Spring 2013

Broad-spectrum antibacterial properties of metal-ion doped borate bioactive glasses for clinical applications

Megan Ottomeyer

Department:

BROAD-SPECTRUM ANTIBACTERIAL PROPERTIES OF METAL-ION DOPED BORATE BIOACTIVE GLASSES FOR CLINICAL APPLICATIONS

by

MEGAN OTTOMEYER

A THESIS

Presented to the Faculty of the Graduate School of the

MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE IN APPLIED AND ENVIRONMENTAL BIOLOGY

2013

Approved by

David Westenberg, Advisor
Delbert Day
Melanie Mormile

ABSTRACT

Bioactive glasses with antimicrobial properties can be implemented as coatings on medical devices and implants, as well as a treatment for tissue repair and prevention of common hospital-acquired infections such as MRSA. A borate-containing glass, B3, is also undergoing clinical trials to assess wound-healing properties. The sensitivities of various bacteria to B3, B3-Ag, B3-Ga, and B3-I bioactive glasses were tested. In addition, the mechanism of action for the glasses was studied by spectroscopic enzyme kinetics experiments, Live-Dead staining fluorescence microscopy, and luminescence assays using two gene fusion strains of *Escherichia coli*.

It was found that gram-positive bacteria were more sensitive to all four glasses than gram negative bacteria, and that a single mechanism of action for the glasses is unlikely, as the rates of catalysis for metabolic enzymes as well as membrane permeability were altered after glass exposure.

ACKNOWLEDGEMENTS

I would like to extend gratitude to my advisor, Dr. David Westenberg, who not only dedicated his time in helping me with my research but also provided guidance throughout my project. I could always count on Dr. Westenberg to assist me in designing experiments or providing encouragement when things didn't go right. I would also like to thank Dr. Delbert Day for serving on my committee, and through his involvement in my project, broadening my knowledge of the materials sciences. Thank you to Dr. Melanie Mormile for serving on my committee, as well as her advice throughout the research process. A special thank you also goes to Dr. Katie Shannon, Terry Wilson, Adam Martin, and Dr. Daniel Forciniti for welcoming me into their laboratories and for allowing me to use their equipment, and for teaching me its proper use. Ali Mohammadkhah should be acknowledged for making all of the bioactive glasses used in my experiments. It should also be noted that the Department of Biological Sciences supported my graduate work by means of providing a Teaching Assistantship within the department.

A special thank you also goes to: Michael Lockett, Brieanna Kroeger, Justin Lovelady, Jean Roussin, Daniel Vincent, Secca Roettenbacher, Shelby Emmett, and Ben Brown.

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 HOSPITAL-ACQUIRED INFECTION

Hospital-acquired infection, also known as nosocomial infection, is a growing 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 wounds, the urinary tract, and the respiratory tract.

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[1]. 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 [1] . 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 [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, *Escherichia coli* and *Pseudomonas aeruginosa* have also been found at surgical implant sites and have been implicated in infections after surgical procedures [2]. The presence of biofilm-forming bacteria pose an increased risk of infection at implant sites, as these bacteria are usually more resistant to antibiotic treatment as well as host defenses [3] and gain protection from surrounding bacteria within the biofilm at the infection site [2]. Other

risk factors can predispose a patient to an implant- associated infection, such as old age and pre-existing health conditions, such as diabetes mellitus, rheumatoid arthritis, or HIV infection [4].

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 [5], adding more complications to infections acquired after surgery or the implantation of a medical device. 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 [6], making prevention of nosocomial infections, especially MRSA a critically important field of research. One area of this research involves the use of biomaterials to prevent infection.

1.2 BIOMATERIALS

Bioactive glass can be defined as a glass that is both biocompataible and surface reactive. This class of biomaterials was originally developed to address the problem of rejection with metal or plastic surgical implants [7]. Since the appearance of Larry Hench's "Bioglass" in 1969[7], the use of bioactive glasses for surgical implants has grown significantly in popularity. In addition, bioactive glasses have been implicated in bone tissue repair, dental and maxillofacial repair, and soft tissue repair[2] and have also been used as drug delivery vehicle for bone disease[5,8,9].

Recently, bioactive glass research has undergone a rapid growth, with the number of papers published in the field doubling between the years 2000 and 2011[10] as seen in Figure 1.1.

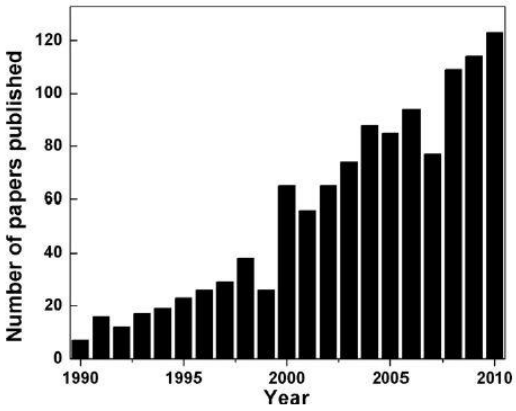

Figure 1.1 Bar graph showing the increase in the number of papers published in the field of bioactive glass from 1990 to 2011[10]

1.2.1 Borate Bioactive Glasses. 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[9].

One benefit of borate bioactive glasses is that their reaction rate can easily be altered by the boron content of the glass[5]. 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[10], and promote wound healing[11].

B3, a novel bioactive glass developed by Dr. Delbert Day and colleagues, is currently undergoing clinical trials for its wound healing abilities[11]. While little is known

about the exact mechanism by which the glass stimulates wound healing, this property of B3 makes it a very promising bioactive glass to be used in clinical applications.

1.2.2 Metal Ions. Through their development, bioactive glasses have had their benefits optimized by changes to their composition. Changes to composition can alter the reaction rates of the bioactive glass and its binding preference to either soft or hard tissue. The addition of metals and other polymers can also be used in combination to change the characteristics of a bioactive glass to make it better suited for its intended use[7].

One such change to composition that has been popular in the field of biomaterials is the addition of metal ions to make the glasses antimicrobial. Silver has long been known to have antimicrobial properties, and has been incorporated into various materials to prevent bacterial growth. In general, silver ions are incorporated into the bioactive glass as silver oxide (Ag_2O) and leach out of the glass to inhibit microbial growth. Incorporation of silver ions into a bioactive glass allow for a slow controlled delivery of the ions, as bioactive glass can have its reaction rate adjusted by changes to its composition[5].

Bellantone and colleagues showed that a silver oxide-doped silicate glass showed bactericidal activity against *S. aureus*, *E. coli*, and *P. 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[12]. 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 in this study that while reactivity of

bioactive glasses in water results in a slight increase in pH, the increase in pH alone is not sufficient to kill the bacteria in solution[12].

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* [13-16], *Bacillus anthracis*[14], *Pseudomonas pyocyanea*[14], *P. aeruginosa*[12,16], *Candida albicans*[16], *Salmonella* sp.[14], *Streptococcus* sp.[14], and *S. aureus*[12,16,17]. Silver ions in solution have also shown antimicrobial efficacy against *P. aeruginosa, S. aureus,* and *C. albicans* when in combination with natural products[18]. Additionally, silver nanoparticals have been incorporated into polymers and have also shown antimicrobial effects[15,19]. These studies show that silver is a reliable antimicrobial component of biomaterials, and is likely to have bactericidal capabilities if integrated into a novel bioactive glass such as B3.

While addition of silver is a reliable means of introducing bactericidal capabilities to bioactive glass, the use of other metal ions is gaining popularity. 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[20]. Doping bioactive glass with other metal ions, such as yttrium, selenium, and iodine also show potential for antimicrobial activity.

1. 3 MECHANISMS OF ACTION

1.3.1 Silver. Since silver-doped 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[2], sulfydryl, amino, or hydroxyl functional groups[12], competing with copper ions as a

cofactor in transport or enzymatic reactions[12], general toxicity[12,21], disruption of aliphatic carbon-hydrogen bonds by insertion of carbenes mediated by silver ions[22], DNA damage by arene-purine hydrophobic interactions mediated by silver ions[23], direct binding to DNA[2], increased permeability and disruption of cell membranes[2], and inhibition of respiratory[2] or signaling[21] enzymes.

1.3.2 Borate. 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[24]. While the antimicrobial capabilities of borate-based biomaterials are not well documented, borate chemistry in solution has been studied. From these studies, various mechanisms for borate- mediated bactericidal action can be reasonably proposed. NMR-spectroscopy experiments by Kim and colleagues have shown that borate can esterify to diol groups in close proximity to one another. This binding was shown on NAD^+, NADH, and ribose, at a wide range of pH values with the most binding occurring at more basic pH values[25]. The results found by these NMR spectroscopy studies were supported by capillary electrophoresis studies done by Ralston and Hunt. These experiments showed that borate does indeed bind weakly and reversibly to biological ligands such as NAD^+ and cis-diol functional groups[26].

The proposed reaction can be found in Figure 1.2. Binding of borate to NAD^+ and NADH may suggest that borate can compete with dehydrogenase enzymes for these molecules and alter their reaction equilibria[25,27]. In addition, binding to ribose or pyridine moieties may be a mechanism by which borate can mediate DNA damage[25,27]. Binding to ribose would disrupt the DNA backbone, causing kinks or breaks that would result in disorderly DNA replication or transcription[25] and interactions between borate and

pyridine nucleotides would cause similar DNA damage[26]. In addition, material scientists have also noticed this behavior of borate to bind hydroxyls at basic pH values. Since this behavior is not seen in silicate based glasses, it was called the "borate anomaly" and is still being studied.[28]

Figure 1.2 Mechanism proposed by Kim et al.[25] by which borate esterifies to cis-diol groups

Throughout these studies, the toxicity of borate has become a concern if borate were to be used in humans. Loomis and Durst proposed in 1992 that borate binding to ribonucleotides may play a role in borate toxicity in humans[29]. While borate was shown to have the potential to be toxic to the reproductive system in a study by Chapin and Ku[30], that toxicity has been shown to only occur at very high borate concentrations and over long periods of time[5]. In addition, it was shown that the "dynamic" nature of body fluid *in vivo* prevents borate toxicity[9].

2. PREVIEW OF EXPERIMENTS

From these findings, it is clear that the presence of borate as well as various metal ions may enhance the bactericidal activity of 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. In addition, the mechanism of action by which the bioactive glasses kill bacteria was also studied. Live-Dead staining fluorescence microscopy was used to determine if the glasses can induce changes in membrane permeability or membrane damage. Spectroscopic enzyme kinetics experiments was used to determine changes in enzyme reaction equilibria after exposure to B3, and a gene fusion strain of *E. coli* was utilized to assess DNA damage mediated by the bioactive glasses.

3. STATEMENT OF OBJECTIVES

The objectives of these experiments were to study the antimicrobial activity of four novel bioactive glasses, B3, B3-Ag, B3-Ga, and B3-I and to elucidate a potential mechanism of action for the bactericidal ability of the glasses.

4. EXPERIMENTAL

4.1 CULTURE MEDIA AND REAGENTS

Table 4.1 Recipes for reagents

Reagent	Recipe	Solvent	Additional Treatment
Trypticase Soy Broth (TSB) [Difco]	30g TSB	1000ml purified H2O	
Trypticase Soy Agar [Difco]	30g TSB, 15g Agar	1000ml purified H2O	
100mM Tris Buffer [Difco]	12.114 Tris base	1000ml purified H2O	pH to 7.5 with HCl
100mM Potassium Phosphate Buffer [Difco]	.0136g Potassium Phosphate	1000ml purified H2O	pH to 7.5 with KOH
Glass Suspension (See Table 3)	.2g Glass Powder	1ml sterile purified H2O	
Live-Dead Staining Buffer [Biovision]	1ul Live-Dye, 1ul Propidium Iodide	1ml staining buffer solution	
Dichloroindophenol (DCIP)	.125g DCIP	250ml purified H2O	
10 mM Phenazine Methosulfate (PMS)	.77g PMS	250ml purified H2O	

4.2 BACTERIAL STRAINS

All bacterial strains used in the following experiments are described in Table 4.2. All strains were obtained from Missouri S&T's teaching laboratory culture collection except where indicated. Strains marked with an asterisk (*) were purchased from the ATCC. The bacteria were incubated at 37ºC with shaking and fresh cultures were made weekly from stock strains kept refrigerated at 4ºC.

Table 4.2 Bacterial strains used in experiments[31]

Organism	**Gram Stain**	**Clinical Relevance**
Shigella flexneri	negative	Causative agent of dysentery
Shigella sonnei	negative	Causative agent of dysentery
Salmonella typhimurium	negative	Causative agent of gastroenteritis, most frequent agent of food poisoning
Proteus vulgaris	negative	Found in fecal matter
Proteus mirabilis	negative	Found in fecal matter
Moraxella catarrhalis	negative	One causative agent of meningitis, often found in mucus membranes and venereal discharge
Vibrio natriegens	negative	related to *Vibrio cholerae*
Enterococcus faecalis	positive	Urinary tract infections, subacute endocarditis
Clostridium difficile	positive	diarrhea, intestinal disease
Staphylococcus epidermidis 12228	positive	chronic skin infections, bacterial endocarditis from ventriculo-atrio shunts/implants
Staphylococcus epidermidis 35984*	positive	Clinical isolate, effective biofilm forming strain
Staphylococcus aureus (MRSA)*	positive	Implicated in skin infections, respiratory disease, food poisoning, drug resistant MRSA infections, wound infections, meningitis, and osteomyelitis
Escherichia coli 25922	negative	Impicated in urinary tract infections and enteric disease
Pseudomonas aeruginosa	negative	Causative agent of wound infections, burn infections, and urinary tract infections

4.3 GLASS COMPOSITION

Bioactive glass powders were prepared by Dr. Delbert Day and Ali Mohammadkhah of the Missouri S&T Department of Materials Science. The composition of each glass used is provided in Table 4.3.

Table 4.3 Glass compositions

	B3	B3-Ag	B3-Ga	B3-I
Compound	Percent weight	Percent weight	Percent Weight	Percent weight
B_2O_3	53%	52%	52%	53%
CaO	20%	20%	20%	20%
K_2O	12%	12%	12%	10*%
Na_2O	6%	6%	6%	6%
MgO	5%	5%	5%	5%
P_2O_5	4%	4%	4%	4%
Ag_2O	0%	1%	0%	0%
Ga_2O_3	0%	0%	1%	0%
I	0%	0%	0%	2%*

*This is an estimate of the percent weight. 2%wt of the K_2O in the glass came from KI.

4.4 LIVE-DEAD STAINING ASSAY

50ul of glass suspension was added to 1 ml of overnight broth culture, and the mixture was immeiately centrifuged at 1100 rpm for 10 minutes. After removal of the supernatant, the pellet was re-suspended in 500ul of staining buffer and incubated at

37°C for at least 15 minutes. After incubation, a slide was prepared by spotting 5ul 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 4000x using FITC, TxRed, and DIC filters. Channel images were captured with a Hamamatsu digital camera (Figure 4.1), and the amount 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, 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.

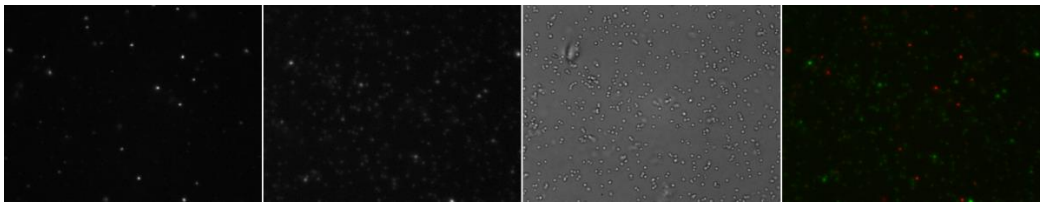

Figure 4.1 Representative image of Live-Dead staining assay for MRSA after 24 hour incubation. From left to right: TxRed channel, FITC channel, DIC channel, combined image. Images are at 4000x

4.5 CELL EXTRACT PREPARATION

Two liters of TSB were inoculated with *E.coli* and incubated with shaking at 37°C for at least 24 hours. The broth culture was then centrifuged at 5000 rpm at 4°C for 10 minutes. The pellets were suspended each in 20ml of TSB and centrifuged again at 5000 rpm at 4°C for 10 minutes and the final pellet was stored at 4°C.

The pellet was re-suspended in 7ml Tris-buffer solution. The suspension was exposed to 15,000psi by using a French Press to lyse the cells. The French press procedure was repeated. The lysate was stored at 4°C.

The lysate was centrifuged at 8000 rpm for 15 minutes at 4°C. The supernatant was then aliquotted into 1ml and .5ml tubes and stored at 4°C and the pellet was discarded.

4.6 SUCCINATE DEHYDROGENASE ASSAY

One ml quartz cuvettes were prepared based on Table 4.4. Two cuvettes were made per sample number. One cuvette was used as a reference, the other was used as the experimental cuvette. 20µl of glass suspension was added to experimental cuvettes.

Table 4.4 Succinate Dehydrogenase assay cuvette preparation

Compound	1	2	3	4	5	6
50mM Tris-SO4 (pH 7.5)	500µl	500µl	500µl	500µl	500µl	500µl
1M Succinate	15µl	15µl	15µl	15µl	15µl	15µl
Sterile, MilliQ H2O	0µl	20µl	30µl	40µl	50µl	60µl
.1M KCN	15µl	15µl	15µl	15µl	15µl	15µl
E.coli extract	20µl	20µl	20µl	20µl	20µl	20µl

The cuvettes were placed in a 37°C incubator for approximately 3 minutes. After incubation, the samples were placed into the UV Spectrometer and the machine was zeroed. To the experimental cuvette,.05% (w/v) Dichloroindophenol (DCIP) and 10mM Phenazine Methosulfate (PMS), as shown in Table 4.5, were added simultaneously and mixed quickly by pipetting. The absorbance was then monitored for 2 minutes at a wavelength of 600nm.

Table 4.5 Succinate Dehydrogenase assay preparation

Compound	1	2	3	4	5	6
PMS	60µl	40µl	30µl	20µl	10µl	0µl
DCIP	30µl	30µl	30µl	30µl	30µl	30µl

4.7 MALATE DEHYDROGENASE ASSAY

One ml quartz cuvettes were prepared based on Table 4.6. Two cuvettes were made per sample number. One cuvette was used as a reference, the other was used as the experimental cuvette. 50 µl of glass suspension were added to experimental cuvettes.

Table 4.6 Malate Dehydrogenase assay cuvette preparation

Compound	Cuvette 1	Cuvette 2	Cuvette 3	Cuvette 4
100mM Potassium Phosphate Buffer, pH=7.5	500µl	500µl	500µl	500µl
β-NADH	100µl	100µl	100µl	100µl
Sterile H2O	300µl	300µl	300µl	300µl
Oxaloacetic acid	(200mM) 50µl	(100mM) 50µl	(50mM) 50µl	(40mM) 50µl

The cuvettes were placed into the UV Spectrometer and the machine was zeroed at 340nm. To the experimental cuvette, 50 µl of cell extract was added and mixed quickly by pipetting. The absorbance was monitored for 2 minutes at a wavelength of 340nm.

4.8 WELL DIFFUSION ASSAY

100ul of overnight broth culture was plated in triplicate on TSA. To make wells in each plate, a sterile, plastic straw 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, 20µl of molten agar was added and allowed to cool to seal the bottom of each well. 50µl of glass suspension was then added on top of the agar in each well, leaving .01g glass powder in each well. The plates were then incubated at 37°C. After incubation, the radius of inhibition was measured by measuring the distance

between the edge of the well to the beginning of bacterial growth on the plate. (Figure 4.2)

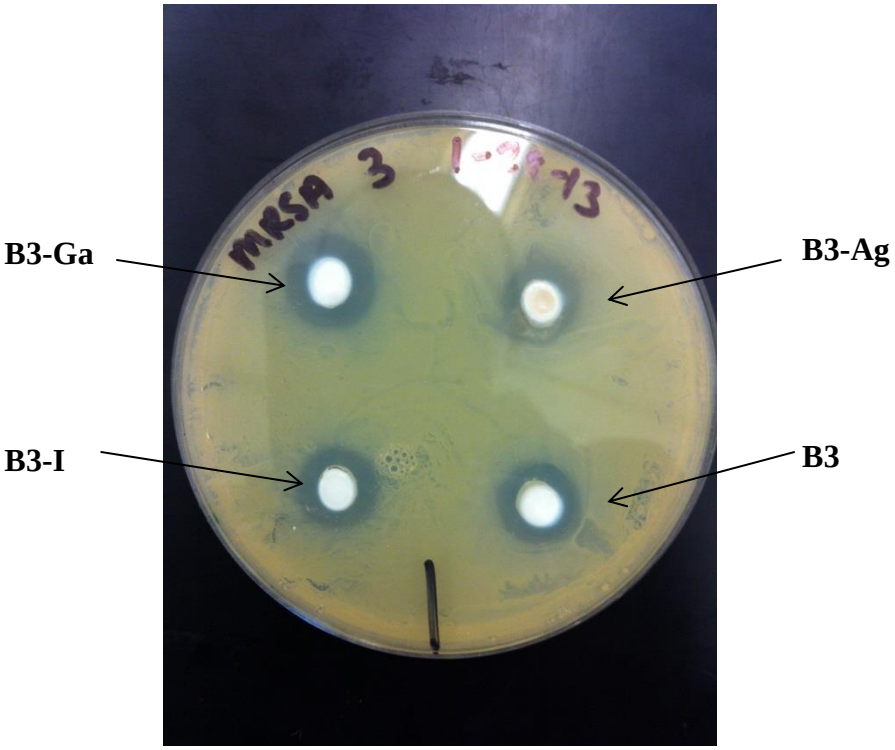

Figure 4.2 Representative image of well diffusion assay for MRSA

A similar assay using sterilized disks of filter paper was also carried out. 10µl of each glass suspension was spotted onto separate disks of filter paper, and the disks were laid on top of 100µl overnight broth culture plates on TSA. The plates were then incubated for at least 24 hours at 37°C. After incubation, the radius of inhibition was

measured by measuring the distance between the edge of the disk to the beginning of bacterial growth on the plate.

4.9 LUMINESCENCE ASSAY

100µl of overnight broth culture was added to the wells of a microtitration plate. 20µl of glass solution was added to experimental wells and the plate was incubated at room temperature for 15 minutes. The luminescence of each well was read by a BMG LabTech Omega plate reader.

5. RESULTS AND DISCUSSION

5.1 BROAD-SPRECTRUM ANTIBACTERIAL PROPERTIES

To determine the sensitivities of various bacteria to B3, B3-Ag, B3-Ga, and B3-I bioactive glasses, well diffusion assays were carried out on 15 clinically relevant bacterial species. The radius of inhibition of each glass for each bacterial species was measured and compared among all of the bacteria types. The disk diffusion assay was also performed, but did not yield repeatable results. Overall, the gram positive organisms (*S. epidermidis* and *S. aureus* MRSA) were more sensitive to all of the glasses than the gram negative organisms (Figure 5.1). MRSA as well as S. epidermidis 12228 showed the most significant growth inhibition by all four glasses tested. *P. aeruginosa, E. cloacae, P. vulgaris,* and *S. flexneri*, all gram-negative organisms, showed no sensitivity to any of the glasses. Some gram negative organisms, however, did show some significant sensitivity to the glasses. These organisms include *V. natriegens, S. sonnei, S. marscescens,* and *M. catarrhalis*.

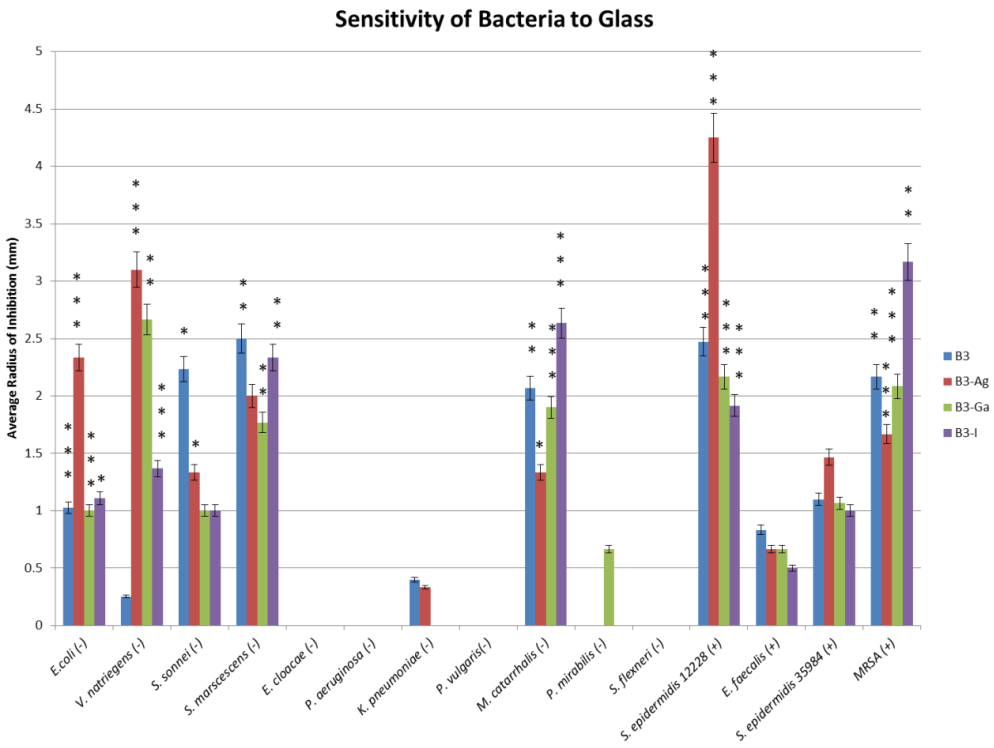

Figure 5.1 Results of well diffusion assays. Error bars represent a standard error of 5%. Blue bars represent B3, red bars:B3-Ag, green bars: B3-Ga,and purple bars: B3-I. Control plates for each organism showed radii of inhibition of 0 for all glasses (***p<.005; **p<.01; *p<.05)

The difference in sensitivity by the various organisms could be due to many different factors. Without knowing the exact mechanism of action for the glass, it is difficult to say exactly why some bacteria are more sensitive to each glass than others. However, given the differences in sensitivity to the glasses between gram positive and gram negative organisms, it is reasonable to believe that reduced sensitivity to the glass maybe related to the presence of an outer membrane. While results of this type of assay did not elucidate the antibacterial mechanisms of the glass, they did provide guidance in choosing organisms to study for further assays.

5.2 ENZYME KINETICS

A study done by Houlsby suggests that borate may act as an antimicrobial compound by inhibiting metabolic enzymes that are critical to cell function[24]. While it is proposed that borate may inhibit these enzymes, the exact mechanism of this inhibition or the enzymes involved is unknown. NMR spectroscopy studies done by Kim found that borate can esterify to the diol groups on nicotinamide dinucleotide in its reduced form (NADH)[25]. Since NADH is used as a cofactor in multiple steps of the TCA cycle and other metabolic reactions, it is reasonable to predict that borate can inhibit NADH-dependent reactions by binding NADH.

The kinetic properties of two metabolic enzymes, succinate dehydrogenase and malate dehydrogenase, were studied. The kinetics of each enzyme's reaction was measured by spectrophotometric means using cell extracts of *E.coli* exposed to the borate-containing bioactive glass, B3.

5.2.1 Succinate Dehydrogenase. Succinate dehydrogenase is an enzyme involved in the catalysis of the oxidation of succinate to fumarate in the TCA cycle. Since succinate dehydrogenase is not dependent on NADH, it would be predicted that the enzyme would not be affected if the action of borate is to interact with NADH. Any effect by borate would therefore indicate an additional or alternate mechanism for the inhibition of metabolic enzyme catalysis by B3.

The maximum velocity (V_{max}) and Michaelis affinity constant (K_m) values of succinate dehydrogenase were determined from the spectrophotometric data represented in a Lineweaver-Burke plot (Figure 5.2). With exposure to B3, the K_m value and the V_{max} value appeared to decrease. The K_m value decreased from -.00031 to -.0002. and the V_{max} value decreased from .037 O.D./second to .012 O.D./second (Table 5.1).

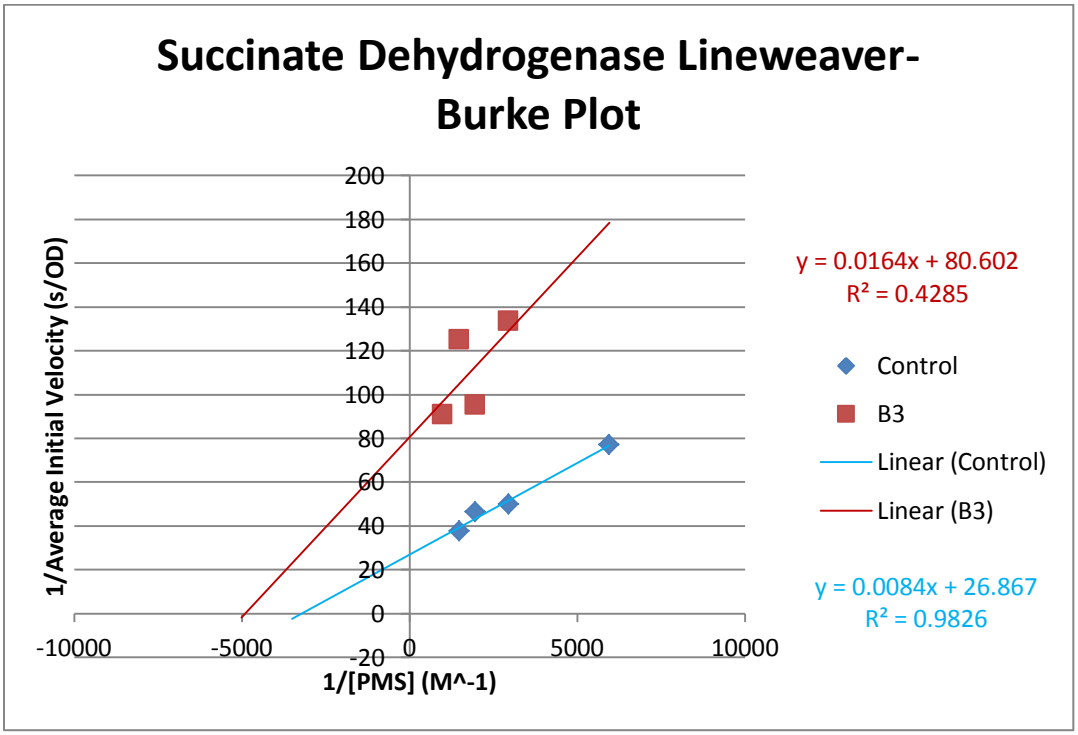

Figure 5.2 Lineweaver-Burke plot for Succinate Dehydrogenase. Blue line: control, red line: treated with B3

Table 5.1 V_{max} and K_m values for Succinate Dehydrogenase

Value	Control	B3
Km	-0.00031	-0.0002
Vmax	0.0372	0.0124

The decrease in V_{max} after exposure to B3 shows that the reaction catalyzed by succinate dehydrogenase proceeds at an overall slower pace after exposure to the glass. These data support the previously proposed hypothesis that borate may inhibit metabolic enzymes such as succinate dehydrogenase. The reason for the modest decrease in K_m values after exposure to B3 is not as clear. A decrease in K_m represents a greater affinity of the enzyme for its substrate after exposure to B3. This suggests that binding of succinate dehydrogenase to its substrate would be enhanced rather than hindered by exposure to B3, as it would be more tightly and easily bound to its substrate as the reaction proceeds. These findings might mean that exposure to B3 allows the enzyme to bind its substrate more tightly, but oxidize that substrate more slowly. The slowed reaction rate may be due to this tighter binding, as the enzyme may not be able to release the fumarate quickly as it is produced.

From these inconclusive data, it is unclear as to how succinate dehydrogenase is inhibited by B3 bioactive glass. However, a decrease in the V_{max} value after B3 exposure is suggestive that B3 is indeed an inhibitor of succinate dehydrogenase, and further research is needed to elucidate the exact mechanism for this inhibition. Other effects on the reaction, such as changes in pH or osmotic effects may be able to explain the results that were seen. In addition, borate may interact with FADH, the cofactor used by

succinate dehydrogenase, differently than NADH. One reason for a difference could be that FADH is covalently bound to the enzyme and not free in solution. The covalent binding may block sites on the molecule where borate could esterify to diol groups. The use of other methods besides spectrophotometry to measure reaction rate may be useful to determine this mechanism or more clearly study the effects of B3 on K_m and V_{max} values of succinate dehydrogenase, as the bioactive glass is optically active and may skew spectrophotometry data[12].

5.2.2 Malate Dehydrogenase. Malate dehydrogenase is also a TCA cycle enzyme and catalyzes the conversion of malate to oxaloacetate by oxidation coupled with the reduction of NAD to NADH. This reaction is reversible, so oxaloacetate can also be used as a substrate for the enzyme's catalysis. Changes in malate dehydrogenase kinetics after B3 exposure would be suggestive of an NADH-dependent inhibitory activity of borate on the enzyme, as malate dehydrogenase is NADH-dependent.

The maximum velocity (V_{max}) and Michaelis affinity constant (K_m) values of malate dehydrogenase were determined from the spectrophotometric data represented in a Lineweaver-Burke plot (Figure 5.3). Once again, the K_m value and the V_{max} value appeared to decrease with exposure to B3. The K_m value decreased from 2139.5 to 170.5 and the V_{max} value decreased from .109 O.D./second to .0254 O.D./second (Table 5.2). The changes in K_m and V_{max} values are compared in Figure 5.4.

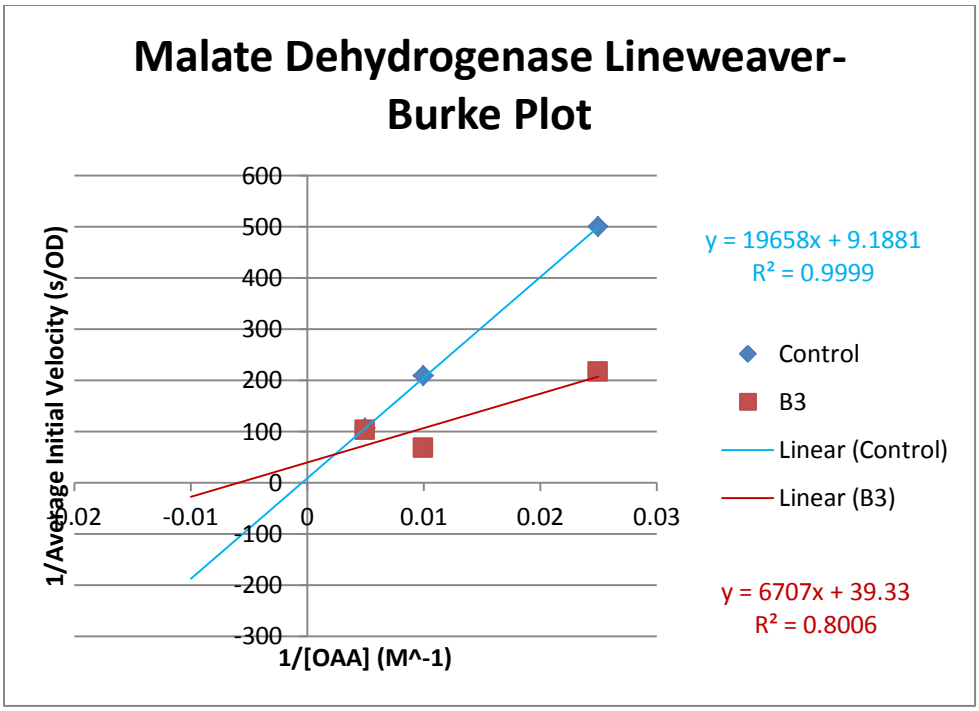

Figure 5.3 Lineweaver-Burke plot for Malate Dehydrogenase. Blue line: Control, red line: B3

Table 5.2 V_{max} and K_m values for Malate Dehydrogenase

Value	Control	B3
Vmax	0.109	0.0254
Km	2139.5	170.5

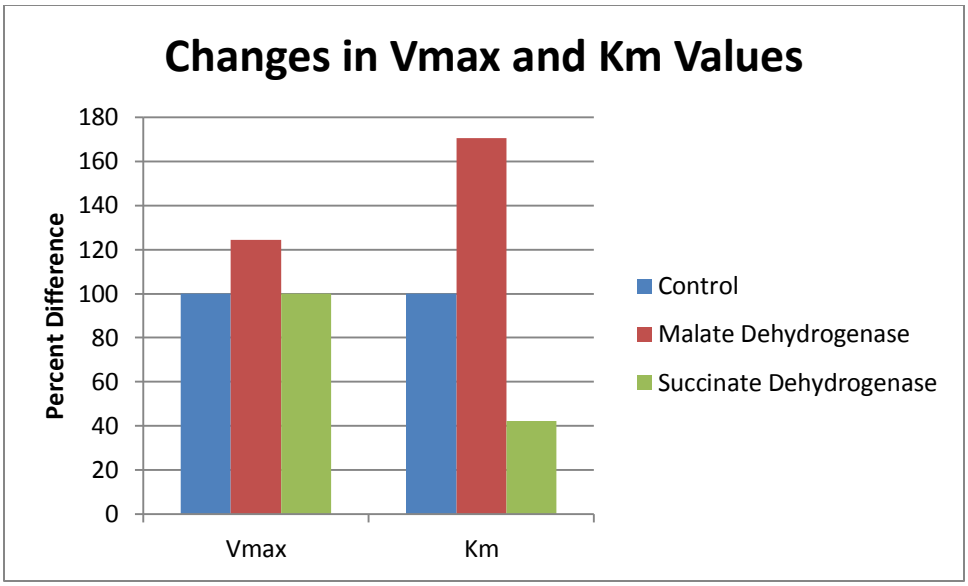

Figure 5.4 Difference in changes of Km and Vmax between Malate Dehydrogenase and Succinate Dehydrogenase

Like with succinate dehydrogenase, a decrease in K_m is unusual, and suggests enhanced binding of the enzyme to its substrate due to a greater affinity of malate dehydrogenase to oxaloacetate. However, the V_{max} value is prominently decreased with exposure to B3, indicating that the reaction catalyzed by malate dehydrogenase proceeds at a slower rate in the presence of borate. These findings support the hypothesis that borate can esterify to NADH, and thus slow the rate of reaction by competing with malate dehydrogenase for NADH binding. While the enzyme itself may bind more tightly to its substrate, it still requires NADH as its cofactor to function; hence binding of NADH by borate would still slow the reaction rate even with a greater affinity of malate dehydrogenase to its substrate.

Like with succinate dehydrogenase, optical activity of B3 may skew spectrophotometric data so other methods for measuring reaction rate may be useful in

determining the mechanism of borate inhibition of malate dehydrogenase. In addition, the use of NMR spectroscopy to determine if the borate specifically in B3 binds NADH would confirm esterification of borate to NADH as an inhibitory mechanism for NADH-dependent metabolic enzyme reaction catalysis.

5.3 LIVE-DEAD STAINING FLUORESCENCE MICROSCOPY

Multiple authors suggest that disruption of membrane integrity may be a mechanism of action for both silicate and borate based bioactive glasses[2, 23]. However, it is unknown whether borate alone, or borate in synergy with various metal ions such as silver, is enough to disrupt bacterial cell membranes. To investigate the ability of B3, B3-Ag, B3-Ga,and B3-I bioactive glasses to disrupt bacterial membranes, Live-Dead staining and fluorescence microscopy were used to observe various bacterial species after exposure to the various glasses.

Live-Dye was used to stain live cells, as it is cell permeable and is taken up by live cells so that they fluoresce green. Propidium iodide was used to stain dead cells, as it is not cell permeable and will only stain cells red if their membranes are damaged.

5.3.1 15-Minute Incubation. The viability of bacterial cells determined by Live-Dead staining appeared to decrease markedly for the gram-positive organisms tested, but did not decrease for the gram-negative organisms tested. The percent viability of MRSA decreased by an average of 31.7% for B3, 48.9% for B3-Ag, 59.5% for B3-Ga, and by 91.9% for B3-I after a 15-minute incubation with each glass (Figure 5.5). Vancomycin is often the antibiotic used to treat MRSA infections. To determine whether the B3 glasses were more effective than Vancomycin, the percent viability of MRSA incubated with Vancomycin was also determined using Live-Dead staining. Treatment with Vancomycin

only decreased percent viability by 27.4%, which is less than any of the glasses tested. This suggests that all of the B3 glasses are more effective at killing MRSA than the current antibiotic used to treat MRSA.

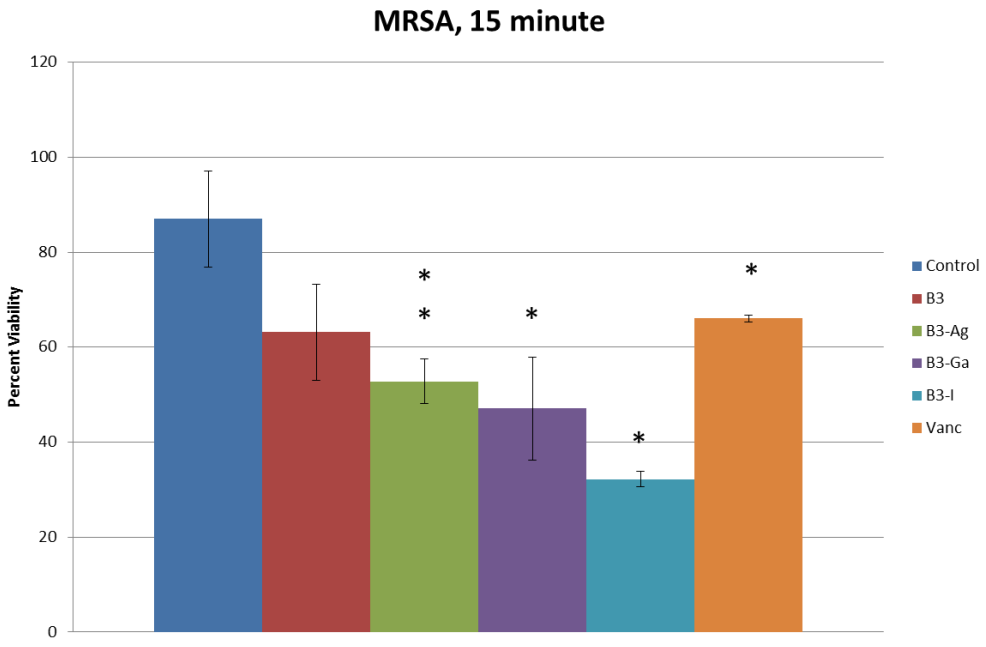

Figure 5.5 Live-Dead staining data for MRSA after 15-minute incubation. Error bars represent standard deviation (***p<.005; **p<.01; *p<.05)

Likewise, the percent viability of *S. epidermidis* also decreased after a 15 minute incubation with each glass. The percent viability decreased by an average of 29.7% for B3, 28% for B3-Ag, 97% for B3-Ga, and 87.7% for B3-I (Figure 5.6).

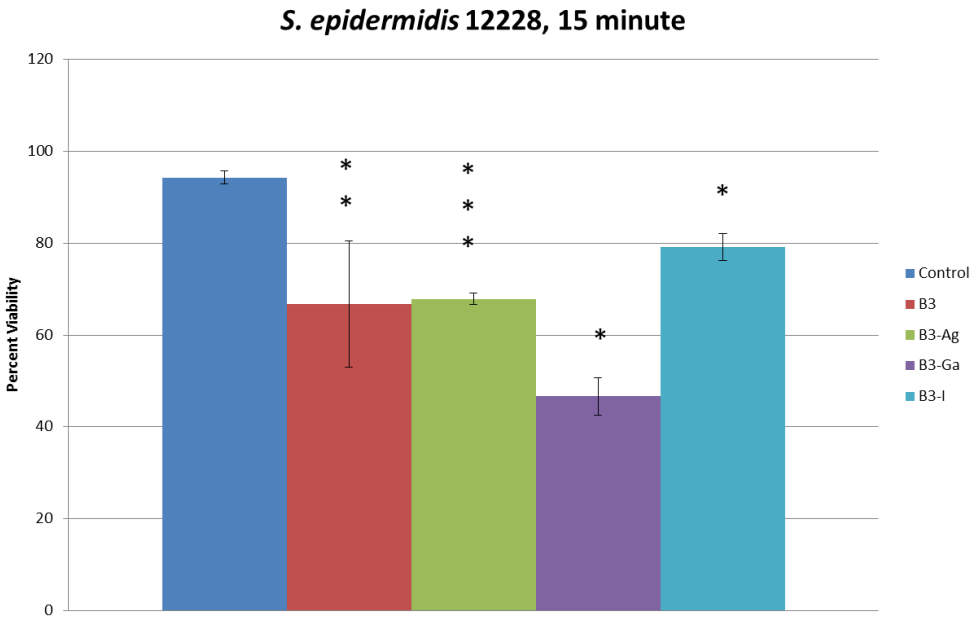

Figure 5.6 Live-Dead staining data for *S. epidermidis* after 15 minute incubation. Error bars represent standard deviation(***p<.005; **p<.01; *p<.05)

Gram negative organisms, however, did not show such a marked decrease in percent viability after exposure to the glass. 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 percent viability of *E.coli* only decreased by an average of .43% for B3 and by 2.15% for B3-Ag after a 15-minute incubation with each glass, neither of which were statistically significantly different from controls (Figure 5.7).

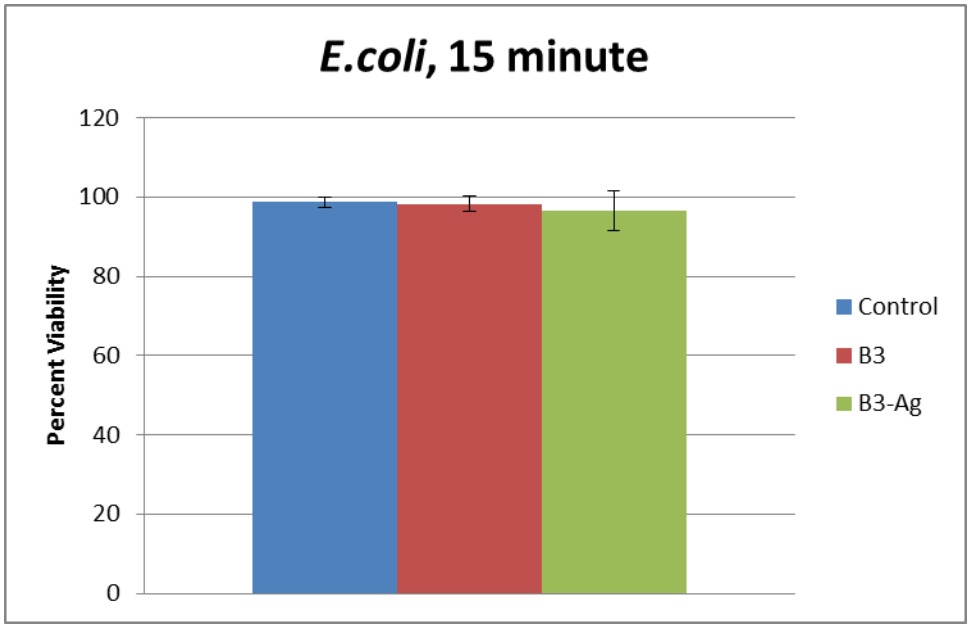

Figure 5.7 Live-Dead staining data for *E. coli* after 15 minute incubation. Error bars represent standard deviation

A similar effect was seen for *P. aeruginosa* after a 15-minute incubation period with each glass. The percent viability of *P.aeruginosa* decreased by only an average of 4.3% for B3, 2.7% for B3-Ag, 7.3% for B3-Ga, and 1.5% for B3-I (Figure 5.8).

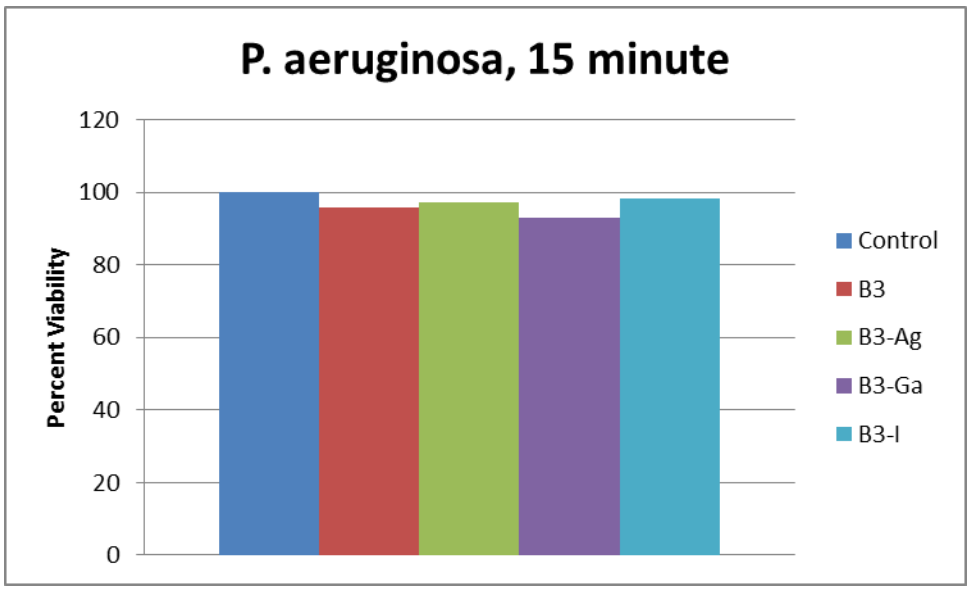

Figure 5.8 Live-Dead staining data for *P. aeruginosa* after 15 minute incubation

While there were some decreases in percent viability for both *E.coli* and *P.aeruginosa*, the decreases were not nearly as large as those for MRSA and *S. epidermidis* and were not statistically significant. These data suggest that gram-positive bacteria are more sensitive to each glass than gram negative bacteria, and this finding is supported also by the well-diffusion data (Figure 5.1).

5.3.2 24-Hour Incubation. To investigate if the viability of bacteria is affected by each glass given a longer exposure time, a 24-hour incubation with the glass was performed on MRSA, *S. epidermidis*, *E.coli*, and *P. aeruginosa*. Over a 24-hour incubation, the average percent viability of MRSA decreased by 43.6% for B3, 36.4% for B3-Ag, 15.5% for B3-Ga, 48.4% for B3-I, and 29.1% for Vancomycin (Figure 5.9). There was an evident decrease in percent viability for B3 with a longer incubation period,

but the decrease was not as evident and was by a smaller margin for Vancomycin. For B3-Ag, B3-Ga, and B3-I however, the decrease in percent viability after 24 hours of incubation was less than that of the 15 minute incubation period. These findings suggest that B3 is able to maintain its antimicrobial activity, but the addition of metal ions to B3 allow for the glass to kill bacteria more rapidly, but hinder the ability of the glass to maintain its antibacterial capabilities. A similar experiment with a 24-hour incubation period was performed using *S. epidermidis* (Figure 5.10).

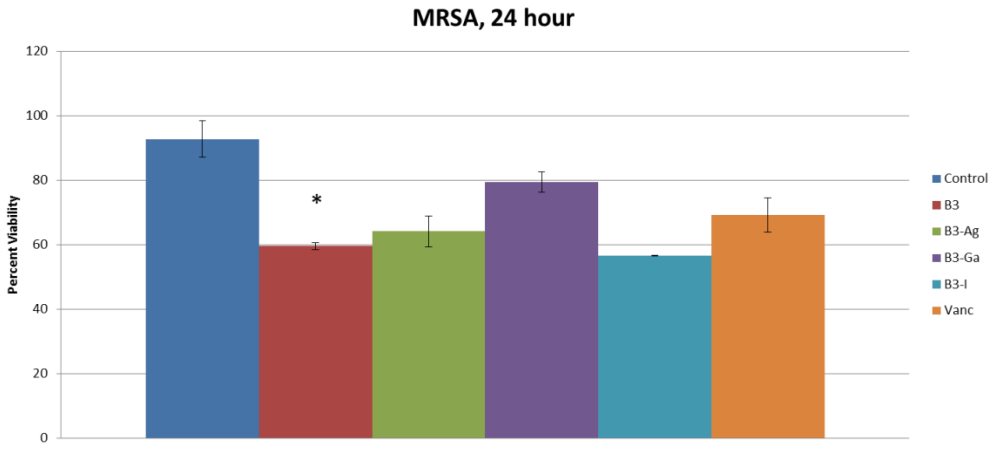

Figure 5.9 Live-Dead staining data for MRSA after 24 hour incubation. Error bars represent standard deviation(***p<.005; **p<.01; *p<.05)

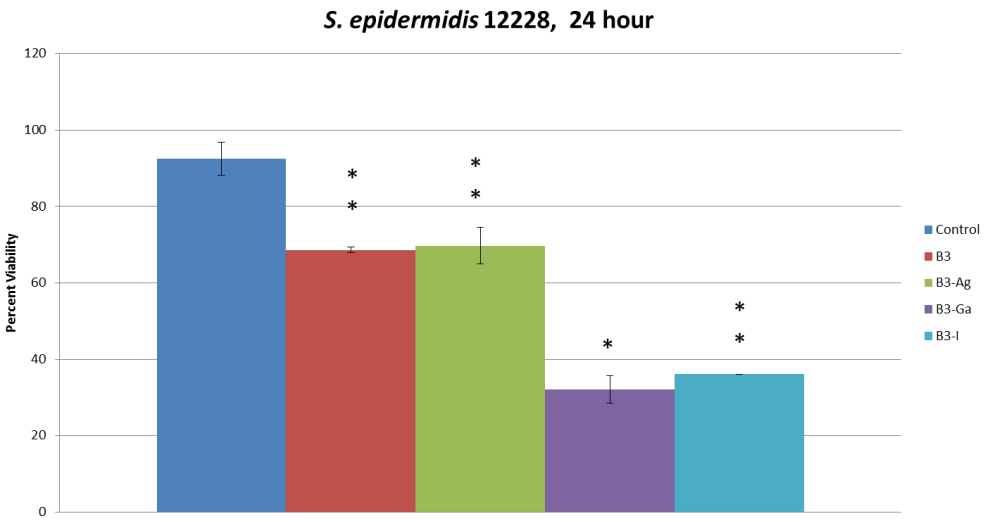

Figure 5.10 Live-Dead staining data for *S. epidermidis* after 24 hour incubation. Error bars represent standard deviation(***p<.005; **p<.01; *p<.05)

The percent viability for *E. coli* actually increased for B3 after a 24 hour incubation with the glass. While this increase was only by 1.4%, it was still very unusual for the bacteria to be more viable in the presence of the glass than not in the presence of the glass. While it is unlikely that B3 provides a growth advantage to the cells, it is more likely that the minimal antimicrobial ability of B3 is not maintained over a 24 hour incubation for *E. coli*. 24 hour exposure to B3-Ag, however, results in a 2.4% decrease in percent viability for *E. coli*, which is a marginally greater decrease than with only a 15 minute exposure to the glass, but is not statistically significant (Figure 5.11). These results suggest that the killing mechanism for B3 glass and its metal-ion containing derivatives may be different between gram positive and gram negative bacteria, as the presence of metal ions allows for the maintenance of killing ability over time for gram negative bacteria, but hinders the maintenance of antibacterial activity over time for gram

positive bacteria. In addition, it appears that even after a 24 hour incubation, gram negative bacteria are less sensitive to B3 and B3-Ag than gram positive bacteria.

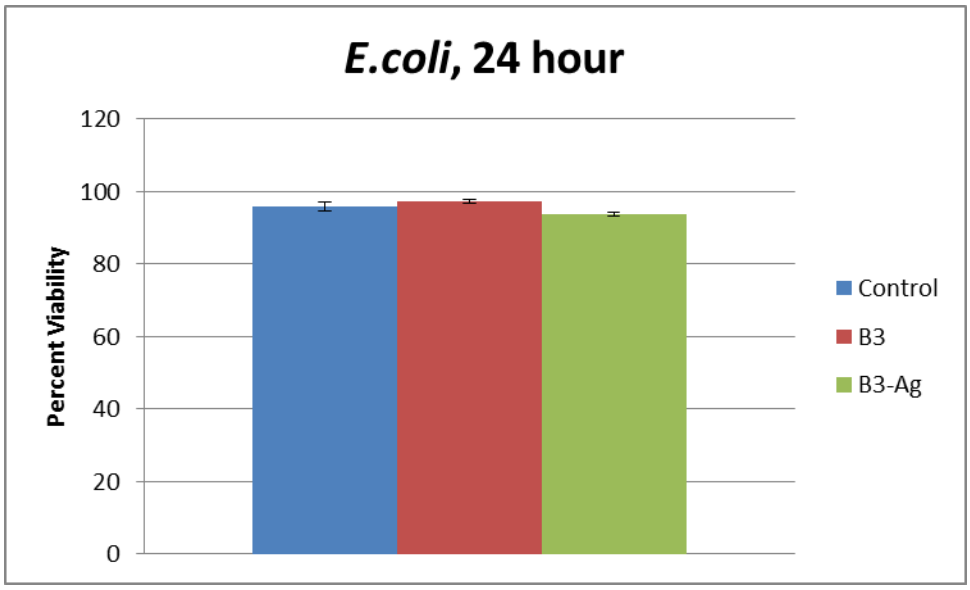

Figure 5.11 Live-Dead staining data for *E. coli* after 24 hour incubation. Error bars represent standard deviation

Overall, the data gathered from Live-Dead staining fluorescence microscopy shows a clear difference between sensitivity to B3 glass and its metal-ion doped derivatives by gram positive and gram negative bacteria. Gram negative bacteria appear to be less sensitive overall to all of the glasses. A decrease in percent viability similar to that shown in a gram positive bacterial species can only be attained in a gram negative bacterial species (*P. aeruginosa*) after a 3 day incubation (Figure 5.12), rather than a 15 minute incubation with the glass. While these decreases were evident, they were not statistically significant.

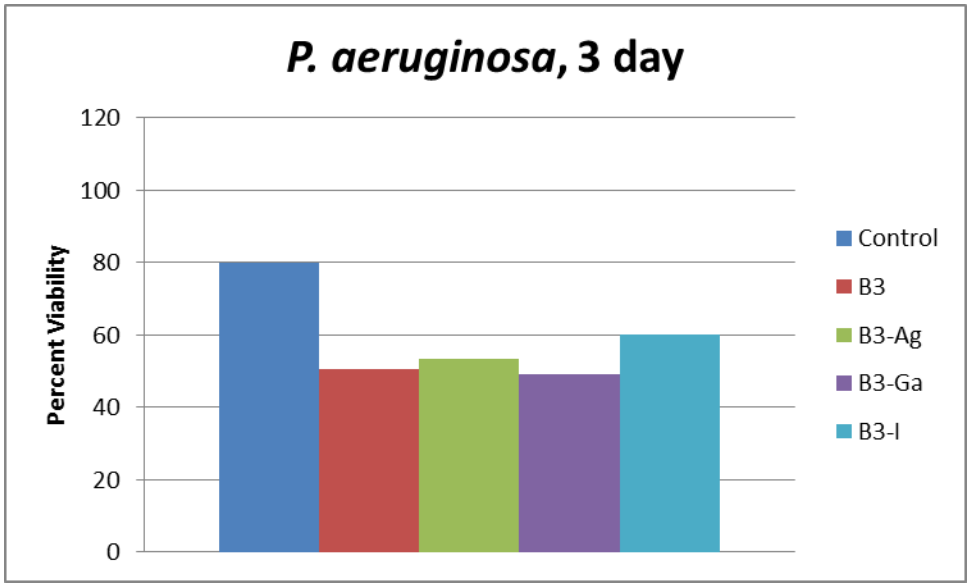

Figure 5.12 Live- Dead staining for *P. aeruginosa* after 3 day incubation

To support these results, multiple additional experiments could be conducted. For instance, a time-course study could be done to show the rate of killing by each glass for both a gram positive and gram negative species. In addition, more bacterial species could be tested to strengthen the claim that overall, gram negative bacteria are less sensitive to the glass than gram positive bacteria. More specific molecular assays could also be conducted to determine the biochemical mechanisms by which each glass kills gram positive bacteria quickly and gram negative bacteria over long incubation times.

5.4 LUMINESCENCE ASSAYS

5.4.1 DNA Damage Assay. As previously mentioned, a study done by Kim et al. found that borate forms ester bonds to cis diol functional groups, suggesting that borate may be able to bind to the sugar-phosphate backbone of DNA and damage DNA. In addition, Johnson et. al. reported that borate can bind pyridine nucleotides, which could act as another means of DNA damage mediated by borate. To assess whether B3, B3-Ag, B3-Ga, or B3-I bioactive glasses are capable of damaging DNA the DPD2794 strain of *E.coli* were exposed to each of the glasses. The DPD2794 strain contains a gene fusion plasmid with the *recA* promoter fused to the *lux* operon. The RecA pathway is a DNA repair pathway, and responds to DNA damage within the cell. Using *lux* as the reporter gene, the bacteria luminesce when the *recA* pathway is activated, making luminescence an indirect measure of DNA damage.

After a 15 minute incubation with B3, B3-Ag, B3-Ga, and B3-I, less luminescence was recorded for the treated cells, than the control group, which had no exposure to glass (Figure 5.13).

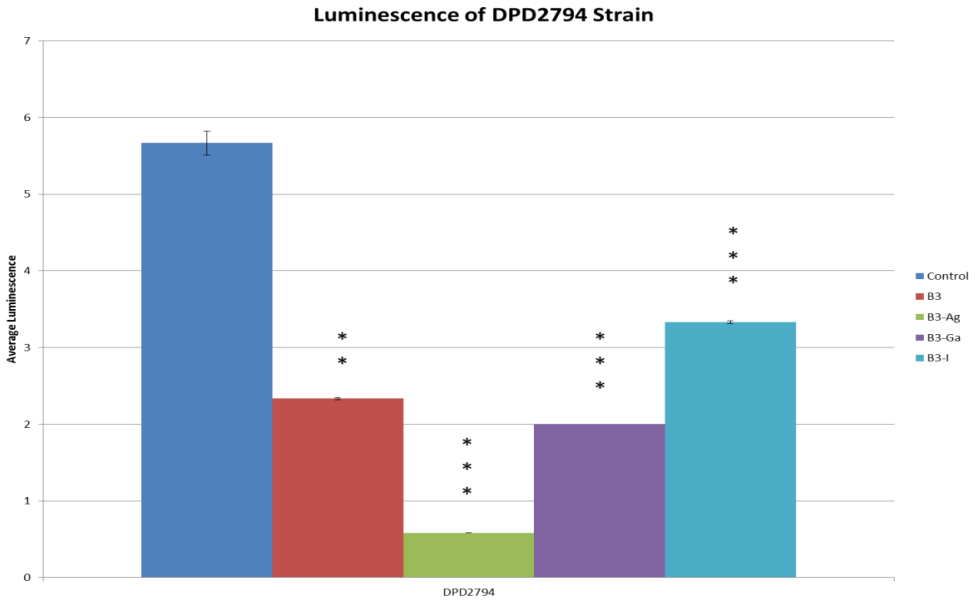

Figure 5.13 Plate reader data for DPD2794 after 15 minute incubation. Error bars represent standard deviation (***p<.005; **p<.01; *p<.05)

These results are surprising, however they may be explained. As previously mentioned, the borate and the metals ions in bioactive glasses have been proposed to disrupt important metabolic functions in bacteria as a mechanism for its antibacterial activity. It is reasonable then to propose that the borate or metal ions present in the bioactive glass may also disrupt regulatory or repair functions in the cell, such as the *recA* pathway. This could explain the lack of luminescence observed when the mutant strain was exposed to each glass, as the glass may have disrupted activation of *recA*, resulting in less luminescence. In addition, the bacteria may also be dying before the *recA* genes are able to become activated, resulting inless luminescence after treatment with the glass. It is also possible that the incubation time may not be long enough for *lux* activity to be expressed to its fullest potential. Changes to experimental design that would keep

exposure time to the glass short enough to not kill the bacteria before *recA* gene expression to take place but long enough to where *lux* genes can be activated may be able to correct for potential problems and yield more reasonable results.

5.4.2 Metabolic Damage Assay. To further investigate the effects of B3, B3-Ag, B3-Ga, and B3-I on bacterial cell metabolism, the TV1061 strain of *E. coli* was exposed to each glass. The TV1061 strain is also a fusion strain with *lux* as the reporter gene. In this strain, the *lux* operon is fused to the *grpE* heat-shock promoter, which is activated by metabolic changes and cytotoxicity. Luminescence of this strain after exposure to each glass was then used as an indirect measure of heat shock promoter activation. Exposure to the glass with this strain yielded somewhat different results than the DPD2794 strain. Exposure to B3 for 15 minutes resulted in the same amount of luminescence as the control, while exposure to B3-Ag, B3-Ga, and B3-I resulted in less luminescence than the control (Figure 5.14).

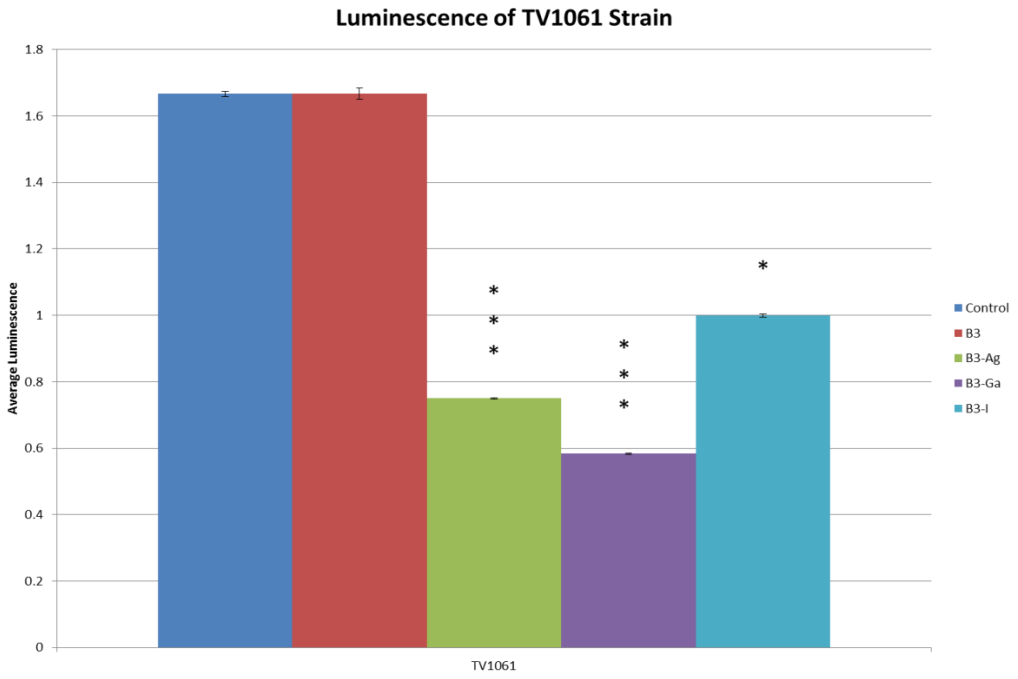

Figure 5.14 Plate reader data for TV1061 after 15 minute incubation. Error bars represent standard deviation (***p<.005; **p<.01; *p<.05)

These data suggest that the metal ions present in B3-Ag, B3-Ga, and B3-I may disrupt the activation of the heat-shock promoter *grpE*, while borate alone in B3 may not be sufficient to disrupt the *grpE* pathway, but cause enough of a metabolic insult to the cell for *grpE* to be activated.

Though the results of the luminescent assays done on the DPD2794 and TV1061 strains of *E. coli* are unclear, these findings provide insight as to further experiments that could be done to elucidate the antibacterial mechanisms of action for B3, B3-Ag, B3-Ga, and B3-I. For instance, using a more direct way to measure DNA damage besides *recA* activation may be useful in determining if the glasses are specifically damaging DNA, or

just inhibiting activation of a repair or regulatory pathway. Also, using a gram positive mutant that may be more sensitive to the glass rather than a gram negative mutant may also be helpful in attaining clearer results.

6. CONCLUSIONS

The results of this research show that the B3, B3-Ag, B3-Ga, and B3-I bioactive glasses are effective at inhibiting the growth of and killing bacteria. Gram-positive bacteria appear to be more sensitive to all of the bioactive glass compositions, as they are killed faster by the glasses than gram-negative bacteria.

The bactericidal capabilities of the glasses are also shown to be independent of the presence of a metal ion, as B3 has antimicrobial properties. This suggests that the borate in the glass alone is sufficient to inhibit bacterial growth.

Live-Dead staining fluorescence microscopy showed that each type of glass is capable of disrupting the cell wall/membrane structure of gram-positive bacteria, but not able to disrupt the cell wall/membrane structure of gram-negative bacteria. This suggests that the outer membrane found only on gram-negative bacteria can protect these bacteria from the antibacterial effects of the glass. These assays also showed that B3, B3-Ag, B3-Ga, and B3-I are more effective at killing MRSA than Vancomycin after a 15 minute incubation. Vancomycin is the current treatment for MRSA infection and can cause serious global side-effects, as it is given to a patient intravenously. These results show that all four glass compositions tested may be a potential topical treatment for MRSA infection, as an alternative or adjuvant to systemic Vancomycin therapy.

In addition, B3 was shown to affect the kinetics of two metabolic enzymes, Succinate dehydrogenase and Malate dehydrogenase by slowing the rate of catalysis by these enzymes and increasing the affinity of the enzymes to their substrates.

At this point, it is unclear whether each glass is able to damage bacterial DNA or activate heat shock protein pathways. However, both remain possible potential mechanisms of action for the bioactive glasses.

Overall, it is clear that B3, B3-Ag, B3-Ga, and B3-I are effective at killing gram-positive bacteria, including MRSA. These glasses can then be implemented as coating on medical devices or implants to reduce the risk of hospital-acquired infection.

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VITA

Megan Ottomeyer was born in Saint Louis, Missouri February 7, 1992. She graduated from Affton High School at the age of seventeen in 2009. From high school, Megan pursued a Bachelor of Science degree in Biological Sciences at Missouri University of Science and Technology with a minor in Chemistry. Before graduating with her Bachelor's degree in May of 2012, Megan served as President of the Phi Sigma Biological Sciences Honor Society and was admitted into the College of Osteopathic Medicine at Kansas City University of Medicine and Biosciences. After deferring her admission to KCUMB for one year, Megan began a Master's degree program at Missouri University of Science and Technology in the summer of 2012. She completed her Master's program in May of 2013, under the guidance and direction of Dr. David Westenberg, Dr. Melanie Mormile, and Dr. Delbert Day. Megan will continue her education beginning in August of 2013 at KCUMB in hopes of becoming a Doctor of Osteopathic Medicine in either the field of Orthopedic Surgery or Emergency Medicine.