

01 Sep 2016

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Recommended Citation

M. Ottomeyer et al., "Broad-Spectrum Antibacterial Characteristics of Four Novel Borate-Based Bioactive Glasses," *Advances in Microbiology*, vol. 6, no. 10, pp. 776-787, Scientific Research, Sep 2016.

The definitive version is available at <https://doi.org/10.4236/aim.2016.610076>



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Broad-Spectrum Antibacterial Characteristics of Four Novel Borate-Based Bioactive Glasses

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How to cite this paper: Ottomeyer, M., Mohammadkah, A., Day, D. and Westenberg, D. (2016) Broad-Spectrum Antibacterial Characteristics of Four Novel Borate-Based Bioactive Glasses. *Advances in Microbiology*, 6, 776-787.

<http://dx.doi.org/10.4236/aim.2016.610076>

Received: May 30, 2016

Accepted: September 3, 2016

Published: September 6, 2016

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Abstract

Bioactive glasses have been developed for medical applications in the body for bone and tissue repair and regeneration. We have developed a borate-containing bioactive glass (13-93B3, referred to as B3), which is undergoing clinical trials to assess its wound-healing properties. To complement the healing properties of B3, metal ion dopants have been added to enhance its antimicrobial properties. Bioactive glasses doped with silver, gallium or iodine ions were found to have broad spectrum antimicrobial effects on clinically relevant bacteria including MRSA. While the B3 glass alone was sufficient to produce antibacterial effects on select bacteria, adding dopants enhanced the broad-spectrum antibacterial properties: Live-Dead staining fluorescence microscopy suggests cell membrane integrity is disrupted in gram positive bacteria exposed to the glass compounds, but not gram negative bacteria, indicating multiple mechanisms of action for each glass formulation.

Keywords

Bioactive Glass, Antibacterial Properties, Metal Ions

1. Introduction

Hospital-acquired infection, also known as nosocomial infection, is a major health care problem that results in longer hospital stays, an increase in the cost of healthcare, and health risks in patients. Common sites for nosocomial infections include medical implants [1] [2] wounds, the urinary tract and the respiratory tract [2].

The presence of infection is not only a problem at the site of the medical implant, but in the surrounding structures and tissues. Chronic bone infections like osteomyelitis can result from infection in surrounding soft tissue [3], adding more complications to infections acquired after surgery or the implantation of a medical device. Because of

their biocompatibility, bioactive glasses show promise as materials that can be used for medical implants and may reduce the risk of infection.

Bioactive glass can be defined as a glass-ceramic material that is both biocompatible and surface reactive. This class of biomaterials was originally developed to address the problem of rejection associated with metal or plastic surgical implants [4]. In addition, bioactive glasses have been developed for bone and tissue repair, dental and maxillofacial repair [5], and soft tissue repair [1] and have also been used as drug delivery vehicles for bone disease and infection [3] [6] [7].

Recently, bioactive glass research has undergone rapid growth, with the number of papers published in the field doubling between the years 2000 and 2011 [8]. The use of borate-based bioactive glasses as an alternative to silicate-based bioactive glasses has also been an emerging trend in the field of biomaterials. Since borate bioactive glasses do not yet have the well-established history of silicate bioactive glasses, there is still little known about their benefits and mechanisms in biological applications [7].

One benefit of borate bioactive glasses is that their reaction rate can easily be altered by the boron content of the glass [3]. Easily changing the reaction rate of the glass is beneficial, as this allows for the surface-reactivity of the glass to be tailored to its specific use on a case-by-case basis. In addition, borate glasses have been shown to have added biological effects. For instance, borate-based bioactive glasses have been shown to promote cell proliferation, cell differentiation [8], and promote wound healing [9]. Borate alone has also been shown to have antimicrobial properties, and has actually been used as an ancient remedy to soothe skin infections and wounds [10]. While the antimicrobial capabilities of borate-based biomaterials are not well documented, borate chemistry in solution has been studied, and potential mechanisms include energy depletion by binding NAD and NADH [11] [12], and initiating DNA damage by binding to ribose groups [11]-[13].

Glass 13-93B3 (B3), a novel bioactive borate glass, is currently undergoing clinical trials for its wound healing abilities [9]. While little is known about the exact mechanism by which the glass stimulates wound healing, this property of B3 makes it a promising borate-based bioactive glass to be used in clinical applications. The addition of metal ions to B3 enhances wound healing, angiogenesis and nerve cell growth.

In addition to modifying their reactive properties, modifying bioactive glasses with metal ions can also make the glasses antimicrobial. Silver has long been known to have antimicrobial properties, and has been incorporated into various materials to inhibit bacterial growth. Silver ions are typically incorporated into the bioactive glass as silver oxide (Ag_2O) which leaches out of the glass to inhibit microbial growth. Because bioactive glass is porous [13] and can have its reaction rate adjusted by changes to its composition [4], incorporation of silver ions into a bioactive glass allows for a slow, controlled delivery of the ions over an extended period of time..

Bellantone and colleagues showed that a silver oxide-doped silicate glass showed bactericidal activity against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* over a 20 hour incubation period. Their study also showed that levels of free

silver in solution decreased over time, meaning that the bacteria were taking up the silver ions before dying [14]. These findings suggest that the silver ions have bactericidal effects by interacting with intracellular components of the bacteria, rather than interaction with the cell wall or outer membranes of the cells. In addition, it was noted that reactivity of bioactive glasses in water results in a slight increase in pH yet the increase in pH alone is not sufficient to kill the bacteria in solution [14].

Other studies have also shown antimicrobial activity of silver-doped bioactive glasses against various bacteria. Overall, the presence of silver ions in bioactive glass has bactericidal properties against *E. coli* [14]-[17], *Bacillus anthracis* [15], *Pseudomonas pyocyanea* [15], *P. aeruginosa* [13] [16], *Candida albicans* [16], *Salmonella* sp. [15], *Streptococcus* sp. [15], and *S. aureus* [13] [16] [18]. Silver ions in solution have also shown antimicrobial efficacy against *P. aeruginosa*, *S. aureus*, and *C. albicans* when in combination with natural products [19]. Additionally, silver nanoparticles have been incorporated into polymers and have also shown antimicrobial effects [17] [20] [21]. These previous studies show that silver is a reliable antimicrobial component of silicate based bioactive glasses, and is likely to have bactericidal capabilities if integrated into a novel bioactive glass such as B3.

Since silver-doped silicate bioactive glasses are well-studied, there are various proposed mechanisms for how silver inhibits bacterial growth and gives bioactive glass bactericidal capabilities. Such proposed mechanisms include complexing with thiol [1], sulfhydryl, amino, or hydroxyl functional groups [13], competing with copper ions as a cofactor in transport or enzymatic reactions [13], general toxicity [13] [22], disruption of aliphatic carbon-hydrogen bonds by insertion of carbenes [23], DNA damage by arene-purine hydrophobic interactions [23], direct binding to DNA [1], increased permeability and disruption of cell membranes [1], and inhibition of respiratory [1] or signaling [21] enzymes.

While the addition of silver is a historically reliable means of introducing bactericidal capabilities to silicate based bioactive glass, the use of other ions is gaining popularity [24]. Valappil and colleagues showed a decrease in *P. aeruginosa* viability after exposure to a gallium-doped bioactive glass, as well as a reduction of biofilm growth [25] [26]. Doping bioactive glass with other ions, such as yttrium, selenium, and iodine also show potential for antimicrobial activity [21] [24]. Gallium doped sol-gel phosphate-based glasses have also shown promising antimicrobial activity against *S. aureus* and *Streptococcus mutans* [27] and phosphate based glasses doped with both silver and gallium have been shown to be effective against *P. aeruginosa* biofilm formation [25]. Further studies have revealed that antimicrobial effects of gallium are likely intracellular and involve disruption of bacterial iron uptake [27] [28].

From these findings, it is clear that the presence of borate as well as various ions, including gallium and iodine, may enhance the bactericidal activity of borate bioactive glasses. The broad-spectrum antimicrobial activity of the borate bioactive glass, B3, doped with silver, gallium, and iodine as well as B3 alone was tested against a variety of clinically relevant pathogens. In addition, the mechanism of action by which the bioac-

tive glasses worked against both gram-negative and gram-positive bacteria was further studied by Live-Dead fluorescence microscopy.

2. Methods

Bacterial Strains

All bacterial strains used in the following experiments are described in **Table 1**.

Glass Composition

Bioactive glasses were prepared in the Missouri S & T Department of Materials Science and ground to a particle size of less than 45 micrometers. The composition of each glass used is given in **Table 2**.

Well Diffusion Assay

100 ul of overnight broth culture grown in Trypticase Soy Broth (TSB) (Difco Labs, Detroit, MI) was plated in triplicate on Trypticase Soy Agar (TSA) (Difco Labs, Detroit,

Table 1. Bacterial isolates used in experiments.

Organism	Clinical Relevance	Source*
Gram-negative organisms		
<i>Enterobacter cloacae</i>	Abdominal infections, abscesses, urinary tract infections	Difco (23355)
<i>Escherichia coli</i>	Enteric disease, urinary tract infections	ATCC (25922)
<i>Klebsiella pneumoniae</i>	Urinary tract infections, pneumonia	Difco (13883)
<i>Moraxella catarrhalis</i>	Respiratory tract infections, meningitis	Carolina (154928)
<i>Proteus mirabilis</i>	Urinary tract infections	Carolina (155239)
<i>Proteus vulgaris</i>	Wound infections, urinary tract infections	Carolina (155240)
<i>Pseudomonas aeruginosa</i>	Wound infections, burn infections, pneumonia, urinary tract infections	Difco (27853)
<i>Serratia marcescens</i>	Respiratory tract infections, urinary tract infections, conjunctivitis	Difco (8100)
<i>Shigella flexneri</i>	Enteric disease, abdominal infections	ATCC (12022)
<i>Shigella sonnei</i>	Enteric disease, abdominal infections	ATCC (25931)
<i>Vibrio natriegens</i>	<i>Vibrio</i> species, simulant for <i>Vibrio cholera</i>	ATCC 14048
Gram-positive organisms		
<i>Enterococcus faecalis</i>	Urinary tract infections, subacute endocarditis	Carolina (155600)
<i>Staphylococcus aureus</i> (MRSA)	Skin infections, wound infections, abscesses, surgical infections, osteomyelitis, enteric disease	ATCC (BAA-44)
<i>Staphylococcus epidermidis</i>	Chronic skin infections, bacterial endocarditis from ventriculo-atrio shunts/implants	Difco (12228)
<i>Staphylococcus epidermidis</i>	Biofilm forming clinical isolate from medical implant	ATCC (35984)

*ATCC (American Type Culture Collection) (ATCC number); Carolina (Carolina Biological Supply) (Carolina Biological Supply item number); Difco (Difco Bactrol Disks, Difco Laboratories) (ATCC number).

Table 2. Compositions of bioactive glasses used.

Component	Percent Weight			
	B3	B3-Ag	B3-Ga	B3-I
B ₂ O ₃	53%	52%	52%	53%
CaO	20%	20%	20%	20%
K ₂ O	12%	12%	12%	10%
Na ₂ O	6%	6%	6%	6%
MgO	5%	5%	5%	5%
P ₂ O ₅	4%	4%	4%	4%
Ag ₂ O	-	1%	-	-
Ga ₂ O ₃	-	-	1%	-
I	-	-	-	2%

Compositions of bioactive glasses used.

MI) plates to make wells in each plate, a sterile, plastic tube was dipped into 70% ethanol for 10 seconds, allowed to air dry, and then plunged into the TSA plate and removed. The plug of TSA was then discarded. To the well, 20 µl of molten agar was added and allowed to cool to seal the bottom of each well.

Glass suspensions were prepared by suspending 100 mg of glass powder in 500 mL sterile saline solution. 50 µl of glass solution (10 mg glass) was added to each well. The plates were incubated at 37°C for 24 hours. After incubation, the distance between the edge of the well to the beginning of bacterial growth on the plate was measured to determine the zone of inhibition.

A 2-way ANOVA of the sensitivity data comparing the size of the zone of inhibition of each glass formula to control for each bacterial species was performed to determine differences in sensitivity of different bacteria to the four glass formulas.

Live-Dead Staining Assay

Samples were prepared in accordance with the Bio-Rad Live-Dead staining kit. 50 µl of glass solution (prepared as described above) was added to 1 ml of overnight broth culture, and the mixture was centrifuged at 1100 rpm for 10 minutes. After removal of the supernatant, the pellet was re-suspended in 500 µl of staining buffer (including Propidium Iodide and Live-Dye™) and incubated at 37°C for 15 minutes. After incubation, a slide was prepared by spotting 5 µl of the solution on a glass microscope slide and covered with a glass coverslip. The slide was then viewed using the Olympus IX51 inverted microscope at 4000× using Fluorescein Isothiocyanate (FITC), Texas Red (TxRed), and Differential Interference Contrast (DIC) filters. Channel images were captured with a Hamamatsu digital camera, and the number of live cells was determined by counting the number of green cells, which were viewed through the FITC channel. The number of dead cells was determined as the number of red cells, which were viewed in the TxRed channel. The total number of cells was the sum of live and dead cells for the given field, and percent viability was determined as the percentage of

live cells over the total number of cells. Changes in viability compared to controls were analyzed using a one-way ANOVA with $\alpha = 0.05$.

3. Results

Based on the well diffusion assay, gram positive and gram negative bacteria showed sensitivity to each glass, however, the glasses appeared to have a greater antimicrobial effect on gram positive species. The borate in the B3 glass alone was sufficient to inhibit growth for *E. coli*, *Shigella sonnei*, *Vibrio natriegens*, *Staphylococcus epidermidis* 12,228, *Serratia marscesens*, and methicillin resistant *S. aureus* (MRSA), as the zone of inhibition was significantly greater than the B3 glass control composition (**Figure 1**). Addition of ions to the B3 glass had varied effects on antimicrobial activity. Growth of *Moraxella catarrhalis* was inhibited significantly only with the Iodine-containing glass formulation (**Figure 1**). The addition of iodine to the B3 glass formulation improved or maintained the antimicrobial properties of B3 against MRSA, *S. marscesens*, *S. epidermidis*, and *E. coli* (**Figure 1**). However, Iodine addition decreased the antimicrobial properties of B3 against *V. natriegens* and *S. sonnei* (**Figure 1**). The addition of silver to B3 drastically improved antimicrobial activity against *S. epidermidis* 12,228 but was actually the least effective glass formulation against MRSA.

Live-Dead staining fluorescence microscopy was used to further evaluate the potential mechanism of action of each glass formulation against gram negative and gram positive bacteria. This method uses differential dyes in order to differentiate cells with intact cell membranes (live) from cells with disrupted cell membranes (dead). LiveDye selectively binds intact membranes and fluoresces green under a FITC filter while PI intercalates intracellular DNA and is viewed as red through a TxRed filter. PI does not pass through intact cell membranes to bind DNA, so it will only be visible if the cell membrane has been disrupted.

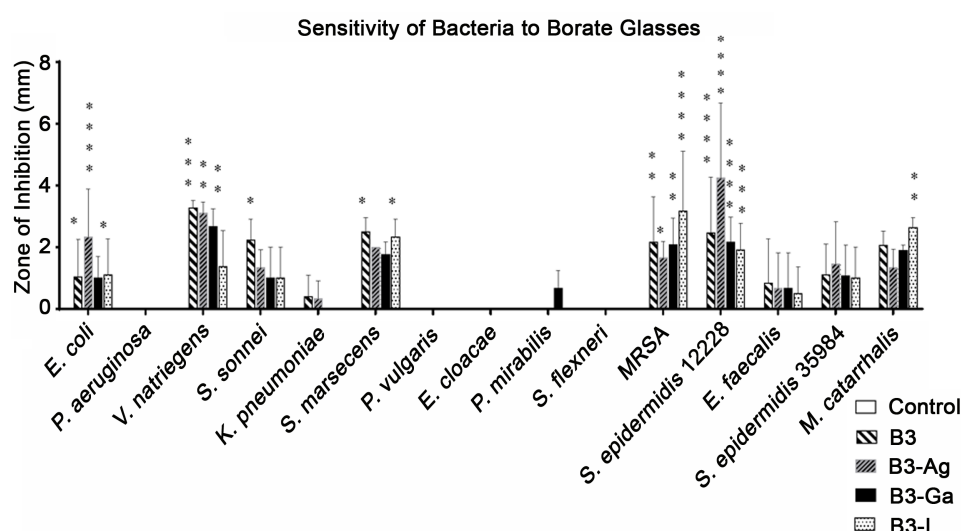


Figure 1. Sensitivity of bacteria to borate glasses. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

The viability of bacterial cells determined by Live-Dead staining appeared to decrease markedly for MRSA and *S. epidermidis* 12,228, but did not decrease significantly for *E. coli* with exposure to glass compositions. MRSA and *S. epidermidis* 12,228 treated with B3-Ga and B3-I had significant decreases in viability compared to controls after 15-minute incubation (Figure 2 and Figure 3). After 24-hour incubation, MRSA viability significantly decreased with contact with the B3, B3-Ag, and B3-I glasses, but interestingly, did not have a significant decrease in contact with the B3-Ga glass (Figure 2).

Likewise, the percent viability of *S. epidermidis* also decreased after 24 hour incubation with each glass, most significantly with B3-Ga and B3-I. However only contact with B3, B3-Ag, and B3-Ga resulted in significant decreases in viability after a 15 minute incubation period (Figure 3).

Given that *E. coli* was most sensitive to B3 and B3-Ag in well-diffusion assays, only these two glasses were used in Live-Dead staining microscopy assays. The viability of *E. coli* based on the Live-Dead staining did not decrease significantly with any of these glass formulations after either a 15 minute or 24 hour incubation period (Figure 4).

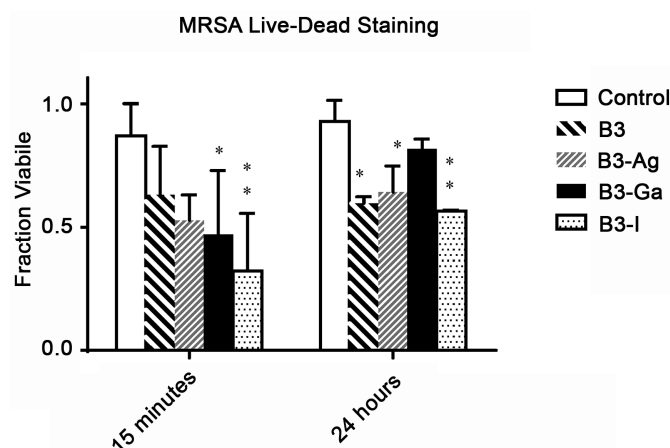


Figure 2. Live-dead staining of MRSA. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.



Figure 3. Live-dead staining of *S. epidermidis* 12,228. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

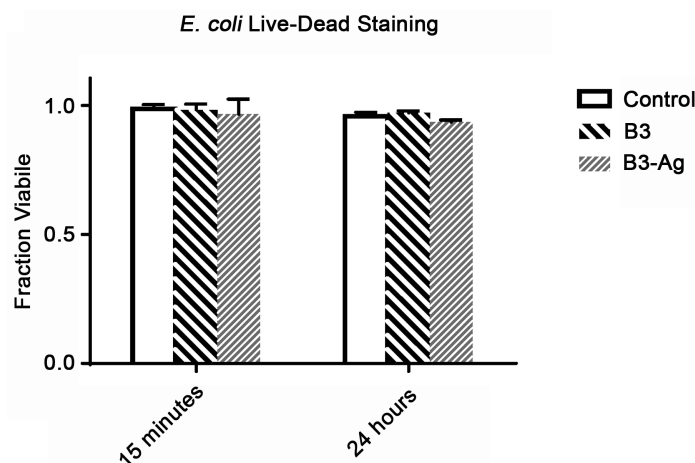


Figure 4. Live-dead staining of *E. coli*. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

4. Discussions

One of the most serious nosocomial pathogens plaguing hospitals today is Methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA infection has many risk factors, including recent surgery or hospital stay, the presence of a medical implant, such as a catheter and undergoing dialysis [2]. Recent surgery or the presence of a medical implant can be said to be the most significant risk factors, as the presence of a surgical implant decreases the minimum infectious dose of MRSA by 100,000 fold [3]. While there are numerous risk factors for MRSA infection, otherwise healthy individuals with no risk factors can also acquire MRSA infections, making MRSA not only a nosocomial pathogen, but also a community-associated pathogen [2].

Other nosocomial pathogens besides MRSA are also relevant when considering recent surgery or the presence of a medical implant as a risk factor. For instance, *E. coli* and *P. aeruginosa* have been found at surgical implant sites and have been implicated in infections after surgical procedures and urinary catheters [4]. The presence of biofilm-forming bacteria poses an increased risk of infection at implant sites, as these bacteria are usually more resistant to antibiotic treatment as well as host defenses [29] and gain protection from surrounding bacteria within the biofilm at the infection site [1]. Other risk factors that can predispose a patient to an implant-associated infection include old age and pre-existing health conditions, such as diabetes mellitus, rheumatoid arthritis, or HIV infection [30]. In addition to complications such as osteomyelitis, other factors contribute significantly to the seriousness of nosocomial infections. For instance, one study reported that MRSA infections were responsible for more deaths in the United States than AIDS [31] making prevention of nosocomial infections, especially MRSA, a critically important field of research.

Overall, the glass formulations tested in the present work appeared to have a broad spectrum of antimicrobial activity, inhibiting growth of both gram negative and gram positive organisms. Borate (B_2O_3) alone in the B3 formulation was sufficient to inhibit growth of *E. coli*, *S. sonnei*, *V. natriegens*, *S. epidermidis* 12,228, *S. marcescens*, and

MRSA, while the addition of other ions to the B3 glass had varied effects on antimicrobial activity and specific sensitivities of bacteria (Figure 1). This suggests borate based bioactive glasses may have inherent antimicrobial activity, which could be considered an advantage to their use as an alternative to silicate based bioactive glasses. However, the addition of ions to the B3 glass resulted in lack of significant difference in growth inhibition compared to controls for *S. sonnei*, suggesting addition of these ions provided a protective factor for reducing the effect of B3 glass against this species. Conversely, the addition of ions showed increased antimicrobial activity of the B3 glass for most of the other susceptible species (Figure 1). Addition of silver ions was especially effective in increasing antimicrobial activity of B3 against *E. coli* and *S. epidermidis* 12,228, while the addition of gallium ions reduced the antimicrobial activity against *E. coli* (Figure 1). *M. catarrhalis*, however, was sensitive only to the B3 glass that contained iodine ions (Figure 1). This suggests that gallium and iodine are potential candidates for use in bioactive borate glasses in order to provide antimicrobial coverage to pathogens that are not sensitive to silver.

MRSA was sensitive to all four glass formulations, but least sensitive to B3-Ag. Since silver has long been used as an antimicrobial agent, the resistance mechanisms employed by MRSA to resist multiple antibiotic drugs may also allow the bacteria to partially resist the antimicrobial effects of B3-Ag glass (Figure 1).

Differences in sensitivity based on glass formulation suggest varied mechanisms of action of each formulation and against different types of bacteria. To further evaluate cell membrane disruption as a potential mechanism of action, Live-Dead Staining Fluorescence microscopy was used to determine changes in viability after 15 minute and 24 hour incubations with the different glass compositions. The Live-Dead staining allowed for discrimination between cells with intact cell membranes and cells with cell membrane disruption that would lead to cell death. Viability determined by Live-Dead staining was decreased for the gram positive bacteria, including MRSA (Figure 2) and *S. epidermidis* 12,228 (Figure 3), after exposure to the glass formulations, but not for the gram-negative bacteria, *E. coli* (Figure 4). This data suggests that while *E. coli* is sensitive to most of the glass formulations as seen in the well-diffusion assay (Figure 1), its growth is not inhibited by the glass disrupting the cell membrane. Further, Live-Dead staining analysis of viability supported that the glass formulations exert antimicrobial activity on gram positive bacteria by a mechanism that disrupts cell membrane integrity. Differences in the effect of each glass formulation on viability depending on incubation time also reveals subtle differences in glass kinetics and duration of action, allowing for a better understanding of how each glass formulation may behave *in vivo*.

While each of the glass formulations were not effective against all of the bacteria tested, the group of the bacteria found to be sensitive to the glass represent a sizeable portion of clinically relevant pathogens capable of causing nosocomial infections. The primary clinical application of these materials is as glass fibers for wound repair. The use of glass ground to less than 45 micrometers was chosen to simulate the approximate diameter of these fibers and mimic the behavior of the glass when it contacts bodily

fluids. Other potential uses of these borate-based glass formulations may be as coatings for medical devices commonly associated with nosocomial infections in order to prevent infection by inhibition of bacterial growth. This can be accomplished by incorporating the 45 micrometer size particles into polymers and coatings to be applied to surfaces. The bioactive nature of these glass formulations also allows for their use as bone grafts and tissue repair, allowing for a dual-purpose of the glass to both bind to and react with bone while preventing or treating bone infections. The advantage of using these antibacterial materials will be to minimize the occurrence of infections associated with standard clinical procedures and reduce the need for antibiotics following procedures.

Further work to improve the efficacy of the glass formulations against clinically relevant bacteria, as well as fungi, and to more clearly elucidate the mechanism of action for each formulation, is vital in guiding treatment decisions with clinical use of these materials. Additionally, a B3 formulation including silver, gallium, and iodine in one glass composition could be useful in making the materials have a wider spectrum of antimicrobial activity as well as potentially preventing future resistance to the glass formulations.

Acknowledgements

Thank you to Dr. Katie Shannon, Terry Wilson, Brianna Kroegger, Justin Lovelady, and Michael Lockett for their contributions to this research.

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