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A microbial infected in vitro wound model treated through microdialysis

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A MICROBIAL INFECTED *IN VITRO* WOUND MODEL TREATED THROUGH MICRODIALYSIS

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ABSTRACT

A MICROBIAL INFECTED *IN VITRO* WOUND MODEL TREATED THROUGH MICRODIALYSIS

By Gina T. Giannini

Skin injuries are vulnerable to *Staphylococcus aureus* infections in nosocomial environments and may result in suppuration of wounds, systemic disease and toxic shock. An *in vitro* wound model was developed using Sykes-Moore chambers composed of biomaterials used for wound treatment (1% alginate, dialyzed "Hyfil" hydrogel, "Zyderm II" collagen) and a fibroblast cell line. The chambers were inoculated with *S. aureus* and antibiotics were introduced through *in situ* microdialysis tubing. Fluid and biomaterial samples were obtained at 0, 2, 8, 24 h and analyzed for *S. aureus* protein A secretion, *S. aureus* concentration and fibroblast viability. Chambers containing each biomaterial were compared. There was an inverse relationship between post-infection *S. aureus* concentration and fibroblast survival. *Staphylococcus aureus* and protein A concentrations were related, but may have been obscured due to differential interactions between protein A and the biomaterials.

ACKNOWLEDGEMENTS

I would like to express my grateful appreciation to my advisor, Dr. John T. Boothby, for his advice, patience, thoughtfulness and guidance during this research. He has had a great impact towards the pursuit of my scientific goals. I would like to extend my sincere gratitude to Dr. Eric E. Sabelman who gave me the great opportunity to pursue this project. His advice, guidance, kindness, patience and the opportunities he has given me have been a great influence towards the pursuit of my scientific endeavors. Thanks to Dr. Sabine Rech for her advice, kindness and support. Finally, I will be forever grateful to my parents for their encouragement, love and support.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page(s)

GENERAL INTRODUCTION

Many people every year in the United States suffer from debilitating or life-threatening conditions caused by significant skin loss due to injury from burns and chronic ulcers. Loss of skin integrity increases vulnerability to microbial infection even when treated with biomaterial grafts. Development of an *in vitro* wound model system will help enable the study of local events involved in wound healing and the study of drugs introduced to wounds in a controlled way. The purpose of this research is to develop an *in vitro* biomaterial-protected wound infection model that can be used to study microbial activity and antimicrobial treatment through microdialysis.

This thesis consists of this general introduction, two chapters and a literature cited section. This general introduction briefly describes the general topic and organization of the thesis. Chapter I describes the physiology of wounds and treatments, *Staphylococcus aureus*, the microdialysis model system and biomaterial graft composition. Chapter II describes the research in journal format for submission to the Journal of Biomaterials Research Part A as a self-standing report including an abstract, an introduction, materials and methods, results, a discussion and references. The last section gives the literature cited throughout the thesis.

CHAPTER I

WOUNDS, MICRODIALYSIS, *STAPHYLOCOCCUS AUREUS*, BIOMATERIALS AND WOUND MODELS

1.1 Wounds

Wounds provide an optimal environment for bacterial infection. The ability of bacteria to inhabit wounds is dependent on wound type, depth, location, quality and the host immune response. Infected wounds are often unable to heal, traumatic for the patient and increase treatment costs. This is a burden not only to the patient but also to the health care system. The treatments for chronic wounds account for approximately 1-2% of the total health care budget in Western societies as a result of prolonged hospitalization.[1,2]

Wound infections can persist subsequent to conventional treatment. Clinically diagnosed wounds exhibit signs and symptoms of infection including increased pain, increased exudates and the inability to heal.[3]

Wounds can be classified as being acute or chronic. Acute wounds arise from external trauma to the skin such as burns and surgical incisions, and are typically self-limiting or require minimal antibacterial treatment. When properly treated, the wound is able to heal through a progressive process. Wound healing consists of four main phases that include a coagulation phase, an inflammation phase, a proliferative phase and a remodeling phase. In the first phase, coagulation initiates hemostasis by exposing

platelets to fibrin and collagen if blood vessels have been damaged. Once the platelets degranulate, the inflammatory phase is initiated. The release of kinins and prostaglandins increase small vessel permeability leading to edema. Growth factors released include platelet factor 4, platelet derived growth factor, epidermal growth factor, transforming growth factor ß, insulin-like growth factor 1 and vascular endothelial growth factor. Additional serum- and cell-derived extracellular molecules accumulate at the wound site establishing a complex matrix. This matrix enhances cell migration during wound repair, and facilitates the accumulation of cytokines and growth factors released from platelets.[1,3,4,6]

In contrast, chronic wounds are unable to successfully proceed through the phases required for wound healing as a result of compromised conditions. Chronic wounds result from prolonged inflammation. The inflammatory response is composed of humoral and cellular responses regulated by cytokines. Cytokines promote the chemotaxis, proliferation and differentiation of inflammatory wound cells (B-cells and T-cells) and non-inflammatory wound cells such as fibroblast, epithelial and vascular endothelial cells. Persistent inflammation occurs because of elevated cytokine levels. Predisposing conditions to chronic wound formation include leg ulcers, foot ulcers and decubitus ulcers. Other confounding factors such as age, obesity, smoking, nutrition and immunosuppression increase the propensity for chronic wound formation and duration.[1,7]

Introduction of bacteria into wounds may result from environmental or endogenous microflora, and consists of three conditions. The first condition is bacterial

contamination, which is the presence of non-proliferating bacteria within wound tissue. This condition does not impact the healing process. The second condition, colonization, is the presence of multiplying bacteria adhered to the wound without tissue damage. A close association between a patient's normal microflora and bacterial colonization of wounds has been observed. Once colonized, wounds that are hypoxic may be predisposed to a third condition, microbial proliferation.[1,3]

Hypoxia is the result of insufficient blood circulation. Blood circulation aids in providing oxygen, nutrients and immune cells to the injury site, which can reduce bacterial colonization and proliferation. Infection may result if microbial virulence factors overcome the host's immune system. Infection is the presence of multiplying bacteria and subsequent wound exacerbation. Factors that determine wound condition include the degree of tissue trauma, the amount of blood circulation to the wound, the host immune system, bacterial abundance and the expression of microbial virulence factors.[1,3]

There is increasing evidence that bacteria, including *Staphylococcus aureus*, live in biofilm communities in chronic wounds. Biofilms are complex communities of aggregated bacteria embedded in a self-secreted extracellular polysaccharide matrix (EPS) attached to tissue or an inanimate surface. Biofilms form when free-living bacteria attach to a solid surface with subsequent formation of microscopic colonies. The EPS provides a protective barrier for bacteria against host defenses. Another microbial defense is quorum sensing, a communication network between bacteria. This

communication network also allows bacteria to acquire alternative phenotypes resulting in altered expression of virulence factors, a more sessile growth pattern and slower metabolic rates. Bacteria in a biofilm are up to 1500 times more resistant to antibiotic therapy compared to free floating bacteria. This resistance is due to the ability of the quiescent bacteria in biofilms to metabolically inhibit the uptake of antibiotics through mutation or via the acquisition of plasmids or transposons. Antibiotics are dependent on high metabolic rates and active cellular division in order to be effective. A greater study of biofilm formation is needed to understand this protective bacterial barrier to antimicrobial treatments.[3,8,9]

Wound infection management is needed to promote healing and prevent biofilm formation. Biomaterial dressings that promote a moist wound environment aid in minimizing wound infections by increasing neutrophil activity and reducing the amount of necrotic tissue. Occlusive dressings seal wounds and facilitate reestablishment of normal skin flora. Topical antimicrobials including silver, iodine and chlorhexidine minimize microbial density in infected wounds and may promote healing. Infections that spread beyond the localized wound area are best treated with systemic antibiotics. Early evaluation of wound infections may lead to more effective treatments.[3]

Wound infections may be characterized by sampling extracellular fluid. Extracellular fluid consists of plasma exudates, soluble tissue and molecules involved in wound healing that have penetrated the active wound environment. Therefore, extracellular wound fluid samples may accurately represent the biochemical and/or cellular environment of the wound.[2]

1.2 *Staphylococcus aureus*

Staphylococcus aureus is a circular-shaped Gram-positive bacterium usually grouped in grape-like clusters. When grown in blood agar plates *S. aureus* forms round and golden yellow colonies. Pathogenic strains convert fibrinogen to fibrin. *Staphylococcus aureus* frequently colonizes the nasal passage, skin, throat, vagina and perianal area and can cause tissue damage.[10,11,12]

Staphylococcus aureus is one of the major causes of nosocomial infections afflicting half a million patients in the United States. *Staphylococcus aureus* infections include superficial skin lesions, serious skin infections, food poisoning, toxic shock syndrome and urinary tract infection. *Staphylococcus aureus* infections consist of five stages of pathogenesis that include colonization, local infection, sepsis, metastatic infection and toxinosis. Non-proliferating organisms can progress to an infection when *S. aureus* attaches to host cells or tissues. The microorganism also expresses surface proteins that facilitate attachment to host proteins enabling local infections to spread systemically. *Staphylococcus aureus* also secretes toxins associated with diseases such as the pyrogenic toxic antigens that induce toxic shock syndrome, and exfoliative toxins associated with scaled-skin syndrome that cause peeling of the skin.[10,11,12]

Staphylococcus aureus infections are prevalent in those afflicted with diabetes mellitus, patients with intravenous catheters, intravenous drug users and the immunocompromised. *Staphylococcus aureus* has demonstrated antibiotic resistance to many antibiotics including penicillin, methicillin and vancomycin. Antibiotic resistance

in chronic wounds especially is an important health issue. The first two cases of vancomycin-resistant *S. aureus* were isolated from chronic wound patients. Approximately half of leg and foot ulcers from hospitalized patients were infected with methicillin-resistant *S. aureus* (MRSA). A greater percentage of patients who received prior antibiotic treatment had wounds infected with MRSA compared to patients who did not have prior antibiotic treatment. Risks suggested to increase exposure to MRSA include cross-contamination of wounds from the patients themselves, long-term use of antibiotics, prior hospitalization and severity of illness. The spread of *S. aureus* including antibiotic resistant *S. aureus*, may be curtailed by hand washing, wearing disposable gloves and disposable garments.[11,12]

1.3 Microdialysis

Microdialysis is the passive diffusion of molecules through a membrane under the influence of an osmotic gradient. The earliest techniques developed in the 1960's were used to obtain intracerebral fluid and blood plasma with a volume no greater than 1 milliliter (ml). The method was adapted to study concentrations of neurotransmitters and glucose metabolism in brain tissue of animals and humans. Microdialysis was further developed for a wide range of research areas for both *in vitro* and *in vivo* experiments requiring continuous sampling of biological fluids. Evaluation of tissue concentrations of exogenous fluids to test drug penetration and distribution, and sampling for endogenous compounds from the interstitial cell fluid of various tissues in both experimental and clinical settings can also be performed. This technique may be especially useful to

determine amounts of a substance in the tissue environment that may not coincide with levels in the blood.[2,13,14,15,16]

Microdialysis can be utilized to deliver a treatment while simultaneously used to evaluate the effectiveness of treatment in a wound site. Typically, a microdialysis system for *in vivo* analysis consists of a pump, probe and microvial for sample collection. The probe, a thin semipermeable tube (0.15-0.30 millimeters in diameter), allows the diffusion of molecules. Fluid delivered in this way enters the probe and diffuses into the surrounding medium and can deliver treatments or obtain samples.[2,13,14]

Samples collected from the probe are known as the dialysate. The concentration of endogenous or exogenous molecules detected in the dialysate will be a fraction of the true concentration in the environment surrounding the probe known as the periprobe fluid. The perfusate diffusing through the probe will exhibit a different molecular composition compared to the periprobe fluid. Factors that affect sample composition are the diffusion of a measured substance across the probe, the environment surrounding the probe, and the interaction between the substance and the environment. The difference in the concentration of a substance between the dialysate and the periprobe fluid is known as relative recovery. Relative recovery for *in vivo* conditions based on the diffusion of the perfusion fluid across the microdialysis membrane is dependent on temperature, membrane pore size, membrane area and concentration gradient, the composition of the perfusion fluid, flow rate and matrix composition. The size of the molecules relative to the membrane pore size and the length of the tubing affect the relative recovery.

Therefore, if the molecules have a molar mass lower or equal to the membrane pore size, the relative recovery will be greater. Since the membrane length also affects the relative recovery, the longer the microdialysis tubing, and the greater the relative recovery. The concentration and flow rate of the perfusate diffusing across the tubing affects the relative recovery. A slow flow rate causes a greater relative recovery due to the absorption rate of the membrane. Finally, the dialysis material composition is a factor in relative recovery because of the cellular structures, the connectivity of spaces and accumulation of macromolecules. In situations where relative recovery will not reach 100%, the microdialysis probe is calibrated before determining concentrations of substances in the periprobe fluid.[2,13,14,17,18]

Microdialysis probes vary in shape and construction depending on the location of intended insertion. The first probes used were linear in shape consisting of either a hollow-fiber tube or a long-length linear probe. The inception of hollow fiber probes impacted the use of microdialysis in humans *in vivo* enabling continuous sampling of a substance. The advantage with the hollow fiber tube was the small diameter allowing for minimal tissue invasiveness. Probes have varying pore sizes, which allow molecules of different molecular weights to diffuse through the microdialysis membrane. Microdialysis probes are now used in various tissues and organs such as the brain, muscle, heart, lung, skin, gastric mucosa, adipose tissue and selected solid tumors.[2,13]

Important advantages of the microdialysis technique are the ability to measure substances of interest directly from a specific site, delivering drugs directly to the desired

site and retrieving repeat samples for a prolonged period of time with minimal fluid loss. For example, microdialysis was used successfully for monitoring tissue histamine concentrations in human burn wounds of various thicknesses by testing the wound fluid from the burn site instead of analyzing tissue biopsies. Also, the safety of microdialysis has been exhibited in compromised individuals such as neonates and diabetics. The safety of microdialysis in clinical applications has been demonstrated for long-term subcutaneous glucose monitoring (4-16 days) where the prevention of excessive blood loss and discomfort during neonatal intensive care was critical. In diabetic patients, foot ulcers are a serious complication associated with neuropathy, vascular disease or infections. The use of microdialysis has shown promise in analyzing the metabolites in diabetic foot ulcers compared to healthy subcutaneous tissue. Microdialysis perfusion treatment may prevent the unnecessary pain and trauma from amputation.[2,15,18,19]

Microdialysis is a promising technique utilized in sampling a variety of tissues and in evaluating treatments. Microdialysis is minimally invasive, allow for continuous sampling with minimal fluid loss, and is simple and cost effective. Future applications of this technique include studying disease status and progression, and developing new diagnostic and therapeutic approaches.[2,13]

1.4 Biomaterials

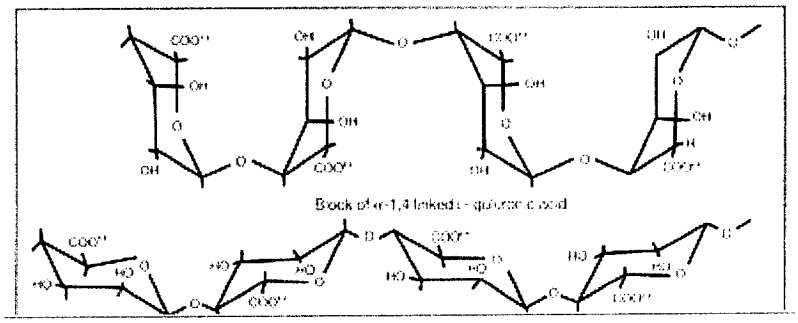
Alginate, extracted from seaweed and kelp, is composed of 1,4-beta-D-mannuronate and alpha-L-guluronate residues covalently linked to form unbranched polymers in different sequences (Fig. 1A). The biomaterial forms a three-dimensional

network when calcium interacts ionically with the guluronic residues. The calcium ions from the calcium alginate are replaced by sodium ions in wound exudates. This ion exchange causes the biomaterial to become a hydrophilic gel. Alginate forms a gel within the wound, thereby maintaining a moist wound environment while absorbing excess wound exudates.[21,22,23,28]

Hydrogel is a three dimensional synthetic gel network composed of hydrophilic polymer chains (Fig. 1B). The biomaterial absorbs wound exudates causing it to swell into the wound area. Simultaneously, the biomaterial maintains a moist wound environment. A cell-line incorporated into the biomaterial enables the study of cell interactions in a three dimensional matrix. This is also known as a cell-seeded biomaterial.[3,24,25,26,29]

Zyderm II is a sterile collagen implant composed of purified bovine-based collagen (65mg/ml in phosphate buffered saline along with 0.3% lidocaine) (Fig. 1C). The biomaterial is used intradermally for correction of facial contour irregularities, and is chemically equivalent to collagen wound dressings. The collagen molecule is a triple-helix structure composed of three intertwined peptide chains that form a matrix between cells. The versatile and naturally occurring biomaterial forms a structured framework aiding in tissue regeneration. Collagen absorbs wound exudates while maintaining a moist wound environment. Collagen is capable of forming a three-dimensional lattice-like structured matrix that allows cells to move between collagen strands, forming a cell-seeded matrix and eventually integrating with the host tissue.[3,27,28]

A



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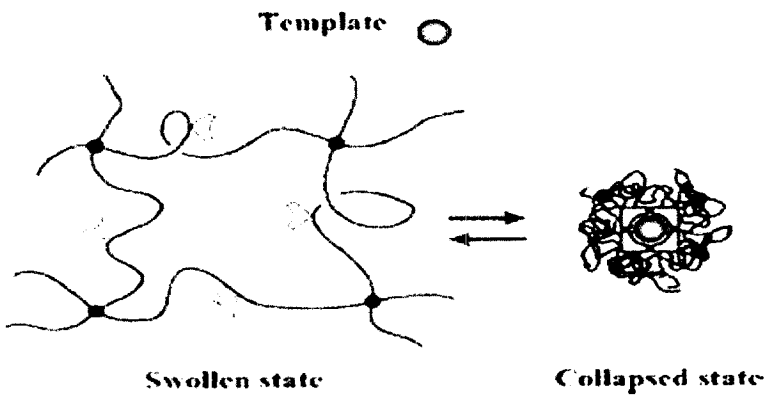

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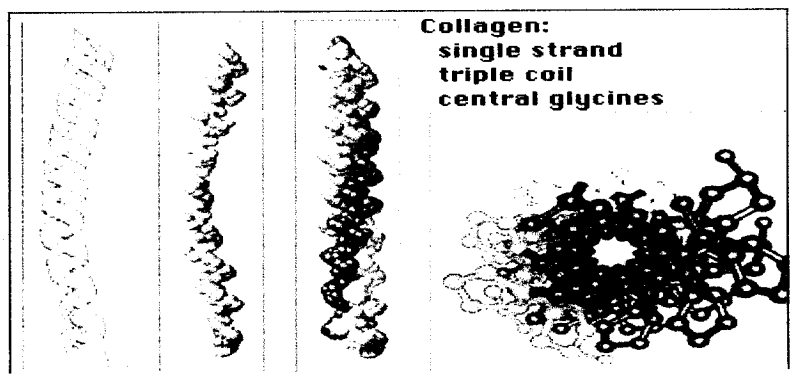

Figure 1. (A) Alginate chemical structure, (B) Hydrogel structure in a non-swollen state and a swollen state when in an aqueous environment, and (C) Collagen chemical structure.

1.5 Wound models: *In Vitro* vs. *In Vivo*

Although the most accurate representation of wound healing is the use of human wounds, the investigation of the wound healing process is dependent on the use of models. Many *in vivo* wound models are affected by the nutritional status of the patient, blood circulation, age, infecting microbe composition and initial wound treatment. Animal models have also been used for *in vivo* wound healing studies, but differences in wound healing between animals and humans are problematic. Difficulties using *in vivo* wound models have led to the development and use of *in vitro* models. *In vitro* wound models exhibit a controlled wound environment. These models can be designed to standard wound dimensions enabling the study of local events involved in wound healing and the study of drugs introduced into the wounds in a controlled fashion. The ability to replicate experimental wound conditions allows for a better understanding of the factors involved in wound healing that can be further studied by *in vivo* analyses.[30]

Wound healing *in vitro* assays have been carried out in tissue culture for years to observe cell behavior, including monitoring the migration and proliferative capacities of different cells under various cell conditions. These assays begin with growing cells to obtain a confluent monolayer. The continuous monolayer is mechanically disrupted by destroying a group of cells to simulate an *in vivo* wound. The wounded area is then microscopically observed over time to determine the rate at which the neighboring cells have filled in or healed the damaged area. The results may be conveyed by obtaining photomicrographs to quantitatively determine the repopulation rate.[31]

Besides mechanical disruption, electrical means have also been used to study the wound and healing process. This method is based on a process to monitor cell behavior referred to as electric cell-substrate impedance sensing (ECIS). In this process, cells are grown on a gold film electrode located on the bottom of a tissue culture well. A counter electrode completes the circuit through standard tissue culture medium. A weak AC signal in the milliampere range at a high frequency is applied to this system. The cells cause changes in this system's impedance. This impedance changes have been used to monitor cell attachment and spreading, cell locomotion and micromotion, and changes in cell morphology in response to physical and biochemical changes, toxicity measurement of compounds, signal transduction and the invasive activities of transformed cells in culture.[31]

In vitro models currently include engineered epidermal equivalents consisting of normal, human-derived keratinocytes that model the human epidermis. Also demonstrated in these engineered epidermal equivalents is the stratum corneum that serves as a protective skin barrier. These epidermal equivalents are used for testing skin irritation of chemicals and also to study the epidermal responses to mechanical stimuli such as the formation of pressure ulcers. Wounded dermal equivalent models are valuable for studying the role of fibroblasts during wound remodeling.[32,33]

In vitro co-culture models are used to investigate the influence of keratinocytes on fibroblast proliferation and fibronectin synthesis. These models are composed of

epithelial sheets cultured above fibroblast monolayers physically separated by grids. This model allows for the complete diffusion of cytokines between keratinocytes and fibroblasts.[34]

Data from these models have indicated that keratinocytes produce soluble factors that down-regulate fibroblast proliferation. Cytokines and growth factors produced by keratinocytes include interleukin (IL-1), -6, -7, -8, -10, -12, -15, -18 and -20, interferon-α, -β and-γ, platelet-derived growth factor, transforming growth factor-β, and tumor necrosis factor-α. After injury, IL-1 and IL-6 production by keratinocytes is increased. IL-6 production also occurs in fibroblasts and IL-1 production by fibroblasts is increased in response to IL-1. IL-1 and -6 secreted by keratinocytes at the wound edge stimulate the production of keratinocyte growth factor by fibroblasts, which promote keratinocyte proliferation. The role of keratinocytes in cytokine production is being increasingly recognized and may be critical in the development of future wound therapies.[34]

In vitro wound model systems permit replication of experimental wound conditions and enhance the investigation of factors involved in wound healing.

CHAPTER II

DEVELOPMENT OF AN *IN VITRO* BIOMATERIAL-PROTECTED WOUND INFECTION MODEL TO STUDY MICROBIAL ACTIVITY AND ANTIMICROBIAL TREATMENT THROUGH MICRODIALYSIS

Journal of Biomedical Materials Research
Part A
Giannini et al.: Development of an *In Vitro* Biomaterial-Protected Wound Infection Model to Study Microbial Activity and Antimicrobial Treatment through Microdialysis

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ABSTRACT

Skin injuries provide a favorable environment for microbial infection if left untreated. This is problematic especially in nosocomial situations having a high prevalence of *Staphylococcus aureus* that can cause suppuration of wounds, systemic disease and toxic shock. A wound model provides an understanding about the interactions between microbial activity, host tissue, therapeutic treatments and wound biomaterials.

An *in vitro* wound model was developed using Sykes-Moore chambers composed of one of three biomaterials used for wound treatment (1% Alginate (ALG), dialyzed "Hyfil" hydrogel (DHG), "Zyderm II" collagen (CGN)) and seeded with fibroblasts. The chambers were inoculated with *S. aureus* and half were later treated with antibiotics through *in situ* microdialysis tubing. The chambers were monitored by obtaining fluid samples and biomaterial samples at specific time intervals (0, 2, 8 and 24 h) and were analyzed for (1) *S. aureus* protein A (SPA) concentration, (2) viable *S. aureus* numbers and (3) fibroblast numbers and viability. Chambers containing each biomaterial with and without antibiotics were compared to controls. There was an inverse relationship between post-infection *S. aureus* numbers and fibroblast viability. *Staphylococcus aureus* numbers were usually consistent with SPA concentration, but may have been potentially confounded due to SPA interactions with the biomaterials.

KEY WORDS Wound model system, Biomaterials, *Staphylococcus aureus*

INTRODUCTION

Wounds provide an optimal environment for bacterial infection. Infected wounds are often unable to heal, traumatic for the patient and result in increased treatment costs. This is a burden to the patient and to the health care system. Wounds can be classified as being acute or chronic. Acute wounds arise from external trauma to the skin and are typically self-limiting or require minimal antibacterial treatment. In contrast, chronic wounds are unable to successfully heal as a result of compromised conditions such as the introduction of bacteria. Infection may occur if microbial virulence factors overcome the host's immune system and may lead to the formation of biofilms. Biofilms are complex communities of aggregated bacteria embedded in a self-secreted extracellular polysaccharide matrix attached to tissue. Treatments for chronic wounds such as these accounts for approximately 1-2% of the total health care budget in Western societies as a result of prolonged hospitalization.[1,2,3,8,9]

Loss of skin integrity increases vulnerability to microbial infection and biofilm formation. These wound infections may be minimized with the aid of biomaterial dressings that promote a moist wound environment. Examples of biomaterial dressings include occlusive dressings that seal wounds and help reestablish normal skin flora. Additional wound treatments are topical antimicrobials including silver, iodine and chlorhexidine that minimize microbial density in infected wounds. Early evaluation of wound infections may lead to more effective treatments.[3]

Wound infections may be defined by sampling extracellular fluid. Extracellular

fluid consists of plasma exudates, soluble tissue and molecules involved in wound healing that have penetrated the active wound environment. Therefore, extracellular wound fluid samples may precisely represent the biochemical and cellular environment of the wound.[2]

Microdialysis, a technique developed in the 1960's, is currently used to continuously sample biological fluids such as extracellular wound fluid. Evaluation of these fluids may be especially useful to determine amounts of a substance, and sampling for endogenous compounds wound tissue environment. Microdialysis can be utilized to deliver a treatment to a desired site while simultaneously used to evaluate the effectiveness of treatment in a wound site with minimal fluid loss. Important advantages of the microdialysis technique are the ability to measure substances non-invasively, allowing for continuous sampling with minimal fluid loss, and is simple and cost effective.[2,13,14,16]

The investigation of the wound healing process is dependent on the use of models. Many *in vivo* wound models are affected by the nutritional status of the patient, blood circulation, age, infecting microbe composition and initial wound treatment. Difficulties using *in vivo* wound models have led to the development and use of *in vitro* models. *In vitro* wound models exhibit a controlled wound environment. These models can be designed to standard wound dimensions enabling the study of local events involved in wound healing and the study of drugs introduced into the wounds in a controlled fashion. The ability to replicated experimental wound conditions allows for a better understanding

of the factors involved in wound healing that can be further studied by *in vivo* analyses.[30] The purpose of this research is to develop an *in vitro* biomaterial-protected wound infection model that can be used to study microbial activity and antimicrobial treatment through microdialysis.

MATERIALS AND METHODS

Fibroblast (L-929) cells

Mus Musculus L-929 fibroblast cells (American Tissue Cell Culture, ATCC: CCL-1) were cultured to >90% confluent in T75 cm^2 cell culture flasks, containing complete Dulbecco's Modified Eagle's Medium (cDMEM) without antibiotics supplemented with 10% fetal bovine serum (Hyclone), 1% HyQ L-glutamine (Hyclone) and 0.1% non-essential amino acids (Gibco). Approximately $5x10^5$ cells/ml were detached using 1x trypsin-EDTA, washed, and resuspended in cDMEM without antibiotics. Cell concentration and viability were determined microscopically using 0.4% Trypan blue at a 1:2 dilution in a hemocytometer. Cell suspensions with ≥80% viability were used.

Biomaterials

Three biomaterials were evaluated (1) 1% alginate (ALG) (Sigma); (2) dialyzed hydrogel (DHG) "Hyfil" (B. Braun Medical Inc.) and (3) collagen (CGN) "Zyderm II" (Inamed Co.). Biomaterials were mixed at a low shear rate with fibroblast cell suspensions at the start of the experiment. ALG was prepared by dissolving low

viscosity alginate in warmed phosphate buffered saline without calcium and magnesium at a 1:100 ratio until well dispersed. DHG was prepared by injecting approximately 3 ml of undialyzed hydrogel into six inch pieces of autoclaved dialysis membrane. The sterile DHG filled membranes were aseptically dialyzed using Spectrapor membrane tubing (molecular weight cutoff, 6,000 to 8,000; Spectrum Medical Industries, Inc) in DMEM without additives for two days at room temperature. CGN at 65 mg/ml was used as prepared by the manufacturer (Inamed Co.).

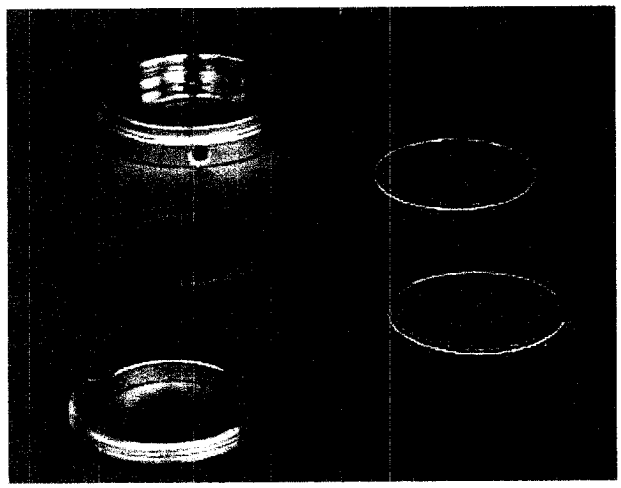
Staphylococcus aureus

Staphylococcus aureus (ATCC: 25923) was incubated for 18 h at 37°C in Tryptic Soy Broth, and diluted to $5x10^7$ CFU/ml for use. Viable *S. aureus* numbers were determined by standard plate count using Tryptic Soy Agar plates (TSA).[11]

Sykes-Moore chambers/Experimental Conditions

Sykes-Moore chambers having a 2.83 cm^2 viewing area and a maximum volume of 0.75 ml were used to construct the *in vitro* wound model (Fig. 1).[35,36]

A



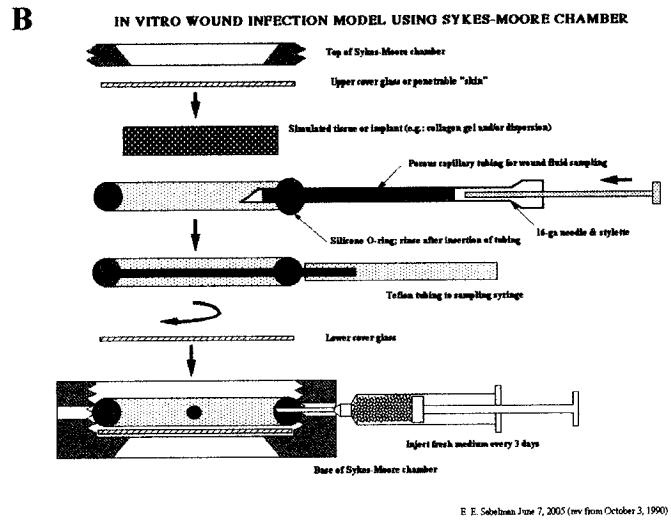


Figure 1. Sykes-Moore chamber components (A), Sykes-Moore chamber set-up, Sykes-Moore chamber set-up (B).

Duplicate Sykes-Moore chambers containing fibroblast-seeded ALG, DHG or CGN from 7 averaged experiments were compared. Each chamber consisted of a top half and a bottom half. Chambers were assembled with round plastic coverslips placed on the inside of the two chamber halves and separated by silicone O-rings. The coverslip placed on the inside end of the top half of the chamber was punctured and overlaid with Tegaderm (3M) to simulate a wound environment. Tegaderm is a simulated skin dressing that allows moisture vapor and oxygen exchange while providing a moist wound environment for enhanced healing. Between the silicone O-rings, hollow fiber microdialysis tubing was looped inside, and the two ends of the tubing extended outside of the chamber. A 1/2 inch 30 gauge needle was inserted in each end of the tubing and sealed with silicone glue. The needles allowed for fluid sampling and introduction of cDMEM. This

assembly was autoclaved prior to the incorporation of the cell-seeded biomaterials (ALG, DHG and CGN). Approximately, 0.65 ml of biomaterial was seeded with approximately $1x10^5$ fibroblast cells in 0.35 ml cDMEM at a 2:1 ratio (v/v). Once the chambers were autoclaved, approximately 0.50 ml of each fibroblast-seeded biomaterial was evenly dispersed inside the chambers and the two halves of the chambers were sealed (Fig. 2). Also, duplicate chambers of each biomaterial were assembled and incubated overnight at 37°C, 96-7% humidity and 4.9% CO_2 to ensure cell attachment to the biomaterials.

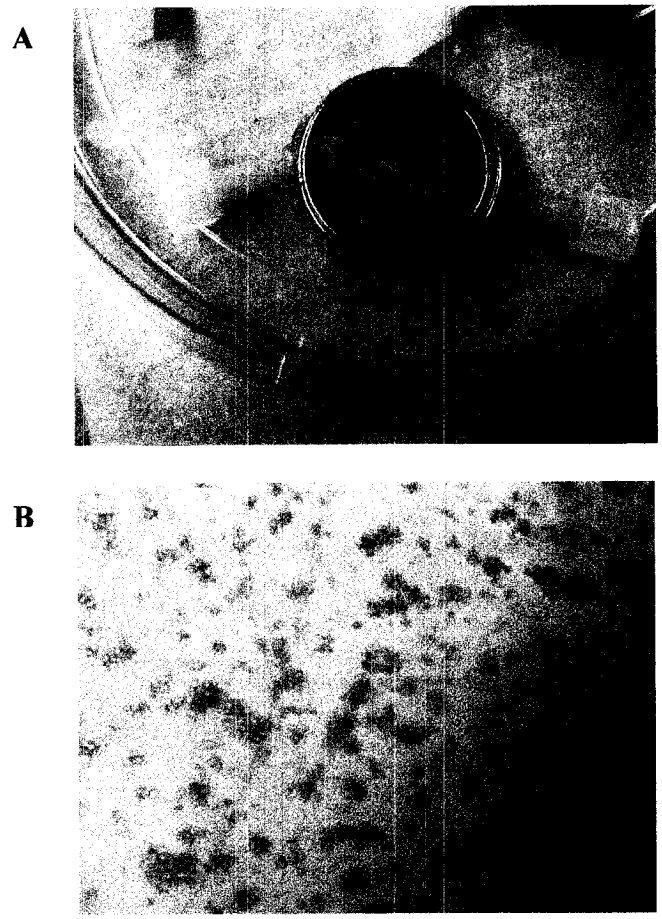


Figure 2. A digital picture of an assembled Sykes-Moore chamber (A), a photomicrograph (10X) of a DHG chamber seeded with a fibroblast cell line (L-929) (B).

Test and control chambers containing ALG, DHG or CGN were sampled at 0, 2, 8 and 24 h. Control chambers included (1) cDMEM with and without 1x antibiotics (BioWhittaker: 100x Penicillin/Streptomycin Amphotericin B), (2) fibroblast cells with and without antibiotics, (3) fibroblast cells and *S. aureus* with and without antibiotics, (4) *Staphylococcus aureus* with and without antibiotics, (5) fibroblast cells with and without antibiotics, (6) each biomaterial and cDMEM with and without antibiotics and (7) each biomaterial with fibroblast cells with and without antibiotics.

Once the fibroblast cells attached to the biomaterials, the chambers were inoculated with an *S. aureus* suspension ($1x10^4$ CFU in 0.05 ml) in a designated area of the chamber through the Tegaderm dressing. The chambers were incubated at 37°C, 96-7% humidity and 4.9% CO_2.

The chambers were monitored by obtaining microdialysis fluid samples (0.3-0.5 ml) from the tubing and by obtaining biomaterial samples from chambers opened at each time (0, 2, 8 and 24 h). When microdialysis samples were taken, appropriate fluid medium (cDMEM with or without antibiotics) of equal volume was simultaneously introduced into the chambers. Samples were analyzed for (1) *Staphylococcus aureus* SPA, (2) *S. aureus* numbers and (3) fibroblast numbers and viability.

Protein A concentration

Staphylococcus aureus SPA concentration was derived by ELISA.[37] Briefly, microwell plates were coated with 1x rabbit IgG (100 μl/well) (Sigma) and incubated overnight at 20° C. The plates were washed once with 0.05% Tween 20 and triplicate wells of Cowen strain SPA standards (0 ng/ml-250 ng/ml), or microdialysis samples (100

μl/well) were added and incubated for 1 h at 37°C. Then the plates were washed once with 0.05% Tween 20 and a 1:20,000 dilution of protein A-alkaline phosphatase (Sigma) (100 μl/well) was added to the appropriate wells followed by another 1 h 37° C incubation. After washing three times with 0.05% Tween 20, p-nitrophenyl phosphate (PNPP) alkaline phosphatase substrate (Sigma) (100 μl/well) was added followed by another 37°C incubation for 1 h. Plates were immediately read by a microplate autoreader (Bio-Tek EL311SX Instruments, Inc.) at 405nm. Data from the SPA standards were used to derive a standard curve. The SPA standards aborbances (y-axis) were plotted against the known concentration of SPA standards (x-axis) using Microsoft Excel. From these data a linear regression line and correlation value (R^2) were determined. The linear regression line equation was used to determine the concentration of SPA in the microdialysis samples. SPA concentrations from ALG and DHG chambers with and without antibiotics were averaged from seven repeated experiments. The SPA concentrations from CGN chambers with and without antibiotics were averaged from two repeated experiments.

RESULTS

SPA concentration

ELISA results for SPA standards indicated a linear dynamic range from 10-100 ng/ml (Fig. 3).[38]

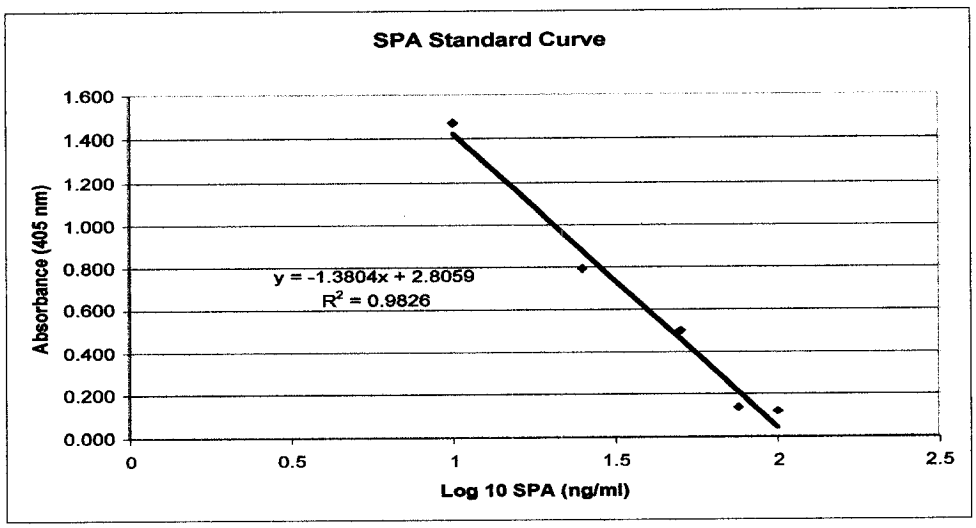


Fig. 3 ELISA results for SPA standards showing a linear dynamic range 10-100 ng/ml.

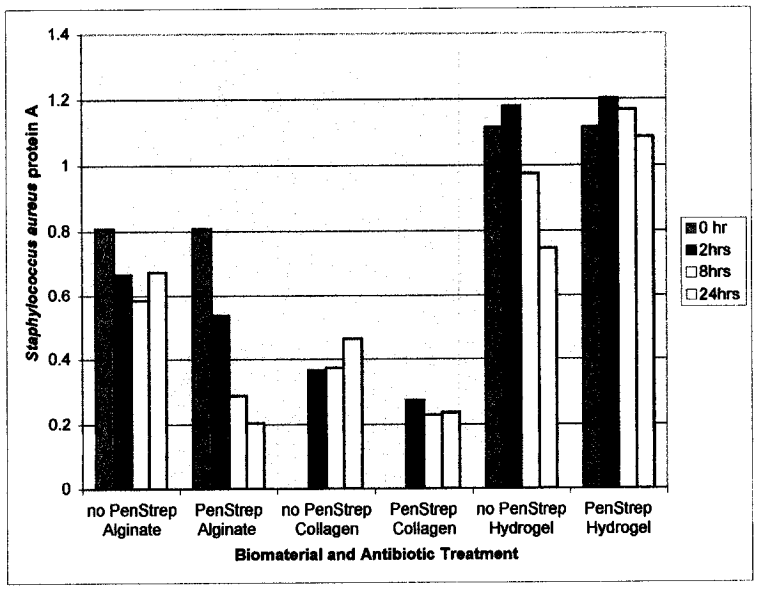
SPA was detected at nanogram concentrations (ng/ml) from microdialysis samples obtained from chambers composed of each of the three biomaterials (Fig. 4A). Preliminary experiments adding known amounts of SPA to control Sykes-Moore chambers confirmed 85% SPA detection via microdialysis sampling (data not shown).

All three biomaterials without antibiotics showed a relatively constant SPA concentration throughout the experimental observation period (Fig. 4A). All three biomaterials with antibiotics showed an overall reduced detection of SPA. Control chambers confirmed that either fibroblast cells or antibiotics alone did not affect SPA concentrations in the microdialysis samples (data not shown).

Staphylococcus aureus viability

Chambers containing all three biomaterials were either treated or not treated with antibiotics through the microdialysis tubing beginning after 2 h of *S. aureus* infection and until experimental termination. The TSA plate results from all three biomaterial samples obtained from those chambers not treated with antibiotics resulted in a continual increase in *S. aureus* numbers (Fig. 4B). In contrast, chambers treated with antibiotics resulted in a steep decrease in *S. aureus* numbers. Control chambers resulted in no *S. aureus* detected on the TSA plates (data not shown).

A



B

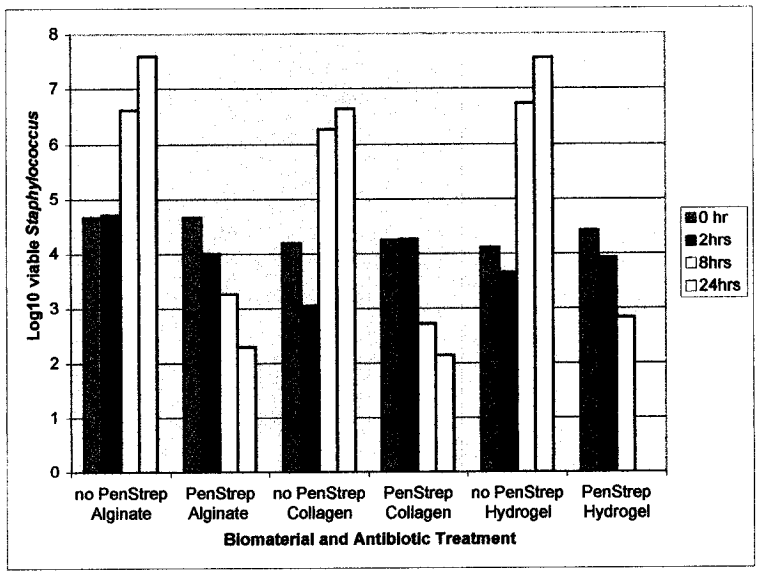
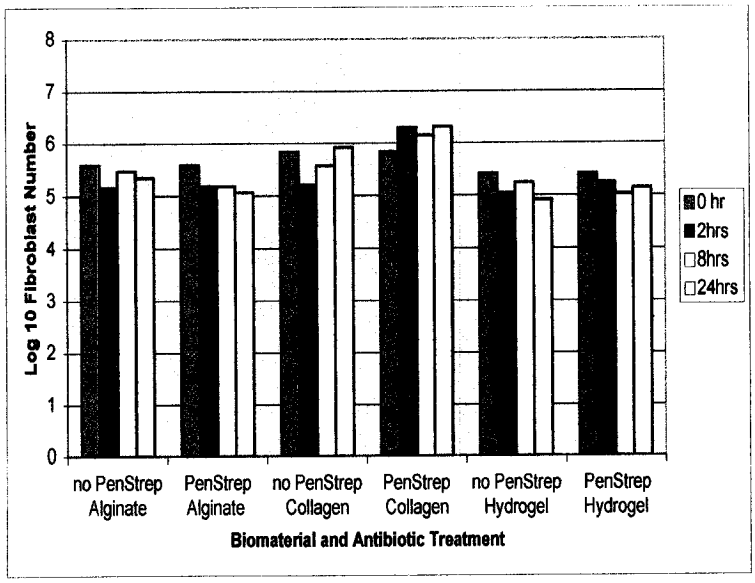


Figure 4. *Staphylococcus aureus* protein A (SPA) concentration (ng/ml) (0 h not done for CGN) (A) and numbers (B) in Sykes-Moore chambers containing fibroblast cells and wound dressing biomaterials (1% alginate, dialyzed hydrogel, collagen) with and without antibiotics at 0, 2, 8 and 24 h after inoculation.

Fibroblast Numbers and Viability

Total (viable and nonviable) fibroblast numbers in all three biomaterial chambers remained relatively unchanged after *S. aureus* inoculation (Fig. 5A). Fibroblast percent viability for all biomaterial chambers continually declined throughout experimental observation but the decline was not as marked in chambers containing antibiotics (Fig. 5B). Fibroblast percent viability in DHG controls declined moderately during the experimental observation period regardless of antibiotic presence (data not shown). ALG and CGN controls maintained relatively constant fibroblast percent viability throughout experimental observation (data not shown).

A



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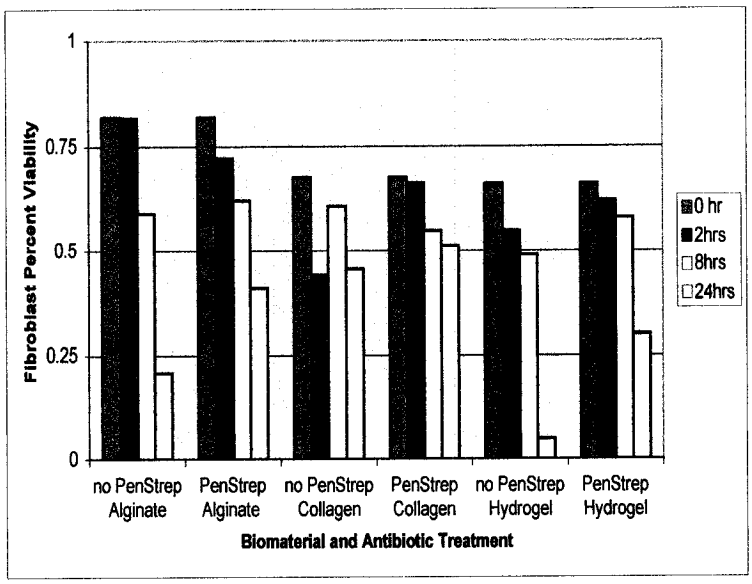

Figure 5. Fibroblast numbers (A) and viability (B) in Sykes-Moore chambers containing fibroblast cells and wound dressing biomaterials (1% alginate, dialyzed hydrogel, collagen) with and without antibiotics at 0, 2, 8 and 24 h after inoculation.

DISCUSSION AND CONCLUSIONS

Although the most accurate representation of wound healing is the use of human wounds, the investigation of the wound healing process is dependent on the use of models. Many *in vivo* wound models are affected by the nutritional status of the patient, blood circulation, age, infecting microbe composition and initial wound treatment. Animal models have also been used for *in vivo* wound healing studies, but differences in wound healing between animals and humans are problematic. Difficulties using *in vivo* wound models have led to the development and use of *in vitro* models. *In vitro* wound models permit the study of a controlled wound environment. These models can be designed to standard wound dimensions enabling the study of local events involved in wound healing, characterize infection processes, and enable microbial identity, viable numbers and expression of virulence factors. Also, models enable continuous monitoring and the study of drugs introduced into the wounds in a controlled fashion via microdialysis.[30]

Microdialysis was developed for a wide range of research areas for both *in vitro* and *in vivo* experiments requiring continuous sampling of biological fluids. This technique may be especially useful to determine amounts of compounds in tissues that may not coincide with levels in the blood. Important advantages of the microdialysis technique are the ability to measure substances of interest directly from a specific site, delivering drugs directly to the desired site and retrieving samples over time with minimal fluid loss. Microdialysis is a promising technique utilized in sampling a variety

of tissues and in evaluating treatments. Microdialysis is minimally invasive, allows for continuous sampling with minimal fluid loss, and is simple and cost effective. The microdialysis technique will enable the study of disease status and progression, and development of new diagnostic and therapeutic approaches to monitor and treat wounds.[2,13,14,16]

In this study, an *in vitro* wound model system was designed and used to evaluate microbial activity and cellular responses in the presence of three biomaterials (1% alginate, dialyzed hydrogel, collagen) seeded with fibroblast cells. The microdialysis technique enabled introduction of antibiotics and model wound sampling. This wound model system incorporated one microorganism (*S. aureus*), one cell line (L-929 fibroblast cells), one 1x antibiotic treatment (100x Penicillin/Streptomycin Amphotericin B), and one virulence factor (SPA). The results indicated that this wound model system enabled the evaluation of fibroblast cell numbers and viability, as well as *S. aureus* numbers and a virulence factor (SPA) concentration. Observations from this study showed the effect of microdialysis-introduced antibiotics on *S. aureus* numbers.

Staphylococcus aureus numbers declined in the presence of antibiotics at the dosage tested in chambers containing all biomaterials while fibroblast numbers were largely unchanged. In contrast, fibroblast viability declined at a similar rate over time in all chambers containing *S. aureus* regardless of the presence of antibiotics, when compared to controls. This may have been due to the continual presence of *S. aureus* metabolic by-products and toxins, or competition for nutrients. The finding that in

control chambers SPA remained constant after antibiotic treatment indicated the absence of a mechanism for binding or clearing SPA despite reduced *S. aureus* numbers.

The low SPA concentrations detected in the CGN chambers compared to ALG and DHG chambers may be due to SPA binding. *Staphylococcus aureus* binds in a specific and saturable manner to various extracellular matrices such as CGN, and may enable the bacterium to bind tissue proteins more effectively. This can be a significant factor in wound pathogenesis. The inhibition of CGN binding was noted when *S. aureus* was treated with trypsin, revealing that the binding site has a protein component. The development of agents that competitively adhere to CGN binding sites may reduce the *S. aureus*-CGN bond and aid resolution of infection. The wound model system described here may be useful in studying the interactions between microorganisms and their virulence factors within cell-seeded biomaterials.[39]

The wound model system can also be further developed to study microbial interactions, expression of virulence factors and development of antibiotic resistance within a wound. Evaluation of host responses such as inflammatory products, cytokines and local immune components can also be made. Novel wound therapies such as bacteriophage therapy for antibiotic resistant infections can be studied using this model system.[40,41] In conclusion, this *in vitro* wound model system can be expanded to evaluate polymicrobial interactions and virulence factors, as well as a variety of host responses. Knowledge gained through *in vitro* wound models, such as the one described in this study can accelerate development of better methods to monitor and treat wounds.

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We give thanks to San Jose State University and Veterans Affairs Palo Alto Health Care System for graciously offering additional materials for this work. This research was funded in part by the Albert and Dorothy Ellis Research Fellowship awarded to from the San Jose State University Foundation awarded to Gina T. Giannini.

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