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Design of a Wound Dressing that Inhibits Microbial Infection

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ABSTRACT

Infected wounds are one of the most prominent health problems today, and many different methods of treatment exist. However, these treatments have their limitations and are often costly. By combining organic essential oils with a pre-existing silver antimicrobial, we can provide a more effective, less expensive wound dressing for treatment of infected wounds. After a series *in vitro* and *in vivo* tests for antimicrobial efficacy, these experiments validate the combination of actives which inhibit and kill specific wound pathogens in a hydrocolloid wound dressing. We used these results to design a three-layered, two-component dressing, to diffuse antimicrobials and to inhibit growth of bacteria as well as to remain mechanically stable in a wound environment.

1. Introduction

Each year, treatment of wounds becomes a more pressing medical issue as the need for wound care affects a large number of the population worldwide. Wounds cost the United States health system $20 million per year (Jackson 2006). These wounds include incisional, acute, and chronic all of which commonly may become infected to cause further complications. Chronic wounds can be very painful and can lead to secondary infections and complications, which can result in death. Recently, a study conducted at the University of Texas stated that approximately 5 million Americans suffer from chronic wounds each year. Chronic wounds, which usually result from progressive tissue degradation are termed "non-healing" wounds and easily result in an infection if not treated properly. Infections therefore result in increased cost for all parties involved (Dowsett, 2004). Incisional wounds along with deep lacerations are also problematic and play a large role in the annual cost spent on wound healing products. Although incisional wounds rarely take as long to heal as chronic wounds, they still pose trouble with proper closure and scar tissue buildup, which can in turn affect one's quality of life. Incisional wounds also run the risk of becoming infected, again causing the need for further treatment.

The need for proper wound care has been a concern throughout time in United States healthcare organization, but often the proper treatment is too costly for many disadvantaged patients. Treatments for wound care cost $4 billion per year in the United States alone (Jackson, 2006). Antiseptics, antibiotics and silver dressings have been providing wound care, however each of these treatments is resistant to various microbes commonly found in wounds so they do not provide an effective result. Newer technologies and treatment methods using silver as the antimicrobial component, such as in the Acticoat™ dressing (Smith & Nephew)

and Actisorb Silver 220™ dressing (Johnson & Johnson), have recently been developed and each have shown promising results in more immediate wound healing as well as inhibiting and killing a broader range of microbes compared to dressings without the silver constituent (Thomas, 2002; Smith & Nephew, 2005).

Each silver dressing on the market has its advantages and disadvantages. Although most of the current models kill and inhibit the growth of a variety of wound pathogens, there are still many limitations that can be addressed and improved upon. In one study, it was concluded that many of the dressings use more than five times the concentration of silver necessary to eradicate most microbes (Demling, 2002). The overabundance of silver is costly in both dollars and in materials. Also, although silver is effective against many wound pathogens, it is limited in microbial resistance. Additionally, many wounds, especially those that are infected, have a potent aroma, which can be embarrassing to the patient and make it hard for the medical attendant to treat the area. Silver dressings do not provide relief to this problem. The lack of pain reduction is also a severe limitation to the current models. Often chronic wounds are exceedingly painful and can take months or even years to completely heal. Similarly, incisional wounds can stretch the surrounding skin and result in painful scar tissue. Constantly applying and removing the dressing can damage the newly forming tissue as well as allow for more contaminants to enter the wound environment.

Due to the previously described limitations of the current silver models, there is a strong need for a next generation wound dressing. Essential oils, also known as terpenes, have been researched for several years due to their high levels of antimicrobial activity. These essential oils are organic compounds that originate from plants so there are many different varieties currently being studied. Plants release these oils as a defense mechanism from any

intruding organisms such as bacteria or fungi (Llusia, 2000). Extensive work has been completed using thirty-two formulations of these compounds by Gary Ostroff, Ph.D (Ostroff, 2005). Ostroff and colleagues were able to determine the strong antimicrobial properties of the various terpenes by testing them against ten wound pathogens. Many of the terpenes were broadly active against the bacterium including *Staphylococcus aureus, Streptococcus pyogenes* and *Candida albicans*. On the other hand, terpenes were not as effective against wound pathogens such as *Pseudomonas aeruginosa*. However, research has shown that silver is quite effective against these bacteria (Ostroff, 2005). Therefore, the possibility of incorporating both silver and terpenes into a dressing to inhibit a broader distribution of wound pathogens could be an exciting solution to creating the next generation of wound care dressings (Wright, 2002).

Terpenes not only possess antimicrobial properties but due to their organic nature, they are also less costly than silver and many of these compounds provide a pleasant smell that could provide relief to foul odors of infected wounds. These terpenes are also beneficial to a wound dressing as some have an added capability of reducing pain as in the case of Eugenol, which is used on the gums of teething infants (Jorkjend, 1990; Ostroff, 2005).

The goal of this project is design a novel wound dressing based on the current models in the field. We hope to address and overcome the limitations of these antimicrobial dressings resulting in a more optimal dressing. Once our dressing has been constructed, we will determine whether or not the dressing effectively decreases the amount of bacteria in the wound and if aids in the wound healing process. A team approach will be used in combination with a thorough engineering design process to accomplish the aforementioned goals. The dressing will include the innovative feature of terpenes, which should provide a broader

microbial inhibition and reduction in cost. In order to verify and validate the claim that the dressing facilitates enhanced wound healing, a full-thickness wound healing model will be analyzed to determine the optimal concentration of terpene in an *in vivo* environment. Bioluminescence technology as well as visual assessment and photographs will be taken to evaluate the length, width, area, and color of the wound throughout the testing period.

While conducting preliminary animal testing to determine proper concentrations as well as assess wound healing capabilities, the wound healing dressing will simultaneously be constructed. By including silver and terpenes, this system will significantly increase the range of bacterial inhibition compared to the current technologies. We will use diffusion disc, zone of inhibition, and biocompatibility assays to determine important parameters of our dressing. These experiments will allow us to create a superior dressing to the current models by comparing our dressing to the present data.

This dressing will be especially beneficial to patients who suffer from infected wounds by providing relief not only in the realm of pain management and of olfaction but also in improvements in cost and in overall treatment time.

2. Background and Literature Review

In order to develop an antimicrobial wound dressing which is superior to current wound dressing products, it is vital to understand the anatomy of the skin, the healing process of chronic and acute wounds, the available antimicrobials used in wound care, and the most often used preclinical wound healing models.

2.1 The Skin

The skin is part of the integumentary system along with sweat and oil glands, hairs, and nails. Skin is often called the integument, which means "covering". It covers the entire human body and makes up about 7% of the total body weight in the average adult (Marieb: Chapter 5, 2001). Skin is viscoelastic and therefore is pliable but it also has the ability to remain mechanically strong when confronting external agents and movements.

Skin is made up of two major layers; the epidermis and dermis and is supported by the hypodermis (see Figure 1). The outermost layer is called the epidermis and is composed of epithelial cells. The epidermal layer acts to protect the middle layer called the dermis. The thicker, vascularized dermis layer of the skin provides nutrients for the outer layer. The dermis layer is tough, leathery, and is comprised of fibrous connective tissue which contains fibroblasts, macrophages, mast cells, white blood cells, collagen, elastin, and reticular fibers (Marieb: Chapter 5, 2001). The hypodermis is the innermost layer and is made up of fats, loose connective tissue, and elastin and provides insulation and padding for the skin (Wake Forest, 2005).

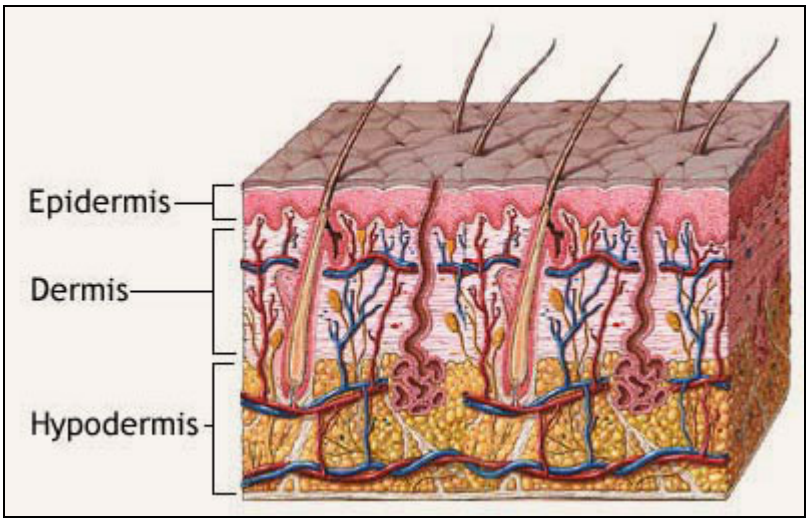


Figure 1: Layers of the Skin (Geneve Cosmeceuticals, 2005)

The skin is the primary barrier between the body and the environment, which functions to maintain water content within the body and acts as the body's first line of defense against foreign invaders. As long as the epidermis remains intact, the keratinized epithelial cells provide a mechanical barrier to keep microorganisms out. However, when body tissues are injured this mechanical barrier is broken, permitting microbial invasion into the underlying tissues. A second line of defense is the immune system, whose role is to keep infectious organisms out of the body. Yet, when pathogens do invade the body, this system provides antibodies using bone marrow ("B") and thymus ("T") cells. The immune system prevents the spread of foreign invaders to neighboring tissues and it also eliminates pathogens and cellular debris (Marieb: Chapter 20, 2001).

The inflammatory response initiates the necessary steps for tissue repair and regeneration. It is the immune system that aides in beginning the wound healing process (Marieb: Chapter 20, 2001; Harding, 2002). Those who are immunosuppressed rely on another agent to aid in starting and progressing with the wound healing process since the body

is unable to start this process alone. Oftentimes a wound is so severe that even healthy patients are unable fight infection and the patient requires additional treatment (Ratner, 2004).

It is important that any implant or wound dressing attract the proper type of cells or receptors to the surface of the skin. These cells and receptors are important because they are the ones that will trigger certain effects such as blood clotting, and will promote certain responses such as initiation of macrophages. Without the proper cellular components and receptors, the dressing will not allow for healing and tissue regeneration (Ratner, 2004). For example, seeding a synthetic collagen matrix with keratinocytes for a skin graft allows for the graft to be more readily accepted by the body because keratinocytes are a natural component of the body and will promote advantageous biological functions for the collagen (Harrison et al., 2006). Without the kerotinocyte cells on the surface, the graft may be rejected by the body and it will take longer to heal and regenerate new tissue.

2.2 Acute and Chronic Wounds

Acute wounds are wounds that heal within the expected timeframe of ten to fourteen days, without complications (Cardiff University, 2005). Acute wounds include incisions or lacerations, which heal best when the ruptured skin is in contact with itself during the healing process. On the other hand, chronic wounds can take months or even years to heal. A main cause of this delay in wound healing is the existence of micro-organisms in the wound, which can prolong the inflammation phase (Dowsett, 2004). With this type of wound, the skin heals from the edges of the wound bed inward to fill the large gap.

The cause of chronic wounds can vary. Some chronic wounds originate from acute injuries that develop infections or do not receive the proper treatment in the early stages of wound healing. The majority of chronic wounds are the result of progressive tissue

degeneration over long periods of time due to various vascular, venous, arterial, or metabolic diseases. Tumors, radiation damage, and pressure ulcers can also result in chronic wounds (Ellermann, 2005). Chronic wounds are demanding on the patient as well as the physician. They are often termed "nonhealing" wounds and require intensive wound care treatments. Wounds of this nature hinder the quality of life for millions in the United States alone (Harding, 2002).

As soon as a wound arises, the dynamic wound healing process is put in motion. This process is complex and multiple metabolic pathways and cellular activities overlap one another. Therefore, it is often hard to determine where one stage of the wound healing process stops and another one begins. However, the process is commonly divided into three phases: inflammation, proliferation, and maturation (Figure 2) (Clark, 1996).

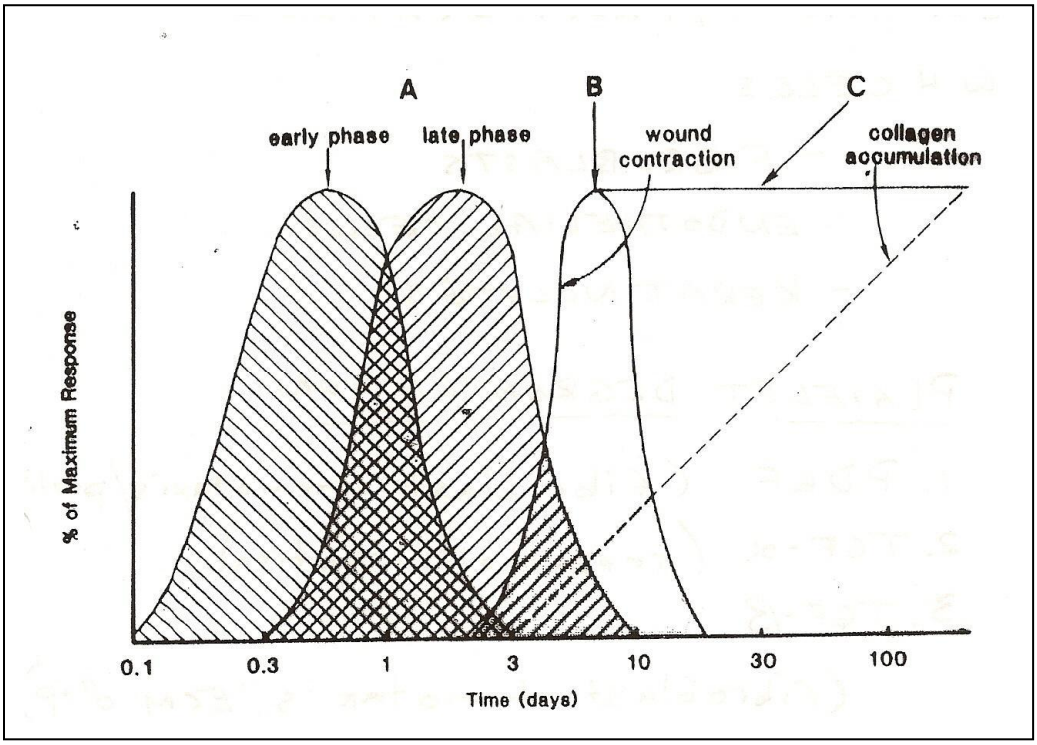


Figure 2: Three Phases of Wound Closure
A) early and late inflammation, B) proliferation (formation of granulation tissue and reepithelialization), and C) maturation (Clark, 1996).

In the inflammatory phase, which begins immediately after a wound has occurred, the patient may experience redness, heat, swelling, pain, and loss of function of the area. At the same time, the coagulation cascade, as well as growth factors, begins to take effect to reduce the loss of fluid from the wound via a fibrin clot formation in order to achieve homeostasis (Marieb: Chapter 20, 2001). Platelets are blood cells that continuously slip and slide in the blood and do not adhere to any other bodies or cells as long as prostacyclin is coating the vessel walls. However, when collagen is exposed outside of the damaged vessel, the platelets bind to the damaged area and degranulate. This results in the release of the mediators serotonin, adenosindiphosphate (ADP), and thromboxane A_2, which cause more adhesion of the platelets, resulting in a platelet plug, vasoconstriction, and activation of plasma proteins called clotting factors. This is the intrinsic activation process (Marieb: Chapter 20, 2001).

A second clotting pathway, called the extrinsic pathway is also activated when a wound is introduced to the skin. The damaged tissue releases thromboplastin, which in turn activates plasma proteins Factor VII followed by Factor X to begin coagulation. At this point, the extrinsic and intrinsic cascades merge into one unified process. These activation pathways rely heavily on calcium ions. Without these ions, blood cannot clot and the wound will continue to bleed, hindering the wound healing process. When calcium ions are present, Factor X can create a complex that clips part of the prothrombin molecule at protein sites Arg^{273} - Thr^{274} and then at Arg^{322} - Ile^{323} which produces active thrombin, an important clot promoter. Thrombin then cleaves soluble fibrinogen into insoluble fibrin, which polymerizes with the aid of plasma protein Factor XIII to create a meshwork clot (Rosing, 1985.)

Once the clot has formed, capillary diameter increases to create space for cellular infiltration. Many other cells are also involved in the wound healing process such as

platelets, which are the first cells employed at the wound site after the coagulation processes occurs. They are a source of growth factors, such as transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF) in the wound, which start the activation of fibroblasts and other mesenchymal cells (Ratner, 2004). Growth factors are peptides, which act on endothelial cells, fibroblasts, and inflammatory cells to stimulate the processes of wound healing. Prostaglandins then release cytokines, which cause fever production as well as pain (Marieb: Chapter 20, 2001; Martin 2005)

Within hours after the initial wound, a large angiogenic response is activated. During this response the epidermal layer starts to repair the damaged tissue by the migration of keratinocytes from the edges of the wound toward the center of the wound. Sheets of keratinocytes move forward at the border between the wound dermis and the fibrin clot. The connective tissue that is located deeper in the wound is replaced by activated fibroblasts at the edge of the wound that proliferate and move to the wound bed to form a granulation bed. (Martin, 2005)

Another class of cells, called macrophages, produces the cytokine interleukin-1 (IL-1) and they promote proliferation and angiogenesis, as well as protect the wound from microbial invasion. After about 24-36 hours, circulating monocytes that have entered the wound reach their maximum number and mature into macrophages. The macrophages exude substances such as basic fibroblast growth factors (bFGF), which stimulates growth for endothelial cells and fibroblasts as well as IL-1. A lack in the number of macrophages would cause an increased risk of infection resulting in a severe alteration in the wound healing process with poor debridement and insufficient fibroblast production and angiogenesis. At the end of the inflammatory phase, the wound becomes more mononuclear with less macrophages, which

defines the next phase called the proliferative phase (Harding, 2002). Additionally, helper T cells, which are a class of mononuclear leukocytes that produce and secrete the cytokine interleukin-2, stimulate more T cells to aid in the immunogenic response (Marieb: Chapter 20, 2001).

Approximately two to three days after the injury, the proliferation stage begins with the arrival of fibroblasts into the wound site, which then produce various growth factors to enhance wound healing such as: bFGF, transforming growth factors (TGF- β), platelet derived growth factors (PDGF), endothelial, and keratinocyte growth factors. The proliferation of the previous growth factors then allows glycosaminoglycans (GAGs) and proteoglycans to be synthesized. These are the foundation for new extracellular matrix of granulation tissue as well as collagen (Marieb: Chapter 20, 2001). Collagen is a large component of acute wound connective tissue. After the emission of collagen molecules, the fibroblasts assemble the molecules into fibers, which are then cross-linked and organized into collagen bundles. Production of collagen continues for six weeks and accounts for increasing tensile strength of the wound site (Madden, 1971).

Once the new extracellular matrix, collagen, and capillaries have formed, the fibrin clots begin to degrade. The granulation tissue continues to be produced until the entire wound is covered, at which time fibroblast migration and proliferation is discontinued. The glycosaminoglycans then act to inhibit fibroblast activity and begin the maturation stage in the wound healing process (Clark, 1996).

During the maturation phase, the collagen is remodeled into a more organized structure to increase the wound's tensile strength. As scar tissue forms, type III collagen is replaced by type I collagen until the ratio between the two is 4:1 for healthy skin, the higher

amount being type III collagen (Marieb: Chapter 20, 2001). Type I collagen is made up of broad fibrils of low hydroxylysine protein which are distributed throughout the skin and account for 90% of body collagen, whereas Type III collagen is also distributed throughout the skin but is made up of high amounts of hydroxyproline protein (Saltzman, 2004). Eventually, the wound is closed by the movement of epithelial cells from the wound edge to holes in the defect. Finally, the wound fibroblasts differentiate into myofibroblasts, which contain actin muscle fibrils that bind together to draw the wound edges closer resulting in a healed wound (Grinnell, 1994).

This wound healing process is similar in both acute and chronic wounds. However, often times an acute model does not represent that of chronic wounds (Dowsett, 2004). An acute wound goes through the previously mentioned sequential and timely wound healing process and results in restored function and anatomy; however, with chronic wounds, the chronological process is disrupted causing a lengthier and often incomplete healing process. Chronic wounds lack restoration in anatomical and functional integrity (Harding, 2002). They can often get caught in the first two phases of wound healing and this can cause them to take months or years to heal as a result of prolonged inflammation and proliferation which may lead to elevated extracellular matrix molecules, indicating an unregulated wound and cellular dysfunction (Dowsett, 2004).

Many factors can contribute to a decrease wound healing. These factors include wound infection, repeated trauma, tissue hypoxia, debris, and necrotic tissue. Other factors are labeled systemic causes such as malnutrition, the use of certain medicines, immunodeficiency, and diabetes mellitus (Harding, 2002; Dowsett, 2004). The most common reason for poor wound healing is infection, which can affect all three stages of the wound healing process.

Once a wound has become infected, wound healing is delayed. In turn, necrosis can occur, which increases the chances of the wound growing in size and severity (Kingsley, 2001).

There are numerous microbes that replicate in a wound and cause infection. In both acute and chronic wounds, *Staphylococcus aureus and Pseudomonas aeruginosa* are two aerobic pathogens that are often associated with postponed wound healing as well as infection. Research has shown that *S. aureus* is one of the most problematic pathogens in when dealing with wound infections. In one study of acute wounds, 14 out of 61 wounds showed that they were infected and identified that the most prevalent wound pathogen was *S. aureus* (Bowler, 1999). *Enterococcus fecalis* is another pathogen that is often common in wounds and can lead to infection as well as other sicknesses especially to the intestines, which can compound the insult of the wound by further suppressing the immune system (Lu, 2002).

It is both costly for patients as well as healthcare providers to afford consistent wound care. Patients suffer in a reduced quality of everyday life and insurance companies experience a financial burden (Dowsett, 2004). Each type of wound varies in treatment, and each treatment also varies in cost. Therefore, there is a demand for a more cost effective, long-lasting antimicrobial wound dressing to satisfy the needs of both patients and healthcare providers.

2.3. Silver

Various forms of silver are currently be used in wound dressings. Although costly, silver has proven to be quite effective as an antimicrobial agent. Silver is classified as a nearly inert metal and therefore ideally, this material does not react with the human body in its non-ionized form. However, in its ionized form, silver becomes bioactive. The transformation from non-ionized to ionized silver, results from the presence of moisture such as that found in

wound exudate. As silver ionizes, silver ions are released along with other ions that are biologically active. These ions then bind with proteins, which can be found on the surfaces of various cells as well as in bacteria and fungi (Dowsett, 2004).

There are four main reasons why scientists believe that silver is a successful antimicrobial agent. The first reason is that silver interferes with bacterial ion release and disrupts electron transport. Second, silver has a tendency to bind to bacterial DNA, which is beneficial since the interaction can hinder cellular replication of the bacteria. Third, the presence of silver causes damage to the receptor function and structural integrity as it interacts with bacterial cell membranes. Finally, silver allows for insoluble and ineffective compounds to form, which can in turn hinder microbial activity (Ruszczak, 2004; Brett, 2006).

Additionally, many limitations of the current silver dressings stem from confusing and conflicting ideas about silver as an antimicrobial in general. In a 2006 article entitled, "A discussion of silver as an antimicrobial agent: alleviating the confusion", the author states that the makers of the current dressings often supply information about the dressings that conflict with data found in literature therefore, clinicians are unsure about what to believe (Brett, 2006). It is currently used in many different applications for its antimicrobial properties, so these various trials cannot all be taken for truth.

Some of the negative aspects of silver as an antimicrobial were very crucial to creating a successful wound dressing, therefore the team wanted to make sure that all of the limitations of the current models were addressed. Some of the limitations were that silver may not be affective against all microbes or all strains within a specific pathogen and the test medium used plays a role in the silver dissociation properties (Brett, 2006). Additionally, rapid silver

delivery is necessary at high doses to be truly effective. However, research has shown that silver is not cytotoxic so high concentrations should not be harmful to the user (Brett, 2006).

2.3.1 Silver Sulfadiazine

For the past few decades, silver sulfadiazine has been the standard treatment for burns and other chronic wounds. In 1968, Fox first synthesized the compound from silver nitrate and sodium sulfadiazine for an increased effectiveness as well as minimal pain upon application. Since its discovery, silver sulfadiazine has been praised for its antibacterial properties. However, recent studies show that the compound may actually hinder and delay the wound-healing process. This new finding can possibly be reversed using growth factor treatments, but it is now important to look at other forms of silver to find the next generation of wound healing agents (Lee, 2005).

2.3.2 Pure Silver

Pure silver has also been studied for use in wound healing. In a study conducted by Robert H. Demling, M.D. and Leslie DeSanti at Brigham & Women's Hospital, the objective was to determine if exposure to pure silver would increase healing and re-epithelialization along partial-thickness wounds. The team compared a silver delivery system with that of xeroform, an eight ply gauze dressing with an antibiotic solution. Each patient was treated with an antibiotic solution on one graft area and the pure silver delivery system on another area. By day seven, in all of the silver areas re-epithelialization was complete and the rate of healing was increased by 40%. Therefore this study concluded that silver used in a moist wound environment increases the healing rate and re-epithelialization rate compared to typical antibiotics (Demling, 2002).

2.3.3 Nanocrystalline Silver

Nanocrystalline silver has been used as a form of silver for wound care management. In one study, the aim was to determine if nanocrystalline silver had the physical properties to act as a barrier against methicillin-resistant *Staphylococcus aureus* (MRSA), which is a common bacteria found in many wound sites. In initial experiments using MRSA suspension and colony culture, it was shown that the silver acts as an effective and efficient antimicrobial agent against the spreading of MRSA. A similar test, which "delineated the MRSA load on the upper side of the dressing and wound bed" every time the dressing was changed, resulted in the silver preventing nearly 95% of the MRSA from spreading. Therefore, nanocrystalline silver dressings are being looked at even more closely as antimicrobial wound healing agent. Not only does it provide safe and effective containment of the bacteria and increased healing, but it also provides cost benefits to the healthcare system and even more importantly, to the patients. The results of this study may greatly benefit patients in which systemic antibiotics often fail to reach secondary infections such as those with diabetes or those with peripheral arterial occlusion. Therefore such a dressing may increase the effectiveness of antibiotic treatments and reduce therapeutic treatments (Strohal, 2005).

It is important to note that in all of the studies conducted, MRSA, *S. aureus* and *P. aeruginosa* strains were not resistant to silver and silver not only has antimicrobial properties but also has shown signs of having anti-inflammatory characteristics (Dowsett, 2004).

2.4 Silver Products

Recently, it has been estimated that the total market for silver dressings is about $40 million annually. This number is nowhere near as high as the numbers for devices such as cardiac stents. However, some of the largest companies in the medical device industry are the

ones showing the greatest interest in the wound healing market and therefore the desire for silver dressings is on the rise (Kerber, 2005). A study was conducted evaluating the slow release silver dressings and the cost involved with daily to weekly dressing changes. This study determined that after rats were treated with various dressings, each of the three different products of dressings were all equally effective over time. Therefore, each company that claims to have the most effective dressing has been proven wrong, that silver itself acts as an effective antimicrobial. Consequently, the race to produce the least expensive yet most effective dressing is currently in progress (Heggers, 2005).

2.4.1 Arglaes™ Antimicrobial Silver Barrier

One of the current wound healing dressings on the market is the Arglaes Antimicrobial Silver Barrier. This product uses controlled release of antimicrobial silver to effectively kill a wide variety of bacteria and fungi that are commonly found in wounds, as well as to help stop the migration of bacteria that already exists. One of the advantages of this design is that it can be worn for up to seven days, which reduces the number of dressing changes, minimizing pain for the patient upon removal as well as the time necessary for patient care (Medline Industries, Inc., 2005).

The Medline product comes in a film form, which is ideal for managing post-operative incisions and donor sites. They have also commercialized a product called the Arglaes™ Island, which is beneficial for managing fluid and bioburden. The Island features a calcium alginate pad, used for fluid management, along with the controlled release silver. Finally, this product comes in a powder form, which can be easily combined with other dressing systems for fluid management and bioburden reduction. This is beneficial for wounds that are of any

shape or size, especially those that are difficult to bandage as well as grafted wounds (Medline Industries, Inc., 2005).

Figure 3: Arglaes Antimicrobial Barrier (Medline Industries, 2005)

The Arglaes Antimicrobial Silver Barrier can be used for pressure, leg, or diabetic foot ulcers, partial- and full-thickness wounds, central lines, donor sites, surgical wounds, lacerations as well as first and second degree burns. On the other hand, it should not be used on third degree burns, as an implant, or on dry wounds as well as those that are covered with necrotic, black tissue. The device should also not be used by those that have a known allergy to silver or combined with topical antibiotics (Medline Industries, Inc., 2005).

2.4.2 Silverlon™

Another silver wound dressing currently on the market is the Silverlon™ dressing created by Argentum. This dressing consists of silver nitrate deposited onto nylon fibers and attached to another fiber layer. This dressing is effective in killing wound pathogens, but unfortunately the use of silver nitrate causes the patients skin to turn black, which is not aesthetically pleasing. Another limitation is that it is hard to control the amount of silver applied as well as released from the dressing so it does not deliver a constant level of effective killing (Canada, 2005).

2.4.3. Actisorb Silver 220™

Johnson & Johnson's silver wound dressing is the Actisorb Silver 220™ and it contains activated carbon, which is impregnated with metallic silver. This is a highly porous bandage constructed from silver-impregnated charcoal cloth sandwiched between two non-woven nylon layers. This charcoal is beneficial as it absorbs odor in the wound, but it also poses a drawback in that it causes the patient's skin to turn black and over time often leaves debris in the wound bed (Thomas, 2002).

When Actisorb Silver 220™ is placed onto the wound; the dressing adsorbs toxins, amines, and fatty acids, which are primarily responsible for the generation of wound odor. This dressing attracts bacteria to the surface of the wound, and the silver kills the pathogenic organisms. This product is best designed for chronic wounds including fungated lesions, faecal fistulae, infected pressure sores, and heavily exuding leg ulcers. Actisorb™ can be very beneficial if it is used properly. However, it should not be used on those who have known allergies to nylon and should not be used with excess ointment as it can greatly reduce the effectiveness of the dressing. Actisorb Silver 220™ can be applied directly to the wound if it is appropriate. It can then be covered with a secondary dressing or held in place with a bandage or cloth. This Johnson & Johnson product can be worn for up to seven days and the outer absorbent layer can be changed as often as needed. For infected or heavy exuding wounds, dressing changes may initially need to be more frequent (Thomas, 2002).

In order to ensure sterility upon use, this product is presented in a peel pouch, which is sterilized by gamma irradiation. The combination is produced under carefully controlled conditions using heat and a treated fine viscous fabric. Actisorb™ is enclosed in a sleeve,

which is composed of non-woven nylon. It is then sealed along each edge to ease handling and decrease fiber and particle loss. Therefore, this product should be used in its manufactured state and should not cut down to various shapes and sizes (Thomas, 2002).

Many clinical trials have been conducted to test the efficacy of this product. Test results have shown that this dressing is able to kill and inhibit common wound pathogens. However, less killing was observed compared to Smith & Nephew's Acticoat 7™ (Dowsett, 2004).

2.4.4 Acticoat 7™

Acticoat 7™, marketed by Smith & Nephew, is considered by some as the "gold standard" of sliver wound dressings (Supp, 2005). The product has been shown *in vitro* to be effective against nearly 150 micro-organisms as well as demonstrated resistance to prevalent wound pathogens such as Methicillin Resistant *Staphoylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Enterococcus fecalis* to name a few (Smith & Nephew, 2005).

Figure 4: Acticoat 7™ (London Health Sciences Centre, 2001)

This dressing is made up of five layers that are ultrasonically welded together, which increases the strength and integrity of the product. The product is marketed in a laminated peel pouch, sealed with a cover, and sterilized with gamma irradiation. Two of the layers are

fine silver-coated mesh of high-density polyethylene, which surrounds the core layers, which are non-woven rayon and polyester. Finally, the innermost portion is another layer of silver-coated polyethylene mesh. A vapor disposition process is used to apply the silver onto the polyethylene mesh, which in turn results in nanocrystalline silver. It is in this form (nanocystalline) that silver exhibits its greatest antimicrobial activity against gram-positive and gram-negative bacteria as well as yeasts and fungi (Thomas, 2004).

This product is marketed for partial- and full-thickness wounds such as leg ulcers, pressure sores, and various other chronic wounds (Thomas, 2004). The preparation for this product is fairly simple – Acticoat 7™ should be moistened with sterile water for 2 minutes to activate the dressing. Saline should not be used because it contains chloride ions, which when added to silver cause silver chloride to form as well as a white precipitate that can greatly affect the efficacy of the sustained silver release of the dressing. By moistening the dressing with sterile water, this will allow for a moist wound-healing environment which increases the silver's efficacy. This dressing can be cut to the right shape and size depending on the wound (Smith & Nephew, 2005), which is not permitted by Johnson & Johnson's dressing. However, like Actisorb Silver 220™, Acticoat 7™ also needs to be covered with a secondary dressing that is appropriate for the wound type. The product can be worn for up to seven days depending on how heavily it exudes fluid. Topical antimicrobials and oil-based products should not be used with Acticoat 7™, as drying out may occur and then hinder the healing process (Thomas, 2004). One of the major benefits of the dressing is that it provides quick and immediate effects, which are often within thirty minutes of application (Smith & Nephew, 2005).

One thing that is important to note is that Acticoat™ cannot be used with magnetic resonance imaging (MRI) due to silver's metallic properties. Also, it may promote some staining or discoloration of the wound as the silver oxidizes and turns black. This causes no harm as even healthy skin may turn brown, yellow, or black where the dressing has overlapped. Some patients also experience a stinging sensation upon application (Smith & Nephew, 2005). This can be minimized by draining the excess water before placing the dressing on the wound site. However, if the patient continues to feel pain, it could be a silver allergy or the patient could be experiencing argyria and the use of the product should be discontinued.

This product has been used in a variety of clinical test settings. In a matched-pair randomized analysis the ease-of-use, comfort, and antimicrobial efficacy were determined for burn patients. The results of the tests demonstrated that the use of Acticoat 7™ resulted in less pain as well as easy application. This product kills more rapidly compared to other silver wound dressings on the market including Actisorb Silver 220™. Smith and Nephew's device demonstrated the lowest minimum inhibitory and bactericidal concentrations (Dowsett, 2004). It was also determined in clinical tests that this product had the fastest antimicrobial effect in an *in vivo* setting. This is due in part to the sustained release profile used to manufacture the dressing as well as the use of a high concentration of silver (Dowsett, 2004).

2.4.5 Additional Silver Models

There are quite a few other silver wound healing dressings on the market today. However, they are not as widely recognized either due to the size and name of the company that created the dressing or the lack of current clinical data.

Aquacel® Ag is composed of Hydrofiber®, which is impregnated with silver. This product contains a gel and non-woven pad for fluid management. It can be used for up to one week and is beneficial for chronic wounds (Dowsett, 2004). Contreet® is another silver dressing that uses ionic silver and polyurethane foam to deliver a controlled release of the antimicrobial agent. Like many of the other current models, this dressing can also last for up to seven days (Dowsett, 2004).

2.4.6 Current Model Summary

Table 1 demonstrates the advantages and disadvantages of the major current silver dressings on the market. It is evident that each of the models contains an antimicrobial component, which is silver. The other constituents of the dressing include a breathable layer for oxygen permeability, a transport layer that holds the antimicrobial, and a final layer for fluid management in order to maintain optimal moisture for wound healing.

Table 1: Evaluation of Current Silver Wound Dressings

	Advantages	Disadvantages
Arglaes™ Antimicrobial Silver Barrier	• Controlled release • Can be worn for 7 days • Good for donor sites, 1st and 2nd degree burns, chronic wounds	• Not applicable for 3rd degree burns • Not good for dry wounds or those with necrotic tissue
Silverlon™	• Effective in killing a variety of wound pathogens	• Silver nitrate turns skin brown/black • Cannot control amount of silver released. Therefore not a constant level of effective killing
Actisorb Silver 220™	• Highly porous • Odor reducer • Good for chronic wounds • Can be worn for 7 days	• Turns skin black • Nylon can cause allergic reaction • Cannot be cut to size or shape
Acticoat 7™	• Effective against 150+ micro-organisms	• Cannot be used with saline • Cannot be use with MRI

	• Good for partial and full-thickness wounds • Easy to prepare • Can be cut into proper shape and size • Can be worn for 7 days • Quick effects within 30 minutes	• Turns skin black • Can cause stinging sensation

Although many of the current silver dressings on the market are thriving, there is still some concern from the individuals using and prescribing these products (See Appendix A). Therefore, there is a strong need to identify these drawbacks and further improve upon the current technology (Dunn, 2005).

2.5 Essential Oils

Over the past several years, essential oils have become popular in various applications. Essential oils are extracted from different varieties of plants and are known for their broad antimicrobial properties (Burt, 2004; Ostroff, 2005). They have been extracted from familiar plants such as oregano, lemongrass and thyme, to name a few. These oils, containing terpenes, are non-toxic because of their organic nature (Ostroff, 2005) and are present in the plants natural environment as a defense mechanism against bacteria and fungi (Llusia, 2000). Essential oils are affected by their environment as decreased temperature, pH and oxygen levels allow for improved antimicrobial response (Burt, 2004).

Overall, over ninety-seven plant oils have been reported and many more are still being discovered. The effectiveness of the compounds is tested through a series of *in vitro* assays which yield a number known as the minimum inhibitory concentration (MIC). This is the lowest concentration where the active (i.e. terpene) is able to achieve growth inhibition of the pathogen it is being testing against such as *Staphylococcus aureus* (Mann, 1997). This

primary assay is often followed by another series of antimicrobial assays to measure the actual percent of pathogenic kill in the MIC concentration. In a study conducted in Australia with 52 essential oils, only three formulations were proven to be effective against inhibiting *P. aeruginosa* while 40 terpenes inhibited the growth of *Candida albicans* and *Staphylococcus aureus.* The latter pathogens were proven to be the most vulnerable to these antimicrobial oils in the primary assays (Hammer et al., 1999).

Another study was completed analyzing the antimicrobial properties of essential oils against *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli 0157:H7*, *Shigella dysenteria*, *Bacillus cereus*, and *Staphylococcus aureus*. Here it was shown that the gram-negative organisms are less susceptible to antimicrobial killing by essential oils than gram-positive bacteria. In these assays, the effective minimum inhibitory concentrations (MIC) varied between 0.05-5 microliters *in vitro*. In food applications, the effective concentration is higher, at 0.5-20 microliters. Essential oils target the bacterial cell and their hydrophobic nature attacks the cell contents and makes cell membranes and mitochondria permeable (Burt, 2004). Since these oils are hydrophobic they can be encapsulated to make them easier to handle, as well as to allow them to solubilize in solution and provide a sustained release (Bishop, 1998). For example, Eden Research plc, has developed a technology of encapsulating the oils into the membrane of a yeast particle which does not effect the antimicrobial activity of the oils as they are still effective in inhibiting pathogenic growth (Ostroff, 2005).

One essential oil that has been used in many burn wound healing applications is Thymus oil from a thyme plant. Often burn wounds emit concentrations of nitric oxide which not only leads to thrombosis but also to other physiological effects. A study was done

submitting 5 different groups of rats to a burn and treating them with silver sulfadiazine, thymus oil, olive oil, and silverdine. Rats treated with thymus oil and silver sulfadizine were most promising as thymus oil protected the tissues from reaching harmful nitric oxide levels (Dursan et al., 2003).

Essential oils are also effective in flavorings and odor emissions. For example, thymol is also effective in reducing unpleasant odor, along with carvacrol as shown through livestock wastes in agricultural applications. Fermentation of waste excreted from cattle was observed in a flask and 1 g of both carvacrol and thymol were added to determine the control of odor emissions. This combination of terpene was extremely effective in reducing odor from livestock waste (Varel, 2001). This consideration of is also important to wound healing, as many wounds and burns exude odors due to bacterial infection or burnt skin, respectively.

The uses and applications of essential oils are endless as their major applications are in agriculture, food borne pathogens, and oral health, amongst others. Terpene antimicrobial activity is promising in effectiveness and in its low cost for treatments in the wound healing market.

2.6 Animal Models

A number of wound healing animal models have been used to test the effect of antimicrobial wound dressings. These *in vivo* studies often follow *in vitro* experiments and test the validity and effectiveness of the dressings.

Various companies such as Jackson Laboratories and Charles River Laboratory are popular suppliers of animals used in animal models. They can be custom ordered to suit the experiment. Animal models can be ordered with medical devices or procedures being performed, such as castration, removal of the thyroid gland etc., depending on the necessary

protocol. Other models available are those ordered already infected with certain strains of bacteria such as *S. aureus* (Charles River, 2005).

Another consideration is nude mice which have a knocked out immune system, absent of antibodies. Oftentimes a common type of mouse is the F1 generation hybrid mouse. These mice are advantageous in that they are identical in genetics and phenotype; they also have a higher longevity and higher survival under stress as well as are known for their larger litters. This model is often extremely beneficial in serving as a host for tissue transplants for their parent strains. The F2 generation has more genetic mutations and targeted knockout genes and most of them are only similar in genetics, but not identical. Therefore, this constant genetic makeup serves as another control in the experiment (Jackson Laboratories, 2005).

Currently a project is underway at Tufts studying the cellulitis model of *S. aureus* in hairless mice. The cellulitis form is first cultured as normal, washed/spun with phosphate buffered saline (PBS) and then beads are added to this culture. This is then injected between the skin and the muscle in the mouse and creates a localized wound once infused into the tissue.

Another study was completed to observe cultured skin substitutes using athymic mice and infected wounds inoculated with *P. aeruginosa* at $1*10^5$ cfu/wound. This wound was then tested in collaboration with Acticoat™ to validate its effectiveness (Supp, 2005).

Accurate wound healing models have been hard to come by as there are many regulations and ethical reasons behind the concept. The most accurate wound model to date is in swine as their morphology and functions of their skin are most comparable to that of human skin (Singer et al, 2000). Swine have been widely used as wound healing models also due to their ability to allow for various studies on multiple sites of their skin rather than only

one model having one wound site. The porcine model also reduces variation as it is one animal receiving different treatments, rather than a series of models receiving different treatments which leads to variation between animals as each are genetically different (Olson, 2000). Usually smaller animals such as rat and mice are used as the initial experiment, and then once the proof of concept is shown in small *in vivo* models, then a larger model can be implemented, and if all goes well, then humans are the next step.

A key factor in wound healing models is the type of wound that is formed on the animal. In severe wounds such as burns, the graft site to culture new skin is often an area which potentially experiences delays in wound healing, and can even be deadly for the patient (Olson, 2000). Graft sites were made on the abdomen and thorax of six pathogen-free pigs and 72 same size wounds were created using an etched grid. This experiment allowed for three different treatment groups on each pig and all pigs were clinically monitored for evidence of infection and dressing moistness. Therefore, this extensive study was conducted over a period of 10 days and only six pigs needed to be evaluated. If a smaller animal model had been chosen, it would have required more attention per mouse and the amount of wounds would have had to be dramatically decreased to a smaller number of mice as each mouse can only contain one wound. From this porcine study, scientists were able to correlate that the healing time in the animal model will be similar to that of human models. The dressings tested in this study have reduced concerns of the physicians as they have provided an ideal healing environment for donor sites (Olson, 2000).

Another of many porcine studies used a 2.5 cm by 2.5 cm burn site for the wound application to pigs to analyze the wound healing with a various occlusive dressings. Again, a small number of animals were needed, as this study only used 2 and the scientists were able to

create four identical sets of 20 burns on each pig. Again, the scientists were able to run the experiment against various treatments yet keeping the animal constant to reduce variation. Interestingly, the choice of a porcine model in the use of studying burn wounds is limited to the actual wound response as pigs do not form blisters. Therefore, with this data, it is hard to correlate the burn site with that of humans, as in most cases severe burns do form a blister as a wound response (Singer et al, 2000).

Again, wound healing models vary not only in animal, but also in metrics used to assess the animal, the wound site, and the effect of the treatment. In smaller individual rodent models, it is easier to assess different parameters of the wound. For example, in a rat model, different cross-linking agents in a hydrogel dressing were measured using parameters such as mechanical properties, water-vapor-transmission, swelling, degradation against enzymatic/cellular agents, and inflammatory reactions (Chang, 2003). The latter parameters would be more difficult to measure in larger animals as there would be many parameters that are different per wound that cannot be measured if they are all on one animal.

On average it takes between 3 months and one year to get a protocol submitted and approved for testing. Therefore, we looked into various protocols that had already been approved in hopes to work off of theirs after brief modifications. We were able to discover many wound experts and ones who had published animal data from the 2000 to present and were within the United States (see Appendix FF). One of the most prominent names is Dr. Michael Hamblin of Mass General Hospital. Upon contact, he agreed to collaborate with us in using his protocol and his laboratory to perform an *in vivo* wound healing experiment. Dr. Hamblin's research is focused on the new field of Photodynamic Therapy (PDT). PDT is where a dye is combined with light in an oxygenated environment to kill harmful human cells

and pathogens. This light treatment targets lysosomes, mitochondria and the plasma membrane in mammalian cells as a site for photodynamic inactivation in cells such as cancerous growths (PDI). Gram positive bacteria have been proven to be more affected by the treatment in comparison to Gram negative bacteria. There is no proven explanation for this reaction, however it is proposed that the differences include membrane permeability barriers, enzyme and cell size (Hamblin, 2004).

In Hamblin's model, bacteria used in PDT are also genetically altered with a *lux* flouresecent gene plasmid to allow for the cells to be viewed by a biolouminescent charge-coupled device camera (CCD) (Hamblin et al, 2003). The *lux* gene is found naturally in many aquatic mammals and consists of luciferase. Bacterial luciferase uses oxygen and long-chain fatty proteins to produce blue-green light (max emission at 490nm) (Eckstein et al, 1990). The lux operon used in these experiments originates from *P.luminescens* in the prescence of mamammalian animal models and is usually present in the form *lux* CDABE (Demidova, 2005). However, in studying a Gram-positive strain of *S. aureus*, the *lux* gene order is altered for stability to *lux* ABCDE. The infected animal is then imaged in the CCD camera, which consists of a light-tight chamber where the animal remains stationary on a stand and the CCD camera detects the light emitted from the mouse and a computer connection obtains the image and analyzes the data. A grayscale image is obtained after a few seconds, and after a two minute reading, the bioluminescent signal is read from the wound. This color image is superimposed onto the grayscale image and then the magnitude of the regions intensity is read using an area function (Demidova, 2005).

The use of PDT is only used for localized infections due to its targeted light delivery; however, burns, infected wounds and tissue infections were studied in BALB/c mice to prove

the positive effect of this treatment (Hamblin, 2004). In Dr. Hamblin's initial PDT application he proved that *S. aureus* is dramatically decreased with this treatment. Dr. Hamblin and his research team also completed a study to treat *P. aeruginosa* infeceted wounds as it was topically applied to the wound and followed by illumination. Here treatment was extremely successful as all of the control mice died after 5 days, and 90% of the PDT-treatment mice survived. Therefore, the PDT-treatment surpassed the control silver nitrate treatment in enhancing wound healing.

2.7 Material Requirements

There are many aspects that need to be carefully considered when trying to create an ideal wound dressing. Some of these features have been tested at Instron ® Corporation (Appendix R and S) to provide us a better understanding of the current products on the market. The dressing must provide bacterial and mechanical protection as well as maintain the proper moisture at the interface between the wound and the dressing (Hom, 1999). Therefore the device must promote fluid and gaseous exchange. A wound with excessive amount of fluid can hinder the wound healing process by providing an environment that is too moist and also ideal for bacterial replication. On the other hand, a dry wound can result in painful cracking and bleeding in the wound bed (Harding, 2002).

Management of an exuding wound is a key in the design of a proper dressing. For example, if necrotic tissue reaches a mushy, liquid-like state, the wound exudate level will increase. Therefore, it is important that the dressing have good fluid retention and absorptive properties while remaining non-adherent to the wound in a normal environment as well as under pressure. If the dressing is suited for and can withstand many everyday situations, cost

can be improved because wear time will increase due to better protection of the wound (Hom, 1999; Harding, 2002).

Other keys to creating a wound dressing include safety and patient acceptability. The device must be sterile, non-allergic, non-toxic, and non-sensitizing to the patient as well as the medical attendant. To satisfy the needs of the patient, the device should provide pain relief and absorb wound odor. Many patients have stated that the healing process can be decreased if the wound smells foul due to bacterial growth or if the dressing causes skin staining (Holloway, 2002). Finally, the dressing should be easy to use so that it can be quickly applied by medical personnel, should not require frequent removal, it should be available in a variety of shapes and sizes to cover a broad range of wounds and areas of the body, and it should be covered under health insurance, and therefore must be cost effective (Hom, 1999).

2.7.1 Lyophilization

In order to improve the mechanical properties of the certain materials, they are often lyophilized or freeze dried, a method is currently used to fabricate collagen scaffolds (O'Brien, 2004). Freeze-drying was approved by the FDA in 1996 as a means of creating dermal substitutes for burn patients (O'Brien, 2004). The technique results in a highly porous hydrocolloid sponge and is beneficial as it allows for a more homogeneous scaffold, gel, or hydrocolloid to be fabricated while generating less deviation in pore size throughout the structure. The pore size is determined by the rate at which the network is cooled and can therefore be customized according to what material properties one desires (O'Brien, 2002).

2.8 Wound Dressing Categories

In the United States alone, there are nearly twenty different types of material categories for wound dressings (Ruszczak, 2004). Table 2, shown below demonstrates the some of the number and variety of dressing categories currently in the United States.

Table 2: Current Material Categories for Wound Dressings in the US (Ruszczak, 2004).

Wound Dressings (Material Categories)	No. Products
Gauzes (woven and nonwoven)	33
Gauzes (impregnated)	25
Gauzes (nonadherent)	13
Gauzes (packing/debriding)	17
Compression bandages and system	24
Specialty absorptives	17
Composites	20
Wound fillers	10
Wound cleansers	28
Synthetic foams	26
Contact layers	7
Hydrocolloids	41
Hydrogels (amorphous)	30
Hydrogels (wafers)	19
Hydrogels (impregnated gauze)	14
Alginates	26
Collagen	9

2.8.1 Tissue Adhesives (Incisional)

Tissue adhesives are a painless way of closing incisional wounds for up to seven days, which might otherwise require stitches or staples. Products of this nature are usually liquid bandages that contain cyanoacrylate derivatives such as enbucrilate, bucrylate, and mecrylate. When in contact with a fluid, the materials react exothermically to polymerize. The polymerization results in a flexible, sturdy, water resistant adhesive. However, the special care is necessary to ensure that none of the adhesive diffuses past the wound borders. Also, tissue adhesives are only good for minor lacerations and cannot be used for chronic wounds or those that exude fluids (Reece, 2001). Therefore, the use of tissue adhesives would not allow us to enhance the current silver wound dressing market.

2.8.2 Silicones

Silicones are one type of dressing which is biologically and chemically inert so that an immune response is not induced. They are primarily composed of cross-linked polymers that are then reinforced with a mesh or fabric (Kestrel Health Information., 2005). This material can be porous or non-permeable and is manufactured as sheets or gels that work as non-adhesives. Silicones can reduce scar tissue as well as scar discoloration by flattening the tissue and increasing its elastic properties. This material is beneficial for incisional wounds and can be used in conjunction with skin substitutes (Reece, 2001).

2.8.3 Barrier Films

Barrier films are versatile in that they can be applied as a fluid and then polymerize into a film. These are mostly comprised of polyurethane or polylactate and provide protection for the wound from moisture that can cause loosening of the dressing as well as cause new

tissue from stripping. Barrier films are considered non-cytotoxic and provide some pain relief (Hom, 1999).

2.8.4 Foams

Foams are used in some of the less notable current silver models including Contreet®. Polyurethane porous sponges are the key component of this material. The sponges have small openings and allow this material to retain fluid. Foams can be easily compliant with other materials and are considered the most adaptable dressings out of others on the market. The outer layer acts as a barrier to microbes and is beneficial for both partial- and full-thickness wounds and is usually waterproof (Worley, 2005). Adhesive borders are an option for this material or it can be transparent (Kestrel Health Information., 2005). This material is absorbent and can used on many types of wounds. Foams can provide some cushioning, which is beneficial for certain areas of the body such as the back and the buttocks. Finally, this material is available in nearly every shape and size so it can accommodate many wound types (Worley, 2005).

However, some foams may require a secondary dressing and cannot be used on dry wounds, as the wound healing process may become inhibited and the risk of infection may increase. If foams are cut to a desired shape and size, there is an increased chance that the material may lose its barrier properties. For proper adhesion, foams require 0.5 inches to 1 inch overlap onto intact skin. This could potentially be a problem for burn victims who may not have viable skin for this product as well as the fact that most foams have strong adhesive properties and should not be used on weak or damaged skin (Hom, 1999).

2.8.5 Gauze (Impregnated and Non-impregnated)

Gauze dressings are the most widely used dressings in wound care due to their practical cost and wide variety of shapes and sizes. They manage fluid by capillary action and dressings of this nature can be packaged as a roll, strip, ribbon, or pad. These dressings can be separated into woven or non-woven categories and their absorbency depends on thickness as well as composition (Kestrel Health Information., 2005). Usually this material is composed of cotton, rayon, polyester, or cellulose as well as other materials. However, cotton is not used as often as some of the other materials due to the increased cost effectiveness of synthetic fibers. Gauze ranges in use from cleansing, wiping, absorbing, and protecting new skin formation. Gauze has the option of being impregnated with other materials such as saline, hydrogels, and antimicrobial agents. There is also a category of "smart" gauze that is non-adherent to the wound bed (Worley, 2005).

Although gauze is widely used, it does not provide the best wound healing environment even if the material is moist. Oftentimes the dressing dries out and even if it is remoistened, pain and damage can result when the dressing is removed. Gauze also lacks in thermal insulation and requires numerous dressing changes compared to new products on the market. Furthermore, there are advantages and disadvantages to using either woven or non-woven fibers. Woven gauze products are more absorbent than non-woven materials but there is more of a chance for the fibers to migrate into the wound. On the other hand, non-woven fibers have less of a chance of irritating the wound but unfortunately they retain less wound fluid (Worley, 2005).

2.8.6 Hydrogels

Hydrogels come in many forms including amorphous, impregnated, and sheets (Kestrel Health Information., 2005). Amorphous hydrogels lack a concrete shape and are made up of polymers and water as well as other additives including collagen, aloe vera, and alginates. This type of hydrogel does not contain cross-linked fibers unlike some of the other forms (Worley, 2005). The primary role of an amorphous hydrogels is to maintain a moist healing environment for the wound and to rehydrate the wound if it were to become dry. In this form, the hydrogel is beneficial for both partial- and full-thickness wounds. In addition an amorphous hydrogel can be converted into the impregnated form by saturating non-woven sponges as well as gauzes with an amorphous hydrogel (Kestrel Health Information., 2005).

Hydrogel sheets use cross-linking to create three-dimensional networks. They are mostly comprised of water in a bioinert matrix (Worley, 2005) along with hydrophilic polymers, which are not soluble in water but rather swell in the presence of aqueous solutions. Hydrogel sheets are beneficial to patients who suffer from partial- and full-thickness wounds in that the dressing is comfortable, porous, absorptive, and can easily be removed (Kestrel Health Information., 2005).

In general, hydrogels have natural adhesive properties, which allow them be secured with tape alone which makes this type of dressing easy to use by most caregivers. As previously mentioned, hydrogels come in a variety of forms depending on the use and preference and all forms cool and soothe almost immediately when in contact with the wound (Worley, 2005). In a sense, hydrogels can be considered ideal for a wound dressing since they can be used throughout all stages of wound healing because they maintain a moist environment and reduce pain through cooling the wound site upon application (Hom, 1999).

Hydrogels also have some disadvantages in that they cannot be used on wounds that exude heavily or those that are infected as maceration of the skin is a risk especially in the healing skin surrounding the wound (Hom 1999; Worley, 2005). When using an amorphous hydrogel, it should be noted that the dressing can become decreasingly viscous as the gel moves to equilibrium with body temperature (Worley, 2005).

2.8.7 Hydrocolloids

Hydrocolloids first became commercially available in the early 1980s (Worley, 2005) and were much more complex compared to hydrogels due to their increased number of elements including pectin, gelatin, adhesives, polymers, polyisobutylene, carboxymethylcellulose (CMC), and alginate (Hom, 1999; Worley, 2005). They are considered "interactive dressings" because they change in physical state as fluids are slowly absorbed by particle swelling, which results in a soft gel forming on the wound bed that can decrease pain (Hom, 1999; Worley, 2005). It is hypothesized by some experts that the resulting gel contains natural growth factors and also allows for bacterial growth inhibition (Worley, 2005).

There are many advantages to using a hydrocolloid in a wound dressing. Hydrocolloids are available in many shapes and sizes as well as gels, fibers, sheets, powders, and pastes (Worley, 2005) and are therefore beneficial for wounds that are in a unique shape and form (Kestrel Health Information., 2005). Also, they come in a variety of thicknesses which is directly related to the fluid management of the material (Kestrel Health Information., 2005). Some hydrocolloids are manufactured with tapered edges to decrease the chance of rolling, while others can be produced with an adhesive outer dressing that can act as a barrier against bacterium as well as against fluids from the external environment (Worley, 2005).

Hydrocolloids maintain a moist environment, which promotes wound healing and can be used on both acute and chronic wounds (Hom, 1999).

On the other hand, hydrocolloids are not recommended for use on infected wounds and almost always require a secondary dressing (Hom, 1999) even though their adhesives are stronger compared to other dressings (Worley, 2005). Additionally, hydrocolloids initially need to be changed frequently until the exudate levels are low enough so that the dressing can be left on for seven days. Due to the fact that frequent dressing changes are an option, it is also possible that the underlying would bed could be damaged more easily due to the stronger adhesives and increased dressing changes (Worley, 2005). Finally, a healthy wound can be mistaken for an infected wound when using a hydrocolloid since they emit a natural odor, which is comparable to an infected wound odor if the dressing comes in contact with certain proteins in the wound fluid (Worley, 2005). Therefore, it is important to use other means of characterizing a wound as healthy or infected when using a hydrocolloid dressing.

Hydrocolloids are one of the most widely used types of wound dressings today. They have not been known to cause any adverse side effects or allergic reactions and are easy to use. Most only require changing every 3-5 days and they do not disturb the wound bed when removed (Thomas, 1992). Hydrocolloids are therefore advantageous as they cut down on the cost of materials and on nurse care by reduced dressing changes since they successfully heal the wound after a few dressing changes (Kim, 1996). Easy removal allows for a less painful treatment as well as one for a diverse application of severe wound types and burns. One of their early applications that is still used today is to apply a hydrocolloid to a wound bed undergoing maggot therapy. Maggots require oxygen, and also need a barrier to keep them in

the wound and hydrocolloids have been proven effective in providing a healthy environment for both the maggots and the wound (Sherman, 1997).

Hydrocolloids are currently in competition with new dressing formulations known as "hydropolymer" dressings. These new dressings are favorable in the aspects of reduced dressing leakage and reduced odor characteristics as compared with traditional hydrocolloid dressings. However, both dressings were tested in diabetic leg ulcer wound and both were equally effective in wound healing (Thomas, et al, 1997).

2.8.8 Carboxymethylcellulose

A common material used in synthesizing a hydrocolloid is carboxymethylcellulose (CMC) which is prevalent in wound dressings and in food applications. CMC is considered to be water soluble which means it dissolves in water, so in most applications the CMC requires a cross-linking agent to stabilize the gel. A few other additions to CMC have been polyacrylamide gels, chitin, chitosan, copolymers and mixtures of these various cross-linkers (Queen, et al, 1996). On average these cross-linking agents are added between the ranges of 0.01% of weight to 20% of weight.

Sodium Carboxymethylcellulose often requires the addition of a plasticizer to stabilize the gel and make it less brittle, therefore propylene glycol and pectin can be used towards this application. Glycerol can also be substituted in a volume of 7% of the total weight (Barnes et al, 2005). We must keep in mind that the main component of the gel must be water, and it should be at least 70% of the formulation. Many of these gels can be sterilized by wet heat in tubes before they are poured into their casting trays or onto the wound site. These hydrocolloid formulations on average swell 10 grams of liquid per gram of hydrocolloid foam (Qin et al, 1999).

Since hydrocolloids have a wide variety of applications, they must not interfere with the bodys natural functions. For example, some CMC gels are particularly effective in applications to the eye, where this area is usually very sensitive to outside materials (Cini et al, 1995). Therefore, it is important our hydrocolloid has a neutral pH and is not acidic as it is packed into many wound sites (Qin et al, 1999). Oftentimes, human growth factors may also be incorporated into the formulation which allows for more stability in a moist environment, more biocompatibility, and also adheres better to contoured wounds (Cini et al, 1995).

2.8.9 Alginates

Natural polysaccharide fibers derived from seaweed are the primary constituent of alginate dressings. They also contain the primary structure of guluronic (G) and mannuronic (M) acid. G alginates are more secure and therefore rope or ribbon-like. On the other hand, M alginates are in a gel form and are soft. In turn, these dressings are produced as non-woven, non-adhesive pads or ribbons (Hom, 1999).

Alginates are characterized by having biodegradable and highly absorbent properties (Hom, 1999). By using a process called ion exchange, the dressing forms a moist gel when it comes in contact with the wound exudate (Kestrel Health Information., 2005), which minimizes the chance of bacterial contamination and wound discharge (Heenan, 1998). Therefore, the dressing should be dry prior to its application (FMC Corporation, 2005). By chemical ion exchange, alginates not only control the amount of fluid in the wound but there has also been evidence to support a bioactive effect by triggering macrophages, which in turn increase wound healing (Hom, 1999).

In the past few years, alginates have become one increasingly popular as wound dressings as they are manufactured as sheets and cavity dressings (FMC Corporation, 2005),

and they are soft and easy to shape, as well as easily manipulated for non-uniform wounds (Kestrel Health Information., 2005). Alginates can be used on wounds with heavy exudate (Kestrel Health Information., 2005) as well as surgical wounds and have shown to be less likely to encounter a microbial attack compared to other various carbohydrates (FMC Corporation, 2005).

One study set out to compare calcium alginate dressings with conventional gauze dressings in a controlled, randomized setting. Thirty-four patients with abscess cavities were analyzed and the results were quite promising. Not only were the calcium alginate dressings easier to remove than the gauze dressings, but they also decreased the overall pain experienced by the patient (National Prescribing Centre, 1999). In a similar study, ninety-two patients with pressure ulcers were observed to compare calcium alginate dressings to dextranomer paste, which serves as a wound healing paste. The test was conducted over an eight week period and the results showed that significantly more patients with the calcium alginate experienced a decrease in wound area and increased healing rates compared to the paste group (National Prescribing Centre, 1999).

Like all wound components, alginates have their drawbacks. Most alginates must be used in conjunction with a secondary dressing, which can add to the complexity of the overall device (Kestrel Health Information., 2005). In addition, alginate dressings should not be used on dry or infected wounds (Hom, 1999). Also, there are many forms of alginate and each type has its own unique properties making the proper choice of alginate a hard decision to make (FMC Corporation, 2005).

2.9 Material Description

The materials that were described in the previous sections have different advantages and disadvantages when used in wound healing dressings. Each material's evaluation was used later in the design process to determine the best material for each layer of the dressing. Table 3 below describes the advantages and disadvantages of materials that are currently used in various wound healing dressings.

Table 3: Advantages/ Disadvantages of Material

Detailed Description of Materials	Advantages	Disadvantages
Hydrogels (Kestrel Health Information., 2005; Worley, 2005) • SPENCO 2nd skin moist gel pad • Mueller More Skin • 3M Tegaderm Wound filler	• Water based, can activate antimicrobials • Can be impregnated • Natural adhesive • Good for wound hydration • Absorption properties • Easy to use and apply • Cooling and soothing properties • Sterile • Good for partial and full-thickness wounds • Variety of forms	• Not good for heavily exuding wounds • If in the amorphous form, gels decreases in viscosity at body temperature
Hydrocolloids (Kestrel Health Information., 2005; Worley, 2005) • DuoDerm CGF • 3M Tegasorb Hydrocolloid Dressing • CMC	• Drainage control • As it absorbs, matrix forms soft gel over wound • Gel contains natural growth factors • Can include outer layer for bacterial and viral barrier • Available in many forms, shapes, and sizes • Good for moderate amounts of exudate • Some have tapered edges to prevent rolling • Good external barrier properties	• Strong adhesive can damage skin • Natural smell when in contact with certain proteins can be misleading that an infection is present • Not to be used on infected wounds
Foams (Kestrel Health Information., 2005; Worley, 2005)	• Porous • Drain wound while keeping it moist • May have adhesive component	• Cannot be used on dry wounds • Needs 0.5" to 1" of intact skin for adhesion • Barrier properties can

• Polyurethane Foam • 3M Foam Dressing	• Outer layer normally waterproof and is a barrier against microbes • Very adaptable • Can provide cushioning • Good for a variety of wound types • Comes in many shapes and sizes	decrease if cut • Some may have strong adhesives that can damage fragile skin or stick to the wound bed
Alginate (Heenan, 1998; Hom, 1999; National Prescribing Centre, 1999; FMC Corporation, 2005;Kestrel Health Information., 2005) • Calcium Alginate	• Controls amount of fluids • Triggers macrophages • Soft • Easy to shape • Good for wounds that need contouring • Good for surgical and wounds with heavy exudates • Least likely to undergo microbial attack compared to other carbohydrates • Decreases pain • Decreases wound healing time	• Needs secondary dressing • Not to be used on dry or infected wounds • Many types with many properties: not all suitable for wound dressings

3. Approach

Once the background research is completed and evaluated, the design team can concentrate on the specifics of the project. The steps that first must be taken are to define the project hypothesis, assumptions and aims. These definitions identify the project objectives and anticipated outcomes of the design.

3.1 Project Hypothesis

The objective of this project is to develop an antimicrobial wound dressing that will inhibit and kill the growth of wound pathogens using a combination of silver and essential oils. The current wound dressings approved by the Food and Drug Administration (FDA) have various forms of silver incorporated in their design. However, there are no current wound dressings that include a combination of silver and essential oils as the active antimicrobial agents. The current silver dressings are expensive and are limited by their microbial resistance in that they do not inhibit the growth of all wound pathogens.

The hypothesis of the design is that the combination of silver and essential oils will increase the efficacy of the antimicrobial property of the dressing while decreasing the overall cost. The combination is hypothesized to be effective against a broader range of wound pathogens and overall becoming more effective than the current silver wound dressings.

3.2 Project Assumptions

The project hypothesis indicates that the design of a silver and essential oils combination wound dressing will increase the efficacy of the antimicrobial properties and eliminate wound pathogen replication in a shorter time period than the current silver wound dressings. However, some assumptions must first be made:

- Dressing will only be tested against three chosen wound pathogens
- The efficacy of both silver and terpene can be predicted through supporting data from *in vitro* assays
- Silver chloride is insoluble and may form a white precipitate yet provide effective antimicrobial properties
- Silver will be effective in becoming encapsulated in the terpene combination
- Eugenol and Thymol (ET) are an effective terpene combination for inhibiting growth of a broad range of wound pathogens

3.3 Project Aims and Specification

The goal of this project is to develop the described antimicrobial wound dressing which is more effective and less expensive than current available silver wound dressings. The specific aims of this project include:

- To produce a silver and essential oil combination that is effective against a broad range of wound pathogens.
- To increase the wound healing process in infected wounds.
- To eliminate pain and odor from the wound.
- To create a wound dressing using materials that have similar mechanical properties and flexibility of the skin.
- To develop an antimicrobial dressing whose cost is equivalent or less than current silver wound dressings.
- To generate and conduct analysis to assess the hypothesis.

4. Design

Before we began designing the anti-microbial wound dressing, we needed to identify the objectives of each stakeholder involved in the project. The major stakeholders include the designers (students), Eden Research plc (Kerry Walker and Gary Ostroff), the user (doctors, registered nurses and patients), and also insurance companies.

We used a design process to create a revised client statement that would provide a solution to the objectives and constraints identified by the stakeholders. The initial problem statement provided to the design team is listed below:

Design and develop an implantable bandage that inhibits microbial infection.

This statement was vague and the objectives needed to be more concise and clearly defined. The design team revised the statement after discussing with each stakeholder their desires and constraints of the project in order to develop a clear and focused client statement.

To gain a broad understanding of the basis of our objectives, we researched current wound dressing models and evaluated the functions and limitations of each design (Chapter 2). We also gained information from interviews with each of our clients. For each stakeholder, we began by first discussing their desired objectives and functions for the wound dressing. However, we needed to quantify the desires of the stakeholders so we could integrate their weighted goals into our Revised Client Statement.

4.1 Clarification of Design Goals

This section describes the different design techniques we used to develop our Revised Client Statement. This process began with a brainstorming session, which led to the creation

of a Pairwise Comparison Chart completed by each stakeholder. Several steps followed the comparison chart which led us to our Revised Statement.

4.1.1 Brainstorming Functions, Objectives, Constraints

The first task we had to complete was to clearly and qualitatively determine the requirements of the project. After interviews with the stakeholders and current product research, the project team was able to generate the overall project goals. The functions of the device were established along with the objectives, and constraints of the product. The general goals of the project are listed in the table below.

ANTI-MICROBIAL
- Inhibits growth/ kills wound pathogens
- Decreases wound healing time

SAFETY OF DEVICE
- Meets FDA standards
- Does not hinder healing process

COST OF DEVICE
- Costs less than current silver wound dressings

EXPANDABLE
- Can be used with other antibiotics/medicines

ALTERABLE
- Change size depending on wound dimensions

FLEXIBLE
- Similar properties to skin
- Strong in flexure

EASY TO APPLY/REMOVE
- Sterilized and packaged
- Removal does not damage healing tissue

RELIABLE
- Quick response system on contact
- Minimize dressing changes

Figure 5: Overall List of Project Goals

The project goals were differentiated between objectives, functions, and constraints and labeled as such. An indented objectives, functions, and constraints list was created and is illustrated below (Figure 6).

PROJECT OBJECTIVES:

1. Wound dressing should increase wound healing
    a. Should kill/ inhibit microbial activity
2. Wound dressing should be safe for patient
    a. Meets FDA standards
    b. Reliable
        i. Does not interfere with healing process
    c. Durable
3. Wound dressing should be easy to use
    a. Ease of application
    b. Ease of removal
4. Wound dressing should be practical to make
    a. Inexpensive- cost less than current silver wound dressings
    b. Must be able to be sterilized
    c. Simple design

PROJECT FUNCTIONS:

1. Reduce odor
    a. Terpenes
2. Minimize pain
    a. Eugenol, a terpene that has been proven to be a natural pain reliever
3. Long lasting
    a. Limit number of dressing changes
        i. Current wound dressing models used for 7 day time periods
4. Permeability rate
    a. Oxygen required for healing process
5. Provide moisture
    a. Moisture to activate silver delivery

PROJECT CONSTRAINTS:

1. Cost of production
2. Timeline of project
    a. Design and prototype must be completed by April
3. Testing limited to certain wound pathogens
4. Use of certain forms of silver
    a. Silver nitrate light sensitive
    b. Silver nitrate soluble
    c. Silver chloride insoluble

Figure 6: Indented Objectives, Functions, and Constraints List

The indented objectives, functions, and constraints list allowed us to clearly define our goals and categorize them appropriately. We further broke down the constraints and elaborated on their definition and application to our wound dressing as shown below in Figure 7.

CONSTRAINTS

Cost for production
- Must be feasible to produce and manufacture, can be easily scaled up for larger output

Timeline of project
- Project must be completed by April

Does not hinder wound healing
- All components are activated and it does not interfere with the natural wound healing process and tissue regeneration
- Does not induce a secondary immune response

Testing against specific pathogens
- Its efficacy has been proven in wound pathogens and in wound models

Timeline for production
- The dressing must be easy to use and ready for testing of efficacy no later than January
- The dressing should be easy to manufacture

Figure 7: Design Constraints

The functions of the project, which are defined as what the dressing design must accomplish, need to be clearly determined before the design process can begin. In Figure 8, the functions are listed with an explanation. Only after the constraints and functions are fully established can the design process continue.

FUNCTIONS

To act as an antimicrobial

- Kill and inhibit common wound pathogens
- Cut down on infection and length of wound healing process

To provide oxygen to the wound (breathable)

- Oxygen required for healing process
- High moisture-vapor transmission rate (MVTR) (Koch, 2001)
- Provide enough protection to foster wound healing as well as protect the wound from the outside environment

To provide transport of antimicrobials

- Reservoir for antimicrobials
- Provides moisture to activate and transport antimicrobials

To manage fluids

- Provide moisture to the wound to allow for wound healing
- Enough moisture to activate silver
- Remove excess moisture which could decrease wound healing and could result in exudate buildup

To reduce odor

- Mask and decrease the odor of the wound site while acting as an antimicrobial
- Increase patient comfort and security

To relieve pain

- Provide pain relief without prescription medicines which would increase the cost and responsibility for the patient
- Reduce the number of dressing changes
- Avoid adhesives which may disturb surrounding healthy tissue as well as the wound bed making the dressing harder to remove

To be long lasting

- Limit the number of dressing changes
- Structurally sound and components are durable for at least 1 week which is comparable to current models
- Provides sustained release of antimicrobials and active compounds over the duration of usage

Figure 8: Design Functions

4.1.2 Development of Revised Client Statement

The results of the indented objectives, functions, and constraints list gave us a clear understanding of the basic goals of the design project. However, the list did not illustrate which objectives were most important to consider in the design process. Since we have design constraints such as time and cost, we used design tools to establish which objectives were most important to take into account when developing the wound dressing. We created an objectives tree, which is a hierarchical list that branches from the top-level design goal to

sub-objectives. The objectives tree is located in Appendix B. The top-level design goals included increase wound healing time, safety, ease of use, and practical to make.

After the objectives tree had been developed, a Pairwise Comparison Chart was developed for each tier of the objectives tree and used to rank the design objectives. Each stakeholder completed Pairwise Comparison Chart, ranking which of the objectives were most important according to their needs. The Pairwise Comparison Chart (Figure 9) works by comparing each of the objectives against one another to determine the rank of importance. Starting on the left column and working across, rank the one objective versus another objective all the way along the row. The ranking system works as follows: 1 = yes, more important, 0.5 = equally important, 0 = no, less important. For example: Increased wound healing is (more/equal to/less) important than Safety etc.

The Pairwise Comparison Chart was completed by the MQP design team, Professor George Pins, Drs. Kerry Walker and Gary Ostroff from Eden Research plc, and also Lisa Szocik, a Medical Esthetician. This process provided us with varied responses because each stakeholder has different interests and primary goals the wound dressing must address. We also interviewed Dr. Raymond Dunn of UMASS Medical School, Department of Plastic Surgery (see Appendix A) and although we did not ask him to fill out a Pairwise Comparison Chart, his input as a stakeholder contributed to our revised client statement.

PAIRWISE COMPARISON CHART

1st Tier

Goals	Kills/Inhibits Microbes	Reliable	Durable	Easy to apply	Easy to Remove	Cost	**Score**
Kills/Inhibits Microbes	X						
Reliable		X					
Durable			X				
Easy to apply				X			
Easy to remove					X		
Cost						X	

2nd Tier

Goals	Increase wound Healing	Safety	Easy to Use	Practical to Make	**Score**
Increase wound Healing	X				
Safety		X			
Easy to Use			X		
Practical to make				X	

3rd Tier

Goals	Includes terpenes	Flexible	Simple Design	**Score**
Includes terpenes	X			
Flexible		X		
Simple Design			X	

Figure 9: Pairwise Comparison Chart

The results from the Pairwise Comparison Chart were compiled from each stakeholder (Appendix C-H) and were useful tools to determine the different needs and desires of each client. The design team took in to consideration that each stakeholder does not carry the same magnitude when the weighted objectives were established.

Each stakeholder has their own goals and constraints. Therefore, it was important for our group to determine which stakeholders hold the most weight so that the team could concentrate on a more focused project. Through our background research and after careful consideration, Dr. Kerry Walker of Eden Research plc was determined to be the most important stakeholder and given the most weight (35%). This was due to her vast knowledge in wound care as well as the fact that Eden was providing the monetary funding to sponsor this project. Dr. Gary Ostroff was given the next highest weight as he is a pioneer of working with terpenes as antimicrobials. He is also very knowledgeable in wound healing. The MQP team ranked in the middle of the stakeholders as far as weight. The team has had experience with both prototype design techniques as well as with wound care. However, the team lacks extensive knowledge in wound management.

Finally, nurse practitioners, medical personnel (Lisa Szocik) and Professor George Pins were assigned the least amount of weight, respectively. George Pins acted as an outside advisor in this activity and therefore his responses were ranked the lowest. Our dressing is to be created so that it is easy to use for not only medical personnel but also everyday caregivers that may not possess in depth knowledge of the sensitivity of wound care. Therefore, we did not rank stakeholders in this category very high. We did however consult a variety of doctors and medical personnel to better understand the limitations of current products (Appendix A).

Accordingly, a weighted objectives tree was developed, which was based on the responses given in the Pairwise Comparison Charts. The weighted objectives tree is used to illustrate the hierarchical layout of the objectives. Appendix I shows each objective with its two weighted values. The left value is the weight compared to the other objectives on that tier out of 100, while the second number, more importantly gives the relative weight of the objective compared to all of the objectives. Safety and decrease wound closure time were the top objectives based on the second value of the weighted objectives tree. The objectives are ranked based on the critical second value, which is shown in Table 4.

Table 4: Weighted Objectives

OBJECTIVE	WEIGHTED %
Tier 1	
Safety	43.1
Decrease wound closure time	32.8
Easy to use	13.8
Practical to make	10.3
TOTAL	100
Tier 2	
Delivers bioactive agent	16.7
Reliable	15.1
Mechanical strength	14.6
Flexible	13.4
Ease of removal	8.39
Pain reducer	8.04
Odor reducer	8.04
Low cost	6.17
Ease of application	5.41
Simple design	4.13
TOTAL	100

Through the design techniques previously explained, we were able to generate a revised client statement. The revised client statement states:

Design and develop an anti-microbial wound dressing that will effectively inhibit and kill the growth of wound pathogens using a silver based dressing. Screen the anti-microbial effects both in vitro through designing a series of engineering and biochemical assays to evaluate the efficacy of these bandages. In vivo studies will be conducted using animal models to assess the rate of healing in incisional wounds. The dressing should decrease the surface area of the wound indicating healing. The dressing must minimize pain, decrease odor, last at least 7 days to reduce the number of dressing changes as compared to current models. This dressing must not interfere with healing process and the removal of the dressing should not harm surrounding healthy tissue. The dressing must be easy to apply by medical staff and once applied be flexible and durable in that it can withstand and support the normal mechanical strength of the skin. The dressing should have tensile stress and strain values similar to 3.61 MPa and 4.9%, respectively. Finally, this dressing must be produced at a cost less than current silver wound dressings.

4.1.3 Brainstorming of Layers

After the revised client statement was developed, brainstorming of the dressing layers could begin. Literature research provided an understanding of the layers that were required for a wound healing dressing. Based on our knowledge of the current wound dressings and our revised client statement, the layers required were proposed (see Figure 10).

It was determined that the dressing must have a layer closest to the wound that would be biocompatible and absorb the excess exudate from the wound. A transport layer would be used to hold the antimicrobial agents, which also act as pain and odor reducers, and allow for them to actively diffuse into the wound. The second fluid layer would be used to activate the silver components and facilitate the diffusion of agents from the transport layers. Lastly, the dressing would need to have an outer layer to protect the wound from contamination while also allowing the delivery of oxygen to the wound.

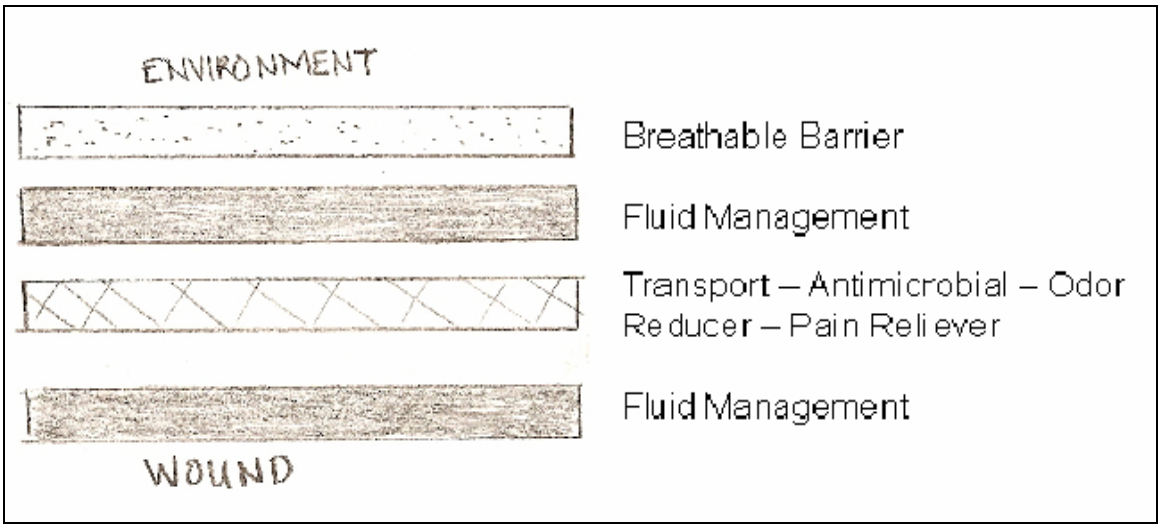


Figure 10: Brainstorm of Dressing Layers

A 3-D image of the basic dressing layers was created using Computer Aided Design (CAD) to give a more detailed look at the structure of the design (see Figure 11).

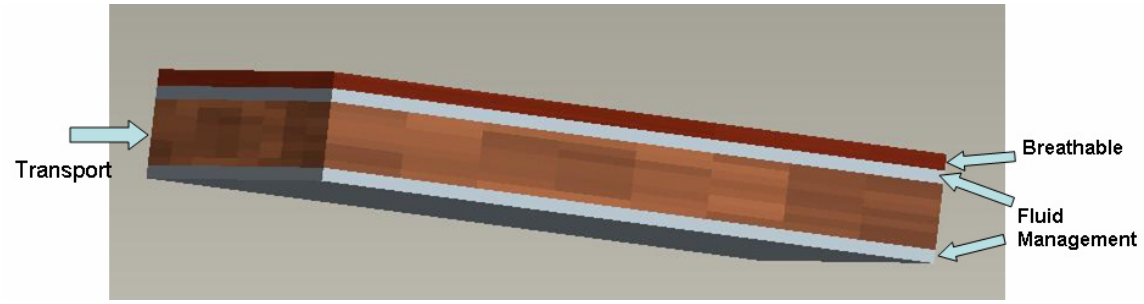


Figure 11: 3-D Drawing of Dressing Layers

4.1.4 Morphological Chart

The morphological chart is an organized chart often used to illustrate various possible methods to accomplish each necessary function (Dym, 2004). During our brainstorming session for possible variations of effective wound healing dressings, we came up with means to accomplish each function as seen below in Table 5.

Table 5: Morphological Chart

FUNCTION	**POSSIBLE MEANS**								
To act as an antimicrobial	Silver	Terpene	Triple antibiotic ointment	Combination of Silver and Terpene					
To provide oxygen to the wound (breathable)	Gauze Outer Layer	Bioclusive	Silk	Medical Tape	Omnifix	Andovers Powerflex	Ace Bandage	Coverlet O.R.	
To provide transport of antimicrobials	Moore Medical Corp.	PU foam	Mesh HDPE	Spenco 2nd skin moist gel pad	Duoderm CGF	Hydrofiber	Mueller More Skin	3M Tegasorb Hydrocolloid Dressing	3M Tegaderm Wound filler
To manage fluids	3M Foam Dressing	Non-woven Nylon	Non-woven Rayon/ polyester	Calcium Alginate	Agar				
To reduce odor	Charcoal	Terpenes							
To relieve pain	Eugenol	Prescribed pain relievers							

We then took each of the possible means and created a detailed list of each of their advantages and disadvantages, which will later help us in determining the best possible means to accomplish our functions (Tables 6 – 11).

Table 6: Evaluation of Antimicrobial Agents

To act as an antimicrobial	**Advantages**	**Disadvantages**
Silver (Demling, 2002; Dowsett, 2004; Strohal, 2005)	• Effective against many microbes found in wounds • Activated in moist environment (i.e. wound site)	• Microbial resistance • Light sensitive • Can turn skin colors • May form a precipitate
Terpene (Ostroff, 2005)	• Antimicrobial properties • Effective against different microbes than silver • Many combinations • Some smell pleasant • Some have pain relief capabilities	• Not effective against all microbes found in wounds • Can be toxic in high concentrations • Hydrophobic if not encapsulated
Triple Antibiotic Ointment (Mayo Clinic, 2005)	• Used often with acute wounds • Effective against surface pathogens	• Cannot be used internally • Makes wound oily • Can hinder wound healing • Effective for surface wounds • Microbial resistance

Combination of Silver and Terpene (Demling, 2002; Dowsett, 2004; Ostroff, 2005; Strohal, 2005)	• Most broad range of antimicrobial activity • May reduce odor • Many provide pain relief • Activated by natural moisture in wound site	• Can potentially be harmful in high concentrations • Silver can turn skin brown/black • Silver is often light sensitive • May form a precipitate • More difficult loading technique

Table 7: Evaluation of Means to Provide Oxygen Transport (breathable)

To provide oxygen to the wound (breathable)	Advantages	Disadvantages
Gauze outer layer (Kestrel Health Information., 2005; Worley, 2005)	• Most widely used dressing in wound care • Good for absorption and protection • Can be impregnated with antimicrobial dressings or hydrogels • Variety of shapes and sizes • "Smart" gauze won't adhere • Least expensive dressing • Commercially available	• Some are cotton based and are not as cost effective and synthetic fibers are better • Does not create best healing environment • Painful to take off if dries out, even if remoistened • Poor thermal insulator • If woven, fibers in wound • If non-woven, less absorbent
Bioclusive (Thomas et al, 1988)	• Transparent • Moisture vapor permeable • Hypoallergenic • Viral barrier	• Semi-strong adhesive • Causes fluid build up • Low breathable properties
Silk (Jin, 2002)	• Good in tension and compression • Beneficial in wound closure • Used for tissue scaffolds • Non-degradable in the short term	• Expensive • In order to achieve beneficial properties must be refined to remove sericin
Medical tape (Karwoski, 2003; Carver, 2005)	• Good for security purposes	• Minimal breathable capabilities • Strong adhesives can disturb wound bed and healthy skin upon removal
Omnifix (MEDCO School First Aid, 2006)	• Breathable • Porous • Not too strong of an adhesive • Cost effective ($12/box) • Absorptive • White – aesthetically pleasing	• Only stretches in one direction • Cotton based (not as durable as compared to other dressings)
Andover's Power Flex (Carver, 2005; Andover Coated Products, 2006)	• Waterproof • Sticks to itself	• Lacks in breathable capabilities

Ace Bandage (O'Meara, 2002; Carver, 2005; Antibody, Inc., 2006)	• Can easily secure dressing to certain body parts (extremities) • Can have rubber component so no metal clips are necessary	• Minimal breathable capabilities • Weak in fluid transport as it hinders fluids from entering and exiting
Coverlet O.R. (Carver, 2005; Worley, 2005)	• Gauze adhesive	• Adhesive may be too strong for fragile skin
Polyurethane Foam (Dowsett, 2004)	• Can be loaded with silver • Allows for silver to be dispersed homogeneously • Foam acts as a protection for the wound bed from harmful contact	• Too porous and may allow for bacterial ingrowth

Table 8: Evaluation of Means of Transporting Antimicrobial Agent

To provide transport of antimicrobials	**Advantages**	**Disadvantages**
Moore Medical Corporation (Hom, 1999; Carver, 2005)	• Non-adherent pad	• Not very durable or long lasting
Polyurethane Foam (Dowsett, 2004)	• Can be loaded with silver • Allows for silver to be dispersed homogeneously • Foam acts as a protection for the wound bed from harmful contact	• Too porous and may allow for bacterial ingrowth
Mesh HDPE (Thomas, 2004; Smith & Nephew, 2005)	• Used in Acticoat™ • Durable • Allows for some absorption • Can be easily loaded with antimicrobials	• More difficult to manufacture • Larger degradation rate
SPENCO 2nd skin moist gel pad (Surgical Materials Testing Lab, 2002).	• 80% water, 4% PEO • Won't stick to skin • Just add water to re-hydrate • Cool – must be stored in a cool place • Soothing • Becomes part of skin	• Only breathable in one direction
DuoDerm CGF (Chakravarthy, 2004)	• Flexible • Hydrocolloid composition • comfortable	• Adhesive skin contact layer
Hydrofiber® (Dowsett, 2004)	• Can be impregnated with silver • Provides moisture to activate silver	• Used in current silver dressings that are not as widely recognized (i.e. Aquacel® Ag)
Mueller More Skin (Carver, 2005; Mueller Sports Medicine, Inc., 2006)	• Hydrogel • Can load particles in pores of gel	• May be difficult to incorporate other layers
3M Tegasorb Hydrocolloid	• Sterile wound dressing	• Hydrocolloid adhesive

Dressing (3M United States, 2005)	• Hypoallergenic • Hydrocolloid adhesive • Clear adhesive film covers and keeps liquids, bacteria, and viruses out • Outer film is breathable • High moisture transmission rate • Can wear from up to 7 days	could damage healthy tissue upon removal
3M Tegaderm Wound filler (3M United States, 2005)	• Amorphous hydrogel • Provides moisture • Sterile • Easy to apply • Good for ulcers and surgical wounds	• Filler needs to be placed in other material • More complex for production

Table 9: Evaluation of Means to Manage Fluid

To manage fluids	Advantages	Disadvantages
3M Foam Dressing (3M United States, 2005)	• Fast wicking • Does not swell • Polyurethane foam • Highly breathable • Barrier to outside pathogens • Dressing can stay in contact and prevents leakage	• Must be used with 3M Tegaderm Transparent Dressing
Non-woven nylon (Thomas, 2002)	• Found in Actisorb Silver 220™ • Absorbs excess fluid	• Can cause an allergic response
Non-woven rayon/polyester (Thomas, 2004; Smith & Nephew, 2005)	• Can catch excess silver flakes • Absorbs excess fluid • Used in Acticoat™ • Non-woven so no fibers in wound site	• Less absorption compared to woven counterpart
Calcium Alginate (Hom, 1999)	• Very absorbable • Biodegradable • Contains seaweed (suggestion of Lisa) • Activates macrophages • Contains constituents of mannuronic acid and guluronic acid	• Secondary dressing necessary • Cannot be used on dried out wounds • Poor choice for infected wounds
Agar	• Porous • Much data if used in agarose form	• Insufficient data • Possibly unsafe • Can induce an immune response

Table 10: Evaluation of Means to Reduce Odor

To reduce odor	Advantages	Disadvantages
Charcoal (Thomas, 2002)	• Decreases odor by adsorbing toxins, amines, and fatty acids	• Turns skin black • Somewhat complex and difficult to work with
Terpenes (Ostroff, 2005)	• Variety of smells • Also have antimicrobial properties	• Some smells may have adverse effect

Table 11: Evaluation of Means to Relieve Pain

To relieve pain	Advantages	Disadvantages
Eugenol (Ostroff, 2005)	• Terpene • Has antimicrobial properties	• Toxic in high doses
Prescribed pain relievers	• Known to decrease pain when used alone	• Could interfere with wound healing

4.2 Preliminary Design

Once we have defined means to accomplish each function, these must then be ranked according to their ability to meet the objectives and constraints. These design alternatives are then ranked according to defined metrics. These metrics were based on quantitative and qualitative measurements of the objectives for the wound dressing. The metrics were used to give each of the possible means a score for which design option was the best at accomplishing most of the objectives. This allows us to see which idea is most favorable, or even narrow down the options to the top three to give us a better area of focus in creating our model prototype design. These metrics allowed us to analyze the overall weighted scores according to the objectives raking from the objectives tree to decide which method is most favorable and in turn create conceptual designs as shown in Figure 12 – 16.

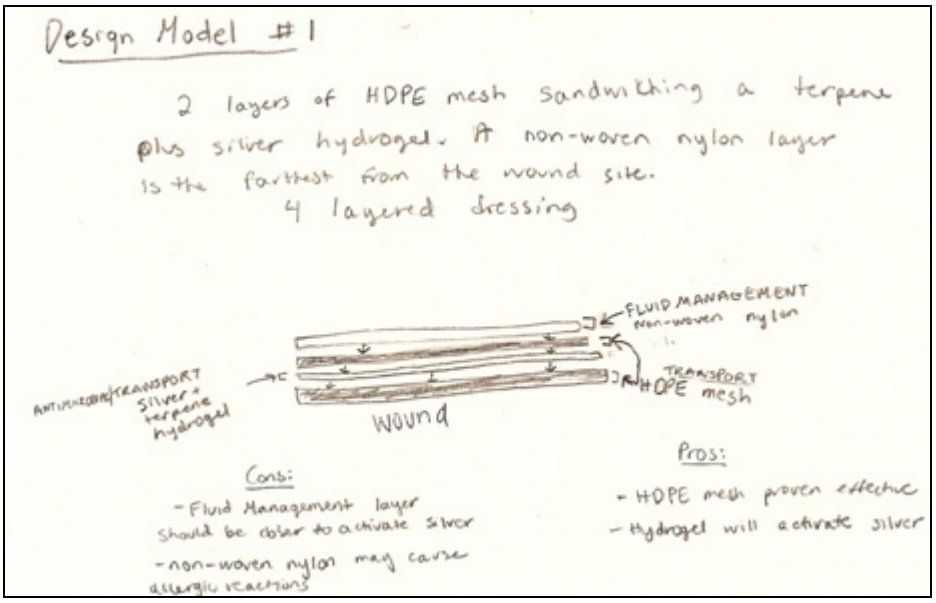


Figure 12: Conceptual Design #1

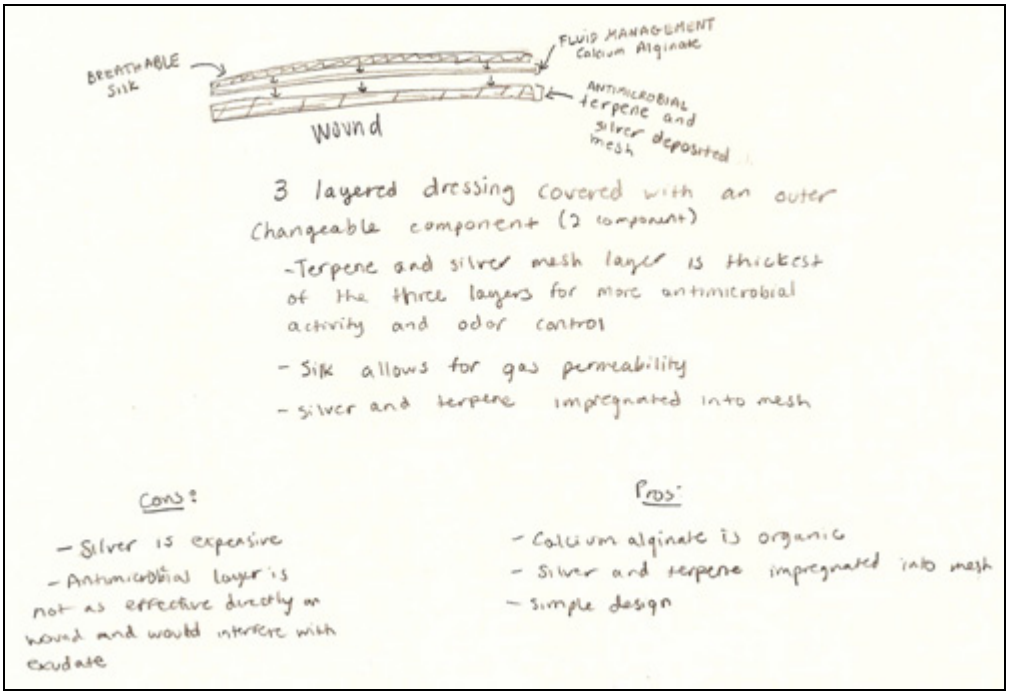


Figure 13: Conceptual Design #2

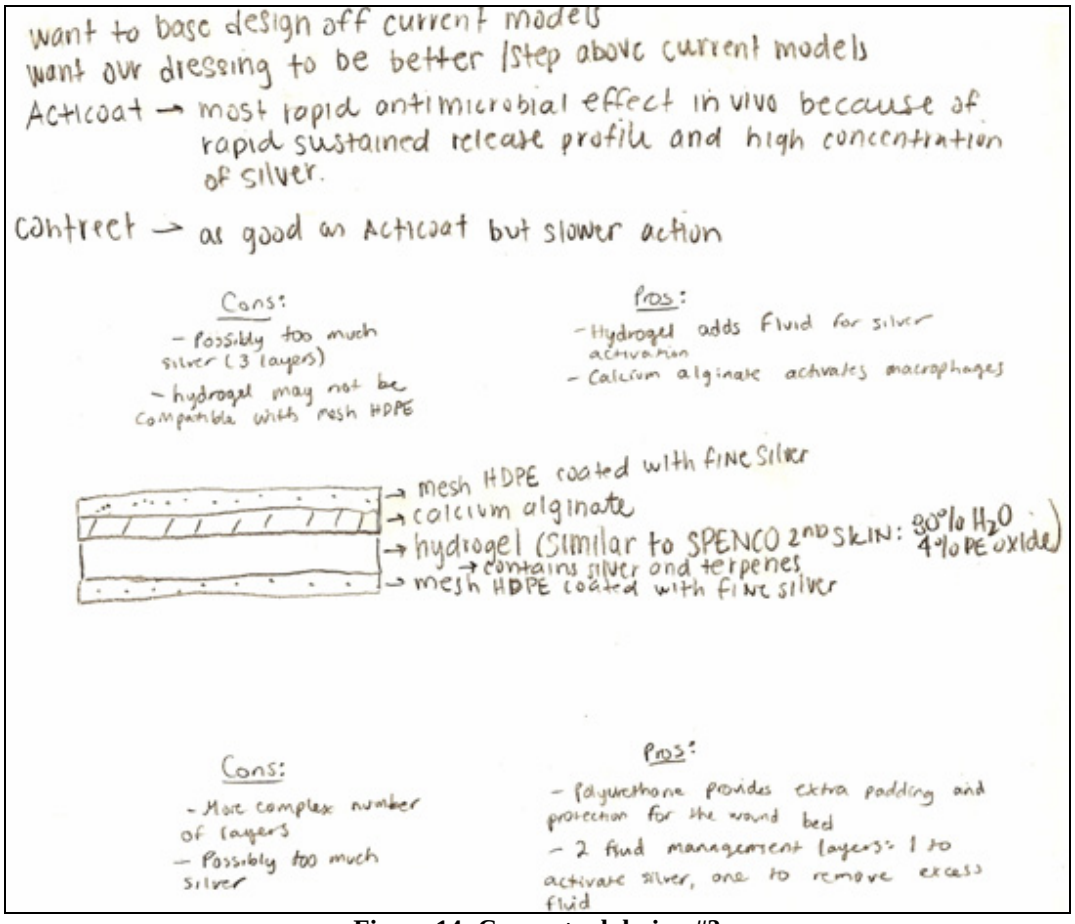


Figure 14: Conceptual design #3

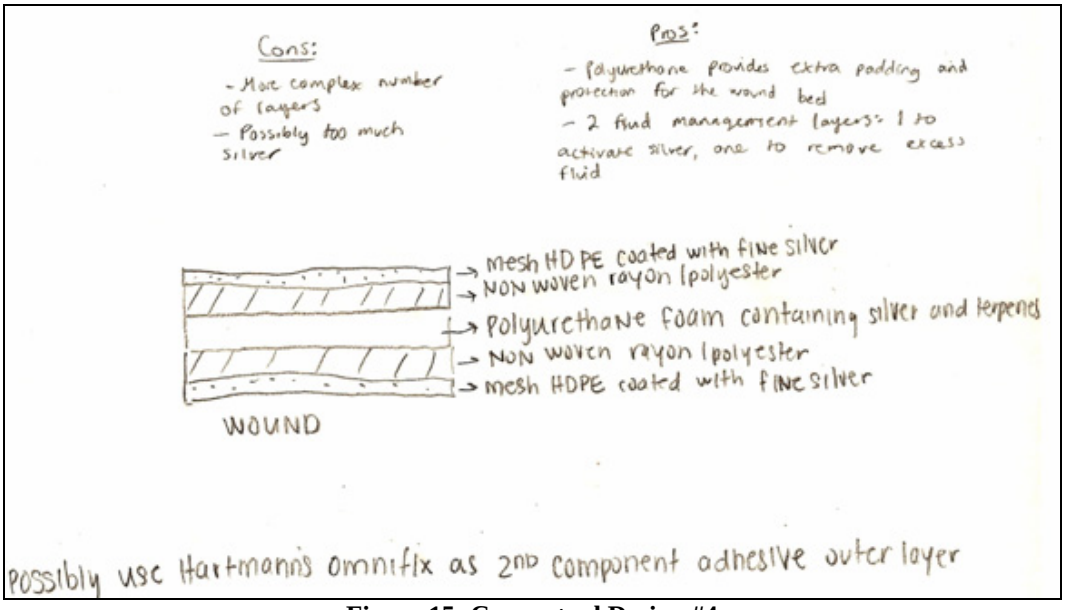


Figure 15: Conceptual Design #4

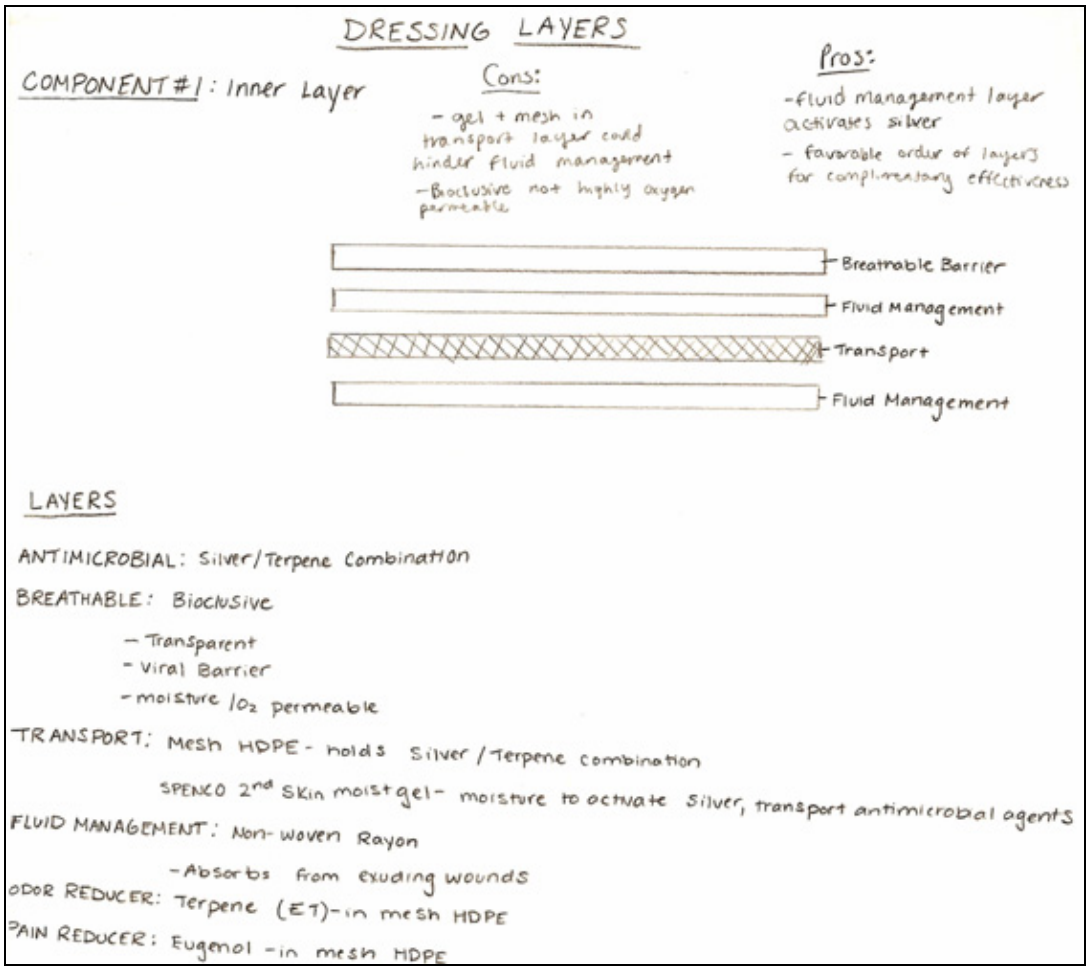


Figure 16: Conceptual Design #5

4.2.1 Metrics

In creating the metrics, we further analyzed and defined the objectives we had identified from the design statement (Appendix J). Metrics were scored on a scale of 1 to 3, 1 being the worst, and 3 being the most favorable. These rankings allow us to determine the most favorable design options by being able to compare each option with their ability to achieve the certain function. Therefore, the best design has the top overall score as it ranks the highest in all areas. For example, if the antimicrobial components of silver and terpene each have a history of providing successful antimicrobial action, then the combination of silver and terpene should provide a more cost efficient bandage than current models using just silver.

Therefore, the combination of actives may be our most favorable option, leading us to further research and consider into our design.

Metrics were defined and justified to decide on a constant method of scoring for each design possibility (Appendix K). For example, a justification for flexible is that the dressing would be ranked according to the fact that it should not constrict any normal motions or movements of the skin. This dressing should exemplify the normal longitudinal stresses, transverse stresses and flexure properties of the skin and if achieved, it will receive a high score of a 3. If these values are unfavorable and below that of the skin, then the score received will be a 1.

4.2.2 Selection Matrices

A selection matrix, also referred to as a decision matrix, was used to rate each possible design alternative against predefined constraints and objectives. These scores are then added to obtain an overall number, the highest overall number being most favorable (Dym, 2004). This matrix determines the option that best meets the objectives set out by the stakeholders. The first step is to evaluate the means and if they satisfy the constraints. If the constraint is met, the box receives a "Y" for "yes" and can then be evaluated further against all of the objectives. If the constraint is not met, then the box receives an "N" for "no" and does not receive any further evaluation as it is no longer evaluated against any objectives. If the method does not apply to the objective to be evaluated against, then the box receives an "X" if it is not applicable. For example, in our evaluation, the aspect of the antimicrobial layer components such as silver and terpene cannot be evaluated against the objective of flexible as their physical properties are not essential to this layer as actives and also would not be able to be tested as they are just constituents of the antimicrobial layer. The score received for

satisfying each objective is based on the metrics, which is then multiplied by the weight of the objectives which has been previously determined by the client and stakeholder evaluation of the Pairwise Comparison Charts. This product of the weights and metrics provides an overall score of the means being evaluated. The design with the highest number suggests that this option is most effective in accomplishing all of the objectives and is a strong candidate for the final design.

All of the objectives were considered in the selection matrix as even though some did not apply to a specific layer, they applied in other layers or other components of the dressing. Therefore, since our objectives tree was split into two tiers, each totaling 100%, and the highest possible score received is a 3, we took the overall tier total of 200 and multiplied it by 3 to yield a total maximum score of 600. Therefore, the score closest to 600 is the most effective method in deciding components of the dressing. The tables below demonstrate the previously described process as well as show the final overall scores of each of the means.

The first layer evaluated is the antimicrobial layer which was scoring each option against the objectives as shown in Table 12. In this category, triple antibiotic ointment was eliminated because it has not been tested against a wide range of wound pathogens nor has it been used in various severe wound healing applications. It is a topical ointment used for small cuts and lesions, but not necessarily for chronic wounds or ulcers which are our target wounds for the dressing. The ointments oils could be detrimental to wound healing (Thomas, 2004).

The other three options were then scored, yielding the most favorable result of the combination of silver and terpene as they have been proven through *in vitro* assays that they are effective in killing and inhibiting growth of most pathogens (Ostroff, 2005). The terpenes kill certain wound pathogens, and the silver also kills certain wound pathogens but is more

resistant to wound microbes, both antimicrobials being different in their breadth of antimicrobial activity. Therefore, where the terpenes couldn't kill, the silver compensates, and vice versa. In addition, the use of two different antimicrobial agents reduces the risk of microbial resistance. Therefore, this option scored the highest and also received the top score out of all of the antimicrobial options. This is a favorable choice in that current wound dressings use only silver to act as an antimicrobial, not a combination of two different agents.

Table 12: Antimicrobial Design Selection Matrix

Design Constraints	**Silver**		**Terpene**		**Triple antibiotic Ointment**		**Combination silver and terpene**			
C: Cost for Production	Y		Y		Y		Y			
C: Time	Y		Y		Y		Y			
C: Doesn't hinder wound Healing	Y		Y		Y		Y			
C: Testing against Specific wound Pathogens	Y		Y		N		Y			
C: Timeline for Production	Y		Y		Y		Y			
Design Objectives (weight %)	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**
O: Decreases Wound closure time (32.8)	2	75.6	2	75.6			3	98.4		
O: Safety (43.1)	2	86.2	2	86.2			2	86.2		
O: Easy to use (13.8)	X		X				X			
O: Practical	2	20.6	2	20.6			2	20.6		

to Make (10.3)										
O: Delivers bioactive agent (16.7)	2	33.4	2	33.4			3	50.1		
O: Reliable (15.1)	2	30.2	2	30.2			2	30.2		
O: Dressing mechanical strength (14.6)	X		X				X			
O: Easy to apply (5.41)	X		X				X			
O: Easy to remove (8.39)	X		X				X			
O: Low Cost (6.17)	1	6.17	3	18.5			2	12.3		
O: Provides pain relief (8.04)	1	8.04	3	24.1			2	16.1		
O: Reduces odor (8.04)	1	8.04	3	24.1			2	16.1		
O: Flexible (13.4)	X		X				X			
O: Simple Design (4.13)	2	8.26	3	12.4			1	4.1		
Total (200*3)		**276.5**		**301.0**				**334.1**		

For the fluid management layer selection matrix (Table 13), agar was eliminated as there was no strong data to prove it had been used in fluid management applications, only in testing the efficacy of antimicrobial agents. This option may not be favorable in that it is poured onto plates to solidify bacterial growth medium and does not manage fluids unless it is heated to liquid form. Therefore, agar does not have the tendency to absorb or provide fluid to the wound environment. Non-woven nylon was also eliminated from the options as it hinders wound healing by often causing an inflammatory or allergic response to many patients who are prone to nylon allergies (Thomas, 2002). Therefore, the most favorable choice was the

calcium alginate although the others were not far behind this option. The calcium alginate was safer than the other methods as it is found in seaweed so like terpene, it is an organic, natural material (Hom, 1999). Calcium alginate is currently a very popular material used in wound dressings because it is very absorptive and can provide some cushioning, which is beneficial to patients with wounds on their back and buttocks (Krestel Health Information, 2005).

Table 13: Fluid Management Design Selection Matrix

Design Constraints	**3M foam dressing (non-adhesive)**		**Non-woven rayon/polyester**		**Calcium Alginate**		**Agar**		**Non-woven Nylon**	
C: Cost for Production	Y		Y		Y		Y		Y	
C: Time	Y		Y		Y		Y		Y	
C: Doesn't hinder wound Healing	Y		Y		Y		N		N	
C: Testing against Specific wound Pathogens	Y		Y		Y		Y		Y	
C: Timeline for Production	Y		Y		Y		Y		Y	
Design Objectives (weight %)	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**
O: Decreases Wound closure time (32.8)	1	32.8	2	75.6	3	98.4				
O: Safety (43.1)	2	86.2	2	86.2	3	129.3				
O: Easy to use (13.8)	3	41.4	2	27.6	1	13.8				
O: Practical to Make (10.3)	2	20.6	2	20.6	2	20.6				

O: Delivers bioactive agent (16.7)	X		X		X					
O: Reliable (15.1)	X		X		X					
O: Dressing mechanical strength (14.6)	2	29.2	2	29.2	2	29.2				
O: Easy to apply (5.41)	X		X		X					
O: Easy to remove (8.39)	X		X		X					
O: Low Cost (6.17)	2	12.3	2	12.3	1	6.17				
O: Provides pain relief (8.04)	X		X		X					
O: Reduces odor (8.04)	X		X		X					
O: Flexible (13.4)	2	26.8	2	26.8	2	26.8				
O: Simple Design (4.13)	X		X		X					
Total (200*3)		**249.3**		**278.3**		**324.3**				

The next functional layer to be determined was to act as an odor reducer (Table 14). Often severely infected wounds release a powerful odor which is often embarrassing for the patient and also shows a sign of delayed wound healing (Dowsett, 2004). The charcoal option is currently used in Johnson and Johnson's model, Actisorb™ Silver 220 as an odor reducer, but has the drawback of impregnating the wound with charcoal pieces after leaving the dressing on for a long period of time (Thomas, 2002). Since terpenes are organic, most of the

formulations have pleasant aromas as many of the essential oils are used in aromatherapy (Szocik, 2005). Not only do terpenes provide a pleasing scent to overpower the infection, they also act as an antimicrobial (Dursan, 2003; Ostroff, 2005), having two properties that are favorable in the design of a wound dressing to inhibit microbial infection.

Table 14: Odor Reducer Design Selection Matrix

Design Constraints	**Charcoal**		**Terpenes**					
C: Cost for Production	Y		Y					
C: Time	Y		Y					
C: Doesn't hinder wound Healing	Y		Y					
C: Testing against Specific wound Pathogens	Y		Y					
C: Timeline for Production	Y		Y					
Design Objectives (weight %)	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**
O: Decreases Wound closure time (32.8)	1	32.8	3	98.4				
O: Safety (43.1)	2	86.2	2	86.2				
O: Easy to use (13.8)	X		X					
O: Practical to Make (10.3)	2	20.6	2	20.6				
O: Delivers bioactive agent (16.7)	1	16.7	2	33.4				

O: Reliable (15.1)	2	30.2	2	30.2				
O: Dressing mechanical strength (14.6)	X		X					
O: Easy to apply (5.41)	X		X					
O: Easy to remove (8.39)	X		X					
O: Low Cost (6.17)	2	12.3	3	18.5				
O: Provides pain relief (8.04)	1	8.04	3	24.1				
O: Reduces odor (8.04)	3	24.1	3	24.1				
O: Flexible (13.4)	X		X					
O: Simple Design (4.13)	2	8.26	3	12.4				
Total (200*3)		**239.2**		**347.9**				

In choosing the component with the most desirable pain reducing properties, we eliminated the original option of acetaminophen (active ingredient in Tylenol) that we had initially considered, as acetaminophen cannot be considered part of the dressing as it is proven most effective in oral medicines (Micromedex, 2006). There has been no previous research completed using acetaminophen in wound dressings. In this decision matrix (Table 15), eugenol, the terpene component of clove oil, was the most favorable result with a score of 311.9 as it is currently used to relieve pain during the teething stage of infant development (Ostroff, 2005). Once again, the eugenol not only provides pain relief to the wound site, but it also provides antimicrobial properties as it kills and inhibits microbes and has the pleasant

aroma of cloves (Ostroff, 2005). Prescribed pain relievers are often used in combination with the wound dressing to help ease pain in the wound. This would cause the dressing to be more complicated if it must be used in combination with pain relievers since the dressing itself does not ease pain.

Table 15: Pain Reducer Design Selection Matrix

Design Constraints	**Prescribed Pain Relievers**		**Eugenol**							
C: Cost for Production	Y		Y							
C: Time	Y		Y							
C: Doesn't hinder wound Healing	Y		Y							
C: Testing against Specific wound Pathogens	Y		Y							
C: Timeline for Production	Y		Y							
Design Objectives (weight %)	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**
O: Decreases Wound closure time (32.8)	1	32.8	3	98.4						
O: Safety (43.1)	3	129.3	2	86.2						
O: Easy to use (13.8)	X		X							
O: Practical to Make (10.3)	X		X							
O: Delivers bioactive agent (16.7)	1	16.7	3	50.1						

O: Reliable (15.1)	3	45.3	2	30.2						
O: Dressing mechanical strength (14.6)	X		X							
O: Easy to apply (5.41)	X		X							
O: Easy to remove (8.39)	X		X							
O: Low Cost (6.17)	1	6.2	3	18.5						
O: Provides pain relief (8.04)	2	16.1	2	16.1						
O: Reduces odor (8.04)	X		X							
O: Flexible (13.4)	X		X							
O: Simple Design (4.13)	2	8.3	3	12.4						
Total (200*3)		**254.7**		**311.9**						

In considering materials for the breathable oxygen permeable layer, the material must allow for oxygen transport in and out of the dressing to allow for proper wound healing (see Tables 16 and 17). Medical tape was immediately eliminated from consideration as it hinders wound healing by being too adhesive to use on a damaged tissue wound site. When removing this tape, healthy or wounded skin could potentially be removed with the tape due to its high adhesive properties (Karwoski, 2003). We also eliminated silk from the decisions as it is a very expensive material so it would produce a costly dressing. The top choice for this

category was Coverlet™ O.R. which is a gauze dressing currently used in hospitals to wrap wound sites. This dressing had a weighted score of 397 as it is favorable consideration in accomplishing all respective objectives.

However, after performing mechanical testing at Instron Corporation in Nowood (Appendix R), Covelet™ O.R. was ruled out as it has an extremely high adhesive strength compared to similar materials. By using the results obtained at Instron, we were able to further evaluate these materials as well as other materials we later decided to investigate to finally choose polyurethane foam as a strong candidate for this functional layer.

Table 16: Breathable Design Selection Matrix

Design Constraints	**Gauze outer Layer**		**Silk**		**Medical Tape**		**Omnifix**			
C: Cost for Production	Y		N		Y		Y			
C: Time	Y		Y		Y		Y			
C: Doesn't hinder wound Healing	Y		Y		N		Y			
C: Testing against Specific wound Pathogens	Y		Y		Y		Y			
C: Timeline for Production	Y		Y		Y		Y			
Design Objectives (weight %)	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**
O: Decreases Wound closure time	1	32.8					2	65.6		

(32.8)										
O: Safety (43.1)	2	86.2					1	43.1		
O: Easy to use (13.8)	3	41.4					2	27.6		
O: Practical to Make (10.3)	X						X			
O: Delivers bioactive agent (16.7)	X						X			
O: Reliable (15.1)	X						X			
O: Dressing mechanical strength (14.6)	2	29.2					1	14.6		
O: Easy to apply (5.41)	2	10.8					3	16.2		
O: Easy to remove (8.39)	3	25.1					2	16.8		
O: Low Cost (6.17)	3	18.5					2	12.3		
O: Provides pain relief (8.04)	X						X			
O: Reduces odor (8.04)	X						X			
O: Flexible (13.4)	3	26.8					2	13.4		
O: Simple Design (4.13)	3	12.4					2	8.3		
Total (200*3)		**283.2**						**217.9**		

Table 17: Breathable Design Selection Matrix Continued

Design Constraints	**Andover's Powerflex**		**Ace Bandage**		**Coverlet O.R.**		**Bioclusive**			
C: Cost for Production	Y		Y		Y		Y			
C: Time	Y		Y		Y		Y			
C: Doesn't hinder wound Healing	Y		N		Y		Y			
C: Testing against Specific wound Pathogens	Y		Y		Y		Y			
C: Timeline for Production	Y		Y		Y		Y			
Design Objectives (weight %)	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**
O: Decreases Wound closure time (32.8)	2	65.6			3	98.4	2	65.6		
O: Safety (43.1)	2	86.2			2	86.2	2	86.2		
O: Easy to use (13.8)	2	27.6			2	27.6	2	27.6		
O: Practical to Make (10.3)	X				X		X			
O: Delivers bioactive agent (16.7)	X				X		X			
O: Reliable (15.1)	X				X		X			
O: Dressing mechanical strength (14.6)	3	43.8			2	29.2	2	29.2		
O: Easy to apply (5.41)	2	10.8			2	10.8	3	16.2		

O: Easy to remove (8.39)	1	8.4			2	16.8	2	16.8		
O: Low Cost (6.17)	2	12.3			2	12.3	2	12.3		
O: Provides pain relief (8.04)	X				X		X			
O: Reduces odor (8.04)	X				X		X			
O: Flexible (13.4)	2	13.4			2	13.4	2	13.4		
O: Simple Design (4.13)	2	8.3			2	8.3	2	8.3		
Total (200*3)		**276.4**				**303.0**		**189.4**		

The final layer and one of the most important layers is the transport layer (see Tables 18 and 19). This layer is responsible for holding the antimicrobial materials and active compounds. We were able to rule out the 3M Tegaderm™ wound filler as it can actually extend the amount of time it takes for the wound to heal, compared to actual dressings and is also harder to manufacture. It acts as a topical ointment to place into a wound bed and was not used in incisional or severe wounds so it was eliminated from our matrix (3M United States, 2005). The 3M Tegasorb™ Hydrocolloid dressing prevailed in the transport category with a score of 379.1 which was followed by the Spenco™ Second Skin hydrogel. Both of these products are currently used in wound healing models, proving their safety and efficacy in allowing normal wound healing (Surgical Materials Testing Lab, 2002).

Table 18: Transport Layer Design Selection Matrix

Design Constraints	**Moore Medical Corp.**		**Mesh HDPE**		**Spenco 2nd Skin**		**Duoderm CGF**		**PU foam**	
C: Cost for Production	Y		Y		Y		Y		Y	
C: Time	Y		Y		Y		Y		Y	
C: Doesn't hinder wound Healing	Y		Y		Y		Y		Y	
C: Testing against Specific wound Pathogens	Y		Y		Y		Y		Y	
C: Timeline for Production	Y		Y		Y		Y		Y	
Design Objectives (weight %)	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**
O: Decreases Wound closure time (32.8)	1	32.8	1	32.8	3	98.4	1	32.8	1	32.8
O: Safety (43.1)	2	86.2	2	86.2	2	86.2	2	86.2	2	86.2
O: Easy to use (13.8)	2	27.6	1	13.8	3	41.4	2	27.6	2	27.6
O: Practical to Make (10.3)	X		X		X		X		X	
O: Delivers bioactive agent (16.7)	X		X		X		X		X	
O: Reliable (15.1)	X		X		X		X		X	
O: Dressing mechanical strength (14.6)	2	29.2	3	43.8	1	14.6	3	43.8	3	43.8
O: Easy to apply (5.41)	1	5.4	2	10.8	3	16.2	2	10.8	1	5.4

O: Easy to remove (8.39)	3	25.2	2	16.8	3	25.2	2	16.8	2	16.8
O: Low Cost (6.17)	2	12.3	2	12.3	3	18.5	3	18.5	2	12.3
O: Provides pain relief (8.04)	X		X		X		X		X	
O: Reduces odor (8.04)	X		X		X		X		X	
O: Flexible (13.4)	2	26.8	2	26.8	3	40.2	3	40.2	2	26.8
O: Simple Design (4.13)	2	8.3	2	8.3	1	4.1	1	4.1	2	8.3
Total (200*3)		**253.8**		**251.6**		**344.8**		**280.8**		**260.0**

Table 19: Transport Layer Continued

Design Constraints	**Hydrofiber**		**Mueller More Skin**		**3M Tegasorb Hydrocolloid Dressing**		**3M Tegaderm Wound Filler**			
C: Cost for Production	Y		Y		Y		Y			
C: Time	Y		Y		Y		Y			
C: Doesn't hinder wound Healing	Y		Y		Y		Y			
C: Testing against Specific wound Pathogens	Y		Y		Y		Y			
C: Timeline for Production	Y		Y		Y		N			
Design Objectives (weight %)	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**		
O: Decreases Wound closure time	2	65.6	1	32.8	3	98.4				

(32.8)										
O: Safety (43.1)	2	86.2	2	86.2	3	129.3				
O: Easy to use (13.8)	2	27.6	2	27.6	2	27.6				
O: Practical to Make (10.3)	X		X		X					
O: Delivers bioactive agent (16.7)	X		X		X					
O: Reliable (15.1)	X		X		X					
O: Dressing mechanical strength (14.6)	2	29.2	2	29.2	3	43.8				
O: Easy to apply (5.41)	2	10.8	2	10.8	2	10.8				
O: Easy to remove (8.39)	2	16.8	2	16.8	1	8.4				
O: Low Cost (6.17)	2	12.3	2	12.3	2	12.3				
O: Provides pain relief (8.04)	X		X		X					
O: Reduces odor (8.04)	X		X		X					
O: Flexible (13.4)	2	26.8	3	40.2	3	40.2				
O: Simple Design (4.13)	2	8.3	2	8.3	2	8.3				
Total (200*3)		**283.6**		**264.2**		**379.1**				

X = does not apply

4.3 Proposed Final Design

With these selection matrices, we are able to choose a final design to consider for each layer. Also, these matrices allow us to choose the second best option if we come across limitations of the top scoring material, or if it ends up being out of the scope of the project cost-wise or production-wise. From the selection matrix, it is suggested that we make a dressing from a combination of silver plus terpene, use a hydrocolloid for transport, and use Coverlet™ O.R. for a breathable gauze layer as shown in Figure 17. However, Instron® mechanical testing allowed the group to rule out Coverlet™ O.R. and as a second choice we considered polyurethane foam as it has a very desirable adhesive strength (Appendix R). The usage of these terpenes also provides an odor reducing option as well as a pain reliever and an antimicrobial. Therefore, the incorporation of these terpenes with silver should set our dressing above the others, accomplishing our design goals.

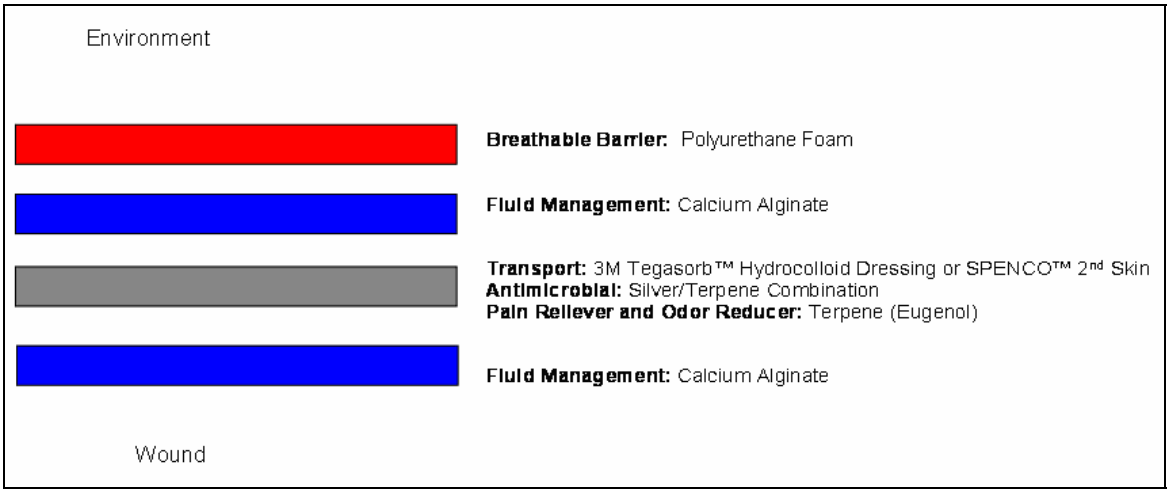


Figure 17: Conceptual Design with Materials

4.4 Modifications

Upon further review, professional input, and quantitative analysis we were able to modify our proposed final design into a dressing that can be easily manufactured. We realized that our proposed design had many layers and was quite complex compared to other current silver wound dressings. Therefore, we were able to reduce the number of functional layers to three instead of our original five, while still complying with our quantitative functions and constraints.

4.4.1 Transport Layer Modifications

We began our studies by choosing carboxymethylcellulose (CMC) and alginate as the main materials in making a hydrocolloid based on the hydrocolloids on the market. With both materials, we produced 0.5%, 1%, 2%, 3% w/w gel formulations by dissolving each material in 45 mL of sterile water (see Appendices U and T, respectively). In this experiment we also added sodium azide to the formulation to act as an antimicrobial in the gel. To one group of both CMC and Alginate gels, we added 7% w/w glycerol, to a second group of both gels we added 6.6 mg/ml amount of terpene to supply antimicrobial properties, and a third group of gels lacking any additives acted as the negative control. For the alginate gels, 10 ml of 1% sodium nitrate was added to allow for cross-linking. These gels were then air dried under a sterile hood to form a film.

Figure 18: Dry Alginate and CMC hydrocolloids,
(top row –alginate control, middle row – alginate + terpene, bottom row – CMC control, left to right are 3%, 2%, 1%, 0.5% formulations by row)

Figure 19: Dry 3% Alginate + Calcium Nitrate –
brittle and curled gel, non-homogeneous

After the hydrocolloid films were made, we ran a water absorption test to measure the amount of swelling and fluid absorption per sample (see Appendix V). This experiment was run in duplicate so two small samples of hydrocolloid were cut from the large gel and placed in a tube with 1 mL water and weighed at different time points. Again, the 0.5% CMC was undesirable as it couldn't be removed from the tray because it was still wet in areas that had

not dried. The remaining CMC samples (1%, 2%, and 3%) were rubbery and stretchy and remained mechanically stable before being put into the water. All of the alginate samples were brittle and the groups without terpene dissolved when submerged in the water. The CMC hydrocolloids showed high yields of water absorption, however, the negative controls dissolved on contact. From this experiment we concluded that the 1%, 2% and 3% CMC formulations were most favorable in the creation of a wound dressing as they absorbed the most fluid and did not dissolve as easily as alginate.

From the water absorption test previously described, we were able to rule out alginate as a component for our antimicrobial layer, and we proceeded with CMC. Since 1% CMC was the lowest concentration that was mechanically stable, we tested 3 different concentrations, 1%, 1.5% and 2%. In order to overcome the brittle properties of the CMC formulations, a plasticizer was added to each formulation. This experiment served to test the efficacy of glycerol plus terpene in CMC. We kept the terpene concentration and the glycerol concentration constant, but this time we cast larger gels in 4 inch by 6 inch plastic containers. We had four different groups per CMC concentration, a CMC + glycerol only group, a CMC + terpene only group, a CMC + glycerol + terpene group, and a CMC only negative control. (see Appendix X).

Once these hydrocolloids were dry, we ran the water absorption test, which demonstrated that CMC began to dissolve in water over time. Overall the 1% CMC + glycerol + terpene had the most consistent results, however, was not much more favorable than the other CMC groups (see Appendix Y). The addition of glycerol proved to overcome the brittle nature of the CMC formulations, but unfortunately the CMC's stability in water did not

improve. From this test we were able to determine we must alter a step in the manufacturing process to create a more stable hydrocolloid in water.

Lyophilization is another method for drying the CMC hydrogel, which can allow for more a homogeneous hydrocolloid and can possibly improve the stability of the formulation. We cast 6 gels into plastic containers and after approximately 1 hour of freezing at -80°C, the frozen gels were placed in a lyophilizer overnight to be freeze-dried (see Appendix BB). For this experiment we used the same concentrations as described above when we air dried them. After freeze-drying, the hydrocolloids became more foamy and firm, much more desirable physical and mechanical properties than those seen by air drying.

After the water absorption of the lyophilized CMC hydrocolloids, we concluded that the CMC alone absorbed the most water by almost 2 fold, as compared with the glycerol and terpene formulation. We concluded that the glycerol amount may be too high as it produced sticky gels and also did not play an effect on the absorption of the formulations (see Appendix Y).

Although the physical properties of the lyophilized CMC hydrocolloids were desirable, the CMC was still not stable in water as observed through running the water absorption test. We hypothesized that the addition of a cross-linking agent would produce a stable CMC formulation. Based on literature, chitosan was first selected to act as a cross-linker stabilizing the matrix, and two gels were cast incorporating chitosan into the formula (Queen, et al, 1996). To one tray 1% w/w chitosan was added to 1% CMC, and to another tray, 0.5% chitosan was added to 2% CMC. The chitosan cross-linked the CMC on impact and therefore made it hard to mix. Also, throughout the gel there were strings of fibers that formed from the reaction between the chitosan and CMC, again causing a non-homogeneous

gel. However, when water absorption was performed, the chitosan containing CMC gels did not dissolve and was more promising than the previous CMC only control gels tested.

Although the chitosan created a more stable CMC hydrocolloid, the cross-linking occurred on impact and the non-homogeneous formulation was undesirable to work with. We next evaluated another thermal gelling hydrocolloid, agarose with CMC formulation to provide act as a stabilizer to reduce CMC dissolving during water contact (Queen, et al. 1996). We added molten agarose in 5 different concentrations (0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5% w/w) to the CMC + glycerol + terpene formulation in a 50ºC water bath to avoid gelling with agarose. Upon gelling and freezing these gels were then lyophilized to again test their stability (Appendix PP).

After the water absorption test, we concluded that 1.5% CMC plus 0.2% or 0.3% w/w agarose were stable and absorbed the most water (Appendix QQ). From this experiment we chose 0.3% w/v agarose as a stabilizer for the CMC formulations.

The initial glycerol concentration as a plasticizer had been chosen from the literature and an optimal concentration in the CMC formulation had not been identified. When the glycerol concentration was 7% w/w, the CMC formulations did not have suitable characteristics because of its sticky nature. Therefore, we tested 4 different glycerol amounts with the previously determined CMC plus agarose formulations, keeping all parameters constant except the glycerol level (Appendix RR). The purpose of this experiment was to identify the glycerol concentration that created a CMC formulation with the most desirable physical properties. CMC plus agarose formulations were prepared with 1.75%, 3.5%, 5.25%, and 7% w/w glycerol and again lyophilized to produce dry hydrocolloid materials.

The water absorption test demonstrated that 1.75% w/w glycerol containing CMC formulations worked best. By calculating the weight of the sample, and knowing the original weight of the gel, we were able to determine the overall percent water absorption. Characterization of other wound dressings from the literature indicated that water absorption should be between 13g-20g of water/wt absorbed by the hydrocolloid (Queen et al. 1996). Water absorption characterization of our dry CMC materials demonstrated that CMC containing 1.75% w/w glycerol absorbed water between these ranges and was also the lowest amount of glycerol tested. This concentration of glycerol resulted in dry CMC materials that were effective in absorbing water and had sufficient flexibility without being sticky (Appendix SS). This experiment allowed us to identify that 1.75% w/w glycerol was the most effective glycerol level, and also demonstrated again that 0.3% agarose stabilizes the dried CMC gel when wetted.

4.4.2 Fluid Management Contact Layer Modifications

We determined the need for a dressing layer between the skin and the CMC active layer for two reasons; 1) to provide a biocompatible skin contact layer and 2) to prevent the actives from migrating into the wound bed and possibly hindering wound healing. Based upon literature research we chose to prepare a calcium alginate film.

Our initial work used alginate cross-linked with calcium nitrate. However, the MSDS of Calcium Nitrate indicates that calcium nitrate is a skin irritant. Therefore, since we did not have success with the alginate and calcium nitrate, and also because the calcium nitrate would have been toxic to the wound, we tried casting the gels again using calcium chloride (see Appendix CC). We identified the amount of calcium chloride necessary to crosslink different

concentrations of alginate. The 0.5% w/w alginate was too soft, and continued to test the 1%, 1.5% or 2% w/w alginate in further formulation experiments. The lowest concentration of calcium chloride to crosslink the alginate gels was 0.25% w/v.

Initially the team set out to discover the best combination of sodium alginate and calcium chloride to produce an effective skin contact and active barrier layer (see Appendix CC). The calcium chloride is necessary to cross-link the sodium alginate to form insoluble calcium alginate. Dry sodium alginate films of 1.5% and 2.0% were produced and soaked in varying amount of calcium chloride for 24 hours to form insoluble calcium alginate gels. After cross-linking, the calcium alginate gels were washed at 20 minute intervals for 1 hour to remove the excess sodium and unreacted calcium chloride, producing calcium alginate gels. These formulation experiments provided the group with very important results: 1.5% alginate provided better mechanical properties than 2.0% alginate, and in order to obtain the best cross-linking the amount of calcium chloride should be between 13.75g – 27.5g of 0.5% w/w calcium chloride per 55g of 1.5% alginate gel.

To ensure the plasticity of the calcium alginate gel it was hypothesized that this layer would benefit from the addition of glycerol. To test this hypothesis, an additional formulation experiment was conducted to finalize the best combination of sodium alginate and calcium chloride, as well as determine the importance of glycerol as a constituent of the layer, and if so, over what concentration range (Appendix DD). The experiment described in Appendix DD allowed the team to conclude the best overall gels included 1.75% and 3.5% glycerol and 0.5% calcium chloride. These results allowed us to determine the final formulation of the alginate layer, composed of 1.5% w/w sodium alginate, 1.75% w/w

glycerol, which coincides with the amount of glycerol in the CMC layer for homogeneity and stability purposes, and 0.5% calcium alginate.

4.4.3 Secondary Dressing Modifications

We were able to use the results from Instron Corporation (Appendix R) as well as the terpene resistance assay described in the Methodology section to choose polyurethane (PU) foam as our outermost breathable barrier. PU foam also has fluid absorptive capabilities (Worley, 2005) therefore we were able to eliminate one of the calcium alginate layers because it's functionality was fluid management and PU foam can act both as a breathable barrier and remove excess exudate when necessary (Worley, 2005).

4.5 The Final Design

Medical grade carboxymethylcellulose (CMC) was chosen as the primary constituent of the active transport layer. This hydrocolloid promotes a moist environment, which allows for better wound healing (Hom, 1999) as well as serving as a matrix to carry and release the actives. The CMC layer also swells when placed on the wound, which is beneficial as it supplies a significant amount of padding for pain relief. A dressing that provides a form of cushioning can be used on nearly any part of the body including the buttocks and back, which are hard to dress areas if there is insufficient padding. Through further research and testing as shown in Appendices RR and PP, a plasticizer (glycerol) and gelling agent (agarose) respectively were also added to this layer to obtain the necessary physical and mechanical properties. (See Figure 20)

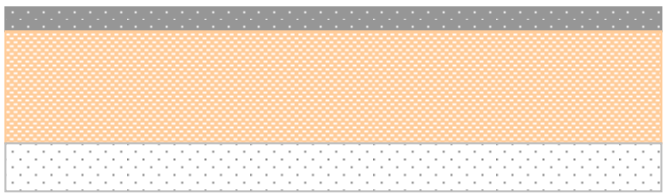


Figure 20: Final Dressing Prototype

The actives contained with in the CMC layer were 6.6 mg/ml YP-ET and 41.8 µg/ml YP-silver chloride. These values were determined by research of current silver wound dressings as well as *in vitro* and *in vivo* antimicrobial experiments. For example, the terpenes had been tested in meat which is closer to an *in vivo* environment, and yielded MIC values of 6,600 ppm (6.6 mg/ml). This was further tested in an *in vivo* animal study and validated this value (see Section 6.6, page 134). The silver was half of the amount of silver dressings currently use on the market (70-100 ppm) so the terpene can compensate for this loss. There was still significant activity of silver at 41.8 ppm as shown through the corrected zone of inhibition assay (see Section 6.9). Additionally, the concentration of YP-silver chloride was chosen based on the team's previously stated objective, which was to decrease the cost of the dressing by reducing the amount of silver. YP-ET also provides pain relief and order reduction, which were two other objectives that the team set out to accomplish.

The third layer of the team's dressing is a contact layer of calcium alginate, which is multifunctional. This layer absorbs fluid and changes physical states to create a soft gel on the wound, which can decrease pain (Hom, 1999; Worley, 2005) as it is easier to remove the dressing without disturbing the underlying wound bed. Additionally, this layer maintains the actives contained in the CMC layer so that a sustained release profile is achieved and the actives do not flood into the wound site. Glycerol was also added to this layer to increase the

homogeneity and stability of the overall dressing as the CMC layer also required the same plasticizer to smooth out the dressing.

The secondary dressing that will be used in conjunction with our primary dressing is the PU Foam covering, which was previously described. Figure 21 demonstrates the final design prototype, which can now be considered for possible animal studies to determine the overall efficacy of the dressing in an *in vivo* setting.

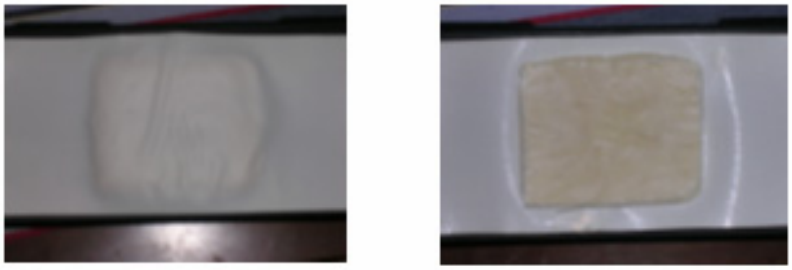


Figure 21: Final Dressing Prototype

5. Methodology

In the methodology section, there will be a detailed discussion of the materials used to construct the wound dressing, how each component was constructed, and how the functionality of the components as well as the efficacy of the entire dressing was determined. Further, we will discuss the animal experimentation including both Phase I and Phase II and the various qualitative and quantitative observations necessary to determine the efficacy of our dressing design.

5.1 Antimicrobial Production

The antimicrobial agents were produced and encapsulated in yeast cell wall particles (YP). This section explains how the silver and terpenes were encapsulated into YP.

5.1.1 Eugenol Thymol (ET)

The terpene formulation of choice was Eugenol and Thymol (ET), 50% of each. A previous study tested 31 combinations of the terpenes: citral, geranial, eugenol, thymol and L-carvone. (Ostroff, 2005) ET was one of the 31 YP encapsulated terpene combinations screened in an *in vitro* assay. Each of the 31 combinations was tested with an initial concentration of 4 mg/ml against ten wound pathogens: *S. aureus*, methicillin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermis*, methycillin-resistant *Staphylococcus epidermis* (MRSE), *E. faecalis*, Vancomycin-Resistant *Enterococcus* (VRE), *P. aeruginosa, Streptococcus pyogenes, Escherichia coli, and Candida Albicans.*

5.1.2 YP- Silver Nitrate

Silver nitrate was purchased and encapsulated into particles following the protocol found in Appendix L. After completing the protocol, an YP- Silver Nitrate powder was formed.

5.1.3 YP- Silver Chloride

YP-Silver chloride was produced following the protocol found in Appendix L. The YP- Silver chloride was also in powder form.

5.2 Antimicrobial Activity

Once the YP encapsulation process of ET terpene, silver nitrate, and silver chloride was completed, the efficacy against wound pathogens needed to be determined. To make sure the materials had effectively been encapsulated, they were tested against three selected wound pathogens; *E. faecalis*, *P. aeruginosa,* and *S. aureus. E. faecalis* was chosen because silver formulations have been shown to be less effective against this species of bacteria. Terpenes are not extremely effective in inhibiting or killing the growth of *P. aeruginosa*. However, terpenes become effective against these pathogens at higher concentrations, such as 2000ppm or higher. Both terpenes and silver formulations are effective against *S. aureus.* Due to time constraints, only three strains of wound pathogens could be tested; however, the three chosen pathogens provide a range to test for antimicrobial efficacy. The hypothesis is that the combination of YP-silver and YP-terpene will be more effective then YP-silver or YP-terpene alone.

A liquid broth 96 well plate-based antimicrobial screening assay was used to determine the Minimal Inhibitory growth Concentration (MIC) activity of YP- Silver nitrate,

YP-silver chloride, and YP-ET. The purpose of this assay was to determine if the silver formulations and terpene were active, which would be verified by whether the antimicrobial agent inhibited the growth of the three pathogens. The inhibition of the pathogens was determined from optical density readings by comparing the optical density of the plate after 24 hours of microbial growth to the original reading at the start. The minimum inhibition concentration (MIC) was defined as the lowest concentration of active that inhibited optical density greater than or equal to 75 percent of average of the positive, bacteria only, control wells. The inhibition due to the three selected antimicrobials was further observed through the use of a metabolic indicator dye, resazarin. The purple dye changes to pink in the presence of metabolic activity, which visually shows the wells with inhibited bacterial growth (see Appendix M).

5.3 Synergy Experiments

A synergy assay was run in a 96 well plate to test for synergy between different concentrations of YP- silver nitrate and YP-ET and YP-silver chloride and YP-ET against *E. faecalis*, *P. aeruginosa,* and *S. aureus* (Appendices N – P). This experiment was run to test the hypothesis that the combination of silver and terpene would be more effective than either antimicrobial agent alone. If the silver concentration required to effectively inhibit the growth of *E. faecalis* was lowered by the addition of inexpensive YP-terpenes, it would support the goal of reducing the amount of silver required for antimicrobial activity. This would in turn reduce not only the cost of the actives, but also reduce the final cost of the antimicrobial dressing.

To test this hypothesis, silver and terpene were diluted serially in microtiter plates to determine a concentration at which both antimicrobials were effectively killing alone and in

combination. Although this experiment was repeated for all three wound pathogens, the experiment with *E. faecalis* will be described as a representative example. The MIC's for YP-silver and YP-ET alone from the primary screening MIC assays were used as the basis for the MIC synergy assay. In order to ensure that the actives will be above the MIC, a start concentration of higher than the MIC is selected. The MICs from the primary assay against *E. faecalis* were that YP-ET had an MIC of 500 ppm, YP-Silver Nitrate had an MIC of 250 ppm, and YP- silver chloride had an MIC of greater than 250 ppm. In the synergy assay, the concentration of the initial solution added to the first well for both YP-silver and YP-terpene must be made at a concentration of four times the desired initial concentration because of the duplicate serial dilutions performed during the assay and to account for the volume of bacteria added to inoculate the microplate.

For both YP- silver nitrate and YP-silver chloride the first well was designed to contain a concentration of 1000ppm, so an initial concentration of 4000 ppm was produced and 100 µl was added to the first well of Row A. For each bacterium, plates 1 and 2 contained YP-silver nitrate and plates 3 and 4 tested YP-silver chloride. The silver was serially diluted across Row A, but no active was added to the last column. Next YP-ET was added at a start concentration of 4000 ppm to achieve the intended 1000ppm. The terpene was diluted serially down the first column, but no active was added to the last row. *E. faecalis* was added to every well and the plates were incubated overnight. This assay was repeated for *P. aeruginosa,* and *S. aureus*, however, the concentration of silver and terpene varied depending on the primary assay results. Additionally, this synergy assay was repeated using a combination of bacteria, *E. faecalis* and *P. aeruginosa* mixed together, to test the

hypothesis that the combination of antimicrobials is more effective than each active alone against a broad range of mixed pathogens.

5.4 Terpene Resistance

Since terpenes can dissolve some materials, such as polystyrene, the materials that were considered for the layers of the dressing had to be tested for their resistance to terpenes. A simple protocol using the free and encapsulated terpene ET was developed to test four materials; 3M Tegasorb™, Coverlet O.R., calcium alginate and Polyurethane foam. The protocol can be found in Appendix Q.

5.5 In Vivo Vehicle Formulation

For the *in vivo* experiment, the vehicle for the actives was chosen in a simple particle settling experiment, which showed that CMC remained homogeneous at 24 hours. Therefore, we had to decide on the concentrations of actives to be tested. As previously mentioned, based on a meat experiment for food borne pathogen applications, meat was inoculated with bacteria and then terpenes were added to the meat and the residual media was serially diluted in a 96-well plate. This assay yielded an MIC terpene concentration of 6.6 mg/ml, which is 6 times the normal MIC. Therefore, we decided this would be our middle value and should chose significantly higher and lower values than this benchmark. We increased this concentration by almost 4 times to 24 mg/ml to act as our high value. This value is 24 times the normal MIC. Then we chose a value that is significantly lower than 6.6 mg/ml so we chose 1.5 mg/ml, slightly above the normal MIC. Therefore, these choices made up the medium, high and low concentrations of terpene, respectively. This experiment will test which concentration is most

effective in an *in vivo* environment. These formulations were then made up by adding the terpene to 0.75% CMC vehicle to act as the animal experiment formulations.

To test the different vehicles, we referred back to the antimicrobial dressing layer and choice of CMC or alginate. Our next step was test the concentration of the both options and their settling properties in a particle settling experiment to determine which could be used in the *in vivo* experiment, and also to test the ability to homogenize if left to rest for a 24 hour time point (see Appendix W). Different concentration vehicles were made by diluting 1% CMC and also diluting 1% alginate into 1%, 0.75%, 0.6%, 0.45%, 0.3%, 0.15%, 0.1%, 0% formulations, with a uniform amount of commercial grade terpene (YP-ET) per tube. All tubes were vortexed and left on the bench top overnight.

Since the YP-terpene particles had not settled in formulations containing 0.45% and higher CMC in the particle settling experiment, we chose to move forward with CMC as the vehicle for the animal experiment. We made up more 0.45%, 0.6%, 0.75% and 1% CMC and using a 3 mL syringe, created a ribbon of vehicle onto an orange peel. An orange peel can be thought of to have similar properties to that of skin, so we chose to use it as a surface to place the ribbon. From this test we determined that the 0.75% CMC was most desirable for our animal study as YP-terpene particles did not settle at 36 hours and also stayed put on the skin once the ribbon left the syringe. The 1% also stayed put on the skin, however, the terpene was not mixed very well in the syringe due to its high viscosity. A drop of the formulations was placed on a paper towel and their spreading was measured after 10 minutes. The 1% and 0.75% CMC had moved the least. (Appendix Z).

Since we were able to determine that 0.75% CMC remained most homogeneous, it was mixed with 3 different concentrations of YP- terpene to be used for the animal

experiment (see Appendix EE). Here these formulations contained a high (24 mg/ml), medium (6.6 mg/ml) and low dose (1.5 mg/ml) of YP-terpenes to be tested *in vitro* for activity before beginning the animal study. Once they have been proven for antimicrobial activity, we can proceed with applying the formulations onto the wound of the animal.

5.5.1 *In Vivo* Formulation Antimicrobial Activity

We had to prove that the actives maintained their antimicrobial effectiveness in the *in vivo* vehicle formulation composed of 0.75% CMC and the three concentrations of YP-ET described in section 5.5. YP-silver chloride was not tested i*n vivo* because the antimicrobial properties of silver are known and it is widely used in current silver dressings. The main concern of our *in vivo* testing was to assess YP-terpenes antimicrobial properties and in an infected wound model.

The measurement of antimicrobial activity of the YP-ET formulated in the *in vivo* vehicle was carried out using the *in vitro* MIC assay against *S. aureus*. This assay was conducted before the animal study to ensure that the terpenes in the vehicle would be active during the *in vivo* study and was also repeated after the study to prove the samples were still active at the end of the *in vivo* study (Appendix TT).

5.6 In Vivo Testing

Once we had formulated the actives in vehicle to be used *in vivo*, we then based our experiment off of Dr. Hamblins past protocol using *P. aeruginosa* and other non-published experiments (Hamblin et al, 2003). Many of Dr. Hamblin's studies had been performed *in vitro* first and then proceeded to be tested *in vivo*. As mentioned, the first phase of the experiment is to genetically alter the bacteria (*Staphylococcus aureus*) with a *lux* fluorescent

gene, culturing it, and plating it in the dark overnight, taking an optical density reading and then testing it for bioluminescence (Demidova, 2005). Some of this bacterial stock was set aside for the *in vivo* study.

The backs of male BALB/c mice were shaved using Nair hair removal and marked using colored markers. This mouse species was chosen due to prior research, and due to their size and anatomy. Mice were anesthetized by intraperitoneal injection of ketamine-xylazine cocktail (90 mg/kg ketamine, 10 mg/kg xylazine) for surgery and also for bioluminescent imaging. A full thickness wound was created on the skin by tracing a box onto the mouse's back, and then cutting the first layer of skin using scissors and a forceps. There were no signs of bleeding in any of the wounds once they had been created and mice were euthanized when their conditions had reached sepsis or extremely visible agitation and infection of the wound site (Hamblin et al, 2003). Bioluminescent *S. aureus* was inoculated into the wound at $5*10^6$ CFU in 50 µl and then imaged for bioluminescence after 20 minutes. At this time point, active antimicrobial was topically applied to the wound at 0.1 ml per wound. This was spread into a thin layer by hand. The YP-terpene treatment groups consisted of three previously formulated concentrations of YP-terpene, high (24 mg/ml), medium (6.6 mg/ml) and low (1.5 mg/ml) groups (n=4). In the control groups (n=2), two mice received a vehicle only treatment, another two mice received no treatment, only an infected wound, and lastly, one of the control mice received a wound only, no infection (Figure 22). After 20 minutes, the mouse was imaged again with the bioluminescent camera and black and white and the color images were superimposed to gain an idea of the active effectiveness. The active application or vehicle only application was repeated daily in respective groups after bioluminescent images were

taken daily. During this whole experiment, mice were monitored for signs of sepsis or severe irritation or aggravated behavior.

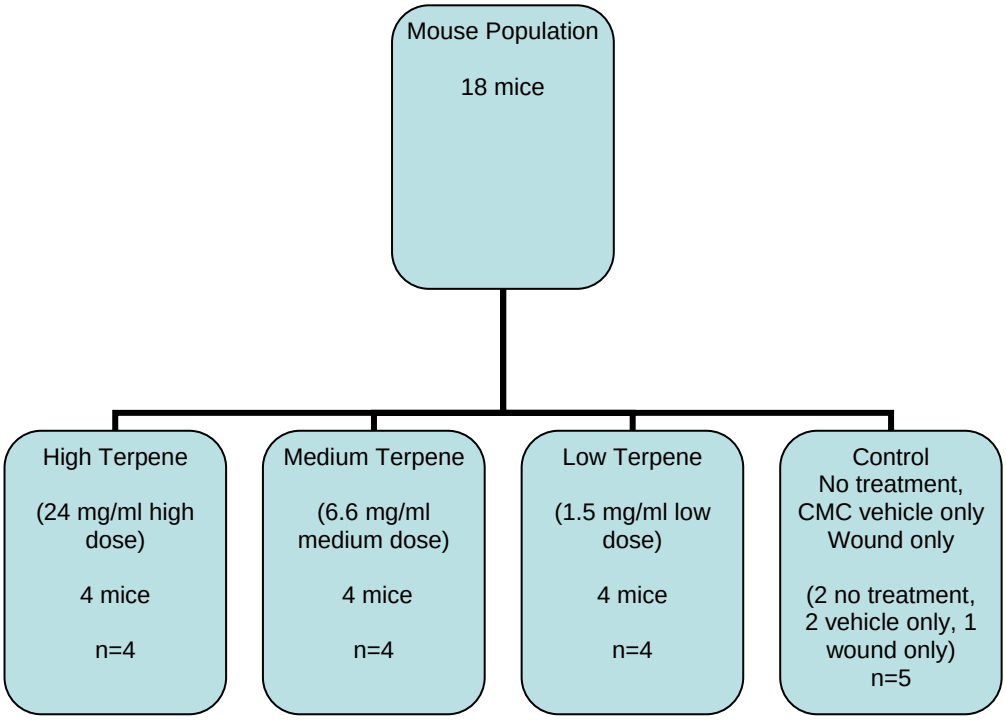


Figure 22: Distribution of animals for the *in vivo* experiment

After Time 0, the protocol was simplified to first take a light image using a still-life digital camera. Next the mouse is placed on a stand in the ARGUS camera setup and a black and white image is recorded. (Figure 23)

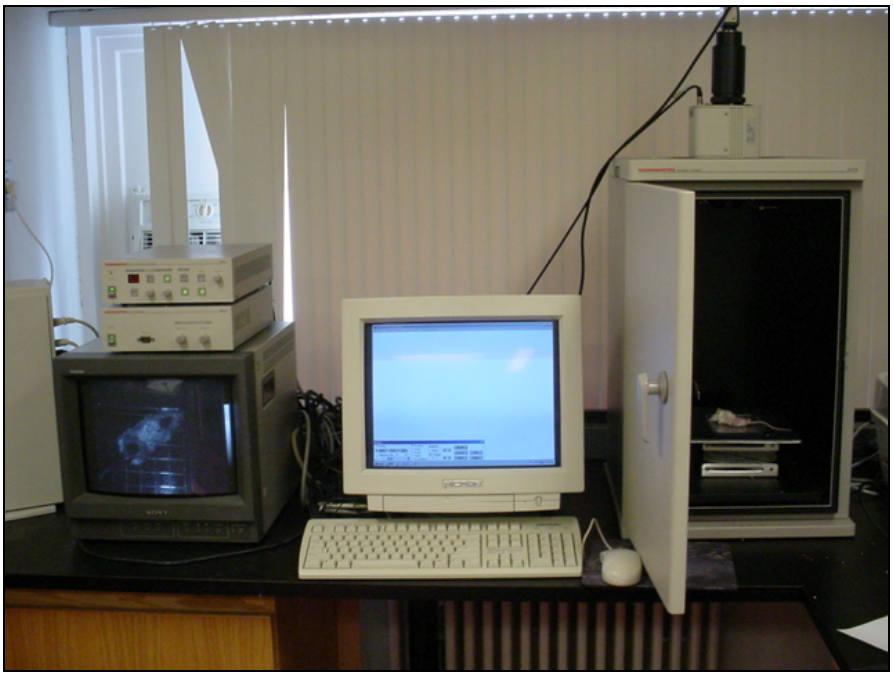


Figure 23: Bioluminescent ARGUS camera setup. Left is the visual screen, imager and intensity controls, center is the computer for ARGUS commands, and to the right is the camera stand light-tight box.

Here the black and white image is read with the ARGUS software with the luminescence on, which freezes a black and white image of the mouse on the stand. Next the mouse must be imaged for fluorescence. The memory must be switched to memory two, and pick the photon counting option under the Imaging drop down menu. A color image of the mouse will be taken by counting the amount of bioluminescent photons in each area within the range of the camera. This will show up as a rainbow of colors around the wound site. The overall analysis is assessed with the superimpose option, which superimposes the color image on top of the black and white image. This can then be further assessed by using the area analysis option, and drawing a selected area to measure the bioluminescent intensity. This value is dependent on the bit size of the image. By multiplying the intensity gained at a

certain bit range, the numbers are standardized through a series of factors which are multiplied by the intensity to yield a series of intensities consistent to each other.

Table 20: Ranges of bit sizes of bioluminescent images and their respective multiplicative factor

Bit range	3	4	5	6	7	8	9	10	11	12	13	14
Multiplicative Factor	1	2	4	8	16	32	64	128	256	512	1024	2048

After seven days of repetitive treatment and imaging, the treatment was stopped, and animals were to be assessed at day ten of the study. After 10 days, the animals were euthanized using a ten-fold increase in anesthetic and animals were properly disposed of in a -60ºC freezer, as indicated by the Animal Care Facility.

5.7 Materials of Construction

Various materials were considered and were tested *in vitro* for use in an antimicrobial wound dressing. As previous described (Chapter 4) many of the materials that the team first looked at had limitations that including cost, manufacturability, and functionality. Therefore, after careful consideration through research and *in vitro* assays the final materials of the antimicrobial wound dressing include:

Secondary Layer

- Polyurethane Adhesive Foam

Active Layer

- 1.5% Medical Grade Carboxymethylcellulose (CMC)
- 0.3% Agarose
- 1.75% Glycerol

- 6.6 mg/ml YP-ET
- 41.8 µg/ml YP-Silver Chloride

Contact Layer

- 1.5% Sodium Alginate
- 1.75% Glycerol
- 0.5% Calcium Chloride

5.8 Dressing Assembly

The assembly of the team's antimicrobial wound dressing prototype is quite simple. The dressing can be manufactured in bulk in nearly any standard chemical or biological laboratory using inexpensive, readily available materials.

The calcium alginate layer is the first to be constructed by creating a dry sodium alginate-glycerol film, which is then soaked in calcium chloride for 24 hours and washed with water to remove the excess sodium and unreacted calcium chloride. After washing, the calcium alginate film is cut to size and placed in a freezer (See Appendix JJ) to create a stable base on which the active layer will be poured on top of.

Next, the active layer is constructed (See Appendix KK) using the materials listed in Section 5.7. A 50°C water bath is used to warm all of the materials for this layer as agarose begins to solidify at temperatures below 40°C. After the materials have reached temperature equilibrium in the bath, 1.5% w/w CMC, 1.75% w/w glycerol, 6.6mg/ml YP-ET, and 41.8µg/ml YP-silver chloride are respectively added to a 100ml glass bottle. After the addition of all of the materials, the bottle must mixed to obtain a homogeneous mixture. Then, agarose is added to achieve a 0.3% w/w final concentration to the bottle and vortexed to mix all of the ingredients once again. The sealed bottle containing the CMC active formulation is kept in the heated water bath until use.

To assemble the dressing, the CMC, glycerol, YP-ET, YP-silver chloride, and agarose mixture is poured over the frozen alginate film to form the two layer dressing. The tray containing the two layer formulation is frozen at -80°C freezer for approximately 1 hour and lyophilized (freeze-dry) for at least 24 hours as described in Appendix KK. After 24 hours, the dry two layer dressing is removed from the lyophilizer, and the polyurethane foam adhesive secondary dressing is firmly attached so that the excess adhesive overlaps the dressing evenly. The dressing can applied easily to a wound and remain in place for at least 7 days.

5.8.1 Lyophilizing (Freeze-Drying)

In order to obtain the material properties that were previously specified, the team sought to lyophilize or freeze-dry the mixture for more desirable properties (see Table 21). The overall process of freeze-drying takes approximately 24 hours. The desired mixture is placed into the lyophilizer at -40 °C. As the amount of water in the mixture decreases due to a drop in chamber pressure, a continuous interpenetrating network is formed, which has improved thermo-stability meaning the hydrocolloid or gel is less soluble and won't fall apart when in contact with the wound bed (Koken, 2005).

Table 21: Evaluation of Drying Techniques

	Advantages	Disadvantages
Lyophilizer	- more homogeneous - takes 24 hrs to cycle - produces foamy material - can control porosity by choosing different temperatures for the pre-freezing process	- Max limit to number of trays lyophilizer can hold - gel must be frozen before entering the lyophilizer
Conventional Air Drying	- large amount of gels can be dried at once	- takes at least 48 hours to completely dry - non-homogeneous drying - resultant gel is brittle without addition of plasticizer

5.9 Primary Dressing Characterization

Once the primary dressing, consisting of the contact and active layers, was assembled, tests were conducted to determine its effectiveness. The dressing had to be tested to determine if it was an effective barrier against contamination and could inhibit the growth of wound pathogens.

5.9.1 Antimicrobial Barrier Assay

The antimicrobial barrier assay is used often in research to validate the effectiveness of a wound dressing. It has been used by groups such as Holder et al, 2003 to determine if a prototype dressing is an effective antimicrobial layer, by inhibiting microbes on the surface of the dressing to enter the wound bed and increase the chance of bacterial infection from an outside source. A punch biopsy was used to create discs of the prototype dressings as well as sterile filter paper (negative control). Five disc specimens from each sample were placed on a separate Mueller-Hinton agar plate as shown in Figure 24. A drop of *S. aureus,* which was calibrated using a 0.5 McFarland Standard, was placed on the top of each disc as shown in Appendix MM. The plates were placed in an incubator and discs were removed at 1, 2, 4, 8, and 24 hours, respectively to look for bacterial growth under the dressing.

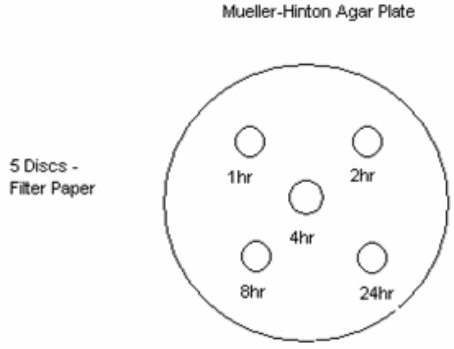


Figure 24: Antimicrobial Barrier Assay Plate Set-up

5.9.2 Disc Diffusion Assay

In order to determine the optimal concentration of YP-ET for the prototype dressing as well as if the active contained within the CMC layer truly does diffuse out of the dressing and into the wound bed, a disc diffusion assay was conducted (Holder et al, 2003). A disc diffusion assay was also performed to determine the final concentration of YP-silver chloride needed in the dressing in the same manner used to assess the YP-terpene value (Appendix LL). *P. aeruginosa* and *S. aureus* were grown on Mueller-Hinton agar plates for 24 hours. The plates were then visually sectioned off into four areas and a disc created using a punch biopsy of either dressing or sterile filter paper was added to each section as shown in Figure 25.

The plates were then incubated for 24 hours as described by the protocol in Appendix LL. Upon removal from the incubator, the radius of clearing was measured for each disc. Any clear zones of 1mm or greater indicate that the active released from the dressing and diffused onto the plate. Additionally, clear zones would conclude that the concentration of active was high enough to kill and inhibit the growth of microbes.

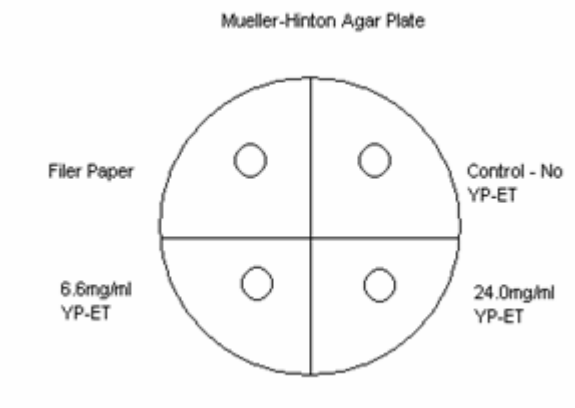


Figure 25: Disc Diffusion Assay Agar Plate Set-up

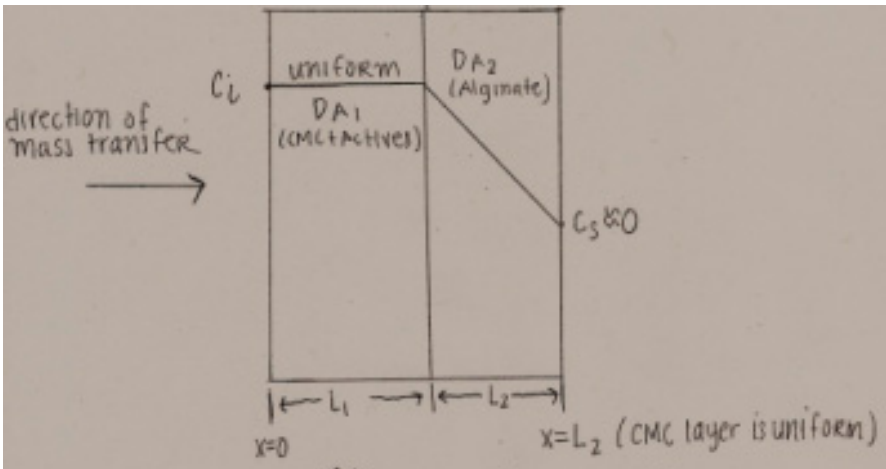
5.9.3 Corrected Zone of Inhibition

This assay will be used to determine the duration of antimicrobial efficacy of the dressing. The dressing will be cut to an appropriate size and placed on a lawn of bacteria, such as *P. aeruginosa* or *S. aureus*, grown on a Petri dish. The dishes will be incubated for 24 hours and the zone of inhibition measured. The piece of dressing will then be applied to a new lawn of the same bacteria, incubated again for 24 hours, and the zone of inhibition measure as shown in Appendix NN. This process will continue for 7 days or until the dressing no longer produces an antimicrobial effect.

5.9.4 Transport Calculations

In order to determine the transport properties of our prototype we modeled our dressing as diffusion through a 2-phase medium (Figure 26). We also had to make a variety of mass transfer assumptions including that the reaction:

- Is in an unsteady-state
- No accumulation of product occurs
- No convective force is present
- The diffusion of water controls the release of the actives (YP-silver chloride and YP-ET)
- Diffusion in other directions besides the one specified are negligible
- The diffusion across the first phase (CMC layer) is constant and uniform as it contains the actives
- No bulk flow
- No chemical reaction



- $\frac{\partial C_A}{\partial t} + u\frac{\partial C_A}{\partial x}^{\nearrow 0} = D\frac{\partial^2 C_A}{\partial x^2} + D_A^{\nearrow 0}$

  No bulk flow; No reaction

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2}$$

$$\left.\frac{\partial C}{\partial x}\right|_{x=0,\,t} = 0 \text{ (symmetry)}$$

$C(x=L, t) = C_s$ (surface concentration is specified)

Initial conditions $c(x, t=0) = C_i$

where C_i = initial concentration and
C_s = concentration at the 2 slab surfaces at time > 0.

- How the concentration of actives changes with time:

$$\frac{C - C_s}{C_i - C_s} = \frac{4}{\pi}\cos\frac{\pi x}{2L_2}\, e^{-D_{A_2}\left(\frac{\pi}{2L_2}\right)^2 t}$$

where c = concentration of actives after time, t
$C_s = 0$
C_i = initial concentration of YP-ET or YP-Silver Chloride
$x = L_2$ = thickness of alginate contact layer
D_{A_2} = diffusion coefficient through alginate layer
t = time (seconds)

Note: D_{A_2} is assumed to be $4.5 \times 10^{-10}\ m^2/s$, which is the diffusion coefficient of urea through an agar gel. Due to the lack of current data of the diffusion of YP-ET and YP-Silver Chloride through alginate, the team used correlations and a similar model to determine D_{A_2}.

- Solve for C:

  $C_S \approx 0$ therefore can be neglected

$$C = C_i \left[\frac{4}{\pi} \cos\frac{\pi L_2}{2L_2} e^{-D_{A_2}\left(\frac{\pi}{2L_2}\right)^2 t} \right]$$

- Using YP-ET:

  $C_S = 0$

  $C_i = 6.6\ mg/ml$

  $x = L_2 = 0.21\ mm = 2.1 \times 10^{-4}\ m$

  $D_{A_2} = 4.5 \times 10^{-10}\ m^2/s$

  $t = 1800, 86400, 259200, 432000, 604800$ seconds
  (30min, 1day, 3days, 5days, 7days)

- Using YP-Silver Chloride

  $C_S = 0$

  $C_i = 41.8\ ppm = 41.8\ \mu g/ml$

  $x = L_2 = 2.1 \times 10^{-4}\ m$

  $D_{A_2} = 4.5 \times 10^{-10}\ m^2/s$

  $t = 1800, 86400, 259200, 432000, 604800$ seconds

Figure 26: Diffusion Calculations

5.10 Secondary Dressing Characterization

Polyurethane foam was selected for the secondary dressing to be used in combination with the primary dressing we constructed. The material properties of the foam were tested and compared to other secondary dressing products. The test protocols that were used to test multiple secondary dressings are described in the following sections.

5.10.1 Instron® Mechanical Testing- Peel Test Configuration

An Instron peel test was used to determine the adhesive strength for various secondary dressing candidates as shown in Appendix R. When conducting a test of this nature it is important to use a configuration that is similar to the environment in which the material will later be used. The results of this test will then be used to aid the team in quantitatively determining the most viable option for the outer component of the dressing to ensure that the adhesive will not disturb the underlying wound bed upon removal or fall off prematurely.

Using the ASTM standard D3330, a 3345EH electromechanical test frame configured with a 50 N load cell and 250 N capacity pneumatic grips at 70 psi with 25 x 25 mm flat metallic faces were used for this test. A variable angle peel fixture was set at 135 degrees to simulate an individual pulling the specimen off the skin. The complete test configuration is shown in Figure 27. The test methodology called for a test speed of 5 mm/min. The set up of the variable angle sled is shown in Figure 28. A piece of leather was used as the substrate to simulate skin as suggested by Instron employees. Figure 29 shows a close up of a test specimen during the peel test. The test specimens were all cut into rectangular strips having a

length of 100 mm, a width of 25 mm to ensure consistency among each sample group and individual specimens.

The most common method for characterizing adhesives is a basic peel test. Bluehill® 2 (Instron® software) can easily perform this test method. This test method was used to evaluate the adhesive strength of various specimens.

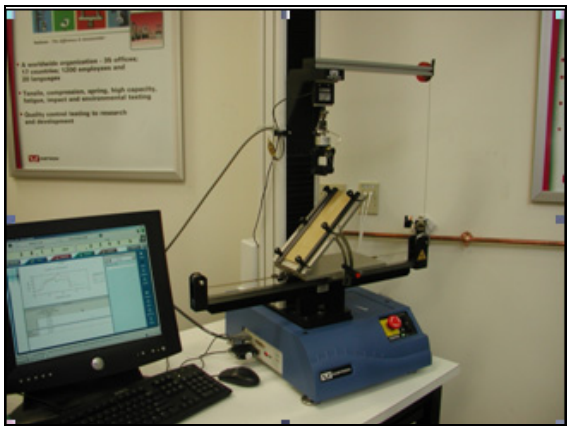


Figure 27: Complete test configuration for 135 degree peel test

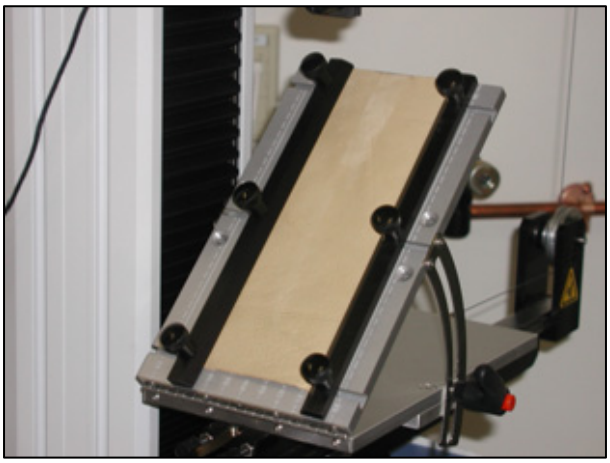


Figure 28: Close up of sled with leather substrate

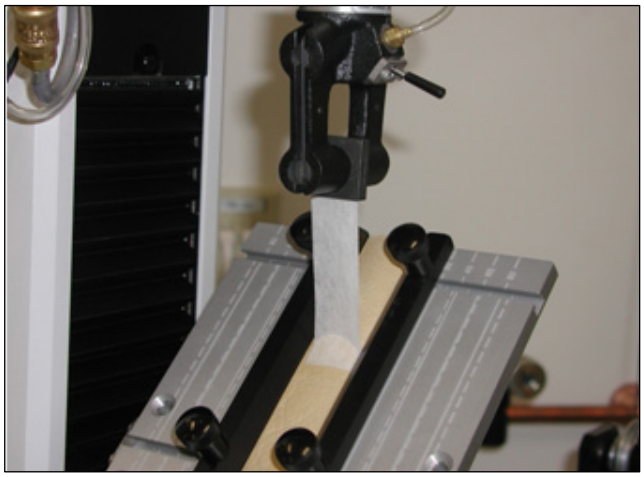


Figure 29: Close up of adhesive specimen during peel test.

5.10.2 Instron® Mechanical Testing- Tensile Test Configuration

This testing is necessary to aid the team in reaching its project goal, which is to create a dressing that enhances the current silver dressings that are on the market. Therefore, Acticoat™ 7 ("Gold Standard") and SilvaSorb™ were tested in tension to determine their mechanical properties (Appendix S). These two products were not tested using the peel fixture as they to do have any adhesive properties.

Using the ASTM standard D882, a 5567 electromechanical test frame configured with a 50 N load cell and 250 N capacity pneumatic grips at 70 psi with 25 x 25 mm flat metallic faces were used for this test. The complete test configuration is shown in Figure 27. Using the ASTM standard, the test speed was calculated to be 24.5 mm/min using A=BC where A is the test speed, B is the initial distance between the grips (50.8mm), and C is the initial strain rate (0.5mm/mm*min). Figure 30 shows a close up of a specimen being pulled to failure and Figure 27 shows the specimen after the test. Figure 32 demonstrates a close up of the other specimen tested.

The samples were prepared as indicated by the manufacturer's guide: the Acticoat™ 7 was moistened with sterile water and then drained for two minutes (Smith & Nephew, 2005) and SilvaSorb™ was moistened with saline to simulate making contact with the skin as the wound is to be washed out with saline prior to use (AcryMed, 2005). Each specimen was cut precisely into a rectangular strip, which was 115 mm long and 25 mm wide.

Bluehill® 2 can easily perform this test method. This test method was used to evaluate the maximum extension and load, tensile stress and tensile strain at the maximum load, maximum tensile strain, and modulus of the specimens.

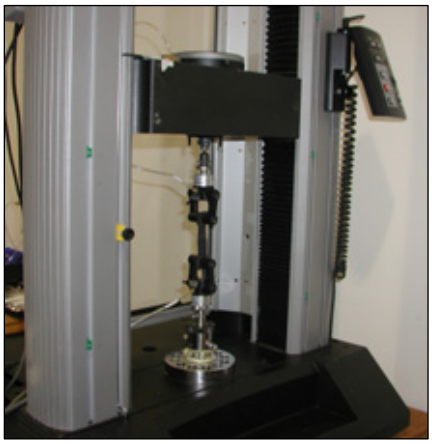


Figure 30: Complete test configuration for tensile test.

Figure 31: Specimen being pulled to failure.

Figure 32: SilvaSorb™ specimen during tensile test

Figure 33: Acticoat™ 7 specimen after testing.

6. Results

This section describes the results found following the methods and procedures previously explained. Results for antimicrobial activity test and terpene resistance test are described in detail.

6.1 Antimicrobial Formulations

As described in the methods section, three formulations of antimicrobial were encapsulated and tested *in vitro* to determine if each had antimicrobial properties against wound pathogens and the concentrations at which each was effective.

6.1.1 Eugenol Thymol (YP-ET)

In most strains, YP-ET showed signs of inhibited growth and in most cases the most favorable MIC. YP-ET demonstrated a broad range of growth inhibition against the different pathogens and therefore, when it is incorporated in a wound dressing would be effective against a variety of wounds infected with different pathogens. Eugenol has also shown to posses odor reducing and pain reducing properties, making it a positive consideration for our experiment. Therefore, due to this preliminary data, we predict this terpene formulation to be most effective in inhibiting microbial growth in the wound.

6.2 Antimicrobial Activity

After the silver nitrate, silver chloride, and YP-ET terpene had been encapsulated following the protocols described in the previous section. The antimicrobial activity of these three agents was determined and the results from the test against *E. faecalis*, *P. aeruginosa,*

and *S. aureus* are displayed in Appendix M. All three of the antimicrobial agents showed activity against the selected wound pathogens.

The data indicated that although all three antimicrobial agents showed activity, there were differences in their effectiveness. As expected, the two silver formulations were not extremely effective against *E. faecalis*; however, the formulations were effective against *P. aeruginosa,* and *S. aureus.* Also as anticipated, YP-ET was most effective at inhibiting the growth of *S. aureus*, but also showed inhibiting of *E. faecalis* and *P. aeruginosa.* The silver formulations could compensate where the YP-ET had lower inhibition, and the YP-ET could compensate where the silver had lower inhibition. The results of the primary assay support the hypothesis that the combination of YP- silver nitrate, YP-silver chloride and YP-ET should be more efficient than any of the three alone.

6.3 Synergy Experiments

The results from the synergy assays showed the antimicrobials inhibited growth against *E. faecalis*, *P. aeruginosa,* and *S. aureus*. The table below lists the MIC for terpene, silver nitrate, silver chloride for the four plates for each of the bacteria that were tested. The values in the table demonstrate that each combination of silver and terpene resulted in a lower MIC concentration of the active than compared to the actives alone.

Table 22: Average Synergy MIC Values

	MIC (ug/ml)		
TREATMENT	Ef	Pa	Sa
YP-ET	1000	1000	1000
YP-ET w/ YP-Silver nitrate	250	500	70.6
YP-ET w/ YP-Silver chloride	250	500	62.5
YP-Silver nitrate	250	31.3	15.6
YP-Silver nitrate w/ YP-ET	125	15.6	11.7
YP- Silver chloride	250	62.5	15.6
YP-Silver chloride w/ YP-ET	125	31.3	7.81

All three antimicrobial agents demonstrated growth inhibition. The MICs were close to the hypothesized values. For example, terpenes were previously found to be less effective against *P. aeruginosa* and the MIC values from the synergy experiment were higher when compared to the terpene MIC values for *E. faecalis*, and *S. aureus.*

When the MIC values of the combinations of YP-ET and YP- silver are compared the MIC values for each active alone, the values of the combination of actives are equivalent or in most cases lower. In both the primary and synergy assay the MIC of terpenes against *P. aeruginosa* was 500 ppm because of its resistance to terpenes. The MIC of YP-silver nitrate and YP-silver chloride was equivalent or lower in the synergy assay. The results support the hypothesis that the combination of silver and terpenes will be more effective at inhibiting a broader range of bacteria. The synergy assay proved that the combination of silver and terpenes was effective against the three chosen strains of bacteria. The assay also demonstrates that the concentration of silver required to inhibit the growth of bacteria can be reduced with the addition of terpenes.

The synergy assay that was conducted with the combination of bacteria, *E. faecalis* and *P. aeruginosa*, demonstrated that the encapsulated forms of silver and terpenes inhibited the growth of the bacteria. Due to resistance of bacteria, YP-silver nitrate and YP-silver chloride alone would have not been effective against *E. faecalis.* The combination of YP-ET with the encapsulated silver forms provided the same antimicrobial effectiveness than either of active alone. Table 23 shows the MIC values from the synergy experiment against the combination bacteria. When YP-ET was added to silver, the silver MIC value was lowered. For the case of YP-silver nitrate, alone the MIC was 62.5ppm, but with the addition of YP-ET the MIC values was reduced to a range of 15.63- 31.25ppm as shown in Figure 28.

Table 23: Synergy MIC Values for Combination Bacteria

	Plate 1	Plate 2	Plate 3	Plate 4
MIC Terpene alone	500	500	500	2000
MIC Silver Nitrate alone	62.5	62.5	-	-
MIC Silver Chloride alone	-	-	31.25	62.5
MIC Terpene w/ Silver Nitrate	125	250	-	-
MIC Terpene w/ Silver Chloride	-	-	125	125
MIC Silver Nitrate w/ YP-ET	15.63	31.25	-	-
MIC Silver Chloride w/ YP-ET	-	-	15.63	31.25

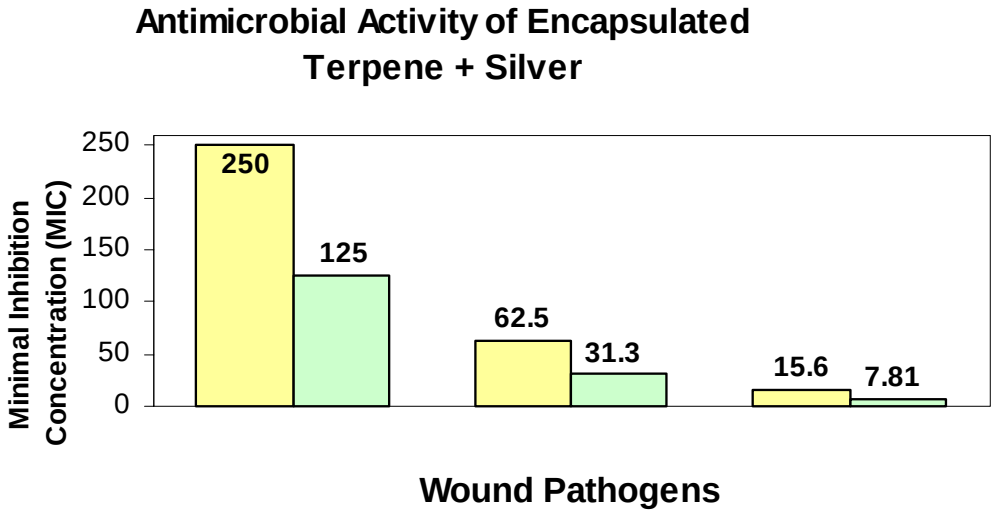


Figure 34: **Antimicrobial Activity of the YP-ET and YP-Silver Chloride**

6.4 Terpene Resistance

After completing the terpene resistance test, which was explained in Section 5, the materials were further evaluated. Any material that was dissolved by terpenes was eliminated and no longer eligible for consideration for the final dressing. The results from the terpene resistance test are shown in Table 24.

Table 24: Terpene Resistance Test

Material	Control (water)	YP-ET	Free ET
Tegasorb	No Change	No Change	Fell Apart
Calcium Alginate	No Change	No Change	No Change
Coverlet™ O.R. Inner	No Change	No Change	No Change
Coverlet™ O.R. Outer	No Change	No Change	No Change
Polyurethane Foam	No Change	No Change	Doubled in size

The results from the terpene resistance test eliminated the use of 3M Tegasorb™ in the dressing because of its deterioration when in contact with free ET. However, 3M Tegasorb™ did not show changes from YP-ET. Once the 3M Tegasorb™ samples were removed from the tubes, it retained the liquid with little drainage from material.

Figure 35: Terpene Resistance Test- 3M Tegasorb™

The calcium alginate did not change in the presence of terpene; however, the free terpene was absorbed less than the YP-ET and water. The free ET has an oily consistency making it more difficult to absorb by the calcium alginate. After removal from the tube, the calcium alginate did not retain as much liquid as did 3M Tegasorb™. The control sample, containing water, appeared to maintain the largest volume of liquid.

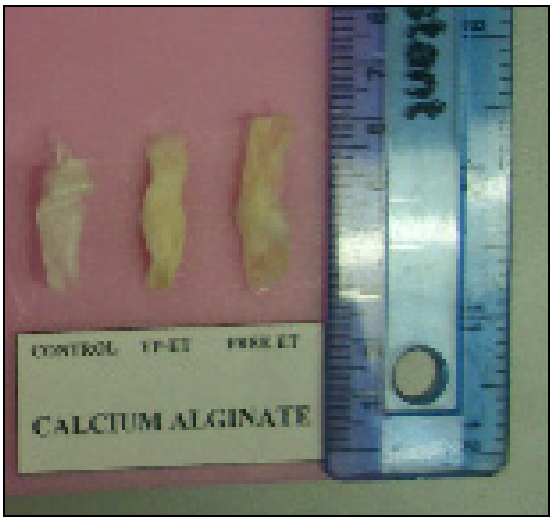


Figure 36: Terpene Resistance Test- Calcium Alginate

Both the outer section, which is an adhesive material and the inner section, which is a gauze material of Coverlet™ O. R. were tested for their resistance to terpenes. The adhesive outer layer demonstrated no changes in the presence of YP-ET or free ET. After removal from the liquid, the sample of Coverlet™ O. R. that had been soaked in YP-ET exhibited the most adhesive ability when compared to the other two samples. The control sample also retained some adhesive properties, while the sample soaked in free ET was least adhesive.

Figure 37: Terpene Resistance Test- Coverlet™ O. R. Outer Section

The inner section of the Coverlet™ O. R., which has gauze-like properties, was not affected by the terpenes. All three samples absorbed the liquid and displayed no physical changes from the YP-ET or from the free ET.

Figure 38: Terpene Resistance Test- Coverlet™ O. R. Inner Section

The polyurethane foam was highly absorptive in the free-terpene solution, but did not appear to absorb any water or any of the YP-ET solution as the surface seems to repel water. In the free terpene, the sample doubled in size yet in the others there was no physical change.

Control Encap.Terpene Free Terpene
Figure 39: Terpene Resistance Test – Polyurethane Foam sample

The results collected from the terpene test eliminated the use of 3M Tegasorb™ in the final design of the dressing. The results of the other samples provided positive feedback in the use of calcium alginate, Coverlet™ O. R., or Polyurethane Foam in the dressing.

6.5 In Vivo Vehicle Formulation

Interestingly, in the particle settling experiment, after 24 hours all of the alginate concentrations separated into a cloudy layer and a liquid layer, the terpene fully settling to the bottom of the tube. The 1% alginate resulted in 23% settling occur, and each respective concentration ranged between 14% and 23% settling, not favorable for delivery of antimicrobials. On the other hand, the 0.45% CMC and higher were effective in remaining homogeneous and showed little to no signs YP-terpene settling. Therefore, we can conclude that alginate is not an effective vehicle for the animal experiment, and also it is not an effective material for delivery of actives to the wound bed as we can deduce that the antimicrobials will settle deep into the wound after 24 hours (see Appendix W).

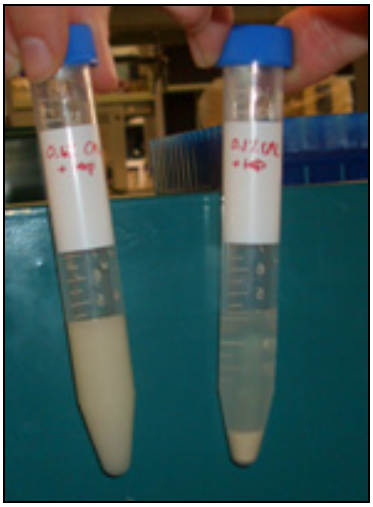


Figure 40: Evidence of particle settling
Left: 0.6% CMC + terpene, right: 0.1% CMC + terpene

6.5.1 *In Vivo* Vehicle Assay Results

The *in vivo* vehicle was tested *in vitro*, as described in the methods section, to determine if the antimicrobials were still active after the suspension in CMC. The results from this test prove that the YP-ET suspended in CMC retained its antimicrobial properties and was still effective in inhibiting the growth of *S. aureus.* Table 25 shows the MIC Values for the three concentrations of YP-ET in CMC, CMC alone, and three concentrations of YP-ET with no vehicle.

Table 25: *In Vivo* Vehicle MIC Values

Sample	MIC µg/ml
High YP-ET 24.0 mg/ml in CMC	250
YP-ET 24.0 mg/ml	250
Med YP-ET 6.6 mg/ml in CMC	206
YP-ET 6.6 mg/ml	412.5
Low YP-ET 1.5 mg/ml in CMC	375
YP-ET 1.5 mg/ml	375
CMC only	No kill
YP-ET 4.0 mg/ml	500

The table demonstrates that although the MIC values change depending on the concentration of YP-ET, all of the formulations show inhibition of bacterial growth. As expected, the CMC alone, which has no antimicrobial benefit, did not inhibit bacterial growth. Since the YP-ET suspended in CMC retained its antimicrobial activity in the *in vitro* assay, the formulations will be further tested in an *in vivo* wound model.

6.6 In Vivo Testing

Overall, all of the terpene groups showed positive wound healing results evidenced by a decrease in wound area over time (Figure 35). These terpene groups had the greatest reduction in percent change in the wound area as compared with the control group of vehicle

only. Here, the control group was shown to get worse by increasing in wound area, and then gradually getting better at a later time point. In the terpene treatment groups, the wounds immediately show improvements in wound area and wound closure, as the wounds in all groups decrease in area over time (see Appendix GG).

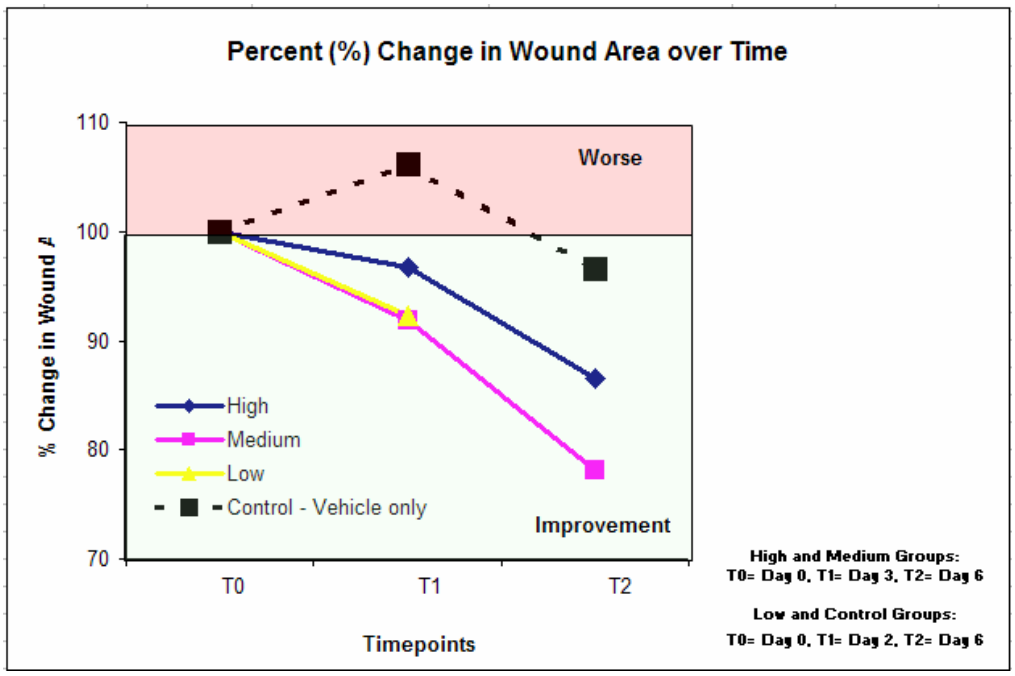


Figure 41: Percent change in wound area over time in animal groups

After ten days, where treatment had been stopped for three days, the wounds showed signs of bacterial growth. The mice had suppressed immune systems, so they therefore cannot fight of any remaining bacteria on their own. Fortunately, the mice which had received terpene treatment had a lower bacterial count and lower bioluminescent intensity of bacteria as compared with those who had been treated.

Interestingly, the mice weights varied fluctuated at three different time points of measurement as shown in Appendix HH. At the middle time points, all mice had lost weight,

signs of their change in feeding and activity, reflective of the pain or fighting of the bacteria in the wound site. Fortunately, by the third time point, many of the mice had gained weight in the terpene groups, correlated to wound healing and pathogen inhibition. However, in all of the control groups, the mice had lost weight over time, and even at the third time point, there was no indication of further weight gain.

When analyzing the bioluminescent data, again, the terpene groups showed the most promising results as the bacterial load was decreased to a few small dots of color if not completely eradicated by day 7 (Figure 42; Appendix OO). In the control (vehicle only) mouse, the animal did not recover from anesthesia so we submitted the mouse to testing to determine what the cause of death was. A swab of the heart was streaked onto a plate and after 24 hours of incubation, the whole plate bioluminesced, indicating death due to a systemic infection of bioluminescent *S. aureus*. The lighter the color on the spectrum means the higher fluorescence in J/cm^2, as indicated by the color bar to the side of the figure (Figure 42). The best visual inhibition of growth was indicated by the high concentration of terpene, whereas the least inhibition was indicated by the no treatment group (Figure 42).

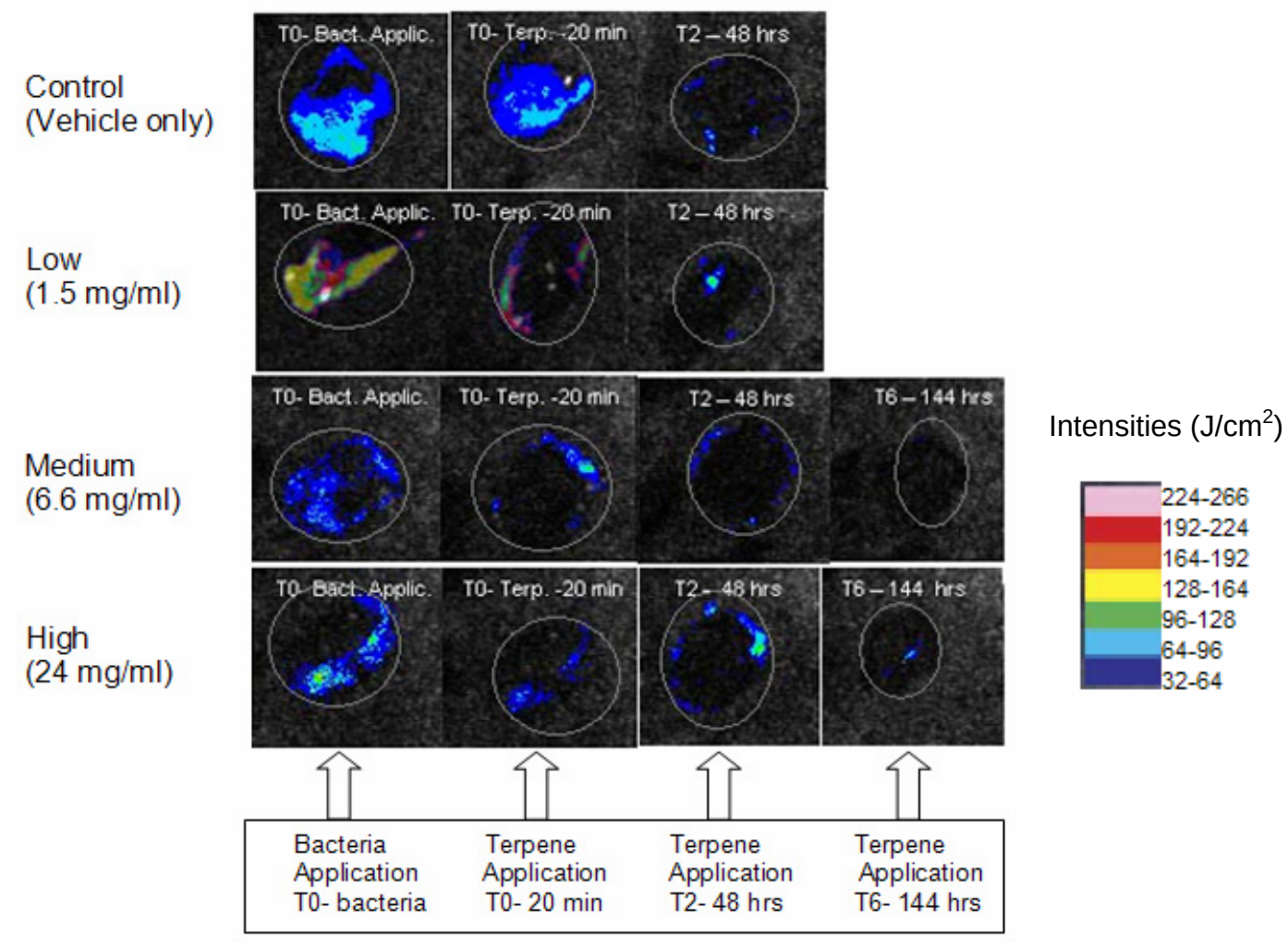


Figure 42: Bioluminescent images at four time points for terpene and control treatment groups

When further analyzing these images, an area was isolated around the wound side and ARGUS software used calculated the bioluminescent intensities as a function of the bit size. These numbers allow us to numerically analyze the bacterial inhibition at each daily reading (Appendix II). Again, the terpene groups yielded similar results, except the low concentration of terpene peaked before decreasing over time. The vehicle only control gradually decreased the bioluminescence, indicating slow inhibition of bacteria, however was not as instant as the terpene treatment groups and increased toward the end of treatment. The lowest values of

intensity were reached by the high and medium terpene treatment groups. The no treatment control showed that the infection in the wound increased dramatically over time, indicating the mouse was not able to fight the infection on its own.

6.7 Material of Construction

The materials selected are listed next to each of the functional layers in the figure below. The primary dressing contains the interfacial barrier and actives layer. The materials selected for the interfacial barrier are calcium alginate and glycerol. The actives retention layer includes CMC, glycerol, and the antimicrobial actives. The second component of the dressing is the outer barrier which is composed of PU foam.

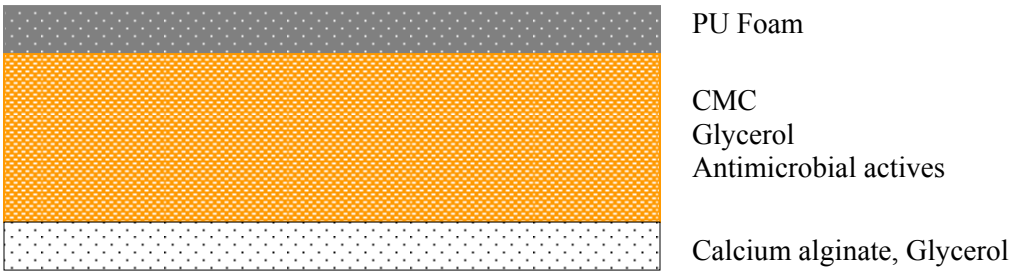


Figure 43: Materials Selected for Final Dressing

6.8 Dressing Assembly

The steps for the dressing assembling the dressing are described in this section.

Step 1: Cast alginate layer

This step involved the cross- linking of the alginate with $CaCl_2$. This layer was rinsed with water to remove excess sodium and excess calcium chloride to produce the calcium alginate formulation. The calcium chloride was cut to fit the casting tray, shown in Figure 44.

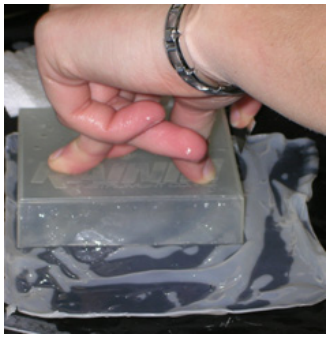


Figure 44: Calcium Alginate Cut to Size

Once the calcium alginate was cut, it was placed in the casting container and prepared for the actives to be added. Figure 45 shows the calcium alginate layer cast and ready for next layer to be produced.

Figure 45: Calcium Alginate Cast

Step 2: Create CMC active layer

The second layer, the actives layer, was prepared by weighing out the components of 1.5 % CMC, 1.75% glycerol, 0.3% agarose, 6.6 mg/ml YP-ET, and 41.8 µg/ml YP-silver chloride. As previously described, each of these components was kept in a 50°C water bath prior to weighing. Once each component was measured, the entire formulation was immediately poured on top of the calcium alginate layer. Figure 46 shows the addition of the actives layer to the calcium alginate layer.

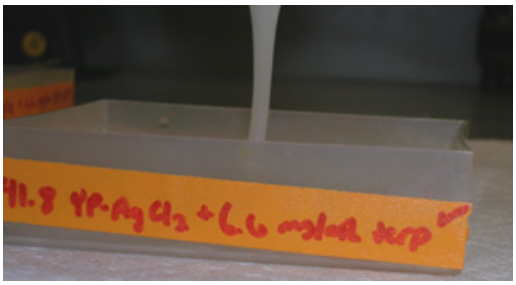


Figure 46: Addition of Actives Layer to Calcium Alginate

Step 3: Lyophilze dressing layers

After the CMC actives layer was added to the calcium alginate layer, the dressing was frozen at -80°C for one hour. The freezer shown in Figure 47 was used to freeze our dressing.

Figure 47: -80°C Freezer

The frozen dressing was lyophilized for 24 hours using the lyophilizer shown in Figure 48. This lyophilizing process created a dressing with more homogeneous characteristics and more desirable properties.

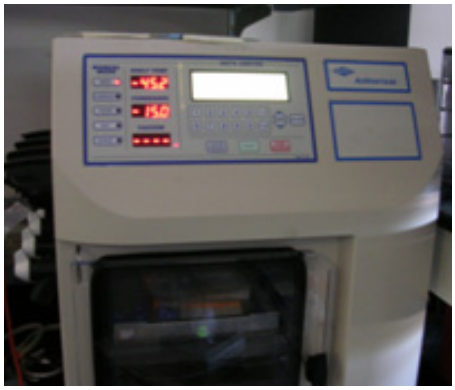


Figure 48: Lyophilizer

Step 4: Apply the secondary dressing and place dressing on wound area

Once the lyophilization process was finished, the primary dressing consisting of the contact and actives layer was ready to be used with the secondary dressing. The final primary dressing formulation is shown below.

Figure 49: Final Primary Dressing

The primary dressing was adhered to the secondary dressing, PU Foam. This was the final step in producing our antimicrobial dressing, which is shown in Figure 50.

Figure 50: Final Antimicrobial Dressing

The final dressing, consisting of both the primary and secondary dressing, was complete and could be placed on a wound site. The adhesive properties of PU Foam secured the dressing to the wound area. An example of the dressing applied to a human arm is illustrated below.

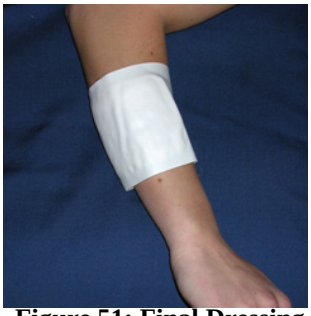


Figure 51: Final Dressing

6.9 Primary Dressing Characterization

The following results demonstrate the outcome of the test conducted on the primary dressing using varying concentrations of YP-ET and YP-silver chloride. The results proved that the concentrations of actives we selected for our final design were most effective when compared to the primary dressings with varying concentrations.

6.9.1 Antimicrobial Barrier Assay - YP-ET

This assay allowed the team to determine that the prototype dressing does act as an antimicrobial barrier to *S. aureus*. It is important that the dressing act as an antimicrobial barrier in order to keep microbes out of the wound site originating from an external environment. If external microbes were able to enter the wound, they could possibly hinder wound healing as well as increase the chance of infection. Table 26 demonstrates that bacterial growth was observed under the sterile filter paper discs (negative control) at 24 hours. On the other hand, our dressing formulations were effective antimicrobial barriers as no growth was seen under these dressings at any of the specified time points.

Table 26: Results of Antimicrobial Barrier Assay

	Bacterial Growth Under Dressing at Time Points				
Plate 1 (Sa)	**1h**	**2h**	**4h**	**8h**	**24h**
Filter Paper (5 pieces)	None	None	None	None	Yes – radius of disc
Plate 2 (Sa)					
Control (5 pieces)	None	None	None	None	None
Plate 3 (Sa)					
6.6 mg/ml YP-ET (5 pieces)	None	None	None	None	None
Plate 4 (Sa)					
24 mg/ml YP-ET (5 pieces)	None	None	None	None	None

6.9.2 Disc Diffusion Assay

By using the criteria set forth by the disc diffusion assay protocol found in Appendix LL (Holder et al, 2003), it was determined that the prototype dressing with YP-ET as the antimicrobial did provide antimicrobial actives to the plates of bacteria in concentrations high enough to kill pathogenic growth. Table 27 provides the radii of clearance for each pathogen and dressing type.

Table 27: Average Radius of Clearing
Including Area under Dressing during Disc Diffusion Assay

Sa	Average Radius of Clearing (mm)
Filter Paper	0
Control	0
6.6 mg/ml YP-ET	2.83
24 mg/ml YP-ET	3.13

Pa	Average Radius of Clearing (mm)
Filter Paper	0
Control	0
6.6 mg/ml YP-ET	2.02
24 mg/ml YP-ET	2.28

The results above show that no clearance was observed with either the sterile filter paper or the control dressing without YP-ET when using both *P. aeruginosa* and *S. aureus*. On the other hand, kill occurred when using the dressings with 6.6mg/ml and 24.0 mg/ml. increased kill was seen when the higher concentration of terpene was present in the dressing. Additionally, the dressings with the active were more effective against *S. aureus* than *P. aeruginosa*, which supports *in vitro* data previously conducted. Finally, by using the results from the *in vivo* animal study along with the results demonstrated here, the team was able to select 6.6 mg/ml as the final YP-ET concentration for the prototype dressing.

By repeating the disc diffusion assay using the dressing with the combined antimicrobial agents, YP-ET and YP-silver chloride, the team was able to determine that not only is the combination of YP-ET and YP-silver chloride effective, but they also inhibit more pathogens than either of the two constituents alone as shown by an increased zone radius of clearing in Table 28.

Table 28: Average Radius of Clearing Including Area under Dressing during Disc Diffusion Assay Using YP-ET and YP-Silver Chloride

Sa	Average Radius of Clearing (mm)	Pa	Average Radius of Clearing (mm)
Filter Paper	0	Filter Paper	0
Control – 13.6ppm YP-Silver Chloride	4.01	Control - 13.6ppm YP-Silver Chloride	4.63
6.6 mg/ml YP-ET, 13.6 ppm YP-Silver Chloride	5.70	6.6 mg/ml YP-ET, 13.6 ppm YP-Silver Chloride	5.99
6.6 mg/ml YP-ET, 41.8 ppm YP-Silver Chloride	6.07	6.6 mg/ml YP-ET, 41.8 ppm YP-Silver Chloride	9.15
6.6 mg/ml YP-ET, 70.0 ppm YP-Silver Chloride	6.15	6.6 mg/ml YP-ET, 70.0 ppm YP-Silver Chloride	5.52*

* Zone had more depth than width.

Furthermore, this assay allowed the team to choose the final YP-silver chloride concentration for the dressing. The low concentration (13.6 ppm) was quite effective in the present assay. However, the team chose 41.8 ppm for the silver active component, which is nearly half of the currently used concentration, which ranges from 70-100 ppm (Smith & Nephew, 2005). The medium concentration of YP-silver chloride was the best choice for the dressing as it was very effective at inhibiting both Sa and Pa when used with 6.6 mg/ml YP-ET. This prototype dressing provided inhibition both in the radial direction as well as through the agar plate. The high concentration "ate" through the agar and therefore, the team speculates that this may be harmful to a patient's skin. Additionally, the low concentration was ruled out as it provided slightly less inhibition than the medium dose and as the team experienced with YP-ET, it is necessary to use a higher concentration *in vivo* to acquire the desired bacterial inhibition.

6.9.3 Corrected Zone of Inhibition

The results of the corrected zone of inhibition assay using the YP-ET prototype were inconclusive. However, they did give the team some valuable insight about the capabilities

and limitations of the current dressing. The active was released from the dressing and did provide killing and inhibition for over 72 hours before the assay was terminated, which proves that the dressing is capable of providing a sustained release of the antimicrobial. Additionally, the data on day 1 demonstrates that the higher the concentration of terpene equals more inhibition, which is what the team had hypothesized. As the days progressed, the data was less and less accurate as the dressing was difficult to move and small portions of the dressing were lost during the transfer from plate to plate using sterile tweezers.

The assay was supposed to run for 7 days as that is the lifespan of most silver dressings currently on the market, yet the dressing swelled considerably and was very hard to transfer from day to day. By day 4, the dressings could no longer be placed on a new plate of bacteria.

As a result of this assay, we determined that when the punch biopsy was taken and the dressing was placed on the Mueller-Hinton agar plate, the CMC layer swelled over the alginate layer. Therefore, we came up with the solution of using bottle caps to cast the gels to use for the next phase of this assay. This way the alginate layer would keep the CMC layer sealed on the sides and reduce swelling caused by the punch biopsy pieces. The swelling that was observed using the biopsy pieces is uncharacteristic of the whole dressing as it is surrounded by the alginate contact layer on the sides.

Table 29: Results of Corrected Zone of Inhibition Assay using YP-ET Prototype Dressing Against Sa

	Radius of Inhibition (mm)		
Plate 1 (Sa)	**1 day**	**2 days**	**3 days**
Filter Paper	0	0	0
Control	0	0	0
6.6 mg/ml YP-ET	1.06	2.03	2.72
24 mg/ml YP-ET	1.63	0.14	Not enough dressing left

Table 30: Results of Corrected Zone of Inhibition Assay using YP-ET Prototype Dressing Against Sa

	Radius of Inhibition (mm)		
Plate 2 (Sa)	**1 day**	**2 days**	**3 days**
Filter Paper	0	0	0
Control	0	0	0
6.6 mg/ml YP-ET	1.47	0.48	0.52
24 mg/ml YP-ET	1.57	1.52	0.14

Additionally, the team performed the same assay using the final prototype dressing, which contained both YP-ET and YP-silver chloride to determine the longevity of the overall dressing. The results demonstrated that the dressings were effective for 7 days and still provided considerable inhibition on Day 7 as shown in Table 31.

Table 31: Results of Corrected Zone of Inhibition Assay using YP-ET and YP-Silver Chloride Prototype Dressing Against Sa

	Radius of Inhibition (mm)			
Plate 1 (Sa)	**1 day**	**3 days**	**5 days**	**7 days**
Filter Paper	0	0	0	0
Control - 13.6ppm YP-Silver Chloride	4.52	4.06	3.85	3.27
6.6 mg/ml YP-ET, 13.6 ppm YP-Silver Chloride	5.73	5.56	4.02	3.98
6.6 mg/ml YP-ET, 41.8 ppm YP-Silver Chloride	6.02	5.68	5.43	4.69
6.6 mg/ml YP-ET, 70.0 ppm YP-Silver Chloride	6.20	6.01	5.73	4.71

The team was then able to us the disc diffusion results as well at the corrected zone of inhibition results to finalize our selection of 41.8 ppm as the concentration of the YP-silver chloride active.

6.10 Secondary Dressing Characterization

The following sections describe the results of the secondary dressing characterization tests that were previously explained.

6.10.1 Instron® Mechanical Testing- Peel Test

The polyurethane foam and the island foam showed similar results and the hydrocolloid demonstrated only a slightly lower adhesive strength. A very low adhesive strength could result in the dressing prematurely falling off. On the other hand, Coverlet™ O.R. has a considerably higher adhesive strength and should be taken into consideration as one of our objectives is easy removal. The high adhesive strength shown by Coverlet™ O.R. could be detrimental to the underlying wound bed upon removal.

6.10.2 Instron® Mechanical Testing- Tensile Test

Acticoat™ 7 was considerably higher in all categories except tensile strain at maximum load and maximum tensile strain when compared to SilvaSorb™. SilvaSorb™ exhibits more elastic material properties than Acticoat™ 7. Both dressings should be taken into consideration as we try to enhance silver dressings which are the "Golden Standards" of the current wound dressings.

7. Analysis

7.1 ANOVA Analysis for Instron® Mechanical Testing

The results of a ANOVA statistical test performed at 10:12 on 26-JAN-2006

Source of Variation	Sum of Squares	d.f.	Mean Squares	F
between	179.8	3	59.92	630.7
error	0.7600	8	9.5006E-02	
total	180.5	11		

The probability of this result, assuming the null hypothesis, is 0.000

Group A (PU Foam): Number of items= 3
2.10 2.20 2.24
Mean = 2.18
95% confidence interval for Mean: 1.768 thru 2.589
Standard Deviation = 7.129E-02
Hi = 2.24 Low = 2.10
Median = 2.20
Average Absolute Deviation from Median = 4.633E-02

Group B (Island Foam): Number of items= 3
2.01 2.32 2.79
Mean = 2.37
95% confidence interval for Mean: 1.964 thru 2.784
Standard Deviation = 0.396
Hi = 2.79 Low = 2.01
Median = 2.32
Average Absolute Deviation from Median = 0.262

Group C (Hydrocolloid): Number of items= 3
1.17 1.56 1.73
Mean = 1.49
95% confidence interval for Mean: 1.077 thru 1.897
Standard Deviation = 0.288
Hi = 1.73 Low = 1.17
Median = 1.56
Average Absolute Deviation from Median = 0.187

Group D (Coverlet™ O.R.): Number of items= 3
10.7 10.8 11.3
Mean = 10.9
95% confidence interval for Mean: 10.51 thru 11.33
Standard Deviation = 0.368
Hi = 11.3 Low = 10.7
Median = 10.8
Average Absolute Deviation from Median = 0.227

Figure: Error Bar Plot

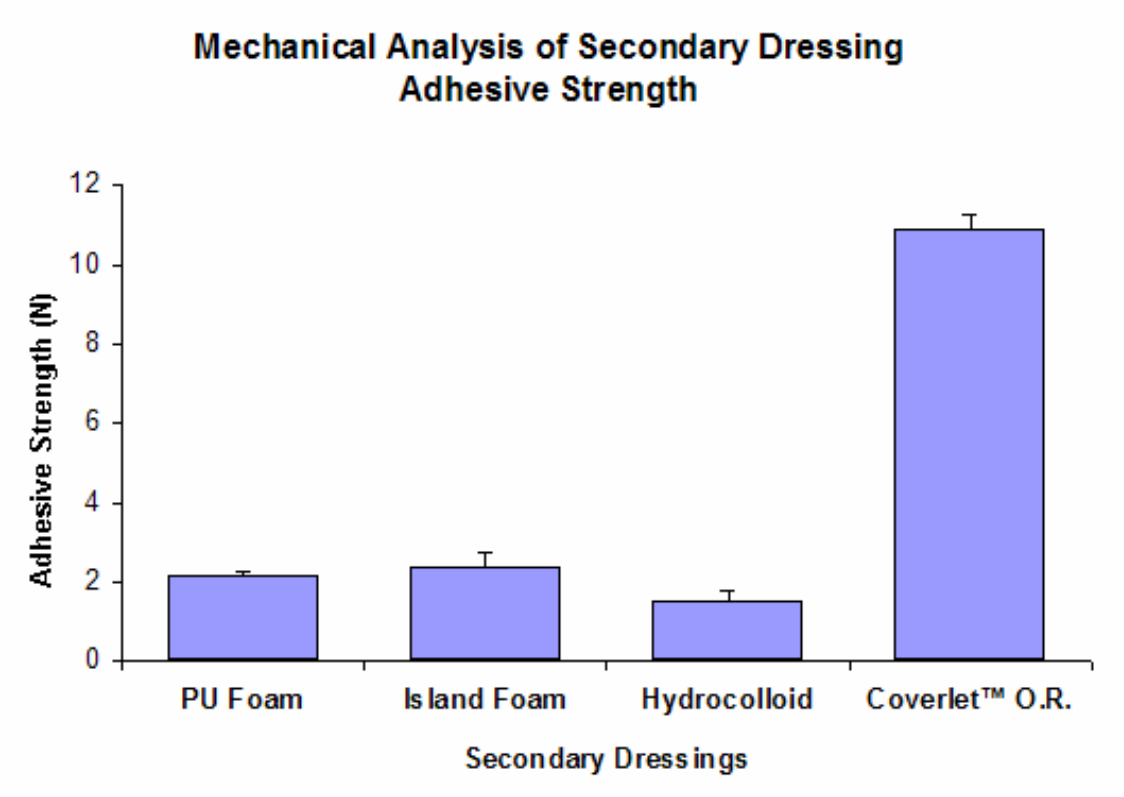


Figure 52: Instron Adhesive Strength Testing of Secondary Dressing

The above plot, Figure 51, demonstrates that there is no significant statistical difference between samples PU Foam, Island Foam, or Hydrocolloid. However, there are notable statistical differences between PU Foam, Island Foam, and Hydrocolloid vs. Coverlet™ O.R. The adhesive strength of Coverlet™ O.R. was much greater than the other samples and was therefore ruled out from our selection for the outer dressing layer as one of our objects was ease of remove. Coverlet™ O.R. has such a high adhesive strength that, upon removal, it could damage the underlying wound bed and hinder successful wound healing.

8. Conclusions

The activity of terpenes as antimicrobial agents against wound pathogens was confirmed by the *in vitro* assays conducted. These results demonstrated the ability of terpenes to inhibit a wide range of wound pathogens. The encapsulated terpene formulation, YP-ET, was determined to be effective in both *in vitro* assays and *in vivo* studies. The use of terpenes did not inhibit healing of the infected wound model and did not have a negative effect on the health of the animal. The study concluded that the terpenes were able to inhibit the growth of *Staphylococcus aureus* in the *in vivo* model, while decreasing the wound area resulting in enhanced wound healing.

The antimicrobial combination of YP-ET and silver chloride proved to be more effective than either agent alone. As a result of the addition of YP-ET, the antimicrobial combination proved effective against pathogens that were resistant to silver alone, supporting our initial hypothesis. Therefore, our antimicrobial combination is able to inhibit the growth of a broader range of wound pathogens. The addition of terpenes also decreased the concentration of silver required to inhibit the growth of wound pathogens, which in turn, should reduce the overall cost of our dressing design compared to current dressings which use high silver concentrations.

The antimicrobial dressing is safe to use because it was constructed with CMC and alginate materials, which are currently used in numerous wound dressing formulations. The dressing allows for the delivery of the antimicrobial agents to the wound site for a period of seven days. The final dressing prototype also prevents contamination of the wound from the outside because it acts as an antimicrobial barrier. The secondary dressing, Polyurethane Foam, provides mechanical stability to the wound area. The adhesive properties of the foam

were tested and determined to be acceptable for our application. The prototype proved to be an effective antimicrobial dressing and with further refinement, could be made into a commercial wound dressing.

9. Recommendations

The results demonstrate that YP-ET is effective *in vivo* not only in decreasing the amount of bacteria present over time but also in reducing wound area. Additionally, the actives and dressing proved to be successful in an *in vitro* environment. However, there are still improvements that can be made to the dressing to increase its usability and its chance of one day competing with current silver wound dressings.

For example, silver chloride, the silver active chosen by the team, is insoluble and when it comes in contact with sodium chloride, which is often present in the wound, a white precipitate can form on the outside of the patient's skin. This film could potentially hinder wound healing and is not aesthetically pleasing. It should be noted though that our dressing is as effective in killing and inhibiting microbes as the current ones and it is possible that the team's dressing may be more cost effective using this form of silver.

Long-term resistance of our dressing formulation to terpene is also a concern that needs to be investigated when determining ways to package the dressing as it is known that terpenes cause many plastics especially polystyrene materials to deteriorate (Ostroff, 2006). Furthermore, terpene resistance may decrease the shelf life of the dressing therefore, more testing is necessary to address this topic.

The team also recommends that more extensive mechanical analysis of the dressing be completed to assess its properties. It is very important to ensure that everything has been done to make sure the dressing is mechanically stable, durable, and strong, yet flexible in order to foster wound healing. Additionally, the dressing prototype has not been tested against a wide range of microbes. Due to time and material constraints, the team focused on three of the most common wound pathogen, which leaves room for further studies against others. Finally,

promising but insufficient data has been collected *in vivo*. Due to the complexity of an animal study, the team only tested the YP-ET active *in vivo*. Therefore, it is suggested that the animal study be repeated with the YP-silver and combination YP-ET and YP-silver actives.

Furthermore, future *in vivo* testing should be done using the final dressing formulation containing both actives to truly determine the worth of the design. Also our terpene and silver concentrations were based on *in vitro* studies using *S. aureus*, however, in the *in vivo* animal study a bioluminescent *S. aureus* was used. Tests should be completed to compare the two *S. aureus* strands used and to validate the chosen concentrations of antimicrobial actives.

10. Glossary

Bioluminescent- the production of light as a result of chemical reactions of a living organism.

Inhibition - To decrease, limit, or block the growth and action of bacterial cells

Microbe – a small living organism such as a bacteria cell

Minimal inhibition concentration (MIC) - lowest concentration of antimicrobial that inhibits 80% of bacterial growth.

Parts per million (ppm) - equivalent to μg/ml, used to describe the concentration of silver in current wound dressing products.

Terpene- organic oils with antimicrobial properties and microbial resistance.

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APPENDIX A: Interview with Dr. Raymond Dunn

Dr. Ray Dunn from UMASS Medical School Department of Surgery and plastic surgery provided us with professional and experienced insight to our wound project. We interviewed him on the following questions which are preceded by his responses.

1.) **What kind of wounds are you most used to treating?**

Dr. Dunn: All wounds, none treated in particular, Arterial, Venous ulcers, Diabetic ulcers, Pressure Ulcers.

   a. **How do you usually treat them?**

Dr. Dunn: They are usually treated by first being cleaned of dead compromised tissue followed by dressing management or an operation, depending on the severity of the wound.

2.) **What do you look for in a dressing when applying it to a wound?**

Dr. Dunn: The dressing must provide a moist wound environment and address the needs of the wound once it has been evaluated. The current dressings do a great job in keeping the wound moist and absorb as well as retain fluid.

3.) **How do you feel about the current wound dressings (particularly the silver dressings)?**

Dr. Dunn: All wounds are colonized with bacteria so it is often hard to distinguish a difference between the normal presence of bacteria or if the

wound has undergone further infection. The dressing should help to keep bacterial growth under control and not grow too much. The wound itself is not usually tested, just the exudate amount.

a. Where are they lacking?

Dr. Dunn: The silver based hydrogel dressing seems to be most effective but the actual areas that need to be worked on depends on the cause/type of wound.

b. What wounds are they least effective against?

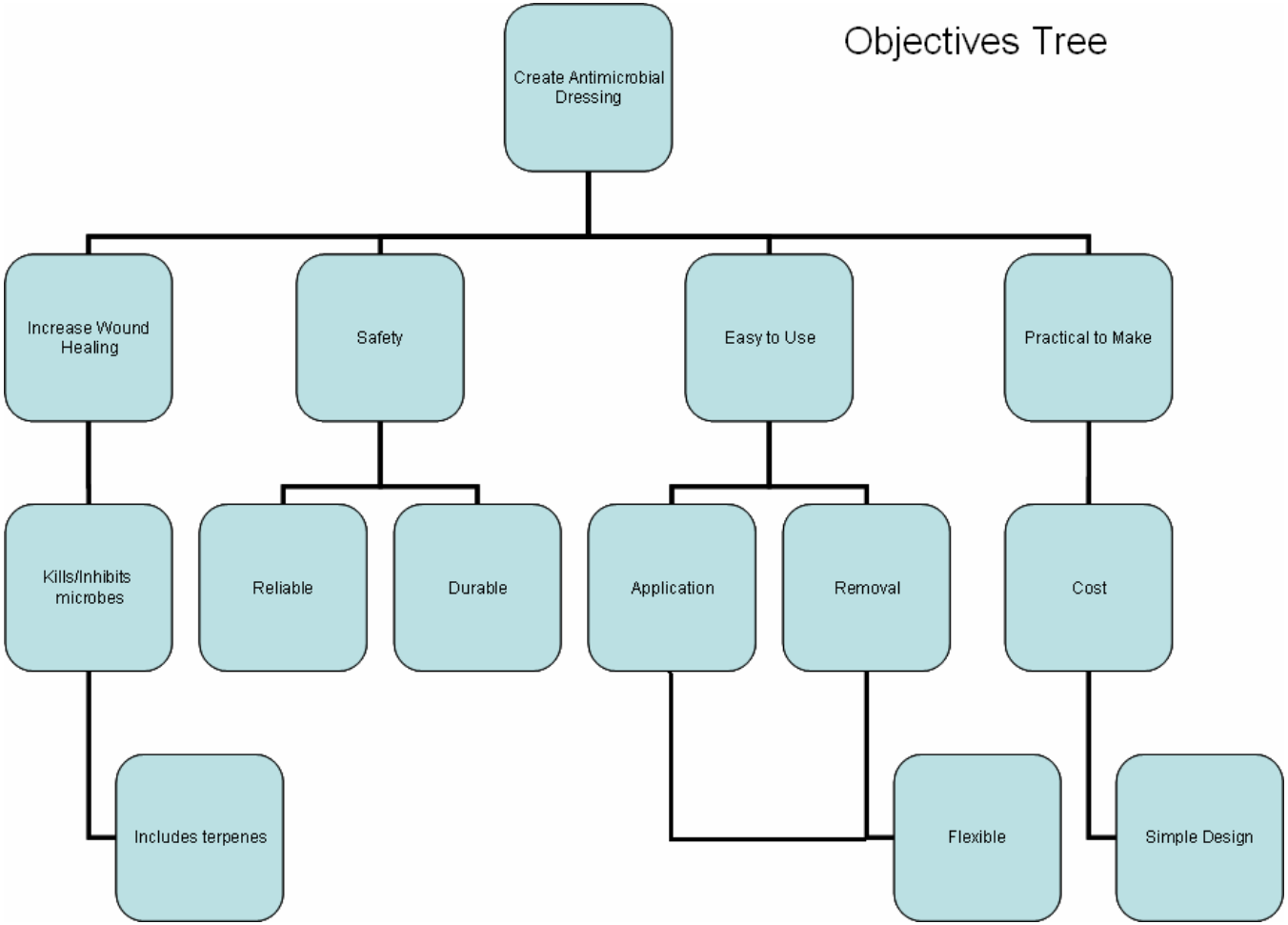
Dr. Dunn: infected wounds and wounds with necrotic tissue.

4.) How often are additional treatments necessary?

Dr. Dunn: 25% of the time, the usual additional treatment is surgery to remove tissue debris.

Dr. Dunn not only provided us with insight to the dressing market and improvements, but also to our animal experiment. He advised us to avoid choosing diabetic foot ulcers for our wound model as they are not only difficult to mimic, but also hard to heal. With this type of wound, they require more care and the dressing must be changed every 1-3 days due to the severity of the wound. Another constraint to using a diabetic ulcer model is that we would need a large sample size, which is costly to produce.

APPENDIX B: Initial Objectives Tree



APPENDIX C: Pairwise Comparison Chart- MQP Team

1st Tier

Goals	Increase wound Healing	Safety	Easy to Use	Practical to Make	**Score**
Increase wound Healing	X	0	1	1	**2**
Safety	1	X	1	1	**3**
Easy to Use	0	0	X	0	**0**
Practical to make	0	0	1	X	**1**

2nd Tier

Goals	Kills/Inhibits Microbes	Reliable	Durable	Easy to apply	Easy to Remove	Cost	**Score**
Kills/Inhibits Microbes	X	1	1	1	1	1	**5**
Reliable	0.5	X	1	1	1	1	**4.5**
Durable	0.5	0.5	X	1	1	1	**4**
Easy to apply	0	0	0	X	0.5	1	**1.5**
Easy to remove	0	0	0	0.5	X	1	**1.5**
Cost	0	0	0	0	0	X	**0**

3rd Tier

Goals	Includes terpenes	Flexible	Simple Design	**Score**
Includes terpenes	X	1	1	**2**
Flexible	0	X	1	**1**
Simple Design	0	0	X	**0**

APPENDIX D: Pairwise Comparison Chart- Prof. George Pins

1st Tier

Goals	Increase wound Healing	Safety	Easy to Use	Practical to Make	**Score**
Increase wound Healing	X	0.5	1	1	**2.5**
Safety	0	X	1	1	**2.0**
Easy to Use	0	0	X	1	**1**
Practical to make	0	0	0	X	**0**

2nd Tier

Goals	Kills/Inhibits Microbes	Reliable	Durable	Easy to apply	Easy to Remove	Cost	**Score**
Kills/Inhibits Microbes	X	1	1	1	1	0.5	**4.5**
Reliable	0	X	0.5	1	1	0	**2.5**
Durable	0	0.5	X	0.5	0.5	0	**1.5**
Easy to apply	0	0	0.5	X	0.5	0	**1.0**
Easy to remove	0	0	0.5	0.5	X	0	**1**
Cost	0.5	1	1	1	1	X	**4.5**

3rd Tier

Goals	Includes terpenes	Flexible	Simple Design	**Score**
Includes terpenes	X	1	1	**2.0**
Flexible	0	X	0.5	**0.5**
Simple Design	0	0.5	X	**0.5**

APPENDIX E: Pairwise Comparison Chart- Gary Ostroff

1st Tier

Goals	Increase wound Healing	Safety	Easy to Use	Practical to Make	**Score**
Increase wound Healing	X	1	0.5	1	**2.5**
Safety	0	X	1	0.5	**1.5**
Easy to Use	0.5	0	X	0.5	**1**
Practical to make	0	0.5	1	X	**0.5**

2nd Tier

Goals	Kills/Inhibits Microbes	Reliable	Durable	Easy to apply	Easy to Remove	Cost	**Score**
Kills/Inhibits Microbes	X	1	1	1	1	1	**5**
Reliable	0	X	0.5	1	1	0.5	**3**
Durable	0	0.5	X	0.5	0.5	0.5	**2**
Easy to apply	0	0.5	0.5	X	0.5	0	**1.5**
Easy to remove	0	0	0	0.5	X	0	**0.5**
Cost	0	0.5	0.5	1	1	X	**3**

3rd Tier

Goals	Includes terpenes	Flexible	Simple Design	**Score**
Includes terpenes	X	1	1	**2**
Flexible	0	X	0.5	**0.5**
Simple Design	0	0.5	X	**0.5**

APPENDIX F: Pairwise Comparison Chart – Kerry Walker

1st Tier

Goals	Increase wound Healing	Safety	Easy to Use	Practical to Make	**Score**
Increase wound Healing	X	0	0.5	0.5	**1.0**
Safety	1	X	1	1	**3**
Easy to Use	0.5	0	X	0.5	**1.0**
Practical to make	0	0	0.5	X	**0.5**

2nd Tier

Goals	Kills/Inhibits Microbes	Reliable	Durable	Easy to apply	Easy to Remove	Cost	**Score**
Kills/Inhibits Microbes	X	0.5	0.5	1	1	0.5	**3.5**
Reliable	0.5	X	0.5	1	0.5	0.5	**3.0**
Durable	0	0.5	X	1	0.5	0.5	**2.5**
Easy to apply	0	0	0	X	0	0	**0**
Easy to remove	0	0.5	0	1	X	0.5	**2.0**
Cost	0.5	0.5	0.5	1	0.5	X	**3.0**

3rd Tier

Goals	Includes terpenes	Flexible	Simple Design	**Score**
Includes terpenes	X	0	1	**1**
Flexible	1	X	1	**2**
Simple Design	0	0	X	**0**

APPENDIX G: Pairwise Comparison Chart – Lisa Szocik

1st Tier

Goals	Increase wound Healing	Safety	Easy to Use	Practical to Make	**Score**
Increase wound Healing	X	0.5	1	1	**2.5**
Safety	0.5	X	1	1	**2.5**
Easy to Use	0	0	X	0.5	**0.5**
Practical to make	0	0	0.5	X	**0.5**

2nd Tier

Goals	Kills/Inhibits Microbes	Reliable	Durable	Easy to apply	Easy to Remove	Cost	**Score**
Kills/Inhibits Microbes	X	0.5	0.5	1	1	0.5	**3.5**
Reliable	0.5	X	0.5	1	0.5	0.5	**3.0**
Durable	0.5	0.5	X	1	0	0.5	**2.5**
Easy to apply	0	0	0	X	0.5	0	**0.5**
Easy to remove	0	0.5	1	1	X	0.5	**3.0**
Cost	0.5	0.5	0.5	1	0.5	X	**3.0**

3rd Tier

Goals	Includes terpenes	Flexible	Simple Design	**Score**
Includes terpenes	X	1	1	**2**
Flexible	0	X	0.5	**0.5**
Simple Design	0	0.5	X	**0.5**

APPENDIX H: Pairwise Comparison Charts - Weighted Scores

1st Tier

Goals	Professor Pins Final Score	Gary Ostroff Final Score	Lisa Szocik Final Score	Kerry Walker Final Score	MQP Team Final Score	**Weighted Score**
Decrease wound healing time	2.5	2.5	2.5	1	2	**2.1**
Safety	2	1.5	2.5	3	3	**2.4**
Easy to Use	1	1	0.5	1	0	**0.7**
Practical to Make	0	0.5	0.5	0.5	1	**0.5**

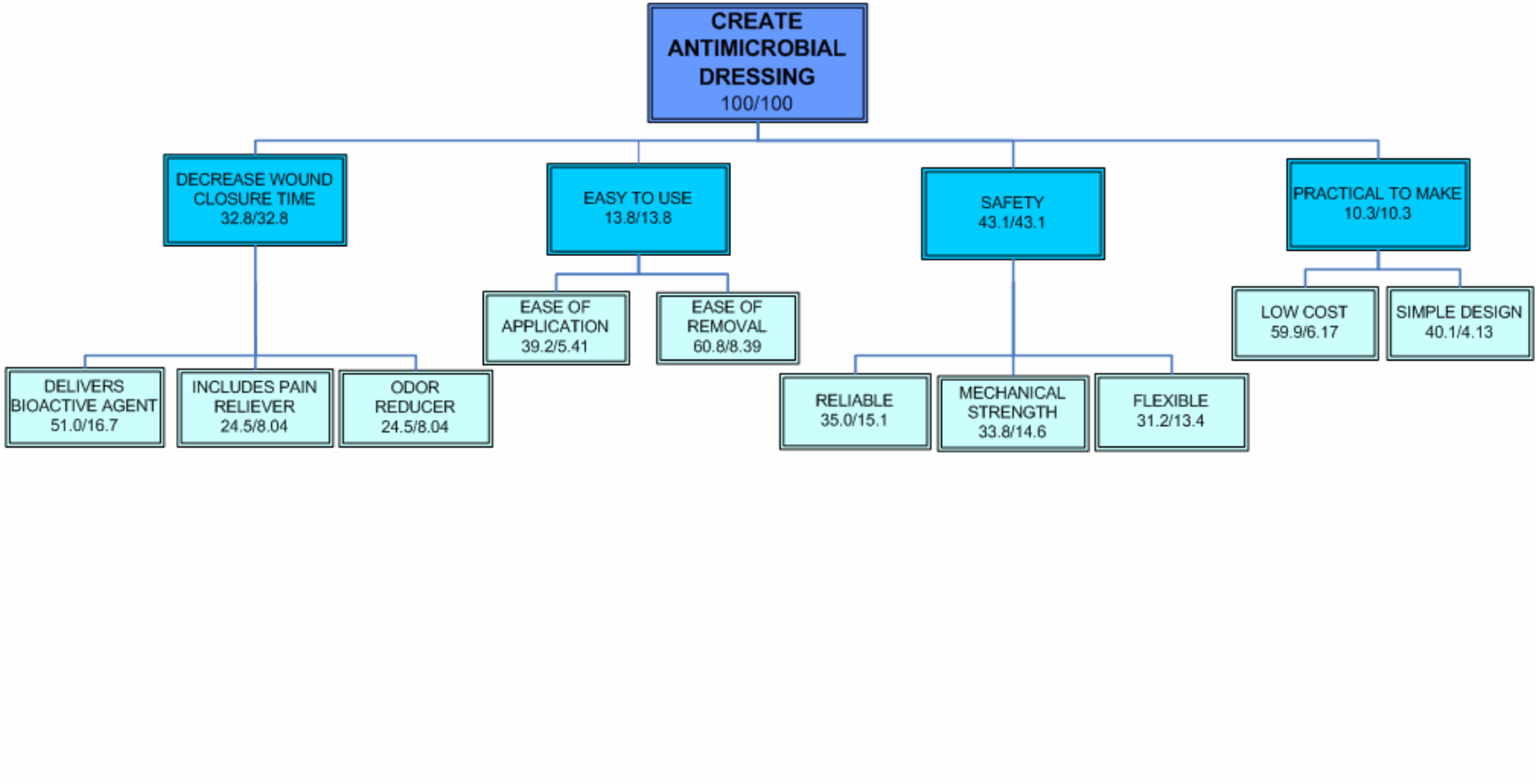
2nd Tier

Goals	Professor Pins Final Score	Gary Ostroff Final Score	Lisa Szocik Final Score	Kerry Walker Final Score	MQP Team Final Score	**Weighted Score**
Delivers bioactive agent	4.5	5	3.5	3.5	5	**4.3**
Reliable	2.5	3	3	3	4.5	**3.2**
Mechanical strength	1.5	2	2.5	2.5	4	**2.5**
Easy to apply	1	1.5	0.5	0	1.5	**0.9**
Easy to remove	1	0.5	2	2	1.5	**1.4**
Low cost	4.5	3	3	3	0	**3.3**

3rd Tier

Goals	Professor Pins Final Score	Gary Ostroff Final Score	Lisa Szocik Final Score	Kerry Walker Final Score	MQP Team Final Score	**Weighted Score**
Pain Reliever/ Odor Reducer	2	2	2	1	2	**1.8**
Flexible	0.5	0.5	0.5	2	1	**0.9**
Simple Design	0.5	0.5	0.5	0	0	**0.3**

APPENDIX I: Weighted Objectives Tree



APPENDIX J: Metrics

Increases wound healing

Objective: *Decrease wound closure time*
Units: Ranking the amount of wound healing on a scale of 1 (worst) to 3 (best)
Metric: Measure the degree of wound healing on a scale of 1 to 3, assign the following ratings to wound healing: 1 is a score for a wound in which the size of the wound gets larger and larger surface area, 2 for which the size of the wound remains the same, and 3 is the wound site gets smaller and shows signs of closure.

Objective: *Delivers bioactive agent*
Units: Ranking the effectiveness of the agents on a scale of 1 (worst) to 3 (best)
Metric: Measure the minimum inhibitory concentration (MIC) of the antimicrobial agents. On a scale of 1 to 3 assign the following ratings killing/inhibiting microbes: 1 has a higher MIC and the least inhibition, 2 has some inhibition, and 3 is the lowest MIC and high inhibition.

Objective: *Includes pain reliever*
Units: Ranking the amount of wound healing on a scale of 1 (no) or 3 (yes)
Metric: 1 is no it does not reduce pain, 2 is neutral or no indication, and 3 is yes it does reduce pain

Objective: *Odor reducer*
Units: Ranking the amount of wound healing on a scale of 1 (no) or 3 (yes)
Metric: 1 is no it does not reduce odor, 2 is neutral or no indication, and 3 is yes it does reduce odor

Safety

Objective: *Safety*
Units: Ranking the amount of safety on a scale of 1 (worst) to 3 (best)
Metric: Measure the amount safety in the wound by determining the level of wound healing. On a scale of 1 to 3, assign the following ratings to safety, 1 is the safety is worse than current models, 2 the safety is the same as current models, 3 the safety is better than the current models.

Objective: *Reliable*
Units: Ranking the amount of reliability on a scale of 1 (no) to 3 (yes)
Metric: Measure the amount of reliability in terms of effectiveness in killing pathogen in assays. On a scale of 1 to 3, assign the following ratings to reliable, 1 is low amounts of kill, 2 is average/substantial amounts of kill, and 3 is highest amounts of kill

Objective: *Mechanical strength*
Units: Ranking the amount of safety on a scale of 1 (worst) to 3 (best)

Metric: Measure the mechanical properties through Instron testing to ensure the dressing matches that of natural skin. On a scale of 1 to 3, assign the following ratings to durable, 1 is low mechanical properties as compared with skin, 2 is average/substantial properties compared with skin, 3 is favorable and accurate mechanical properties compared with skin

Easy to Use

Objective: *Easy to use*
Units: Ranking the amount of ease of use on a scale of 1 (worst) to 3 (best)
Metric: Measure the ease of use of the dressing through the steps required for preparation, amount of re-application, amount of re-activation. On a scale of 1 to 3, assign the following ratings to easy to use, 1 being more steps as compared to current models, and 2 being similar amount of steps compared to current models, and 3 being the least amount of steps as compared to current dressing.

Objective: *Easy to apply*
Units: Ranking the amount of ease of use on a scale of 1 (worst) to 3 (best)
Metric: Measure the ease of use of the dressing application. On a scale of 1 to 3, assign the following ratings to easy to apply, 1 being the longest amount of time, 3 being the shortest amount of time.

Objective: *Easy to remove*
Units: Ranking the amount of ease of removal on a scale of 1 (worst) to 3 (best)
Metric: Measure the ease of use of the dressing removal by observing the amount of disturbed tissue around the wound site. On a scale of 1 to 3, assign the following ratings to easy to remove, 1 being much tissue removed, 3 being minimal disturbance of tissue (no tissue on dressing).

Objective: *Flexible*
Units: Ranking the amount of flexibility on a scale of 1 (worst) to 3 (best)
Metric: Measure the amount of mechanical testing and flexibility using the Instron machine. On a scale of 1 to 3, assign the following ratings to flexibility, 1 being the mechanical flexibility does not match that of skin, 2 being the mechanical flexibility is within a few units to that of the skin, and 3 being the mechanical flexibility is representative of the units of the skin.

Practical to Make

Objective: *Practical to make*
Units: Ranking the cost on a scale of 1 (worst) to 3 (best)
Metric: Measure the production of our bandage versus that of the other silver dressings on the market. On a scale of 1 to 3, assign the following ratings to practical to make, 1 being harder than current models, require multiple steps, 2 being equal steps and materials to the current models, 3 being easier with less steps and materials than the current models

Objective: *Low Cost*
Units: Ranking the cost on a scale of 1 (worst) to 3 (best)
Metric: Measure the cost of our bandage versus that of the other silver dressings on the market. On a scale of 1 to 3, assign the following ratings to cost, 1 being more expensive than current models, 2 being equal to the current models, 3 being less expensive than the current models

Objective: *Simple Design*
Units: Ranking the simplicity of the design on a scale of 1 (worst) to 3 (best)
Metric: Measure the amount of simplicity of the dressing by measuring the amount of materials used compared with the current models. On a scale of 1 to 3, assign the following ratings to a simple design, 1 being more materials than current models, 2 being the same amount of materials than current models, 3 being fewer materials than current models.

APPENDIX K: Metric Justifications

Decreases wound closure time: The dressing must be effective in wound healing and will be evaluated by determining if the is remaining the same, or getting worse. Observations such as inflammation, swelling, color of skin, and amount of exudate are used to assess the healing process. A skin color ranging from black to pink indicates a severely infected wound to a healing wound, respectively. To examine if the infection is spreading, the inflammation around the wound is measured and the length and width and overall area of the wound is recorded at each observation. The status of the wound healing can also be measured by examining the amount of exudate, and the color and viscosity of the exudate.

Delivers bioactive agent: The dressing must deliver a bioactive agent to the wound site to act as an antimicrobial to fight against wound pathogens. A swab technique will be used to determine the volume and type of bacteria in the exudate. The surface contamination is not a problem for the swabbing technique because the dressing needs to be effective against a broad range of microbes. The antimicrobial agents will be considered effective if they kill and inhibits the pathogens as indicated by the swab.

Includes pain reliever: The dressing must deliver pain relief during the duration of time of usage. The incorporation of pain relievers will make the dressing more effective against a broader range of wound pathogens. Agitated behavior will be observed for pain and distress.

Reduces odor: The dressing must reduce odor during the duration of time of usage. The incorporation of an odor reducer will make the dressing superior to other models.

Safety: The wound dressing must be safe for the user, in that it must not hinder the wound healing process by providing an effective barrier from outside pathogens as well as hold in moisture to facilitate wound healing. The dressing should limit rashes and secondary infection to avoid allergic reaction or immune response. The physical state of the animal and the wound healing level will be assessed for a level of safety.

Reliable: The dressing must be reliable, which can be determined by the amount of bacteria to assess the efficacy of the antimicrobial agents. Growth inhibition and cidal assays will be completed to test the efficacy of the antimicrobial agents.

Mechanical strength: The dressing must exhibit strengths and flexibility similar to that of the skin.

Easy to use: The dressing would be ranked according to attention through observation, reapplication and remoistening. Ideally the dressing would require minimal observation, no re-application and no further re-moistening after initial application, which would receive the highest score.

Easy to apply: The most effective dressing would be available for use directly out of the package and can be applied directly to the wound without any additional time for application. For example, Acticoat™ is shipped sterile and in tact and needs to be activated through adding water to the dressing.

Easy to remove: The dressing would not cause any additional stresses on healthy tissue surrounding the wound site as well as in the wound bed. For example, many of the current dressings consist of a two-component system of which the outer layer is composed of a breathable fabric, which should not adhere to the skin and cause further tissue damage.

Flexible: This dressing should not constrict any normal motions or movements of the skin. This dressing should exemplify the normal longitudinal stresses, transverse stresses and flexure properties of the skin.

Practical to make: The dressing is not a hassle to produce and could be scaled up for larger quantities of production. Ideally this dressing would use less or equivalent numbers of materials as compared to the other dressings on the market. This dressing must also be easily sterilized according to materials used, for example, certain materials will require easier sterilization processes than others.

Low Cost: The dressing after production and care is around the ballpark of the current silver dressings on the market. Ideally our dressing would require less dressing changes and less home care providing an overall cheaper dressing. For example, with the current wound dressing Silverlon, it is currently the cheapest on the market with a total cost of ~$55 including dressing changes and total nursing cost over a 1 week period (Argentum Medical, 2004).

Simple Design: The device consists of an effective amount of layers, each serving aiding in providing a wound healing property to the dressing. For example, most dressings on the market such as Acticoat™ and Actisorb Silver 220™ which consist of a breathable non-woven layer, followed by one layer for transport of oxygen and fluid management to keep the wound healthy and provide a desirable environment for wound healing, and the main component being the antimicrobial layer which consists of silver. These layers are all manufactured together and do not require any additional materials or adhesives to keep the dressing compact.

APPENDIX L: Silver Protocol

Eden - Produce free and YP encapsulated Silver Nitrate, Silver Chloride and Silver Thiosulfate

Purpose: To produce free and YP encapsulated silver nitrate, silver chloride and silver thiosulfate

Materials:
1. Silver nitrate (BDH 99.5%)
2. 5M Sodium chloride
3. YGP - Levpan, YGMP - SAF-Mannan (Biospringer)

Methods:

Prepare silver nitrate

1. Use BDH silver nitrate from bottle. Keep dark to prevent light induced oxidation

Prepare YGP and YGMP silver nitrate

1. Prepare 0.5 mg silver nitrate/ ul water - keep dark
2. Weigh 400 mg YGP and YGMP into plastic tubes
3. Add 250 ul silver nitrate per tube
4. Mix to obtain crumbly dough in reduced light
5. Freeze, lyophilize in the dark the remaining material
6. Store dry product at room temperature in the dark
7. Yield - YGP $AgNO_3$ (_____mg) 55.5% $AgNO_3$ w/w
   -YGMP $AgNO_3$ (_____ mg)
   55.5% $AgNO_3$ w/w

Prepare YGP and YGMP silver chloride

1. Prepare 0.5 mg silver nitrate/ ul water - keep dark
2. Weigh 100 mg LEV and SAF-Mannan into round bottom polypropylene tubes
3. Add 250 ul silver nitrate per tube
4. Mix to obtain crumbly dough
5. Add 10 ml 5M NaCl with rapid mixing
6. Collect insolubles by cfg
7. Wash 3X with cold water to remove NaCl
8. Wash 3X with ethanol
9. Wash 2x with acetone
10. Dry in the dark - Cycle 1 load contains 55.5% silver nitrate silver equivalents w/w
11. Store dry product at room temperature in the dark
12. Yield - YGP AgCl (_____ mg) 55.5% $AgNO_3$ w/w
    -YGMP AgCl (_____ mg)
    55.5% $AgNO_3$ w/w

APPENDIX M: YP-Silver Assay

Eden - Antimicrobial Properties of YP-Silver Chloride, YP-Silver Nitrate, and YP-ET vs. Select Wound Pathogens

Purpose: To test YP-silver salt formulations and YP-ET against Sa, Pa, Ef

Materials

Tube	description	Stock Materials	MIC ug/ml
1	YP-Silver Chloride	Prepare 1 mg/ml in BHI and LB	
2	YP-Silver Nitrate	Prepare 1 mg/ml in BHI and LB	
3	YP-ET	Stock at 40 mg/ml terpene. Dilute 1/10 in BHI and LB to 4 mg/ml terpene	

Microbes	Medium	Temp	Conditions
S. aureus	LB	37C	aerobic
E. faecalis	BHI	37C	aerobic
P. aeriginosa	LB	37C	aerobic

Method:

1. Prepare silver and terpene formulations in BHI and LB as indicated above
2. Grow microbes under conditions described above. Dilute 1:1000, or as appropriate in medium for plate inoculation
3. Set up Primary Growth Inhibitory MIC assay as follows
   a. Add 100 ul of indicated medium (BHI or LB) to indicated wells.
   b. Add 100 ul of YP-silver or YP-terpene test dilutions in indicated medium (number keyed to list above) to Row A wells
      Add 100 ul indicated medium to well A10, A11 and A12

No growth
rtial Inibition
Growth

   c. Perform 1:1 dilutions by transferring 100 ul serially from Row A to Rows B, etc...as indicated.
   d. Add 100 ul 1:1000 dil of indicated microbes to all wells, except 10A-H and 11A-H. Add 100 ul of indicated sterile medium to 10A-H and 11A-H.
   e. Read To A620 in microplate reader. Incubate plate at 37C o/n under indicated growth conditions. Read A620 in microplate reader.
   f. Determine MIC = ≥75% inhibition of growth
   g. Add 50 ul 50 ug/ml Resazarin. Incubate for 30 min. Score color change. Image on scanner

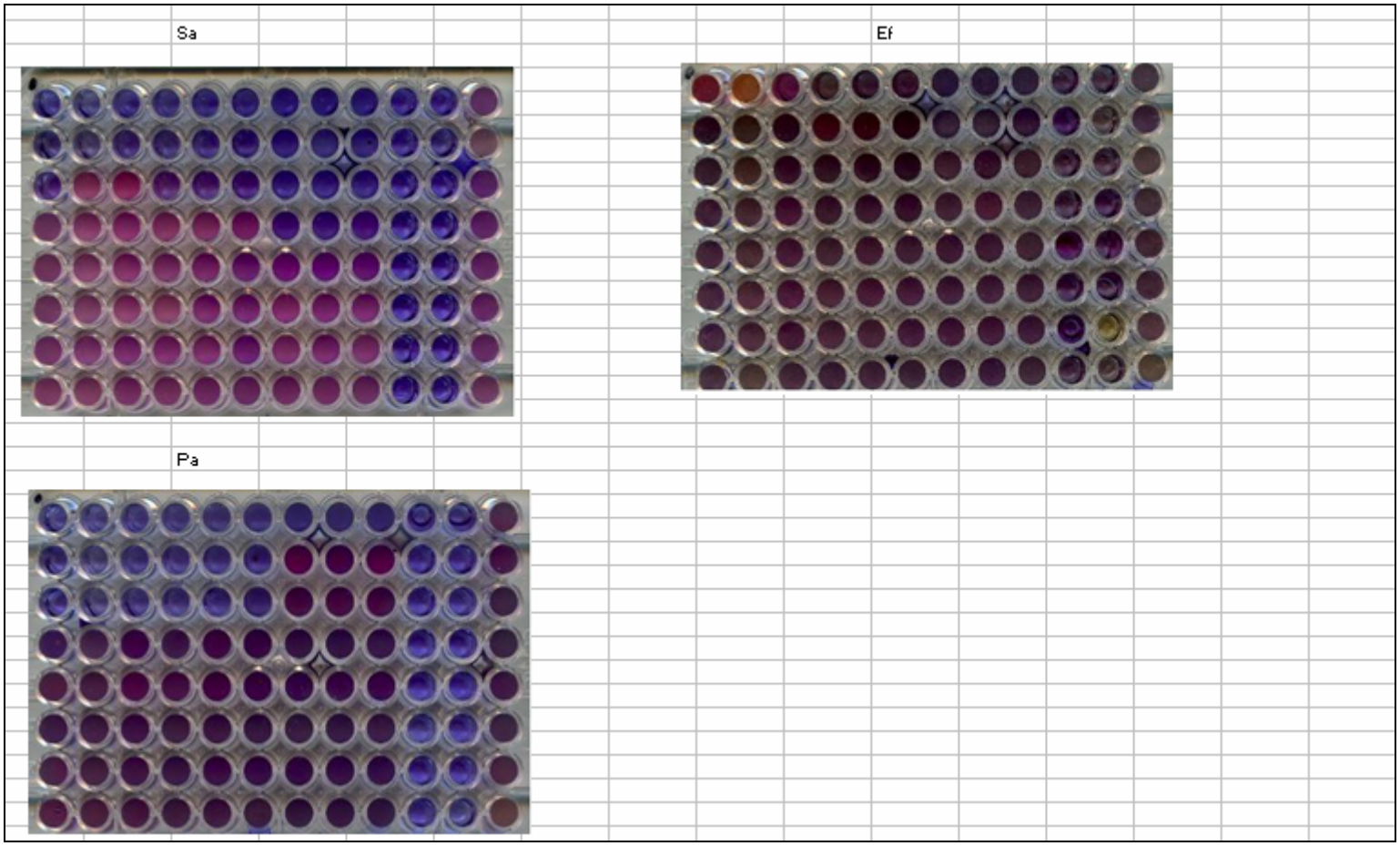
Plate A	Test Formulation	**1**	**1**	**1**	**2**	**2**	**2**	**3**	**3**	**3**	-	-	+	
Sa	Microbe	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	**LB**	**LB**	*Sa*	
LB	Tube										Control	Control	Control	
Silver	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12	GI
ug/ml	ug/ml	Row	**62.5**	**125**	**125**	**62.5**	**62.5**	**62.5**	**250**	**250**	**250**	-	-	+	**MIC**
250	1000	A	-0.017	-0.032	-0.03	-0.006	-0.01	-0.011	-0.032	-0.034	-0.025	-0.002	0.003	0.671	(ppm)
125	500	B	-0.041	-0.049	-0.032	0.003	0.002	-0.001	0.004	0.018	0.014	0.005	-0.002	0.594	
62.5	250	C	-0.008	0.377	0.387	0.087	0.07	0.054	0.009	0.026	0.013	0.004	0	0.619	
31.25	125	D	0.55	0.436	0.45	0.473	0.476	0.516	0.37	0.318	0.363	0.004	0.007	0.619	
16.125	62.5	E	0.597	0.473	0.485	0.503	0.506	0.522	0.491	0.484	0.495	0.004	0.001	0.617	
8.06	31.25	F	0.581	0.475	0.473	0.506	0.498	0.528	0.529	0.539	0.531	0.003	0.004	0.62	
4.03	16.125	G	0.587	0.467	0.486	0.47	0.478	0.509	0.526	0.53	0.533	0.003	0.004	0.626	
2.02	8.06	H	0.64	0.608	0.615	0.614	0.621	0.63	0.659	0.638	0.635	0.005	0.007	0.667	
		Discard	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	
													Avg + control	0.6	
													20% cut-off	0.125825	

Plate B	Test Formulation		**1**	**1**	**1**	**2**	**2**	**2**	**3**	**3**	**3**	-	-	+	
Ef		Microbe	***Ef***	***Ef***	***Ef***	***Ef***	***Ef***	***Ef***	***Ef***	***Ef***	***Ef***	**BHI**	**BHI**	***Ef***	
BHI		Tube										Control	Control	Control	
Silver	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12	GI
ug/ml	ug/ml	Row	**>250**	**>250**	**250**	**250**	**250**	**250**	**500**	**500**	**500**	-	-	+	**MIC**
250	1000	A	0.668	0.451	0.052	-0.024	-0.002	-0.028	-0.028	-0.029	-0.038	0.001	0.002	0.55	(ppm)
125	500	B	0.662	0.69	0.691	0.63	0.722	0.557	0.011	0.02	0.01	0.001	0.002	0.516	
62.5	250	C	0.581	0.615	0.609	0.624	0.644	0.646	0.464	0.463	0.467	0.006	0.002	0.52	
31.25	125	D	0.529	0.548	0.548	0.558	0.557	0.571	0.49	0.507	0.498	0.005	0.006	0.52	
16.125	62.5	E	0.521	0.532	0.533	0.533	0.534	0.531	0.503	0.512	0.511	0.001	0.001	0.55	
8.06	31.25	F	0.523	0.532	0.525	0.527	0.529	0.531	0.505	0.513	0.511	0.001	0.004	0.549	
4.03	16.125	G	0.528	0.539	0.524	0.524	0.525	0.529	0.515	0.518	0.511	0.003	0.005	0.562	
2.02	8.06	H	0.57	0.556	0.553	0.531	0.545	0.545	0.532	0.516	0.524	0.006	0.006	0.574	
		Discard	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	
													Avg + control	0.542625	
													20% cut-off	0.108525	

Plate C	Test Formulation		**1**	**1**	**1**	**2**	**2**	**2**	**3**	**3**	**3**	-	-	+	
Pa		Microbe	***Pa***	***Pa***	***Pa***	***Pa***	***Pa***	***Pa***	***Pa***	***Pa***	***Pa***	**LB**	**LB**	***Pa***	
LB		Tube										Control	Control	Control	
Silver	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12	GI
ug/ml	ug/ml	Row	**62.5**	**62.5**	**62.5**	**62.5**	**62.5**	**62.5**	**500**	**500**	**500**	-	-	+	**MIC**
250	1000	A	-0.048	-0.059	-0.061	-0.027	-0.035	-0.048	-0.05	-0.046	-0.053	0.003	0.002	0.902	(ppm)
125	500	B	-0.042	-0.057	-0.052	-0.014	-0.011	-0.009	0.423	0.428	0.518	0.002	0.002	0.915	
62.5	250	C	-0.015	-0.016	-0.014	-0.004	0.001	0.176	0.548	0.531	0.552	-0.009	0.003	1.006	
31.25	125	D	0.677	0.653	0.912	0.56	0.857	0.76	0.692	0.663	0.736	0.005	0.005	0.944	
16.125	62.5	E	0.884	0.854	0.827	0.807	0.77	0.738	0.589	0.683	0.666	0	0	0.92	
8.06	31.25	F	0.793	0.777	0.769	0.73	0.739	0.73	0.705	0.7	0.685	0.004	0.001	0.934	
4.03	16.125	G	0.884	0.878	0.798	0.766	0.746	0.727	0.734	0.727	0.713	0.001	0.002	1.066	
2.02	8.06	H	1.076	1.08	0.97	0.889	0.92	0.891	0.842	0.771	0.803	0.003	0.005	1.154	
		Discard	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	
													Avg + control	0.380125	
													20% cut-off	0.136025	

MIC (ppm)

	Mixture	***Sa***	***Ef***	***Pa***
1		**125**	**>250**	**62.5**
2		**62.5**	**250**	**62.5**
3		**250**	**500**	**500**


Sa
Ef
Pa

APPENDIX N: Synergy Experiment- *Enterococcus faecalis*

MQP- Evaluate Synergy between Silver Compounds and YP- ET Against Ef

PURPOSE: To determine if there is synergy between terpene formulations and silver formulations

MATERIALS	**Ef**		wt (mg)	vol (ul)	conc	
	1. YP-AgNO3 - prepare 1 mg/ml in BHI	Prepared powder form	8	2000	4 mg/ml	Input weight in mg and the formula will calculate the volume of LB to add in ul
	use material you made					
	2. YP-AgCl - prepare 1 mg/ml in BHI	Prepared powder form	8	2000	4 mg/ml	
	use material you made					
	3. YP-ET- prepare 25mg/ml in BHI	Commercial product	100	4000	25 mg/ml w/w = 4 mg/ml terpene	
	use commerical product YP-2ESL	160 mg/ml terpene				

METHOD:

PLATE 1 **YP-AgNO3 + YP- ET**

AgNO3 dilution

1. Add 100 ul of BHI:DMEM to all wells
2. Add 100 ul of **YP-AgNO3 (1 mg/ml)** to column 1, rows A- H

Transfer 100 ul from column 1 to column 2, column 2 to 3, etc. Discard 100 ul from column 11. Add nothing to column 12.

	Silver concen	**1000.00**	**500.00**	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0**
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
1000	A												
500	B												
250	C												
125	D												
62.5	E												
31.25	F												
15.625	G												
0	H												

ET dilution

3. Add 100 ul of **YP-ET (4mg/ml)** to row A, column 1-11.

Transfer 100 ul from row A to row B, row B to row C, etc. Discard 100 ul from row G. Add nothing to row H

Silver dilution factor		**1000.00**	**500.00**	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0**
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
1000	A												
500	B												
250	C												
125	D												
62.5	E												
31.25	F												
15.625	G												
0	H												

6. Add 100 ul 1/1000 diluted Ef to all wells in the plate. 45 ul/45 ml= BHI
7. Read To A620 in microplate reader.
8. Incubate plates at 37C o/n.
9. Read A620 in microplate reader.
10. Add 50 ul resazurin - incubate 1 hr. Scan color change.
11. Determine MIC = >75% inhibition of growth

PLATE 2 **YP-AgNO3 + YP- ET**

Repeat plate 1

PLATE 3 **YP- AgCl + YP-ET**

AgCl dilution

1. Add 100 ul of BHI:DMEM to all wells
2. Add 100 ul of **YP-AgCl (1 mg/ml)** to column 1, rows A- H

Transfer 100 ul from column 1 to column 2, column 2 to 3, etc. Discard 100 ul from column 11. Add nothing to column 12.

Silver dilution factor		**1000.00**	**500.00**	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0**
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
1000	A												
500	B												
250	C												
125	D												
62.5	E												
31.25	F												
15.625	G												
0	H												

ET dilution

3. Add 100 ul of **YP-ET (4mg/ml)** to row A, column 1-12.

Transfer 100 ul from row A to row B, row B to row C, etc. Discard 100 ul from row G. Add nothing to row H

Silver dilution factor		**1000.00**	**500.00**	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0**
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
1000	A												
500	B												
250	C												
125	D												
62.5	E												
31.25	F												
15.625	G												
0	H												

6. Add 100 ul 1/1000 diluted Ef to all wells in the plate. 45 ul/45 mls BHI
7. Read To A620 in microplate reader.
8. Incubate plates at 37C o/n.
9. Read A620 in microplate reader.
10. Add 50 ul resazurin - incubate 1 hr. Scan color change.
11. Determine MIC = >75% inhibition of growth

PLATE 4	YP- AgCl + YP-ET												
	Repeat Plate 3												
RESULTS:													
PLATE 1	Silver dilution factor		**1000.00**	**500.00**	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0**
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	1000	A	-0.055	-0.065	-0.074	-0.062	-0.058	-0.079	-0.033	-0.034	-0.031	-0.052	-0.024	-0.062
	500	B	-0.027	-0.006	-0.016	-0.004	-0.012	-0.011	0.024	0.122	-0.005	-0.002	0.244	0.538
	250	C	-0.027	0.002	-0.01	0.002	0.4	0.397	0.398	0.431	0.424	0.408	0.609	0.548
	125	D	-0.01	0.014	0.006	0.375	0.453	0.481	0.498	0.547	0.555	0.579	0.812	0.849
	62.5	E	-0.001	0.007	0.01	0.535	0.551	0.534	0.501	0.62	0.752	0.732	0.901	0.937
	31.25	F	-0.013	0.007	0.001	0.529	0.53	0.536	0.563	0.569	0.771	0.836	0.879	0.935
	15.625	G	-0.007	0	-0.007	0.579	0.553	0.525	0.553	0.559	0.773	0.74	0.93	0.957
	0	H	-0.003	-0.002	-0.009	0.592	0.602	0.57	0.548	0.614	0.777	0.839	0.972	0.883
													Avg	0.698125
														0.1745
PLATE 2	Silver dilution factor		**1000.00**	**500.00**	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0**
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	1000	A	-0.068	-0.047	-0.067	-0.062	-0.067	-0.082	-0.076	-0.06	-0.077	-0.066	-0.072	-0.079
	500	B	-0.025	0.002	0.001	0.001	-0.004	-0.011	0.004	0.054	-0.011	0.001	0.085	0.57
	250	C	-0.035	-0.005	-0.009	0.049	0.438	0.393	0.419	0.425	0.392	0.378	0.488	0.527
	125	D	-0.013	0.004	0.002	0.553	0.511	0.454	0.472	0.472	0.445	0.585	0.757	0.817
	62.5	E	0.008	-0.003	0	0.567	0.498	0.509	0.484	0.538	0.469	0.567	0.752	0.917
	31.25	F	-0.003	-0.002	-0.001	0.579	0.52	0.508	0.531	0.514	0.498	0.569	0.843	0.947
	15.625	G	-0.001	-0.008	0	0.533	0.539	0.514	0.537	0.613	0.49	0.698	0.831	0.896
	0	H	0.008	-0.008	-0.005	0.607	0.602	0.563	0.546	0.556	0.523	0.562	0.809	1.004
													avg	0.699875
														0.17497

PLATE 3	Silver dilution factor		1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	1000	A	-0.103	-0.075	-0.098	-0.028	-0.012	-0.092	-0.078	-0.063	-0.048	-0.109	0.012	-0.098
	500	B	0.336	0.181	0.134	0.127	0.144	0.026	-0.033	0.163	-0.02	0.056	0.118	0.566
	250	C	-0.152	-0.046	-0.005	-0.027	0.099	0.416	0.436	0.378	0.402	0.376	0.447	0.501
	125	D	-0.086	-0.129	-0.015	-0.002	0.528	0.454	0.518	0.523	0.552	0.562	0.838	0.801
	62.5	E	-0.1	-0.05	-0.062	0.515	0.508	0.492	0.512	0.526	0.584	0.536	0.88	0.849
	31.25	F	-0.161	-0.105	-0.111	0.327	0.533	0.54	0.518	0.541	0.589	0.69	0.864	0.855
	15.625	G	-0.043	0.121	0.049	0.056	0.579	0.518	0.546	0.539	0.696	0.615	0.87	0.862
	0	H	-0.026	-0.101	-0.087	-0.042	0.329	0.577	0.58	0.569	0.618	0.693	0.876	0.946
													Avg	0.66025
														0.16505

PLATE 4	Silver dilution factor		1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	1000	A	-0.053	-0.072	-0.081	-0.139	-0.137	-0.115	0.007	-0.022	-0.042	0.054	-0.082	-0.035
	500	B	-0.404	-0.203	-0.153	-0.164	-0.169	-0.045	0.07	0.034	0.028	0.039	0.064	0.208
	250	C	0.092	0.055	-0.004	0.033	0.054	0.363	0.399	0.409	0.387	0.424	0.406	0.419
	125	D	0.017	0.119	-0.015	0.038	0.482	0.488	0.404	0.368	0.463	0.587	0.713	0.757
	62.5	E	-0.013	0.006	0.064	0.586	0.546	0.526	0.507	0.546	0.619	0.657	0.843	0.854
	31.25	F	0.061	0.05	0.114	0.568	0.524	0.528	0.508	0.57	0.535	0.742	0.864	0.911
	15.625	G	-0.05	-0.183	-0.122	0.022	0.549	0.551	0.539	0.563	0.602	0.727	0.888	1.011
	0	H	-0.037	-0.006	-0.025	-0.046	0.056	0.631	0.593	0.552	0.556	0.704	0.731	0.943
													Avg	0.6335
														0.15837

	Plate 1	Plate 2	Plate 3	Plate 4
MIC Terpene	125	125	62.5	62.5
MIC Silver Nitrate	250	250	-	-
MIC Silver Chl	-	-	250	250

PLATE 1

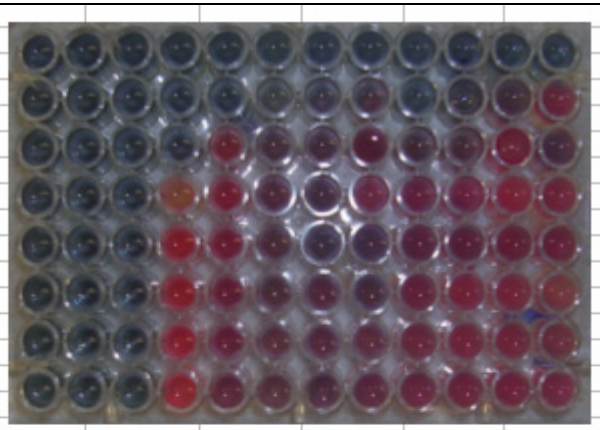


PLATE 3

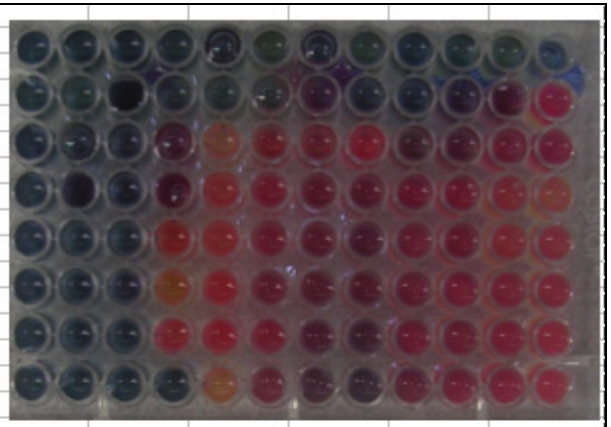


PLATE 2

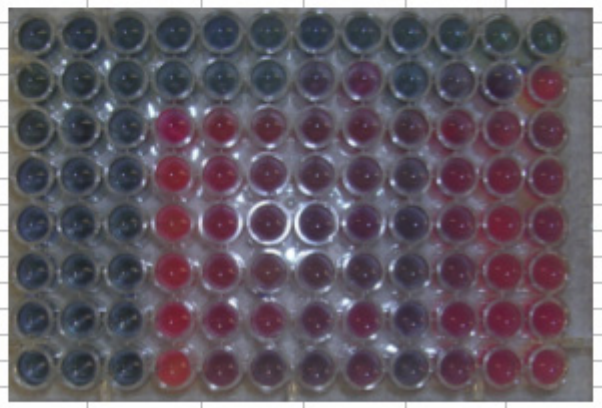
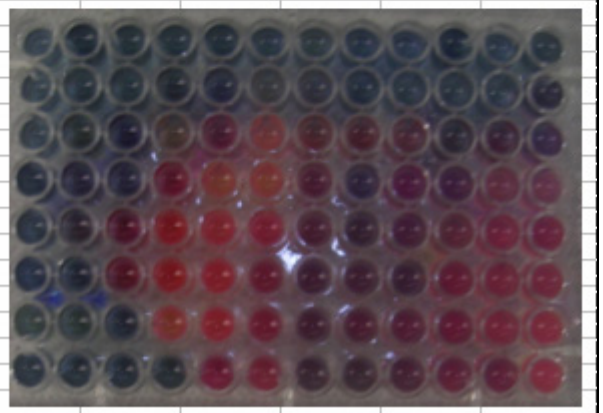


PLATE 4



APPENDIX O: Synergy Experiment- *Pseudomonas aeruginosa*

MQP- Evaluate Synergy between Silver Compounds and YP- ET Against Pa 20606

PURPOSE: To determine if there is synergy between terpene formulations and silver formulations

MATERIALS	**Pa**		wt (mg)	vol (ul)	conc	
	1. YP-AgNO3 - prepare 1 mg/ml in LB	Prepared powder form	2	2000	1 mg/ml	Input weight in mg and the formula will calculate the volume of LB to add in ul
	use material you made					
	2. YP-AgCl - prepare 1 mg/ml in LB	Prepared powder form	2	2000	1 mg/ml	
	use material you made					
	3. YP-ET- prepare 25mg/ml in LB	Commercial product	400	8000	25 mg/ml w/w = 4 mg/ml terpene	
	use commerical product YP-2ESL	160 mg/ml terpene			**8mg/ml**	

METHOD:

PLATE 1 **YP-AgNO3 + YP- ET**

AgNO3 dilution

1. Add 100 ul of LB:DMEM to all wells
2. Add 100 ul of **YP-AgNO3 (1 mg/ml)** to column 1, rows A- H

Transfer 100 ul from column 1 to column 2, column 2 to 3, etc. Discard 100 ul from column 11. Add nothing to column 12.

	Silver concen	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0.49**	**0.24**	**0**
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
2000	A												
1000	B												
500	C												
250	D												
125	E												
62.5	F												
31.25	G												
0	H												

ET dilution

3. Add 100 ul of **YP-ET (4mg/ml)** to row A, column 1-11.

Transfer 100 ul from row A to row B, row B to row C, etc. Discard 100 ul from row G. Add nothing to row H

Silver dilution factor		**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0.49**	**0.24**	**0**
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
2000	A												
1000	B												
500	C												
250	D												
125	E												
62.5	F												
31.25	G												
0	H												

6. Add 100 ul 1/1000 diluted Pa to all wells in the plate. 45 ul/45 mls LB
7. Read To A620 in microplate reader.
8. Incubate plates at 37C o/n.
9. Read A620 in microplate reader.
10. Add 50 ul resazurin - incubate 1 hr. Scan color change.
11. Determine MIC = >75% inhibition of growth

PLATE 2 **YP-AgNO3 + YP- ET**

Repeat plate 1

PLATE 3 **YP- AgCl + YP-ET**

AgCl dilution

1. Add 100 ul of LB:DMEM to all wells
2. Add 100 ul of **YP-AgCl (1 mg/ml)** to column 1, rows A- H

Transfer 100 ul from column 1 to column 2, column 2 to 3, etc. Discard 100 ul from column 11. Add nothing to column 12.

Silver dilution factor		**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0.49**	**0.24**	**0**
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
2000	A												
1000	B												
500	C												
250	D												
125	E												
62.5	F												
31.25	G												
0	H												

ET dilution

3. Add 100 ul of **YP-ET (4mg/ml)** to row A, column 1-12.

Transfer 100 ul from row A to row B, row B to row C, etc. Discard 100 ul from row G. Add nothing to row H

Silver dilution factor		**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0.49**	**0.24**	**0**
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
2000	A												
1000	B												
500	C												
250	D												
125	E												
62.5	F												
31.25	G												
0	H												

6. Add 100 ul 1/1000 diluted Pa to all wells in the plate. 45 ul/45 mls LB
7. Read To A620 in microplate reader.
8. Incubate plates at 37C o/n.
9. Read A620 in microplate reader.
10. Add 50 ul resazurin - incubate 1 hr. Scan color change.
11. Determine MIC = >75% inhibition of growth

RESULTS:													
PLATE 1		Silver concen	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0.49**	**0.24**	**0**
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	2000	A	-0.153	-0.16	-0.153	-0.178	-0.182	-0.224	-0.147	-0.143	-0.155	-0.147	-0.163	-0.145
	1000	B	-0.008	0.006	0.013	0.014	0.016	0.008	-0.009	-0.018	-0.033	-0.046	-0.031	-0.045
	500	C	0.008	0.016	0.017	0.025	0.016	-0.009	-0.021	0.232	0.318	0.301	0.317	0.324
	250	D	0.002	0.024	0.014	-0.002	0.232	0.364	0.406	0.449	0.442	0.417	0.425	0.499
	125	E	-0.006	0.005	0.005	0.036	0.667	0.573	0.513	0.537	0.527	0.591	0.493	0.588
	62.5	F	-0.022	-0.007	-0.001	0.27	0.614	0.563	0.611	0.559	0.558	0.548	0.577	0.727
	31.25	G	-0.017	-0.007	0.003	0.013	0.696	0.621	0.593	0.605	0.625	0.619	0.62	0.697
	0	H	-0.016	-0.013	-0.002	0.009	0.452	0.758	0.681	0.739	0.736	0.76	0.797	0.833
													Avg	0.43475
														0.1087
PLATE 2		Silver concen	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0.49**	**0.24**	**0**
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	2000	A	-0.181	-0.206	-0.208	-0.216	-0.173	-0.178	-0.221	-0.203	-0.222	-0.221	-0.209	-0.22
	1000	B	-0.037	-0.004	0.002	0.004	0.001	-0.013	-0.003	-0.078	-0.038	-0.06	-0.039	-0.069
	500	C	0.004	0.025	0.03	0.022	0.013	-0.009	0.346	0.201	0.301	0.24	0.31	0.292
	250	D	0.002	0.011	0.025	0.028	0.301	0.387	0.468	0.402	0.442	0.438	0.486	0.564
	125	E	-0.015	-0.002	0.001	0.006	0.594	0.572	0.571	0.499	0.6	0.527	0.623	0.587
	62.5	F	-0.01	-0.012	-0.002	0.003	0.756	0.556	0.573	0.629	0.576	0.679	0.583	0.738
	31.25	G	-0.019	-0.017	-0.001	0.002	0.712	0.649	0.604	0.586	0.615	0.589	0.626	0.799
	0	H	-0.02	-0.025	-0.008	-0.002	0.47	0.768	0.712	0.749	0.766	0.732	0.67	0.701
													Avg	0.424
														0.106

PLATE 3	Silver dilution factor		250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0.49	0.24	0
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	2000	A	-0.139	-0.119	-0.102	-0.131	-0.107	-0.21	-0.155	-0.24	-0.129	-0.137	-0.114	-0.142
	1000	B	0.002	0.044	0.045	0.051	0.036	-0.034	0.031	-0.023	0.011	0.029	-0.027	-0.019
	500	C	0.009	0.042	0.045	0.047	0.033	-0.02	0.006	0.226	0.253	0.373	0.277	0.338
	250	D	0.005	0.018	0.021	0.373	0.395	0.411	0.434	0.425	0.463	0.493	0.479	0.522
	125	E	-0.047	0.002	0.011	0.666	0.59	0.531	0.557	0.538	0.664	0.605	0.614	0.613
	62.5	F	-0.035	-0.012	0.009	0.781	0.62	0.534	0.544	0.627	0.563	0.587	0.659	0.705
	31.25	G	-0.047	-0.017	-0.003	0.752	0.691	0.5	0.613	0.578	0.586	0.599	0.614	0.75
	0	H	-0.043	-0.023	-0.007	0.768	0.798	0.58	0.726	0.653	0.725	0.748	0.739	0.722
													Avg	0.436125
														0.109

PLATE 4	Silver dilution factor		250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0.49	0.24	0
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	2000	A	-0.201	-0.145	-0.156	-0.192	-0.19	-0.224	-0.193	-0.272	-0.186	-0.192	-0.252	-0.191
	1000	B	-0.08	0.014	0.007	0.016	0.029	-0.097	0.005	-0.022	-0.021	0.045	0.03	0.025
	500	C	0.015	0.038	0.043	0.036	0.003	-0.055	0.347	0.192	0.314	0.306	0.314	0.394
	250	D	-0.003	0.022	0.015	0.339	0.498	0.362	0.419	0.38	0.405	0.379	0.391	0.505
	125	E	-0.03	0.008	0.01	0.673	0.606	0.592	0.575	0.539	0.64	0.603	0.331	0.603
	62.5	F	-0.026	-0.014	0.012	0.761	0.633	0.474	0.564	0.593	0.611	0.556	0.554	0.658
	31.25	G	-0.037	-0.03	-0.009	0.372	0.669	0.54	0.594	0.607	0.672	0.648	0.707	0.722
	0	H	-0.042	-0.021	-0.008	0.715	0.774	0.602	0.729	0.696	0.659	0.705	0.596	0.481
													Avg	0.399625
														0.0999

	Plate 1	Plate 2	Plate 3	Plate 4
MIC Terpene	500	500	500	500
MIC Silver Nitrate	31.25	31.25	-	-
MIC Silver Chl	-	-	62.5	62.5

PLATE 1

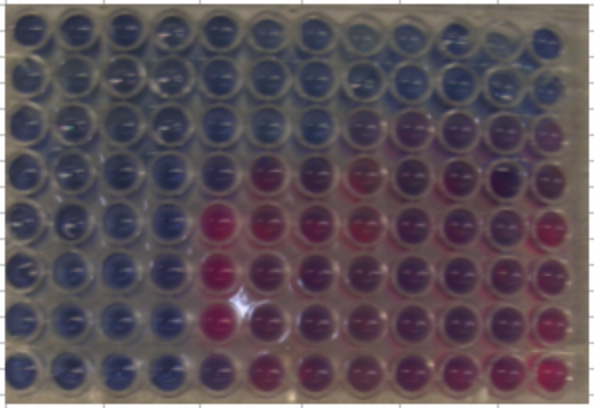


PLATE 3

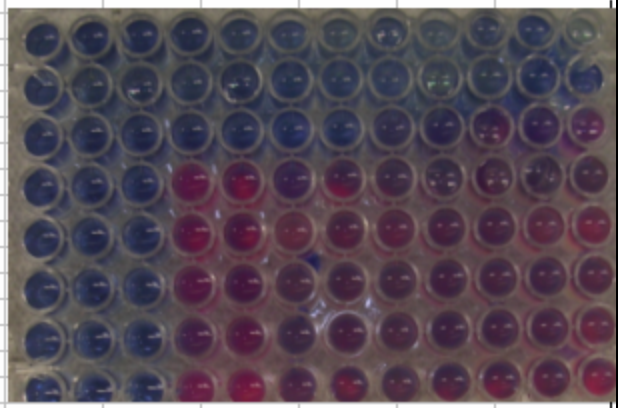


PLATE 2

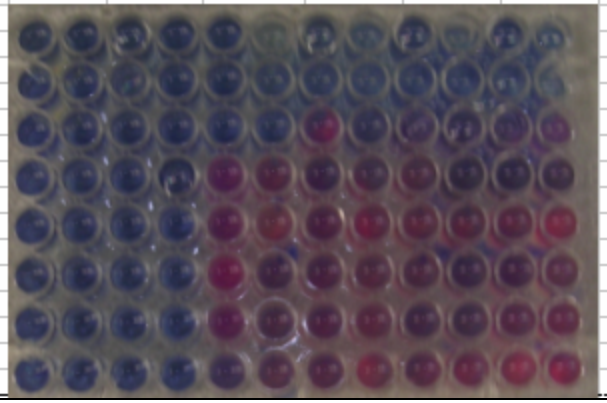
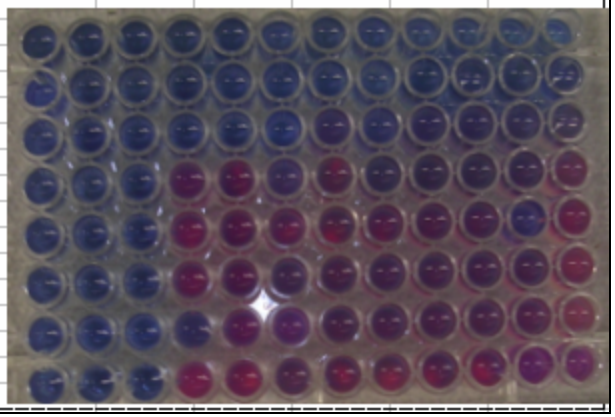


PLATE 4



APPENDIX P: Synergy Experiment: *Pseudomonas aeruginosa & Enterococcus faecalis*

MQP- Evaluate Synergy between Silver Compounds and YP- ET Against Ef and Pa together

PURPOSE: To determine if there is synergy between terpene formulations and silver formulations

			wt (mg)	BHI vol (ul)	conc	
MATERIALS	**Ef + Pa**					
	1. YP-AgNO3 - prepare 4 mg/ml in BHI	Prepared powder form	8	2000	4 mg/ml	Input weight in mg and the formula will calculate the volume of LB to add in ul
	use material you made					
	2. YP-AgCl - prepare 4 mg/ml in BHI	Prepared powder form	8	2000	4 mg/ml	
	use material you made					
	3. YP-ET- prepare 25mg/ml in BHI	Commercial product	400	8000	25 mg/ml w/w = 4 mg/ml terpene	
	use commerical product YP-2ESL	160 mg/ml terpene			**8mg/ml**	

METHOD:

PLATE 1 **YP-AgNO3 + YP- ET**

AgNO3 dilution

1. Add 100 ul of BHI to all wells
2. Add 100 ul of **YP-AgNO3 (1 mg/ml)** to column 1, rows A- H

Transfer 100 ul from column 1 to column 2, column 2 to 3, etc. Discard 100 ul from column 11. Add nothing to column 12.

		Silver concen	**1000.00**	**500.00**	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0**
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	2000	A												
	1000	B												
	500	C												
	250	D												
	125	E												
	62.5	F												
	31.25	G												
	0	H												

ET dilution

3. Add 100 ul of **YP-ET (4mg/ml)** to row A, column 1-11.

Transfer 100 ul from row A to row B, row B to row C, etc. Discard 100 ul from row G. Add nothing to row H

Silver dilution factor		**1000.00**	**500.00**	**250.00**	**125.00**	**62.50**	**31.25**	**15.63**	**7.81**	**3.91**	**1.95**	**0.98**	**0**
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
2000	A												
1000	B												
500	C												
250	D												
125	E												
62.5	F												
31.25	G												
0	H												

6. Prepare 1/1000 diluted Ef together with Pa in BHI by adding 25 ul of each microbe to 25 mls BHI

7. Add 100 ul of diluted bacteria to each well

9. Read To A620 in microplate reader.

10. Incubate plates at 37C o/n.

11. Read A620 in microplate reader.

12. Add 50 ul resazurin - incubate 1 hr. Scan color change.

13. Determine MIC = >75% inhibition of growth

YP-AgNO3 + YP- ET

PLATE 2 Repeat plate 1

YP- AgCl + YP-ET

PLATE 3 **AgCl dilution**

1. Add 100 ul of BHI:DMEM to all wells
2. Add 100 ul of **YP-AgCl (1 mg/ml)** to column 1, rows A- H

Transfer 100 ul from column 1 to column 2, column 2 to 3, etc. Discard 100 ul from column 11. Add nothing to column 12.

Silver dilution factor		1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
2000	A												
1000	B												
500	C												
250	D												
125	E												
62.5	F												
31.25	G												
0	H												

ET dilution

3. Add 100 ul of **YP-ET (4mg/ml)** to row A, column 1-12.

Transfer 100 ul from row A to row B, row B to row C, etc. Discard 100 ul from row G. Add nothing to row H

Silver dilution factor		1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0
	Tube												
Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
conc	Row												
2000	A												
1000	B												
500	C												
250	D												
125	E												
62.5	F												
31.25	G												
0	H												

6. Prepare 1/1000 diluted Ef together with Pa in BHI 2.5 ul/25 mls BHI
7. Add 100 ul of diluted bacteria to each well 12.5 ul/25 mls BHI
9. Read To A620 in microplate reader.
10. Incubate plates at 37C o/n.
11. Read A620 in microplate reader.
12. Add 50 ul resazurin - incubate 1 hr. Scan color change.
13. Determine MIC = >75% inhibition of growth

RESULTS:

PLATE 1	Silver concen	1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0
	Tube												
	Terpene Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc Row												
	2000 A	-0.195	-0.194	-0.2	-0.186	-0.2	-0.19	-0.179	-0.06	-0.099	-0.162	-0.157	-0.146
	1000 B	-0.018	0.016	0.058	0.029	0.052	0.066	0.035	0.032	0.019	0.035	0.037	0.012
	500 C	0.017	0.038	0.048	0.027	0.028	0.024	0.021	0.031	0.031	0.086	0.041	0.007
	250 D	0.017	0.029	0.034	0.023	0.03	0.025	0.027	0.218	0.189	0.503	0.668	0.745
	125 E	0.003	0.023	0.025	0.019	0.022	0.079	0.354	0.422	0.39	0.934	0.845	0.899
	62.5 F	-0.001	0.012	0.017	0.018	0.016	0.437	0.451	0.434	0.449	1.034	1.008	1.044
	31.25 G	-0.001	0.011	0.012	0.008	0.01	0.496	0.468	0.466	0.605	1.084	1.093	1.19
	0 H	-0.013	0.003	0.005	0.008	0.005	0.501	0.498	0.485	0.684	1.159	1.157	1.233
			MIC of YP silver nitrate alone = 62.5 ppm										0.623
			Can reduce silver to 15.63 ppm by adding 250 ppm YP-ET										0.1246

PLATE 2	Silver concen	1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0
	Tube												
	Terpene Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc Row												
	2000 A	-0.159	-0.124	-0.132	-0.274	-0.176	-0.11	-0.185	-0.057	-0.218	-0.183	-0.05	-0.188
	1000 B	-0.017	0.017	0.021	0.047	0.018	0.021	-0.001	0.023	0.03	-0.004	-0.003	-0.007
	500 C	0.036	0.044	0.047	0.048	0.014	0.039	0.025	0.028	0.035	0.029	0.012	0.015
	250 D	0.021	0.027	0.031	0.021	0.025	0.025	0.019	0.295	0.384	0.606	0.314	0.711
	125 E	0.012	0.028	0.021	0.018	0.021	0.371	0.405	0.41	0.602	0.847	0.823	0.839
	62.5 F	0.004	0.017	0.027	0.014	0.026	0.464	0.445	0.433	0.752	1.042	0.979	1.029
	31.25 G	0.003	0.012	0.014	0.01	0.018	0.492	0.478	0.462	0.685	1.085	1.058	1.101
	0 H	-0.004	0	0.003	0.001	0.008	0.481	0.468	0.457	0.654	1.14	1.134	1.239
													0.592375
													0.118475

PLATE 3	Silver dilution factor		1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	2000	A	-0.248	-0.264	-0.186	-0.163	-0.233	-0.139	-0.163	-0.379	-0.239	-0.029	0.05	0.007
	1000	B	-0.063	-0.01	-0.027	0.003	-0.011	0.011	0.025	0.038	0.038	0.068	0.054	-0.021
	500	C	-0.037	0.043	0.022	0.019	0.024	0.033	0.037	0.051	0.057	0.061	0.043	0.522
	250	D	-0.022	0.031	0.025	0.017	0.01	0.016	0.017	0.027	0.03	0.328	0.689	0.74
	125	E	-0.017	0.019	0.008	-0.003	-0.004	0.007	0.032	0.404	0.419	0.826	0.847	0.773
	62.5	F	-0.009	0.006	0.001	-0.008	-0.012	-0.002	0.331	0.441	0.41	1.01	1.052	0.988
	31.25	G	-0.023	0.002	-0.009	-0.013	-0.024	-0.013	0.449	0.46	0.449	1.055	1.112	1.105
	0	H	-0.025	-0.014	-0.026	-0.046	-0.035	-0.009	0.491	0.474	0.48	1.046	1.17	1.185
				MIC of YP silver chloride alone = 47 ppm										0.662375
				Can reduce silver to 15.63 ppm by adding 125-250 ppm YP-ET										0.132475

PLATE 4	Silver dilution factor		1000.00	500.00	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95	0.98	0
		Tube												
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12
	conc	Row												
	2000	A	-0.266	-0.246	-0.139	-0.302	-0.227	-0.125	-0.145	-0.092	-0.14	-0.189	-0.188	-0.202
	1000	B	-0.063	-0.017	-0.023	0.061	0.008	0.002	-0.033	0.036	-0.036	-0.002	0.004	0.165
	500	C	0.015	0.037	0.014	0.024	0.021	0.028	0.018	0.04	0.025	0.012	0.003	0.394
	250	D	0.002	0.024	0.017	0.013	0.007	0.018	0.018	0.298	0.146	0.556	0.569	0.647
	125	E	0.002	-0.005	0.007	-0.001	-0.003	0.012	0.297	0.456	0.627	0.851	0.91	0.746
	62.5	F	-0.025	-0.017	-0.013	-0.014	-0.004	0.168	0.459	0.651	0.983	1.084	1.054	0.91
	31.25	G	-0.029	-0.024	-0.017	-0.013	0.015	0.472	0.509	0.958	1.092	1.094	1.044	0.962
	0	H	-0.015	-0.028	-0.036	-0.037	-0.033	0.205	0.492	0.482	0.829	1.2	1.136	1.102
				Conclude: YP-ET can reduce silver by 3-4 fold										0.5905
						MIC VALUES								0.1181

	Plate 1	Plate 2	Plate 3	Plate 4
MIC Terpene alone	500	500	500	2000
MIC Silver Nitrate alone	62.5	62.5	-	-
MIC Silver Chloride alone	-	-	31.25	62.5
MIC Terpene *w/* Silver Nitrate	125	250	-	-
MIC Terpene *w/* Silver Chloride	-	-	125	125
MIC Silver Nitrate *w/* ET	15.63	31.25	-	-
MIC Silver Chloride *w/* ET	-	-	15.63	31.25

PLATE 1

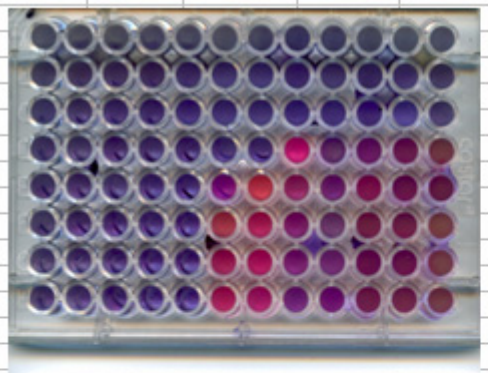


PLATE 3

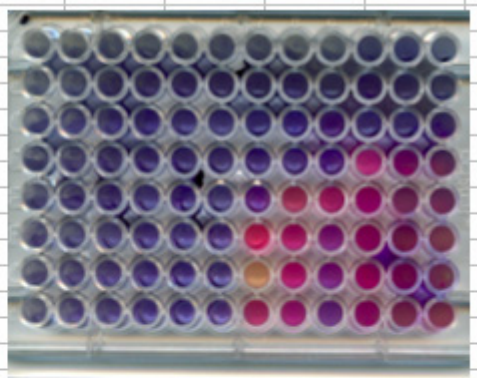


PLATE 2

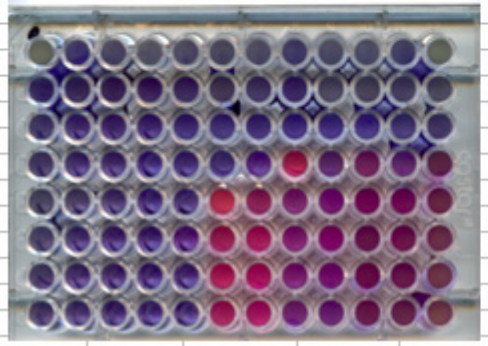
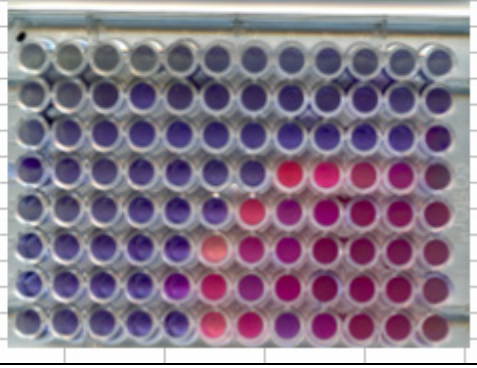


PLATE 4



APPENDIX Q: Terpene Resistance Test Protocol

Material Resistance to Terpenes

Purpose: To determine whether the selected materials are resistant to terpenes.

Materials:
1. Terp (4 mg/ml) and YP-terp (25 mg/ml = 4 mg/ml terp) stock concentrations
2. Calcium Alginate
3. Tegasorb
4. Coverlet O.R. Inner/ Outer

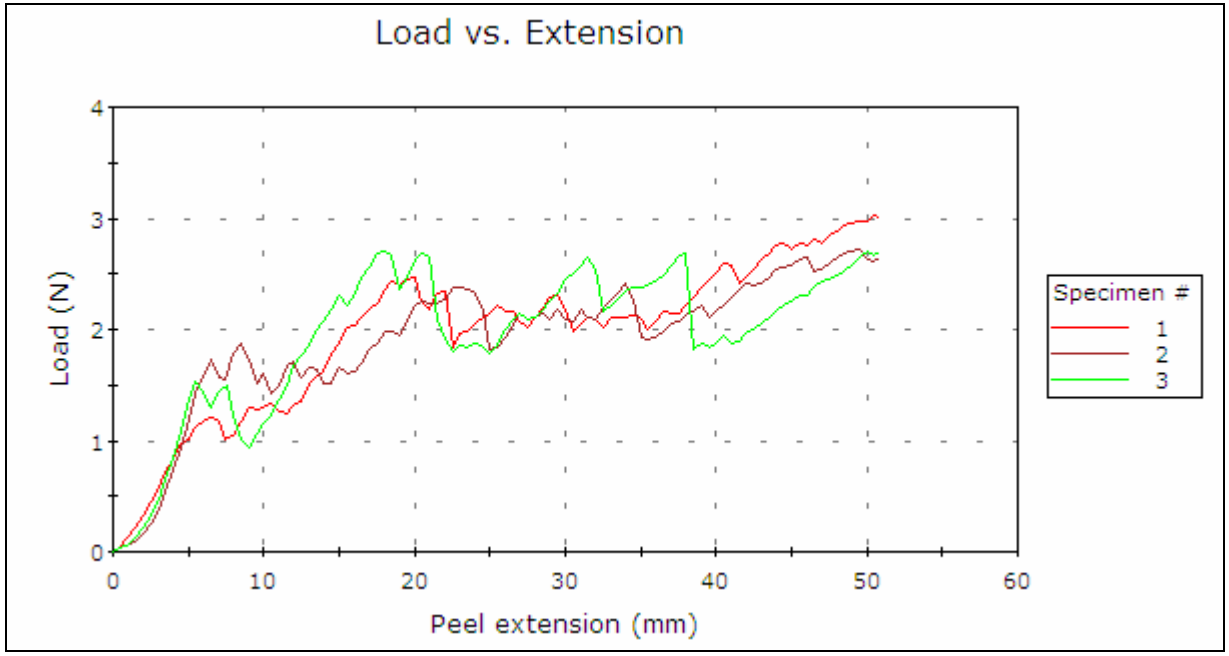
Protocol:
1. Label three tubes for each material; Control, YP-ET, Free ET
2. Place material in ucfg tube
3. Add 500ul of liquid to indicated ucfg tube
4. Let material soak in liquid for 7 days.
5. Observer changes.

Results:

Material	Control	YP-ET	Free ET
Calcium Alginate	No change	No change	Less absorbed
Tegasorb	No change	No change	Fell apart
Coverlet O.R- Inner material	No change	No change	No change
Coverlet O.R- Outer material	No change	No change	No change
P. U. Foam-Inner material			
P. U. Foam- Outer material			

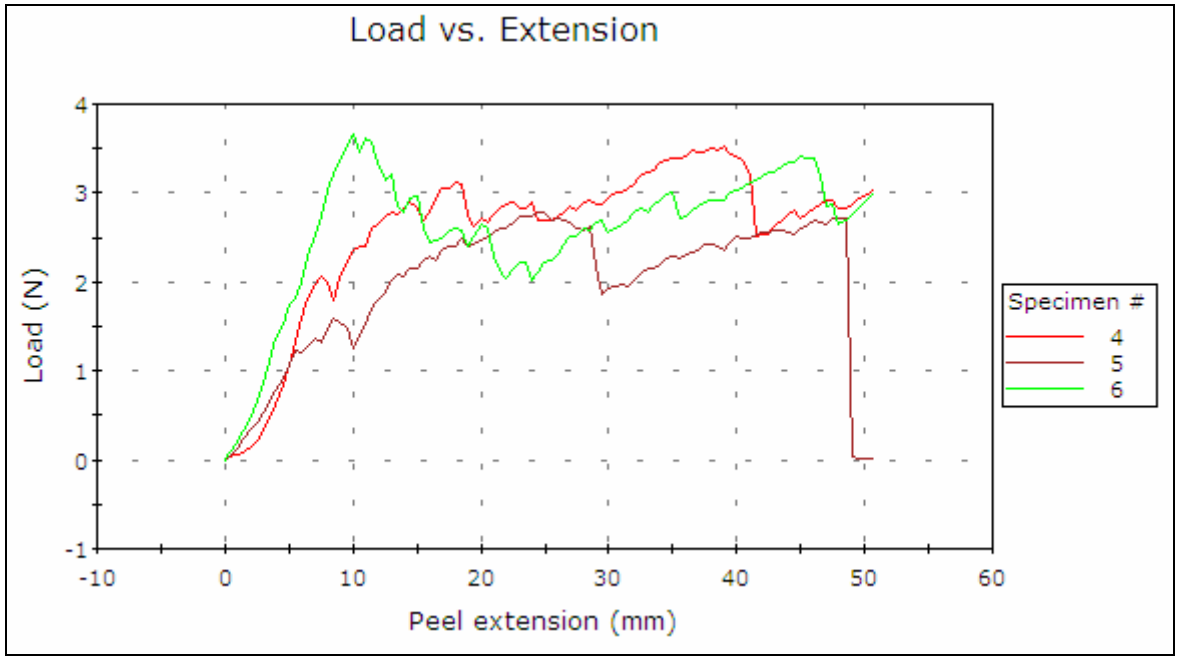
APPENDIX R: Instron® Mechanical Testing- Peel Test

Specimen 1: Polyurethane Foam



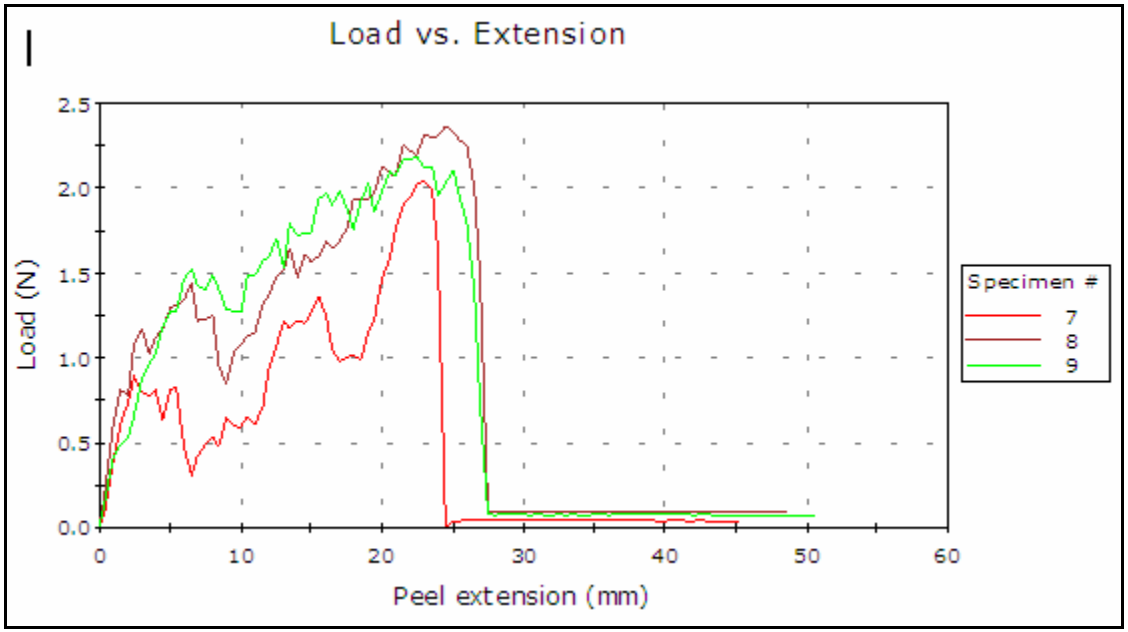
	Specimen label	Adhesive Strength (N)
1	PU Foam	2.197
2	PU Foam	2.100
3	PU Foam	2.239
Mean		2.179
STD		0.072

Specimen 2: Island Foam



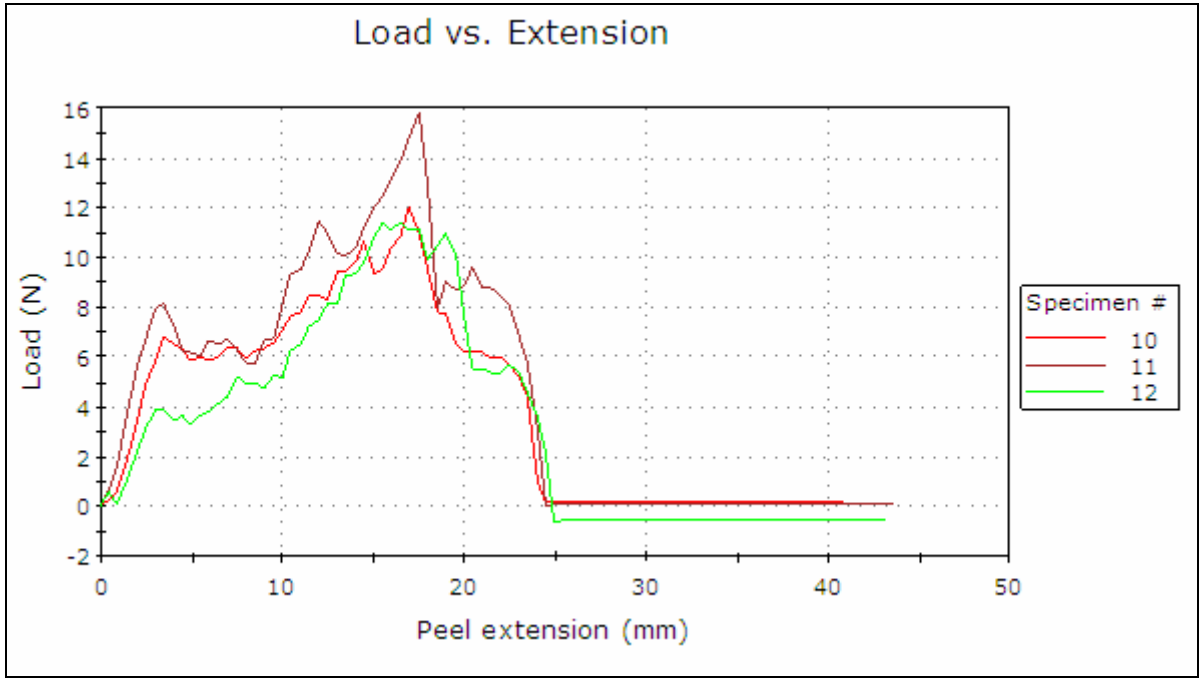
	Specimen label	Adhesive Strength (N)
4	Island Foam	2.795
5	Island Foam	2.317
6	Island Foam	2.010
Mean		2.374
STD		0.396

Specimen 3: Hydrocolloid



	Specimen label	Adhesive Strength (N)
7	Hydrocolloid	1.170
8	Hydrocolloid	1.560
9	Hydrocolloid	1.731
Mean		1.487
STD		0.288

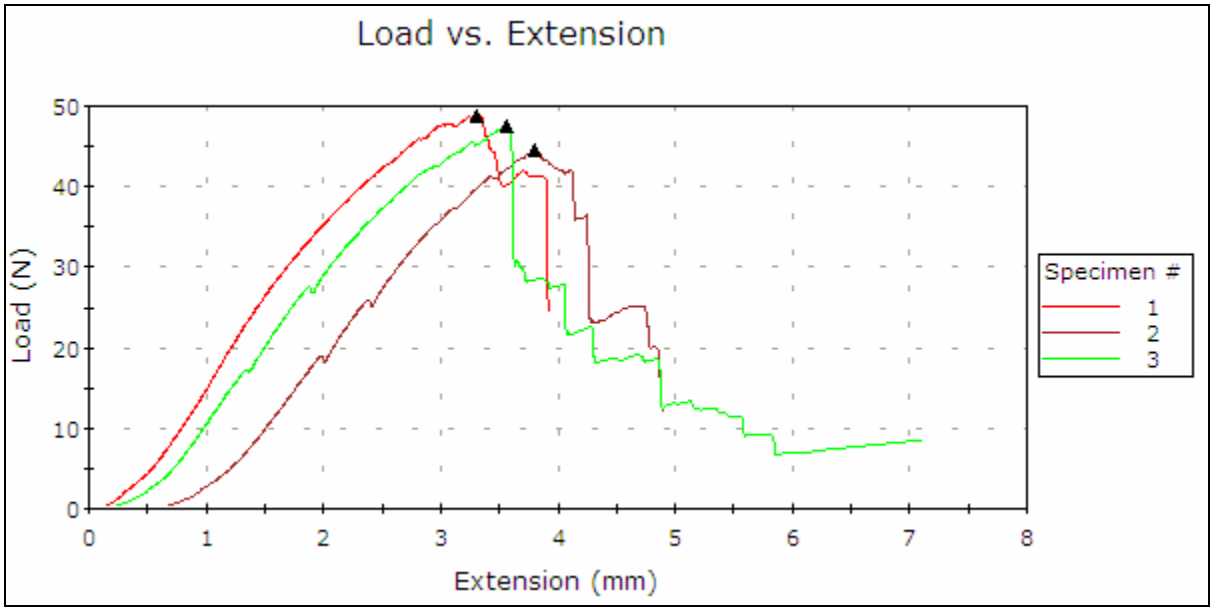
Specimen 4: Coverlet™ O.R.



	Specimen label	Adhesive Strength (N)
10	Coverlet™ O.R.	10.661
11	Coverlet™ O.R.	11.341
12	Coverlet™ O.R.	10.755
Mean		10.919
STD		0.369

APPENDIX S: Instron® Mechanical Testing- Tensile Test

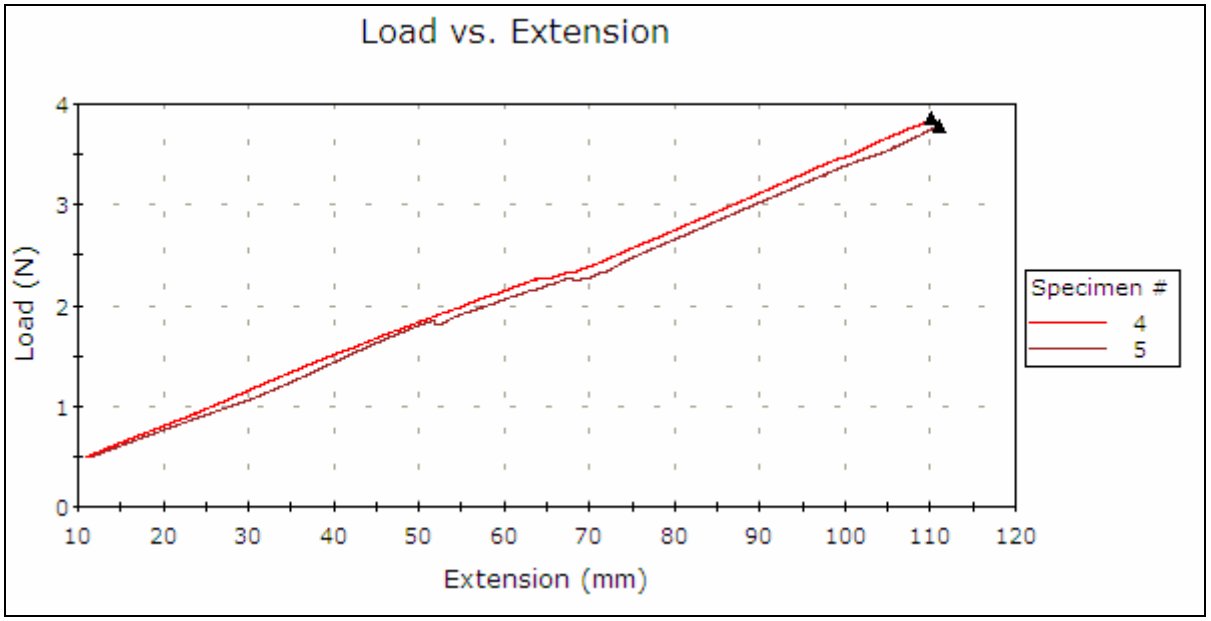
Specimen 1: Acticoat™ 7



	Specimen label	Max Extension (mm)	Max Load (N)	Tensile stress at Max Load (MPa)
1	Acticoat™ 7	3.917	48.824	3.764
2	Acticoat™ 7	4.899	44.425	3.425
3	Acticoat™ 7	7.110	47.390	3.653
Mean		5.309	46.880	3.614
STD		1.636	2.244	0.173

	Specimen label	Tensile strain at Max Load (%)	Max Tensile strain (%)	Max Tensile stress (MPa)	Modulus (MPa)
1	Acticoat™ 7	3.161	3.763	3.764	183.442
2	Acticoat™ 7	3.092	4.186	3.425	187.229
3	Acticoat™ 7	3.306	6.847	3.653	175.340
Mean		3.186	4.932	3.614	182.003
STD		0.109	1.671	0.173	6.074

Specimen 2: SilvaSorb™



	Specimen label	Max Extension (mm)	Max Load (N)	Tensile stress at Max Load (MPa)
4	SilvaSorb™	110.222	3.855	0.297
5	SilvaSorb™	110.951	3.784	0.292
Mean		110.587	3.819	0.294
STD		0.515	0.050	0.004

	Specimen label	Tensile strain at Max Load (%)	Max Tensile strain (%)	Max Tensile stress (MPa)	Modulus (MPa)
4	SilvaSorb™	89.431	89.431	0.297	0.339
5	SilvaSorb™	89.015	89.015	0.292	0.304
Mean		89.223	89.223	0.294	0.322
STD		0.294	0.294	0.004	0.025

APPENDIX T: Alginate Hydrocolloid Protocol

Alginate Hydrocolloid Protocol 12406

Purpose: Create and test variability in concentrations in a hydrocolloid as part of a dressing and each of the components individual effect

Materials:
- Alginate powder ~1 g total weight
- Sterile water
- Calcium Nitrate ~ 10%
- Sodium Azide ~ 0.1 mg
- Glycerol Powder
- Commercial grade YP-ET
- Magnetic stir bar/stir plate

n=3 1.) Obtain alginate. Make 3 sets of 0.5%, 1%, 2%, 3% solution by adding 225 mg, 450 mg, 900 mg, 1.35 g algin. to 45 mL sterile water respectively.

2.) Add 90 ul 1M sodium azide to the CMC mixture to reach 2 mM sodium azide final concentration

n=1 3.) To one group (1 set = 4 gels), add 7% glycerol (3.5 g)

n=1 To another group (1 set = 4 gels) add 2.1 g YP-ET (160 mg/ml terp) for a final conc. of 6.6 mg/ml

n=1 To the third group (1 set = 4 gels), no additive - to act as a negative control

4.) Add a stir bar and cover the tubes and place it on the rotator to dissolve the powder overnight

5.) Once dissolved, heat the tubes in a warm water bath at 50 degrees C for ~ 5 minutes, to make the hydrocolloid less viscous

6.) Pour the warmed liquid into a weigh trays.

7.) Dissolve 20 mg of calcium nitrate powder in 180 mL sterile water

8.) Place magnetic stir bar in bottle and place on stir plate until dissolved.

9.) Pour 10 mls 1% calcium nitrate over the alginate gels and let solidify at room temperature in a sterile hood overnight.

Tube distribution:

n=1 1 set of no additive for neg control (4 tubes)

n=1 1 set of 0.5%, 1%, 2%, 3% alginate + glycerol (4 tubes)

n=1 1 set of 0.5%, 1%, 2%, 3% alginate + YP-ET (4 tubes)

Glycerol Plasticizer concentration:

Barnes, Scott and Ding, Jim Jian Ling. Calendered Hydrocolloid dressing: US Patent 6923982. Published 02 August 2005. www.freepatentsonline.com/6923982

APPENDIX U: CMC Hydrocolloid Protocol

CMC Hydrocolloid Protocol 12406

Purpose: Create and test variability in concentrations in a hydrocolloid as part of a dressing and each individual effect in a hydrocolloid

Materials: Carboxymethylcellulose powder ~1 g total weight
Sterile water
Sodium Azide - 1M
Glycerol
Commercial Grade YP-ET

n=3 1.) Obtain CMC. Make 3 sets of 0.5%, 1%, 2%, 3% solution by adding 225 mg, 450 mg, 900 mg, 1.35 g CMC to 45 mL sterile water respectively.
2.) Add 90 ul 1M sodium azide to the CMC mixture to reach 2 mM sodium azide final concentration
n=1 3.) To one group (1 set = 4 gels), add 7% glycerol (3.5 g)
n=1 To another group (1 set = 4 gels) add 2.1 g YP-ET (160 mg/ml terp) for a final conc. of 6.6 mg/ml
n=1 To the third group (1 set = 4 gels), no additive - to act as a negative control
4.) Add a stir bar and cover the tubes and place it on the rotator to dissolve the powder overnight
5.) Once dissolved, heat the tubes in a warm water bath at 50 C for ~ 5 minutes, to make the hydrocolloid less viscous
6.) Pour the warmed liquid into weigh trays.
7.) Let solidify/dry under sterile hood overnight

Tube distribution:
n=1 No additive for neg control (4 tubes)
n=1 1 set of 0.5%, 1%, 2%, 3% CMC + glycerol (4 tubes)
n=1 1 set of 0.5%, 1%, 2%, 3% CMC + YP-ET (4 tubes)

GlycerolPlasticizer concentration:
Barnes, Scott and Ding, Jim Jian Ling. Calendered Hydrocolloid dressing: US Patent 6923982. Published 02 August 2005. www.freepatentsonline.com/6923982

APPENDIX V: Water Absorption Test

Water Absorption Test 13006

Purpose: Test the amount of fluid absorptive capacity in each film

Materials: Hydrocolloid samples
Microfuge tubes
24 mL sterile water (1 mL/tube)

Procedure:
1.) Weigh 24 empty microfuge tubes and label according to hydrocolloid sample, concentration, and additive
2.) Cut a small sample of hydrocolloid and place in microfuge tube - weigh and record scale reading
3.) Fill microfuge tubes with 1.0 mL sterile water
4.) Leave at room temperature for 5 minutes
5.) Extract surrounding water from the tube and weigh on the scale
Record final volume of water remaining in the sample
SET 2

Hydrocolloid	Empty Tube (g)		Tube+Sample (g)		Final Sample (g)		Weight Sample	Final Vol. (g)
0.5 % CMC -	1.0051		1.0054		1.2121		0.003 0.207	0.204
1% CMC -	0.9999		1.0017		1.2588		0.0018 0.2589	0.2571
2% CMC -	0.9992		1.0022		1.1454		0.003 0.1462	0.1432
3% CMC -	0.9964		1.0176		1.7113		0.0212 0.7149	**0.6937**
0.5 % CMC + glyc.	0.9954		-		-		-	-
1% CMC + glyc.	0.9982		1.0122		1.1978		0.0140 0.1996	0.1856
2% CMC + glyc.	0.9934		1.0492		1.2169		0.0558 0.2235	0.1677
3% CMC + glyc.	0.9996		1.0624		1.7359		0.0628 0.7363	**0.6735**
0.5 % CMC + YP-ET	1.0043		1.0122		1.249		0.0079 0.2447	0.2368

1% CMC + YP-ET	0.9997		1.0075		1.696		0.0078 0.6963	0.6885
2% CMC + YP-ET	1.0008		1.014		1.509		0.0132 0.5082	0.495
3% CMC + YP-ET	0.9995		1.0204		1.98		0.0209 0.9805	**0.9596**
0.5% Alg.+ CaN2 -	0.9996		1.0056		1.1849		0.0060 0.1853	0.1793
1% Alg.+ CaN2 -	0.9967		1.0013		1.4557		0.0046 0.4590	0.4544
2% Alg. +CaN2 -	0.9996		1.0163		1.6941		0.0167 0.6945	**0.6778**
3% Alg. + CaN2 -	0.9952		1.0067		1.3995		0.0115 0.4043	0.3928
0.5% Alg.+ CaN2 + glyc.	0.9976		1.0444		1.1973		0.0468 0.1997	0.1529
1% Alg.+ CaN2 + glyc.	0.994		1.0299		1.129		0.0359 0.1350	0.0991
2% Alg. +CaN2 + glyc.	1.0029		1.0465		1.1212		0.0436 0.1183	0.0747
3% Alg. + CaN2 + glyc.	1.002		1.0649		1.2984		0.0629 0.2335	**0.1706**
0.5% Alg.+ CaN2 + YP-ET	0.9965		1.0098		1.1571		0.0133 0.1606	0.1473
1% Alg.+ CaN2 + YP-ET	1.0013		1.0306		1.3906		0.0293 0.3893	0.36
2% Alg. +CaN2 + YP-ET	0.9983		1.0117		1.2166		0.0134 0.2183	0.2049
3% Alg. + CaN2 + YP-ET	0.9945		1.0123		1.2856		0.0178 0.2911	**0.2732**

Overall:
0.5% CMC + glycerol was too soft and couldn't be removed for sampling
The CMC + Terpene samples were all very swollen in water and were more in tact than the other samples

In all of the alginate gels, the negative controls and glycerol, much of them dissolved so only in the terpene addition was there evidence of swelling and the gel stayed together

Alone, the 1%, 2%, and 3% CMC + glycerol were stretchy and rubbery,

though the 3% was bubbly

The Alginate+terpene samples were all brittle, especially the higher concentrations

Much of the samples dissolved so it was
hard to absorb accurate amounts of
water

APPENDIX W: Particle Settling Vehicle for *In Vivo* Experiment

Particle Settling Vehicle for *In Vivo* Experiment 13006

Purpose: Test the concentration of vehicles for application of actives in an *in vivo* experiment
Test particle settling and ability to homogenize

Materials: YP-ET
CMC powder
Alignate
1 M Sodium Azide

Methods:

Make up azide diluent - 80 ul azide +40 mls water
Make up 1% hydrocolloid solutions from CMC and alginate in 25 mL of water +50 ul azide
Weigh in indicated volumes of water and 1% hydrocolloid

Tube	Final Alginate Concentration	g 1% Alginate	Final CMC Concentration	g 1% CMC	g Azide Diluent
1	1	4	-	-	0
2	0.75	3	-	-	1
3	0.6	2.4	-	-	1.6
4	0.45	1.8	-	-	2.2
5	0.3	1.2	-	-	2.8
6	0.15	0.6	-	-	3.4
7	0.1	0.4	-	-	3.6
8	0	0	-	-	4
9	-	-	1	4	0
10	-	-	0.75	3	1
11	-	-	0.6	2.4	1.6
12	-	-	0.45	1.8	2.2
13	-	-	0.3	1.2	2.8
14	-	-	0.15	0.6	3.4
15	-	-	0.1	0.4	3.6

Add 165 mg YP-ET to achieve a final concentration of 6.6 mg/ml terpene - the high dose for the animal expt
280 mg/7mL total = 40mg/mL in tube of terpene
Mix by vortex or rotation and leave overnight. Record the amount of settling after 24 hrs
Measure the height of the clear layer and the height of the cloudy liquid layer to find the fraction
of settling that occurred - clear layer/cloudy liquid layer = <10% settling is favorable
Enter height of clear and liquid layers into spreadsheet to calculate % settling
Take a digital photograph of the tubes

Tube	Hydrocolloid	% hydrocolloid	cloudy layer (cm)	liquid layer (cm)	% settling
1	Alginate	1%	1	4.2	23.80952381
2	Alginate	0.75%	0.6	3.9	15.38461538
3	Alginate	0.60%	0.8	4.1	19.51219512

4	Alginate	0.45%	0.8	4	20	
5	Alginate	0.30%	0.6	4	15	
6	Alginate	0.15%	0.5	4	12.5	
7	Alginate	0.10%	0.6	4.1	14.63414634	
8	Alginate	0%	0.5	4	12.5	
9	**CMC**	**1%**	**3.9**	**0**	**none**	
10	**CMC**	**0.75%**	**4.1**	**0**	**none**	
11	**CMC**	**0.60%**	**4.1**	**0**	**none**	*
12	**CMC**	**0.45%**	**3.8**	**0.2**	**none**	thin film on top
13	CMC	0.30%	0.6	4.1	14.63414634	
14	CMC	0.15%	0.7	4.1	17.07317073	
15	CMC	0.10%	0.7	4	17.5	

APPENDIX X: Glycerol + Terpene Combination Test

Glycerol + Terpene Combination test 13106

Purpose: Test and decide on most effective hydrocolloid formulations with glycerol and terpene

Materials: CMC powder
Sterile water
Sodium Azide ~ 0.1mg
Glycerol
Commercial Grade YP-ET
2% CMC stock hydrocolloid (45 mL gel = 46 g)

1.) Obtain CMC, make 1%, 1.5%, and 2% hydrocolloids by diluting stock 2% solution

	Terpene addition (g)	**glycerol (g)**	**grams 2% CMC**	grams water
1% CMC alone	-	-	27.5	27.5
1% CMC +terp	2.1	-	27.5	25.4
1% CMC + glycerol	-	3.5	27.5	24
1% CMC +glycerol + terp	2.1	3.5	27.5	21.9
1.5% CMC alone	-	-	41.25	13.75
1.5% CMC +terp	2.1	-	41.25	11.65
1.5% CMC + glycerol	-	3.5	41.25	10.25
1.5% CMC + glycerol + terp.	2.1	3.5	41.25	8.15
2% CMC alone	-	-	55	0
2% CMC +terp	2.1	-	52.9	0
2% CMC + glycerol	-	3.5	51.5	0
2% CMC +glycerol +terp	2.1	3.5	49.4	0

2.) Cast gels and let dry in hood for 48 hours

3.) Run water absorbtion test to measure amount of volume the gels can hold and dissolving that occurs

APPENDIX Y: Water Absorption Test

Water Absorption Test 20206

Purpose: Test the amount of fluid absorptive capacity in each film

Materials: CMC Hydrocolloid samples
Microfuge tubes
24 mL sterile water (1 mL/tube)

Procedure:
1.) Weigh 24 empty microfuge tubes and label according to hydrocolloid sample, concentration, and additive
2.) Cut a small sample of hydrocolloid and place in microfuge tube - weigh and record scale reading
3.) Fill microfuge tubes with 1.0 mL sterile water
4.) Leave at room temperature for 5 minutes
5.) Extract surrounding water from the tube and weigh on the scale
Record final volume of water remaining in the sample

SET 1

Hydrocolloid	Empty Tube (g)	Tube+Sample (g)	Final Sample (g)	Sample dry (g)	Sample wet (g)	Final Vol. (g)
1% CMC + glyc.	1.002	1.048	1.3504	0.046	0.3484	0.3024
1% CMC+glyc+YP-ET	0.998	1.0024	1.3925	0.0044	0.3945	**0.3901**
1% CMC - control	1.0043	1.0732	1.2403	0.0689	0.236	0.1671
1.5% CMC + glyc.	1.0007	1.061	1.3703	0.0603	0.3696	0.3093
1.5% CMC+glyc+YP-ET	0.9956	1.0357	1.5079	0.0401	0.5123	**0.4722**
2 % CMC + YP-ET	0.9963	1.0095	1.5976	0.0132	0.6013	0.5881
2% CMC + glyc.	0.9945	1.0912	1.4665	0.0967	0.472	0.3753
2% CMC - control	0.996	1.0207	2.0074	0.0247	1.0114	**0.9867**
2% CMC+glyc+YP-ET	1.0025	1.0918	1.6435	0.0893	0.641	0.5517

SET 2

Hydrocolloid	Empty Tube (g)	Tube+Sample (g)	Final Sample (g)	Sample dry (g)	Sample wet (g)	Final Vol. (g)
1% CMC + glyc.	1.0045	1.0796	1.2308	0.0751	0.2263	0.1512
1% CMC+glyc+YP-ET	0.9984	1.0032	1.2486	0.0048	0.2502	**0.2454**
1% CMC - control	0.996	1.0405	1.2484	0.0445	0.2524	0.2079
1.5% CMC + glyc.	0.9977	1.0433	1.3282	0.0456	0.3305	**0.2849**
1.5% CMC+glyc+YP-ET	1.0007	1.0275	1.2172	0.0268	0.2165	0.1897
2 % CMC + YP-ET	1.0025	1.0187	1.5271	0.0162	0.5246	**0.5084**
2% CMC + glyc.	0.9988	1.0643	1.3236	0.0655	0.3248	0.2593
2% CMC - control	0.9945	1.0167	2.005	0.0222	1.0105	**0.9883**
2% CMC+glyc+YP-ET	0.9969	1.0854	1.4786	0.0885	0.4817	0.3932

Chitosan

Hydrocolloid	Empty Tube (g)	Tube+Sample (g)	Final Sample (g)	Sample dry (g)	Sample wet (g)	Final Vol. (g)
1% CMC + 1% Chitosan	1.0045	1.0117	1.5181	0.0072	0.5136	0.5064
1% CMC+1% Chitosan	0.9991	1.0083	1.5181	0.0092	0.519	0.5098
2% CMC + 0.5% Chitosan	1.0043	1.0149	1.552	0.0106	0.5477	0.5371
2% CMC+0.5% Chitosan	0.9997	1.0141	1.6453	0.0144	0.6456	0.6312

APPENDIX Z: Uniformity Test

Purpose: to test the uniformity and ease of each homogeneous formulation and its spreading capabilities

1.) Using a 3 mL syringe, draw up active+ CMC vehicle formulations (1%, 0.75%, 0.6%, 0.45%) into separate syringes

2.) Spread active formulation in a 0.5-100 ul formulation on a small wound site simulation such as an orange piece

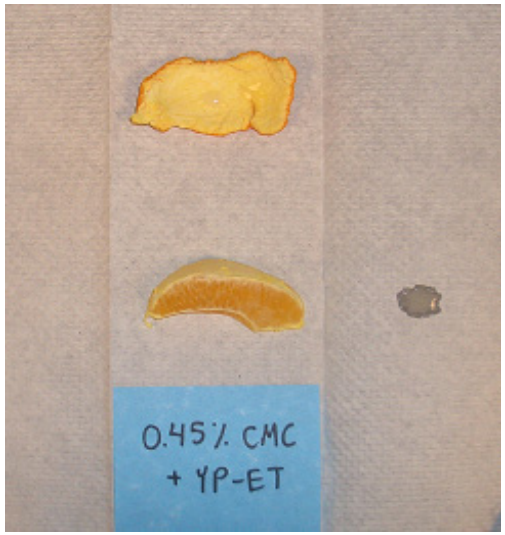
3.) Observe the distribution and diffusion of the ribbon.

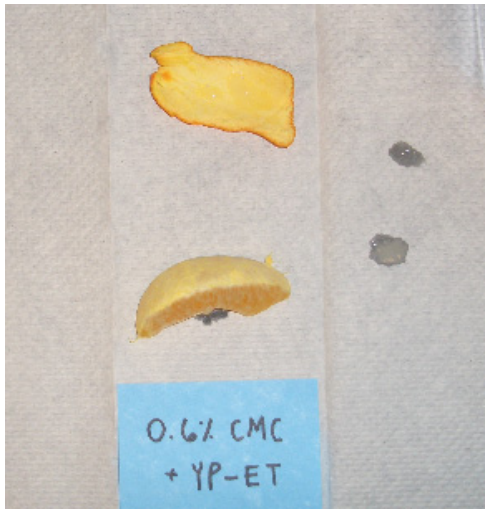
% form.	Observations	Time to leave the tube	Measurement on towel	Notes:	
1%	Stayed put, harder put in syringe	0.4 sec	1.3 cm	not mixed, small amt of settling @ 36 hrs	
0.75%	Stayed put, easy spread	0.35 sec	1.2 cm	didn't settle @ 36 hrs	
0.60%	very liquidy, quickly spread, drips	0.2 sec	2 cm	settled @ 36 hrs	
0.45%	liquidy, stayed in clump	0.15 sec	1.7 cm	settled @ 36 hrs	

4.) Take pictures with a digital camera

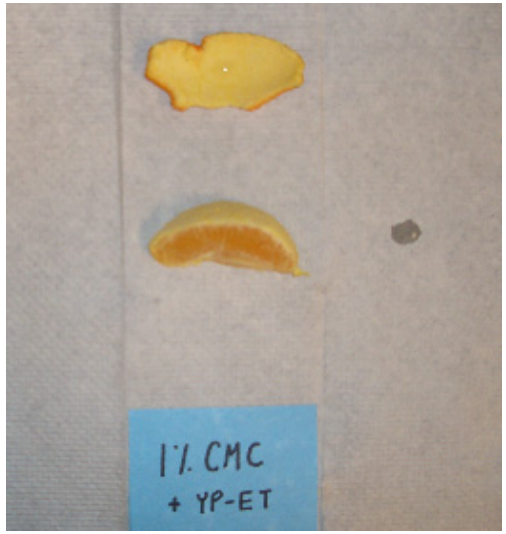
Results:

0.75 % CMC + YP-ET formulation was easiest to apply and stayed in place, and also did not settle after 36 hours.


0.45% CMC
+ YP-ET


0.6% CMC
+ YP-ET


0.75% CMC
+ YP-ET


1% CMC
+ YP-ET

APPENDIX AA: Alginate Hydrocolloid plus Calcium Chloride Protocol

Alginate Hydrocolloid Protocol plus Calcium Chloride 20606

Purpose: Identify CaCl2 concentration to crosslink alginate

Materials: Stock 2% alginate hydrocolloid
and 0.5% ((0.5 ml 2% + 1.5 ml water) alginate solutions
Sterile water
Calcium Chloride - prepare 5% calcium chloride (1 g/20 ml)

Prepare 1.5% (1.5 ml 2% + 0.5 ml water), 1% (1.0 ml 2% + 1.0 ml water)

% [CaCl2]	ul 5% CaCl2	ul Water
0	0	4000
0.1	80	3920
0.25	200	3800
0.5	400	3600
0.75	600	3400
1	800	3200
5	4000	0

1.) Add 1000 ul of each CaCl2 concentration to the labeled Ep tubes
2). Add 250 ul of indicated alginate concentrations to 1000 ul CaCl2
3) Allow to gel overnight
4). Centrifuge. Remove liquid and assess if the alginate has gelled. Identify the lowest concentration of CaCl2 that gels each concentration of aliginate

This will establish the relationship between alginate and CaCl2 to gel

5. Add 1 ml water to the alginate samples that gelled
6. Change the water hourly to remove any free chloride
7. Assess stability of alginate gel after desalting. Identify the lowest concentration of CaCl2 that produces a stable gel

Next: scale up washed calcium alginate production in tip covers using optimized conditions

% [CaCl2]	0.5% Alg	1% Alg	1.5% Alg	2% Alg
0	-	-	-	-
0.1	soft, clear	soft, clear	soft, clear	soft, clear
0.25	soft	less strong	strong	strong
0.5	some dissolved	softer	stable, harder gel	stable, harder gel
0.75	some dissolved	strong	stable, harder gel	stable, harder gel
1	some dissolved	strong	stable, harder gel	stable, harder gel
5	some dissolved	strong	stable, harder gel	stable, harder gel

Eliminate 0.5% Alginate gels because they were not stable in water and after calcium was washed away.

APPENDIX BB : Glycerol + Terpene Combination Lyophilizer Experiment

Glycerol + Terpene Combination Lyophilizer experiment

Purpose: Test and experiment with lyophilizer effect on hydrocolloid formulations with CMC and combinations of glycerol and terp

Materials: CMC powder
Sterile water
2% CMC stock hydrocolloid
Glycerol
Commercial Grade YP-ET

1.) Obtain CMC, make 1%, 1.5%, and 2% hydrocolloids by diluting stock 2% solution

Formulation	Terpene addition (g)	glycerol (g)	grams 2% CMC	grams water
1.5% CMC alone	-	-	41.25	13.75
1.5% CMC + glycerol	-	3.5	41.25	10.25
2% CMC alone	-	-	55	0
2% CMC +terp	2.1	-	52.9	0
2% CMC + glycerol	-	3.5	51.5	0
2% CMC +glycerol +terp	2.1	3.5	49.4	0

2.) Cast gels and freeze at -80C for 2 hours
3.) Load gels into lyophilizer and run standard recipe
Shelf temperature at -45C at 600mTorr vacuum ~1 or 2 hours to stabilize.
It reaches 0C after stopping at -20C to let things stabilize, and reduces the vacuum to below 200mTorr
Let sit for 12 hours while water sublimates and then warms gradually to 20C
Note: The total time depends on the shelf temperature when sample is put in.

4.) Run the water absorption test for absorptive properties of the gel

APPENDIX CC: Alginate + Calcium Chloride Test

Alginate + Calcium Chloride Test

Purpose: To determine the best combination of alginate and calcium chloride.

Materials: Alginate
Calcium Chloride
Water
Gel plates

Methods:

1.) Pour alginate into gel plates by weight using both 1.5% and 2.0% alginate
2.) Let gels dry overnight in 30 degree incubator
3.) After a minimum of 24 hours and there are no signs of wetness on the gel, peel alginate film from plate using a spatula
4.) Add, by weight, specified amount of calcium chloride to empty gel plate
5.) Place film back into plate on the calcium chloride ~1 minute
6.) Remove film once again, flip it over and allow other side to soak
7.) Allow film to soak in calcium chloride solution for a minimum of 24 hours
8.) After 24 hours, pour off CaCl2 solution and add enough water to submerge the gel careful not to disturb the gel. Allow 20 minutes to soak
9.) After 20 minutes, remove old water and replace it with clean water and continue the changing the water and soaking the gels at 20 min. intervals for at least 1 hour
10.) Allow gel to remain in clean water to avoid drying out

Alginate (g)	Alginate (%)	Calcium Chloride (g)	% Calcium Chloride
50	2	75	1.5
53	1.5	53	1
55	1.5	13.75	0.25
55	1.5	27.5	0.5
55	2	13.75	0.25
55	2	27.5	0.5

Conclusions: Need glycerol because gels are currently too wrinkly and lack necessary mechanical properties
Determined the best range for calcium chloride is 13.75g - 27.5g (0.25% - 0.5%) to obtain the best crosslinking
Determined that 1.5% alginate is better than 2.0% alginate

APPENDIX DD: Alginate + Glycerol + Calcium Chloride Test

Alginate + Glycerol + Calcium Chloride Test

Purpose: To determine the best combination of alginate and calcium chloride.

To determine if glycerol is an important constituents for the calcium alginate layer and if so over what range

Materials: 1.5% Alginate
Glycerol
Calcium Chloride
Water
Weigh trays

Methods:

1.) Pour 1.5% alginate into flask by weight according to the calculations below

2.) Add, by weight, varying amounts of glycerol to alginate (final volume of 55 ml)

3.) Vortex mixture and cast into weigh trays and label according to sample

4.) Let gels dry overnight in 30 degree incubator

5.) After a minimum of 24 hours, peel alginate film from tray

6.) Cut alginate to specified weight

6.) Add, by weight, specified amount of calcium chloride and water to weigh tray

7.) Place film back into tray on top of the calcium chloride - soak for ~ 1 minute

8.) Remove film once again, flip it over and allow other side to soak

9.) Allow film to soak in calcium chloride solution for a minimum of 24 hours

10.) After 24 hours, pour off $CaCl_2$ solution and add enough water to submerge the gel careful not to disturb the gel. Allow 20 minutes to soak

11.) After 20 minutes, remove old water and replace it with clean water and continue the changing the water and soaking the gels at 20 min. intervals for at least 1 hour

12.) Allow gel to remain in clean water to avoid drying out

1.5% Alginate (g)	Glycerol (g)	Glycerol (%)
54.125	0.875	1.75
53.25	1.45	3.5
52.375	2.625	5.25
51.5	3.5	7
55	0	0

Note: 5.25% and 7.0% glycerol take at least 5 days to dry, therefore they were ruled out due to time manufacturing time constraints

Glycerol (%)	Wt of Dry Gel (g)	Wt of piece (g)	%of total wt	g of CaCl2 using 55g gel	g CaCl2 for piece	g water for piece
0	0.77	0.14	18.2	27.5	5	5
0	0.77	0.16	20.8	20.63	4.29	7.15
0	0.77	0.21	27.3	13.75	3.75	11.38
1.75	1.64	0.24	14.6	27.5	4.02	4.01
1.75	1.64	0.56	34.1	20.63	7.03	11.7300
1.75	1.64	0.37	22.6	13.75	3.11	9.32
3.5	2.4	0.59	24.6	27.5	6.77	6.76
3.5	2.4	0.77	32.1	20.63	6.62	11.04
3.5	2.4	0.49	20.4	13.75	2.81	8.41

Conclusions: Using metrics (see Calcium Alginate Metrics) the best overall gel contained 1.75% and 3.5% glycerol
The gel also contained 0.5% of calcium
chloride

APPENDIX EE: *In Vivo* Vehicle Formulation Preparation

***In Vivo* Vehicle Formulation Preparation** 21306

Purpose: To demonstrate the *in vivo* antimicrobial effect of the terpene + vehicle formulations in an incisional wound model
Safety study to determine the effect of terpene + vehicle formulations on wound healing

Materials: YP-ET
CMC powder
Sterile water

Terpene:	[Terpene] Final Conc.	[Terpene] Stock	Final [Terpene]	Final Volume	Final Terp weight
High	24 mg/mL	160 mg/mL	150 mg/mL	40 mL	6 g
Medium	6.6 mg/mL	160 mg/mL	41.25 mg/mL	40 mL	1.65 g
Low	1.5 mg/mL	160 mg/mL	9.375 mg/mL	40 mL	0.375 g

Methods:

Preparation of formulations

Make up 2% hydrocolloid stock from CMC and with 8 g CMC, 400 mL sterile water
Weigh in indicated volumes of water to form a 0.75% CMC solution and weigh in YP-ET as indicated to yield 40 mL volumes

Formulation	Terpene (g)	2% CMC (g)	Sterile water (g)	Total Volume
high 24 mg/ml	6	15	19	40 mL
medium 6.6 mg/ml	1.65	15	23.35	40 mL
low 1.5 mg/ml	0.375	15	24.625	40 mL
control	0	15	25	40 mL

Test *in vitro* antimicrobial activity

Test 4 formulations in duplicate for activity in the *in vitro* primary assay against *S. aureus* prior to the animal study
and again after the animal study

APPENDIX FF: Infected Wound Model Research Chart

ANIMALS (species)	WOUND HEALING MODEL	LOCATION	CORRESPONDING AUTHOR	REFERENCE
Pigs	Burns	Worcester, MA	**Tim Roth**	Word of Mouth
	Varies	Worcester, MA	**Mitch Sanders**	Word of Mouth
Mini Pig	Full Thickness	University of Miami School of Medicine, Department of Dermatology, Miami, Florida.		Sullivan, Tory P, Eaglstein, William H, Davis, Stephen C & Mertz, Patricia (2001) **THE PIG AS A MODEL FOR HUMAN WOUND HEALING.** *Wound Repair and Regeneration* **9** (2), 66-76.
	80 C burns to backs and flanks 2 young pigs, 80 burns total infected with Sa	Stony Brook, NY	**Adam J. Singer, MD** Dept. of Emergency Medicine L4-515, Stony Brook, NY 11794-7400 Fax 516-444-3919; asinger@epo.som.sunysb.edu	Adam J. Singer, MD, Mazhar Mohammad, BS, George Tortora, PhD Henry C. Thode, Jr., PhD and Steve A. McClain, MD **Octylcyanoacrylate for the Treatment of Contaminated Partial-thickness Burns in Swine** *Academic Emerg Med.* 2000 Mar;7(3):222-7
Domestic Yorkshire pigs	Partial-thickness 3×3-cm excisional wounds, 4, 7 days	Nashville, Tenn. Dept of Plastic Surgery, Dept of Pathology, and the Department of Cell Biology, Vanderbilt Univ School of Medicine and the Dept of Veterans Affairs.	**Lillian B. Nanney, Ph.D.** Department of Plastic Surgery Vanderbilt Univ School of Medicine S-2221, Medical Center North Nashville, Tenn. 37232-2631 lillian.nanney@mcmail.vanderbilt.edu	**An *in Vivo* Comparison of Topical Agents on Wound Repair.** Plastic & Reconstructive Surgery. 108(3):675-683, September 1, 2001. *Bennett, Laura L. M.D.; Rosenblum, Richard S. M.D.; Perlov,* Cathy B.S.N.; Davidson, Jeffrey M. Ph.D.; Barton, Ronald M. M.D.; Nanney, Lillian B. Ph.D.
	cm × 2 cm × 0.4 mm	Fort Saskatchewan, AB, T8L 3W4 Canada	**Robert E. Burrell**	Merle E. Olson, J. Barry Wright, Kan Lam, Robert E. Burrell **Healing of Porcine Donor Sites Covered with Silver-coated Dressings** Taylor & Francis Health Sciences, part of the Taylor & Francis Group Volume 166, Number 6 / July 6, 2000
	full thickness contaminated with Pa	University of Calgary, Calgary, Alberta, Canada	**Robert E. Burrell**	Wright J.B.; Lam K.; Buret A.G.; Olson M.E.; Burrell R.E. **Early healing events in a porcine model of contaminated wounds: effects of nanocrystalline silver on matrix metalloproteinases, cell apoptosis, and healing** *Wound Repair and Regeneration*, Volume 10, Number 3, May 2002, pp. 141-151(11)
	acute wounds	Stony Brook, NY	**Richard A. F. Clark MD**	http://www.biotech.sunysb.edu/educWork/ibrp/rclark.html

			RAFClark@epo.som.sunyb.edu	http://www.bme.sunysb.edu/bme/people/faculty/fac_core.html#clark
	excisional wound swabbed with Sa	Stony Brook, NY	**Adam J. Singer, MD,** Dept of Emergency Medicine, Stony Brook Univ School of Medicine, Stony Brook, NY 11794-7400.	Singer, Adam J., Nable, Maria, Cameau, Paul, Singer, Daniel D. & Mcclain, Steve A. (2003) **Evaluation of a new liquid occlusive dressing for excisional wounds.** *Wound Repair and Regeneration* 11 (3), 181-187.
	Eight pigsw/ total of 645 partial-thickness wounds			Davis S.C.; Eaglstein W.H.; Cazzaniga A.L.; Mertz P.M. **An Octyl-2-Cyanoacrylate Formulation Speeds Healing of Partial-Thickness Wounds** *Dermatologic Surgery*, Volume 27 Number 9, 1 September 2001 p. 783-788(6)
	5x5x0.03 cm wounds on dorsum 12 excised wounds/pig	University of Utah	**Glenn D. Prestwich** Univ. of Utah - Dept. Medicinal Chem. 419 Wakara Way, Suite 205 Salt Lake City, UT, 84108-1257	Kirker,K.R. BSE; Luo,Y. PhD; Morris,S.E. MD; Shelby,J. PhD; Prestwich,GD. PhD **Glycosaminoglycan Hydrogels as Supplemental Wound Dressings for Donor Sites** *Journal of Burn Care & Rehabilitation*. 25(3):276-286, May/June 2004
	human imitation bite wounds	University of Toronto	SL Avon University of Toronto sylvie-louise.avon@fmd.ulaval.ca	Avon, SL, Wood, RE **Porcine skin as an in-vivo model for ageing of human bite marks** *J Forensic Odontostomatol.* 2005 Dec;23(2):30-9
New Zealand white **Rabbits**	rabbit ear full-thickness dermal ulcers 6 female rabbits	Northwestern University, Chicago, IL	M.J. Lee Division of Plastic and Reconst. Surg. Northwestern University, Chicago, IL	Lee MJ, Roy NK, Mogford JE, Schiemann WP, Mustoe TA **Fibulin-5 promotes wound healing *in vivo*** *J Am Coll Surg.* 2004 Sep;199(3):403-10
Adult Male Harlan Sprague-Dawley **Rats**	Full-thickness, lasting 20 days	Dept of Human Biological Chemistry and Genetics, Univ of Texas Medical Branch, Galveston, Texas, Chrysalis BioTechnology, Inc., b Galveston, Texas		Stiernberg, Janet, Norfleet, Andrea M, Redin, William R, Warner, W. Scott, Fritz, Richard R & Carney, Darrell H (2000) **Acceleration of full-thickness wound healing in normal rats by the synthetic thrombin peptide, TP508.** *Wound Repair and Regeneration* **8** (3), 204-215
	8% full thickness burns infected with Ec, Pa	Animal Studies of Massachusetts General Hospital, Boston, Mass	**H. Shaw Warren** Infectious Disease Unit, 5 floor, Mass General Hospital, 149 13th St., Charlestown, MA 02129 (617) 726-5774 warren@helix.mgh.harvard.edu .	Nathan A. Busch,Emily M. Zanzot, Paul M. Loiselle, Edward A. Carter Jennifer E. Allaire, Martin L. Yarmush and H. Shaw Warren **A Model of Infected Burn Wounds Using *Escherichia coli* O18:K1:H7 for the Study of Gram-Negative Bacteremia and Sepsis** *Infection and Immunity*, June 2000, p. 3349-3351, Vol. 68, No. 6
	20% full thickness burns	US Army Institute of Surgical Research	3400 Rawley E Chambers Ave Fort Sam Houston, TX 78234	Chu CS, McManus AT, Mason AD, Pruitt BA Jr. **Topical silver treatment after escharectomy of infected full thickness burn wounds in rats.**

				2005 May;58(5):1040-6.
	20% TBSA full-thickness burn infected with Pa	US Army Institute of Surgical Research, Texas	**David S. Kauvar, MD** USAISR, 3400 Rawley E. Chambers Ave Fort Sam Houston, Texas 78234–6315	Kauvar, David S. MD; Acheson, Eric MD; Reeder, Joanna BS; Roll, Kristin BS; Baer, David G. PhD **Comparison of battlefield-expedient topical antimicrobial agents for the prevention of burn wound sepsis in a rat model.** *Journal of Burn Care and Rehabilitation*, 26(4), July/August 2005, pp 357-361
	standard contact burn (20% TBSA). infected with Pa, Sa	Department of Surgery, University of Texas Medical Branch Shriners Hospital for Children, Burns Hospital, TX	**John P. Heggers, PhD** Shriners Hospital for Children 815 Market St., Galveston, TX 77550	Heggers, John PhD, CWS; Goodheart, Rick E. MLT; Washington, Joyce BS; McCoy, Lana MT; Carino, Edith BS; Dang, Thanh BS; Edgar, Pat RN; Maness, Cassie; Chinkes, David PhD **Therapeutic Efficacy of Three Silver Dressings in an Infected Animal Model** *J. Burn Care Rehabil.*, Vol. 26(1), Jan/Feb. 2005, pp 53-56
	Full-thickness, lasting 13 days	Institute for Wound Research Department of Obstetrics and Gynecology Div. of Plastic and Rec. Surgery,Dept. of Surgery University of Florida, Gainesville, Florida.		Chen, Chin, Schultz, Gregory S., Bloch, Melissa, Edwards, Paul D., Tebes, Steve & Mast, Bruce A. (1999) **Molecular and mechanistic validation of delayed healing rat wounds as a model for human chronic wounds.** *Wound Repair and Regeneration* 7 (6), 486-494.
	Linear incision wound	Burn Center, Brigham and Women's and Beth Israel Hospitals, Department of Surgery, Harvard Medical School, Boston, Massachusetts.	**Robert H. Demling**	Demling, Robert H (2000) **Oxandrolone, an anabolic steroid, enhances the healing of a cutaneous wound in the rat.** *Wound Repair and Regeneration* **8** (2), 97-102.
	scalding wound 20% TBSA right and left lateral flanks	Dept of Surgery, University of Michigan Department of Microbiology, University of Iowa Department of Medicine, UCLA School of Medicine Ruhr University Bergmannsheil, Bochum, Germany	**Lars Steinstraesser** Plastic and Reconstructive Surgery Ruhr University Bergmannsheil 49(0234)302-6841 F:49(0234)302-6379 lars.steinstraesser@ruhr-uni-bochum.de	Lars Steinstraesser, Brian F. Tack, Alan J. Waring, Teresa Hong, Lee M. Boo Ming-Hui Fan,DI Remick,Grace L. Su,Robert I. Lehrer,and Stewart C. Wang **Activity of Novispirin G10 against Pseudomonas aeruginosa** **In Vitro and in Infected Burns** Antimicrobial Agents and Chemotherapy, June 2002, p. 1837-1844, Vol. 46, No. 6
Mice	Full thickness 8X12.5mm 5mm unbroken skin between wounds 4 wounds per mouse E.coli injected	Research Animal Care of Mass General Hospital	**Michael R. Hamblin** BAR 314B, Wellman Laboratories of Photomedicine, Mass General Hospital 50 Blossom St, Boston, MA 02114-2698	Michael R. Hamblin, David A. O'Donnell,Naveen Murthy, Christopher H. Contag,and Tayyaba Hasana **Rapid Control of Wound Infections by Targeted PhotodynamicTherapy Monitored by *In Vivo* Bioluminescence Imaging** *Photochemistry and Photobiology*: Vol. 75, No. 1, pp. 51–57., 2001

		hamblin@helix.mgh.harvard.edu	
several models of infections in wounds and soft-tissue abcesses	Harvard Medical School, Boston, MA,	**Michael R. Hamblin** 617 726 6182	Tatiana N Demidovaa, Faten Gada, Touqir Zahraa, Kevin P Francisd and Michael R Hamblin **Monitoring photodynamic therapy of localized infections by bioluminescence imaging of genetically engineered bacteria** *Journal of Photochemistry and Photobiology B: Biology* Volume 81, Issue 1 , 3 October 2005, Pages 15-25
excisional wounds Infected with Ec, Pa	Research Animal Care of Mass General Hospital	**Michael R. Hamblin**	Hamblin, Michael R.; O'Donnell, David A.; Zahra, Touqir; Contag, Christopher H.; McManus, Albert T.; Hasan, Tayyaba **Targeted photodynamic therapy for infected wounds in mice** *Proc. SPIE* Vol. 4612, p. 48-58, 2002
Burns 2X2 cm Infected with Pa	University of Cincinnati College of Medicine	**Daniel J. Hassett** Dept of Molecular Genetics, Biochemistry and Microbiology, Univ of Cincinnati College of Medicine 231 Albert Sabin Way, Cincinnati, OH 45267-0524 (513) 558-1154 Daniel.Hassett@UC.Edu	Gee W. Lau, Bradley E. Britigan and Daniel J. Hassett **Pseudomonas aeruginosa OxyR Is Required for Full Virulence in Rodent and Insect Models of Infection and for Resistance to Human Neutrophils** *Infection and Immunity*, April 2005, p. 2550-2553, Vol. 73, No. 4
incisional model 2cm infected with Spy, Se, Sa	SmithKline Beecham Pharmaceuticals Collegeville, Pa	**Valerie Berry**	Valier Berry, Roni Page, Jennifer Satterfield, Christine Singley, Rob Straub, Gary Woodnutt **Comparative efficacy of gemifloxacin in experimental models of pyelonephritis and wound infection** *Journal of Antimicrobial Chemotherapy* (2000) 45, Suppl. S1, 87-93
Burns	Texas Tech Univ, Texas	**John A. Griswold MD** Department of Surgery, Texas Tech University Health Sciences Center 3601 4th Street, Lubbock, TX 79430, , USA	Kendra P. Rumbaugh Ph.D, Abdul N. Hamood Ph.D. and John A. Griswold M.D **Cytokine induction by the P. aeruginosa quorum sensing system during thermal injury** *Journal of Surgical Research* Volume 116, Issue 1 , January 2004, Pages 137-144
Injection	Baltimore, MD	**Eric Nuermberger** 1 410 502 0580	Eric Nuermbergera, Kris Helkeb and William R. Bishaia **Low-dose aerosol model of pneumococcal pneumonia in the mouse: utility for evaluation of antimicrobial efficacy** *International Journal of Antimicrobial Agents* Volume 26, Issue 6 , December 2005, Pages 497-503
wound, approx 1-cm in diameter, was created on the dorsal	Salt Lake City, UT	**Glenn D. Prestwich** Dept of Medicinal Chemistry,	Kelly R. Kirkera, Yi Luob, J. Harte Nielsonc, Jane Shelby and Glenn D. Prestwich

		The University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, UT 84108 1-801-585-9051	**Glycosaminoglycan hydrogel films as bio-interactive dressings for wound healing** *Biomaterials* Volume 23, Issue 17 , September 2002, Pages 3661-3671
infection in excisional mouse wounds		**Michael R. Hamblin**	Michael R. Hamblin, Touqir Zahra, Christopher H. Contag, Albert T. McManus, and Tayyaba Hasan **Optical Monitoring and Treatment of Potentially Lethal Wound Infections *In Vivo*** *The Journal of Infectious Diseases*, volume 187 (2003), pages 1717–1726
infected wounds, burns, and soft tissue infections in mice	Wellman Center for Photomedicine, Mass General Hospital, Boston,	**Michael R. Hamblin**	Demidova TN, Hamblin MR. **Photodynamic therapy targeted to pathogens.** *Int J Immunopathol Pharmacol.* 2004 Sep-Dec;17(3):245-54.
infected burn and bacteria observ.	Dept Medicine/Infectious Diseases, Univ. of Florida Shriners Hospital for Children, Cincinnati, Ohio Dept. of Microbiology, Harvard Medical, Boston	**Reuben Ramphal** Dept of Medicine/Infectious Diseases, P.O. Box 100277, JHMHC, Univ. of FL Gainesville, FL 32610. . Ph: (352)392-2932 F:(352)392-6481 E-mail: ramphr@medmac.ufl.edu	Shiwani K. Arora, Alice N. Neely, Barbara Blair, Stephen Lory, and Reuben Ramphal **Role of Motility and Flagellin Glycosylation in the Pathogenesis of Pseudomonas aeruginosa Burn Wound Infections** *Infection and Immunity*, Vol. 73, No. 7, July 2005, p. 4395-4398
partial-thickness and full-thickness wounds infected with Pa	Department of Research, Shriners Hospitals for Children, Cincinnati, Ohio 45229-3095, USA	**Steven T. Boyce, Ph.D** Research Department Shriners Hospital for Children 3229 Burnet Ave, Cincinatti, OH stboyce@shrinenet.org	Supp AP, Neely AN, Supp DM, Warden GD, Boyce ST **Evaluation of cytotoxicity and antimicrobial activity of Acticoat Burn Dressing for management of microbial contamination in cultured skin substitutes grafted to athymic mice** *J Burn Care Rehabil.* 2005 May-Jun;26(3):238-46
Sprague Dawley and diabetic 3 cm Skin punch biopsy	Departments of Microbiology and Immunology Surgery, Burn and Shock Trauma Institute Loyola University Medical Center, Maywood, Illinois	**Luisa A. DiPietro** Loyola University Medical Center 2160 S. 1st Ave., BSTI, Building 110 Maywood, IL 60153 ldipiet@lumc.edu	Julia V. Dovi, Li-Ke He and Luisa A. DiPietro **Accelerated wound closure in neutrophil-depleted mice** *Journal of Leukocyte Biology.* 2003;73:448-455
Full-thickness	Section of Plastic Surgery, University of Michigan Medical Center, 1500 East Medical Center Drive, 2130 Taubman Center, Ann Arbor, MI 48109-0340	**Riley S. Rees, MD, FACS** Fax: (734) 763-5354 rreese@umich.edu	Rees, Riley S., Adamson, Belinda F. & Lindblad, William J. (2001) **Use of a cell-based interactive wound dressing to enhance healing of excisional wounds in nude mice.** *Wound Repair and Regeneration* **9** (4), 297-304.
full-thickness, third-degree burns infected with Pa	Infectious Disease Division, Massachusetts General Hospital and	**JA Fishman**	Wilkinson, Robert A. BS; Fishman, Jay A. MD **Effect of thermal injury with Pseudomonas aeruginosa infection on**

		Harvard Medical School Boston, MA		**pulmonary and systemic bacterial clearance.** *Journal of Trauma-Injury Infection & Critical Care.* 47(5):912, Nov. 1999
	mouse oocytes	WPI	**Eric Overstrom**	**Genetic strain variations in the metaphase-II phenotype of mouse oocytes matured *in vivo or in vitro***
	burns		**Laurence Rahme**	Fred Ausubel (works with Microbes)
	tissue transplants on athymic mice	Department of Chemical and Biological Engineering State University of New York at Buffalo	**S.T. Andreadis** Bioengineering Laboratory, 908 Furnas Hall State University of New York Buffalo, NY 14260 sandread@eng.buffalo.edu	Geer DJ, Swartz DD, Andreadis ST **Biomimetic Delivery of Keratinocyte Growth Factor upon Cellular Demand for Accelerated Wound Healing *in Vitro* and *in Vivo*** *Am J Pathol.* 2005 Dec;167(6):1575-86
	10mm diameter dorsal skin wounds	Section of Endocrinology, Diabetes, and Nutrition Department of Medicine Boston University School of Medicine Boston, MA	**J.D. Safer** Endocrinology, Diabetes, & Nutrition Boston University School of Medicine 715 Albany Street, Room M-1016 Boston, Massachusetts 02118 jsafer@bu.edu	Safer JD, Crawford TM, Holick MF **Topical thyroid hormone accelerates wound healing in mice** *Endocrinology*. 2005 Oct;146(10):4425-30. Epub 2005 Jun 23
	tissue transplants on athymic mice	Shriners Burns Hospital	**Dorothy M. Supp, Ph.D.** Adjunct Research Assistant Prof. Shriners Burns Hospital dsupp@shrinenet.org (513) 872-6000 Fax: (513) 872-6072	Supp DM, Bell SM, Morgan JM, and Boyce ST **Genetic modification of cultured skin substitutes by transduction of keratinocytes and fibroblasts with platelet derived growth factor A** Wound Repair Regen 2000; 8:26-35
	3 mm in diameter biopsy punch acute wound	Loyola University Medical Center, Maywood, Illinois Schering-Plough Research Institute Kenilworth, NJ Dana-Farber Cancer Institute, Boston, Massachusetts	**Dr. Luisa A. DiPietro** Burn and Shock Trauma Institute Loyola University Medical Center ldipiet@luc.edu	Quentin E.H.Low, Iulia A. Drugea, L.A. Duffner, D.G. Quinn, D.N. Cook Barrett J. Rollins, Elizabeth J. Kovacs and Luisa A. DiPietro **Wound Healing in MIP-1{alpha}-/- and MCP-1-/- Mice** *American Journal of Pathology.* 2001;159:457-463
***db/db* mice**	Full-thickness	*Laboratory of Microvascular Research and* Vascular Tissue Engineering, Institute of Reconstructive Surgery, New York University Medical Center, New York, New York.	**Geoffrey C. Gurtner, MD** New York University Medical Center, 560 First Avenue, TH-169, New York NY 10016. Fax: (212) 263-0481 geoffrey.gurtner@med.nyu.edu	Galiano, Robert D., Michaels, V, Joseph, Dobryansky, Michael, Levine, Jamie P. & Gurtner, Geoffrey C. (2004) **Quantitative and reproducible murine model of excisional wound healing.** *Wound Repair and Regeneration* **12** (4), 485-492.
	Full-thickness	*Department of Cell Biology and Molecular* Medicine, New Jersey Medical School, UMDNJ,	**S. Joseph Leibovich, PhD,** *Fax: (973) 972-7489*	Masters, Kristyn S. Bohl, Leibovich, S. Joseph, Belem, Paula, West, Jennifer L. & Poole-Warren, Laura A. (2002)

		185 South Orange Avenue, Newark, NJ 07103-2714	leibovic@umdnj.edu.	**Effects of nitric oxide releasing poly(vinyl alcohol) hydrogel dressings on dermal wound healing in diabetic mice.** *Wound Repair and Regeneration* **10** (5), 286-294.
Smad3 null mice	Full-Thickness	Department of Dermatology and Skin Surgery Roger Williams Medical Center, Elmhurst Building, 50 Maude Street, Providence, RI 02908	**Dr. Vincent Falanga,** Professor and Chairman Fax: (401) 456-6449 vfalanga@bu.edu.	Falanga, Vincent, Schrayer, David, Cha, Jisun, Butmarc, Janet, Carson, Polly, Roberts, Anita B. & Kim, Seong-Jin (2004) **Full-thickness wounding of the mouse tail as a model for delayed wound healing: accelerated wound closure in Smad3 knock-out mice.** *Wound Repair and Regeneration* **12** (3), 320-326.
Transgenic Mice	transgenic mouse strain with the MMP-13 or the COL1A2 promoter laser wounds, scalpel wounds	Department of Pathology Vanderbilt University School of Medicine Nashville, Tennessee 37232-2561	**Jeffrey M. Davidson, PhD** Department of Pathology Vanderbilt Univ. School of Medicine C-3321 Medical Center North, Nashville, TN 37232-2561 jeffrey.m.davidson@vanderbilt.edu	Nanjun Wu*, E. Duco Jansen†, and Jeffrey M. Davidson **Comparison of mouse matrix metalloproteinase 13 expression in free-electron laser and scalpel incisions during wound healing** *Journal of Investigative Dermatology*, Vol. 121 p. 926 (2003)
Rodents	Inflammatory response	Case Western University	**Horst von Recum** **Michael J. Cima**	Word of Mouth
	Varies	Worcester, MA	**Mitch Sanders**	Word of Mouth
	Burns	University of Iowa	**G. Patrick Kealey, MD**	**Methamphetamine-associated burn injuries: a retrospective analysis.** J Burn Care Rehabil. 2004 Sep-Oct;25(5):425-9.
N/A	Burns	Burns Service and Shriners Burns Institute	**Dr Ronald Tompkins**	Word of Mouth; Dr. J Fisherman
	Burns	Department of Physiology, Loyola University Center, 2160 South First Ave., Maywood, IL 60153, United States.	**Richard Gamelli**	Word of Mouth

APPENDIX GG: *In Vivo* Animal Model- Wound Measurement

MGH WOUND ANIMAL STUDY (FEB. 18 - FEB. 25)

WOUND AREA (mm^2)

Treatment	Cage #	Mouse	1st timepoint	2nd timepoint	Final timepoint	Difference
High	1	yellow	122.82	97.7284	76.9232	
			112.64	96.8438	92.9961	
			106.64	97.266	90.552	
			114.0333333	**97.2794**	**86.82376667**	27.20956667
High	1	purple	101.2476	106.9285	86.5348	
			116.1276	107.7234	91.8826	
			109.1111	108.0576	103.3032	
			108.8287667	**107.5698333**	**93.90686667**	14.9219
High	1	blue	99.83	EXPIRED	EXPIRED	EXPIRED
			82.7113			
			97.9932			
			93.5115			0
High	2	purple-green	103.356	96.8272	88.6788	
			109.8251	104.4903	92.6276	
			100.1806	99.9075	95.5636	
			104.4539	**100.4083333**	**92.29**	12.1639
Medium	2	black with pink	98.6884	96.714	69.3116	
			88.572	92.2031	70.2402	
			91.854	94.119	70.8152	
			93.03813333	**94.34536667**	**70.12233333**	22.9158
	2	Yellow w/ pink	EXPIRED	EXPIRED	EXPIRED	EXPIRED
						0
Medium	3	blue	102.7242	79.2642	62.9909	
			117.6184	80.0934	79.692	
			104.0778	78.9488	75.2335	
			108.1401333	**79.43546667**	**72.6388**	35.50133333
Medium	3	green	104.6261	96.558	80.1905	
			126.1212	108.1106	78.3232	
			103.6575	100.0278	83.9644	
			111.4682667	**101.5654667**	**80.82603333**	30.64223333
Medium	3	pink	88.638	106.6891	84.7308	
			105.3315	87.248	94.2624	
			85.95	98.6272	101.9649	
			93.3065	**97.52143333**	**93.6527**	-0.3462

Low	4	pink with green	91.2924 101.9342 86.0952 **93.10726667**	87.4375 93.2928 78.2624 **86.3309**	EXPIRED	6.776366667
Low	4	yellow	116.1594 107.1534 98.5842 **107.299**	95.571 88.4317 81.2842 **88.42896667**	EXPIRED	18.87003333
Low	4	blue with green	91.8162 107.2305 117.1924 **105.4130333**	95.3304 95.1142 109.725 **100.0565333**	EXPIRED	5.3565
Low	5	blue-green	100.975 88.1624 108.0777 **99.0717**	101.472 91.451 103.74 **98.88766667**	EXPIRED	0.184033333
C-vehicle	5	pink with black	83.4067 88.4588 100.0296 **90.6317**	101.9769 92.5931 95.8518 **96.80726667**	EXPIRED	-6.175566667
C-vehicle	5	pink	96.1644 118.1862 90.809 **101.7198667**	106.0477 108.2697 107.9148 **107.4107333**	97.864 83.304 97.6752 **92.94773333**	8.772133333
C-no treat.	6	yellow	101.5404 108.9165 103.5095 **104.6554667**	102.9766 100.362 101.2128 **101.5171333**	89.67 98.4932 84.8232 **90.99546667**	13.66
C- no treat	6	pink with blue	94.024 96.4712 93.7368 **94.744**	104.6583 101.1512 110.8968 **105.5687667**	98.388 105.742 112.9887 **105.7062333**	-10.96223333
C-wound	6	green	84.2607 92.379 76.8379 **84.49253333**	88.0686 84.7008 82.5792 **85.1162**	70.4781 96.88 100.488 **89.28203333**	-4.7895

APPENDIX HH: *In Vivo* Animal Model- Weight

WEIGHT (g)

Treatment	Cage #	mouse	1st weigh (start)	2nd weigh (middle)	3rd weigh (end)
High	1	yellow	20.38	17.8	18.36
High	1	purple	22.4	16.64	17.76
High	1	blue	21.64	EXPIRED	EXPIRED
High	2	purple-green	21.68	18.38	18.94
Medium	2	black with pink	20.96	18.7	19.3
Medium	3	blue	22	18.58	19.5
Medium	3	green	19.84	16.44	19.12
Medium	3	pink	21.44	19.12	19.24
Low	4	pink with green	21.94	17.82	EXPIRED
Low	4	yellow	21.92	EXPIRED	EXPIRED
Low	4	blue with green	21.42	19.0	EXPIRED
Low	5	blue-green	22.2	EXPIRED	EXPIRED
C-vehicle	5	pink with black	20.4	EXPIRED	EXPIRED
C-vehicle	5	pink	20.6	18.84	16.58
C- no treat.	6	yellow	21.68	18.78	17.9
C- no treat.	6	pink with blue	20.42	18.36	19.02
C- wound	6	green	23.02	19.74	20.34

APPENDIX II: *In Vivo* Animal Model- Bioluminescent Values

		Bioluminescence		
High	Cage 1	Blue		
	Date		Total Intensity	Pixels
	21806		262428	3166
	21806		409684	3378
	21906		331560	3575
	22006		120156	4706
High	Cage 1	Purple	Total Intensity	Pixels
	21806		141094	4254
	21806		98532	4966
	21906		204126	4966
	22006		69464	4966
	22106		115492	4966
	22206		32332	4966
	22306		27395	3006
	22406		22456	3006
	22806		113880	3477
High	Cage 1	Yellow	Total Intensity	Pixels
	21806		166912	3806
	21806		69776	3806
	21906		24864	3806
	22006		89004	3806
	22106		93085	3806
	22206		16302	3806
	22306		21617	3806
	22406		21578	3806
	22806		82292	3806
High	Cage 2	black w/pink	Total Intensity	Pixels
	21806		110446	3806
	21806		130688	3806
	21906		77100	3806
	22006		110388	3806
	22106		30254	3806
	22206		27911	3806
	22306		19150	3806
	22406		20374	3806
	22806		48215	3806
Medium	Cage 2	Purp w/green	Total Intensity	Pixels
	21806		220712	3806
	21806		133416	3806
	21906		138512	3806
	22006		79560	3806
	22106		140036	3806
	22206		73568	3806
	22306		53292	3806
	22406		68120	3806
	22806		146484	3477

Medium	Cage 3	Blue	Total Intensity	Pixels
	21806		143568	3806
	21806		329280	3806
	21906		238652	3806
	22006		81528	4010
	22106		37700	4254
	22206		21046	4254
	22306		25346	4254
	22406		19601	4254
	22806		33998	2274
Medium	Cage 3	Green	Total Intensity	Pixels
	21806		421856	22774
	21806		511696	2774
	21906		556368	2238
	22006		225608	5194
	22106		51264	2974
	22206		42146	4486
	22306		38396	3382
	22406		35168	3382
	22806		96070	3382
Medium	Cage 3	Pink	Total Intensity	Pixels
	21806		217594	4718
	21806		191348	4718
	21906		467008	2418
	22006		106144	4434
	22106		48812	3218
	22206		25075	4254
	22306		32974	3253
	22406		10878	1446
	22806		66374	3354
Low	Cage 4	blue w/green	Total Intensity	Pixels
	21906		2579392	4098
	21906		8597248	6138
	22006		251672	4706
	22106		480976	4706
	22206		45028	2078
	22306		euthanized	
Low	Cage 4	Pink w/green	Total Intensity	Pixels
	21906		3420512	5336
	21906		2904960	4372
	22006		231712	4372
	22106		110512	2659
	22206		44592	1359
	22306		euthanized	
Low	Cage 4	Yellow	Total Intensity	Pixels
	21906		243280	4018
	21906		382752	4132
	22006		69268	3378
	22106		48008	3170
	22206		euthanized	

Low	Cage 5	Blue-green	Total Intensity	Pixels
	21906		2352640	4126
	21906		788400	7236
	22006		99664	2676
	22106		29048	1738
	22206		expired	
C- vehicle	Cage 5	Pink	Total Intensity	Pixels
	21906		418176	3179
	22006		177696	1738
	22106		38014	3168
	22206		74560	1738
	22306		23060	1738
	22406		48360	1738
	22506		89640	3107
	22806	expired	whole plate	
C-vehicle	Cage 5	Pink w/black	Total Intensity	Pixels
	21906		1528176	4364
	22006		165744	3168
	22106		139784	3663
	22206		euthanized	
C - no treat.	Cage 6	Green	Total Intensity	Pixels
	21906			
	22006		272192	2791
	22106		47000	2791
	22206		49512	3070
	22306		109944	2791
	22406		62780	2791
	22506		22672	2791
	22806		69784	1919
C- no treat.	Cage 6	Pink w/blue	Total Intensity	Pixels
	21906		2709568	3793
	22006		91056	1419
	22106		91152	4710
	22206		58500	3454
	22306		108052	4014
	22406		156840	3688
	22506		82572	3688
	22806		103488	2762
C- wound	Cage 6	Yellow	Total Intensity	Pixels
	21906		48212	4026
	22006		60820	3354
	22106		100412	3354
	22206		38848	2670
	22306		18184	735
	22406		133856	735
	22506		198616	3926
	22806		400224	1216

APPENDIX JJ: Final Dressing Construction Using YP-ET

Alginate + Glycerol + Calcium Chloride Layer + CMC + Glycerol + Agarose + YP-ET Layer Dressing Construction

Purpose: Create final dressing prototype using YP-ET antimicrobial agent

Materials: CMC powder
Alginate powder
Sterile water
Agarose 1% - 0.5g in 50mL water, boil 30 seconds, until all powder is melted
Glycerol
Commercial Grade YP-ET - must vortex before each use

Methods:

Alginate Layer

1.) Obtain and make 1.5% Alginate (900 ml total vol.) and pour into 1 liter bottle
2.) Add, by weight, 1.75% glycerol (15.75g) to alginate in bottle
3.) Vortex mixture and pour ~55g into large gel plates keeping the volume per plate constant by weight
4.) Let gels dry overnight in 30ºC incubator
5.) After a minimum of 24 hours and there are no signs of wetness on the gel, peel alginate film from plate using a spatula
6.) Add, by weight, specified amount of calcium chloride (0.5%) to empty gel plate
7.) Place film back into plate on the calcium chloride ~1 min
8.) After 1 minute remove film once again, flip it over and allow other side to soak
9.) Allow film to soak in calcium chloride solution for a minimum of 24 hours
10.) After 24 hours, pour off $CaCl_2$ solution and add enough water to submerge the gel careful not to disturb the gel. Allow 20 minutes to soak
11.) After 20 minutes, remove old water and replace it with clean water and continue the changing the water and soaking the gels at 20 minute intervals for at least 1 hour
12.) Allow gel to remain in clean water to avoid drying out
13.) Using a razor, cut clean calcium alginate gels to size of smaller plates by tracing along the edge the blade
Note: Be sure there is a tight seal between the edge of the gel and the tray edge. If necessary cut the gel larger than the actual plates.
14.) Place in freezer for ~20 minutes to solidify the alginate layer

APPENDIX KK: Final Dressing Construction Using YP-ET Continued

Glycerol + Terpene + Agarose CMC Prototype 32806

Purpose: Test and decide on most effective terpene concentrations with varied amounts
Determine the stability of prototype dressing by pouring the CMC onto the alginate layer

Materials: CMC powder
Sterile water
Agarose 2% - 2g in 100 mL water, boil until all powder is melted
Glycerol
Commercial Grade YP-ET - vortex before each use!
1.5% CMC stock
2% CMC stock
silver 1 mg/ml (20 mg diluted in 20 mL) water - homogenize using polytron

Methods:
1. Prepare 2% CMC and allow to mix overnight
2.) Add 2 g of agarose powder to 100g water - boil to dissolve agarose
3.) Place all materials into the warmed 50C water bath including bottles and water
4.) Measure out indicated volumes (round to 2 decimal places) into glass 100 mL bottle, taring after each material

	3	2	1	4	5	
Hydrocolloid	**YP-ET (g)**	**glycerol (g)**	**2% CMC (g)**	**2% agar. (g)**	**silver (g)**	**water**
1.5% CMC + 0.3% agar. + 1.75% glycerol + 41.8 ppm silver control	0	0.963	41.250	8.25	2.3	2.238
1.5% CMC + 0.3% agar. + 1.75% glyc. + 6.6 mg/ml YP-ET + 41.8 ppm silver	2.27	0.963	41.250	8.25	2.3	0.000

5.) Mix throughouly by vortex or by shaking the bottle until all particles are evenly distributed.
6.) Cast gels on top of thin alginate layer, freeze at -80C for ~ 1 hr and then lyophilize on standard recipe for 24 hrs.
7.) Run water absorption test to measure amount of volume the gels can hold and stability of the gels

APPENDIX LL: Disc Diffusion Assay

Disc Diffusion Assay

Purpose: To determine the optimal concentration of YP-ET for the prototype dressing by analyzing the antimicrobial activity of different concentrations on various microrganisms

Materials:
5 mm biopsy punch
S. aureus
P. aeruginosa
Mueller-Hinton Agar Plates
0.5 McFarland Standard
prototype dressing
sterile filter paper (negative control)

Method:

1.) Punch out discs of protype dressing and sterile filter paper using 5 mm biopsy punch
2.) Grow microrganisms overnight
3.) Dilute microrganisms to the density of a 0.5 McFarland standard
4.) Pour microrganisms over the surface of agar plates and decant excess
5.) Allow plates to dry
6.) Place dressing discs on the surface of the agar (2 microbes @ n=2 and sterile filter papers (negative control) @ n=2)
7.) Incubate overnight at 35 degrees C
8.) Measure clear zones around the disc

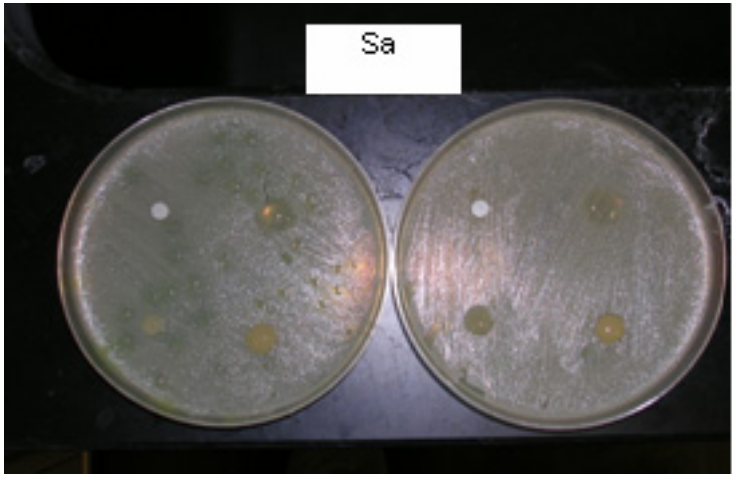
*Any clear zones of 1 mm radius or greater means that actives have released from the dressing and migrated through the agar matrix. Also, the active was in high enough concentration to inhibit the growth of the microbes in the clear zone.

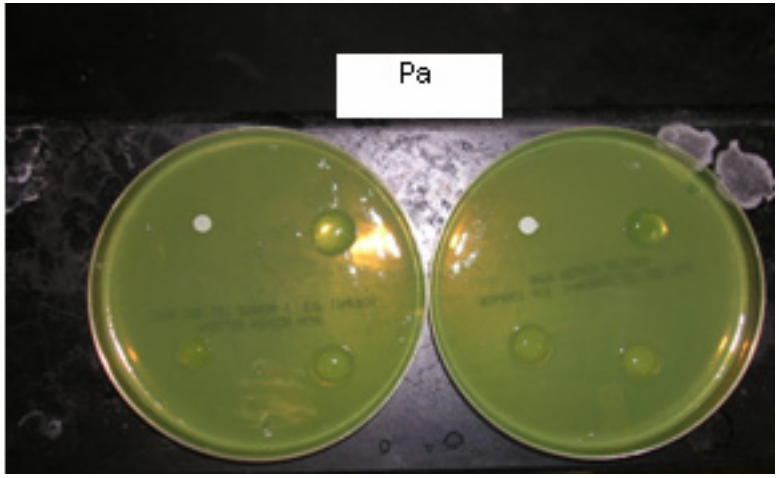
Plate 1 (Sa)	Zone Radius (mm)
Filter Paper	
Control	
6.6 mg/ml YP-ET	
24 mg/ml YP-ET	

Plate 2 (Sa)	Zone Radius (mm)
Filter Paper	
Control	
6.6 mg/ml YP-ET	
24 mg/ml YP-ET	

Plate 3 (Pa)	Zone Radius (mm)
Filter Paper	
Control	
6.6 mg/ml YP-ET	
24 mg/ml YP-ET	

Plate 4 (Pa)	Zone Radius (mm)
Filter Paper	
Control	
6.6 mg/ml YP-ET	
24 mg/ml YP-ET	





APPENDIX MM: Antimicrobial Barrier Assay

Antimicrobial Barrier Assay

Purpose: To determine if the prototype dressing is effective as an antimicrobial barrier, which does not allow microbes on the surface to enter the wound dressing

Materials:
S. aureus
Muller-Hinton Agar plates
0.5 McFarland Standard
prototype dressing
sterile filter paper (negative control)

Method:

1.) Grow microrganisms overnight

2.) Punch out discs of protype dressing and sterile filter paper using 5 mm biopsy punch

3.) Place squares on the surface of agar plates

4.) Innoculate test bacteria on top of the squares of dressing and filter paper

4a.) Dilute microrganisms to the density of a 0.5 McFarland standard

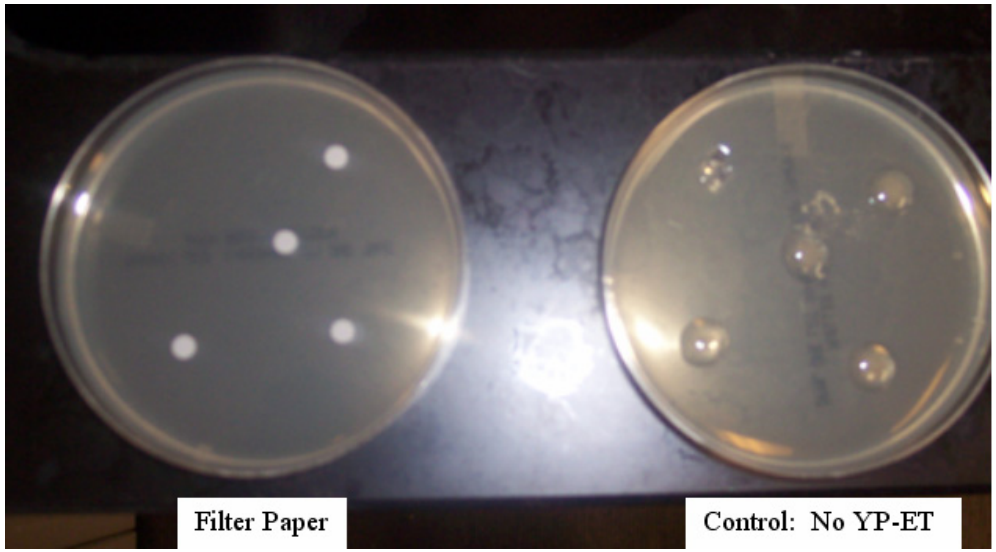
4b.) Pour microrganisms over the surface of the dressing squares

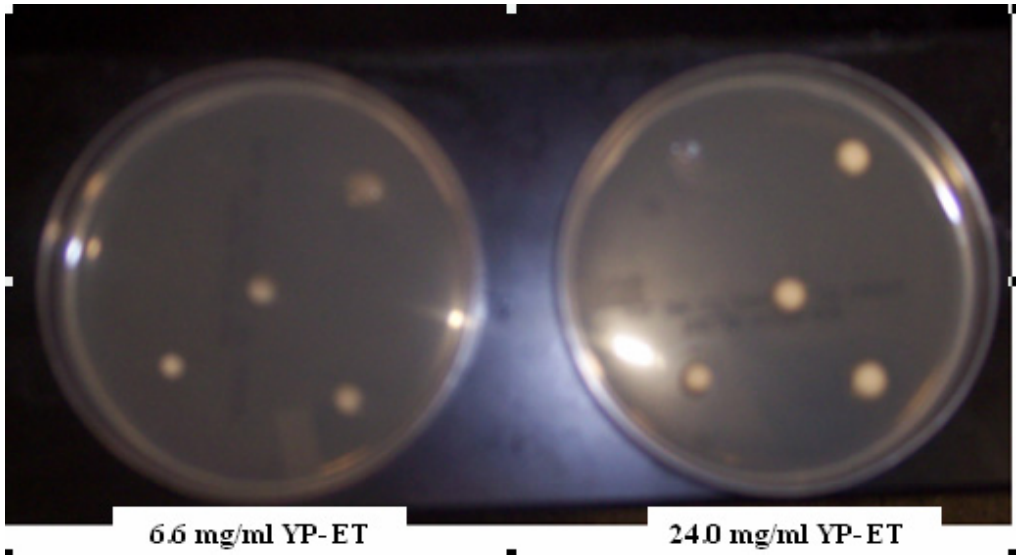
4c.) Allow plates to dry

5.) Incubate at 35 degrees C for a total of 24 h, removing one square of dressing and filter paper at 1, 2, 4, 8, and 24 h.

6.) Observe for evidence of microbial growth in the area, which was under the square that is removed at each time point

	Bacterial Growth Under Dressing at Time Points				
Plate 1 (Sa)	**1h**	**2h**	**4h**	**8h**	**24h**
Filter Paper (5 pieces)					
Plate 2 (Sa)					
Control (5 pieces)					
Plate 3 (Sa)					
6.6 mg/ml YP-ET (5 pieces)					
Plate 4 (Sa)					
24 mg/ml YP-ET (5 pieces)					


Filter Paper
Control: No YP-ET


6.6 mg/ml YP-ET
24.0 mg/ml YP-ET

APPENDIX NN: Corrected Zone of Inhibition Assay

Corrected Zone of Inhibition Assay

Purpose: To determine if a single prototype dressing is effective in killing bacteria for a minimum of 7 days

Materials:

S. aureus
Muller-Hinton Agar plates
0.5 McFarland Standard
prototype dressing
sterile filter paper (negative control)

Method:

1.) Punch out discs of prototype dressing and sterile filter paper using 5 mm biopsy punch

2.) Grow microrganisms overnight

3.) Dilute microrganisms to the density of a 0.5 McFarland standard

4.) Pour microrganisms over the surface of agar plates and decant excess

5.) Allow plates to dry

6.) Incubate overnight at 35 degrees C

7.) Place dressing squares and filter paper squares on each agar plate.

8.) After 24 h, remove the squares, examine the plates to determine if colonies reside under the dressing and filter paper

9.) Place the SAME squares on a NEW/FRESH plate.

10.) After 24 h, remove the squares, examine the plates to determine if colonies reside under the dressing and filter paper

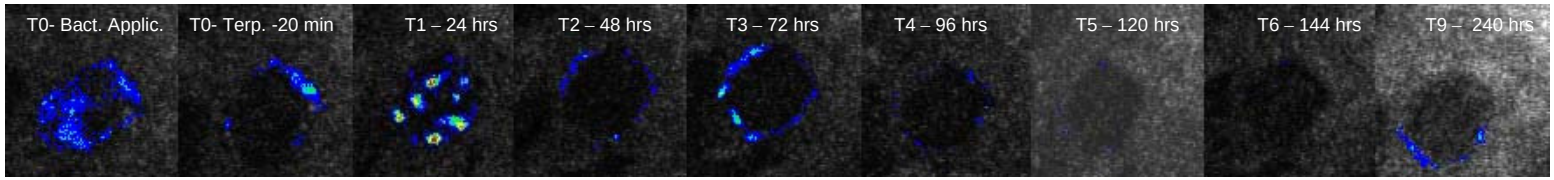
11.) Continue the same procedure for 7 days

Plate 1 (Sa)	**Amount of Kill Under Dressing Square at Time Points**						
Filter Paper	**1 day**	**2 days**	**3 days**	**4 days**	**5 days**	**6 days**	**7 days**
Control							
6.6 mg/ml YP-ET							
24 mg/ml YP-ET							

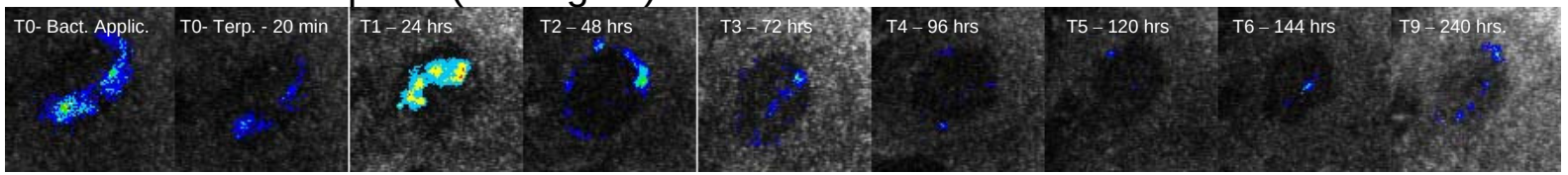
Plate 2 (Sa)	**Amount of Kill Under Dressing Square at Time Points**						
Filter Paper	**1 day**	**2 days**	**3 days**	**4 days**	**5 days**	**6 days**	**7 days**
Control							
6.6 mg/ml YP-ET							
24 mg/ml YP-ET							

APPENDIX OO: *In Vivo* Experiment- Bioluminescent Pictures

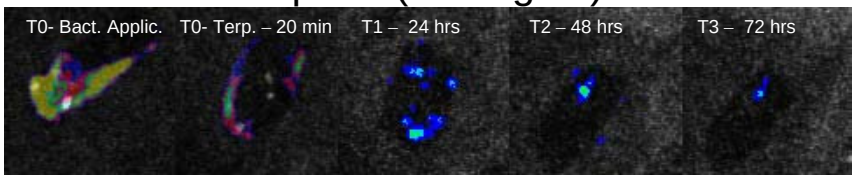
High Dose Terpene (24 mg/ml)



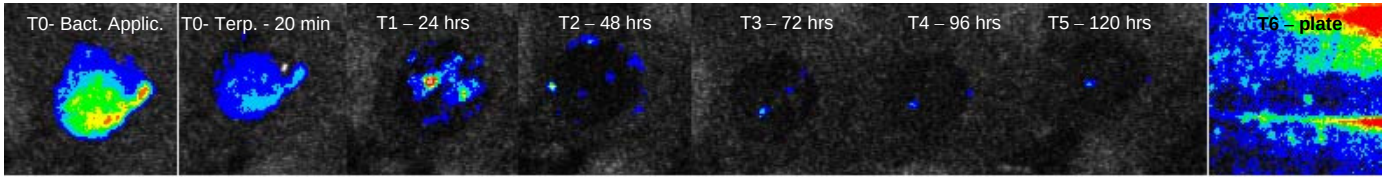
Medium Dose Terpene (6.6 mg/ml)



Low Dose Terpene (1.5 mg/ml)

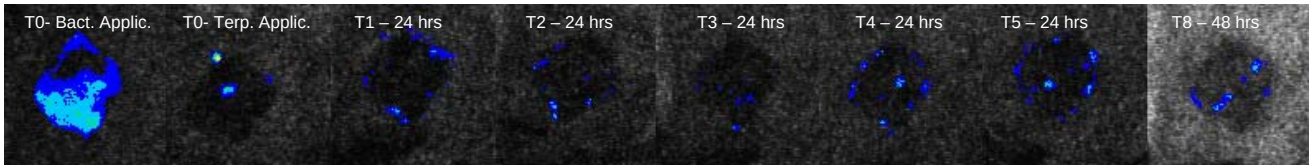


Control – Vehicle only

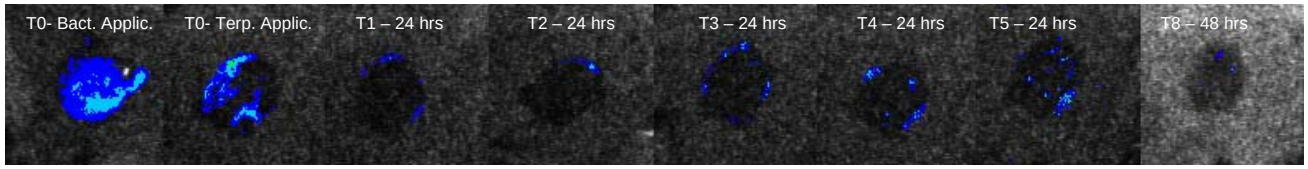


Signs of a systemic infection – heart swab

Control – No treatment



Control – Wound only (no infection)



APPENDIX PP: CMC Hydrocolloid plus Agarose

CMC Hydrocolloid plus Agarose 21406

Purpose: Identify Agarose concentration to stabilize CMC hydrocolloid

Materials: Stock 2% CMC hydrocolloid - heat to 50C
Sterile water - heat to 50C
Agarose - prepare 2 % agarose by suspending 0.75 gm in 50 ml water. Boil until dissolved. Cool in 50C water bath

Prepare 1.5% CMC gels with varying agarose concentrations

% [Agarose]	ml 2 % Agarose	% CMC	ml 2% CMC	ml sterile water
0	0	1.50%	11.25	3.8
0.1	0.75	1.50%	11.25	3.0
0.2	1.5	1.50%	11.25	2.3
0.3	2.25	1.50%	11.25	1.5
0.4	3	1.50%	11.25	0.8
0.5	3.75	1.50%	11.25	0.0

Methods:

1.) Add amounts of sterile water, CMC and agarose to tubes in 50C water bath

2.) Mix to form uniform suspension

3) Cast into small weigh boats. Allow to gel at room temperature and dry to form a film overnight

4.) Run the water absorption test for assessment of dissolving that may occur.

This will establish the relationship between agarose and CMC to produce a stable film

Appendix QQ: Agarose and CMC Water Absorption

Agarose+CMC Water Absorption Test 21506

Purpose: Test the amount of fluid absorptive capacity in each film
Measure the effect of agarose on the stability of the CMC hydrocolloid

Materials: CMC Hydrocolloid + Agarose samples
Microfuge tubes
24 mL sterile water (1 mL/tube)

Methods:
1.) Weigh 24 empty microfuge tubes and label according to hydrocolloid sample, concentration, and additive
2.) Cut a small sample of agarose+CMC hydrocolloid and place in microfuge tube - weigh and record scale reading
3.) Fill microfuge tubes with 1.0 mL sterile water
4.) Leave at room temperature for 5 minutes
5.) Extract surrounding water from the tube and weigh on the scale
Record final volume of water remaining in the sample

SET 1

Hydrocolloid	Empty Tube (g)	Tube+Sample (g)	Final Sample (g)	Sample dry (g)	Sample wet (g)	Final Vol. (g)
0% Agarose + 1.5% CMC	0.9957	1.006	-	0.0103	-	-
0.1% Agarose + 1.5% CMC	0.9958	1.0017	1.2317	0.0059	0.2359	0.23
0.2 % Agarose + 1.5% CMC	0.9965	1.0083	1.3965	0.0118	0.4	0.3882
0.3% Agarose + 1.5% CMC	0.9973	1.006	1.3213	0.0087	0.324	0.3153
0.4% Agarose + 1.5% CMC	0.9995	1.0102	1.3405	0.0107	0.341	0.3303
0.5% Agarose + 1.5% CMC	0.9995	1.0107	1.3474	0.0112	0.3479	0.3367
0.4% Agarose only control	0.9924	0.9982	1.0806	0.0058	0.0882	0.0824
0.3% Agarose only control	0.9965	0.9998	1.0567	0.0033	0.0602	0.0569

SET 2

Hydrocolloid	Empty Tube (g)	Tube+Sample (g)	Final Sample (g)	Sample dry (g)	Sample wet (g)	Final Vol. (g)
0% Agarose + 1.5% CMC	1.0039	1.0104	-	0.0065	-	-
0.1% Agarose + 1.5% CMC	1.0008	1.0068	1.2482	0.006	0.2474	0.2414
0.2 % Agarose + 1.5% CMC	0.9971	1.0073	1.3735	0.0102	0.3764	0.3662
0.3% Agarose + 1.5% CMC	0.9998	1.0095	1.4072	0.0097	0.4074	0.3977
0.4% Agarose + 1.5% CMC	1.003	1.011	1.2917	0.008	0.2887	0.2807
0.5% Agarose + 1.5% CMC	1.0002	1.0116	1.3646	0.0114	0.3644	0.353
0.4% Agarose only control	0.9999	1.0034	1.0848	0.0035	0.0849	0.0814
0.3% Agarose only control	1.0006	1.0012	1.1011	0.0006	0.1005	0.0999

Results: 0.2%, and 0.3% Agarose + 1.5% CMC were most stable with the highest amount of water absorption

Appendix RR: Glycerol Amount Variation Test

Glycerol + Terpene + Agarose CMC Combination test 22006

Purpose: Test and decide on most effective glycerol concentrations with varied amounts
Determine the stability of agarose in the CMC hydrocolloid

Materials: CMC powder
2% CMC stock hydrocolloid
Sterile water
Agarose 1% - 0.5g in 50 mL water, boil 30 sec, until all powder is melted
Glycerol
Commercial Grade YP-ET - vortex before each use!

Methods:

1.) Obtain CMC, make 1.5% hydrocolloid by diluting stock 2% solution

2.) Place all materials into the warmed 50C water bath including bottles and water

3.) Measure out indicated volumes (round to 2 decimal places) into glass 100 mL bottle, taring after each material

	4	3	1	2	5
Hydrocolloid	**Terpene addition (g)**	**glycerol (g)**	**grams 2% CMC**	**grams water**	**grams 1% agarose**
1.5% CMC + 0.3% agarose alone	-	-	41.25	13.585	0.165
1.5% CMC + 0.3% agarose+YP-ET	2.1	-	41.25	11.485	0.165
1.5% CMC + 1.75% glycerol+0.3%agarose+YP-ET	2.1	0.875	41.25	10.61	0.165
1.5% CMC + 3.5%glycerol + 0.3% agarose+ YP-ET	2.1	1.75	41.25	9.735	0.165
1.5% CMC+5.25% glycerol+0.3% agarose+YP-ET	2.1	2.625	41.25	8.865	0.165
1.5% CMC +7% glycerol+0.3%agarose+YP-ET	2.1	3.5	41.25	7.985	0.165

4.) Mix throughouly by vortex or by shaking the bottle until all particles are evenly distributed.

5.) Cast gels, freeze at -80C for ~ 1 hr and then lyophilize on standard recipe for 24 hrs.

6.) Run water absorbption test to measure amount of volume the gels can hold and stability of the gels

Appendix SS: CMC Lyophilization Water Absorption

Lyophilization CMC+glycerol+agarose Water Absorption Test

Purpose: Test the amount of fluid absorptive capacity in each film

Materials: CMC Hydrocolloid samples
Microfuge tubes
24 mL sterile water (1 mL/tube)

Methods:

1.) Weigh 12 empty microfuge tubes and label according to hydrocolloid sample, concentration, and additive

2.) Cut a small sample of lyophilized hydrocolloid and place in microfuge tube - weigh and record scale reading

3.) Fill microfuge tubes with 1.0 mL sterile water

4.) Leave at room temperature for 5 minutes

5.) Extract surrounding water from the tube and weigh on the scale
Record final volume of water remaining in the sample

SET 1

gel (g)	Hydrocolloid	Empty Tube (g)	Tube+Sample (g)	Final Sample (g)	Sample dry (g)	Sample wet (g)	Final Vol. (g)
0.7	1.5% CMC + 0.3% agarose alone	0.9969	1.0144	1.5131	0.0175	0.5162	0.4987
1.24	1.5% CMC + 0.3% agarose+YP-ET	1.0044	1.0335	1.5288	0.0291	0.5244	0.4953
2	1.5% CMC + 1.75% glycerol+0.3%agarose+YP-ET	0.9942	1.0258	1.3626	0.0316	0.3684	0.3368
2.44	1.5% CMC + 3.5%glycerol + 0.3% agarose+ YP-ET	0.996	1.0447	1.3171	0.0487	0.3211	0.2724
3.27	1.5% CMC+5.25% glycerol+0.3% agarose+YP-ET	0.9964	1.0646	1.4257	0.0682	0.4293	0.3611
4.14	1.5% CMC +7% glycerol+0.3%agarose+YP-ET	0.9965	1.0825	1.4774	0.086	0.4809	0.3949

SET 2

Hydrocolloid	Empty Tube (g)	Tube+Sample (g)	Final Sample (g)	Sample dry (g)	Sample wet (g)	Final Vol. (g)

1.5% CMC + 0.3% agarose alone	1.0029	1.025	1.6399	0.0221	0.637	0.6149
1.5% CMC + 0.3% agarose+YP-ET	1.0003	1.0209	1.6021	0.0206	0.6018	0.5812
1.5% CMC + 1.75% glycerol+0.3%agarose+YP-ET	0.9984	1.0288	1.3427	0.0304	0.3443	0.3139
1.5% CMC + 3.5%glycerol + 0.3% agarose+ YP-ET	1.0008	1.0423	1.4088	0.0415	0.408	0.3665
1.5% CMC+5.25% glycerol+0.3% agarose+YP-ET	0.994	1.0693	1.4075	0.0753	0.4135	0.3382
1.5% CMC +7% glycerol+0.3%agarose+YP-ET	0.999	1.0625	1.4391	0.0635	0.4401	0.3766

we want the % absorption by weight to be in the range of 13 g - 22 g based on current CMC wound dressings

therefore, any value higher than that is not favorable and we would ideally like to be within this range

Adding the lowest conc. of glycerol would therefore be most favorable, so 1.75% glycerol

Need glycerol to not absorb too much water, CMC alone is too high of an amt based on

APPENDIX TT: Antimicrobial Properties of *In Vivo* Vehicle Formulation

Eden - Antimicrobial Properties of *In vivo* Vehicle formulations vs. Sa 21306

Purpose: To test the vehicle formulation we prepared has retained its antimicrobial activity against Sa for *in vivo* studies before and after animal study

Materials:	Tube - description	Stock Materials	[terpene]	wt mg	ul sterile water		MIC ppm
1	0.75% CMC high	15 g 2% CMC + 19g H20 + 6g YP-ET	24 mg/ml	use directly		use neat	
2	0.75% CMC high	15 g 2% CMC + 19g H20 + 6g YP-ET	24 mg/ml	use directly		use neat	
3	YP-ET	160 mg/ml terpene	24 mg/ml	0	0.0		
4	0.75% CMC medium terp	15 g 2% CMC + 23.35 g H20 + 1.65g mg YP-ET	6.6 mg/ml	use directly		use neat	
5	0.75% CMC medium terp	15 g 2% CMC + 23.35 g H20 + 1.65g mg YP-ET	6.6 mg/ml	use directly		use neat	
6	YP-ET	160 mg/ml terpene	6.6 mg/ml	0	0		
7	0.75% CMC low terp	15 g 2% CMC + 24.625 g H20 + .375g YP-ET	1.5 mg/ml	use directly		use neat	
8	0.75% CMC low terp	15 g 2% CMC + 24.625 g H20 + .375g YP-ET	1.5 mg/ml	use directly		use neat	
9	YP-ET	160 mg/ml terpene	1.5 mg/ml	0	0		
10	0.75% CMC only		0 mg/ml	use directly		use neat	
11	YP-ET only	160 mg/ml terpene	4 mg/ml	0	0.0		
12	LB only	100 ul	0	0	1000		

Formula is ul = mg of sample X 160 mg/ml / [terpene] mg/ml

Microbes	Medium	Temp	Conditions
S. aureus	LB	37C	aerobic

Method:	1. Use CMC formulations directly. Dilute YP-ET commercial in sterile LB as indicated above to get 6.3 mg/ml terpene													
	2. Grow microbes under conditions described above. Dilute 1:1000, or as appropriate in medium for plate inoculation													
	3. Set up Primary Growth Inhibitory MIC assay as follows													
	a. Add 100 ul of 2X LB to Row A, and 100 ul 1X LB to rest of wells.													
	b. Add 100 ul of samples (number keyed to list above) in Row A wells													
No growth	c. Perform 1:1 dilutions by transferring 100 ul serially from Row A to Rows B, etc...as indicated. Discard 100 ul from Row H													
artial Inibition	d. Add 100 ul 1:1000 dil of indicated microbes to all wells													
Growth	e. Read To A620 in microplate reader. Incubate plate at 37C o/n under indicated growth conditions. Read A620 in microplate reader.													
	f. Determine MIC = ≥75% inhibition of growth													
	g. Add 50 ul 50 ug/ml Resazarin. Incubate for 30 min. Score color change. Image on scanner													
Before		Formulation Tube	1	2	3	4	5	6	7	8	9	10	11	11	
Plate A		Test Formulation	**YP-ET 24**	**YP-ET 24**	**YP-ET 24**	**YP-ET6.6**	**YP-ET6.6**	**YP-ET6.6**	**YP-ET1.5**	**YP-ET1.5**	**YP-ET1.5**	**CMC only**	**YP-ET4**	-	
Sa		Microbe	***Sa***	***Sa***	***Sa***	***Sa***	***Sa***	***Sa***	***Sa***	***Sa***	***Sa***	***Sa***	***Sa***	***Sa***	
LB			CMC	CMC	-	CMC	CMC	-	CMC	CMC	-	CMC	-	Control	
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12	GI
	ug/ml	Row											-	-	**MIC**
		A													(ppm)
		B													
		C													
		D													
		E													
		F													
		G													
		H													
		Discard	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	

Results		Formulation Tube	1	2	3	4	5	6	7	8	9	10	11	11	
Plate A		Test Formulation	YP-ET 24	YP-ET 24	YP-ET 24	YP-ET6.6	YP-ET6.6	YP-ET6.6	YP-ET1.5	YP-ET1.5	YP-ET1.5	CMC only	YP-ET4	+	
Sa		Microbe	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	*Sa*	
LB			CMC	CMC		CMC	CMC		CMC	CMC		CMC		Control	
	Terpene	Column	1	2	3	4	5	6	7	8	9	10	11	12	GI
	ug/ml	Row													**MIC**
		A	-0.267	-0.278	-0.189	-0.022	-0.029	-0.005	0.158	0.234	0.533	1.27	0.056	1.021	(ppm)
		B	-0.109	-0.206	-0.202	0.142	0.106	0.126	0.548	0.603	0.648	1.274	0.34	0.974	
		C	0.152	0.104	0.107	0.191	0.167	0.159	0.753	0.75	0.75	0.777	0.656	0.961	
		D	0.172	0.182	0.156	0.447	0.59	0.65	0.737	0.748	0.701	1.298	0.702	0.922	
		E	0.536	0.379	0.531	0.758	0.761	0.729	0.748	0.751	0.748	0.746	0.733	0.934	
		F	0.764	0.652	0.739	0.727	0.741	0.748	0.755	0.754	0.734	0.752	0.769	0.927	
		G	0.903	0.789	0.785	0.77	0.756	0.756	0.73	0.749	0.754	1.234	0.762	0.938	
		H	0.966	0.903	0.916	0.941	0.933	0.933	0.932	0.91	0.905	0.918	0.912	0.956	
		Discard	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	100 ul	
													Avg + control	0.954125	
													20% cut-off	0.190825	

Sample	MIC ug/ml
High YP- ET 24 mg/ml	500
YP-ET only- 24 mg/ml	500
Med YP-ET 6.6mg/ml	412.5
YP-ET only- 6.6 mg/ml	412.5
Low YP-ET 1.5 mg/ml	>375
YP-ET only- 1.5mg/ml	>375
CMC only	
YP-ET 4mg/ml	1000