DEVELOPMENT AND VALIDATION OF AN *IN VITRO* PORCINE SKIN MODEL OF BACTERIAL BIOFILMS IN CHRONIC WOUNDS

By

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To my family

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Chronic wounds are a major source of patient suffering and expenditure in home care. It has been accepted that biofilms are a major culprit responsible for the non-healing of chronic wounds. To better understand biofilm characteristics in chronic wounds for such disease control, it is imperative to invent a model which more closely mimics the mini-ecological environment of a human body. Our study established a novel in vitro bacterial biofilm model of the porcine skin for chronic wound healing. This model has been validated using different disinfectants and dressings from commercial markets. Our study showed the iodine dressing killed the bacteria in biofilms, whereas the silver dressings only partially inhibited bacterial growth in the biofilms. This model can be applied in the efficacy assessment of antimicrobial agents and dressings for chronic wound healing.

CHAPTER 1
LITERATURE REVIEW: MICROBIAL BIOFILM MODELS FOR CHRONIC WOUND

Introduction

Many bacteria species are important pathogens for human diseases. Even benign bacterial species (commensal and derived from the environment), when found on intact skin, often become opportunistic pathogens when they are found on skin compromised by trauma (e.g., surgical, burn, laceration) or disease (e.g., diabetes, dermatitis, ischemia). The presence of bacteria, particularly as bacterial biofilms, has been suggested as one of the main factors contributing to the non-healing of chronic wounds (Edwards and Harding, 2004; James et al., 2008; Jones et al., 2004). Chronic wounds are a major source of patient suffering and expenditure in home care.

Treatment and prevention of infection caused by the presence of biofilm on medical devices have been based on *in vitro* or *in vivo* studies of biofilm formation and the response of biofilm to various antimicrobial agents. The more recently established link of bacterial biofilm to chronic wounds has lead to the desire to asses the efficacy of antimicrobial agents and dressings to eradicate bacterial biofilm in chronic wounds. To better understand biofilm characteristics in chronic wounds for such disease control, it is imperative to establish a model which more closely mimics the mini-ecological environment of a human body.

Importance of Chronic Wound Healing and Biofilm Presence in Chronic Wounds

Chronic wounds recalcitrant to healing are an increasingly grave worldwide problem. They include predominantly diabetic foot ulcers (DFU), pressure ulcers (PU), and venous leg ulcers (VLU), which lead patients to suffering pain, impaired mobility, reduced quality of life, and frequent amputations. More than 1% of the population in developed countries has been estimated to experience a chronic wound during their lifetime, which increases with the number

of lifestyle diseases such as obesity, diabetes, and cardiovascular diseases (Gottrup, 2004). For instance, 15% of diabetic patients will develop lower extremity ulcers and 14-24% of patients with DFUs will eventually undergo amputation (Association, 1999; Reiber, 1996). 1% of the world's population suffer painful inflictions of VLU (Trent et al., 2005). The moneytary expenditure on chronic wounds is enormous and a financial toll worldwide. In 2004, the total cost of DFU rose to $10 billion, including direct expenses (about 4% of the total personal health spending) and another $5 billion in indirect expenses (disability, nursing homes, etc.). In the U.S., chronic wounds affect roughly 3 million people and the frequency is increasing at exponential rates, doubling every 4-5 years (Brian, 2008; Talsma, 2007). As a consequence, chronic wound healing is of significant importance to human health as well as heathcare costs.

It is widely accepted that biofilms are a major culprit responsible for chronic wounds (Costerton et al., 1999; Donlan and Costerton, 2002; Parsek and Singh, 2003). Multiple bacterial species were observed to reside in chronic wounds. For example, chronic VLUs have been reported to be colonized by *Staphylococcus aureus* (in 93.5% of the investigated ulcers), *Enterococcus faecalis* (71.7%), *Pseudomonas aeruginosa* (52.2%), coagulase-negative *staphylococci* (45.7%), *Proteus* species (41.3%), and anaerobic bacteria (39.1%) (Gjodsbol et al., 2006). *S. aureus* is almost universally present in all chronic wounds (Colsky et al., 1998; Valencia et al., 2004); and *P. aeruginosa*-infected wounds appeared larger in terms of area than other wounds without *P. aeruginosa* (Halbert et al., 1992; Madsen et al., 1996). These bacteria in chronic wounds form a structured community called biofilm. Bacterial biofilms are an extracellular matrix-enclosed structure consisting of a bacterial population that adheres to each other and/or surfaces or interfaces (e.g., solid-liquid; liquid-air) (Costerton et al., 1999; Davis et al., 2008; James et al., 2008). Bacteria embedded in biofilms are physiologically different from

planktonic (free-floating) ones. Planktonic bacteria reversibly adhere to surfaces, such as open wounds or medical devices. In response to environmental signals, they become sessile (irreversibly attached) and secrete a protective matrix consisting of self-synthesized extracellular polymeric substance (EPS), differentiate and form microcolonies, and finally build up to form complex biofilms (Costerton et al., 1999; Davies et al., 1998; Donlan and Costerton, 2002; Gilbert et al., 1997). The bacteria within biofilms are notoriously resistant to host defenses, antibiotics, disinfectants, and biocides, which leads to difficulty in eradication of the biofilm from chronic wounds by conventional antibiotic therapy ;(Costerton et al., 1999; Edwards and Harding, 2004). Therefore, investigating bacterial biofilms in chronic wounds is pertinent for understanding how to tip the physiological balance toward healing.

Importance of Bacterial Bioflim Presence on Medical Devices

Bacterial biofilms are a leading culprit in chronic nosocomial infections. Among all nosocomial infections , biofilm-based infections contribute more than 60% and lead to an increase in patients' hospitalization by 2 to 3 days and additional costs of over $1 billion per year (Archibald and Gaynes, 1997). For example, bacterial biofilms on prosthetic valves cause endocarditis and 70% mortality in patients whose heart valves have been replaced (Hyde et al., 1998).

Early studies of patients receiving artificial hearts revealed that bacteria colonized the implant surfaces and exacerbated the infection surrounding damaged tissues, which was the principal barrier to the extended use of this medical implant (Gristina et al., 1988). Similar colonies of bacteria embeded in glycocalyx-matrix were also observed on urinary catheters by scanning and transmission electron microscopy (Nickel et al., 1989). These colonized bacteria were considered bacterial biofilm (Davey and O'Toole G, 2000). Further evidence that biofilms were formed on implants comes from the investigation of silicone rubber voice prostheses, which

were inserted in a non-sterile environment. The biofilms formed on these implants were found to contain 79 bacterial and 39 yeast strains (Neu et al., 1994). Later studies on artificial throat implants revealed that biofilm infection caused the deterioration of voice prostheses (Busscher et al., 1998). Many studies on bacterial biofilms have been aimed at developing more effective approaches for the prevention of bacterial biofilm formation on medical devices with the goal that chronic nosocomial infections will significantly decrease or perhaps even disappear clinically.

Characterization of Bacterial Biofilms in Chronic Wounds

Although bacterial biofilms can be formed on a variety of surface interfaces, they develop preferentially on inert surfaces and dead tissues (Davey and O'Toole G, 2000; Hall-Stoodley et al., 2004). Biofilm formation occurs commonly on medical devices and fragments of dead tissue such as sequestra of dead bone (Lambe et al., 1991). Regardless of their location or diversity, all microbial biofilms have a typical common pattern of developmental process including attachment, colonization, maturation, and dispersion as described in more detail below (Costerton et al., 1999; Davey and O'Toole G, 2000; Donlan and Costerton, 2002; Hunter and Beveridge, 2005; Spoering and Gilmore, 2006). During attachment, bacteria sense nutritional and other chemical and physical environmental signals (e.g., quorum sensing) and assemble a critical number of organisms for their irreversible attachment. After the attachment becomes permanent, bacteria change the original free-floating, planktonic growth state into the attached sessile state. They phenotypically differentiate (for examples. lose their flagella-driven motility), excrete extracellular polysaccharides and / or other molecules (e.g., DNA) to form the EPS matrix, increase expression of cell surface adhesions, recruit new members, and form microcolonies on the surface. These microcolonies are further enlarged through bacterial proliferation and additional recruitment, finally forming the complex architecture of a mature

biofilm. Mature biofilms are dynamic microbial populations that respond to various environmental signals that induce highly regulated bacterial cell death (Rice and Bayles, 2008) or dispersion where single motile cells are produced, or biofilm fragments break off, and are dispersed into the surrounding environment. These individual cells or biofilm fragments 'seed' other surfaces, re-initiating the process.

Bacterial biofilms have an elaborate three-dimensional structure that allows their survival in a variety of harsh environments (O'Toole et al., 2000). The structural complexity of biofilms was thought to be analogous to tissues of higher organisms (Costerton, 1995). The two remarkable features of biofilm structure are the organization of interstitial channels and the distinct locations of constituent cells (subpopulations) with different patterns of gene expression in biofilms (Watnick and Kolter, 2000). These structural features are believed to provide constituent cells in the biofilm a lifeline through which oxygen is available and water and nutrients are transported to different locations (de Beer et al., 1994; Stoodley et al., 1994); metabolic products and signal molecules are exchanged (Costerton, 1995); and potentially toxic metabolites and wastes are removed (Costerton et al., 1995). Thus, highly dense cells in biofilms overcome the potential limitation of nutrients and oxygen as well as toxicity of metabolic products (Parsek and Greenberg, 2005; Xu et al., 1998).

Another important feature is the diversity and dynamics of bacterial clusters within biofilms in response to environmental changes. The molecular mechanism underlying this adaptation is to change gene expression patterns through communication among cells (Tolker-Nielsen and Molin, 2000). Quorum sensing is the major form of communication that coordinates the multicellular behavior of bacteria in biofilms (Davies et al., 1998; Fuqua et al., 1994; Reading and Sperandio, 2006). The signals from quorum sensing have been shown to regulate

many physiological processes, including swarming, bioluminescence, antibiotic synthesis, conjugated plasmid transfer and the expression of virulence factors (de Kievit and Iglewski, 2000; Reading and Sperandio, 2006; Van Delden and Iglewski, 1998) and has been suggested to be involved in communication between bacterial and eukaryotic host cells (Sperandio et al., 2003).

The common components of biofilms include water, accounting for approximately 95–97% of the biofilm mass; microbial cells, accounting for about 2–5% of the biofilm mass; EPS, accounting for 1–2% of the biofilm mass; and other substances such as proteins, DNA, RNA, and ions, accounting for approximately 2% of the biofilm mass (Costerton, 1995; Sutherland, 2001a). Among the various types of EPSs that may be found in bacterial biofilm matrix, polysaccharides are characteristic components of most bacterial biofilms (Branda et al., 2005). Exopolysaccharides provide the extracellular matrix, the 'gel' framework, which immobilizes the bacteria, creating the three-dimensional structures. Apart from the structural component, EPS also plays other major roles such as providing a protective barrier against antibiotic agents and a nutritional reserve in many cases (Sutherland, 2001a, b).

Although biofilms in chronic wounds share the general biofilm properties discussed above, they also have their own characteristics because of their specific environment. The biofilms are formed on human bodies at various locations including live, damaged or dead tissues. During the initial stages of normal wound healing, a fibrin and fibronectin plug is formed to hold the damaged tissue together and to provide a matrix for recruitment of inflammatory cells such as neutraphils and macrophages (Blakytny and Jude, 2006). Bacterial biofilm easily absorb a large amount of water and abundant nutrients. In addition, they grow in the favorable temperature of approximately 37 °C. Therefore, biofilms in chronic wounds commonly have a large mass of

microbial cells and extracellular matrix. Chronic wound bed biofilms face both cellular and humoral immune defenses. Bacterial cells are protected by the EPSs of the biofilm matrix from phagocytosis and other immunological factors (e.g., antimicrobial peptides) (Edwards and Harding, 2004; Vuong et al., 2004). How bacterial biofilms specifically interact with all the processes involved in wound healing are still being determined, however, it is clear that their presence negatively affects wound healing. It is believed that the persistent presence of bacterial biofilm results in an excessive inflammatory response (Edwards and Harding, 2004; Jones et al., 2004). The increased release of pro-inflammatory molecules and enzymes into the tissues around the biofilms, in addition to the possible release of bacterial toxins, lead to the collateral tissue damage (e.g., necrosis) which promotes biofilms to enlarge into adjacent tissues (Costerton et al., 1999; Jones et al., 2004; Madianos et al., 2005).

All these properties of biofilms mentioned above in chronic wounds offer researchers an opportunity for potential applications and careful considerations in designing models to mimic the biofilm formation process in chronic wounds.

Biofilm Models for Chronic Wound Healing

Chronic wound healing is a complex biochemical and cellular process related to the general phenomenon of growth and tissue regeneration (Beckert et al., 2007). A number of barriers, including poor perfusion, white cell dysfunction, poor nutrition, and repetitive pressure, among others, restrict the healing of chronic wounds. These limitation factors have been extensively investigated through various established models (Falanga, 2005; Krasner, 1995; Schultz et al., 2003). Biofilms in chronic wounds are also a major barrier to healing. However, many basic questions concerning this barrier remain unaddressed. For example, what is the precise role of biofilms in delaying wound healing? To what the extent of the cellular regeneration processes accompanying the healing may inadvertently provide nutritional support

for sustaining biofilm viability? To answer these questions, it is imperative to establish suitable experimental models. The ethical restriction leads to unacceptability of investigations of new substances or wound dressings in humans. Thus, applicable wound healing models outside of humans are required. In this part, at first the general considerations of a wound healing model design are presented, then an overview of *in vivo, in vitro, and ex-vivo* models for wound healing, followed by considerations of biofilm model design used in investigating chronic infection associated with biofilm on medical devices, and finally focus on the criteria for biofilm models for chronic wounds are presented.

General Considerations of Wound Healing Models

The three main factors that should be considered when developing experimental models to generate data to be applied to promote wound healing are 1) experimental purpose, 2) methods used (technology), and 3) organism selections. The 'ideal' wound healing models will meet numerous requirements: similarity with human skin physiology, reproducible results, conducive to a large number of trials, and reasonable economic expectation. Unfortunately, no existing model for wound healing fulfills all these requirements. Different models have different characteristics. The advantage of *in vitro* models is the direct observation of environmental or pharmacological agent effects on specific targets (e.g., cultured cells). *In vitro* model systems are generally rapid and simple with less expenditure and minimal ethical considerations, compared with *in vivo* wound models. Additionally, the effects of different pharmacological agents or factors at various concentrations can be revealed simultaneously without disturbance of the inherent heterogeneity of *in vivo* models. Furthermore, in vitro models simplify mechanisms of pharmacological agent actions, which are complicated *in vivo*. The major disadvantage is that the results from *in vitro* models are unlikely to be completely applied to humans. In contrast to *in vitro* models, the advantage of *in vivo* models for wound healing is the high similarity between

wounded tissues and wounds found in the clinical practice. The disadvantage is the difficulty in directly examining each tissue component. For example, in *in vivo* human skin wound models, only small wounds can be produced—and even this may have ethical considerations (Gottrup et al., 2000).

Cultured tissue (*ex-vivo*) models for the investigation of wound healing reported in the literature have used various normal tissues as a wound bed, notably the skin tissue of animals including the mouse, rat, hamster, rabbit, and pig, as well as human (Ahn and Mustoe, 1990; Barker et al., 1994; Mustoe et al., 1991; Sullivan et al., 2001). The *ex-vivo* tissue model has several of the advantages of an *in vitro* model such as reproducibility and its amenability to multiple trials. Different shapes of the wound can be made according to various experimental purposes. The *ex vivo* model has one of the advantages of an *in vivo* wound healing model in that the tissue explants are viable and can be used to evaluate growth and 'healing' to some extent. The *ex-vivo* tissue model for wound healing is often used for evaluation of different types of dressings, dermal substitutes, cell and tissue transplantation, and pharmacological agents (Brandner et al., 2006; Brandner et al., 2008; Gottrup et al., 2000).

Porcine Skin Models in the Study of Chronic Wounds

Mice, rats and hamsters are small, sold for a low price and easy to handle. However, their skins are too thin to use as a human wound healing model. The pig skin has been used as the model system for human wound healing and has shown the greatest similarity to human skin (Schmook et al., 2001; Sullivan et al., 2001). However, the laboratory pig is quite expensive to keep. As a consequence, the *in vivo* experiments with a large number of trials, such as drug screening, are costly and have ethical animal welfare concerns. To avoid these restrictions, Brandner *et al* established a porcine cultured tissue *ex-vivo* wound healing model to evaluate dressing and drugs for wound healing. They used only skins from the inner side of ears directly

from the slaughter houses. The use of the same age and species makes the experiments highly reproducible and avoids animal experiments. Therefore, the porcine *ex-vivo* wound healing model is appropriate for investigation of the growth effects from soluble substances, ointments, creams, and wound dressings used in wound healing (Brandner et al., 2006). This *ex-vivo* model has been used as a bacterial infection wound healing model, but it has not been tested as a model suitable for biofilm growth and assessment. Whether or not tissue viability can be maintained remains unknown while establishing a mature bacterial biofilm.

In developing a biofilm model for chronic wounds an additional two factors must be considered: bacterial strains and surface (substrata). Biofilms are surface-adherent microbial communities. Therefore, choice of bacterial strains and substrate surfaces are the most important factors for biofilm experiments *in vitro* or *in vivo*. In nature, microbial cells within biofilms usually are heterogeneous. However, the known biofilm models often include distinct microorganisms, depending on the specific experimental design. Commonly used bacterial strains, include the Gram negative bacterium *Pseudomonas aeruginosa* and the Gram positive bacteria *Staphylococcus epidermidis* and *Staphylococcus aureus* for models systems for medical biofilm research. *P. aeruginosa* is prevalent in various environments. Additionally, it readily forms biofilms that cause a large number of infections, notably for a wound, burn, lung and catheter (Gjodsbol et al., 2006; Rumbaugh et al., 1999; Singh et al., 2000; Stickler et al., 1988). *S. epidermidis* and *S. aureus* are commensal to the human body such as skin and cause devastating chronic infections in compromised hosts such as cystic fibrosis lung infections (Costerton, 1995, 1999). The type of surface to which biofilm are to be grown on is another factor that must be considered. There is a general rule that bacterial adherence increases as surface roughness increases (Donlan and Costerton, 2002; Picioreanu et al., 2000a, b). The

nutrients, pH, ionic strength, hydrophobicity, and polarity of surfaces also contribute to the attachment (Bruinsma et al., 2003; Bruinsma et al., 2001; Busalmen and de Sanchez, 2001a, b). The suitable parameters of these factors are adjusted to maintain stable bacterial adherence, particularly for biofilm models for medical devices.

In Vitro and *in Vivo* Models in Investigating Chronic Infections Associated with Biofilm on Medical Devices

As mentioned above, biofilms are involved in both medical device related chronic infections and non-healing chronic wounds. To prevent chronic infections, it is imperative to understand how bacterial biofilms are formed on the surface of medical devices and how they expand to human tissues. A large number of in vivo biofilm models for chronic infections have been established, notably for various devices and dental biomaterials. These implants are generally transferred into animal bodies, and after a period time, the implant surfaces are examined for biofilm formation. These in *vivo* models for biofilm development on medical devices have the same disadvantages listed above for the *in vivo* wound healing models. The established *in vitro* biofilm models used to evaluate various antimicrobial agents are based on culturing biofilm on various materials: examples include the modified Robbins device, Calgary biofilm device, disk reactor, CDC biofilm reactor, perfuse biofilm fermentor, and model bladder (Donlan and Costerton, 2002). Considering that biofilm development is clearly regulated by environmental signals, including the type of substrata to which it attaches, data from these types of *in vitro* assessments can only be translated as the effects these test agents may have on biofilm that would be found in wounds with reservation. An *in vitro* model utilizing the growth of bacterial biofilm on porcine tissue explants can lessen the disadvantages and keep many of the advantages of the *in vitro* and *in vivo* biofilm (grown on plastic, metal, etc.) models.

Methods and Techniques for Characterization of Microbial Biofilms to Evaluate Models

Microscopy is an important tool for observation of microbial biofilm growth and differentiation. Although different microscopy methods have distinct characteristics, they all contribute to the evaluation of models in which microbial biofilms are formed.

Electron Microscopy

Although light microscopy was originally used for studies on microbial biofilms, it was rapidly replaced by electron microscopy. Now electron microscopy including transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are often used for the characterization of medical biofilms because they have the advantage of high resolution over light microscopy. Using SEM, Ganderton *et al* observed bacterial biofilms formed on the surface of the Foley bladder catheter, which provided evidence for the presence of nosocomial infections (Ganderton et al., 1992). By SEM, it was observed that biofilms also form on the adaxial surface of cotyledons of mung bean and titanium devices (Fett and Cooke, 2003; Fujimura et al., 2008). Hunter and Beveridge visualized the biofilm of *P. aeruginosa* PAO1 with high resolution by freeze-substitution TEM. They found numerous finer structures that were previously unknown. Their results supported the concept of physical microenvironments within biofilms (Hunter and Beveridge, 2005).

Unfortunately, TEM and SEM require that samples be stained/coated with heavy metals for the observation of samples in a high vacuum. For this staining the samples are fixed and dehydrated, which introduces dehydration artifacts, since biofilms contain a large amount of water. Therefore, SEM and TEM are limited to general structural characterization of medical biofilms. To avoid dehydration artifacts, Mackie *et al* developed a technique that preserves the structure of the biofilm. They used specific antibodies to bind with EPS for stabilization of the biofilm matrix (Mackie et al., 1979). This technique was further applied in TEM for numerous

investigations of the biofilm ultra-structure and molecular localization, sometimes with the aid of immunostaining (Lawrence et al., 2003).

With advent of many novel technologies, SEM has been further developed, creating distinct microscopes: environmental SEM (ESEM) and Cryo-SEM. They allow specimens to be observed without dehydration because the ESEM operates in reduced air pressure rather than a high vacuum and Cryo-SEM operates at the super low temperature through cooling with liquid nitrogen. Specimens are also rapidly frozen by liquid nitrogen. As a consequence, the ice crystals in biofilms preserve the matrix structure. Although ESEM has a lower resolution than Cryo-SEM, it allows completely untreated live biofilms to be examined (Priester et al., 2007). ESEM and Cryo-SEM generally need much less time for their specimen preparation, compared to SEM. Therefore, they are frequently used in biofilm research.

X-ray Microscopy

X-ray microscopy including transmission X-ray microscopy (TXM), soft X-ray scanning transmission X-ray microscopy (STXM), and near-edge X-ray absorption spectroscopy (NEXAFS) microscopy, is a powerful new tool for the investigation of fully hydrated biofilms for various reasons. Soft X rays can penetrate water; have reduced radiation damage (compared to the electron beam of an electron microscopy); provide spatial resolution of better than 50 nm, which is suitable for imaging bacteria and bacterial biofilms; and reveal detailed, quantitative chemical identities in biofilms. Thus Soft X-ray microscopy is a useful tool for the localization and mapping of the distribution of macromolecules in biofilms (Lawrence et al., 2003; Loo et al., 2001). Gilbert *et al* observed microbial biofilm formation at the early developmental stages, using TXM (Allison et al., 1999). Using STXM and NEXAF microcopy, Lawrence *et al* successfully investigated the biochemical basis of the biofilm organization (Lawrence et al., 2003).

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) has become widespread in molecular and cellular investigations partly because it allows the studies of live and hydrated biological specimens, such as fully hydrated microbial biofilms (Lawrence and Neu, 1999). Using CLSM, Psaltis *et al* demonstrate that bacterial biofilms are present in patients with chronic rhinosinusitis (CRS). This evidence further supported the hypothesis that biofilms play a role in the pathogenesis of CRS (Psaltis et al., 2007). The CLSM technique was also applied for studying the effects of surface characteristics of substrata, surrounding environments, and genetic transformation on microbial biofilm formation (Hendrickx and Wuertz, 2004; Mohle et al., 2007).

With the help of complex computer programs, CLSM can generate simultaneous information on the three-dimensional (3D) structure of a biofilm and the localization of different components in the biofilm by use of specific fluorescent dyes to specific bacterial components such as DNA and EPS (Lawrence et al., 2003; Neu et al., 2004). Through this technique, Eaglesham *et al* confirm that water channels, pores, and other structural features of the matrix are present in peripheral regions of natural microbial biofilms. Additionally, higher magnification images revealed the 3-D architectural distribution of specific biofilm components such as filaments of sheathed bacteria projecting outward into the liquid midieu (Eaglesham et al., 2004). Wood *et al* determine the structure of medical biofilms on human tooth surfaces through CLSM and staining of the biofilms with fluorescein in conjunction with fluorescence. Their results show that all the examined biofilms are heterogeneous structures with clearly visible channels and voids. In addition, these voids are fluid-filled (Wood et al., 2000). Kuehn *et al* created a specific computer program suitable for routine measurements of biofilm structures under in situ conditions, using images from CLSM (Kuehn et al., 1998).

Episcopic Differential Interference Contrast Microscopy and Atomic Force Microscopy

In addition to the above-mentioned three major types of microscopy, *Episcopic Differential Interference Contrast (EDIC) Microscopy and Atomic Force Microscopy* (AFM) are also used for biofilm studies, especially for surface characterization. EDIC microscopy provides a rapid, real time analysis of the biofilm formation and growth on a wide variety of substrate surfaces (Keevil, 2003; Rogers and Keevil, 1992; Warner et al., 2008). AFM provides image features with dimensions at the atomic scale up to approximately 100 µm (Chaw et al., 2005; Hansma et al., 2000; Santos et al., 2008). AFM has also been used to assess the strength of bacterial adhesion to various surfaces and substrate coated surfaces (Liu et al., 2006).

Spectroscopy for Biofilm Biology

Although microscopy plays a paramount role in the understanding of biofilm growth and development, spectroscopy also contributes to the understanding of biofilm molecular biology. The types of spectroscopy that are used for microbial biofilm investigation mainly includes infrared and Raman spectroscopy, photoacoustic spectroscopy (PAS), X-ray photoelectron spectroscopy (XPS), nuclear magnetic resonance (NMR) spectroscopy, and reflectance spectroscopy. For all these spectroscopy, Denkhaus *et al* presented a review on their characterization and application in identification of chemical species in microbial biofilms (Denkhaus et al., 2007). Spectroscopy can reveal the presence of a specific metabolite in mature microbial biofilms that are indicative of formed biofilms on the target surfaces in the validated models.

Molecular Technique for Biofilm Gene Expression Analysis

The techniques of modern molecular biology have widely been applied for the characterization of gene expression of constituent cells in microbial biofilms. In recent years, novel techniques of genomics, microarray, and proteomics have been developed that can more

efficiently reveal the spatial and temporal pattern of gene expression during the microbial biofilm formation in model systems (Costerton, 1999; Costerton et al., 2003). The molecular techniques of pyrosequencing, density gradient gel electrophoresis (DGGE), and full ribosome shotgun sequencing have also been recently applied to survey the bacterial diversity of natural biofilm found in various types of chronic wounds and to determine the most common bacterial types and species generally found in each wound type (Dowd et al., 2008). Such information is invaluable in further refining experimental design parameters and wound model development for investigating bacterial biofilm and evaluating antimicrobial agents, dressings, and other factors that may be used to promote wound healing.

CHAPTER 2
IN VITRO PORCINE SKIN WOUND MODEL

Introduction

Biofilms are considered to be structured microbial communities formed on surface interfaces in a protected mode of growth that allows survival in a hostile environment, providing cooperative defense against injury or death by various physical and chemical means, such as phages, phagocytosis, biocides, and antibiotics (Costerton, 1999). The protection of biofilm provides to bacteria resulting in difficulty in effectively removing and/ or inhibiting development of biofilm is a persistent problem in many industrial processes as well as in medicine. For these reasons it is important to explain, predict, modify or counter biofilm development and behavior. Studies *in situ* or *in vivo* are often impractical. Consequently, appropriate *in vitro* biofilm culture and modeling is needed. A major advantage of *in vitro* over *in vivo* biofilm studies is the degree of control over the microbes, environment, nutrient supply, and substrata, with increased options for experimental protocols, sampling and analysis. Advances in understanding the complex process of biofilms in wound healing would benefit from models that mimic closely the physiology of human wounds. To this end, we developed an *in vitro* porcine skin model of bacterial biofilms in chronic wounds.

Many *in vitro* models for bacterial adhesion to biomaterials have been developed. They can be classified as the following: 1) Bacterial adherence to commercially available flat solid surfaces such as plastic culture tubes, petri dishes, or tissue culture plates; 2) Bacterial adherence to a solid substrata placed into culture tubes, petri dishes, tissue culture plates, or chemotaxis chamber; 3) Bacterial adhesion to solid surfaces tested under flow conditions, such as a flow cell perfusion model, rotating disc apparatus, parallel plate flow chamber, radial flow chamber, Robbins device, or Calgary biofilm devise (Donlan and Costerton, 2002). The solid surfaces to

be tested are often built as a part of the circulating or perfusion system, such as the inner wall of tubes or a sample piece fixed inside the flow system.

When designing an *in vitro* model for biofilm study, the following factors should be taken into consideration, including 1) the selection of the microorganism, 2) the selection and preparation of the surface for biofilm adhesion, 3) the conditions used to cultivate microbial biofilm, and 4) the method of sample preparation and analysis used to determine the response of the microbial biofilm to changes in the environment.

Examining the influence of active substances, wound dressings, transplanted cells or the raw materials of ointments and creams on human skin can only be carried out directly on human beings in exceptional cases. The alternatives used until now, such as *in vivo* animal trials, single cell cultures, skin equivalents or human organic skin culture models frequently demonstrate definite disadvantages. Animal trials are often undesirable, limiting, or forbidden by law. Keeping animals is cost prohibitive and the results of animal trials are not always transferable to humans. For example, rodents have a different skin structure from humans and their wound healing differs from that in humans (Galiano et al., 2004). The structure of tissue cell cultures (monolayers) is too simple, including a lack of connective tissue. Skin equivalents, such as artificially cultured skin, are very expensive. The skin material needed to produce human organic skin culture models is a by-product of surgical operations. It is only available in limited amounts and the results achieved are not easily reproducible as a result of the patients' previous diseases or their varying ages. This study solves many of these problems by developing an *in vitro* porcine skin wound model. Porcine and human skins have remarkable similarities and offer the most appropriate model for all types of dermatological and surgical wound investigations (Sullivan et al., 2001). Thus, we hypothesized that porcine skin would make the

most appropriate substratum to use in developing an in vitro cultured bacterial biofilm in wound model. In addition, porcine skin is readily available from various sources (i.e. slaughterhouses) in sufficient quantity. As such, the goals of this research were: 1) to develop an *in vitro* porcine skin model of bacterial biofilms for human chronic skin wounds, and 2) to test active substances (i.e. antimicrobial dressings or solutions) used in wound treatment. The model should: 1) be standardized and reproducible, 2) use readily available skin material with comparatively uniform properties, 3) be cost effective for testing inhibitory or antimicrobial treatments for either inhibiting bacterial biofilm development or killing and or dispersion of exsisting bacterial biofilm that may influence wound healing.

Materials and Methods

Optimization of Porcine Skin Explant Sterilization

The reagents and media used in this study were all purchased from Fisher Scientific and all antibiotics were purchased from Sigma-Aldrich unless otherwise indicated.

Preparation of porcine skin explants

The porcine skins used in developing a standardized sterilization method were supplied by the Meat Processing Center, the Institute of Food and Agricultural Sciences, the University of Florida, Animal Science Building 459, Shealy Drive, Gainesville, FL. The cheek skins were harvested from pigs which had been sacrificed, drained, scalded with boiling water, brushed, depilated (hair removal) with a flame torch, and then processed. The skin was immediately placed in a cooler and transferred to the laboratory. Fat and connective tissues were removed by scalpel. They were immediately used or immersed in liquid nitrogen and stored at a -80 ºC refrigerator for later studies. The whole procedure was completed within two hours of harvesting the skin. Explants were mechanically created. Punch biopsies with a diameter of 8 mm were taken from the pieces of skin (approximately 4.0 mm thick) to be used for testing

sterilization methods and histology. Histologica examination indicated that the epidermis of the scalded porcine skin was missing. Other porcine skin, which came from a commercial abattoir, Chiefland Custom Meat,14053 NW HWY129, Gainesville, FL, were harvested without prior scalding and flame depilation. These skins were stored in a -20 ºC refrigerator for use in the study for optimization of porcine explant sterilization and model validation. More preparative work was needed to use this resource, including: shaving the hair and cleaning the surface of dirt and debris. Although the non-scalded porcine skin had greater microbial contamination, histology revealed it was an improved source of porcine skin. The protocol for sterilization initially developed using the scaled porcine skin was modified to produce comparable sterile explants using the non-scalded porcine skin.

Explant sterilization

The initial explants sterilization test used 10% bleach or 70% ethanol in ddH_2O to disinfect the explants, alone or after a pretreatment wash with phosphate buffered saline [PBS; (Sambrook et al., 1989)] with 5 ppm tween-80, performed at 15, 30, 45, 60, 75, 90 minutes exposure to the disinfect. The results were compared to subsequent tests on explants performed using ethanol, chlorine bleach, hydrogen peroxide, chlorine gas chamber, or antibiotic treatments.

For the sterilization by disinfectant test solutions, five explants were each put into 50 mL tubes containing 10 mL of a disinfectant solution prepared with 1x PBS with 5 ppm tween-80: 1) 70% ethanol, 2) 10% bleach, 3) 70% ethanol followed by a transfer of the explants into 10% Bleach, 4) and 6% hydrogen peroxide solutions. The explants were incubated in the disinfectant solutions at room temperature for 30 and 60 minutes.

For the sterilization by antibiotic test solutions, ten explants were placed into 24-well micro-plate wells. Five wells containing 1mL of Tryptic Soy broth (TSB; Difco^{TM}) with 200

μg/mL gentamicin, and another five wells contain 1 mL of TSB media with 100 mg/mL oxacillin, incubated for 24 hours at 37ºC.

For sterilization by chlorine gas, five explants were placed in a chlorine gas chamber (BEL-ART® Desiccator Shields). The chloride gas is produced from mixing 20 mL Acetic acid with 10 mL Bleach in the reaction chamber. The reaction is stopped by the addition of sodium carbonate. The explants were treated with chloride gas at room temperature for 30 and 60 minutes.

After each treatment as described above, each set of five explants were washed with sterile PBS three times. Four explants were transferred onto plates containing Tryptic Soy Agar (TSA; Difco™). The plates were incubated at 37ºC, for up to one month. During the incubation, microbial growth was visually assessed daily.

Histology analysis

After the sterilization of porcine skin, one explant from each treatment as mentioned above was fixed with 4% formaldehyde in PBS at room temperature for 15 minutes. After three washes with PBS, the explants were dehydrated, embedded in paraffin, and sectioned by Pauletta Sander, a histological scientist working at the University of Florida. Pictures were taken of the sections using the AXIOSKOP2 microscope (Carl Zeiss, Inc).

Optimization of Culturing Bacterial Biofilms on Porcine Skin Explants

Preparation for partial thickness wound beds

Explants from freshly frozen (to facilitate excision) porcine skin were made using an 8 mm (diameter) biopsy punch. Initially, a 3 mm diameter biopsy punch and a scalpel for excision was used to try to create the 'partial thickness wound' in the middle of explants, but the depth of wound was hard to control and labor intensive. The wound bed was not uniform and impossible to inoculate with the same quantity of bacterial culture. The *in vitro* 'wound' borehole on the

explants of porcine skin was mechanically created with a drill (Dremel® MT. Prospect, IL.; #191 1/8" high speed cutter bit). The wound size is 3 mm in diameter and approximately 1.0 mm in depth. The borehole was located at the centre of each explant. The explants with the freshly created wound beds were sterilized with the chlorine gas method as previously described and immediately used for the bacterial biofilm culture.

Bacterial strain

The primary bacterial strain used in this study is the laboratory strain, PA01 of *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* is a Gram negative nonfermentative facultative anaerobic rod-shaped bacterium. It is not only an important opportunistic pathogen and causative agent of emerging nosocomial skin and soft tissue infections but can also be considered a model organism for the study of diverse bacterial mechanisms that contribute to bacterial persistence (Sriramulu et al., 2005). It has even been suggested that the presence of *Pseudomonas aeruginosa* in biofilms is the reason chronic wounds do not heal (Bjarnsholt et al., 2008). The *Staphylococcus aureus* laboratory strain SA35556 was used only for characterization of the biofilm bacterial growth.

Bacterial biofilm culture

Pseudomonas aeruginosa PAO1 and *Staphylococcus aureus* SA35556 were streaked for isolation on TSA plates from frozen stocks and incubated for 16-18 hours at 37°C. A culture tube containing 5mL of TSB was inoculated with an isolated colony and incubated overnight at 37°C. A flask containing 50 mL of TSB was inoculated with 150µL of overnight culture and incubated at 37°C in a water bath (Becton Dickinson and Company, Sparks, MD) at 150 rpm until the culture reached an optical density (OD_{640}) between 0.2 and 0.4 (Log phase). The optical

density was determined using a spectrophotometer (UNICO 1100 Uniten Producrts & Instrument INC).

The freshly sterilized porcine skin explants were inoculated by adding 10 µl of log phase bacterial culture into the 'wound bed' borehole. The explants were placed on appropriate culture media and incubated under the saturated humidity and 5 % CO_2 at 37 ºC. The culture media was daily changed with the freshly made media.(Sambrook et al., 1989)

Sonication and quantification

Each explant was rinsed three times in sterile PBS, soaked in PBS for 10 minutes, and then rinsed three more times with PBS. Each explant was aseptically placed into a 15 mL sterile tube containing chilled 7 mL sterile PBS with 5 ppm tween-80 (to achieve optimal solution depth for sonication). The explants were sonicated using a 23 kHz ultrasonic dismembrator (Fisher Scientific, Model 100, Pittsburgh, PA) for 30 seconds at 20~21 watts with the samples on ice. The setting on the dismembrator tip was adjusted to maintain this target watt output. (With more than 30 samples a time, the setting of dismembrator is typically adjusted from 7 to 9). This liberates bacteria from the biofilm into suspension. Serial dilutions were plated in triplicate on TSA plates and incubated overnight at 37 ºC, 5% CO_2. Colonies were counted from the plates to determine CFU/mL of sonicated explant bacterial suspension. Live and dead staining was used to determine the relative ratio of live to dead bacteria in the bacterial suspensions. LIVE/DEAD ® *Bac*light ™ Bacterial Viability Kits L7007 (Invitrogen Molecular Probes, Inc.) were used according to the manufacturer's recommendation as follows: Equal volumes of Component A and Component B were combined in a microfuge tube and mixed thoroughly. 1 µL of the dye mixture was added for each 0.33 mL of the bacterial suspension, mixed thoroughly, and incubated at room temperature in the dark for 15 minutes. 5 µL of the stained bacterial

suspension was trapped between a slide and an 18 mm square coverslip and observed in a fluorescence microscope.

Microscopy Analysis

Scanning electron microscopy

After three washes with BPS, the explants were fixed in Trump's solution for one hour, washed three times with PBS at 10 minute intervals followed by a single 10 minute wash with distilled water. Each fixed and washed explant was dehydrated in a graded ethanol series of 25%, 50%, 75%, 95%, and 100% at 10 minute intervals then washed twice with hexamethyldidilazane at 5 minute intervals. The explants were then air dried in the fume hood overnight. Each explant was then mounted on a SEM stub and stored in desiccators until sputter coated with gold and palladium using an argon gas sputter coating unit. The borehole of the explants were imaged using a scanning electron microscope (Hitachi S-4000, Tokyo, Japan) with a magnification range of 40× to 20,000×. Images were taken at an accelerator voltage of 6-8 kV and digital images were captured for qualitative analysis.

Cryo-scanning electron microscopy

Unfixed explants were directly mounted on a Cryo-SEM stub, and immediately put into liquid nitrogen for quick freezing. The frozen specimens were cryo-sputter coated. The specimens were imaged using Cryo-SEM (Zeiss 1450EP, Carl Zeiss MicroImaging, Inc. One Zeiss Drive, Thornwood, NY) with a magnification range of 40× to 50,000×.

Environmental scanning electron microscopy

The unfixed explants were directly mounted on an ESEM stub. The samples were immediately imaged using ESEM (Philips XL30 ESEM, Philips Electron Optics, Netherlands).

Confocal laser scanning microscopy

The explants were stained with 100 µl (placed on the 'wound bed') of the *Bac*Light ™ fluorescent dye solution (prepared as recommended by the manufacturer). The images were taken by CLSM. The CLSM microscope used in this study was a Zeiss Pascal LSM5 Confocal Laser Scanning Microscope with the following assessories: Axiovert 200 microscope with 10x, 20x, and 40x objective lenses; Argon-laser (458/488/514 nm); Helium-neon laser (633 nm) (University of Florida, Department of Microbiology and Cell Science). This instrument has differential interference contrast and epifuorescence microscopy capability with digital imaging.

Results

Chlorine Gas Sterilization for 45 Minutes is the Optimal Disinfection Method in the Model System

To avoid interference of other bacteria with the biofilm formation of bacteria of interest, the porcine skin needs to be sterilized. Three methods were tested in this study. The first test was to use different disinfectant solutions: 70% ethanol, 10% bleach, and 6% hydrogen peroxide, which have widely been applied in medical sterilization. After the pretreatment, the porcine skins were immersed in the different disinfectant solutions for 30 and 60 minutes, respectively (addressed in the method). TSA plates showed bacterial growth on the bottom of all explants after the 30 minute 6% hydrogen peroxide treatment the next day, and bacterial growth on all explants after 60 minute 6% hydrogen peroxide treatment after two day incubation. In addition, histological analysis showed that 6% hydrogen peroxide treatment resulted in significant tissue damage. Numerous voids appeared in the treated porcine skin (Figure 2-1O, 2-1P, 2-1S, 2-1T), compared to fixed sections of untreated control tissues (Figure 2-1A, 2-1B, 2-1C, 2-1D). These results indicate that 6% hydrogen peroxide is unfit to the use in the model system because of incomplete sterilization and tissue damage. The one hour 70% ethanol solution treatment

showed no microbial growth on any explants until six days of incubation. In all sterilization experiments where no microbial growth appeared within six days incubation, any microbial growth that appeared after six days was usually fungal. One hour 10% Bleach solution treatment showed no microbial growth on any explants until ten days of incubation. Combination treatment of 70% ethanol followed by 10% bleach, each for 30 minutes, showed no microbial growth on plates for one month (and repeated experiments showed that the order of treatment did not change the results). Histological analysis showed that 70% ethanol and/or 10% bleach was visually indistinguishable from fixed sections of untreated tissue (Figure 2-1E, 2-1F, 2-1G, 2-1H). However, these methods also caused the partial damage of the specimen tissues when the treatment time extended to 60 minutes (Figure 2-1I, 2-1J, 2-1K, 2-1L). The fat extraction by 70% ethanol is the well known reason for this damage. Therefore, the combination treatment of 70% Ethanol followed by 10% Bleach, each for 30 minutes is an efficient method in the model system. However, when the combination treatment was further used in subsequent numerous experiments, the microbial growth often occurred partially due to extra specimens in a limited disinfectant solution.

Antibiotics are widely used for inhibition of microbial growth in various experiments. In our study, gentamicin and oxacillin were chose. The gentamicin and oxacillin antibiotic treatment showed no growth on any explants until six days of incubation. However, oxacillin is an expensive agent and a large amount of oxacillin is required in experiments because of a high concentration (1 mg/mL) for the effective disinfection. Histological analysis showed that the tissue damage appeared on the both fresh and frozen porcine skin treated with gentamicin (Figure 2-2C, 2-2G), as well as the frozen porcine skin treated with oxacillin (Figure 2-2D, 2-

2H). The reason for the damage in the antibiotic solutions is likely to be due to detergent, tween 80, for 24 hour treatment.

The porcine skin with abundant fats makes water not accessable to the tissue without detergent addition. To avoid the potential effect of detergent on the tissue of porcine skin, chlorine gas sterilization was tested in the model system. In addition, chlorine gas sterilization is a simple and cheap method of disinfection. The chlorine gas chamber showed no microbial growth on explants after 30 or 60 minute treatment after one month. Histological analysis showed that both 30 and 60 minute treatments in the chlorine gas chamber were visually indistinguishable from fixed sections of untreated tissue (Figure 2-3). However, subsequent experiments with gas sterilization of 30 explants at a time showed that 30 minute treatment was not effective (showed microbial growth within one month on some explants). It was later observed that 45 minute treatment was sufficient for sterilization of 30 explants at a time and had no microbial growth after one month incubation on TSA plates. Therefore, the 45 minute treatment with chlorine gas is optimal sterilization method in the model system (Figure 2-4).

Determination of Optimal Culture Media for Growing Bacterial Biofilm on Porcine Explants

To mimic the bacterial growth environment found in human exudating chronic wound beds, liquid TSB medium was used initially to culture bacterial biofilms. Liquid medium also maximizes nutritional and water availability. The result showed that the bacteria penetrated through the bottom of the explants and rapidly proliferated in the liquid media. After overnight culture, the media became cloudy. To inhibit the bacterial growth in the liquid media, gentamicin was added to the media. Media was tested that contained a final concentration of 25, 50, 100, and 200 µg/mL gentamicin. The gentamicin concentration of less than 200 µg/mL failed to inhibit bacterial growth into the liquid media, whereas 200 µg/mL bacterial growth in

both the media and the wound bed of the explants. To overcome these problems, solid TSA media (1.5% agar) was tested for culturing bacterial biofilms. The explants became obviously deformed because of tissue desiccation after the 48 hour culture. Therefore, the solid media failed to provide biofilms an adequately moist environment.

For this *in vitro* model, ideally, bacterial biofilms should be restricted to the 'wound bed' without penetrating through the bottom of the explants. In addition, the culture conditions should provide adequate moisture and nutrients for development of bacterial biofilm on the explants. To search for a suitable medium to culture the bacterial biofilm, agar was added to the TSB culture media at 0.25, 0.5, 0.75, 1.0, and 1.5% (weight / volume). The results show that a soft agar 0.5% TSA plate provides adequate moisture and nutrients to grow both *P. aeruginosa* PAO1 (Figure 2-5) and *S. aureus* SA35556 bacterial biofilms. Different concentrations of gentamycin (25, 50, 100, 200 µg/mL) were tested for inhibition of penetration of *P. aeruginosa* PAO1 biofilm. Five explants were placed on each TSA plate made at each concentration of agar and antibiotic combination. Explant cultures were transferred to fresh plates daily and the used plates kept to check for bacterial growth (due to penetration through the bottom of the explants) after 24 hours incubation at 37ºC, 5% CO_2, and saturated humidity. Bacterial colonization appeared on 25 µg/mL gentamicin TSA plates from the four day explants cultures. Only half of explants resulted in bacterial colonization from six day explants cultures on 50 µg/mL gentamicin TSA plates. Results from subsequent assays showed that development of mature *P. aeruginosa* PAO1 biofilm in 3 days in this *in vitro* model. In summary, the medium containing 0.5% agar and 50 µg/mL gentamicin among the 20 media variations with different concentrations of agar and gentamicin was the best for the growth of *P. aeruginosa* PAO1 biofilm in this model system.

Measurement of the Bacterial Biofilm Growth Rate

Freshly sterilized pprcine skin explants were prepared as described above. Two explants were transfered to Trumps (formaldehyde 4%, gluteraldehyde 1%, ddH_2O 95%) (McDowell and Trump, 1976) for SEM imaging (negative control). The explants were aeseptically transfered to soft 0.5% agar TSA with 50 μg/mL gentamicin or 250 μg/mL oxacillin plates and inoculated with 10 μL of log phase bacteria culture, *P. aeruginosa* PAO1 or *S. aureus* SA35556 respectively. The inoculated porcine skin explants were cultured up to 5 days. The porcine skin explant biofilm cultures were transferred to fresh plates once every 24 hour. Each day, three explants were treated for 24 hrs with 2 mL TSB with 200 μg/mL gentamicin (PAO1) or 1 mg/mL oxacillin (SA35556) to kill planktonic bacteria. Each day, six explants (three with antibiotic treatment, three without antibiotic treatment) were transferred to 7 mL PBS with 5 ppm tween-80 and processed via sonication as previously described and quantified by plating serial dilutions of the sonicated explant bacterial suspension on TSA in triplicate. Each day, one explant (without antibiotic treatment) was transferred to Trumps for SEM analysis and another was transferred to 4% formaldehyde for histology. Plate counts were recorded as CFU / mL of bacterial suspension and used to create the biofilm growth curve of PAO1 and SA35556 before and after 24 hour antibiotic treatment. To determine the relative ratio of live to dead bacteria, the bacterial suspension was stained with fluorescent dye from LIVE/DEAD® *Bac*light ™ Bacterial Viability Kit, as described above, and observed under a fluorescent microscope.

The results from the *P. aeruginosa* PAO1 growth curve experiment showed that biofilm bacteria were different from the planktonic bacteria in growth characteristics (Table 2-2, Figure 2-7). The total number of bacteria was always higher than that of the antibiotic tolerant biofilm bacteria (Table 2-1, 2-2; Figure 2-7A). Within the first 24 hours the culture consists primarily of

antibiotic sensitive, presumably mostly planktonic, bacteria. After 24 hours, the rate of biofilm formation accelerates. After 48 hours bacterial growth almost reaches the maximum and the majority of the culture is in biofilm form. However, 72 hour incubation is required to obtain a consistent maximal CFU/mL of mature biofilm on all explant replicates. The same growth characteristics were observed in SA35556, *S. aureus* SA35556 cultures (Table 2-3, 2-4; Figure 2-7B). The reason for the lower growth rate of the biofilm bacteria (the lag) in the first 24 hour culture is likely that the bacteria gradually change their physiological mode from the planktonic to sessile state.

Bacterial strains PAO1 and SA35556 are sensitive to the antibiotics gentamicin and oxacillin respectively. The antibiotic treatment was used to kill planktonic bacteria while most of the biofilm bacteria survive because of the biofilm matrix protection. 50 μg/mL gentamicin was sufficient to inhibit penetration of PAO1 biofilm through the bottom of the porcine skin explants and 200 μg/mL gentamicin effectively limited PAO1 biofilm to the porcine explants submerged in liquid TSB media (no bacterial growth in the media), while 250 μg/mL oxacillin plates and 1 mg/ml oxacillin TSB did not have the effectiveness in restraining SA35556 biofilm to the explant. However, the growth curves for both PAO1 and SA35556, before and after 24 hour antibiotic treatment in liquid media, was strikingly similar (Figure 2-6). This suggests SA35556 in biofilm form is neither killed nor inhibited by oxicillin even at extremely high concentrations. Gentamicin, on the other hand, at sufficient concentration, is able to inhibit PAO1 biofilm growth in liquid media and spread of the biofilm outside the physical limits of the porcine skin explant. Perhaps PAO1 biofilm readily reverts to a susceptible phenotype, in the presence of abundant nutrients despite the presence of antibiotics, when at less stable interfaces (liquid or semi-solid meda) than porcine skin. Perhaps it is liquid flow that facilitates gentamicin

penetration of PAO1 biofilm at less stable liquid and semi-solid media interfaces. Perhaps a percentage of POA1 biofilm cells as well as planktonic are killed by gentamicin. It is clear that the antibiotics kill the susceptible bacterial cells after 1-2 day culturing (Figure 2-6). Figure 2-5 also shows that there is a reduction in CFU/mL after antibiotic treatment even at 3-5 day mature biofilm. It is not clear what percentage of biofilm is killed by antibiotic treatments in addition to planktonics or if the CFU/mL reduction seen after antibiotic treatment of the mature 3-5 day biofilm is simply due to physical debridement of surface bacteria as a result of the gentle post-treatment wash.

Characterization of the Biofilm Structure

To characterize the biofilms formed in the wound beds, four types of microscopy were used for examination of different structural characteristics of the biofilms on porcine skin. For the visualization of live bacterial biofilms forming on the wound beds of the porcine skin we used environmental scanning electron microscopy (ESEM) because this microscopy allows fully hydrated biofilms to be imaged. The explants at different developmental stages were taken out of the plates. After three washes, the explants were directly mounted on stubs for the imaging. The result showed that the living bacterial biofilms covered all the surfaces of the borehole of the porcine skin explant after two days of culture (Figure 2-7B), whereas the control borehole displayed a bare tissue surface (Figure 2-7A). This result was further confirmed by the image taken by scanning electron microscopy (SEM) and cryo-scanning electron microscopy (Cry-SEM). The higher resolution image of SEM clearly visualized the formed biofilms in an entire borehole for the two-day culture explants (Figure 2-7D), while for the one-day culture explants, the biofilms partially covered the boreholes (Figure 2-7E). The biofilms of the four-day culture appeared on the edge of the borehole. Compared to the biofilms of the one day culture, the biofilms of the four day culture are thicker based on the smooth surface (Figure 2-7F).

To visualize the structural detail of the bacterial biofilms, we used SEM to image biofilm bacteria and matrix on the borehole surface because SEM has a higher resolution than ESEM. Before the visualization, the explants were processed by fixation with Trump's solution and dehydration with a serial concentration of ethanol. The image showed that the biofilm matrix covered the wound bed surface. The detail revealed the biofilm bacteria were partially embedded within the matrix, whereas some bacterial were attached on the matrix surface of the three-day culture biofilms (Figure 2-8A). The control borehole showed only the tissue of the porcine skin (Figure 2-8B). One reason for some bacteria attached on the surface is likely to be an artifact, which was generated by the process of the SEM sample preparation: some matrix might be removed during the fixation and dehydration. To avoid this potential artifact, we used Cryo-SEM to further examine the fine structure of the bacterial bioflims because Cryo-SEM also allows a fully hydrated biofilms to be observed. The explants were taken out of the plates. After three washes with sterile water, they were immediately immersed into liquid nitrogen. These images also demonstrated that the biofilm bacteria were embedded within the matrix (Figure 2-9). In addition, the bacterial clusters in the biofilm matrix were clearly visualized (Figure 2-9A). Most of the biofilm bacteria are embedded in the matrix, some are in depth although a few seemingly attach the surface (Figure 2-9B, 2-9C). The bacterial clusters within the matrix are one of the most typical characteristics of mature biofilms (Stewart and Franklin, 2008; Stoodley et al., 2001). Therefore, our results show that the normal biofilms have formed on the wound beds of the porcine skin explants in this model system.

To determine the viability of biofilm bacteria, we used confocal laser scanning microscopy and the *Bac*Light Bacterial Viability Kit L7007 to visualize live bacteria. The CLSM microscope allows the studies of live and hydrated biofilms and localization of different

components in biofilms, using specific fluorescent dyes. The explants from the 1 day, 2 day, and 3 day cultures were taken out of the plates. After the biofilm staining with the fluorescent dye in *Bac*Light Bacterial Viability Kit L7007, the specimens were visualized by CLSM. The results showed that the bacteria within the biofilms are as viable as the planktonic bacteria (Figure 2-10). The viable bacterial clusters were clearly visualized, indicating that even the biofilm bacteria in clusters are also viable (Figure 2-10F).

Conclusion

The bacterial biofilm of the porcine skin for chronic wound healing in this study has been established. The main techniques were described as follows: the porcine skin directly from slaughter house is biopsied into explants of 8 mm in diameter and 3 mm in depth. The wound bed is a borehole that is mechanically created: 3 mm in diameter and 1.5 mm in depth at the centre of the explants. A 10 µl suspension cells in log phase are inoculated into the borehole. The explants are sterilized by the chlorine gas for 45 minutes. After sterilization, the explants are cultured in soft TSA medium with 0.5% agar and 50 µg/mL gentamicin for two to five days under the growth conditions of saturated humidity and 5% CO_2. After three days of culture, the mature bacterial biofilms are formed in the wound beds of porcine skin explants. The main steps are outlined in Figure 2-11.

Table 2-1. Growth rate of *P. aeruginosa* PA01 within the wound beds

	Observation value (CFU/mL)				Standard
Days	X_1	X_2	X_3	Average	deviation
0	5.60E+02	4.50E+02	4.80E+02	4.97E+02	5.69E+01
1	1.30E+06	1.66E+06	1.45E+06	1.47E+06	1.81E+05
2	9.30E+06	7.00E+06	8.30E+06	8.20E+06	1.15E+06
3	4.00E+06	4.70E+06	5.70E+06	4.80E+06	8.54E+05
4	2.21E+06	2.70E+06	2.54E+06	2.48E+06	2.50E+05
5	1.40E+06	1.51E+06	1.64E+06	1.52E+06	1.20E+05

Table 2-2. Growth rate of *P. aeruginosa* PA01 within the biofilms

	Observation value (CFU/mL)				Standard
Days	X_1	X_2	X_3	Average	deviation
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1	5.20E+01	5.30E+01	6.20E+01	5.57E+01	5.51E+00
2	7.80E+04	8.50E+04	9.50E+04	8.60E+04	8.54E+03
3	1.38E+05	1.40E+05	1.45E+05	1.41E+05	3.61E+03
4	3.20E+05	3.40E+05	3.90E+05	3.50E+05	3.61E+04
5	3.90E+05	4.00E+05	4.60E+05	4.17E+05	3.79E+04

Table 2-3. Growth rate of *S. aureus*, SA35556 within the wound beds

	Observation value (CFU/mL)				Standard
Days	X_1	X_2	X_3	Average	deviation
0	2.12E+02	2.00E+02	2.40E+02	2.17E+02	2.05E+01
1	1.65E+06	2.35E+06	2.40E+06	2.13E+06	4.19E+05
2	5.30E+06	7.50E+06	8.50E+06	7.10E+06	1.64E+06
3	2.50E+06	2.90E+06	3.90E+06	3.10E+06	7.21E+05

Table 2-4. Growth rate of *S. aureus*, SA35556 within the biofilms

	Observation value (CFU/mL)				Standard
Days	X_1	X_2	X_3	Average	deviation
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
2	5.60E+04	6.90E+04	8.00E+04	6.83E+04	1.20E+04
3	1.71E+05	1.77E+05	1.44E+05	1.64E+05	1.76E+04

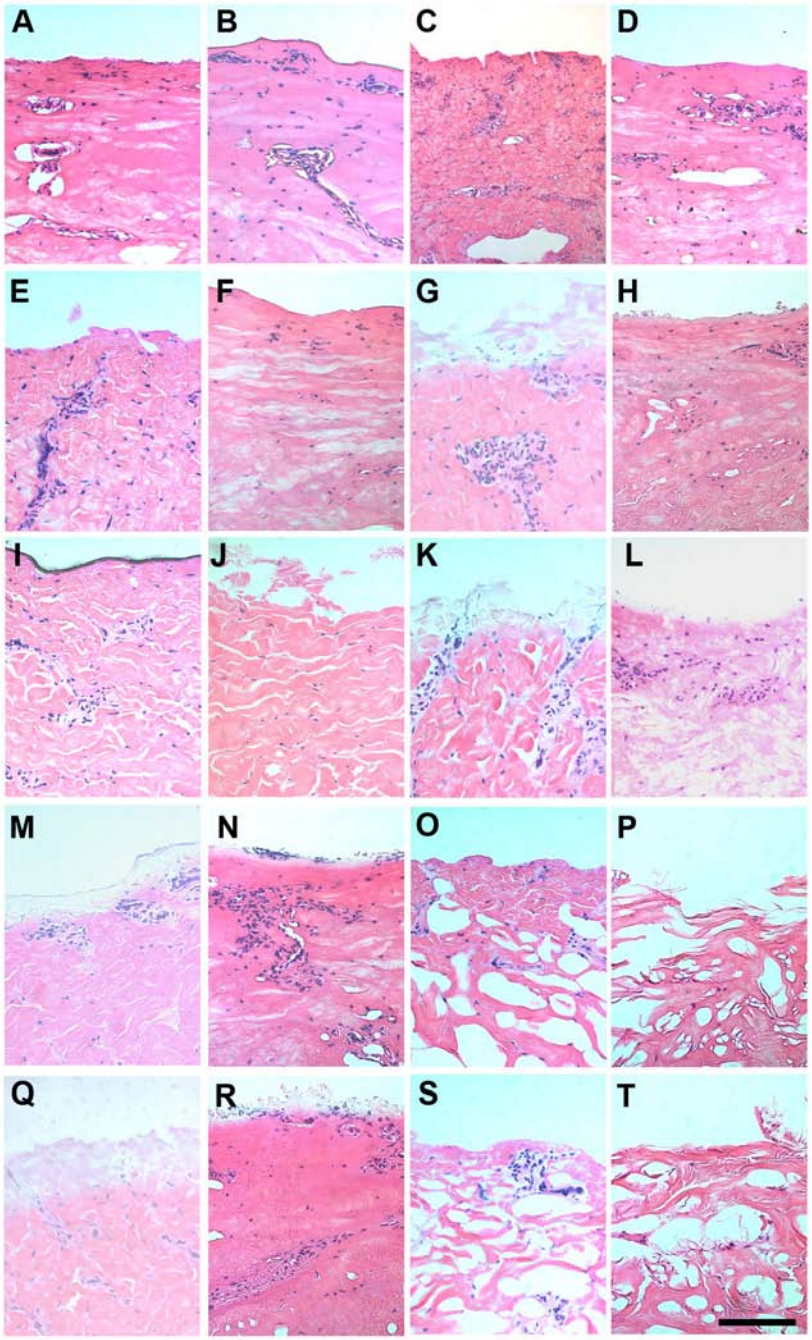

Figure 2-1. Effect of different disinfectants on the porcine skin tissue. A, C, E, G, I, K, M, O, Q, and S: fresh skin. All others: frozen skin. A and B: untreated skin. C and D: buffer solution. E and F: 70% ethanol for 30 minutes. G and H: 10% bleach for 30 minutes. I and J: 70% ethanol for 60 minutes. K and L: 10% bleach for 60 minutes. M and N: 70% ethanol for 30 minutes followed by 10% bleach for 30 minutes. O and P: hydrogen peroxide for 30 minutes. Q and R: 10% bleach for 30 minutes followed by 70% ethanol for 30 minutes; S and T: 6% hydrogen peroxide for 60 minutes. Bar = 100 µm for all pictures.

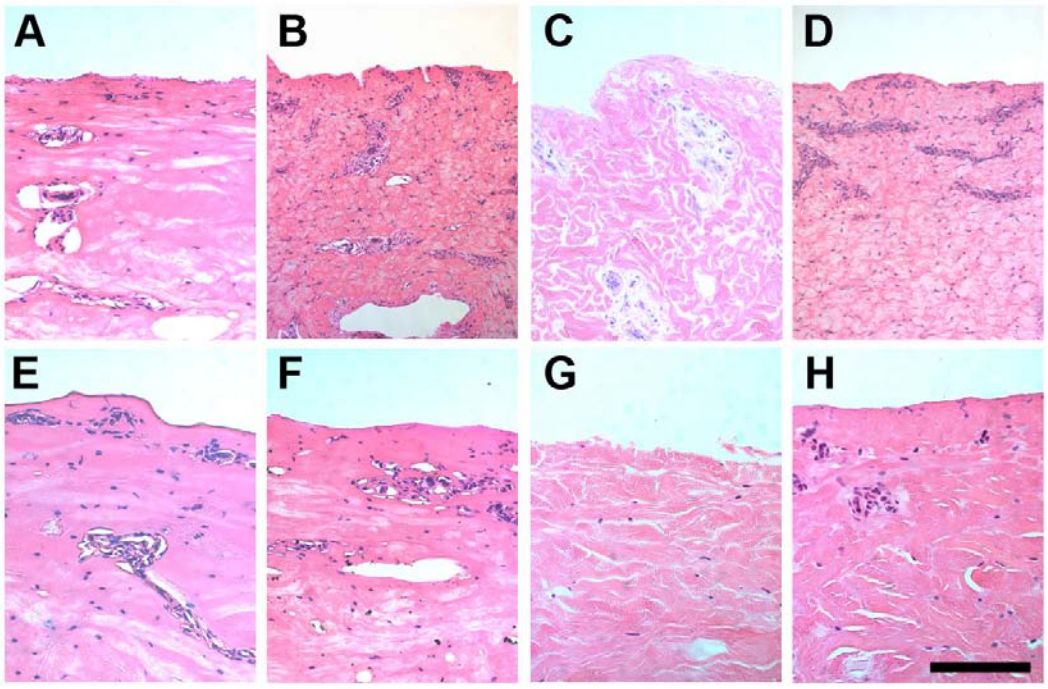

Figure 2-2. Effect of different antibiotics on the porcine skin tissue. A, B, C, and D: fresh skin. All others: frozen skin. A and E: untreated skin. B and F: buffer solution. C and G: 200 µg/mL gentamicin for 24 hours. D and H: 1 µg/mL oxacillin for 24 hours. Bar = 100 µm for all pictures.

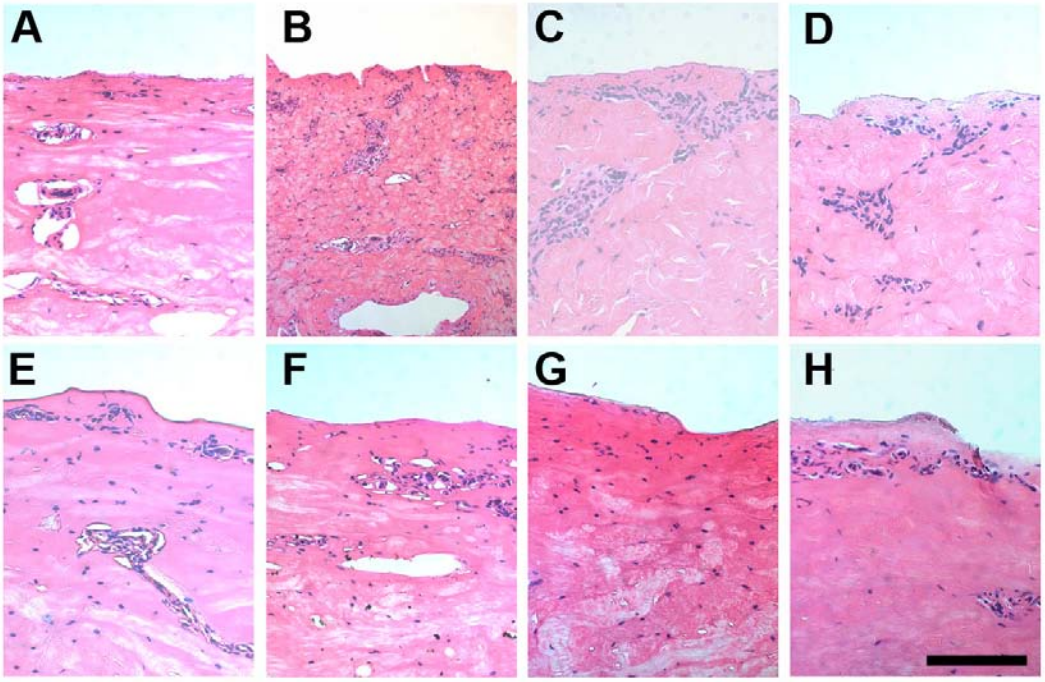

Figure 2-3. Effect of the chloride gas on the porcine skin tissue. A, B, C, and D: fresh skin. All others: frozen skin. A and E: untreated skin. B and F: buffer solution. C and G: chloride gas for 30 minutes. D and H: chloride gas for 60 minutes. Bar = 100 μm for all pictures.

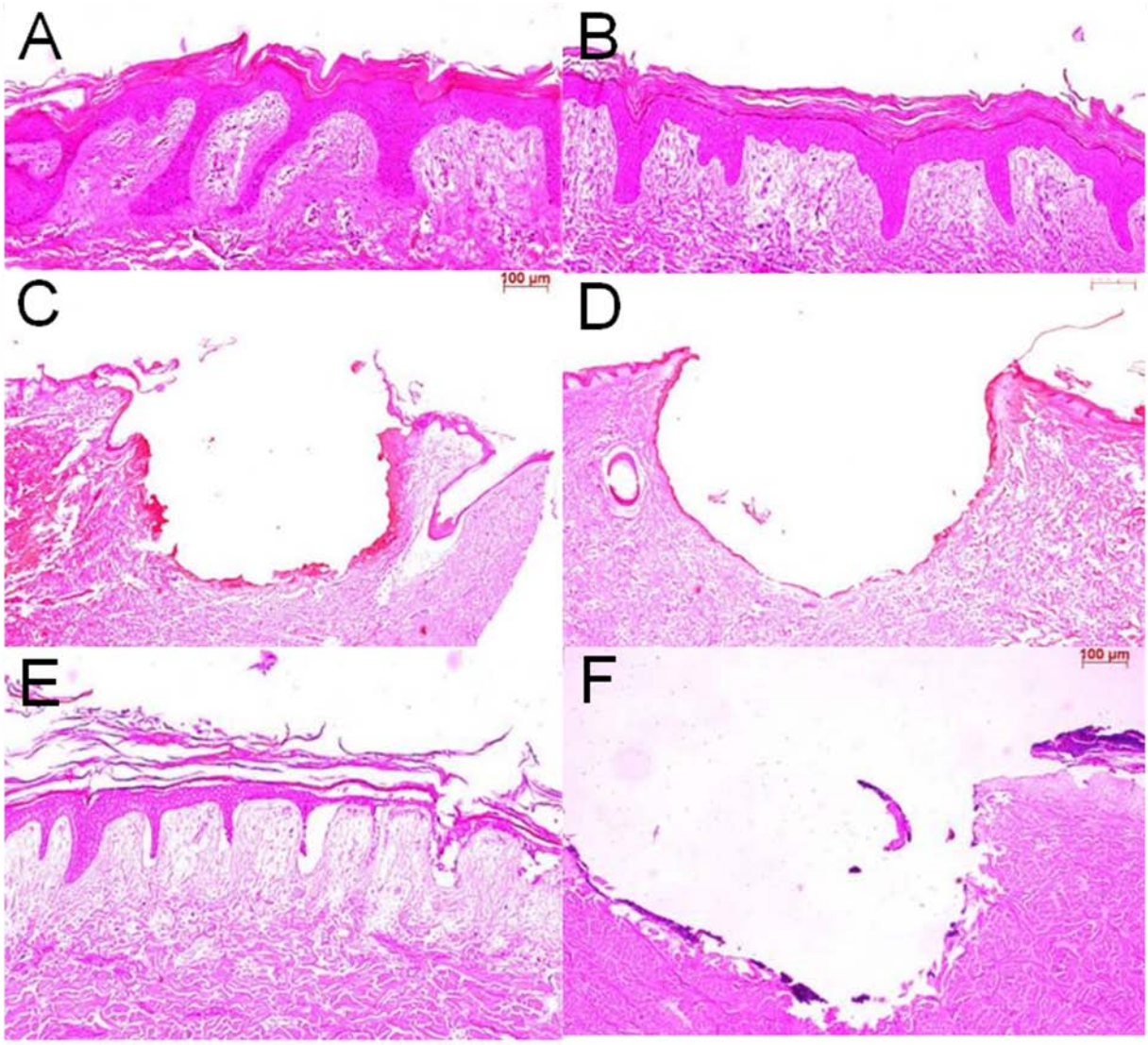

Figure 2-4. The 45 minute treatment with chlorine gas is optimal sterilization method in the model system. Compare before (A, C) and after (B, D) sterilized tissue histology results, there are no change and epidermis keep intact; After 3 day bacteria culture E, F, bacteria cluster are observed along the surface of wound bed. But up layer (stratum corneun) of epidermis look like loose from tissue.

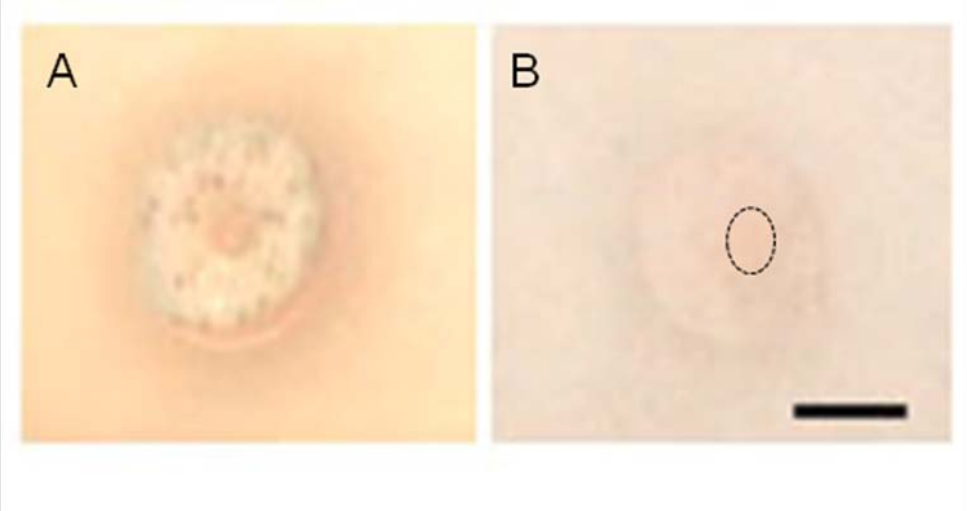

Figure 2-5. Growth inhibition of the bacteria outside of the borehole by 50 µg/mL gentamicin in 0.5 % agar TSB medium. A: 0 µg/mL gentamicin showing a cloudy ring of bacteria surrounding the explant of porcine skin in the medium and claques on the top surface of the explants. B: 50 µg/mL gentamicin showing a light cloudy ring of bacteria in the borehole of the explants in the drawn circle. Bar = 5 mm for A and B.

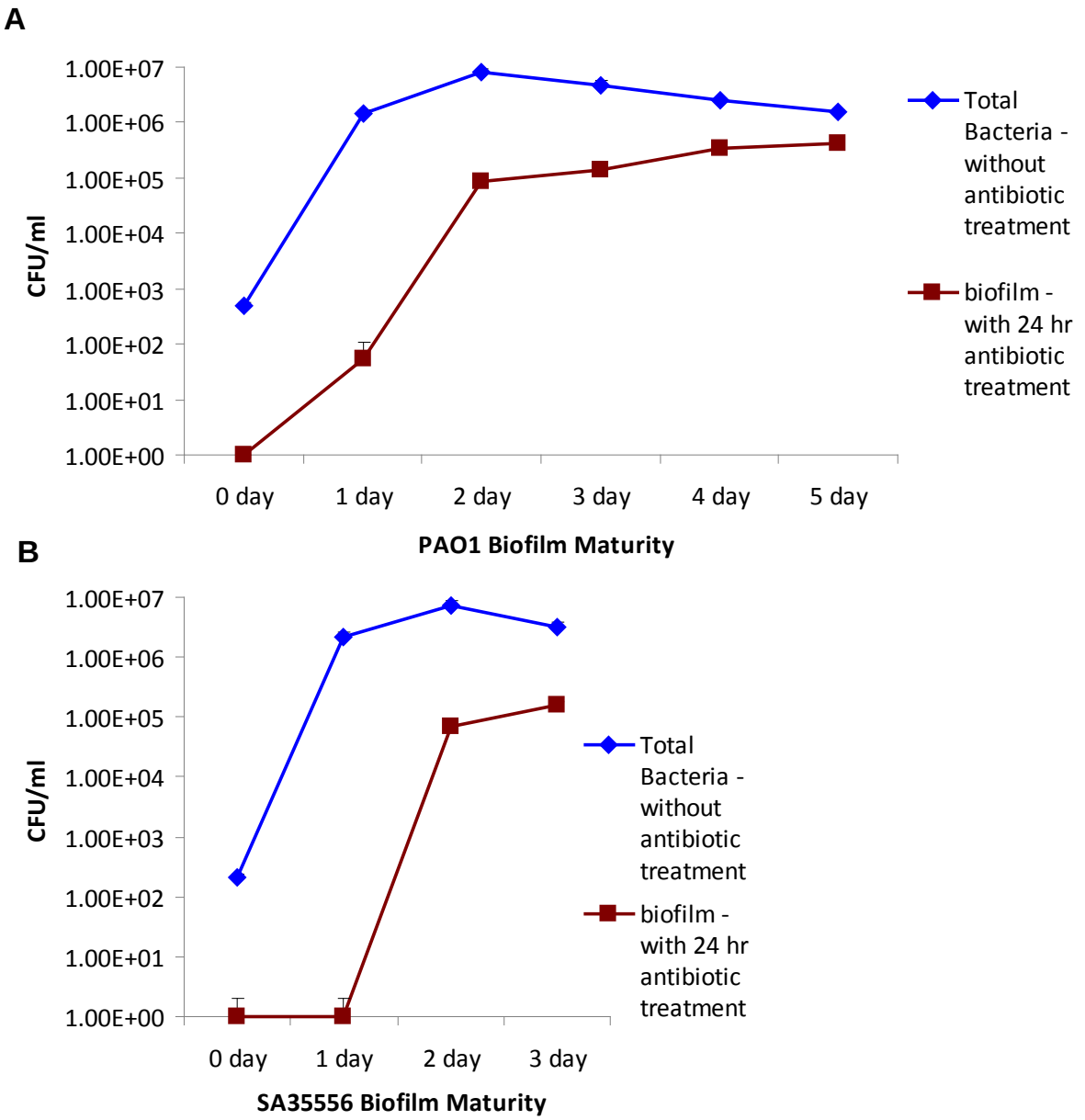

Figure 2-6. Growth curves of biofilm bacteria. A: *P. aeruginosa* PA01. B: *S. aureus* SA35556. The blue curves represent the total bacteria - planktonic and biofilm. The red curve represents the biofilm bacteria present after 24 hour antibiotic treatment.

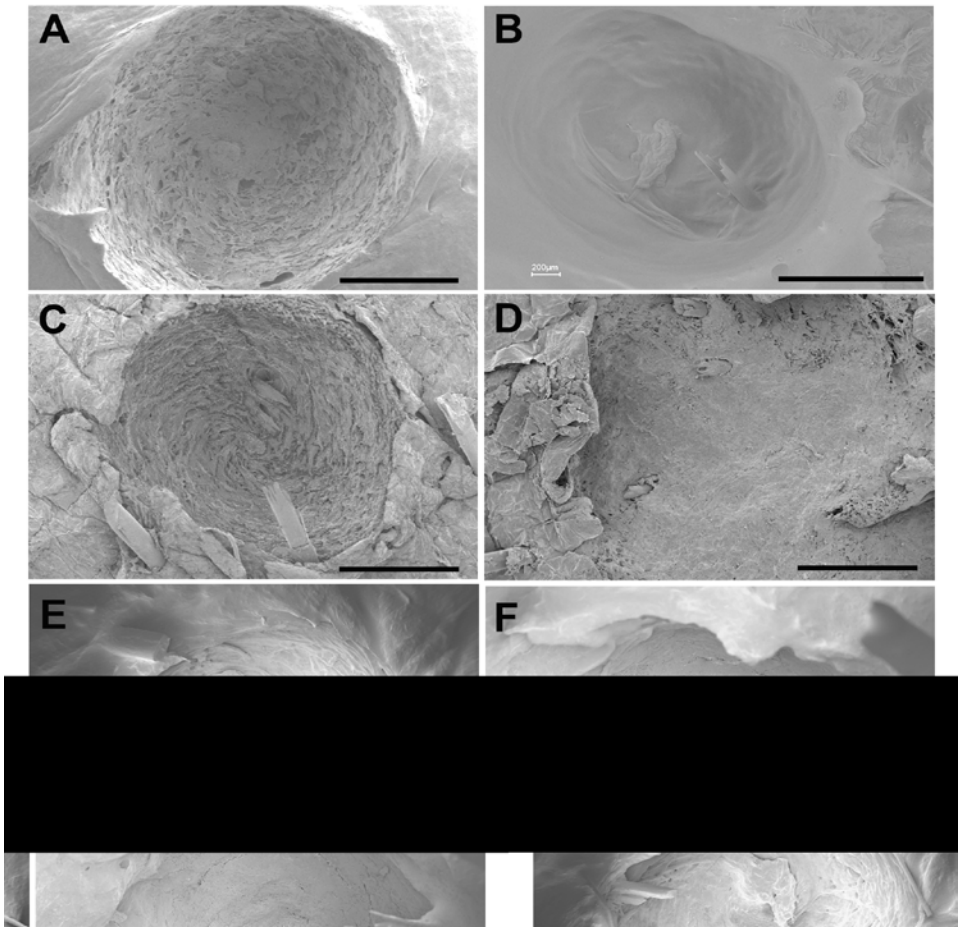

Figure 2-7. Bacterial biofilm formation on the borehole surfaces of the porcine skin explants in the presence of gentamicin. A: SEM showing an original bare borehole. B: ESEM showing the fully covered surface of the borehole by the live bacterial biofilms of the two-day culture. C: Cryo-SEM showing an original bare borehole. D: SEM showing the fully covered surface of the borehole by the dehydrated bacterial biofilms of the two-day culture. E: Cryo-SEM showing the partially covered surface of the borehole by the fully hydrated bacterial biofilms of the one-day culture. F: Cryo-SEM showing the fully covered surface of the borehole by the fully hydrated bacterial biofilms of the four-day culture. All bars = 1mm.

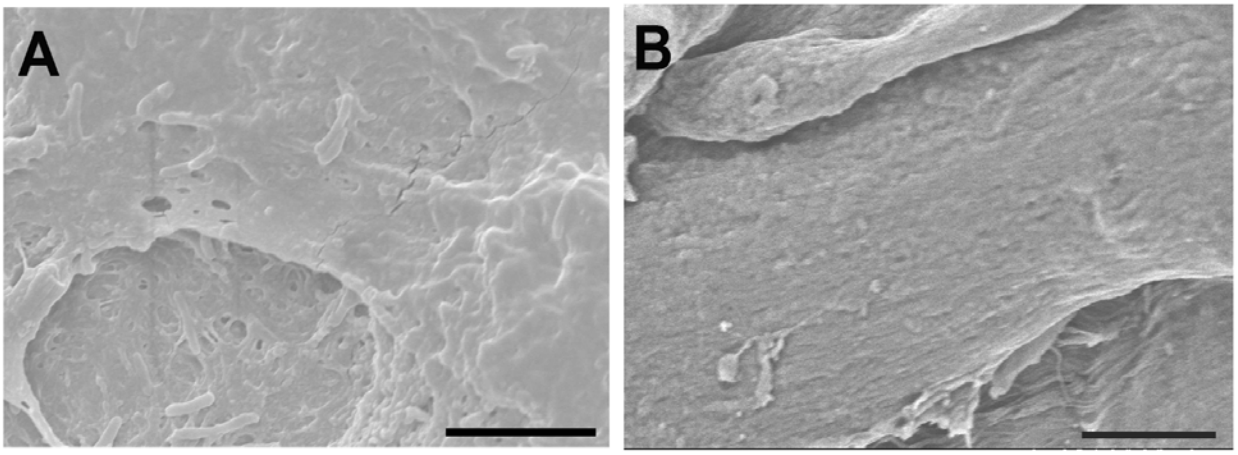

Figure 2-8. Fine structure of mature bacterial biofilms formed on the borehole surfaces of the porcine skin explants gentamicin. A: SEM showing the biofilm bacteria attached on the surfaces of the matrix or partially embedded within the matrix of the three-day culture biofilms. B: SEM showing the tissue surface of a borehole. Bar = 5 µm.

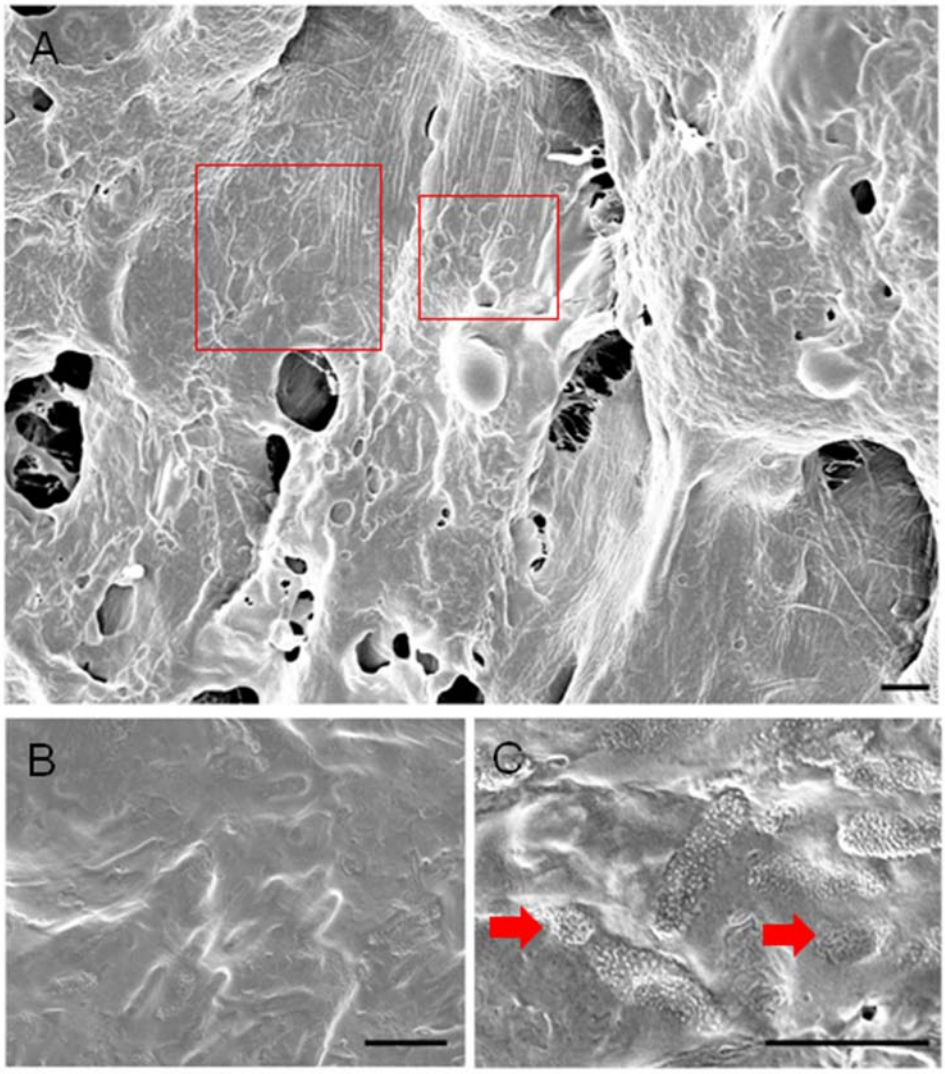

Figure 2-9. Bacterial clusters in the biofilms formed on the borehole surfaces of the porcine skin explants. A: Cryo-SEM showing the bacterial clusters in the formed biofilms of the two-day culture, surrounded by red squares. B: Cryo-SEM showing a large number of the biofilm bacteria embedded in the matrix of the three day biofilms. C: Cryo-SEM showing the biofilm bacteria embedded in depth of the matrix with only the one end out of the matrix, pointed by red arrow. All bars = 2 µm.

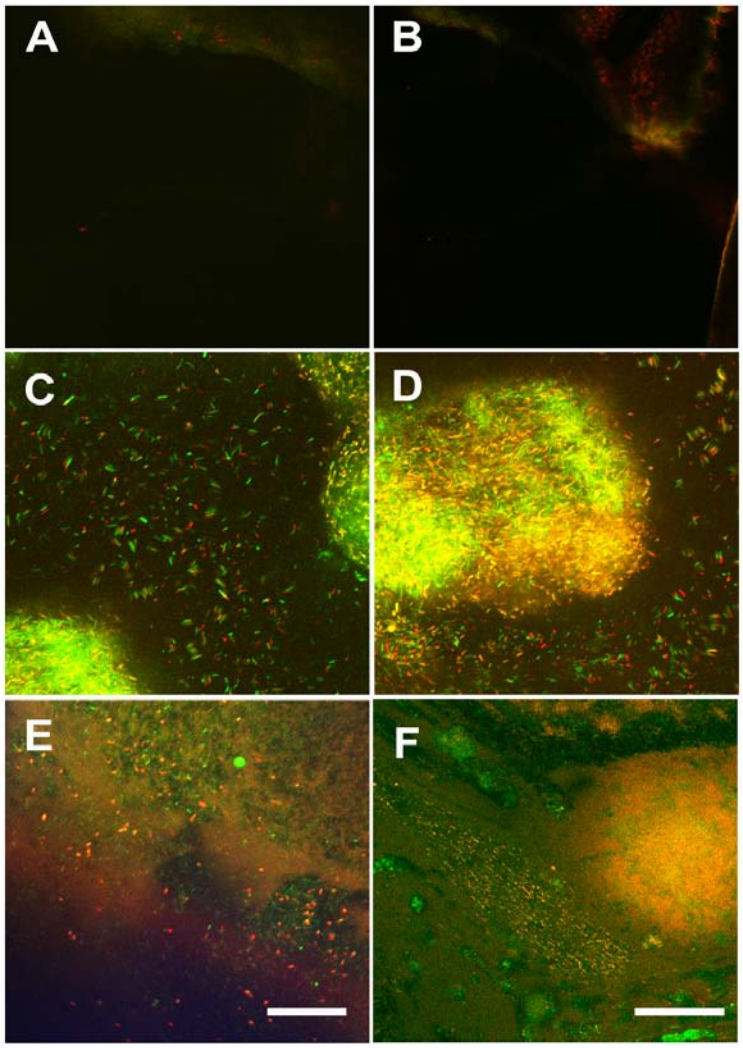

Figure 2-10. Live bacteria in the biofilms formed on the borehole surfaces of the porcine skin explants. A and B: wound bed without inoculation of the bacteria, A 1-day culture; B 4-day culture. C, E: bacteria for the 1-day culture. D, F: bacteria for the 4-day culture. Bars = 50 µm in F; 20 µm in E and all others as the same as E.

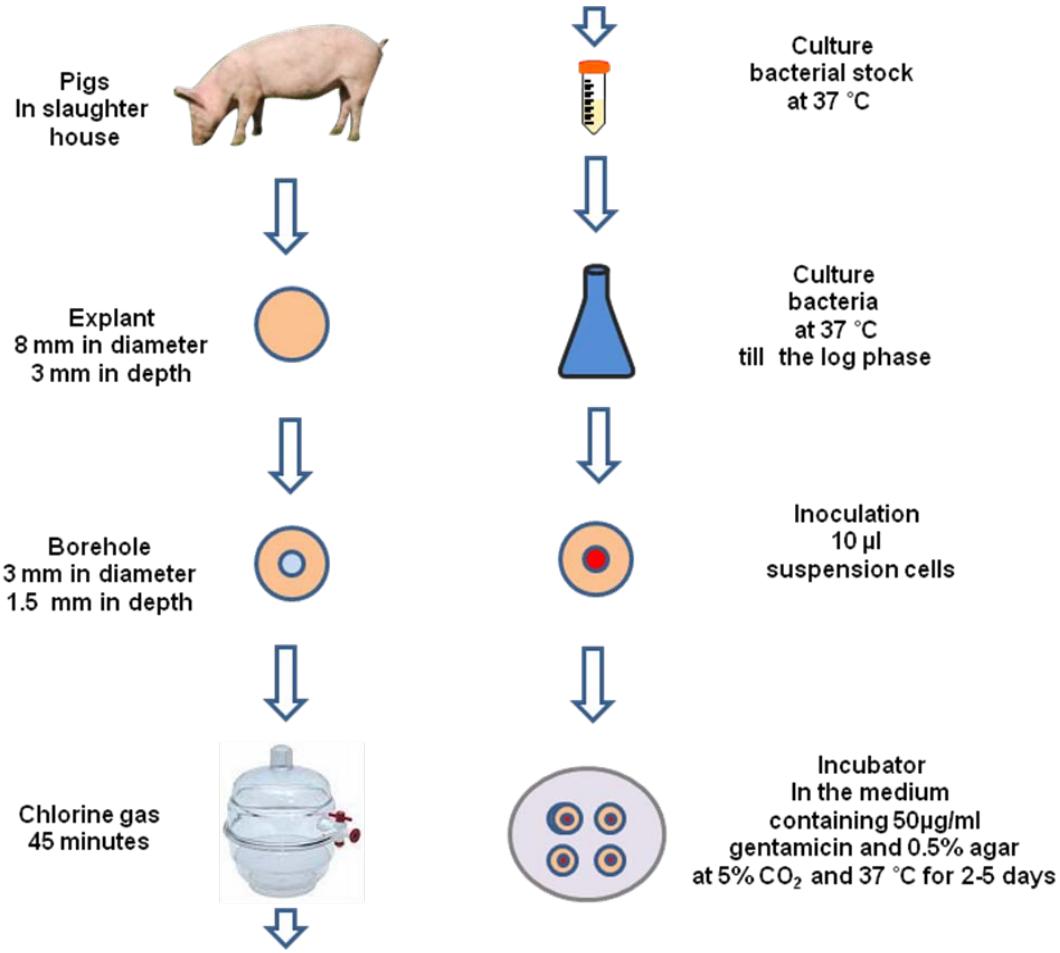

Figure 2-11. Biofilm model of porcine skin

CHAPTER 3
VALIDATATION OF THE *IN VITRO* PORCINE SKIN BIOFILM MODEL

Introduction

Antimicrobial Agents: Antibiotics, Antiseptics, and Disinfectants

The *in vitro* porcine skin biofilm model must be validated for its applicability for the pre-clinical evaluation of antimicrobial agents and dressings in treatment of chronic wounds. Antimicrobial agents can generally be categorized into three major groups: Antibiotics, antiseptics, and disinfectants. These agents may be bactericidal (directly kill bacteria) or bacteriostatic (prevent bacteria from proliferating). These three categories can usually be distinguished by their application. Antibiotics are generally used to kill bacteria within the body (delivered orally, intravenously) but may also be used topically on vulnerable tissue such as mucosal membranes or compromised tissue such as wounds. Antiseptics are generally used on external living tissue (e.g., skin, oral cavity, etc.) to inhibit infection. Disinfectants are generally used on non-living surfaces to destroy microorganisms.

The original definition of antibiotics only included natural antimicrobial molecules produced by microorganisms that are antagonist (bactericidal or bacteriostatic) to the growth of other organisms. Modern pharmaceutical research has lead to the development of synthetic or semi-synthetic antibiotics based on the study of natural antibiotics and their mode of action. They generally have specific targeted activity that results in inhibition of important or essential cellular processes in the bacteria: Protein synthesis, nucleic acid synthesis, cell wall synthesis, inhibits enzyme activity, modifies cell membrane, and interferes with cell membrane permeability (ionophores). A specific bacterial species or strain exposed to a specific antibiotic may have genetically encoded mechanisms of resistance that are constitutively expressed or induced by the presence of the antibiotic. In addition, because antibiotic efficacy depends on

accessibility to its target and are usually only effective on actively growing bacteria, bacteria in biofilms usually have an innate tolerance to the presence of antibiotics due to the unique characteristics of biofilm development which are absent when the same bacteria are growing planktonically. For this reason, bactericidal antimicrobial agents in the form of antiseptics or disinfectants that are generally considered effective were considered the most appropriate substances to first test on *P. aeruginosa* PAO1 biofilm grown using the *in vitro* porcine skin model in order to validate the model.

Two common but effective household and hospital disinfectants used to kill microorganisms are chlorine bleach and Lysol brand disinfectant. Consequently, these bactericidal agents were chosen as disinfectants to be evaluated using the *in vitro* porcine skin biofilm model. Chlorine bleach is a chemical solution made of sodium hypochlorite and acts as a strong oxidizing agent. Lysol brand disinfectant is a chemical solution made of benzalkonium chloride. Benzalkonium chloride belongs to the quaternary ammonium group and is a mixture of various alkylbenzyldimethylammonium chlorides. It acts as a nitrogenous cationic surfactant and is manufactured with various formulations depending on its application (antiseptic or disinfectant) and is generally considered a safe synthetic biocide. It is a broad spectrum antimicrobial agent, effective against a wide range of Gram+ and Gram- bacteria, fungi, and some protozoa, however, *Pseudomonas* species are often resistant (Kaye and Kaye, 2000). Benzalkonium chloride is commonly used to prepare the periurethral area for catheterization and for urinary catheter care (Kaye and Kaye, 2000). CAZS is a bacteriostatic biofilm surfactant solution made by Meditronic. Consequently, it's capabilities as a bacterial suspension solution and dispersion agent, to aid processing of biofilm grown on the explants, was evaluated in an experiment which compared it against PBS and PBS with 5 ppm Tween-80. The results, shown

below, led to it being chosen as the third solution to be evaluated (to determine its bactericidal-bacteriostatic characteristics) and compared to chlorine bleach and Lysol brand disinfectant.

Antimicrobial Dressings

Many antimicrobial dressings are commercially available that are designed to reduce bacterial load and/or inhibit bacterial growth and promote wound healing. Moisture dressings designed to absorb wound exudates and/or maintain a moist wound environment are generally changed daily, up to the length of time proscribed by the manufacturer, depending on the level of wound drainage. Several antimicrobial dressings, and a couple of non-antimicrobial moisture dressing counterparts, were evaluated for their antimicrobial efficacy against *P. aeruginosa* PAO1 biofilm grown using the *in vitro* porcine skin model.

Four types of antimicrobial agents were assessed: Iodine, silver, polyhexamethylene biguanide (PHMB), and Doxycycline. The dressings tested are described by the manufacturers as follows: Iodoflex™ cadexomer iodine pad (Smith & Nephew) is antimicrobial moisture dressing designed to absorb wound exudate (of ulcers, sores, and infected wounds), lower wound pH, and time-release iodine from cadexomer beads. Algisite™ M (Smith & Nephew) is a moisture dressing made of calcium alginate fibers and is designed to absorb wound exudates (of full and partial thickness wounds) for up to three days (or changed daily for heavily exuding or infected wound). Acticoat™ absorbent (Smith & Nephew) is an antimicrobial moisture dressing made of calcium alginate fibers coated in nanocrystalline silver and is designed to absorb wound exudates (of full and partial thickness wounds) and steadily release silver ions (70-100 ppm continuously available) for up to seven days (with an antimicrobial effect minimum of three days). Tegaderm™ AgMesh (3M) is an antimicrobial nonwoven cotton dressing which contains silver sulfate and is designed to release ionic silver on moist or moistened wounds for up to seven days. Aquacel® Ag (ConvaTec®) is an antimicrobial hydrofiber® moisture dressing

designed to absorb wound exudates and steadily release silver ions (1 ppm continuously available) for up to seven days. Curity™ AMD™ (Kendall) gauze PHMB (0.2%) packing strip for exudating wounds, surgical wounds, or wound packing is designed to resist bacterial colonization within the dressing and act as a prophylactic barrier to prevent penetration of external invading bacteria through the dressing. Aquacel® (convatec) is a hydrofiber® moisture dressing of sodium carboxymethlcellulose fibers design to absorb wound exudates for up to seven days. NanoDox™ hydrogel (Nanotherapeutics) with 3% doxycycline monohydrate is designed to provide a moist wound environment and deliver the broad spectrum antibiotic doxycyline which also inhibits matrix metalloproteinases (MMPs).

Iodine is an essential trace element and has been used, depending on the formulation, as a wound antiseptic, disinfectant, and water sanitizer. Iodine is a broad-spectrum antimicrobial agent (including *Pseudomonas* species). Iodine in aqueous solutions may be present in many different species forms. At physiological pH at low concentrations, the only important species are I^-, I_2, I_3^- (Cooper, 2007). Antimicrobial activity of aqueous iodine decreases with increased alkalinity and storage time (Cooper, 2007). Hydrated molecular iodine (I_2) has the highest antimicrobial potential (Cooper, 2007). Iodophores, composed of iodine complexed with organic compounds, are less irritating and allergenic than iodine solutions due to slow release (dispersion) of free iodine from the aggregates into aqueous solutions (Kaye and Kaye, 2000; White et al., 2006). The concentration of free iodine, and therefore activity, depends on the type of iodophore. The most common iodophore in clinical use is polyvinyl-pyrrolidone (povidone iodine; PVP-I) with an available concentration of iodine between 8-12% (w/v) (Cooper, 2007). With sufficient exposure, iodophores are effective disinfectants primarily used on intact skin. Historically, iodine has been considered cytotoxic and use in open wounds has been discouraged,

except for severely infected wounds. However, studies have never adequately determined if cytotoxicity was caused by the iodine or the detergent vehicle (Kaye and Kaye, 2000). In one study recent *in vitro* study using human infant fibroblasts and keratinocytes to evaluate the cytotoxicity of 20 different skin and wound cleansers, they found that 10% povidone, PVP-I surgical scrub, and 3% hydrogen peroxide were all 100 times less toxic than household bath soaps but 1000 times more toxic than saline (Wilson et al., 2005). Newer slow release fomulations, such as from cadexamer beads (Iodoflex™ dressing), have been approved for use in open wounds. Cadexomer iodine has been shown to accelerate wound healing, particularly chronic venous leg ulcers (Cooper, 2007).

Silver has been recognized for its antimicrobial properties for centuries. Silver resistant bacteria have been reported since the 1950's, including *Pseudomonas aeruginosa* strains / iosolates, but are currently relatively rare (Brett, 2006; Cooper, 2007; Franke, 2007). Silver is a broad-spectrum antimicrobial agent and is generally recognized as safe with minimal side-effects (Brett, 2006; White, 2006). The silver ion concentration required to kill bacteria is still debated, however, the literature supports bactericidal activity at levels of ppm (Brett, 2006; White, 2006). Silver in the form of silver nitrate is more astringent and irritating and used rarely today (White, 2006). Silver dressing should not be moistened with saline due to precipitation of silver ions. One study by Milliken & Company (the makers of SelectSilver™) showed that regardless of the dressing type or the form of silver on the dressing (including Acticoat™ 7 with Silcryst Nanocrystals™, Aquacel® Ag, and Tegaderm™ AgMesh), without organic loading, silver release in simulated wound fluid (142 mM NaCl, 2.5 mM $CaCl_2$) maintained an equilibrium concentration of 0.5 ppm (Canada et al., 2007). However, with organic loading (5% BSA) of the simulated wound fluid, the silver dressings released significantly greater amounts of silver that

varied with dressing and time: Acticoat™ 7 released >80 ppm after 1 day exposure, which reduced to ~ 60 ppm after 2 and 3 days exposure; Aquacel® Ag released ~ 40 ppm after 1 day exposure, which reduced to ~ 10 ppm after 2 and 3 day exposure; Tegaderm™ AgMesh released > 20 ppm after 1 day exposure, which reduced to ~ 0 ppm after 2 and 3 days (Canada et al., 2007).

Polyhexamethylene biguanide (PHMB) is a broad spectrum (bacterial and fungal) biocidal antimicrobial agent and disinfectant with no known microbial resistance or negative effects on wound healing (Cazzaniga and M., 2002; Lee et al., 2004; White et al., 2006). PHMB is not considered cytotoxic and is used in skin, wound, water, and contact lens cleansing and disinfection. PHMB acts by associating with the acidic lipid components of microbial membranes to cause phase separation, disrupting microbial membranes at high concentrations (Cazzaniga and M., 2002). Increasing length of the PHMB polymer correlates with increasing antimicrobial efficacy (Cazzaniga and M., 2002). Cazzaniga *et al* did a study where partial thickness wounds on pigs were dressed with gauze containing 0.2 % PHMB then challenged with the addition on top of the dressing of 100 µl of 10^6 *Pseudomonas aeruginosa* (ATCC 27317) bacterial suspension prepared from colonies scraped from overnight culture plates (Cazzaniga and M., 2002). PHMB Gauze dressing was shown to reduce recovery of invading *Pseudomonas aeruginosa* from 10^5 (with control gauze) to 10^2 less bactera and likewise inhibit growth within the dressing (Cazzaniga and M., 2002). However, there was no significant difference of total number of bacteria recovered from the wound with or without PHMB, which included normal epidermal flora (Cazzaniga and M., 2002).

Doxycycline is a broad spectrum semi-synthetic tetracycline antibiotic developed in the 1960's commonly used in the treatment of Lyme disease, ehrlichiosis, Rocky Mountain fever,

Yersinia pestis (bubonic plague), syphilis, chlamydia, pelvic inflammatory disease, chronic prostatitis, sinusitis, acne, rosacea, anthrax and as a prophylaxis against malaria (Todar, 2008). Tetracyclines inhibit bacterial protein synthesis and have been shown to have low toxicity (Todar, 2008). *Pseudomonas aeruginosa* is less sensitive but generally susceptiple to tetracyclines (Todar, 2008).

Materials and Methods

Assess PBS, PBS with Tween-80, and CAZS Surfactant Bacterial Dispersion with sonication Using the *In Vitro* Porcine Skin Explant Model

Sterile porcine explants were inoculated with (in the partial thickness borehole) with 10 µl of log phase growth *P. aeruginosa* PAO1 culture. The PAO1 biofilm was cultured for 4 days on 0.5% TSA with 50 µg / mL Gentamicin. The explants were transferred to fresh plates daily. Mature 4 day biofilm explants were each transferred into 2 mL TSB media with 200 µg / mL gentamicin (24 well microplate) and incubated for 24 hours. The antibiotic treated explants were washed three times by gently removing media from the wells with a pipette, filling each well with 2 mL sterile PBS and soaking for 10 minutes each wash. Each explant was transferred to chilled 7 mL sterile PBS, PBS with 5 ppm tween-80 or CAZS (Biofilm surfactant solution; Meditronic Xomed Inc., Jacksonville, FL. 32216-0980). The explants were each sonicated as previously described. The bacterial suspension was serially diluted 10 fold to 10^{-5} and 100 µl of appropriate dilutions were spread plated onto TSA in triplicate. The plate counts were reported as CFU/mL found in the 7 mL bacterial suspension.

Assess Disinfectant Treatment Efficacy on Biofilm Cultured Using the *In Vitro* Porcine Skin Explant Model

Sterile porcine explants were inoculated with (in the partial thickness borehole) with 10 µl of log phase growth *P. aeruginosa* PAO1 culture. The PAO1 biofilm was cultured for 3 and 4 days on 0.5% TSA with 50 µg / mL Gentamicin. The explants were transferred to fresh plates

daily. Each explant was transferred to a well of a 24 well microplate containing 1mL of disinfectant solution: 10% chlorine bleach (Active ingredient of stock solution: 6.15% sodium hypochlorite), 8% Lysol ® all purpose cleaner solution (Active ingredient of stock solution: 1.1856% Alkyl- dimethyl benzyl ammonium chlorides; Reckitt Benckiser Inc., Parsippany, NJ. 07054-0224), and CAZS (Biofilm surfactant solution; Meditronic Xomed Inc., Jacksonville, FL. 32216-0980). The explants were soaked in the disinfectant solution for 5, 10, 15 minutes. Each explant was rinsed by gently removing the disinfectant and replacing it with 2 mL sterile PBS with 5 ppm tween-80, soaked for 10 minutes, gently pipetted up and down 6 times and removed. The explants were washed three times. The explants were each transferred to chilled 7 mL sterile PBS with 5 ppm tween-80 and sonicated as previously described. The bacterial suspension was serially diluted 10 fold to 10^{-5} and 100 µl of appropriate dilutions were spread plated onto TSA in triplicate. The plate counts were reported as CFU/mL found in the 7 mL bacterial suspension.

Assess Silver Dressing Antimicrobial Efficacy on Biofilm Cultured Using the *In Vitro* Porcine Skin Explant Model

The experimental parameters and dressings tested are listed in Table 3-1. Sterile porcine explants were inoculated with (in the partial thickness borehole) with 10 µl of log phase growth *P. aeruginosa* PAO1 culture. The PAO1 was cultured on the explants placed on soft 0.5% agar TSA with 50 µg / mL gentamicin to produce explants with 4, 2 and 0 day (initial log plaktonic culture innoculant) biofilm. The explants were transferred to fresh TSA with 50 µg/mL gentamicin daily. Three explants of each maturity were transferred to soft 0.5% TSA with gentamicin (total of 6 explants per plate). 1 cm^2 squares of dressing were prepared and placed on all explants on plates. Explants of each combination of dressing type and biofilm maturity (36 explants per day) after 1 day exposure to the dressing were each placed in 15 mL test tube

containing 7 mL sterile PBS with 5ppm tween-80. The Explants were process by sonication as previously described. The bacterial suspension was serially diluted 10 fold to 10^{-5} and 100 µl of appropriate dilutions were plated onto TSA in triplicate. The plates were incubated overnight and plate counts were report as CFU/mL found in the 7 mL bacterial suspension.

Antimicrobial Dressing Strip Assay on Mature Biofilm *In Vitro* Porcine Skin Explant Model

The experimental parameters and dressings tested are listed in Table 3-2. Sterile porcine skin explants were placed on soft 0.5% agar TSA with 50 µg/mL gentamicin (4-6 explants per plate). The explants were inoculated (in the partial thickness borehole) with 10 µl of log phase growth *P. aeruginosa* PAO1 culture. One plate per dressing type per exposure time was prepared. The inoculated explants were cultured for 3 days in saturating humidity and 5% CO_2 at 37 ºC and transferred to fresh plates daily. Mature 3 day biofilm explants were each transferred into 2 mL TSB media with 200 µg / mL gentamicin (24 well microplate) and incubated for 24 hours. The antibiotic treated explants were washed by gently removing media from the wells with a pipette, filling each well with 2 mL sterile ddH_2O or PBS, soaking for 10 minutes and removing the wash. The washed 24 hour antibiotic treated 3 day cultured explants were placed onto soft 0.5% agar TSA with 50 µg/mL gentamicin plates. Aseptically prepared dressing strips (~1.3 cm x 5 cm) were placed on the 3 day biofilm cultured explants. Two layers of Curity AMD and Tegaderm AgMesh were used, placing strips in crisscross layers. 200 µl of NanoDox hydrogel was applied on the 3 day biofilm cultured explants. 80 µl of sterile ddH_2O was pipetted onto the dry dressing strips directly over each explant (except for the NanoDox hydrogel dressing, which is already moist). A sterile glass microscope slide was placed as a weight onto dressing-explants (one per plate). The explants were incubated for 24 and 72 hours. Three explants per experimental condition set were transferred to 15 mL conical tubes containing

chilled 7 mL sterile PBS with 5ppm tween-80 and sonicated as previously described. The bacterial suspension was serial diluted 10 fold to 10^{-6} and 100 µl of appropriate dilutions were spread plated onto TSA in triplicate. The plates were incubated overnight and plate counts were report as CFU/mL found in the 7 mL bacterial suspension. One explant per experimental condition was fixed in 4% formalin for histology.

Results

PBS, PBS with Tween-80, and CAZS Surfactant Dispersion with sonication of 4 Day *P. aeruginosa* Biofilm

Based on the reduction of CFU/ml (Table 3-3; Figure 3-1), the CAZS surfactant was originally determined to be potentially bactericidal. However, subsequent assessment of CASZ treatment of *P. aeruginosa* PAO1 biofilm explant cultures without sonication lead to the determination that the increased kill is likely due to sonication and that CASZ surfactant is likely compromising the integrity of the cell membrane making it more vulnerable to sonication.

Efficacy of Lysol® and Bleach Disinfectant and CAZS Surfactant Treatment on Two, Three and Four Day *P. aeruginosa* Biofilm

To determine the bactericidal efficacy of CAZS surfactant, 8% solution of Lysol® all purpose cleaner and 10% Bleach, two and three day *P. aeruginosa* PAO1 cultured biofilms on explants were treated for 5 or 10 minutes (Table 3-4; Figure 3-2) and four day *P. aeruginosa* PAO1 cultured biofilms on explants were treated for 5, 10 or 15 minutes (Table 3-5; Figure 3-3). CAZS doesn't kill PAO1 biofilm within 15 minutes. Both 8% Lysol® all purpose cleaner and 10% Bleach did not reduce CFU/mL of four day PAO1 biofilm in 10 minutes but did reduce the biofilm approximately 2 logs in 15 minutes. This data suggests that these disinfectant solutions are inefficient bactericides of mature biofilm in tissue within 15 minutes.

Silver Dressing Antimicrobial Efficacy on *P. aeruginosa* Biofilm

The silver dressing Acticoat™ Absorbent reduced 0 day (planktonic early log culture initial inoculant), two day and four day *P. aeruginosa* PAO1 biofilm approximately 1-2 log compared to Algisite™-M after one day exposure (Table 3-6; Figure 3-4). The silver dressing Silverlon® did not appreciably reduce 0 day (planktonic early log culture initial inoculant), two day and four day *P. aeruginosa* PAO1 biofilm. Acticoat™ Absorbent dressing may partially kill of inhibits bacterial growth compared to its counterpart moisture dressing but the results suggests that these silver dressings are inefficient topical bactericides of either planktonic bacterial growth or mature biofilm in tissue.

Antimicrobial Dressing Efficacy on Mature *P. aeruginosa* Biofilm

The iodine dressing Iodoflex™ effectively killed the bacteria in mature 3 day *P. aeruginosa* PAO1 cultured biofilms on explants (Table 3-7, 3-8, 3-9; Figure 3-5, 3-6).

Table 3-1. Parameters of the silver dressing anitimicrobial efficacy assay.

Test parameters	3 replicates of each parameter
Microorganism	*P. aeruginosa*
Biofilm maturity	0 (inoculated, 1 hour incubation), 2, and 4 day cultured explants
Dressings	Algisite™ M (Smith&Nephew) – No silver negative control Acticoat™ absorbent (Smith&Nephew) Aquacel® hydrofiber® 177902 (ConvaTec®; Bristol-Myers Squibb Co.) – No silver negative control Silverlon® WCD-22 (Argentum medical, LLC)
Exposure time to Dressing	1,2,3 days

Table 3-2. Parameters of the antimicrobial dressing efficacy on mature PAO1 biofilm assay.

Test parameters	3 replicates of each experimental condition set
Microorganism	*P. aeruginosa* PAO1
Biofilm maturity	3 day cultured biofilm on explants
Antibiotic treatment of 3 day mature explant biofilm to kill Planktonics	24 hr with 2 ml TSB with 200 µg/ml Gentamicin
Dressings	Algisite™ M (Smith&Nephew) – No silver negative control Acticoat™ absorbent (Smith&Nephew) Aquacel® hydrofiber® 177902 (ConvaTec®; Bristol-Myers Squibb Co) – No silver negative control Aquacel® Ag (ConvaTec®; Bristol-Myers Squibb Co) Tegaderm™ AgMesh (3M Health Care) Iodoflex™ Cadexomer iodine (Smith&Nephew) Curity™ AMD™- 0.2% Polyhexamethylene Biguanide (Kendall) NanoDox™ hydrogel – 3% doxycycline (Nanotherapeutics)
Exposure time to Dressing	24 and 72 hours

Table 3-3. CAZS surfactant dispersion with sonication of four-day *P. aerginosa* biofilms

	Observation value (CFU/mL)				Standard
Treatment	X1	X2	X3	Average	deviation
PBS	1.52E+06	1.54E+06	1.47E+06	1.51E+06	3.61E+04
PBS with Tween 80	9.60E+06	1.05E+07	9.00E+06	9.70E+06	7.55E+05
CAZS	9.50E+03	9.90E+03	1.06E+04	1.00E+04	5.57E+02

Table 3-4. Efficacy comparsion of CAZS and disinfectants treated two or three day cultured biofilms

		Observation value (CFU/mL)				Standard
	Treatment	X1	X2	X3	Average	deviation
Control	A	8.10E+04	7.90E+04	7.20E+04	7.73E+04	4.73E+03
	B	4.70E+04	5.00E+04	5.10E+04	4.93E+04	2.08E+03
CAZS solution	A	1.36E+06	1.34E+06	1.38E+06	1.36E+06	2.00E+04
	B	5.20E+04	5.20E+04	5.50E+04	5.30E+04	1.73E+03
8% Lysol® all	A	1.38E+06	1.43E+06	1.48E+06	1.43E+06	5.00E+04
purpose cleaner	B	5.50E+05	5.50E+05	6.30E+05	5.77E+05	4.62E+04
10% Bleach	A	1.80E+05	2.00E+05	2.40E+05	2.07E+05	3.06E+04
	B	1.70E+06	1.80E+06	1.80E+06	1.77E+06	5.77E+04

Note: A: 2 day biofilm treated with solution for 5 minutes. B: 3day biofilm treated with solution for 10 minutes. CAZS is a citric acid/surfactant solution.

Table 3-5. Efficacy comparsion of CAZS with Lysol and bleach treated four day cultured biofilms for 5, 10 or 15 minutes

	Treatment	Observation value (CFU/mL)				Standard
Disinfectant	(minutes)	X1	X2	X3	Average	deviation
Control	5	3.89E+05	2.90E+05	3.50E+05	3.43E+05	4.99E+04
	10	3.89E+05	2.90E+05	3.50E+05	3.43E+05	4.99E+04
	15	3.89E+05	2.90E+05	3.50E+05	3.43E+05	4.99E+04
CAZS solution	5	5.90E+05	7.30E+05	6.90E+05	6.70E+05	7.21E+04
	10	3.00E+04	3.60E+05	3.30E+05	2.40E+05	1.82E+05
	15	1.56E+05	1.66E+05	1.70E+05	1.64E+05	7.21E+03
8% Lysol® all	5	6.00E+05	7.00E+05	8.60E+05	7.20E+05	1.31E+05
purpose cleaner	10	9.20E+05	1.04E+06	1.14E+06	1.03E+06	1.10E+05
	15	7.40E+03	7.90E+03	8.00E+03	7.77E+03	3.21E+02
10% Bleach	5	3.10E+05	2.90E+05	3.20E+05	3.07E+05	1.53E+04
	10	1.06E+06	1.09E+06	1.24E+06	1.13E+06	9.64E+04
	15	5.50E+03	6.20E+03	6.30E+03	6.00E+03	4.36E+02

Table 3-6. Silver dressing inhibits PAO1 growth

Dressing	Biofilm day	Observation value(CFU/mL) X1	X2	X3	Average	Standard deviation
Acticoat™-Absorbent	0	2.33E+04	7.83E+04	2.53E+03	3.47E+04	3.92E+04
	2	3.12E+06	4.57E+06	1.67E+06	3.12E+06	1.45E+06
	4	4.53E+06	5.60E+06	2.87E+06	4.33E+06	1.38E+06
Siverlon®	0	1.97E+07	6.93E+06	1.26E+07	1.31E+07	6.40E+06
	2	8.07E+07	2.80E+06	1.00E+06	2.82E+07	4.55E+07
	4	5.97E+07	2.53E+07	4.92E+07	4.47E+07	1.76E+07
Algisite™-M	0	2.57E+06	3.28E+06	4.72E+06	3.52E+06	1.10E+06
	2	3.03E+07	2.30E+06	1.83E+07	1.70E+07	1.40E+07
	4	5.30E+08	6.70E+08	6.00E+08	6.00E+08	7.00E+07
Aquacel®	0	2.82E+07	5.97E+07	4.40E+07	4.40E+07	1.58E+07
	2	1.31E+07	7.95E+07	1.07E+06	3.12E+07	4.22E+07
	4	1.25E+08	9.00E+07	1.63E+07	7.71E+07	5.55E+07

Table 3-7. Iodine dressing kills bacteria within mature PAO1 biofilms

Dressing	# explants assessed	Average CFU/ml	Standard deviation
Control	3	1.96E+06	2.97E+05
Algisite™-M	7	1.19E+08	7.51E+07
Aquacel®	3	1.82E+07	1.25E+06
Aquacel®-Ag	8	1.17E+04	1.25E+04
Acticoat™-Absorbent	9	1.65E+04	1.69E+04
Curity™-AMD	5	3.53E+04	3.00E+04
Iodoflex™	5	0.00E+00	0.00E+00

Table 3-8. Iodine dressing one day exposure kills bacteria within the 3 day cultured biofilms

Dressing	Observation Value (CFU/mL)			Average	Standard deviation
	X1	X2	X3		
Algiste™-M	2.98E+08	3.06E+08	2.75E+08	2.93E+08	1.61E+07
Acticoat™-Absorbent	9.33E+04	2.23E+05	6.30E+04	1.26E+05	8.50E+04
Aquacel®	5.73E+06	1.03E+07	1.06E+07	8.88E+06	2.73E+06
Tegaderm™ AgMesh	6.33E+04	2.92E+04	2.70E+04	3.44E+04	1.62E+04
Iodoflex™	0	0	0	0	0
Curity™-AMD	1.98E+06	1.39E+06	2.94E+06	2.10E+06	7.82E+05
Aquacel®-Ag	1.45E+06	1.43E+06	2.18E+06	1.69E+06	4.27E+05
NanoDox™	1.13E+05	2.33E+03	1.13E+04	4.22E+04	6.15E+04

Table 3-9. Iodine dressing three day exposure kills bacteria within the 3 day cultured biofilms

Dressing	Observation Value (CFU/mL)			Average	Standard deviation
	X1	X2	X3		
Algisite™-M	6.48E+09	2.29E+09	3.65E+09	4.14E+09	2.14E+09
Acticoat™-Absorbent	2.20E+06	1.41E+07	6.60E+06	7.63E+06	6.02E+06
Aquacel®	6.60E+07	8.30E+07	7.30E+06	5.21E+07	3.97E+07
Tegaderm™ AgMesh	2.90E+06	3.50E+06	1.61E+07	7.50E+06	7.45E+06
Iodoflex™	0	0	0	0	0
Curity™ AMD	6.40E+05	4.10E+06	5.30E+05	1.76E+06	2.03E+06
Aquacel®-Ag	1.10E+06	5.80E+05	6.70E+05	7.83E+05	2.78E+05
NanoDox™	9.80E+05	9.80E+05	6.30E+06	1.05E+06	3.07E+06

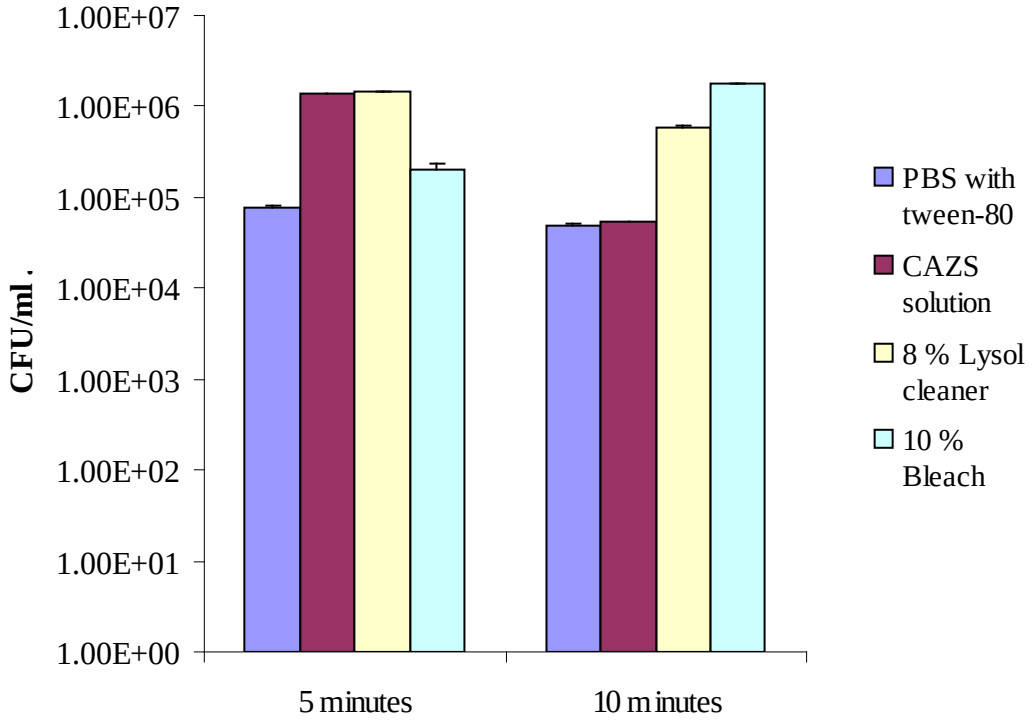

Figure 3-1. Efficacy comparsion of CAZS, 8% lysol cleaner, and 10% bleach solutions treatment of two or three day cultured biofilms

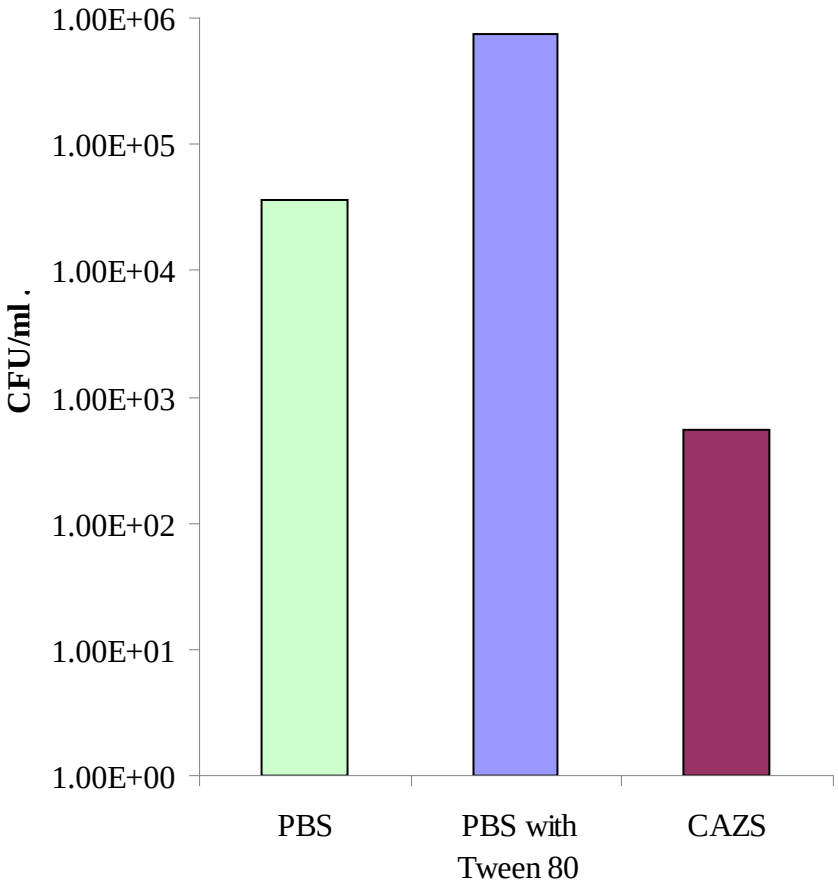

Figure 3-2. CAZS surfactant dispersion of four day *P. aerginosa* biofilms

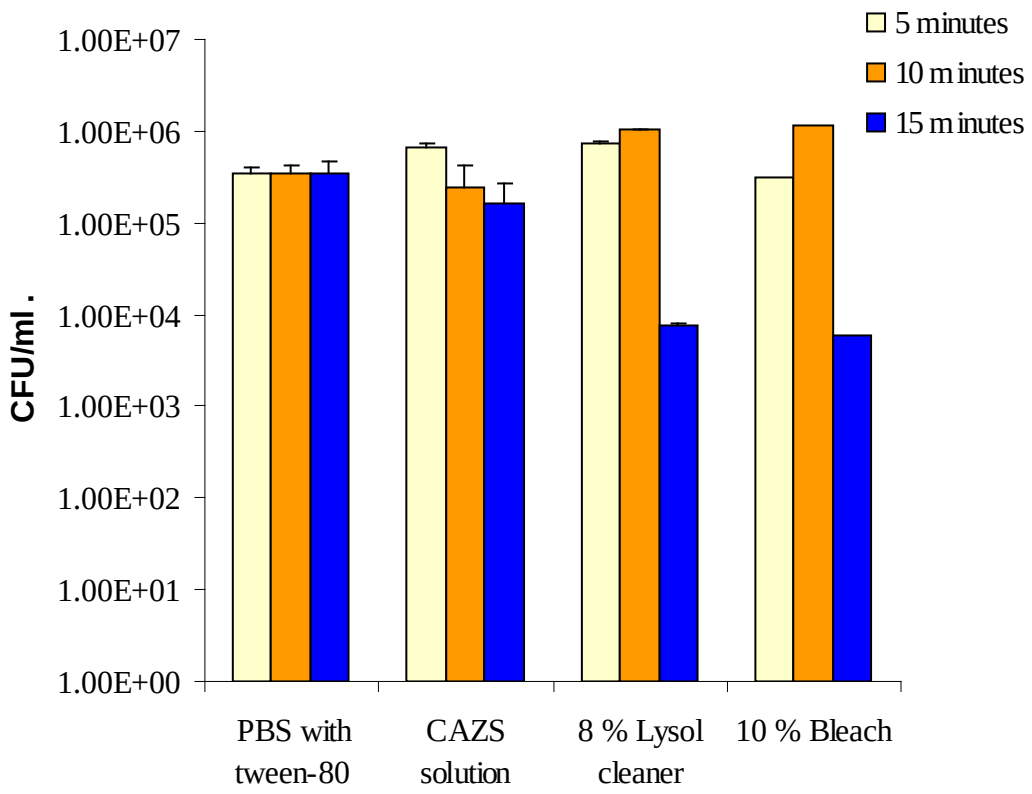

Figure 3-3. Efficacy comparsion of CAZS, 8% lysol cleaner, and 10% bleach solution treated four day cultured PAO1 biofilms for 5, 10 or 15 minutes

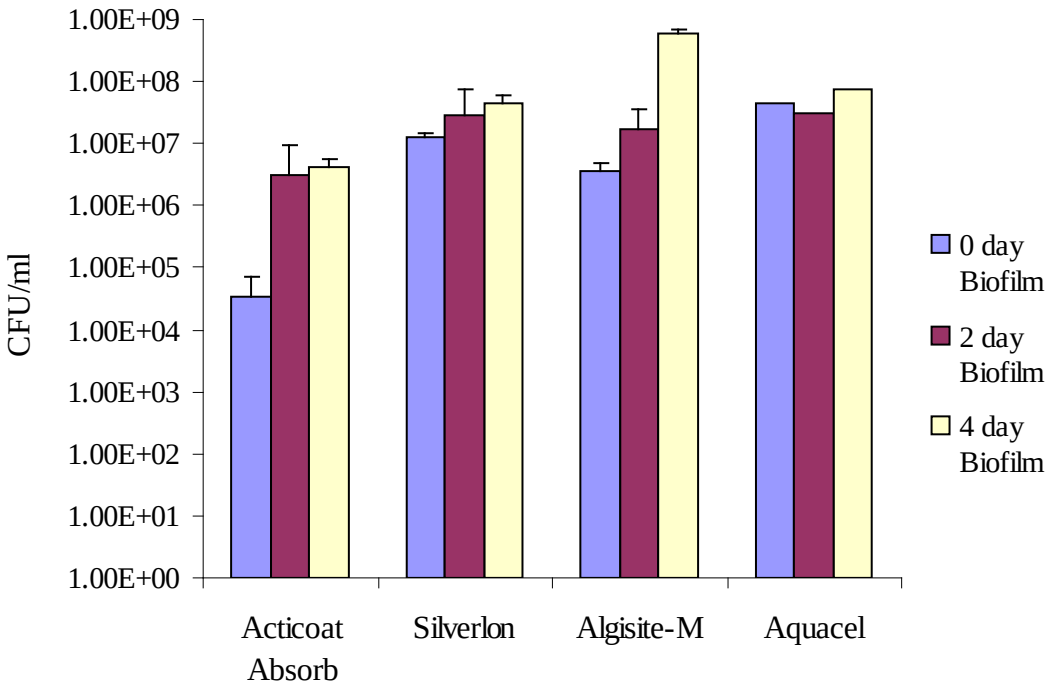

Figure 3-4. One day exposure to Acticoat-Absorb nanocrystaline silver dressing inhibits PAO1 bacterial biofilm growth compared to it's no silver Algisite-M counterpart.

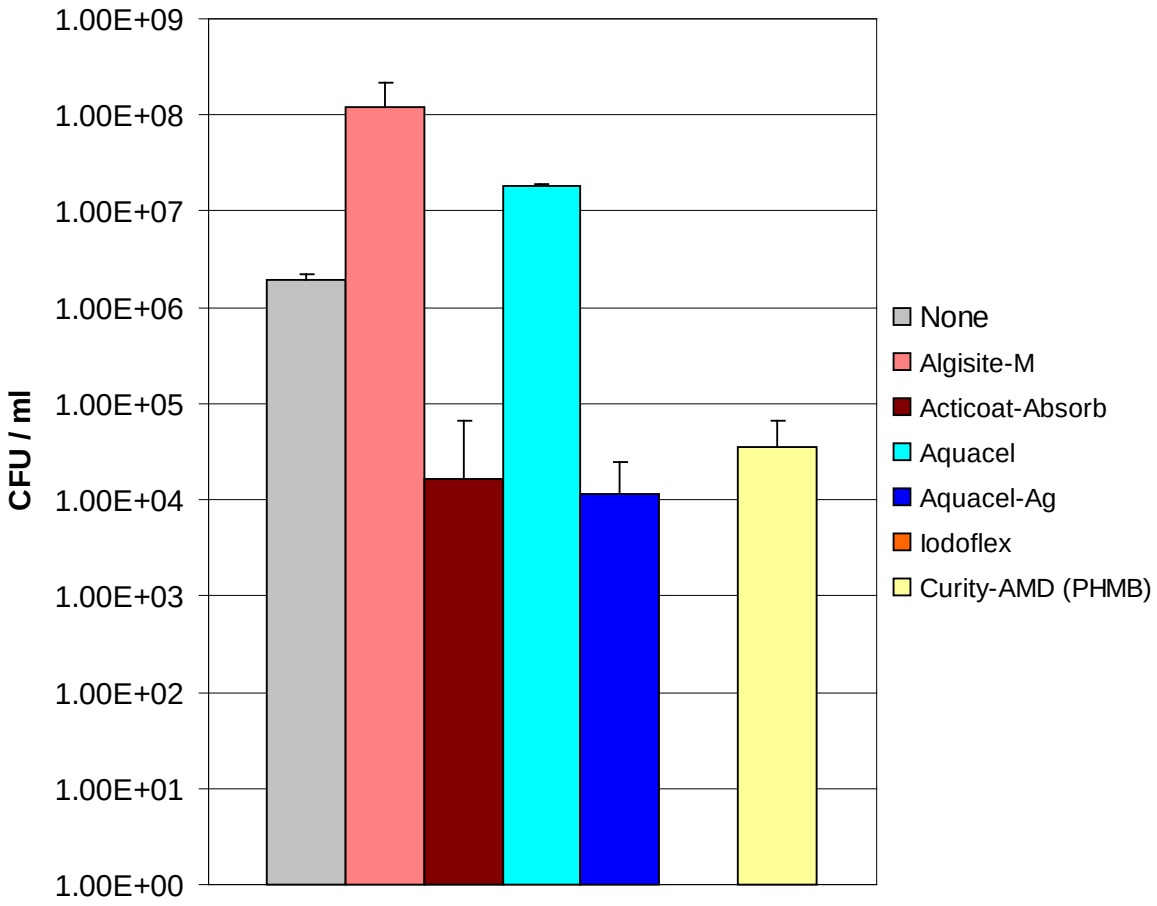

Figure3-5. Cadexomer Iodine dressing kills PAO1 bacteria within the mature biofilm cultured on porcine explants and treated for 24 hours with 200 µg/mL gentamicin. Nanocrystalin silver of Acticoat appears to kill approximately 4 logs compared to its no silver counterpart Algisite-M, but only between 1-1.5 logs compared to no dressing. Silver of Aquacel Ag appears to kill approximately 2 logs compared to its no silver counterpart Aquacel. PHMB appears to kill1-1.5 logs compared to the no dressing control. Algisite appears to promote PAO1 biofilm growth on porcine explants. The dressings placed on top of the explants 'wound bed' absorb moisture, antibiotics, and nutrients from the semi-solid media on which the explants are placed. The comparatively reduced water and nutrient availability may be contributing to the lower level of biofilm growth on the no dressing control.

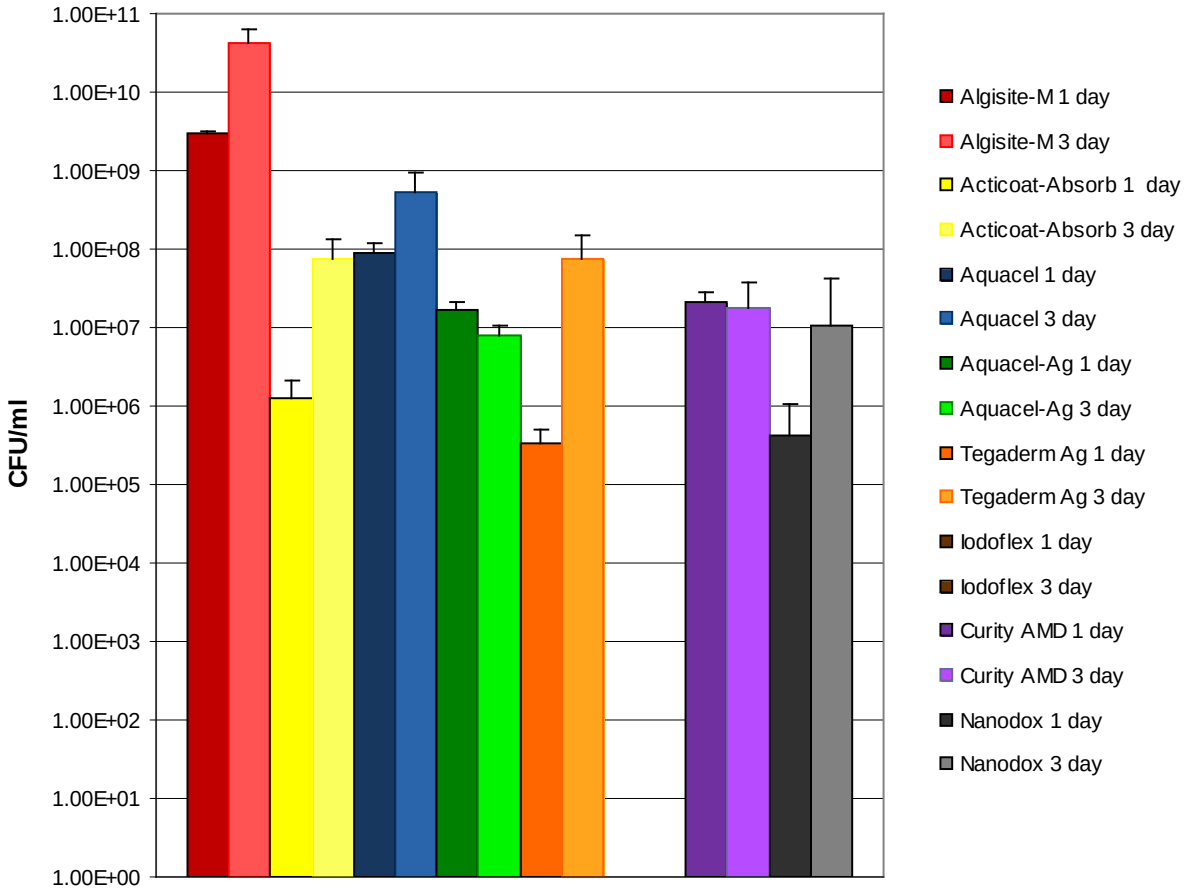

Figure3-6. Antimicrobial dressing efficacy after 1 and 3 day exposure on 3 day mature PA01 biofilm cultured on porcine explants and treated for 24 hours with 200 µg/mL gentamicin. Cadexomer iodine completely kills mature PAO1 biofilm cultured on procine explants after 1 day exposure. Silver and doxycycline kills approximately 1-3 logs of mature PAO1 biofilm cultured on porcine explants after 1 day exposure.

CHAPTER 4
DISCUSSION AND FUTURE WORK

Discussion

Chronic wounds, a major source of patient suffering and expenditure in home care, are an increasingly grave worldwide problem due to their recalcitrantance to healing. Bacterial biofilm is widely accepted to be a major culprit responsible for chronic wounds. The biofilm bacteria are notoriously resistant to host defense, antibiotics, disinfectants, and antiseptics, which leads to difficulty in biofilm eradication from chronic wounds by conventional antibiotic therapy (Costerton *et al*., 1999; Donlan and Costerton, 2002; Parsek and Singh, 2003). Before applying unconventional or novel therapy for chronic wounds, it is imperative to examine the biofilm formation in the chronic wounds. Therefore, our study established an *in vitro* porcine skin model to mimic the biofilm formation in human chronic wounds. Our final goal was to search for a simple marker to identify biofilm presence in chronic wounds using the invented model.

Although chronic wound healing faces numerous barriers such as poor perfusion and white cell dysfunction the presence of biofilms in chronic wounds is a major barrier to healing. Chronic wound healing involves the general phenomenon of growth and tissue regeneration (Beckert *et al*., 2007). Bacterial biofilms growing under this particular mini-environment easily absorb a large amount of water and abundant nutrients. In addition, approximately 37°C of the human body temperature is favorable for their growth. As a consequence, biofilms in chronic wounds commonly have a large mass of microbial cells and extracellular matrix. However, they also face various challenges from both cellular and humoral immune systems. How bacterial biofilms interact with the processes of the wound healing remains unknown. The possibility that cellular regeneration processes accompanying the healing may inadvertently provide nutritional support for sustaining biofilm viability is unaddressed. Understanding of these interactions

would, no doubt, facilitate the development of novel methods to promote chronic wound healing. Unfortunately, our *in vitro* porcine skin model of bacterial biofilms in chronic wounds cannot answer such questions since the artificial wound beds in our model do not involve the immune functions, but our model is an appropriate model for tests of antimicrobial agent efficacy.

The bacterial biofilm model of *in vitro* porcine skin with artificial wound beds in this study closely resembles biofilm formation in human chronic wounds. The porcine skin has been used as the model system for human wound healing and has shown the highest similarity to human skin (Schmook *et al*., 2001; Sullivan *et al*., 2001). Additionally, the porcine skin explants cultured in the soft TSB media were provided ample water and abundant nutrients for the biofilm bacterial growth. Furthermore, our culture condition was at 37°C and 5% CO_2. The porcine skin used in our model directly came from slaughter houses. Such an abundant source allow for a large number of trails such as drug or dressing screens without a large monetary expenditure for laboratory pigs. Our model is different from the porcine cultured tissue *ex-vivo* wound healing model established by Brandner *et al*. They used only skins from the inner side of ears directly from slaughter houses. Because this is tissue culture model for wound healing, to make the experiments highly reproducible, the same age and species of pigs must be used. The porcine *ex-vivo* wound healing model is appropriate for investigation of the growth effects from soluble substances, ointments, creams, and wound dressings used in wound healing (Brandner et al., 2006), but this model has not been tested for biofilm growth and assessment. Whether or not tissue viability can be maintained remains unknown while establishing a mature bacterial biofilm.

We chose *Pseudomonas aeruginosa* and *Staphylococcus aureus* for use in an *in vitro* porcine skin model suitable for biofilm growth and assessment. These two bacterial strains

displaying opposite Gram reactions are universally present in chronic wounds and are often used in various model systems for medical biofilm research (Colsky *et al.*, 1998; Valencia *et al.*, 2004). *P. aeruginosa* prevails in various environments and cause a large number of biofilm infections, notably for a wound, burn, lung and catheter (Stickler *et al.*, 1988; Rumbaugh *et al.*, 1999; Singh *et al.*, 2000; Gjodsbol *et al.*, 2006). *S. aureus* is commensal to the human body such as skin and cause devastating chronic infections in compromised hosts such as cystic fibrosis lung infections (Costerton *et al.*, 1995; Costerton *et al.*, 1999). The use of *P. aeruginosa* and *S. aureus* in this study enhances the similarity with biofilms in human chronic wounds. However, in nature, the biofilm bacteria in chronic wounds are not a single strain. Whether or not the *in vitro* porcine skin model can be suitable for the growth and assessment of biofilms consisting of multiple bacterial strains need to be determined.

The *in vitro* porcine skin model successfully avoids the interference from unwanted endogenous bacteria and fungus through chlorine gas sterilization. The chlorine gas can reach everywhere on the porcine skin explants. The optimal treatment time (45-60 minutes) can kill all microorganisms without significant damage to the porcine skin tissue structure. Although different disinfectants were also tried the sterilization effect was not satisfactory in this study. One reason for partial sterilization is likely that the disinfectant solutions fail to completely penetrate the porcine skin explants because of hydrophobic fats on the explants surface.

Bacterial biofilm formation was successfully restricted within the wound beds in the *in vitro* porcine skin model through the optimal concentrations of gentamicin and agar in the TSB medium. The soft TSB medium containing 50μg/mL and 0.5% agar not only provides bacteria in the wound beds for ample water and abundant nutrients, but also inhibits the bacterial growth outside of the wound beds. Other concentration combinations of gentamicin and agar were

unsatisfactory because of multiple reasons: growth inhibition of bacteria both in the wound beds and the broth media in the presence of a higher gentamicin concentration or bacterial growth both in the wound beds and the broth media in the presence of a lower gentamicin concentration; similar growth inhibition was observed with higher agar concentrations presumably due to insufficient availability of water and nutrients.

Mature bacterial biofilms were able to form within the wound beds of the porcine skin explants. The bacterial biofilm rapidly grows after 1 day culture, then turn slow after 2 day culture, finally matured after 3 day culture. A large amount of biofilms covered the entire wound beds at this time. Many bacterial clusters were embedded within the biofilm matrix. After 4 days of culture, the biofilm bacteria were still alive in the wound beds. No abnormalities were observed through the different microscopy methods used. Our results showed that the *in vitro* porcine skin model can be used to culture mature bacterial biofilms and is inexpensive, reproducible, and conducive to evaluating agents that may be used to promote chromic wound healing.

The bacterial concentration in terms of optical density (OD) is an important factor that affects the biofilm bacterial growth. Bacteria at log phase are in optimal physiological state and exponentially multiple. The different OD values were tested in this study. The result show the OD_{640} value between 0.2-0.4 is optimal for our model. This range of O.D. is often used in other methods for specific purposes. The reason for this range is specific growth environment of in vitro porcine model. After one hour when the suspension cells add into borehole 'wound beds' of the porcine skin, the liquid volume is visually reduced because the water is absorbed by the skin tissues. If OD_{640} is less than 0.2 or more than 0.4 the bacteria (data not shown), the total CFU/mL on the explants after 2-4 day culture does not reach the cell density obtained when

using an inoculum within 0.2-0.4 (Figure 2-5). Therefore, the OD_{640} of less than 0.2 or more than 0.4 is not optimal for our model. The biological basis of this observation is unclear.

We observed that total CFU/mL obtained from biofilm cultured on freshly prepared porcine skin was as much as 4 log greater (e.g., increasing from 10^6 to 10^{10}) than porcine skin frozen more than two weeks (-80ºC) (data not shown). Fresh skin explants maintains tissue structure and contain more water. The suspension cells lose less water. The bacteria on this 'wound beds' easily gain nutrients from the medium through diffusion. However, the frozen skins change in structure and dehydrate. The nutrients in the medium are not readily diffused into the 'wound beds'.

It has been shown that PAO1 biofilm is more resistant that planktonic bacteria to heavy metal (e.g., 600 times more for Cu^{2+}) when grown on polycarbonate in a rotating disk biofilm reactor and that it was likely due to decreased penetration through the extracellular polymeric substance (EPS) matrix of the 10 μm thick biofilm (Teitzel and Parsek, 2003). Our model indicates that silver dressing is ineffective as topical antimicrobial agent on skin. The inability to significantly kill or inhibit growth of *Pseudomonas aeruginosa* PAO1 (which does not carry the silver resistence genes) on either freshly inoculated explants or mature biofilm on skin, contrary to published data using surface (glass slide) cultured *Pseudomonas aeruginosa* biofilm (Percival et al., 2008), may be due to insufficient penetration of silver or differences (e.g., protein expression) in biofilm development on skin. The silver dressing was premoistened with water rather than saline to facilitate silver release and penetration without salt precipitation of the Ag^+. In addition, the requirement of organic loading to facilitate silver release as shown by Canada *et al.* (2007) (addressed in more detail previously) is unlikely to be an issue due to the fact that the biofilm is cultured on porcine skin and that the dressing absorbs the organic media components

as well. Consequently, this lack of efficacy is unlikely to be due to misuse of the dressing. Yet, our model indicates that cadexomer iodine is an effective antimicrobial dressing. The primary question that has to be answered is what factor or factors contribute to its efficacy? Perhaps it is the negative charge of I^- which facilitates its penetration through the overall negatively charged alginate EPS of PAO1 biofilm matrix where Ag^+ may bind to the matrix retarding its access to the bacterial cells. However, published data suggestes that alginic acid neutralizes povidone-iodine (Brown et al., 1995). Perhaps the cadeomer component is a key contributor to cadexomer iodine's effectiveness. Considering that published data assessing the efficacy of iodine on biofilm [e.g., effect of povidone-iodine on *S. epidermidis* cultured on polystyrene microtiter plates (Presterl et al., 2007); effect of Lugols iodine on *P. aeruginosa* cultured in polyxamer hydrogel (Gilbert et al., 1998) indicate that iodine is not effective against bacterial biofilm, further experimental assessments must be done.

Conclusion

The *in vitro* porcine skin model of bacterial biofilms in chronic wounds has been established in this study. The main techniques used are described as follows: the porcine skin directly from slaughterhouses is biopsied into explants of approximately 8 mm in diameter and 3 mm in depth. The machanically created wound bed is a borehole that is 3 mm in diameter and 1.5 mm in depth at the centre of the explants. A 10 µl suspension of bacterial cells in the log phase are inoculated into the borehole. The explants are sterilized by the chlorine gas for 45 minutes. After the sterilization, the explants are cultured on soft TSA medium with 0.5% agar and 50 µg/mL gentamicin (for *Pseudomonas aeruginosa* PAO1) for two to five days under the growth condition of saturate humidity and 5% CO_2. After three days of culture, the normal mature bacterial biofilms are formed within the wound beds of porcine skin. The *in vitro* porcine skin model of bacterial biofilms in chronic wounds has been validated by evaluating the efficacy

of numerous antimicrobial agents and commercially available wound dressings. It is proposed that this model is suitable for large scale screening of novel agents / treatments that may be used to promote wound healing.

Future Work

Further experiments should be done for completion of the *in vitro* porcine skin model of bacterial biofilm in chronic wounds. We consider the following experiments important to complete this model system: 1) biofilm growth conditions using this model must be developed for other medically important chronic wound bacterial species such as *Staphylococcus* and *Escherichia* species, as well as revevant anaerobic species; 2) other media that more closely simulate growth conditions in chronic wound beds (i.e. simulated wound fluid) to culture bacterial biofilms should be assessed using this model; 3) Alternative methods other than antibiotic treatment to limit biofilm growth to the explant and kill planktonic bacteria should be explored; 4) improved microscopic methods for visually assessing biofilm development on porcine skin explants (i.e. histology and cell viability) needs to be developed.

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BIOGRAPHICAL SKETCH

Qingping Yang was born in China. She entered Wuhan University for professional training in laboratory techniques in 1987. Immediately after her graduation, she was offered a position in the College of Life Sciences, Wuhan University in 1989. She worked there until 2003, when she left for the USA. During that period, she completed several training programs as a part-time student. In 1993, Qingping Yang finished a three-year training program in applied electron and appliance technology from the Department of Physics, Wuhan University. In 1998, she was awarded a bachelor's degree in public administration from the Department of Politics and Administration, Wuhan University. In 2003, she passed the two-year post-baccalaureate training in microbiology in the college of Life Sciences, Wuhan University. In June 2007, Qingping Yang began her master training in the Department of Microbiology and Molecular Genetics, the University of Florida.