic effects caused by the nonphysiological concentration of ions dissolved from the glass [4]. Thus, the weak pH effect of CaPSiO II in anaerobic conditions may, at least partly, give a reason for its relatively weak antibacterial properties on anaerobic bacteria compared to its strong bactericidal effect on aerobic bacteria [6].

BAG 13–93 had moderate antibacterial properties. Thus far, its solubility in anaerobic conditions has not been studied. In general, the antibacterial effects of all the BAGs tested were slower and weaker in anaerobic than in aerobic conditions [6].

Practically all systems used for testing antibacterial properties of various substances are very sensitive to the inoculate effect, i.e. the amount of live bacteria exposed to the materials affects the results. To minimize the variation, we stained the bacteria with two dyes (SYTO 9 and PI) and analyzed the stained bacteria with FCM, which could discriminate between live and dead bacteria. This cytometric approach enabled us to equalize the number of live bacteria inoculated in each run thus making the results more reliable.

In some previous studies it has been shown that a BAG has antibacterial properties only if it contains silver [14, 15]. For example, in the study of Catauro et al. [15] the bacteria were in contact with the BAG only for a very short time, and the lack of dissolution of the glass network might explain their results. The antibacterial effect of silver is widely known and it has been used as an antimicrobial agent in the medical field for centuries. Bacteria show a low propensity to develop resistance to silver-based products [16] but some silver-resistant bacteria have been reported and widespread use of silver may result in more bacteria developing resistance analogous to antibiotic-resistant bacteria [17]. The advantage of S53P4, CaPSiO II, and 13–93 is that the constituent chemicals are all found in the body, which may decrease the possibility that bacteria develop resistance to these materials.

The possibility of bacterial adhesion is a remarkable problem concerning the use of prostheses and other medical devices introduced in the body. Several studies have shown that BAGs have bactericidal properties on both aerobic and anaerobic bacteria [4–6]. Therefore, it appears evident that the antibacterial effects of BAGs tested cover a wide selection of clinically important pathogens. Modifications of the surfaces of prostheses and other medical devices, e.g. by coating them with a suitable BAG, may prevent bacterial adhesion and thus prevent the tissues around them from being infected. For example, there are some preliminary results suggesting that coating with a silver-containing bioactive glass limits bacterial attachment to surgical sutures [18].

Conclusions

Two conventional bioactive glasses (S53P4 and 13–93) and a sol–gel derived material (CaPSiO II) were found to inhibit the growth of a wide selection of clinically important anaerobic pathogens. The antibacterial effect of these materials varied between bacterial species but there was no significant difference between grampositive and gramnegative species. BAG S53P4 was the most effective, inhibiting efficiently the growth of all pathogens tested. BAG 13–93 and sol–gel derived material CaPSiO II showed moderate antibacterial effects. In general, the antibacterial effects of all the BAGs tested were slower and weaker in anaerobic than in aerobic conditions. The mechanism of the antibacterial action of BAGs is probably based on a combination of several factors, including high pH and osmotic effects caused by dissolution of the glass network and network-modifying ions. For reliable results, the amount of live bacteria exposed to BAGs at each run was standardized by analyzing the viability of the bacteria with a rapid flow cytometric method.

Acknowledgement This study was supported by the Finnish Funding Agency for Technology and Innovation (TEKES).

References

1. L. L. HENCH and Ö. ANDERSSON, in "An Introduction to Bioceramics" (World Scientific Publishing Co, Singapore, 1993) p. 41
2. L. L. HENCH and H. A. PASCHALL, *J. Biomed. Mater. Res.* **7** (1973) 25
3. J. WILSON, A. YLI-URPO and R. HAPPONEN, in "An Introduction to Bioceramics" (World Scientific Publishing Co, Singapore, 1993) p. 63
4. P. STOOR, E. SÖDERLING and J. I. SALONEN, *Acta Odontol. Scand.* **56** (1998) 161
5. E. KEROSUO, V. O. ROTIMI, S. SURANA, F. KODHAKHAST and J. I. SALONEN, *Dental News* **X** (2003) 33
6. E. MUNUKKA, O. LEPPÄRANTA, M. KORKEAMÄKI, M. VAAHTIO, T. PELTOLA, D. ZHANG, L. HUPA, H. YLÄNEN, J. I. SALONEN, M. K. VILJANEN and E. EEROLA, *J. Mater. Sci. Mater. Med*. In press, DOI 10.1007/s10856-007-3143-1
7. H. ARSTILA, L. FRÖBERG, L. HUPA, E. VEDEL, H. YLÄNEN and M. HUPA, *Glass Technol.* **46** (2005) 138
8. T. PELTOLA, M. JOKINEN, H. RAHIALA, E. LEVÄNEN, J. B. ROSENHOLM, I. KANGASNIEMI and A. YLI-URPO, *J. Biomed. Mater. Res.* **44** (1999) 12
9. I. ALLAN, H. NEWMAN and M. WILSON, *Biomaterials* **22** (2001) 1683
10. Ö. H. ANDERSSON, J. ROSENQVIST and K. H. KARLSSON, *J. Biomed. Mater. Res.* **27** (1993) 941
11. D. ZHANG, E. VEDEL, L. HUPA, H. YLÄNEN and M. HUPA, *Key Eng. Mater.* **284–286** (2005) 481
12. D. ZHANG, E. MUNUKKA, L. HUPA, H. YLÄNEN, M. K. VILJANEN and M. HUPA, *Key Eng. Mater.* **330–332** (2007) 173
13. M. VAAHTIO, E. MUNUKKA, O. LEPPÄRANTA, D. ZHANG, E. EEROLA, H. YLÄNEN and T. PELTOLA, *Key Eng. Mater.* **309–311** (2006) 349
14. M. BELLANTONE, H. D. WILLIAMS and L. L. HENCH, *Antimicrob. Agents Chemother.* **46** (2002) 1940
15. M. CATAURO, M. G. RAUCCI, F. DE GAETANO and A. MAROTTA, *J. Mater. Sci. Mater. Med.* **15** (2004) 831
16. J. J. BLAKER, S. N. NAZHAT and A. R. BOCCACCINI, *Biomaterials* **25** (2004) 1319
17. A. GUPTA and S. SILVER, *Nat. Biotechnol.* **16** (1998) 888
18. J. PRATTEN, S. N. NAZHAT, J. J. BLAKER and A. R. BOCCACCINI, *J. Biomater. Appl.* **19** (2004) 47