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Biohazard neutralization based on lipid technology

Edelmira Bosques

Florida International University

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

BIOHAZARD NEUTRALIZATION BASED ON LIPID TECHNOLOGY

A thesis submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

in

BIOMEDICAL ENGINEERING

by

Edelmira Bosques

2008

To: Interim Dean Amir Mirmiran
College of Engineering and Computing

This thesis, written by Edelmira Bosques, and entitled Biohazard Neutralization Based on Lipid Technology, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

Anthony J. McGoron

Chenzhong Li

Eric T. Crumpler, Major Professor

Date of Defense: July 18, 2008

The thesis of Edelmira Bosques is approved.

Interim Dean Amir Mirmiran
College of Engineering and Computing

Dean George Walker
University Graduate School

Florida International University, 2008

DEDICATION

To my first mentors, my mom Carmen T. Bosques and my dad Leonardo Bosques. Thanks for teaching me the value of the studies, for always believing in my work, and for all your help and loving support through out my career.

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ABSTRACT OF THE THESIS
BIOHAZARD NEUTRALIZATION BASED ON LIPID TECHNOLOGY

by

Edelmira Bosques

Florida International University, 2008

Miami, Florida

Professor Eric T. Crumpler, Major Professor

A rapid detection and neutralization method for biowarfare agents would be a great biodefense in war times. With this purpose, liposomes were developed following the lipid film formation, rehydration, and extrusion procedure as the production method. MgOCl_2 was encapsulated in the liposomes and it was tested with three different bacterium *B. cereus*; *B. thuringiensis*; and *B. subtilis*. For specificity, the liposomes were modified with a polyclonal antibody against *B. cereus* and *B. subtilis*. The liposomes were characterized using a Malvern Zetasizer Instrument, and the study revealed stability of the liposomes stored at 4°C for a period of 15 days. A live/dead assay revealed a significant reduction of bacterium incubated with MgOCl_2 -liposomes. Smaller reduction percentages, but yet significant, were observed with the MgOCl_2 -immunoliposomes. A colony growth assay revealed a significant reduction percentage for empty liposomes, MgOCl_2 -liposomes, and MgOCl_2 -immunoliposomes incubated with *B. thuringiensis*.

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1.0 INTRODUCTION

Beginning on September 18, 2001, several mailed letters containing *Bacillus anthracis* (*B. anthracis*) spores were received at New York and Florida, by some media organizations. In October 2001, some members of the U.S. Congress also received mailed letters containing *B. anthracis* spores. Due to the *B. anthracis* intentional delivery in October 2001; 22 cases of pulmonary anthrax, caused by the inhalation of *B. anthracis* spores were reported. Five of the patients died due to a misdiagnosis of the disease (John A. Jernigan, Stephens et al. 2001). *B. anthracis* spores are acquired by the host through breaks in the skin, by inhalation or by ingestion (Committee to Update Science). It can cause different types of infections, which, if not detected before the fatal stage it will kill the host. The ease of spreading this bacteria spore as an aerosol, with the purpose of causing a massive infection, is attractive to terrorists. In addition, the spores are resistant to environmental changes and can persist in the environment for long periods, causing massive infections. Since all members of the *Bacillus* genus produce the endospores as part of their life cycle, it is impossible to determine the identity of the spores by casual or morphological examination (Swiecki 2006). In case of a bioattack, a fast and accurate way of neutralization is needed to prevent a massive infection.

In this study, liposomes were used as drug carriers of a bacterial neutralizing agent magnesium oxide chlorine (MgOCl_2) with the purpose of enhancing the bactericidal effect of MgOCl_2 on bacterium, aiming the use of this system in the future as a defense against a biohazard. Since their discovery, liposomes have become popular drug carriers systems and have been enhanced in order to target specific sites of drug action such as the brain, cancer, and HIV-infected cells. The liposomes presented in this

study are loaded with a neutralizing agent MgOCl_2 , capable of reducing *Bacillus* bacteria viability. In order to increase the specificity of these liposomes, their surface was modified with a polyclonal antibody specific to the spores and vegetative cells of the bacterium *Bacillus cereus* (*B. cereus*) and *Bacillus subtilis* (*B. subtilis*). The viability of the bacterium *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus subtilis* was studied in the presence of the MgOCl_2 liposomes and MgOCl_2 immunoliposomes, compared to the viability of the bacteria in the presence of free MgOCl_2 .

1.1 Research objectives and specific aims

The primary purpose of this research is to develop a feasible method, capable of neutralizing Gram positive bacteria such as *Bacillus cereus* (*B. cereus*), *Bacillus subtilis* (*B. subtilis*) and *Bacillus thuringiensis* (*B. thuringiensis*). Also, it is proposed that the modified surface of a liposome with a polyclonal antibody against *B. cereus* and *B. subtilis*, has a better neutralization effect than a non targeted liposome. Due to the similarities between *B. cereus* and *B. thuringiensis* to *B. anthracis*, it is expected that the behavior of *B. anthracis* in contact with the immunoliposomes and with the drug loaded liposomes will be similar to that of the aforementioned *Bacillus* species.

Specific Aims:

1. Development of surface modified MgOCl_2 -immunoliposomes with pseudo-specificity toward *B. cereus* and *B. subtilis* bacterium.
2. Determine the efficacy of MgOCl_2 -immunoliposomes compared to MgOCl_2 liposomes and free MgOCl_2 , against bacterial viability.

1.2 Significance of the Study

The significance of this study stems in the attempt to develop a neutralizing immunoliposome system for *Bacillus* bacterium. The neutralizing agent (MgOCl_2) that is able to damage or compromise the membrane cell wall of bacteria was encapsulated within liposomes and immunoliposomes. The development of this neutralization system brings the MgOCl_2 -liposomes and immunoliposomes technology closer to the development of a neutralization system capable of detecting and neutralizing the bacterium in a rapid and effective form. The bactericidal effect observed for the MgOCl_2 immunoliposomes and MgOCl_2 liposomes towards the bacterium *B. thuringiensis*, *B. subtilis*, and *B. cereus* should be similar towards the bacteria *B. anthracis*.

2.0 LITERATURE REVIEW

The Institute of Scientific Medicine affirmed that the United States is not yet prepared to deal with a bioterrorist attack. There are many bacterial threats that could risk citizens' health, for example *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, and *Clostridium botulinum* (Nolen 2003).

Bacillus anthracis (*B. anthracis*), the bacteria that causes the disease anthrax, is the most dangerous bioweapon available to date. The three types of anthrax infections are cutaneous, gastrointestinal, and inhalation of anthrax. Cutaneous anthrax occurs when anthrax spores penetrate a wounded area of the skin; the infection will appear to be an insect bite. In approximately 2 days, the area will become a fluid filled vesicle, which will rupture and form an ulcer. As a peculiar characteristic, it will have a black necrotic area in the center. Approximately 20% of untreated cases of cutaneous anthrax result in death because the infection becomes systemic (Lenski). Gastrointestinal anthrax may be caused by consumption of contaminated meat from infected animals and it is characterized by an acute inflammation of the intestines. Mortality rates are around 25-60% (Weyant, Ezzell et al. 2001). Inhalation anthrax is the most lethal type of anthrax. It is the easiest way of spreading the bacteria in massive amounts. The symptoms of inhalation anthrax are similar to a common cold. If the patient is not treated within 36 hours he would enter into the lethal phase where chances of survival are not encouraging.

Current methods available to determine the presence of *B. anthracis* take up to 48 hours (Zayas 2006). For example, two types of tests performed to detect the presence of *B. anthracis* are the phenotypic identification of *B. anthracis* and antibody based methods. The phenotypic identification method requires blood cultures and requires 1-2

days to provide a definitive identification and cultures from skin or lesions (Eduards 2006). Therefore, attention is focused on *B. anthracis* neutralization in a fast and precise way, with the purpose of biodefense.

2.1 The *Bacillus* genus

The family of *Bacillus* genus distinguishing feature is the production of endospores formed in the bacteria cell. Members of this group are Gram positive bacteria. Gram positive bacterium cell wall thickness is around 15-18 nm and it is composed of linear anionic polymers termed teichoic or teichuronic acids that are covalently linked to the peptidoglycan, giving the membrane cell wall support and protection (Lambert 2002; Kim, Farrah et al. 2007). The membrane wall is a bilayered structure and it has a selective permeable barrier that regulates the passage of substances into and out of the cell. Disorganization of the membrane by undesired or a foreign substance can cause loss of permeability of the membrane; therefore, causing its death (Lambert 2002). Liposomes composition is similar to the membrane cell wall and they can pass easier through it without being recognized as a foreign substance.

All *Bacillus* strains contain special mechanism of survival which is based in the production of endospores, the mechanism is activated in reaction to environmental alterations, such as exhaustion of essential nutrients or stress. The spores are resistant to extreme environmental changes and when the environmental stress is relieved, they germinate and become vegetative cells (K. Todar 2005). This mechanism of survival

makes the elimination of these bacteria even more difficult, and a good bioweapon resource.

The *Bacillus* genus contains some toxins, which are generally water soluble and capable of interacting with the cell membrane. The toxins recognize specific receptors, which usually are lipids or another molecule that bound to the lipid bilayer. Bacterial toxins are able to cross lipid membranes, and induce pores to release cell nutrients or kill target cells by disrupting their membrane. The pore formation involves dynamic interactions between hydrophobic protein and hydrophobic lipid bilayers (Geny and Popoff 2006).

The use of liposomes to carry a bactericidal agent to treat *Bacillus* bacterium will provide a faster neutralization effect because they can pass through the membrane cell wall of the bacteria and the bactericidal toxins permeate the liposomes enhancing the drug release from the liposomes into the cell.

2.1.1 *Bacillus anthracis*

Bacillus anthracis (*B. anthracis*) is the bacteria that cause the disease anthrax. *B. anthracis* has a capsule, a proteinaceous surface layer, and several layers of peptidoglycan outside the surface of the plasma membrane. The capsule of *B. anthracis* contains poly-D-glutamic acid, which is a major determinant of its virulence. Of the *Bacillus* genus strains *B. cereus* and *B. thuringiensis* are the closest relatives to *B. anthracis*. Typically *B. cereus* and *B. thuringiensis* do not contain the poly-D-glutamic acid capsule (Sue, Hoffmaster et al. 2006). *B. anthracis* has some surface labels, which

are the major components of the surface layer proteins EA1 and Sap. These labels can be identified by their specific antibodies anti-EA1 and anti-Sap. In the presence of the capsule, there is an absence of immunolabels; therefore, it is suggested that the cell surface is inaccessible to the EA1 and anti-Sap antibodies. The presence of the antibodies could be due to the synthesis of these proteins prior to the complete coverage of the surface by the capsule or to leakage or bacterial lysis (Mesnage and Tosi-Courtoure 1998). This suggests that targeting the bacteria with an antibody EA1 or anti-Sap needs to be done before the bacterium is covered by the capsule. *B. anthracis* was not used for this thesis due to safety precautions, but the *Bacillus* strains that are described in the following section were used as a model of these bacteria.

2.1.2 *Bacillus thuringiensis*

Bacillus thuringiensis (*B. thuringiensis*) is an anaerobic Gram positive bacterium that is closely related to *B. anthracis* and *B. cereus*. These organisms are capable of producing endospores and differ mainly in their plasmids (Madigan and Martinko 2005). This bacterium has been used as the active ingredient of some insecticides because protein extracts of the bacteria react with the antiprototoxin antibody forming protein crystals of insecticidal protein toxins. These insecticidal proteins are called Cry proteins; these proteins have specific activities against *Diptera*, *Cleoptera*, and *Lepidoptera* species (Du 2005). From previous studies, *Bacillus thuringiensis* Cry4Ba toxin is shown to be capable of permeabilizing liposomes and forming ionic channels in receptor free planar lipid bilayers (Theeraporn Puntheeranurak 2005).

Figure 1. *Bacillus thuringiensis*, microscope image, observe the rod shape of the bacteria (K. Todar 2005).

2.1.3 *Bacillus cereus*

B. cereus is a spore forming bacteria that causes food spoilage and therefore food-borne illnesses (Koo, ForEgeding et al. 1998). This bacterium causes two different types of food poisoning, which are characterized by either emesis or diarrheal type of intoxication (Dietrich 1999). The first type of intoxication is the short- incubation period of 1-6 hours and is characterized by nausea, vomiting, and abdominal cramps. It resembles *Staphylococcus aureus* food poisoning in its symptoms and incubation period. *B. cereus* food poisoning occurs year-round and is without any particular geographic distribution. The short-incubation form is most often associated with fried rice that has been cooked and then held at warm temperatures for several hours. The second type is manifested primarily by abdominal cramps and diarrhea with an incubation period of 8 to 16 hours (K. Todar 2005). This bacterium has two different enterotoxin complexes, the nonhemolytic and hemolysin enterotoxins. Each of them is composed of three components with different molecular masses. The components of the hemolysin BL have been characterized and are required to produce maximum biological activity (Dietrich 1999). The surface protein for *B. cereus* is used for the production of an antibody from

immunized animals and it is purified through SDS-PAGE (Hua 2002). *B. cereus* has an enzyme called Phospholipase C hydrolyzes with phosphatidylcholine, the major component in the liposomes used for this thesis.

2.1.4 *Bacillus subtilis*

Bacillus subtilis (*B. subtilis*) is not classified as a human threat, it may contaminate food but it rarely causes food poisoning. It is commonly found in the soil and has a protective endospore that protects it from extreme environments. *B. subtilis* has become adopted as a model organism; it is used as a Gram positive equivalent of *E. coli*.

2.2 MgOCl₂ Nanoparticles as Bactericidal Agent

Nanotechnology is applying science and engineering knowledge for the design and production of new materials and devices on the nanoscale. Nanotechnology has the goal of increasing the ratio of the surface area per volume present in many nanoscale materials. Nanoparticles used as a bactericidal agent are able to cover the bacteria cells to a high extent bringing the drug's active form, at high concentration, in close proximity to the cell wall membrane where it acts by damaging the cell wall. This nanomaterial characteristic was used as the criteria of selection for liposome encapsulation. MgOCl₂ nanoparticles are capable of damaging the bacterial cell wall because they can surround a higher surface area and create an alkaline environment that destroys the membrane cell wall of the bacteria (Stoimenov 2002). Therefore, MgOCl₂ was encapsulated in the liposomes and tested for bactericidal effect.

Stoimenov used MgOCl_2 nanoparticles prepared through an aerogel procedure (AP) to test its bactericidal and sporicidal activity. AP-MgO nanoparticles possess many properties that are very desirable for a potent disinfectant. AP-MgO nanoparticles have the ability to absorb and retain for longer periods of time (in the order of months) significant amounts of chlorine and bromide. It is suggested that AP-MgO nanoparticles can absorb 20 wt% chlorine. Furthermore, Koper showed that AP-MgO/ Cl_2 formulations are quite active as biocides, more so than free Cl_2 or AP-MgO itself or commercially available microcrystalline MgO (Koper, Klabunde et al. 2002). In previous studies by Stoimenov, it has been proven that AP-MgO/ Cl_2 and AP-MgO/ Br_2 changed significantly the *Escherichia coli*, *Bacillus subtilis*, and *Bacillus Megaterium* cell wall (Stoimenov 2002). Chlorine action is dependent on pH and temperature. From the reaction of chlorine and water, one of the products is HOCl, and pH levels are around 4.0-6.0. Microorganism killed by disinfectants are assumed to follow the CT concept, defined as the product of disinfectant concentration and time (Davis and Cornwell 1991).

2.3 Liposomes and Liposome Technology

Liposomes were discovered by A.D Bangham (Segota and Tezak 2006), who was performing blood clot research on the colloidal behavior of some phospholipids. Bangham found that phospholipids were capable of forming spheres in diluted aqueous solutions, and he described the liposomes as the smallest artificial vesicles that can be produced from natural nontoxic phospholipids and cholesterol (Segota and Tezak 2006).

Since Bangham's discovery, liposomes are considered as highly versatile structures for research due to their many applications.

Liposomes can be used to encapsulate and deliver a chemical dissolved in an aqueous environment. Hydrophilic chemicals are entrapped in the aqueous compartments of the liposomes, whereas hydrophobic chemicals are incorporated into their lipid bilayers (Mugabe, Azghani et al. 2005) (see figure 2). For the purpose of this research a hydrophilic chemical $MgOCl_2$ was used, and the liposomes entrapped it in its aqueous compartment.

Liposomes have permeability properties similar to biological membranes, also they are non toxic and non immunogenic. Therefore, interest in these vesicles towards the study of the interactions between membrane lipids and biomolecule grew due to the possibility of their use as a model of biological membranes (Mugabe, Azghani et al. 2005; Eduards 2006; Segota and Tezak 2006). Liposomes can be characterized by size, number of layers, and surface charge (Segota and Tezak 2006). Liposome size can be controlled by decreasing the total energy in the dispersed system (Sabin, Prieto et al. 2006). Liposomes formed with phospholipids have high stability, but have no thermodynamic stability. They require some energy input provided by sonication, electrophoration or extrusion in order to form vesicles. Initially liposomes consist of several layers (multilamellar vesicles), which are removed as the extrusion process is performed to make them unilamellar encapsulating vesicles of a desired size (Segota and Tezak 2006). The extrusion procedure was the energy input method used for the development of the liposomes in this thesis and filter membranes of 100-80 nm were used in the procedure to make 100nm liposomes.

Phosphatidylcholine, liposomes are zwitterionic lipids, meaning that they have zero net charge and that they will repel each other providing colloidal stability to the phosphatidylcholine liposome system (Malmsten 2002). The membrane of the liposomes made for this thesis is mainly composed of phosphatidylcholine and Biotin-polyethylene glycol (PEG) and is “alloyed” with cholesterol (which is the basic constituent of cell membranes) to improve mechanical stability. This results in a stronger membrane with reduced permeability (Lasic 1998; Sederatou, Tsiourvas et al. 2000). Cholesterol in liposomes has been proven to enhance liposomes recognition due to the organization of the lipids and resulting in a higher lateral motility of the recognizable molecules in the liquid ordered phase (Sederatou, Tsiourvas et al. 2000).

The stability of the liposomes can be enhanced by the addition of a protective agent. In addition, the agent will help avoid aggregation and provide a longer storage time. The cryoprotectant trehalose is a non-reducing glucose disaccharide, which is found in many species of hydrobiotic organism. Trehalose is type of sugar that has been used to maintain the structural integrity and viability of certain biomaterials, and it was proven to be capable of maintaining liposome stability in solution for a longer period of time (Holovati and Acker 2007). Trehalose was used to increase the stability and storage time of the liposomes created for this thesis.

Figure 2. Liposome structure. Hydrophobic drugs can be entrapped within the liposome lipid bilayer. $MgOCl_2$ is a hydrophilic molecule which was used as a neutralizing agent for the purposes of this thesis and was entrapped inside the aqueous area of the liposomes.

Liposomes are of great interest to pharmaceutical companies due the drug encapsulation capacity and the possibility of control of their delivery to a specific target in the body. Drug targeting by antibody-conjugated liposomes (immunoliposomes), see figure 3, represents a technology that has been applied to the targeting of specific sites of drug action, such as the brain, lung, cancer cells or cells of the immune system (Schnyder, Krahenbuhl et al. 2004). Site-specific targeting depends on the labeling of a liposome with an antibody and the high affinity between the antibody and its specific antigen. The use of antibodies in the surface of the liposomes may shorten the detection and neutralization time from days to hours (Koo, ForEgeding et al. 1998). The efficacy of the targeting depends on choosing the correct antibody to label the surface of the liposomes, and the specificity of the antigen against that antibody. The liposomes made for this application contain Biotin PEG- 2000. Biotin is used in many bioanalytical

techniques specifically to form essential irreversible and specific linkages between biological molecules. Avidin and the homologous protein Streptavidin are tetrameric proteins, each can bind up to four molecules of biotin with high affinity (Lo 1999).

Figure 3. Surface modification of a liposome with antibodies to increase specificity.

Streptavidin has a high affinity for biotin ($K_a \sim 10^{15} \text{ M}^{-1}$) (Wolfgang Frey 1996) and will form a high strength bond between these two components. Streptavidin provides four binding sites for the biotin and for an antibody. This will help increase liposome specificity towards a specific target. The antibody used to demonstrate the specificity of the liposomes toward the bacterium in this thesis is a polyclonal antibody. Polyclonal antibodies come from different B cell lines and are a mixture of the immunoglobulin molecules produced by those B cells. Each immunoglobulin differs in the epitope that recognizes and the strength of the interaction. The recognition of the different epitopes in polyclonal antibodies may lead to the cross reaction with different species similar to the target antigen. The antibody to be used in this thesis is the rabbit antibody to *Bacillus* spores and is reactive with spores and vegetative cells of *Bacillus cereus* and *Bacillus subtilis*. As indicated by the manufacturer there is a possibility of cross-reaction with other *Bacillus* species. As a proof of principle, this polyclonal antibody is sufficient to

demonstrate the identification of the bacteria by the MgOCl_2 -immunoliposomes, but for later applications, a monoclonal antibody against the target bacteria should be used.

2.4 m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) crosslinker

MBS is a heterobifunctional cross linker that contains N-hydroxysuccinimide (NHS) ester and maleimide groups that allow covalent conjugation of amine- and sulfhydryl-containing molecule. NHS esters react with primary amines at pH 7-9 to form amide bonds, while maleimides react with sulfhydryl groups at pH 6.5-7.5 to form stable thioether bonds (Pierce Company 2000). MBS was used to add an amine group in the N terminal of Streptavidin. This amine group will bind to the thiolated antibody used for this application of anti *Bacillus* spores.

2.5 2-Iminothiolane (TRAUTS) crosslinker

TRAUTS reagent is a thiolating agent used for the preparation of a disulfide conjugates (R. 1978). It was used to add a sulfhydryl to the amine group in the anti *Bacillus* spores antibody. The addition of the sulfhydryl group in the antibody will permit a conjugation with a Streptavidin protein creating a Streptavidin-antibody conjugate that will bind to the biotin surface of the liposomes created for this thesis.

2.6 HEPES buffer

HEPES is a zwitterionic buffer, that do not bind Magnesium ions and is usually used to maintain pH of cell culture media. It was stored at ambient temperature and protected from light to avoid the production of hydrogen peroxide ions, and cell toxicity (Lepe-Zuniga, Jr. et al. 1987; Probes 2001; Incorporation 2006).

2.7 Size exclusion chromatography (SEC)

SEC is use to separate molecules by size. In general, a column is packed with porous particles of a defined pore size, and the SEC mobile phase is the solvent used to dissolve the sample for purification. The molecules that are too large to penetrate the pores elute in the void volume of the column, and high molecular weight molecules elude first. Smaller particle have a longer residence time since they interact more with the column packing and will elude after the larger molecules and therefore the larger molecules can separate and purified (Mori and Barth 1999).

2.8 Minimum Inhibitory Concentration (MIC) assay

MIC assay is a basic technique used to determine the minimum drug concentration required to kill a bacterial population in a period of 24 hours. The assay consists of adding known concentrations of free MgOCl_2 to a certain volume of bacteria to determine the bactericidal effect compared to the MgOCl_2 -liposomes.

2.9 Fluorescent Microscopy

Fluorescent microscopy is used for a rapid identification of bacterial antigens. A Live/Dead assay kit from Molecular Probes can assess the enzymatic activity of bacterial cells and is used to quantify the amount of live and dead bacteria in a sample. This kit provides two dyes, Syto 9 and propidium homodimer. Propidium homodimer dye penetrates the compromised membrane of the bacteria. The bacterium stained with this dye emits a red fluorescent color whereas the intact bacteria membrane will be dyed with Syto 9 and emit a green fluorescent light. Both can be observed under a fluorescent microscope with adequate filters (Probes 2004). The green fluorescent emission is due to the capacity of living cells to cleave the masked nonfluorescent form of green calcein fluorophore. The reason for the red fluorescence image in dead cells is the failure to exclude the ethidium homodimer dye from their nuclei (Ratner, Hoffman et al. 2004).

3.0 MATERIALS AND LIPOSOMES METHODS

3.1 Materials necessary for the creation of the liposome solution

The materials necessary to make the lipid film are 1,2 Distearoyl-sn-Glycerol Phosphoethanolamine-N- Biotinyl [DSPE-PEG 2000] (from Avanti Polar Lipids) L- α -Phosphatidylcholine (Egg-Chicken) (from Avanti Polar Lipids), Cholesterol (Ovine wool) (from Avanti Polar Lipids) and Chloroform ACS graded (from Fisher Scientific). The lipid film was hydrated with HEPES Buffer 1M (from Gibco Company) and the sugar Trehalose was used to preserve the lipids and the bactericidal agent MgOCl₂ (from Nanomaterials Company).

3.2 Method #1, 100 nm liposome solution preparation

To accomplish the first aim of this thesis, a liposome solution composed of 100 nm sized vesicles was created following Becker's protocol (Becker 2007). This was the first step leading to the accomplishment of Aim 1.

In order to prepare the lipid solution, 37.5 mg of DSPE- PEG 2000 Biotin (solid), 87.5 mg of L- α -Phosphatidylcholine, and 6.5 mg of cholesterol were weighted and dispensed into a round bottom flask. In the fume hood, 50 ml of Chloroform were added and stirred in a round bottom flask for 1 minute to produce a well-mixed solution and to avoid any particle residue. After mixing, the liposome solution was placed in the rotary evaporator (rotovap) under low vacuum to assist the evaporation of the chloroform. The water bath of the rotatory evaporator was set at 40°C, and the water bath of the circulator was set to 14°C. The evaporation of the solvent was assisted by low vacuum, and the

rotation was set to 155 rpms. The average time for the solvent to evaporate was one hour. The lipid film was immersed in liquid Nitrogen three times and it was placed in the lyophilizer for an overnight procedure.

3.3 Liposome film rehydration

The flask containing the lipid film was rehydrated by an addition of 35mL HEPES buffer after the overnight lyophilization. The flask was placed in the rotatory evaporator, and the rotations were set at 100 RPM until the lipid film is completely dissolved in the HEPES (this process takes approximately 45minutes). After the lipid film was completely rehydrated, 13.0 mg MgOCl_2 were added to the liposome solution. The liposome solution containing the MgOCl_2 was placed in the rotatory evaporator at the same conditions previously mentioned for a period of 15 minutes to help the MgOCl_2 dissolve. After the drug was dissolved in the liposome solution, the liposomes were ready for the extrusion procedure (Becker 2007).

3.4 Making 20.0% Trehalose 3mM HEPES solutions

A 3mM HEPES solution was prepared by adding 3 ml of HEPES buffer to a 1000 ml volumetric flask and then filled to the 1000 ml mark with distilled water. The 20.0% trehalose solution was prepared by adding 20.0 mg of Trehalose to 100 ml of 3mM HEPES in an Erlenmeyer flask.

3.5 Extrusion Procedure using the 100 ml extruder

Liposomes are not thermodynamically stable therefore will not form spontaneously (Lasic 1998). Extrusion of the liposomes in a 100 ml extruder provided the required energy to the system for the formation of the vesicles. The 100ml extruder needed to be prepared before the extrusion procedure. The extruder parts were cleaned with water and Alconox anionic detergent and rinsed with 1M HEPES buffer. After the instrument parts were cleaned, an extrusion with Triton X was performed followed by ten HEPES buffer extrusions of 10 ml to remove any liposome residual left in the extruder. After the extruder was cleaned, the water bath was allowed to heat to 42°C and 15 ml of the liposomes were added into the extruder tank. The Argon tank and the valve that allows the flow of the Argon into the extruder (green valve) were opened and the valve that releases the pressure of the tank was closed and the pressure of the Argon tank was increased to 100 psi or to the required pressure for the liposomes to start coming out of the extruder. This process continues until the liposome solution is completely extruded. The extrusion procedure was repeated 10 times with the 100 nm membrane, the membrane was replaced every 5 extrusions. After the tenth extrusion 1 ml of 2% trehalose was added to the extruded liposomes. Ten extrusions were performed with the 80 nm membrane and the membrane was replaced after 5 extrusions, after the tenth extrusion 2 ml of 10% of trehalose were added. Finally, 3 extrusions were performed with a 50 nm membrane and a sample of 1 ml was placed in the Zetasizer Nano ZS (from Malvern Instruments Ltd.) for size measurement. If the size is in the range of 100-80 nm, the liposomes are ready for the purification procedure. Otherwise, 5 more extrusions need to be performed using a 50 nm membrane, until the liposome size matched the

desired size range. This liposome extrusion procedure was established by Michael Becker 2007 (Becker 2007).

3.6 Liposome Purification

Liposome purification was performed by size exclusion chromatography using a Sephadex G-50 column. To make the Sephadex-G50 columns, 25 g of Sephadex were swelled in 250 ml of HEPES (1 Gram swells in 9-11 ml of buffer) at room temperature for 3 hours or at 90°C for one hour. Glass wool was packed inside a 60 ml syringe. It is necessary to pre-treat the glass wool for 15 minutes with HEPES buffer before packing it. The Sephadex-G50 column was placed at low vacuum and 10 ml of liposome solution was added to the column for purification. The purified liposomes were recovered in a 50 ml Erlenmeyer flask and stored in the refrigerator at 4-8°C. After the purification, the liposomes size was measured with a Zetasizer Nano ZS manufactured by Malvern Instruments Ltd.

3.7 Method #2, Streptavidin-Nanoparticle antibody Complex

The development of the Streptavidin- Antibody complex is the second step leading to the accomplishment of Aim 1.

3.8 Streptavidin Coated Nanoparticle- MBS synthesis

A Streptavidin-MBS complex was developed by mixing 40 µl of Streptavidin in 5 ml of a 16.0 mM HEPES solution (4.3 ml of deionized water, and 0.7 mL HEPES) in a

15 ml flask. A 40:1 molar excess of MBS solution was added to the diluted Streptavidin by adding 200 μ l of MBS solution.

3.8.1 MBS 40:1 Molar Excess

Following the instructions from the Pierce company protocol; 3.14 mg of MBS was dissolved in 1 mL of DMSO to make a 10 mM MBS solution. The preparation of the molar excess consists of adding 40 μ l of Streptavidin to the MBS solution. A 40:1 molar excess of MBS solution is prepared by adding 200 μ l of MBS to 40 μ l of Streptavidin solution. The MBS-Streptavidin Complex was purified following the standard protocol of a 30 Centriprep Ultrafiltration Device from Millipore.

3.9 Antibody Thiolation

The antibody needs to be thiolated before it can bind to the available maleimide in the Streptavidin-MBS complex. A 40:1 molar excess of TRAUTS powder, was prepared by dissolving 1 mg of TRAUTS in 200 μ l of HEPES. The antibody (rabbit anti*Bacillus* spores) (40 μ l) was diluted in 2 ml of HEPES 1M. A 40:1 molar excess was added to the diluted antibody with the buffer flask, and it was placed in a gently stirred water bath at room temperature for one hour. After the antibody thiolation, purification by size exclusion chromatography was performed in a Sephadex 25-G column.

3.10 Method #3, Conjugation of the Streptavidin- Antibody Complex

The third and final step for the accomplishment of Aim 1 consists of the formation of the Streptavidin-MBS complex and the conjugation of the complex with the liposomes.

In order to obtain the Streptavidin-Antibody complex, 150 μ l of the Streptavidin-MBS Complex were mixed with 500 μ l of the antibody-TRAUTs into a 15 ml conical tube and vortexed it for 20 seconds. This complex was left in a water bath overnight to permit the conjugation to take place.

The MgOCl_2 -immunoliposomes were made with a 1:1 ratio of the MgOCl_2 liposomes and the Streptavidin-Antibody complex. The MgOCl_2 -immunoliposomes inside the 15 ml flask were placed in a water bath at room temperature, and stirred for 30 minutes to ensure the reaction took place.

Figure 4. Schematic of the biotinylated liposome surface, modified with the Streptavidin- Antibody Complex, where $\times$ represents Streptavidin, $\Uparrow$ represents the anti-*Bacillus* spores antibody, $\square$ and $\blacksquare$ represents the PEG-2000 Biotin.

3.11 BCA Assay

To determine the concentration of proteins (antibody) in the Streptavidin-antibody complex, a BCA assay was performed. This assay is required for the quantification of the total protein attached to the surface of the liposome (Streptavidin and the anti *Bacillus* spores antibody).

The BCA assay is based on the reduction of Cu^{+2} to Cu^{+1} by proteins. The reduction is proportional to the protein present in a solution. The biuret product that is in contact with the Cu^{+1} turns violet is an indicator of protein presence (Pierce Company 2006). The assay comes with two reagents that mixed together in a 50:1, A to B. This mixture is called the working solution. This solution makes the samples change from clear to color purple when protein is present. The manufacturer standard protocol is followed to create the standard solutions in order to obtain the absorbance values in the spectrophotometer. The obtained data was used to determine the protein concentration. The absorbance of the standard and the Streptavidin-Antibody sample was determined in a TECAN spectrophotometer at 595 nm. A standard curve of absorbance vs. concentration was created in Microsoft Excel. With the standard curve, the values of the absorbance obtained for the Streptavidin-Antibody complex were used to find the total protein concentration in this complex.

3.12 Method #4, Bacteria Preparation

In order to test the MgOCl_2 -immunoliposomes to accomplish Aim 2 a standard protocol of bacteria preparation was followed as described in the next section.

3.12.1 Luria Broth Media Preparation

Fresh Luria Broth bacteria media was made by adding 20 g of Luria Broth (LB) to 1000 ml of distilled water into 2000 ml Erlenmeyer flask. This solution was stirred in a stirring plate at high velocity until a homogeneous solution was observed. After the solution was prepared, it was placed into the autoclave equipment for a period of 50 minutes in the liquid cycle.

3.12.2 Agar Preparation

As recommended in the standard protocol for agar preparation, 40 mg of agar powder was added to 1000 ml of dionized water. The solution was well mixed with a magnetic stirrer for 15 minutes. The agar solution was placed in the autoclave for 25 minutes. After removing from the autoclave, the solution was placed in the magnetic stirrer mixer to cool down the solution. After the solution is warm enough, it was poured in the agar plates.

3.12.3 Bacteria Culture

Basic bacteria culture procedures like bacteria streaking and bacteria inoculation was followed. Bacteria stocks were placed in a -80°C freezer; the vials were taken from the freezer; and was warmed in the hands. With a pipette tip, obtain some cells from the freeze stock and streak in the agar plate as shown in figure 5.

Figure 5. Plate streaking sample. The number 1 indicates the streaking starting point

After streaking the desired number of culture dishes, the plates were placed inside an incubator at physiological conditions (37°C) overnight.

3.12.4 Bacteria Inoculation

Sixteen hours after the streaking incubation, the culture dishes were removed from the incubator and placed in the bench top until a selection of one isolated colony was made. The selected colony was scraped with a round tip and then mixed in a 50ml Erlenmeyer flask with 10 ml of Luria Broth. The bacterial solution was placed inside a shaking incubator at 37°C overnight. A bacteria concentration was determined using an Optical Density Spectrophotometer analysis at 600 nm. From this concentration, 4 serial dilutions of 1:10 were made to reach theoretical concentration of 1×10^5 cells/ml.

3.13 Protocol 1, BCA assay

The most important step to develop a successful BCA assay to obtain the unknown amount of protein in solution, is to generate a standard curve of Absorbance vs.

Concentration. The standard solutions were prepared following the instructions provided by the manufacturer in Table 1. Three samples of twenty five microliters each of the standard dilutions were pipetted into a 96-well microplate with 200 μ l of working reagent. The plate was then shaken for 30 seconds and incubated at 60°C for 30 minutes. After 30 minutes of incubation the microplate was cooled at room temperature for 30 minutes. The absorbance reading was measured in the Tecan instrument at 595 nm. An Absorbance vs. Concentration curve was performed with the averages of the measured absorbance values subtracted to the blank standard measurement at 595nm.

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA concentration
A	720 μ l	80 μ l of stock	200 μ g/ml
B	300 μ l	500 μ l of vial A dilution	125 μ g/ml
C	160 μ l	640 μ l of vial B dilution	100 μ g/ml
D	400 μ l	400 μ l of vial C dilution	50 μ g/ml
E	400 μ l	400 μ l of vial D dilution	25 μ g/ml
F	800 μ l	0	0 μ g/ml= Blank

Table 3-1. Dilutions for enhanced BCA standard. Preparation of diluted albumin (BSA) standards dilution scheme for enhanced protocol microplate (working range 200-5 μ g/ml) modified from Thermo Scientific Pierce Protein Research Products, protocol.

3.14 Protocol 2, Minimum Inhibitory Concentration Test

In order to determine the $MgOCl_2$ liposomes effectiveness compared to the free drug at different concentration effectiveness, a Minimum Inhibitory Concentration assay was performed. $MgOCl_2$ was diluted with 1M HEPES buffer to create solutions at concentrations of 2M, 1M, 0.5M, 30mM, 3mM, 1.5mM, 0.75mM, 0.3mM, 0.15mM, and 0.075mM.

B. thuringiensis cultured overnight was diluted 5 times to a theoretical cell concentration of 1×10^5 cells/ ml. Optical density measurements at 600nm were recorded. One hundred and fifty microliters of the diluted bacteria was placed inside a 96-well plate and mixed with 150 μ l of the different $MgOCl_2$ solutions. At time 0, 100 μ l of the bacterial solutions were plated in a Petri Dish. The 96-well plate was placed inside a shaking incubator at physiological conditions for a period of 22 hours. After 22 hours 100 μ l of the 96-well plate were plated in a Petri Dish and incubated at physiological conditions for 8 hours to observe the bacterial growth.

The 96-well plate and Petri dishes were filled and identified as described in Table 2.

Plate Identification	Contents
A	HEPES + <i>B. thuringiensis</i>
B.	2M $MgOCl_2$ + <i>B. thuringiensis</i>
C.	1M $MgOCl_2$ + <i>B. thuringiensis</i>
D	0.5M $MgOCl_2$ + <i>B. thuringiensis</i>
E	30mM $MgOCl_2$ + <i>B. thuringiensis</i>
F	3mM $MgOCl_2$ + <i>B. thuringiensis</i>
G	1.5mM $MgOCl_2$ + <i>B. thuringiensis</i>
H	0.75mM $MgOCl_2$ + <i>B. thuringiensis</i>
A6	0.3mM $MgOCl_2$ + <i>B. thuringiensis</i>
B6	0.15mM $MgOCl_2$ + <i>B. thuringiensis</i>
C6	0.075mM $MgOCl_2$ + <i>B. thuringiensis</i>
D6	$MgOCl_2$ liposomes + <i>B. thuringiensis</i>

Table 3-2. 96-well plate and Petri dish contents for the minimum growth concentration assay.

3.15 Protocol 3, Colony Growth inhibition assay (CGA)

This assay was used to accomplish aim 2 to quantify the effect of the $MgOCl_2$ –immunoliposomes and $MgOCl_2$ liposomes towards *B. thuringiensis*.

Bacillus thuringiensis was streaked and inoculated using standard protocol described above. A 100 μ l sample of each bacterium strain was taken from the flask inoculated overnight, and diluted with 900 μ l of Luria Bertani (LB) broth. Five hundred micro liters of the diluted sample were placed in a 1 cm^3 cuvette. An optical density analysis was taken at 600 nm wavelength to determine the original bacteria concentrations. Four serial dilutions of 1:10 were performed. The diluted bacteria were divided in four 15 ml flasks. Flask number four was filled with 2 ml of the fourth dilution (containing 2.5 ml of third dilution plus 22.5 ml of Luria Broth). A quantity of 1.5 ml of HEPES buffer was added to 15 ml flask number one, the same amount of empty liposomes, MgOCl_2 liposomes, and MgOCl_2 -immunoliposomes were added to flask number 2, 3, and four respectively. The same solutions prepared for *B. cereus* were prepared for the bacteria *B. thuringiensis* and *B. subtilis*. Two 1:10 serial dilutions were performed in order to obtain less than 300 colonies in the plates. The control plates of *B. cereus* and *B. thuringiensis* contain 100 μ l of each of the flasks prepared in four different Petri dishes at time zero. After one hour of incubation, 100 μ l of each flask was plated in four different Petri dishes. After three hours of incubation, 100 μ l of each of the solutions was plated onto four different plates. The same procedure was repeated for *B. thuringiensis*, but only with the difference of control plate containing 100 μ l of each solution. All the plates were incubated overnight at 37°C.

Figure 6. Colony growth assay (CGA) summary procedure. 1. Bacteria Streaking schematic, 2. One isolated colony from the streaked plate is inoculated overnight in LB broth at physiological conditions. 3. Five serial dilutions were performed in order to have approximately 1×10^4 cells/ml, a 1:1 ratio (Bacterial solution: HEPES, Empty liposomes, MgOCl_2 liposomes, MgOCl_2 immunoliposomes and 3mM MgOCl_2 in five different vials). 4. At time 0 hours 100 μl of the solutions prepared in step 3 were plated in petri dishes, the solutions were incubated at physiological conditions for two hours and plating was performed for each hour. In a period of 6-8 hours the colonies become visible and the CFU count was performed.

3.16 Protocol 4, Fluorescent Microscopy

To identify the effect in the membrane cell wall of the bacteria caused by the MgOCl_2 liposomes and the MgOCl_2 immunoliposomes a live/dead assay for cell viability was performed. The bacteria samples in contact with the MgOCl_2 liposomes and the MgOCl_2 -immunoliposomes were dyed with the backlight kit L7012 from Molecular Probes that contain propidium iodide and Syto-9. If the membrane cell wall is damaged, propidium iodide will enter inside the cell and bind to nucleic acid. This will produce the red image as an indication of dead or damaged cell. In contrast, Syto-9 will make a green fluorescent image as a result of the labeling of the dyed nucleic acids in the bacteria without damage in the membrane cell wall. The protocol followed was as provided by

the company. The bacteria was grown overnight. From the bacteria culture, 30 ml of bacteria were grown until an absorbance of 1 AU was observed. After this absorbance was obtained, 25 ml were centrifuged for 15 minutes at 9000g in Sorval RT 6000 Series Centrifuge. The supernatant was decanted and the pellet was resuspended in 2 ml of 0.85% of NaCl (saline solution). The 2 ml of bacterial suspension was divided into two vials. One vial contained 20 ml of Isopropyl Alcohol and the second vial contained 20 ml of 0.85% of saline solution. The vials were incubated for one hour and mixed every 15 minutes. The cells were centrifuged at 9000g for 15 minutes. The supernatant was decanted from both vials and the pellet was re-suspended in 20 ml of the 0.85 % saline solution. The centrifugation procedure was repeated for the last time at the same conditions previously mentioned. The supernatant was decanted and the pellet was re-suspended. The bacterial concentration was adjusted to 1×10^8 cells/ml and it was stained with a mixture of the propidium iodide and Syto 9. It was then observed under the fluorescent microscope.

Figure 7. Schematic representation of MgOCl_2 -immunoliposomes attached to the antigens of *B. subtilis*

4.0 RESULTS

4.1 Liposome Size

Liposome size measurements were performed in the Malvern Zeta Sizer. The liposome size varied between 50 nm and 500nm with averages size of 100-149 nm (Figures 8-17). The liposomes were kept at 4°C and weekly size distribution test readings were performed in the Malvern Zetasizer to determine the stability of the liposomes in solution. The polydispersity index of the empty liposome solution doubled after a week but remained stable for a period of three weeks. The polydispersity index for MgOCl_2 liposomes remained stable for three weeks. In the third week of storage the MgOCl_2 liposomes size increased suggesting liposome swelling or aggregation. On the third week empty liposomes size and polydispersity also increased.

MgOCl_2 liposomes and empty liposomes at 4 weeks of storage at 4°C, showed a polydispersity index (PDI) of above 0.2, and more than one peak in the size distribution curve. The MgOCl_2 liposomes with the antibody attached to its surface showed a polydispersity index of above 0.2 and a second peak on the size distribution curve after one week of storage at 4°C.

Figure 8. Empty liposomes size distribution curve on the 1st day of storage at 4°C. Obtained in the Malvern Zetasizer for liposomes after extrusion and trehalose addition. The polydispersity index corresponding to this curve is 0.059 and the average Z size is 123 nm.

Figure 9. Empty liposome size distribution curve after one week of storage at 4°C. The polydispersity index corresponding to this curve is 0.122 and the average Z size is 143nm.

Figure 10. Empty liposome size distribution curve after 10 days of storage at 4°C. The polydispersity index corresponding to this curve is 0.123 and the average Z size is 122nm.

Figure 11. MgOCl₂ liposome on the 1st day of storage at 4°C. Extruded liposome batch with the addition of trehalose. The polydispersity index corresponding to this curve is 0.093 and the average Z size is 108nm.

Figure 12. MgOCl₂ liposomes stored at 4°C for one week. The polydispersity index corresponding to this curve is 0.101 and the average Z size is 122 nm.

Figure 13. MgOCl₂ liposomes stored at 4°C for 3 weeks. The polydispersity index corresponding to this curve is 0.122 and the average Z size is 146 nm.

Figure 14. Empty liposomes batch 2, on the 1st day of storage at 4°C. Size Distribution curve obtained in the Malvern Zetasizer for liposomes after extrusion and trehalose addition. The polydispersity index corresponding to this curve is 0.180 and the average Z size is 116 nm.

Figure 15. Empty liposomes batch 2, stored at 4°C 4 weeks. Size Distribution curve obtained in the Malvern Zetasizer for liposomes after storage in 4°C for a 4 weeks period. The polydispersity index corresponding to this curve is 0.283 and the average Z size is 50.8 nm.

Figure 16. MgOCl_2 liposomes batch 2, on the 1st day of storage at 4°C. Size Distribution curve obtained in the Malvern Zetasizer for liposomes after extrusion and Trehalose addition, on the 1st day of storage. The polydispersity index corresponding to this curve is 0.299 and the average Z size is 125 nm.

Figure 17. MgOCl_2 liposomes batch 2 stored at 4°C 4 weeks. Size Distribution curve obtained in the Malvern Zetasizer for liposomes after 4 weeks of storage. The polydispersity index corresponding to this curve is 0.653 and the average Z size is 61.4 nm.

Figure 18. MgOCl_2 immunoliposomes 1st day of storage at 4°C. Size Distribution curve obtained in the Malvern Zetasizer for liposomes where the polydispersity index corresponding to this curve is 0.299 and the average Z size is 120 nm.

Figure 19. MgOCl_2 immunoliposomes stored 10 days at 4°C. Size Distribution curve obtained in the Malvern Zetasizer for liposomes after 10 days of storage. The polydispersity index corresponding to this curve is 0.235 and the average Z size is 103nm.

4.2 BCA Assay

The BCA assay standard curve was used to approximate the amount of total protein (Streptavidin plus Anti-*Bacillus* antibody) present after the crosslinking and purification of the Streptavidin-Antibody complex. The average absorbance for the protein solutions after subtracting the average 595 nm absorbance measurement of the blank standard was 0.048. From this value an interpolation in the BCA standard curve was done to obtain a 44.2 $\mu\text{g/ml}$ concentration for the Streptavidin-Antibody complex.

Figure 20. BCA standard curve. The Streptavidin-antibody absorbance and concentration point is represented with the black triangle (44.2 $\mu\text{g/ml}$, 0.0482).

4.3 Minimum Inhibitory Concentration (MIC) assay

B. thuringiensis with a theoretical concentration of 1×10^5 cells/ml was incubated for a period of 22 hours with different MgOCl_2 solution concentrations 2 M; 1 M; 0.5 M; 30 mM; 3 mM; 1.5 mM; 0.75 mM; 0.3 mM; 0.15 mM; 0.075 mM; and MgOCl_2 liposomes. Samples of 100 μl were plated in different Petri dishes at time 0 and after 22 hours of incubation, the bacterial growth was observed. The plates containing high MgOCl_2 concentrations (2 M, 1 M and 0.5 M) showed no bacterial growth. Bacterial growth was observed in the plates that contained the drug concentrations from 30 mM to 0.075mM plates A6, B6, C6, A, F, E, G, and H (Figure 19). The plate containing the MgOCl_2 liposomes incubated with the bacteria (D6) had no bacterial growth, indicative of the efficiency provided by the liposomes.

Figure 21. MIC for *B. thuringiensis* after 22 hours of incubation. Observe plates A6, B6, C6, A, F, E, E, G, H bacterial growth. In plates B, C, D, and D6 you can see through the plate, and no bacterial growth was observed. See table 2 for plate identification.

4.4 Colony Growth Assay (CGA)

A CGA for *B. thuringiensis* was performed as a quantification method for the bactericidal effect of the MgOCl_2 liposomes, and the MgOCl_2 immunoliposomes. A two

sample paired t-test for means was used to compare the reduction effect caused by HEPES buffer; empty liposomes; and 3mM MgOCl₂ to the MgOCl₂ liposomes and MgOCl₂-immunoliposomes effect.

	Test 1	Test 2	Test 3	Test 4	Avg	Stdev
HEPES						
1 hour	0.0	0.0	0.0	0.0	0.0	0.0
2 hours	0.0	0.0	0.0	0.0	0.0	0.0
Empty liposomes						
1 hour	36.2	30.3	78.0	31.9	44.1*	22.7
2 hours	100.0	0.0	0.0	100.0	50.0	57.7
MgOCl₂ liposomes						
1 hour	57.8	55.1	80.0	38.0	57.7 * ⁺	17.2
2 hours	26.5	60.3	40.0	33.8	40.2 *	14.5
MgOCl₂ immunoliposomes						
1 hour	0.0	20.8	52.3	0.0	18.3	24.7
2 hours	0.0	57.7	38.3	0.0	24.0	28.8
3mM MgOCl₂						
1 hour	0.0	0.0	32.0	0.0	8.0 ⁺	16.0
2 hours	0.0	41.7	34.8	0.0	19.1	22.3

Table 4-1. Reduction percentages of four different tests for *B. thuringiensis* CGA, where * means p value < than 0.05 compared to Hepes and + means p value < 0.05 compared to empty liposomes.

(A)

(B)

Figure 22. Colony growth assay (CGA) for test #1 (A) CGA for *B. thuringiensis* after 1 hour (B) CGA for *B. thuringiensis* after 2 hours of treatment with MgOCl_2 solutions, incubated overnight. Where 1, is *B. thuringiensis* in HEPES, 2, is *B. thuringiensis* in Empty liposomes, 3, *B. thuringiensis* in MgOCl_2 immunoliposomes, 4, is *B. thuringiensis* in MgOCl_2 liposomes, and 5, is *B. thuringiensis* in 3mM MgOCl_2 solution.

CGA p values from the t:Test after 1 hour of treatment with bacteria

	<i>B. thuringiensis</i> P value compared to HEPES buffer	<i>B. thuringiensis</i> P value compared to Empty liposomes
HEPES	N/A	0.015
Empty liposomes	0.015	N/A
MgOCl_2 lip	0.034	0.47
Immunoliposomes	0.12	0.010
3mM MgOCl_2	0.20	0.00099

Table 4-2. *B. thuringiensis* CGA P values from a t-Test comparing the effect of MgOCl_2 liposomes, 3mM MgOCl_2 and MgOCl_2 -immunoliposomes vs. the control HEPES and empty liposomes at 1 hour of bacteria treatment.

CGA p values from the t:Test after 2 hours of treatment with bacteria

	<i>B. thuringiensis</i> P value compared to HEPES buffer	<i>B. thuringiensis</i> P value compared to Empty liposomes
HEPES	N/A	0.090
Empty liposomes	0.090	N/A
MgOCl ₂ lip	0.0057	0.40
Immunoliposomes	0.097	0.29
3mM MgOCl ₂	0.092	0.25

Table 4-3. *B. thuringiensis* colony growth assay (CGA) P values from a t-Test comparing the effect of MgOCl₂ liposomes, 3mM MgOCl₂ and MgOCl₂-immunoliposomes vs. the controls HEPES and empty liposomes at 2 hours of bacteria treatment.

CGA p values from the t:Test, comparison of the general effect of liposomes vs. the controls

	<i>B. thuringiensis</i> P value compared to HEPES buffer	<i>B. thuringiensis</i> P value compared to Empty liposomes
HEPES	N/A	0.0272
Empty liposomes	0.00687	N/A
MgOCl ₂ lip	4.87E-05	0.0178
Immunoliposomes	0.0242	0.457
3mM MgOCl ₂	0.0410	0.253

Table 4-4. *B. thuringiensis* colony growth assay (CGA) P values from a t-Test comparing the general effect (combining 1 and 2 hours) of MgOCl₂ liposomes, 3mM MgOCl₂ and MgOCl₂-immunoliposomes vs. the controls HEPES and empty liposomes.

4.5 Live/Dead Assay

The bacterium *B. thuringiensis*, *B. subtilis* and *B. cereus* were incubated with 5 different solutions; HEPES buffer; empty liposomes; MgOCl₂ liposomes; MgOCl₂ immunoliposomes; and 3mM MgOCl₂. The vials containing the different solutions were centrifuged in a Pico Fuge microcentrifuge at 10,000 RPM for 10 minutes. The supernatant was removed and 1 ml of HEPES, Empty liposomes, MgOCl₂ liposomes, MgOCl₂ immunoliposomes, or 3mM MgOCl₂ solutions were re-suspended in each vial.

Three (3) samples of 150 μ l of the re-suspended solution were placed in a 96-well plate and 0.15 μ l of the mixed fluorescent dyes was added to the solutions. The 96-well plate was incubated for 15 minutes. Three microscope slides were prepared with 5 μ l of the dyed solution and observed at 40X under the fluorescent microscope. A total count of 100 live and dead cells was performed and pictures were captured. Reduction percentages at time 0 hours and 1 hour are shown on tables 7, 8, 10, 11, 13, and 14. In order to determine the statistical significance of the reduction percentages obtained, a two sample paired t-test for means was performed. The t-test compared the reduction percentages of the bacteria incubated with the controls, HEPES, and empty liposomes, at time 1 hour versus the reduction effect of the liposomes and free drug at 1 hour of incubation. The tables presenting the p-values for *B. thuringiensis*, *B. cereus* and *B. subtilis* are 9, 12, and 15 respectively.

4.5.1 *Bacillus thuringiensis* live/dead assay results

Average reduction times of *B. thuringiensis* at 0 hour of incubation with the solutions

	Avg. Reduction % HEPES	Avg. Reduction % Empty Liposomes	Avg. Reduction % MgOCl ₂ liposomes	Avg. Reduction % MgOCl ₂ immuno- liposomes	Avg. Reduction % 3mM MgOCl ₂
Test 1	12.3	4.3	15.0	15.0	7.7
Test 2	14.0	20.7	37.0	26.0	11.3
Test 3	28.0	19.0	12.5	18.9	19.5
Avg	18.1	14.7	21.5	20.0	12.8
Stdev	8.6	9.0	13.5	5.6	6.0

Table 4-5. *B. thuringiensis*, live/dead assay reduction percentages averages at time 0 hr. At this time non significant reduction percentages were observed in the t-test performed.

Average reduction times of *B. thuringiensis* at 1 hour of incubation with the solutions

	Avg. Reduction % HEPES	Avg. Reduction % Empty liposomes	Avg. Reduction % MgOCl ₂ liposomes	Avg. Reduction % MgOCl ₂ immuno-liposomes	Avg. Reduction % 3mM MgOCl ₂
Test 1	8.7	7.7	20.0	22.0	13.0
Test 2	10.7	18.7	32.0	27.0	30.0
Test 3	25.3	31.1	43.0	40.0	36.0
Avg	14.9	19.2	31.7* ⁺	29.7* ⁺	26.3 ⁺
Stdev	9.1	11.7	11.5	9.3	11.9

Table 4-6. *B. thuringiensis*, live/dead assay average bacterial reduction percentages at time 1 hr, where * means p value < than 0.05 compared to HEPES and + means p value < 0.05 compared to Empty liposomes

B. thuringiensis live/dead assay p values from the t:Test samples for means at time 1 hour

	<i>B. thuringiensis</i> P value compared to HEPES buffer	<i>B. thuringiensis</i> P value compared to Empty liposomes
HEPES	N/A	0.128
Empty liposomes	0.128	N/A
MgOCl ₂ liposomes	0.0145	0.000554
MgOCl ₂ -Immunoliposomes	0.0071	0.0157
3 mM MgOCl ₂	0.0560	0.0371

Table 4-7. *B. thuringiensis*, P values from a t-test: for means. The reduction percentage caused by the controls (HEPES and empty liposomes) were compared to the effect of the MgOCl₂ liposomes, MgOCl₂-immunoliposomes and 3mM MgOCl₂ after one hour of incubation.

Significant reduction percentages were observed for the MgOCl₂ liposomes; MgOCl₂-immunoliposomes, and 3mM MgOCl₂. The highest reduction percentage was observed in the MgOCl₂ liposomes 31.7%, compared to a 26.0% reduction caused by the free drug and a reduction of 29.0% was observed for the MgOCl₂-immunoliposomes.

4.5.2 *Bacillus cereus*, live/dead assay results

Average reduction times of *B. cereus* at 0 hours of incubation with the solutions

	Avg. Reduction % HEPES	Avg. Reduction % Empty Liposomes	Avg. Reduction % MgOCl ₂ liposomes	Avg. Reduction % MgOCl ₂ immuno- liposomes
Test 1	14.14	14.7	13.0	10.0
Test 2	30.56	10.9	20.5	13.0
Test 3	26.0	10.6	20.2	21.6
Avg	23.6	12.1	17.9	17.3
Stdev	8.5	2.3	4.2	6.0

Table 4-8. *B. cereus*, percentage reduction averages using different solutions at time 0 hr for the live dead assay.

Average reduction times of *B. cereus* at 1 hour of incubation with the solutions

	Avg. Reduction % HEPES	Avg. Reduction % Empty Liposomes	Avg. Reduction % MgOCl ₂ liposomes	Avg. Reduction % MgOCl ₂ immuno- liposomes
Test 1	32.2	26.0	34.6	38.0
Test 2	28.5	34.0	27.7	32.7
Test 3	31.0	32.0	29.0	20.0
Avg	30.5	30.7	30.4	30.2
Stdev	1.9	4.2	3.7	9.3

Table 4-9. *B. cereus*, percentage reduction averages using different solutions at time 1 hr for the live dead assay.

B. cereus live dead p values from the t:Test paired two samples for means

	<i>B. cereus</i> P value compared to HEPES buffer	<i>B. cereus</i> P value compared to Empty liposomes
HEPES	N/A	0.487
Empty liposomes	0.487	N/A
MgOCl₂ liposomes	0.471	0.482
MgOCl₂-Immunoliposomes	0.480	0.479

Table 4-10. *B. cereus*, P values from a t-test: for means, comparing the significance of the effect on the 2 controls HEPES and Empty liposomes vs. the MgOCl₂ liposomes and MgOCl₂ immunoliposomes. The reduction percentage caused by the controls HEPES and empty liposomes at time 1 hr was compared with its reduction percentage after 1 hour of incubation to the effect of the MgOCl₂ liposomes and MgOCl₂-immunoliposomes one hour of incubation.

The reduction percentage of *B. cereus* at time 1 hour in solution with HEPES buffer was compared with the effect of the bacteria in solution with empty liposomes, MgOCl₂- liposomes, and MgOCl₂-immunoliposomes. The statistical analysis revealed a non-significant increase in reduction percentage. The same statistical analysis was performed for the comparison of the effect of empty liposomes at time 1 hour with the aforementioned solutions. The statistical analysis result of this assay revealed a non significant bactericidal effect on *B. cereus* caused by the MgOCl₂ liposomes and MgOCl₂-immunoliposomes. The result for this experiment showed a similar reduction percentages averages (30.5, 30.7, 30.4, and 30.2) for each solution. The reduction percentage observed in the controls can be due to the presence of hydrogen peroxide in the HEPES buffer.

4.5.3 *Bacillus subtilis* live/dead assay results

Average reduction times of *B. subtilis* at 0 hours of incubation with the solutions

	Avg. Reduction % HEPES	Avg. Reduction % Empty Liposomes	Avg. Reduction % MgOCl ₂ liposomes	Avg. Reduction % MgOCl ₂ immuno- liposomes
Test 1	10.0	11.9	16.0	6.0
Test 2	24.0	13.6	7.0	6.0
Test 3	27.0	16.0	21.0	35.9
Avg	20.3	13.8	14.7	16.0
Stdev	9.1	2.1	7.1	17.3

Table 4-11. *Bacillus subtilis* average reduction bacterial percentage at time 0 hr.

Average reduction times of *B. subtilis* at 1 hour of incubation with the solutions

	Avg. Reduction % HEPES	Avg. Reduction % Empty Liposomes	Avg. Reduction % MgOCl ₂ liposomes	Avg. Reduction % MgOCl ₂ immuno- liposomes
Test 1	20.0	28.9	38.0	37.5
Test 2	23.0	18.0	45.7	25.0
Test 3	26.0	23.0	53.0	40.8
Avg	23.0	23.3	45.6* ⁺	34.4 ⁺
Stdev	3.0	5.5	7.5	8.3

Table 4-12. *Bacillus subtilis* average reduction bacterial percentage after 1 hr of incubation with the different solutions, where * means p value < than 0.05 compared to Hepes and + means p value < 0.05 compared to Empty liposomes

The reduction percentages observed at time 0 hours suggests that the HEPES buffer caused the highest reduction percentages, but after the bacteria was incubated for an hour the effect of HEPES on the bacteria remained stable while the effect of the MgOCl₂ liposomes and MgOCl₂ immunoliposomes significantly increased. This

suggests that the reduction effect observed at time 0 hours for HEPES is not persistent and will disappear after time.

***B. subtilis* live dead p values from the t:Test for 1 hour of incubation with bacteria**

	<i>B. subtilis</i> P value compared to HEPES buffer	<i>B. subtilis</i> P value compared to Empty liposomes
HEPES Buffer	N/A	0.476
Empty liposomes	0.476	N/A
MgOCl ₂ liposomes	0.00650	0.0390
MgOCl ₂ -Immunoliposomes	0.0670	0.0400

Table 4-13. *B. subtilis*, P values from a t-test: for means, comparing the significance of the effect on the 2 controls HEPES and empty liposomes vs. the MgOCl₂ liposomes and MgOCl₂ immunoliposomes. The reduction percentage caused by the controls HEPES and empty liposomes at time 1 hr was compared with its reduction percentage after 1 hour of incubation with the effect of the MgOCl₂ liposomes, MgOCl₂-immunoliposomes after 1 hour of incubation.

Reduction percentages of *Bacillus subtilis* at time 0 hours and 1 hour of incubation are presented in table 11 and 12, respectively. The bacterium *B. subtilis* incubated one hour with empty liposomes and HEPES buffer did not showed a significant reduction percentage. The bacteria show a significant increase in the reduction percentage of MgOCl₂ liposomes compared to both controls. The immunoliposomes reduction was 34.4%. The immunoliposomes showed a significant reduction percentage when it was compared to the empty liposomes reduction percentage.

5.0 DISCUSSION

The MgOCl_2 -liposomes and empty liposomes developed for this thesis preserved its size stability for a period of 15 days, stored at 4°C . In figure 8, the empty liposomes size distribution curve varied between 80nm and 500nm with an average size distribution of 123nm, the polydispersity index (PdI) of 0.059 approximate 0.06 is considered to be a monodispersed solution. This result coincides with Lieberman et al. in their 1998 book, where they state that polymer dispersions with a PdI between 0.03 and 0.06 are considered to be monodispersed solutions. The polydispersity index (PdI) doubled (figure 9) showing some instability after one week of storage and remained stable after 10 more days of storage (figure 10).

In figure 11, the MgOCl_2 liposomes size distribution curve varied between 50-500nm with an average size of 108nm. The polydispersity index of 0.093 approximately 0.1, is in the range of 0.1-0.2 which is generally found in colloidal drug carriers (Lieberman, Rieger et al. 1998), suggesting that MgOCl_2 drug was encapsulated within the liposomes. MgOCl_2 liposomes showed more stability than empty liposomes in storage for one week (figure 12) the PdI average size distribution remained stable, on the third week of storage MgOCl_2 liposomes PdI increased also the average size (figure 13) suggesting the starting of liposome degradation.

The size distribution curve of the empty liposomes and MgOCl_2 liposomes showed more than one peak with PdI of 0.283 and 0.653 after 4 weeks of storage at 4°C respectively. The formation of new peaks of large size (800nm) observed in figure 15 suggests that some liposomes were coalescing. In addition, the shift in the peak of the empty liposomes average size distribution curve to the left suggests that many small lipid

particles (in the range of 20-100nm) related to the degradation of the liposomes were in the solution, causing a shift in the size distribution curve to the left. The high polydispersity index (above 0.2) observed in the size distribution curve for the empty liposomes size distribution curve also indicated a broad size distribution, as the particle size range varied from 20-1000 nm (figure15).

In figure 17, the formation of a peak in the area between 10-30 nm with an average size of 17nm, suggested that this peak represents free MgOCl_2 particles, meaning that the liposomes contained MgOCl_2 particles and that it remained within the liposomes for 3-4 weeks days until the liposomes started degrading. This means that particles of lipids or MgOCl_2 nanoparticles made a broader size distribution curve thus affecting the PId and size distribution average values of the size distribution curve (figure 17).

More than one peak in the liposome size distribution curve was observed for the MgOCl_2 -immunoliposomes after 10 days of storage at 4°C. The short life of the MgOCl_2 -immunoliposomes is related to an increase in antibody instability after dilution. Antibodies become unstable after dilution, which in turn makes them oxidize, the oxidation of the antibody may have made liposome degradation hastened. A technical resource article written by Thermo Scientific Company Pierce Products, suggests to add a carrier protein or a filler protein, like Bovine Serum Albumin, to the diluted antibody to protect it against degradation (Pierce 2005). The addition of the filler protein, a protein stabilizing cocktail or a cryoprotectant agent to the MgOCl_2 -immunoliposomes should increase their shelf life, but more studies need to be performed in order to determine the effect of the addition of these agents on the effectiveness of MgOCl_2 -immunoliposomes as a bactericidal agent.

A BCA assay standard curve was used to determine the total protein concentration present in the Antibody-Streptavidin complex which attaches to the surface of the liposome after the dilutions and purification. The theoretical concentration of antibody after dilutions was of 90.0 $\mu\text{g/ml}$ and after the purification the total concentration of the Streptavidin-antibody complex obtained was 44.2 $\mu\text{g/ml}$. A ratio of 1:1 of Streptavidin-Antibody complex to MgOCl_2 liposomes was used to develop the immunoliposome, and the concentration of Streptavidin-Antibody in the liposome solution was approximately 22.1 $\mu\text{g/ml}$. This low concentration might be the cause of the low reduction percentage concentration and given the results obtained for the reduction percentages of the bacteria treated with the MgOCl_2 immunoliposomes a higher concentration of antibody and Streptavidin should be tested in order to find the Streptavidin-Antibody concentration that will give optimum results for bacterium targeting.

The results from the Minimum Inhibitory Concentration assay for *B. thuringiensis* (figure 21) showed that after a period of 22 hours of bacterial incubation with the MgOCl_2 , drug concentrations of 2.0 M, 1.0 M, 0.5 M, and the MgOCl_2 liposomes caused an absolute bacterial growth inhibition. The control plate (HEPES) and MgOCl_2 of 30mM and lower showed bacterial growth after the 22 hours period. The importance of these results is the observation of an absolute bacterial growth inhibition caused by the MgOCl_2 liposomes after the 22 hours of incubation.

Bacillus thuringiensis was incubated with the liposomes to determine the growth inhibition percentage of the bacteria in contact with the liposomes at three different times– 0, 1, and 2 hours in the Colony Growth Assay (CGA). The highest average reduction percentage (58%) of *B. thuringiensis* was obtained after 1 hour of incubation

with MgOCl_2 liposomes. The statistical analysis also revealed that the reduction percentage was significant in comparison with the controls (HEPES and Empty liposomes). The drug concentration inside the liposomes remains unknown. Assuming a 25% drug encapsulation efficiency, the approximated drug concentration of these liposomes was 0.75 mM and the reduction percentage obtained from the bacteria incubated with the 3 mM MgOCl_2 free drug was smaller than the reduction obtained for the MgOCl_2 liposomes. This suggests that the MgOCl_2 liposomes contributed to the MgOCl_2 delivery through the membrane cell wall of the bacteria, causing a higher bacterial reduction within 1 hour. Empty liposomes caused the 2nd highest bactericidal effect, at time 1 hour, this result showed statistical significance in the t-test performed. The average of the bacterial reduction percentage of the empty liposomes increased due to the reduction percentage of test #3 (see table3). The high bacterial reduction caused by the empty liposomes on that particular test could be due to an unexpected degradation of the empty liposomes or a change in pH in the empty liposomes solution, causing cytotoxicity to *B. thuringiensis* cells. The average bacterial reduction effect caused by MgOCl_2 -immunoliposomes was lower than the effect of MgOCl_2 liposomes, since test #1 and #4 showed no bacterial reduction effect. There are several reasons that can explain the low bacterial reduction caused by the MgOCl_2 immunoliposomes, random orientation of the antibodies may have cause lower specificity and lower reduction. Also, the functionality of the antibody may have been affected after the Streptavidin-antibody complex attachment to the liposomes. It is necessary to perform a functionality test on the MgOCl_2 -immunoliposomes in order to determine if the low reduction percentages obtained with the MgOCl_2 -immunoliposomes is due to the lost of functionality of the

antibody. Free MgOCl_2 (3mM) drug did not show a bacterial reduction in 3 of the 4 tests performed, this suggests that 3mM of free MgOCl_2 is not a sufficient amount to cause a bactericidal effect to *B. thuringiensis* in 1 hour of treatment.

In the CGA the highest bacterial reduction effect at 2 hours of incubation was caused by the empty liposomes. The high average reduction percentage in the empty liposomes is the result of the high reduction percentage observed on tests #1 and #4. Due to the high variability obtain within each test in the CGA, a test like a fluorescence-activated cell sorter (FACS) should minimizes the experimental error and should be used in future studies for a better quantification of the bactericidal effect of the MgOCl_2 liposomes and MgOCl_2 -immunoliposomes. Halwani, used FACS to confirm interactions of liposomes with bacterial membranes, and it would be practical for the confirmation of the efficacy of the bactericidal effect for the application in this research (Halwani, Mugabe et al. 2007). In order to get into additional conclusions with a CGA, a larger population of samples should be used to obtain statistical results with smaller variability values.

The bactericidal effectiveness of the MgOCl_2 liposomes observed in the CGA was better for the treatment of *B. thuringiensis* after 1 hour of incubation than after 2 hours. This result was because the bacteria that remained alive after the 1st hour of incubation kept on replicating in the 2nd hour of incubation; therefore, the reduction percentage diminished. This means that MgOCl_2 liposomes are capable of affecting the cell wall of the bacteria, but the concentration of MgOCl_2 inside the liposomes is not sufficient to exterminate all the bacterium present in the solution in a period of 2 hours. Better results

would have been obtained if the cells were incubated only with MgOCl₂ liposomes, instead of in a 1:1 ratio of MgCl₂ liposomes to bacterial solution in LB broth.

It is stated in the product information sheet of Meridian Science Co. that the rabbit anti-*Bacillus* spores antibody used to make the MgOCl₂-immunoliposomes are reactive with *B. cereus* and *B. subtilis* spores and vegetative cells, but the antibody can cross-react with other *Bacillus* species. The MgOCl₂-immunoliposomes incubated with *B. thuringiensis* showed a lower average reduction percentage than the MgOCl₂ liposomes. These results can be due to the presence of the antibody on the surface of the liposomes, since the antibody attached to the surface of the liposomes is not against *B. thuringiensis* a lower interaction between liposomes and the membrane of the bacteria was observed. The low bactericidal reduction observed for the immunoliposomes could be due to a small cross-reaction of *B. thuringiensis* with the antibody on the liposomes or the production of a small quantity of *B. thuringiensis* CryA toxins which cause liposomal membrane permeation promoting the MgOCl₂ release (Du 2005). Also, the low bactericidal effect could be due to the lost of functionality in the antibody after the crosslinking reaction with the Streptavidin and after linking it with the liposomes. For the future use of this MgOCl₂-immunoliposome to detect only *B. anthracis*, a monoclonal antibody specific for the *B. anthracis* poly-D-γ-glutamic acid capsule, will be the best choice.

The live/dead assay results demonstrated statistically significant reduction rates of *B. thuringiensis* compared to HEPES at 1 hour of incubation, with MgOCl₂-immunoliposomes and MgOCl₂ liposomes, with averages reduction percentages of 29.7 and 31.7 respectively. The p value of the MgOCl₂ liposomes (0.014) is larger than the p

value of the MgOCl_2 -immunoliposomes (0.0017), this states that the reduction percentages observed for the MgOCl_2 -immunoliposomes is statistically more significant than the reduction percentage of the MgOCl_2 -liposomes, due to a higher variance observed in the MgOCl_2 -liposomes. The free MgOCl_2 at concentration of 3mM also showed a statistically significant reduction percentage with a p value of 0.036. This p value is large compared to the p values of MgOCl_2 -immunoliposomes and MgOCl_2 -liposomes due to the lower average reduction percentage observed in *B. thuringiensis* treated with the free MgOCl_2 and due to a high variability between the samples. The statistical analysis and the reduction percentages caused by the aforementioned liposomes compared to the HEPES (control) suggests that MgOCl_2 liposomes and MgOCl_2 immunoliposomes are better bactericides for *B. thuringiensis* than 3mM of MgOCl_2 . Since the encapsulation percentage of the liposomes is 25 %, the concentration of MgOCl_2 within the MgOCl_2 -liposomes and MgOCl_2 -immunoliposomes is lower than 3mM. The statistical analysis performed to compare the effect of empty liposomes with MgOCl_2 liposomes (p value 0.00055), MgOCl_2 immunoliposomes (p value 0.016) and 3mM MgOCl_2 (p value 0.037), showed significant reduction percentages for the 3 aforementioned solutions. In this comparison free 3mM MgOCl_2 showed a significant reduction percentage but its average reduction percentage compared to the MgOCl_2 liposomes and MgOCl_2 immunoliposomes is lower.

The live/dead assay for *B. thuringiensis* suggests that the MgOCl_2 -liposomes and MgOCl_2 -immunoliposomes were effective in the drug transportation to the bacteria; this result suggests that a higher bactericidal effect can be accomplished if a higher drug concentration is encapsulated within the liposomes. In order to make sure that an

interaction between the bacteria and the liposomes is actually taking place some signaling assays can be performed such as Atomic Force Microscopy (AFM) or Transmission Electron Microscopy (TEM) can be used to observe the damage on the membrane cell wall of the bacteria caused by the liposomes.

In the live/dead assay comparing the effect of the empty liposomes at time 0 hours compared to 1 hour, the empty liposomes had no harmful effect on the *B. thuringiensis* cells, supporting the hypothesis that the empty liposomes have a minimum effect in bacterial cells cytotoxicity (see appendix J for statistical analysis). This result supports the need of additional experiments to abandon the suspicion of empty liposomes cytotoxicity observed in the colony growth after two hours of empty liposomes incubation with bacterial cells.

B. subtilis live/dead assay average reduction percentages after 1 hour of incubation for the MgOCl_2 liposomes and MgOCl_2 -immunoliposomes were 45.56 and 34.4 respectively. Statistical analysis comparing the effect of HEPES vs. MgOCl_2 liposomes and MgOCl_2 -immunoliposomes showed significant bactericidal effects only in the MgOCl_2 liposomes (p value 0.0065). The non-significant reduction percentage result observed for the immunoliposomes compared to the HEPES is due to a low bacterial reduction percentage observed in test #2 where the reduction percentage of HEPES is similar to the reduction percentage caused by the MgOCl_2 -immunoliposomes. The statistical analysis observed comparing the effect of empty liposomes at 1 hour compared to the MgOCl_2 liposomes and MgOCl_2 -immunoliposomes showed a statistically significant reduction percentage for both solutions suggesting that they acted as good drug carriers for *B. subtilis*.

The higher reduction percentages obtained for the treatment of *B. subtilis* with MgOCl₂-immunoliposomes compared to the effect of the immunoliposomes for *B. thuringiensis* are due to the detection of the antibody attached to the surface of the liposome by the antigens in *B. subtilis*, this identification caused the better drug release than immunoliposomes with *B. thuringiensis*. Moreover, a higher reduction percentage was observed for the treatment of *B. subtilis* with the MgOCl₂- liposomes. This might be caused by a higher sensitivity of *B. subtilis* to the drug, and suggests that the immunoliposomes should be improved by the addition of a higher concentration of the Streptavidin-Antibody complex in order to obtain better reduction percentages results caused by the MgOCl₂-immunoliposomes. A higher reduction percentage caused by the MgOCl₂-immunoliposomes incubation with *B. subtilis* was expected. The antibody attached to the surface of the liposomes is against the bacterium *B. subtilis* and *B. cereus* the unexpected low reduction percentage caused by the MgOCl₂-immunoliposomes could be due to a lost in functionality of the antibody in the crosslinking procedure.

The results obtained in this test for *B. cereus* showed a similar reduction percentage and statistically non significant, between the bacteria treated with HEPES buffer; empty liposomes; MgOCl₂ liposomes; MgOCl₂-immunoliposomes (30.5; 30.7; 30.4; and 30.2 respectively). This result could be due to a low pH of the HEPES buffer and the presence of hydrogen peroxide due to light exposure of the buffer.

In a study by Hamouds 1999, they investigated the sporicidal properties of 2 antimicrobial lipid emulsions, in order to eliminate human toxicity caused by the sporicidal agents by itself. In this study, they observed a high sporicidal effect (98%) on

B. cereus and *B. subtilis* caused by the lipid nanoemulsions after 4 hours of treatment.

In the same study they used transmission electron microscopy (TEM) to observe the physical damage in the bacterial spores caused by their nanoemulsion (Hamouda, Hayes et al. 1999). The use of TEM would be a good approach to corroborate the MgOCl₂ liposomes and MgOCl₂-immunoliposomes interaction with the membrane cell wall of the *Bacillus* bacterium studied in this thesis.

To my knowledge, this is the first study that applies nano-liposome technology to encapsulate MgOCl₂ with the purpose to be treated as a bactericidal agent. Previous studies performed by Stoimenov group with free MgOCl₂ nanoparticles used as a bactericidal agent for the treatment of *E. coli*, *B. megaterium* and *B. subtilis*, showed promising results of the effect on this nanoparticles as a disinfectant. In this particular study AFM imaging and TEM imaging was used to observe the nanoparticles effect on the bacteria. It showed a strong and fast effect on the killing influence of the MgOCl₂ nanoparticle effect on the killing action of both bacterial cells and spores (Stoimenov 2002). In the research performed by Stoimenov (2002) no reduction percentages of bacteria caused by the presence of MgOCl₂-particles were calculated.

With the purpose of applying the MgOCl₂ nanoparticles as biowarfare defense agent, to increase the intracellular drug concentrations through fusion with bacterial cells membranes, MgOCl₂ was encapsulated in liposomes. The attempt of a quantification of its bactericidal effect was performed in our studies. The tendency on the bactericidal effect caused by the MgOCl₂ liposomes toward *B. subtilis* and *B. thuringiensis* coincides with the results that Stoimenov group obtained in the treatment of *B. megaterium* and *B. subtilis* with free MgOCl₂ nanoparticles. Low MgOCl₂ concentrations encapsulated

within the liposomes caused better reduction percentages than higher free drug concentrations. The obtained results are promising, since low concentrations of encapsulated MgOCl_2 within the liposomes caused some bactericidal effect within 2 hours. However, the system has room for improvement, if a higher drug concentration could be encapsulated in the liposomes higher reduction percentages can be achieved in a smaller amounts of time. Also, the functionality of the antibody attached to the liposomes has to be tested in order to determinate the MgOCl_2 -immunoliposomes specificity, in a study by Schnyder 2004, they used immunocytochemistry and determined the cellular uptake of an antibody in a Streptavidin-antibody complex (Schnyder, Krahenbuhl et al. 2004). For better accuracy in the quantification of the reduction percentages caused by the bacteria a Fluorescence Activated Cell Sorting (FACS) system is highly recommended, also methods such as AFM or TEM can be used to observe the damage on the membrane cell wall of the bacteria caused by the liposomes.

6.0 CONCLUSION

In this thesis, liposomes of average size of 130-90 nm were developed, with a shelf life of 15 days. The liposomes were modified with a polyclonal antibody against the *Bacillus cereus* and *Bacillus subtilis* antigens. The drug delivery efficacy of both liposomes was tested in two different forms, a colony growth assay (CGA) and a Live/Dead assay.

The bacterial viability of *B. subtilis* and *B. thuringiensis* tested using a live/dead assay revealed that bacterium treated with the MgOCl_2 liposomes experienced a statistically significant reduction percentage, compared to HEPES and empty liposomes, with average reduction percentages of 45.6 and 31.7 respectively. The results of the bacteria *B. cereus* in the live/dead assay did not show a statistically significant bactericidal effect of the liposomes. MgOCl_2 immunoliposomes showed a lower bactericidal effect toward the bacterium cells, compared to the MgOCl_2 liposomes bactericidal effect, this could be due to a loss of functionality during the crosslinking procedure of the antibody to the liposomes. Immunocytochemistry tests, as well as competition and uptake experiments, should be performed to corroborate the antibody functionality after the crosslinking procedure.

The results of the CGA, revealed a statistical significant bacterial reduction percentage for the MgOCl_2 liposomes at time 1 hour compared to HEPES and empty liposomes. At time 2 hours the CGA revealed a statistical significant bacterial reduction percentage for the MgOCl_2 liposomes compared with HEPES. Non-statistically significant bactericidal results were observed for the MgOCl_2 -immunoliposomes. The non-statistically significance observed in the bactericidal effect of the MgOCl_2 -

immunoliposomes is due to a low bactericidal effect observed in the CGA compared to the HEPES and empty liposomes. Finally, empty liposomes caused a high average reduction percentage since 2 of the 4 tests performed revealed 100 % of bactericidal effect. Due to the high variability observed in the CGA test, further conclusions cannot be drawn regarding to the MgOCl_2 liposomes efficacy, what can be pointed is that the MgOCl_2 -liposomes showed the highest bacterial reduction in each of the tests, compared to the MgOCl_2 -immunoliposomes and 3mM MgOCl_2 free drug.

The results presented in this thesis reveal a tendency of MgOCl_2 liposomes as a bactericidal agent for *B. thuringiensis* and *B. subtilis*, suggesting this is an effective bactericidal agent. Lower reduction percentages were caused by the MgOCl_2 -immunoliposomes compared to the MgOCl_2 liposomes.

In order to obtain better bacterial targeting effect a monoclonal antibody with a concentration higher than 44.2 $\mu\text{g}/\text{ml}$ of the Streptavidin-Antibody complex should be attached to the liposome surface. Further research is still necessary to improve the drug encapsulation efficiency; also for the corroboration of the efficacy of the MgOCl_2 liposomes and MgOCl_2 -immunoliposomes compared to the empty liposomes. In addition the determination of the exact mechanism of MgOCl_2 -liposomes and MgOCl_2 -immunoliposomes between the liposomes and the membrane cell wall of the bacteria is recommended in order to further improve this drug delivery system. Moreover, additional studies such as Fluorescent Activated Cell Sorting (FACS), Atomic Force Microscopy (AFM), and Transition Electron Microscopy (TEM) are recommended in order to confirm liposome interactions with the bacterial membrane.

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APPENDIX

Appendix A. Liposome development

1. Weight 87.5 mg of Phosphatidylcholine, 37.5 mg of PEG 2000 Biotin, and 6.5 mg of Cholesterol.
2. Place the weighted lipids in a round bottom flask and add 50 ml of chloroform in the chemical hood. Mix the lipids with the chloroform until no particles can be observed.
3. Put a piece of parafilm around the neck of the flask before removing the flask from the hood. Place the flask in the water bath of the rotovapor at 40°C, turn on the vacuum, set the flask rotation 155 RPM, and wait one hour for the chloroform to completely evaporate.
4. After the film is made take the round bottle from the rotovapor, use a kimwipe and an elastic rubber to cover the neck of the flask.
5. Dip the flask 3 times in liquid Nitrogen.
6. Place the round bottom flask in the lyophilizer flask and leave it in the lyophilizer over night.
7. After the overnight lyophilization, remove the flask from the lyophilizer and add 35 ml of 1M pH7 HEPES buffer.
8. Set the water bath of the rotovapor to 40°C and the circulator to 14°C.
9. Put the flask in the rotovapor at 100 RPM for 45 minutes verify that there is no film attached to the bottom of the flask.
10. Weight 12.5mg of MgOCl_2 in the scale and add it to the liposome solution.
11. Place the liposome solution on the rotovapor at the same conditions mentioned on step 8 and wait 15 minutes.

Appendix B. Liposome extrusion protocol

1. Clean the 100 mL extruder parts with HEPES before adding the liposome solution in it.
2. Connect the pressure "pipe" with the extruder.
3. Place the supporter and the 100 nm membrane filter in the tank.
4. Make an extrusion with 10 ml of HEPES to remove any residual particles from the extruder.
5. Add 15 mL of the liposomes into the extruder.
6. Open the Argon tank and increase the pressure to 100 psi, open the valve that connects the tank with the extruder (green valve) and close the release pressure valve (black valve), keep on increasing the pressure until liposomes start coming out of the extruder. If the liposomes stop from getting out of the extruder increase the pressure 100 psi until the batch of liposomes is completely extruded.
7. Repeat this extruding procedure five times.
8. After the fifth time change the membrane filter and add 2ml of 2% Trehalose 3mM HEPES. Repeat step 7 and 8 for a total of 15 extrusions.

9. Change the membrane filter to 80 nm and repeat steps 7 and 8. Add 1 ml of 10% Trehalose 3mM solution after every five extrusions, before changing the filter.
10. Measure the liposome size in the nanosizer. If the size of the liposomes is in the range of 120 to 100 nm the liposomes are ready for purification, if not the extrusion procedure will be repeated with a 50 nm membrane filter.
11. To prepare the Sephadex G-50 weight 25 g of Sephadex.
12. Add 250 ml of HEPES to the 25 g of Sephadex.
13. Let the mix swell at room temperature for 3 hours or at 90°C for one hour.
14. While waiting for the Sephadex to swell, place some glass wool in HEPES buffer.
15. When the Sephadex is swelled cut some of the glass wool and place it inside a 10 ml syringe, and fill the syringe with swelled sephadex-G50 to the 7ml mark.
16. Place the column in a 50 ml plastic flask and place it in the centrifuge to spin for 3 minutes at 1000g.
17. Place 2.5 ml of MgOCl_2 liposomes in the column and place the column inside a 50 ml plastic flask.
18. Spin the columns containing the liposomes for 10 minutes at 50g, and for 3 additional minutes at 1000g.

Appendix C. Synthesis of Streptavidin- antibody complex

1. Vortex the antibody for 30 seconds. Dilute it in 2 ml of 1M HEPES pH 7.
2. Dissolve 1 mg of TRAUTS reagent into 200 μl of HEPES.
3. Add a 40:1 molar excess TRAUTS:Antibody.
4. Place it in a stirred water bath at room temperature for one hour.
5. Purify the complex in a Sephadex G-25 column, previously prepared.
6. In order to prepare the MBS dissolve 3.14 mg of MBS into 1mL of DMSO
7. Vortex the streptavidin nanobeads for 30 seconds.
8. Dilute 40 μl of Streptavidin in 5.0 ml of 16.0 mM of HEPES buffer.
9. Add a 40:1 molar excess of MBS:Streptavidin by adding 200 μl of MBS to 40 μl of diluted Streptavidin.
10. Mix 150 μl of Streptavidin- MBS complex with 500 μl of Trauts-Antibody.
11. Gently stir the mixture overnight in a water bath at room temperature.
12. Add a 1:1 ratio of liposomes and Streptavidin-Antibody complex, stir it in a water bath for 1 hour at room temperature.

Appendix D. BCA assay enhanced protocol

1. Pipette 25 μ l of each standard or unknown sample replicate into a microplate 96 well plate.
2. Add 200 μ l of the working reagent (WR) to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 60°C for 30 minute.
4. Cool plate to room temperature (RT) for 30 minutes.
5. Measure the absorbance at 595 nm on a plate reader.

Appendix E. Bacterial preparation

1. The bacteria is located at -80°C freezer, remove the bacteria from the freezer and thaw, by rubbing the vial in your hands for 1 minute.
2. Streak the bacteria in a Luria Bertani agar Petri dish and incubate overnight at physiological conditions.
3. Select an isolated colony for inoculation in 10ml of LB broth in a shaking incubator at physiological conditions overnight.
4. Dilute the overnight culture and measure its absorbance in a spectrophotometer at 600nm.

Appendix F. Colony growth assay

1. Make a 1:10 dilution of the overnight inoculated culture five times for a concentration of (1×10^5 cells/ml). Make an OD 600 nm absorbance measurement.
2. Mix well 500 μ l of the diluted bacteria with 500 μ l of HEPES buffer in vial 1.
3. Repeat step 2 for Empty liposomes, MgOCl₂ liposomes, MgOCl₂-immunoliposomes, and 3mM MgOCl₂.
4. In a previously identified LB agar plate add 100 μ l of the corresponding well mixed solution prepared on step 2 and 3 at time 0 hours, place the vials in a shaking incubator at physiological conditions. Place the plates in the incubator at the same conditions (do not shake the plates).
5. After one hour of incubation, remove the vials from the incubator and repeat step.
6. Place the vials in the shaking incubator at physiological conditions. Place the plates in the incubator at the same conditions (do not shake the plates).
7. After two hours of incubation, remove the vials from the incubator and repeat step.
8. Let the plate incubate for a period of 8 hours, in the incubator. The colonies should be visible at this time, and count the colonies formed.

Appendix G. Colony growth assay pictures

Colony Growth assay pictures, Test 2

Figure 23. *Bacillus thuringiensis* colony growth assay (A) Colony growth assay for *B. thuringiensis* after 1 hour treatment with MgOCl₂ solutions, incubated overnight, where (1), is *B. thuringiensis* in HEPES, (2), is *B. thuringiensis* in Empty liposomes, (3), *B. thuringiensis* in MgOCl₂ immunoliposomes, (4) is *B. thuringiensis* in MgOCl₂ liposomes, and (5), is *B. thuringiensis* in 3mM MgOCl₂ solution (B) Colony growth assay for *B. thuringiensis* after two hours of treatment with MgOCl₂ solutions, incubated overnight. Observe the reduction of CFU's in plate number 3.

Colony Growth Assay Pictures, Test 3

Figure 24. *Bacillus thuringiensis* colony growth assay (A) Colony growth assay for *B. thuringiensis* after 1 hour treatment with MgOCl₂ solutions, incubated overnight, where (1), is *B. thuringiensis* in HEPES, (2), is *B. thuringiensis* in Empty liposomes, (3), *B. thuringiensis* in MgOCl₂ immunoliposomes, (4) is *B. thuringiensis* in MgOCl₂ liposomes, and (5), is *B. thuringiensis* in 3mM MgOCl₂ solution (B) Colony growth assay for *B. thuringiensis* after two hours of treatment with MgOCl₂ solutions, incubated overnight.

Appendix H. Live/Dead assay protocol (adapted from Invitrogen protocol)

1. Concentrate 10 ml of the overnight culture in a centrifuge at 10,000X g for a period of 15 minutes.
2. Remove the supernatant and resuspend the pellet in 10 ml of .85% of NaCl
3. Separate the resuspended 10 ml of cell solution into 5 flasks
4. Centrifuge the solution at 10,000X g for a period of 15 minutes.
5. Remove the supernatant of each vial and resuspend the pellet in 1 ml of HEPES, empty liposomes, MgOCl₂ liposomes, MgOCl₂- immunoliposomes and 3mM MgOCl₂ respectively
6. Adjust the cell concentrations to 1x10⁸ cells/ml
7. Prepare a mixture of 10 µl of component A with 10 µl of component B
8. Add 3 µl of the dye mixture for each ml of bacterial suspension
9. Mix thoroughly and incubate in the dark for 15 minutes
10. Trap 5 µl of the stained solution between a slide and a 18 mm square cover slip
11. Observe the bacteria under the microscope using a 40X lens with the appropriate filter in order to see the stained bacteria.

Appendix I. Two paired t-test results for *Bacillus thuringiensis*, colony growth assay.

Bacillus thuringiensis incubated with HEPES buffer reduction percentages (μ_1) compared to the reduction percentages of *Bacillus thuringiensis* incubated with Empty Liposomes (μ_2)

Two paired t-test for $\mu_1 \neq \mu_2$ $H_0 = \mu_1 = \mu_2$				
t-Test: Paired Two Sample for Means				
μ_1	μ_2		Variable 1	Variable 2
0	36.2	Mean	0	47.0502978
0	30.3	Variance	0	1660.05595
0	78	Observations	8	8
0	31.9	Pearson Correlation	#DIV/0!	
0	100	Hypothesized Mean Difference	0	
0	0	Df	7	
0	0	T Stat	-3.266224	
0	100	P(T<=t) one-tail	0.0068729	
		T Critical one-tail	1.8945775	
		P(T<=t) two-tail	0.0137458	
		T Critical two-tail	2.3646226	

Table I-1 *B. thuringiensis* incubated with HEPES buffer CGA

Bacillus thuringiensis incubated with HEPES buffer reduction percentages (μ_1) compared to the reduction percentages of *Bacillus thuringiensis* incubated with MgOCl₂ liposomes (μ_3).

Two paired t-test for $\mu_1 \neq \mu_3$ $H_0 = \mu_1 = \mu_3$				
		t-Test: Paired Two Sample for Means		
μ_1	μ_3		Variable 1	Variable 2
0	57.8	Mean	0	48.9429568
0	55.06	Variance	0	305.819196
0	80	Observations	8	8
0	38.0	Pearson Correlation	#DIV/0!	
0	26.5	Hypothesized Mean Difference	0	
0	60.3	Df	7	
0	40	t Stat	-7.915946	
0	33.8	P(T<=t) one-tail	4.875E-05	
		t Critical one-tail	1.8945775	
		P(T<=t) two-tail	9.75E-05	
		t Critical two-tail	2.3646226	

Bacillus thuringiensis incubated with HEPES buffer reduction percentages (μ_1) compared to the reduction percentages of *Bacillus thuringiensis* incubated with MgOCl₂ immunoliposomes (μ_4).

Two paired t-test for $\mu_1 \neq \mu_4$ $H_0 = \mu_1 = \mu_4$		t-Test: Paired Two Sample for Means		
μ_1	μ_4	Mean	Variable 1	Variable 2
0	0	Variance	0	21.1295646
0	20.8	Observations	0	626.813714
0	52.3	Pearson Correlation	8	8
0	0	Hypothesized Mean Difference	#DIV/0!	
0	0	Df	0	
0	57.7	T Stat	7	
0	38.3	P(T<=t) one-tail	-2.387076	
0	0	T Critical one-tail	0.0241881	
		P(T<=t) two-tail	1.8945775	
		T Critical two-tail	0.0483762	
			2.3646226	

Bacillus thuringiensis incubated with empty liposomes reduction percentages (μ_2) compared to the reduction percentages of *Bacillus thuringiensis* incubated with MgOCl₂ liposomes (μ_3).

Two paired t-test		t-Test: Paired Two Sample for Means, for $\mu_2 \neq \mu_3$ $H_0 = \mu_2 = \mu_3$		
μ_2	μ_3		Variable 1	Variable 2
36.2	57.8	Mean	47.050298	48.9429568
30.3	55.06	Variance	1660.0559	305.819196
78	80	Observations	8	8
31.9	38.0	Pearson correlation	-0.235225	
100	26.5	Hypothesized Mean Difference	0	
0	60.3	Df	7	
0	40	t Stat	-0.111597	
100	33.8	P(T<=t) one-tail	0.4571378	
		t Critical one-tail	1.8945775	
		P(T<=t) two-tail	0.9142757	
		t Critical two-tail	2.3646226	

Bacillus thuringiensis incubated with empty liposomes reduction percentages (μ_2) compared to the reduction percentages of *Bacillus thuringiensis* incubated with MgOCl₂ immunoliposomes (μ_4)

Two paired t-test		for $\mu_4 \neq \mu_2$ $H_0 = \mu_4 = \mu_2$	Variable 1	Variable 2
μ_2	μ_4			
36.2	0	Mean	47.050298	21.1295646
30.3	20.8	Variance	1660.0559	626.813714
78	52.3	Observations	8	8
31.9	0	Pearson Correlation	-0.453836	
100	0	Hypothesized Mean Difference	0	
0	57.7	Df	7	
0	38.3	t Stat	1.2934604	
100	0	P(T<=t) one-tail	0.1184481	
		t Critical one-tail	1.8945775	
		P(T<=t) two-tail	0.2368962	
		t Critical two-tail	2.3646226	

Bacillus thuringiensis incubated with empty liposomes reduction percentages (μ_2) compared to the reduction percentages of *Bacillus thuringiensis* incubated with 3mM MgOCl₂ (μ_5).

Two paired t-test for $\mu_2 \neq \mu_5$ $H_0 = \mu_2 = \mu_5$		t-Test: Paired Two Sample for Means		
μ_2	μ_5		Variable 1	Variable 2
36.2	0	Mean	47.050298	13.5520833
30.3	0	Variance	1660.0559	356.914559
78	32	Observations	8	8
31.9	0	Pearson Correlation	-0.483473	
100	0	Hypothesized Mean Difference	0	
0	41.7	Df	7	
0	34.8	t Stat	1.8030696	
100	0	P(T<=t) one-tail	0.0571847	
		t Critical one-tail	1.8945775	
		P(T<=t) two-tail	0.1143694	
		t Critical two-tail	2.3646226	

Appendix J. *Bacillus thuringiensis*, live/dead assay t-test: paired two sample for means

Bacillus thuringiensis, incubated with HEPES at time 1 hour ($\mu_{1,1}$) compared to *B. thuringiensis* incubated with empty liposomes at 1 hour of incubation ($\mu_{2,1}$), where $\mu_1 \neq \mu_2$ $H_0 = \mu_{1,1} = \mu_{2,1}$

$\mu_{1,1}$	$\mu_{2,1}$		Variable 1	Variable 2
8.7	7.7	Mean	14.9	19.16667
10.7	18.7	Variance	82.12	137.0533
25.3	31.1	Observations	3	3
		Pearson Correlation	0.929222	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-1.57509	
		P(T<=t) one-tail	0.127957	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.255915	
		t Critical two-tail	4.302656	

Bacillus thuringiensis, incubated with HEPES at time 1 hour ($\mu_{1,1}$) compared to *B. thuringiensis* incubated with MgOCl₂ liposomes at 1 hour of incubation ($\mu_{3,1}$), where $\mu_1 \neq \mu_3$ $H_0 = \mu_{1,1} = \mu_{3,1}$

$\mu_{1,1}$	$\mu_{3,1}$		Variable 1	Variable 2
8.7	20.0	Mean	14.9	31.66667
10.7	32.0	Variance	82.12	132.333
25.3	43.0	Observations	3	3
		Pearson Correlation	0.90555	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-5.7337	
		P(T<=t) one-tail	0.01455	
		t Critical one-tail	2.91999	
		P(T<=t) two-tail	0.0291	
		t Critical two-tail	4.30266	

Bacillus thuringiensis, incubated with HEPES at time 1 hour ($\mu_{1,1}$) compared to *B. thuringiensis* incubated with MgOCl_2 immunoliposomes at 1 hour of incubation ($\mu_{4,1}$), where $\mu_1 \neq \mu_4$ $H_0 = \mu_{1,1} = \mu_{4,1}$

$\mu_{1,1}$	$\mu_{4,1}$		Variable 1	Variable 2
8.7	22.0	Mean	14.9	29.6667
10.7	27.0	Variance	82.12	86.3333
25.3	40.0	Observations	3	3
		Pearson Correlation	0.98693	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-17.038	
		P(T<=t) one-tail	0.00171	
		t Critical one-tail	2.91999	
		P(T<=t) two-tail	0.00343	
		t Critical two-tail	4.30266	

Bacillus thuringiensis, incubated with HEPES at time 1 hour ($\mu_{1,1}$) compared to *B. thuringiensis* incubated with 3mM MgOCl_2 at 1 hour of incubation ($\mu_{5,1}$), where $\mu_1 \neq \mu_5$ $H_0 = \mu_{1,1} = \mu_{5,1}$

$\mu_{1,1}$	$\mu_{5,1}$		Variable 1	Variable 2
8.7	13.0	Mean	14.9	26.3333
10.7	30.0	Variance	82.12	142.333
25.3	36.0	Observations	3	3
		Pearson Correlation	0.77604	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-2.631	
		P(T<=t) one-tail	0.05959	
		t Critical one-tail	2.91999	
		P(T<=t) two-tail	0.11918	
		t Critical two-tail	4.30266	

Bacillus thuringiensis, incubated with empty liposomes at time 0 hours ($\mu_{2,0}$) compared to *B. thuringiensis* incubated with empty liposomes at 1 hour of incubation ($\mu_{2,1}$), where $\mu_{2,0} \neq \mu_{2,1}$ $H_0 = \mu_{2,0} = \mu_{2,1}$

t-Test: Paired Two Sample for Means				
$\mu_{2,0}$	$\mu_{2,1}$		Variable 1	Variable 2
4.3	7.7	Mean	14.6666667	19.14815
20.7	18.7	Variance	80.7777778	137.5844
19	31.1	Observations	3	3
		Pearson Correlation	0.79486676	
		Hypothesized Mean Difference	0	
		Df	2	
		T Stat	-1.089383	
		P(T<=t) one-tail	0.19487609	
		T Critical one-tail	2.91998731	
		P(T<=t) two-tail	0.38975219	
		T Critical two-tail	4.30265573	

Bacillus thuringiensis, incubated with empty liposomes at time 1 hours ($\mu_{2,1}$) compared to *B. thuringiensis* incubated with MgOCl_2 liposomes at 1 hour of incubation ($\mu_{3,1}$), where $\mu_2 \neq \mu_3$ $H_0 = \mu_{2,1} = \mu_{3,1}$

$\mu_{2,1}$	$\mu_{3,1}$		Variable 1	Variable 2
7.7	20.0	Mean	19.1667	31.6667
18.7	32.0	Variance	137.053	132.333
31.1	43.0	Observations	3	3
		Pearson Correlation	0.99822	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-30.024	
		P(T<=t) one-tail	0.00055	
		t Critical one-tail	2.91999	
		P(T<=t) two-tail	0.00111	
		t Critical two-tail	4.30266	

Bacillus thuringiensis, incubated with empty liposomes at time 1 hours ($\mu_{2,1}$) compared to *B. thuringiensis* incubated with MgOCl_2 immunoliposomes at 1 hour of incubation ($\mu_{4,1}$), where $\mu_2 \neq \mu_4$ $H_0 = \mu_{2,1} = \mu_{4,1}$

$\mu_{2,1}$	$\mu_{4,1}$		Variable 1	Variable 2
7.7	22.0	Mean	19.1667	29.6667
18.7	27.0	Variance	137.053	86.3333
31.1	40.0	Observations	3	3
		Pearson Correlation	0.97662	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-5.5035	
		P(T<=t) one-tail	0.01573	
		t Critical one-tail	2.91999	
		P(T<=t) two-tail	0.03147	
		t Critical two-tail	4.30266	

Bacillus thuringiensis, incubated with empty liposomes at time 1 hour ($\mu_{2,1}$) compared to *B. thuringiensis* incubated with 3mM MgOCl_2 at 1 hour of incubation ($\mu_{5,1}$), where $\mu_2 \neq \mu_5$ $H_0 = \mu_2 = \mu_5$

$\mu_{2,1}$	$\mu_{5,1}$		Variable 1	Variable 2
7.7	13.0	Mean	19.1667	26.3333
18.7	30.0	Variance	137.053	142.333
31.1	36.0	Observations	3	3
		Pearson Correlation	0.95416	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-3.4623	
		P(T<=t) one-tail	0.03712	
		t Critical one-tail	2.91999	
		P(T<=t) two-tail	0.07425	
		t Critical two-tail	4.30266	

Appendix K. *B. subtilis*, live/dead assay t-test: paired two sample for means

B. subtilis incubated with HEPES at time 1 hours ($\mu_{1,1}$) compared to *B. subtilis* incubated with Empty liposomes at 1 hour of incubation ($\mu_{4,1}$), where $\mu_1 \neq \mu_2$
 $H_0 = \mu_{1,1} = \mu_{2,1}$.

$\mu_{1,1}$	$\mu_{2,1}$		Variable 1	Variable 2
20.0	28.9	Mean	23	23.2963
23.0	18.0	Variance	9	29.70782
26.0	23.0	Observations	3	3
		Pearson Correlation	-0.54022	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-0.06835	
		P(T<=t) one-tail	0.475862	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.951725	
		t Critical two-tail	4.302656	

B. subtilis incubated with HEPES at time 1 hours ($\mu_{1,1}$) compared to *B. subtilis* incubated with $MgOCl_2$ at 1 hour of incubation ($\mu_{3,1}$), where $\mu_1 \neq \mu_3$ $H_0 = \mu_{1,1} = \mu_{3,1}$.

$\mu_{1,1}$	$\mu_{3,1}$		Variable 1	Variable 2
20.0	38.0	Mean	23	45.57881
23.0	45.7	Variance	9	56.26863
26.0	53.0	Observations	3	3
		Pearson Correlation	0.999834	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-8.68659	
		P(T<=t) one-tail	0.006497	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.012995	
		t Critical two-tail	4.302656	

B. subtilis incubated with HEPES at time 1 hours ($\mu_{1,1}$) compared to *B. subtilis* incubated with MgOC₁₂ immunoliposomes at 1 hour of incubation ($\mu_{4,1}$), where $\mu_1 \neq \mu_4$
 $H_0 = \mu_{1,1} = \mu_{4,1}$.

$\mu_{1,1}$	$\mu_{4,1}$		Variable 1	Variable 2
20.0	37.5	Mean	23	34.42557
23.0	25.0	Variance	9	69.31516
26.0	40.8	Observations	3	3
		Pearson Correlation	0.196785	
		Hypothesized Mean Difference	0	
		Df	2	
		T Stat	-2.39133	
		P(T<=t) one-tail	0.069628	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.139255	
		t Critical two-tail	4.302656	

B. subtilis incubated with empty liposomes at time 1 hours ($\mu_{2,1}$) compared to *B. subtilis* incubated with MgOC₁₂ liposomes at 1 hour of incubation ($\mu_{3,1}$), where $\mu_2 \neq \mu_3$
 $H_0 = \mu_{2,1} = \mu_{3,1}$.

$\mu_{2,1}$	$\mu_{3,1}$		Variable 1	Variable 2
28.9	38	Mean	23.2963	45.57881
18.0	45.7	Variance	29.70782	56.26863
23.0	53.0	Observations	3	3
		Pearson Correlation	-0.55544	
		Hypothesized Mean Difference	0	
		Df	2	
		T Stat	-3.36694	
		P(T<=t) one-tail	0.039014	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.078028	
		t Critical two-tail	4.302656	

B. subtilis incubated with empty liposomes at time 1 hours ($\mu_{2,1}$) compared to *B. subtilis* incubated with MgOCl₂ immunoliposomes at 1 hour of incubation ($\mu_{4,1}$), where $\mu_2 \neq \mu_4$ $H_0 = \mu_{2,1} = \mu_{4,1}$.

$\mu_{2,1}$	$\mu_{4,1}$		Variable 1	Variable 2
28.9	37.5	Mean	23.2963	34.42557
18.0	25.0	Variance	29.70782	69.31516
23.0	40.8	Observations	3	3
		Pearson Correlation	0.718765	
		Hypothesized Mean Difference	0	
		Df	2	
		T Stat	-3.31614	
		P(T<=t) one-tail	0.040077	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.080155	
		t Critical two-tail	4.302656	

Appendix L. *Bacillus cereus*, live/dead assay t-test: paired two sample for means

B. cereus incubated with HEPES at time 1 hours ($\mu_{1,1}$) compared to *B. cereus* incubated with empty liposomes at 1 hour of incubation ($\mu_{2,1}$). where $\mu_1 \neq \mu_2$ $H_0 = \mu_{1,1} = \mu_{2,1}$.

$\mu_{1,1}$	$\mu_{2,1}$		Variable 1	Variable 2
32.2	26.0	Mean	30.54333	30.66667
28.5	34.0	Variance	3.597433	17.33333
31.0	32.0	Observations	3	3
		Pearson Correlation	-0.88182	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	-0.03618	
		P(T<=t) one-tail	0.487212	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.974424	
		t Critical two-tail	4.302656	

B. cereus incubated with HEPES at time 1 hours ($\mu_{1,1}$) compared to *B. cereus* incubated with MgOCl_2 liposomes at 1 hour of incubation ($\mu_{3,1}$), where $\mu_1 \neq \mu_3$ $H_0 = \mu_{1,1} = \mu_{3,1}$.

$\mu_{1,1}$	$\mu_{3,1}$		Variable 1	Variable 2
32.2	34.6	Mean	30.54333	30.43333
28.5	27.7	Variance	3.597433	13.58173
31.0	29.0	Observations	3	3
		Pearson Correlation	0.850641	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	0.082858	
		P(T<=t) one-tail	0.470755	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.941511	
		t Critical two-tail	4.302656	

B. cereus incubated with HEPES at time 0 hours ($\mu_{1,1}$) compared to *B. cereus* incubated with MgOCl_2 immunoliposomes at 1 hour of incubation ($\mu_{4,1}$) where $\mu_1 \neq \mu_4$ $H_0 = \mu_{1,1} = \mu_{4,1}$.

$\mu_{1,1}$	$\mu_{4,1}$		Variable 1	Variable 2
32.2	38.0	Mean	30.54333	30.24667
28.5	32.7	Variance	3.597433	85.66253
31.0	20.0	Observations	3	3
		Pearson Correlation	0.077995	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	0.055242	
		P(T<=t) one-tail	0.480484	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.960968	
		t Critical two-tail	4.302656	

B. cereus incubated with empty liposomes at time 1 hours ($\mu_{2,1}$) compared to *B. cereus* incubated with MgOCl₂ liposomes at 1 hour of incubation ($\mu_{3,1}$) where $\mu_2 \neq \mu_3$
 $H_0 = \mu_{2,1} = \mu_{3,1}$.

$\mu_{2,1}$	$\mu_{3,1}$		Variable 1	Variable 2
26.0	34.6	Mean	30.66667	30.43333
34.0	27.7	Variance	17.33333	13.58173
32.0	29.0	Observations	3	3
		Pearson Correlation	-0.99805	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	0.051517	
		P(T<=t) one-tail	0.481798	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.963596	
		t Critical two-tail	4.302656	

B. cereus incubated with empty liposomes at time 1 hours ($\mu_{2,1}$) compared to *B. cereus* incubated with MgOCl₂ immunoliposomes at 1 hour of incubation ($\mu_{4,1}$), where $\mu_2 \neq \mu_4$
 $H_0 = \mu_{2,1} = \mu_{4,1}$.

$\mu_{2,1}$	$\mu_{4,1}$		Variable 1	Variable 2
26.0	38.0	Mean	30.66667	30.24667
34.0	32.7	Variance	17.33333	85.66253
32.0	20.0	Observations	3	3
		Pearson Correlation	-0.53893	
		Hypothesized Mean Difference	0	
		Df	2	
		t Stat	0.060511	
		P(T<=t) one-tail	0.478626	
		t Critical one-tail	2.919987	
		P(T<=t) two-tail	0.957252	
		t Critical two-tail	4.302656	

Appendix M, Live/Dead Assays

Bacillus thuringiensis live/dead assay pictures

Figure 25. *Bacillus thuringiensis* with HEPES buffer at time 0 hours (A), *B. thuringiensis* incubated with HEPES at time 1 hour green color represent bacterial cells alive.

B. thuringiensis in Empty liposomes time 0hr

(A)

B. thuringiensis in Empty liposomes time 1 hr

(B)

Figure 26. *Bacillus thuringiensis* with empty liposomes at time 0 hours (A), *B. thuringiensis* incubated with empty liposomes at time 1 hour green color represent bacterial cells alive.

Bacillus thuringiensis in MgOCl_2 liposomes time 0 hr

(A)

Bacillus thuringiensis in MgOCl_2 liposomes time 1 hr

(B)

Figure 27. *Bacillus thuringiensis* with MgOCl_2 liposomes at time 0 hours (A), *B. thuringiensis* incubated with MgOCl_2 liposomes at time 1 hour green color represent bacterial cells alive, the red color represents dead cells, observe the increase in red cells after one hour of incubation with the MgOCl_2 liposomes.

Figure 28. *Bacillus thuringiensis* with MgOCl_2 immunoliposomes at time 0 hours (A), *B. thuringiensis* incubated with MgOCl_2 -immunoliposomes at time 1 hour green color represent bacterial cells alive, the red color represents dead cells.

Bacillus thuringiensis in 3mM MgOCl₂ time 0 hours

(A)

Bacillus thuringiensis in 3mM MgOCl₂ time 1 hours

(B)

Figure 29, *Bacillus thuringiensis* with 3mM MgOCl₂ at time 0 hours (A), *B. thuringiensis* incubated with 3mM MgOCl₂ at time 1 hour green color represent bacterial cells alive, the red color represents dead cells.