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The Development of a Novel Multi-dimensional Product for Wound Healing Applications

Necrisha Roach
Virginia Commonwealth University

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THE DEVELOPMENT OF A NOVEL MULTI-DIMENSIONAL PRODUCT
FOR POTENTIAL WOUND HEALING APPLICATIONS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University.

by

NECRISHA N.C. ROACH
Bachelor of Business Administration, Finance and Investments
Baruch College, City University of New York, June, 2006

Director: DORNE R. YAGER
ASSOCIATE PROFESSOR & DIRECTOR OF RESEARCH,
DEPARTMENT OF SURGERY
DIVISION OF PLASTIC SURGERY

Virginia Commonwealth University
Richmond, Virginia
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<th>Definition</th>
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<tbody>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>inhibitory concentration - 50</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>mcg</td>
<td>microgram</td>
</tr>
<tr>
<td>MIC$_{50}$</td>
<td>minimal inhibitory concentration -50</td>
</tr>
<tr>
<td>MMP</td>
<td>metalloproteinases</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>MU</td>
<td>milliunits</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate-oxidase</td>
</tr>
<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PSS</td>
<td>polystyrene sulfonate</td>
</tr>
</tbody>
</table>
TGF-β  transforming growth factor
Abstract

THE DEVELOPMENT OF A NOVEL MULTI-DIMENSIONAL PRODUCT FOR POTENTIAL WOUND HEALING APPLICATIONS

By Necrisha N.C. Roach, B.B.A.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2010.

Major Director: Dorne R. Yager
Associate Professor and Director of Research, Department of Surgery, Division of Plastic Surgery

A characteristic feature of chronic wounds is a prolonged inflammatory response as well as susceptibility to infection. Studies have shown that during the inflammatory response, there is a significant increase in the levels of neutrophil-derived enzymes. The purpose of this work was to determine whether the anionic macromolecule polystyrene sulfonate (PSS) and five of its salt forms, namely PSS-calcium, PSS-chlorhexidine, PSS-doxycycline, PSS-glutathione and PSS-silver are able to inhibit the activity of three of the enzymes whose levels are elevated in chronic wounds: elastase, cathepsin G and myeloperoxidase.

In addition to the enzyme inhibition study, the various formulations’ antimicrobial properties were analyzed by evaluating their ability to inhibit the growth of three common clinical isolates: Staphylococcus aureus, Pseudomonas aeruginosa and Acinetobacter baumannii. It is worthy to note that the structure of PSS makes it a very flexible platform to which other molecules can be added in order to address a variety of “targets” as well as tailor quantitative strength.
The results from this project showed that purified PSS and the various salt derivatives were able to inhibit elastase and cathepsin G activity. In addition, three of the therapeutic cations attached to PSS: silver, doxycycline and chlorhexidine retained their intrinsic antimicrobial properties without having an adverse effect on healthy tissue. In summary, this study demonstrated that PSS possessed an intrinsic ability to inhibit a number of proteases and that it could also be used as a delivery vehicle for other compounds with potential therapeutic value.
Introduction

Phases of Wound Healing

The skin is the largest and one of the most complex organs in the body and damage to it is an inevitable occurrence. Therefore, it is imperative that the skin has evolved mechanisms that allow it to repair itself and restore functionality to the injured area in a timely manner. Although some of the key components of wound healing have been worked out, there is still a great deal to be learnt about this inherently complex process. To that end, the management and treatment of complex wounds is often viewed as an art and not necessarily a science since healthcare professionals often have to rely on their past experiences and intuition in order to plan the right approach to dealing with a specific wound. Prior work has shown that this restorative phenomena occurs in four overlapping stages that can take several days or years to complete, depending on the severity and complexity of the injury.

Hemostasis

The first phase in wound healing is hemostasis which is then followed by inflammation, tissue formation and remodeling. During wounding, in addition to the dermis, the blood vessels and tissue underlying the outermost layer of the skin are also damaged. This results in bleeding at the site that needs to be controlled and ultimately stopped. In order to achieve this, there is instantaneous and transient vasoconstriction of the blood vessels at the site of the injury followed by the formation of a plug in the form a clot. Clots are formed because blood platelets that are usually contained within the lumen of the blood vessels become exposed to tissue in the extracellular matrix causing them to aggregate at the site of hemorrhage (Esmon 1993). After the
bleeding has been controlled, the blood vessels vasodilate and develop gaps in the capillaries (Hynes 1992). The presence of these gaps allow plasma as well as the migrating leukocytes to enter the extravascular space causing edema, a characteristic feature of this stage in the healing process (McLean et al. 1964).

The clot is a multifunctional component because it not only stops the flow of blood, but upon the completion of its formation fibrin, an end product of coagulation is released. Fibrin lays down the foundational matrix in the wound which is later covered with vitronectin and fibronectin. Fibronectin is produced by surrounding fibroblast and epithelial cells and is integral in providing the framework to which migrating cells can attach to so that they can make their way to the wound (Clark et al. 1985; Grinnell 1984). In addition, degranulation of the platelets in the clot releases cytokines and a wide variety of growth factors including PDGF and TGF-β that recruit other cells such as neutrophils and macrophages to the wound site thereby initiating the second phase of healing— inflammation (Clark 2001).

**Inflammation**

Inflammation can be defined as “the breakdown of preexisting tissue scaffolding and cleanup of cellular, extracellular, and pathogen debris.” (Van Der Veen et al. 2009). Neutrophilic migration help establish the infamous hallmarks of inflammation – erythema, pain, swelling and heat. Under normal conditions, leukocytes are not present in tissue, however, immediately after trauma, the endothelial cells at the site begin to express P-selectin and E-selectin which facilitates the tethering and subsequent adhesion of local leukocytes specifically neutrophils. Once they have adhered to the endothelial cell surface via their respective receptors, they are primed making it possible for them to cross the endothelial barrier. When in the tissue, they can
bind to the fibrin-fibronectin-vectronectin matrix using their membrane receptors in order to arrive at the site of injury (Hsieh et al. 1983; Hynes 1992).

Neutrophils, which are also known as polymorphonuclear leukocytes (PMN), are the most abundant circulating leukocyte. The concentration of neutrophils in the blood of healthy humans ranges from 3 to 6 million cells per ml but can increase to 15–40 million per ml in individuals with an infection (Li et al., 2002). Neutrophils constitute the "first line of defense" against infectious agents or "nonself" substances that penetrate the body's physical barriers. Once an inflammatory response is initiated, neutrophils are the first cells to be recruited to sites of infection or injury. Their targets include bacteria, fungi, protozoa, viruses, virally infected cells, tumour cells, and devitalized tissue. They develop in the bone marrow. Mature neutrophils contain cytoplasmic granules (primary or azurophil and secondary or specific) and a lobulated chromatin-dense nucleus with no nucleolus.

Neutrophils are professional phagocytes, they utilize a combination of degradative enzymes that are contained in their granules and a robust respiratory burst to neutralize ingested pathogens. During phagocytosis, cytosolic granules (lysosomes) fuse with the invaginating plasma membrane (around the engulfing microorganism) to form a phagolysosome into which they release their contents, thereby creating a highly toxic microenvironment. It is at this step that the contents of the granules and products of the respiratory burst come together to act on ingested microbes. This degranulation normally prevents release of the toxic components into the extracellular milieu. However, some target may be too large to be fully phagocytosed or they avoid engulfment, resulting in frustrated phagocytosis in which no phagosome is formed. These may be killed extracellularly. However, tissue damage occurs when neutrophil microbicidal
products are released extracellularly to such an extent that host defences (antioxidant and antiprotease screens) in the immediate vicinity are overwhelmed.

The reactive oxygen species produced as a result of the activated NADPH complex’s respiratory burst aid in the killing of microbes. The initial product of the respiratory burst is the relatively unreactive molecule superoxide (O₂). However, this molecule can be utilized by a number of pathways to generate far more reactive reactive oxygen species including hydrogen peroxide (by dismutation), hydroxyl radical (OH), singlet oxygen, and peroxynitrite (ONOO⁻) by reacting with nitric oxide. In neutrophils, using hydrogen peroxide as a substrate, myeloperoxidase can produce hypochlorous acid (Nathan 2002).

After phagocytizing the damaged tissue and the microbes, neutrophils undergo apoptosis and are in turn phagocytized by macrophages that have begun to invade the site. Macrophages appear at the site 48 to 96 hours after the initial injury and like the neutrophils, they contribute to the wound’s debridement, phagocytize the apoptotic neutrophils, continue pathogen killing and release growth factors such as PDGF, TGF-β and FGF which serve to regulate the wound healing process (Adams et al. 1984; Goldman 2004). Activation of the macrophages is an important step in ensuring the wound progresses to the next stage and is not stalled in the inflammatory stage. Prior studies have shown that if these growth factors are not present or their expression is reduced, the healing process is significantly impaired and many of the wounds are unable to progress through the other phases of healing and subsequently become acute (Cooper et al. 1994).

It is worthy to note that although the macrophages are a key component in destroying the microbes that the neutrophils were unable to destroy, the mechanism via which they fulfill this task is slightly different. Although there is some controversy regarding the exact mechanism
involved, the vast majority of researchers seem to agree on the fact that the macrophages involved make a large amount of nitric oxide. The nitric oxide that is synthesized then reacts with peroxide ion oxygen radicals that give rise to an even more potent toxin in the form of peroxynitrite and hydroxyl radicals (Beckman et al. 1990). This lethal combination is usually sufficient to rid the wound of any bacteria that was not previously cleared by neutrophilic activity.

**Tissue Formation and Remodeling**

The inflammatory process does not continue indefinitely but it is regulated by proteins such as PGE2 that shuts down the inflammatory response thereby promoting the proliferative or tissue forming phase (Nathan 2002). In the restorative phase, keratinocytes and mesenchymal cells proliferate, migrate and differentiate so that the provisional matrix formed by the fibrin-vectronectin-fibronectin during hemostasis can be replaced by a more permanent scaffold in the form of fibrillar collagens and proteoglycans thereby restoring the integrity of the injured region (Nwomeh 1998). Furthermore, epithelial cells proliferate in an attempt to repair the damaged blood vessels, re-establish the vascular network, prevent fluid loss and further bacterial infection (Broughton 2006). The epithelial cells are able to form new vessels after they have been stimulated by the growth factor VEGF which is secreted primarily by keratinocytes. It is worthy to note that macrophages, fibroblasts, platelets and neighboring endothelial cells are also able to contribute to angiogenesis (Gospodarowicz et al.1989; Folkman et al. 1992).

Another pair of important growth factors involved in this phase of healing is PDGF and EGF (Lawrence 1998). They are secreted from platelets, macrophages and are responsible for recruiting fibroblasts to the wound site. Once the fibroblasts arrive, the presence of the
aforementioned growth factors causes them to synthesize a temporary matrix composed of collagen III, glycosaminoglycans and fibronectin. In addition to the re-establishing the connective tissue, the integrity of the epithelium has to be restored so that the individual can be protected from further invasion of microbes and other foreign substances. Epithelialization is also closely regulated by growth factors namely growth factor-α, epidermal growth factor and keratinocyte growth factor (Nanney et al. 1996). These growth factors are responsible for activating the epidermal cells that would allow them to migrate to the dermis.

At this point, the injured region has regained most of its normalcy and the remodeling phase begins in an attempt to fine tune the structure of the extracellular matrix and optimize the strength of the skin. Even in optimal conditions, the strength of the scar never reaches that of normal skin, however approximately 80% of the skin’s strength is regained after 6 months (Grinnel 1984).

**Chronic Wounds**

Although the wound healing process is a well orchestrated series of mechanisms that involves a host of proteases, growth factors and enzymes, whose coordinated appearance at the site of injury restores functionality and integrity, if it is not allowed to progress through the various stages in a timely manner the healing process can be delayed indefinitely. This delayed healing predisposes the wound to becoming acute and eventually chronic. Previous studies have shown that one of the hallmarks of chronic wounds is elevated levels of neutrophils which ultimately lead to an increase in proteolytic and degradative enzymes that arrest the wound in the inflammatory phase (Yager et al. 1999). There have been several neutrophilic proteases and
enzymes that have been identified as key players in perpetuating the inflammatory phase specifically elastase, cathepsin G, myeloperoxidase and a host of MMPs.

Elastase is one of the many cationic serine proteases whose level is frequently elevated in chronic wounds and is responsible for the degrading peptide growth factors and fibronectin: one of the proteins involved in the re-construction of the extracellular matrix. Cathepsin G, another serine protease has also been shown to destroy extracellular matrix (Chen, et al. 1997; Grinnell et al. 1996; Hennrick et al. 1997). They both usually cleave their substrate at a valine or alanine residue which is characteristic of most serine proteases. As previously mentioned, under normal circumstances these proteases are important in removing damaged tissue and eliminating pathogens after injury, however because their levels are elevated in chronic wounds, they can bind and cleave healthy tissue. Elastase and cathepsin G are of particular concern because of their broad specificity and subsequent destruction of a wide variety of extracellular matrix components, soluble growth factors and cell surface proteins.

Myeloperoxidase is another neutrophilic enzyme whose levels are elevated in chronic wounds. During the normal immune response, interacts with its substrates hydrogen peroxide and a halide and together they form a ‘powerful antimicrobial system (Van Der Veen et al. 2009). At high concentrations, the MPO-derived oxidants have been implicated in tissue injury. For example, cartilage erosion in rheumatoid arthritis is a result of the activity of activated neutrophils specifically the myeloperoxidase (Schiller 2003).

Chronic wounds are not only problematic to healthcare providers but it is a growing socioeconomic burden that translates into approximately 25 billion dollars spent a year on their treatment (Sen et al. 2009). Although it is possible for an injury resulting in the breaking of the skin to progress to a chronic wound, it is possible for them to form as result of leg, pressure and
diabetic ulcers. Leg ulcers are a common condition among persons who have lower limb arterial or venous insufficiency. Individuals faced with this condition have valves in their lower extremity that are not functioning well and therefore they are unable to provide proper circulation throughout the limb as well as take blood from the extremity back to the heart (Mekkes et al. 2003). As a result, the capillaries are faced with increased tension in an effort to accommodate the increased blood supply in the area.

If the situation is not treated, the capillaries become malformed and eventually become leaky. These leaky capillaries allow the movement of proteins that are usually fond in the plasma to enter the tissue resulting in protein-rich edema (Bollinger et al. 1997). This often leads to the formation of microthrombi blocking the capillaries and subsequently causing the build up of activated leukocytes to the area (Coleridge et al. 1988). This edema eventually leads to localized tissue hypoxia. Hypoxia in the presence of elevated levels of activated leukocytes can promote breakdown of tissue leading to the formation of a venous stasis ulcer (Yager et al. 2002). These types of chronic wounds are initially treated with cleansing, removal of necrotic tissue and compression therapy to help assist with the poor circulation. While these treatments help control the situation, most patients require the placement of stents to provide long term assistance with circulation.

Factors Involved in Chronic Wounds

It has been clearly illustrated that elevated proteolytic activity is associated with delayed healing, however there are other factors that are equally important – infection, age, disease and socioeconomic factors. When an individual is injured and the injury involves the breaking of the skin, there is always the possibility of the wound being invaded by microbes that are part of the
dermal floral (Bowler et al. 2001). This invasion only becomes a concern when the host’s defense system is unable to get rid of the bacteria resulting in colonization and subsequent infection of the wound. A study aimed at understanding the connection between healing and infection revealed that the exudate from pressure ulcers with a bacterial load greater than $10^6$ CFU/ml were faced with the problem of delayed healing (Bendy 1964). A large number of types of bacteria have been implicated in the delayed healing seen with chronic wounds. These include Gram positive bacteria such as *Staphylococcus aureus* and Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Yun et al. 2006).

Nutrition and age also play a role in delaying the healing process. The older a person is, the more fragile their skin which not only makes them more susceptible to wounds, but the healing process is also slower (Marcus et al 2000). In addition studies have shown that there is decreased proliferation of fibroblasts and keratinocytes which are key players in the tissue formation stage (Ashcroft et al. 2002). There is a strong connection between one’s ability to heal and the amount of nutrition that person receives. The wound healing process is an energy intensive one that requires the synthesis and release of a host of proteins which can become a bit of a challenge for someone who is undernourished (Russell 2001; Williams et al. 2003).

In addition, insufficient intake of carbohydrates and fat which are the primary source of energy results in the body relying on the protein catabolism as a source of energy leading to hyoproteinemia. Hyoproteinemia reduces the amount of amino acids available for the synthesis of new protein at the site of injury and it has been shown that insufficient levels of amino acids such as arginine can inhibit the body’s immune response (Barbul et al. 1990). Although proper nutrition is important, there is currently no standardized method to measure nutritional adequacy although serum albumin is often used as an indicator (Yager et al. 2002). Another contributing
factor to chronic wounds is diseases such as cancer and HIV in which the patient’s immune system is compromised (Lord 1997). A compromised immune system means it is difficult for the body to initiate the necessary immune response to ensure proper healing of the wound.

**Treatments: Dressings and Drugs**

Given our understanding of chronic wounds and the various facets that contribute to its perpetuation, it is imperative that more effective treatments need to be developed to target the elevated levels of neutrophils and increased bacterial infection without adversely affecting the newly re-constructed tissue. Dressings are the most popular form of treatment because it is an effective way of closing off the wound to the environment in an attempt to limit further infection and encourage healing. Most dressings contain silver because of its potent antimicrobial activity. However, a universal dressing product cannot be used for all wounds, but it must be tailored based on the nature of the injury (Higgins et al. 1995). Last year, scientists at an Israeli lab developed a copper dressing that was designed to protect the wound from the elements as well as promote angiogenesis (Borkow et al. 2010). While this dressing was shown to work better than the traditional silver dressing, it does not address the problem of elevated levels of neutrophils.

Recently work by Moor, Gould and Vachon confirm that an anionic macromolecule, polystyrene sulfonate (PSS) is able to inhibit elastase activity via electrostatic interactions rendering the enzyme inactive (Moor et al. 2009). In addition, to PSS several cations such as doxycyline, chlorhexidine, and silver have been implicated in promoting the healing process because of their strong antimicrobial potency.

Chlorhexidine gluconate is a cationic biguanide with a very broad spectrum of action against bacteria. The antibacterial action of chlorhexidine is believed to be mediated by its
disruption of bacterial cell membranes (Mohammadi and Abbott, 2009). The major membrane components of prokaryotic cells are phospholipids, and their membranes contain higher amounts of anionic lipids than eukaryotic cell membranes but no cholesterol or sphingomyelin (Mohammadi and Abbott, 2009). Thus, it is likely that cationic biguanide-based antimicrobials will preferentially target bacteria over eukaryotic cells. At high concentrations chlorhexidine is bactericidal, at low concentrations it is bacteriostatic.

Doxycycline a member of the class of antibiotics referred to as tetracyclines and is one of the many bacteriostatic chemicals that are widely used in the medical field to treat infections. It can prevent bacterial growth by inhibiting protein synthesis in the organism. This is achieved by the chemical binding to the 30s ribosomal subunit in the mRNA translation complex. This interaction prevents the amino-acyl tRNA from binding to the A site of the ribosome (Vincente et al. 2010).

Specific Aims

It has been shown that chronic wounds are very complex to treat because there are many factors that contribute and perpetuate their existence. Although there are therapies and treatments available, many of them are not very effective at targeting the enzymes and proteases that are responsible for arresting the wound in the inflammatory stage. PSS was used as the backbone of these formulations because of its large structure and stable structure. The molecule contains an aromatic ring which is very difficult to break because of the strong pi/pi interactions of the carbon atoms. In addition, it has a strong negative charge which allows it to easily form ionic bonds with cations. The flexibility of this compound makes it a very versatile platform on which an array of charged molecules can be attached. The aim of this research is combine PSS, an
anionic macromolecule that has been shown to inhibit proteolytic activity in chronic wounds
with cations that have an antimicrobial property and which may posses the ability to attenuate
the activity of these degradative enzymes encouraging the injury to transition out of the
inflammatory phase and progress towards the final stage of healing.
Materials and Methods

Test Formulations

Six formulations were developed and provided by IASIS Molecular Sciences, LLC, Spokane, Washington. These formulations included PSS and five salt derivatives of the anionic polymer namely: PSS-Calcium, PSS-Chlorhexidine, PSS-Doxycycline, PSS-Glutathione and PSS-Silver. PSS-Doxycycline and PSS-Chlorhexidine were found to be poorly soluble in aqueous solution. Stocks of these two formulations were made up in DMSO, while all of the other formulations were dissolved using distilled water to yield a working stock concentration of 20mg/ml.

Elastase Inhibition Assay

An elastase assay was designed to determine how well the formulations were able to inhibit elastase activity. This assay involved using an Elastase buffer [100mM Hepes (pH 7.5), 500mM NaCl, 0.05% Triton X-100 and distilled water], DMSO, deionized water, an elastase-specific substrate (Methoxysuccinyl-Ala-Ala-Pro-Val-paranitroanilide (Calibiochem, San Diego CA), the formulations and fluid obtained from a pressure ulcer using the VAC (vacuum-assisted closure) device with the approval of the Virginia Commonwealth University IRB.

Comparison of the wound fluid against purified neutrophil elastase revealed a titer of 13 units of elastase activity per ml (where one unit is defined as the amount of enzyme that will hydrolyze 1.0 µmol of MeO-Suc-Ala-Ala-Pro-Val-pNA per minute at 25°C, pH 8.0).
Formulations were incubated in Elastase buffer containing 25mU/ml elastase. Except where noted, formulations were used at a final concentration of 100µg/ml and typical reaction volumes were 500µl. Reactions were incubated for one hour at room temperature (except where noted).

To measure elastase activity, 160µl aliquots were mixed with 40µl of the elastase substrate (600µM final concentration) and incubated at room temperature. Cleavage of the substrate, as reflected by release of p-nitroaniline, was monitored spectrophotometrically at 405nm at 60 second intervals for 10 minutes. Standard curves were generated using the diluted wound fluid. Using the standard curve, the concentrations of elastase remaining in the wound was calculated. All reactions including the standard curve were performed in duplicate.

**Cathepsin G Assay**

The ability of the PSS-formulations to inhibit cathepsin G was determined in a similar manner. This assay involved using the Elastase buffer, cathepsin G substrate (N-Succinyl-A-AP-F-p-NA (Calibiochem, San Diego CA), the PSS formulations, and cathepsin G (MP Biochemicals: #191344, MW 23,500, 2-4 units/mg protein). A working stock of enzyme was made by reconstituting the lyophilized enzyme (100µg) in 1ml of 50% glycerol-50m M sodium acetate-150m M NaCl pH 5.5 for a concentration of 100µg/ml or a minimum of 200 milliunits/ml.

Cathepsin G was diluted with buffer to 2.2 milliunits/ml and PSS-formulations to 2mg/ml in the same buffer. Equal volumes (200µl) of enzyme and PSS-formulation were then mixed and incubated at room temperature for 1 hour.

To measure remaining cathepsin G activity, 160µl aliquots were mixed with 40µl of 2mM cathepsin G substrate (400µM final concentration) and incubated at room temperature.
Cleavage of the substrate, as reflected by release of \( p \)-nitroaniline, was monitored spectrophotometrically at 405nm at 60 second intervals for 10 minutes. Standard curves were generated using dilutions of cathepsin G. Using the standard curve, the concentrations of cathepsin G activity remaining in the samples were calculated. All reactions including the standard curve were performed in duplicate.

**Effects of PSS-Formulations on the Activity of Myeloperoxidase (MPO)**

The ability of the PSS-formulations to inhibit myeloperoxidase was examined by adapting a standard assay. In this assay, \( \text{H}_2\text{O}_2 \) is broken down by myeloperoxidase. The oxygen radical that is produced combines with a hydrogen donor, \( \text{o-dianisidine dihydrochloride (AH}_2 \) which is converted to a colored compound (A).

\[
\begin{align*}
(1) & \quad \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}^- \\
(2) & \quad \text{O}^- + \text{AH}_2 \rightarrow \text{H}_2\text{O} + \text{A}
\end{align*}
\]

Neutrophil myeloperoxidase, 200 milliunits/ml, (EMD catalog # 475911) and 1mg/ml PSS-formulation in a total volume of 240 \( \mu \)l of 50mM sodium phosphate, pH 6.0 were incubated at room temperature. At 2 and 24 hrs, duplicate 50\( \mu \)l aliquots were removed and mixed with 50\( \mu \)l of 800\( \mu \)M \( \text{o-dianisidine dihydrochloride} \) and 50 \( \mu \)l of 0.01% hydrogen peroxide. Dilutions of the myeloperoxidase incubated without a PSS-formulation were used to generate a standard curve. The reaction was followed by measuring the absorbance at 450nm every two minutes for twenty minutes.
Inhibition of Bacterial Growth by PSS-Salts

PSS-silver, PSS-doxycycline and PSS-chlorhexidine were tested for their ability to inhibit the growth of bacteria. For this study, cultures of clinically relevant bacteria were obtained from the VCU Microbiology/Immunology Laboratory, Department of Clinical Pathology. These bacteria, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Staphylococcus aureus*, are commonly associated with non-healing chronic wounds (Yun et. al 2006). Isolated colonies of the individual organisms were picked and used to generate stocks by growing overnight in 5 ml of Lysogeny Broth (LB) media at 37°C at 200-300 RPM.

The minimal inhibitory concentration-50 (MIC\textsubscript{50}) is defined as the minimal concentration of an agent that inhibits the growth of an organism 50%. This is a standard means of assessing the effectiveness of antimicrobials. PSS-formulations were serially diluted (typically two-fold) in quadruplicate in 48-well plates containing LB alone or LB containing 10% fetal calf serum. Overnight cultures of bacteria were diluted 1:1000 in LB and 20µl used to inoculate each well. The initial (T=0) light scattering at 600nm was determined to provide a baseline. The plate was then placed in a 37°C shaker (200 RPM) overnight and read again at 600nm the following day.

Growth as represented by mean (n=4) light scattering at 600nm was plotted as a function of the concentration of the PSS-formulation. From this, the Minimum Inhibitory Concentration required to inhibit the growth of 50% (MIC\textsubscript{50}) of the three different bacteria was determined.
Cytotoxicity Assay

To assess cytotoxicity, human neonatal fibroblasts were seeded onto 88 wells of 96-well cell culture plates, 100µl/well, at a density of 5 x 10^4/ml. After 24hr, the medium was replaced with 100µl of fresh medium containing graded concentrations of the PSS-formulations. Half-log serial dilutions from 800 µg/ml to 13 ng/ml were made. Four replicate wells per concentration were tested. The plates were incubated overnight in a humidified atmosphere (5% CO₂/95% air) at 37°C.

To quantify cell viability, the medium was replaced with fresh medium containing 5% CellTiter 96® AQueous One Solution Reagent (Promega Corp.). This reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). The absorbances at 490nm were read (T=0) and the plates placed back in the incubator for 1 hr and then the absorbances read again. In this assay, the yellow tetrazolium salt is reduced by NADPH or NADH (produced by dehydrogenases) in viable cells to a soluble blue formazan product. The change in absorbance that occurred in 1hr was then determined and plotted as a function of concentration. The Inhibitory Concentration-50 (IC₅₀) value was defined as the concentration allowing 50% survival of cells and was determined graphically.

Biocompatibility Indices

For this study, the Biocompatibility Index of the investigated PSS-formulations was defined as the ratio of each formulation’s IC₅₀ on human dermal fibroblasts and its mean MIC₅₀. This yields a dimensionless value. A Biocompatability Index (BI) greater than 1.0 represents an
agent that preferentially targets microbes. A BI of less than 1.0 would indicate that the agent is relatively cytotoxic.

**Data Analysis**

Except where noted, experiments were repeated a minimum of eight times. Error bars represent one standard deviation.
Results

Elastase Assay

As previously stated, one of the aims of these studies was to test the hypothesis that various salts of the anionic polymer, polystyrene sulfonate (PSS), are able to modulate the activities of various neutrophil-derived cationic proteins. Previous studies have shown that a characteristic feature of chronic wounds is the persistent elevated level of these proteins (Yager 1999) hence it is imperative to not only understand the mechanism that triggers their over expression, but to find a way to decrease their levels. In addition, salts in which the cation is a known antimicrobial were tested for their ability to inhibit the growth of bacteria that are commonly found in the chronic wound environment.

The first experiment was done to determine the effects the formulations had on elastase activity. This was achieved by using an elastase assay in which Methoxysuccinyl-Ala-Ala-Pro-Val-para-nitroaniline was the substrate. This particular substrate is specific to elastase which is an important characteristic given the fact that for these experiments, wound fluid which has numerous proteases, served as the elastase source. As a prelude, a pilot assay was performed to test whether the wound fluid contained elastase activity and that para-nitroanaline was released in a linear dose response manner. As shown in Fig 1. para-nitroanaline was released in a linear dose-response.
Each of the six PSS formulations were tested for their ability to inhibit elastase. Reactions contained 1.0mg/ml of PSS-formulations in a 240µl volume containing 25milliunits of elastase and were incubated for two hours at room temperature before measuring remaining activity. Elastase activity was measured kinetically measuring absorbance at 405nm. Typical results are shown in Figure 2 and are presented as the percentage of activity inhibited. With the exception of PSS-chlorhexidine, all the formulations efficiently inhibited elastase activity (≥ 90%) and the formulations that had a cation attached to them were no more effective than the pure PSS which seems to suggest that none of the cations used significantly contributed to elastase inhibition in the wound fluid. Working stocks (20m/ml) of both the doxycycline and chlorhexidine PSS salts were made with DMSO. PSS-doxyxycline remained in solution when
diluted to 1.0 mg/ml in the aqueous buffer. In contrast, PSS-chlorhexidine formed a large precipitate when added to the aqueous buffer. The compound’s inability to sufficiently dissolve may account for the relatively large error that was observed. Nevertheless, this compound still managed ability to achieve approximately 30% elastase inhibition.

Fig. 2 Formulations were incubated with wound fluid for 1hr. at room temperature. Elastase activity was determined by incubating aliquots with the elastase-specific substrate, methoxysuccinyl-Ala-Ala-Pro-Val-paranitroanilide and measuring anilide release overtime. The maximum slope was compared to a standard in parallel. Elastase activity was greatly reduced by all of the formulations except PSS-Chlorohexidine
Given the fact that the results showed that PSS was able to inhibit elastase activity, further experiments were designed to examine the kinetics of this inhibition. To that end, a time response assay was done using the same protocol as the first experiment except changing the incubation duration to hourly increments. In so doing, one would be able to determine how long the elastase needed to be exposed to the formulations before it was inactivated. The results of this round of experimentation revealed that there was no increase in the level of inhibition after one hour (Figure 3A) which was indicative of the fact that the elastase inhibition was occurring much sooner than hypothesized.

In order to capture the rate of inhibition, the time response assay was repeated, using 10mins increments. (Figure 3B). The results showed that even without preincubating, efficient inhibition (>80%) was observed with maximum inhibition of 90.00% occurring after 20mins but subsequently declining to 80% inhibition. This experiment clearly demonstrated that the kinetics of the elastase-formulation interaction was relatively quick and maybe occurring within a matter of seconds of them meeting. It is worthy to note that the variability in the observed PSS inhibition in latter experiment vs. that of the previous experiments could be as a result of the progressive weakening in the potency of the formulations from the time stock solutions of them were made to the time they were used (all stock solutions were used within a week after which they were discarded). However, despite the lower inhibitory activity observed in the later experiments in which PSS was the only formulation used, the effect was still significant >80%.
Figure 3. Elastase inhibition: time course. PSS was incubated at room temperature with wound fluid for varying times: (A) hour segments, (B) mins. segments. Elastase activity was determined by incubating aliquots with the elastase-specific substrate, methoxysuccinyl-Ala-Ala-Pro-Val-paranitroaniline and measuring aniline release overtime. The maximum slope was compared to a standard in parallel. Elastase activity was greatly reduced after 20 mins, however approximately 16% of elastase activity was regained after 10 mins.
With the initial set of experiments not only confirming that PSS was able to inhibit elastase activity, but it was able to do so after a relatively short period of time, further research was done in the form a dose response assay. In so doing, the results of the assay would help determine the minimum amount of the PSS formulation that would be needed to inactivate the elastase. This experiment was important if future work was going to be done to develop a treatment method that would incorporate these formulations into at a concentration that would be potent enough to inhibit elastase activity without damaging surrounding, healthy tissue. In this assay, the PSS formulation was serially diluted (1 log dilutions) in order to determine the minimum concentration of the salt that was needed to inhibit the elastase. The results revealed that as little as 0.1 microgram of PSS was able to inhibit 82% of elastase activity (Figure 4) using these reaction conditions.

![Elastase Inhibition: PSS Dose Response](image)

Figure 4. Elastase inhibition: PSS dose response. Varying doses of PSS was incubated at room temperature with wound fluid. Elastase activity was determined by incubating aliquots with the elastase-specific substrate, methoxysuccinyl-Ala-Ala-Pro-Val-paranitroanilide and measuring anilide release overtime. Percent inhibition was determined by comparing remaining activity with a standard curve.
Although the results that were obtained up to this point in the project was very reassuring, questions were raised regarding the interaction between the formulation and the elastase. Before progressing further with the effectiveness of the formulations to inhibit other proteases and microbial activity, it was necessary to validate that the observed inhibition was as a result of the formulation binding to the elastase and not the elastase substrate. In the event, the formulation was in fact inhibiting the substrate, then the elastase inhibition observed was not really inactivation of the protease but it was a ‘sequestering’ of the substrate so that it was no longer available to the elastase. To address this, 40ul of the substrate (600uM) was mixed with 160ul of 1mg/ml PSS. This mixture was immediately centrifuged at 15k x g for 30 minutes through a Microcon centrifugal filter unit YM-10 membrane with a 10 kDa cutoff. This membrane would retain the 70 kDa PSS molecule and any substrate that bound to it. Unbound substrate that passed through (smaller than 10 kDa) was then mixed with 25 milliunits of elastase and the release of paranitroanilide monitored at 405nm. This was compared to substrate not mixed with PSS but also passed through a filter. There was no significant difference in the amount of paranitroanilide that was released. This indicates that the observed reduction in activity was due to interactions of PSS with neutrophil elastase and not to protection of the substrate by the PSS molecule.

**Cathepsin G Assay**

Prior studies have shown that like elastase, cathepsin G was another cationic neutrophil-derived serine protease whose levels are elevated in chronic wounds (Yager 1999). Therefore, reducing the proteolytic activity of this enzyme would help the chronic wound progress pass the
inflammatory stage. To that end, the various formulations were added to a working stock of 100µg/ml of purified cathepsin G. The enzyme stock solution was further diluted with buffer to yield a final concentration of 2.2milliunits/ml. It was then incubated with 2mg/ml of the formulations at room temperature for an hour. The results of the cathepsin G assay showed that all of the formulations were able to inhibit >90% of cathepsin G activity, with PSS-chlorohexidine and doxycycline providing complete inhibition of the protease. (Figure 5).

**Figure 5** Purified cathepsin G was incubated for an hour at room temperature with each of the six formulations and then percent inhibition was determined.
Myeloperoxidase Assay

Myeloperoxidase (MPO) is one of the many proteins that are found in the intracellular granules of neutrophils and it is often used as a marker to quantify the degree of inflammation and estimate the accumulation of neutrophils in tissues. Five percent of the total protein of a neutrophil is comprised of MPO. The principal reaction mediated by MPO uses H₂O₂ and Cl⁻ as substrates to generate hypochlorous acid which is subsequently metabolized to hypochlorite and chlorine:

\[
\text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{HOCl} \\
\text{HOCl} \leftrightarrow \text{H}^+ \text{OCl}^- \\
\text{HOCl} + \text{Cl}^- \rightarrow \text{Cl}_2 + \text{OH}^-
\]

It has been hypothesized that elevated levels of MPO with the subsequent production of large amounts of highly reactive oxidants in chronic wounds can severely impair the wound’s ability to heal. MPO is a cationic protein, therefore, the PSS formulations were tested for their ability to inhibit MPO. A MPO assay similar to the previous assays was designed to monitor the protein’s activity. In this assay 200 milliunits/ml of MPO was incubated for two hours and 24 hours with 1mg/ml of the PSS-formulations. Oxidation of o-dianisidine dihydrochloride when H₂O₂ was provided was then determined. The absorbance of the reaction was measured every two minutes for twenty minutes and the results showed that the only formulation that was able to inhibit MPO activity was PSS-Glutathione while the other formulations appeared to increase the apparent activity of MPO (Figure 6).

The results of this experiment clearly illustrate that PSS and most of the PSS formulations were unable to reduce the enzymatic activity of MPO. Further reviewing of the literature revealed evidence that glutathione can react directly with H₂O₂. As a result, what
seemed to be a reduction in MPO activity was more likely a case of substrate depletion. In contrast, several of the other PSS-formulations appeared to “increase” MPO activity. The reasons for this remain unclear.

**Figure 6** Myeloperoxidase Inhibition after 24hr. incubation with the formulations. Values greater than zero indicate increased activity and values less than zero are indicative of inhibition.

**Bacterial Inhibition**

Another important aspect of chronic wound pathology is infection. A large number of bacteria species have been observed in chronic wounds. Infection can impair healing either directly through the actions of bacterial products (e.g., endotoxins), or indirectly through the provoking of an exuberant inflammatory response. Thus, approaches targeting the microbial flora is an attractive strategy for treating these problem wounds. Several of the PSS-formulations have been designed with antimicrobials as the cation. The ability of these
formulations to inhibit planktonic bacterial growth was investigated. For this purpose, these formulations were tested against three clinically isolated bacteria that are commonly associated with non-healing chronic wounds namely *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Staphylococcus aureus*. The bacteria were grown in LB media as well as LB media and 10% fetal calf serum as a means to mimic the physiological environment of the wound. The microbes were incubated overnight with serial dilutions of some of the formulations that contained a cation with known antimicrobial property PSS-chlorhexidine and PSS-doxycycline and resulting growth was plotted as a function of the concentration of the formulations.

A representative result of the data obtained is shown in Figure 7. Further analysis of the data from this experiment was done in an effort to calculate MIC<sub>50</sub>: the concentration of the PSS formulations at which 50% of the microbial growth was inhibited (Table 1). Surprisingly, especially in the case of PSS-silver, presence of serum in the LB had a negligible influence on the observed MIC<sub>50</sub>s. Because silver ions react fairly non-specifically with targets such as thiols, it was expected that the presence of additional organic material in the medium would reduce its effectiveness.

There was some interest regarding whether interactions of PSS with the antimicrobial cations might influence their effectiveness. To examine this, the ability of PSS-doxycycline to inhibit the growth of *Staph. aureus* was compared with doxycycline alone. Based on a weight-mass comparison, the MIC50 for PSS-doxycycline was indeed slightly higher than that observed for doxycycline alone. This slight difference might be attributed to the slightly lower concentration of doxycycline in the PSS-doxycycline reactions.
Figure 7 Representative MIC$_{50}$ Assay of Acinetobacter baumannii and PSS-chlorhexidine. Bacteria were grown in LB media or LB media + 10% fetal calf serum containing serial dilutions of PSS-chlorhexidine or PSS-doxycycline. Growth was monitored by mean light scattering at 600nm and plotted as a function of the concentration of the PSS-formulation.
Table 1 Growth Inhibition of Clinically Relevant Bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Formulation</th>
<th>LB Media</th>
<th>MIC$_{50}$ LB Media + 10% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td>PSS-chlorhexidine</td>
<td>50 mcg/ml</td>
<td>105 mcg/ml</td>
</tr>
<tr>
<td></td>
<td>PSS-doxycline</td>
<td>0.3 mcg/ml</td>
<td>12 mcg/ml</td>
</tr>
<tr>
<td></td>
<td>PSS-silver</td>
<td>3.0 mcg/ml</td>
<td>4.0 mcg/ml</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>PSS-chlorhexidine</td>
<td>105 mcg/ml</td>
<td>108 mcg/ml</td>
</tr>
<tr>
<td></td>
<td>PSS-doxycline</td>
<td>70 mcg/ml</td>
<td>80 mcg/ml</td>
</tr>
<tr>
<td></td>
<td>PSS-silver</td>
<td>2.0 mcg/ml</td>
<td>6.0 mcg/ml</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>PSS-chlorhexidine</td>
<td>9 mcg/ml</td>
<td>80 mcg/ml</td>
</tr>
<tr>
<td></td>
<td>PSS-doxycline</td>
<td>0.2 mcg/ml</td>
<td>0.6 mcg/ml</td>
</tr>
<tr>
<td></td>
<td>PSS-silver</td>
<td>8.0 mcg/ml</td>
<td>9.5 mcg/ml</td>
</tr>
</tbody>
</table>

Cytotoxicity

Although relatively low concentrations of the formulations were needed in order to kill the microbes, further work needed to be done to determine whether these concentrations were also harmful to eukaryotic cells. To that end, a cytotoxicity assay was performed using human neonatal dermal fibroblasts as a source of eukaryotic cells. The fibroblasts were incubated overnight with serial dilutions of the formulations. Viability was then assessed by measuring the conversion of MTS into formazan. Plotting formazan production (reflection of viable mitochondria) as a function of formulation concentration allowed the determination of the Inhibitory Concentration (IC$_{50}$) i.e. the concentration of the formulation that killed 50% of the cells (Table 2).

Table 2: Cell Viability Post Treatment

<table>
<thead>
<tr>
<th>Formulations</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSS</td>
<td>&gt;1000 mcg/ml</td>
</tr>
<tr>
<td>PSS-calcium</td>
<td>&gt;1000 mcg/ml</td>
</tr>
<tr>
<td>PSS-chlorhexidine</td>
<td>20 mcg/ml</td>
</tr>
<tr>
<td>PSS-doxycline</td>
<td>&gt;1000 mcg/ml</td>
</tr>
<tr>
<td>PSS-gluathione</td>
<td>&gt;1000 mcg/ml</td>
</tr>
<tr>
<td>PSS-silver</td>
<td>20 mcg/ml</td>
</tr>
</tbody>
</table>
Compatibility Index

Compatibility indices are very useful tools in figuring out whether or not it would be in the best interest of the patient to administer a new drug. It is calculated by dividing the IC\textsubscript{50} by the MIC\textsubscript{50} and if the resulting number is greater than 1, it means that the treatment in question would kill the microbe causing negligible damage to healthy tissue. On the other hand, if the resulting number is less than 1, it means that the concentration of the drug needed to destroy the microbe is high enough to kill healthy cells.

From the analysis conducted, PSS-doxycycline and PSS-silver consistently had a compatibility indices value greater than 1 (Table 3) suggesting that they were the best treatment options if the formulations were to be incorporated in a product.

The results of the various assays were very promising as they all, except for the MPO experiment, showed that they were able to inhibit the various neutrophilic proteases with which they were incubated. In addition, the results clearly illustrated the multidimensionality of the anionic macromolecule PSS because one can now make a formulation that contains the PSS with various cations attached to it in order to combat the elevated proteolytic environment by simultaneously targeting a wide variety of enzymes.
### Table 3: Compatibility Index

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Formulation</th>
<th>LB Media</th>
<th>LB Media + 10% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>PSS-chlorhexidine</td>
<td>0.400</td>
<td>0.190</td>
</tr>
<tr>
<td></td>
<td>PSS-doxycycline</td>
<td>3333.333</td>
<td>83.33</td>
</tr>
<tr>
<td></td>
<td>PSS-silver</td>
<td>6.666</td>
<td>5.0</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>PSS-chlorhexidine</td>
<td>0.190</td>
<td>0.185</td>
</tr>
<tr>
<td></td>
<td>PSS-doxycycline</td>
<td>14.286</td>
<td>12.500</td>
</tr>
<tr>
<td></td>
<td>PSS-silver</td>
<td>10.0</td>
<td>3.333</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>PSS-chlorhexidine</td>
<td>2.222</td>
<td>0.250</td>
</tr>
<tr>
<td></td>
<td>PSS-doxycycline</td>
<td>5000</td>
<td>1666.667</td>
</tr>
<tr>
<td></td>
<td>PSS-silver</td>
<td>2.5</td>
<td>2.105</td>
</tr>
</tbody>
</table>
Discussion

Chronic wounds are a growing concern because their treatment represents a significant healthcare cost and with the growing age of the population, it is a problem that will continue to grow. To that end, it is imperative that cost-effective therapies be developed to adequately resolve this problem. This study examined the ability of PSS-formulations to inhibit two different serine proteases that are primarily neutrophil-derived and their ability to inhibit myeloperoxidase. In addition, the ability of three of these formulations to inhibit bacterial growth and to what extent they demonstrated cytotoxicity towards human dermal fibroblasts was examined.

The results of study confirmed that PSS was able to inhibit the activity of the cationic serine protease, elastase, (>90%) and the various salt derivatives were just as effective as the anionic macromolecule to which they were attached. One can therefore infer that the cations that were added to the anionic PSS macromolecular anion were not binding to the PSS in a way that prevented it from interacting with elastase that were examined or they themselves were not contributing to the overall protease inhibition. This finding confirmed the work by Sorsa that showed that while tetracyclines like doxycycline were effective at inhibiting MMPs, another neutrophilic protease, the chemical had no effect on elastase activity (Sorsa 1994). It remains to be seen whether PSS by itself or in combination with doxycycline can also inhibit MMPS, in particular, MMP-8 or MMP-9. Both of these MMPs are expressed primarily by neutrophils.

It was also shown that a relatively low concentration of the formulations was needed and the observed inhibition happened shortly after the protease was exposed to the salts. The fast acting inhibition is an encouraging sign because it means that if any of these formulations were to be added to a chronic wound, it can quickly attenuate the destructive activity of the enzyme.
The formulations were also able to reduce the activity of Cathepsin G, another cationic serine protease whose levels are elevated in chronic wounds. In the case of cathepsin G, there were two formulations namely PSS-chlorhexidine and PSS-doxycycline that were more effective than the other salts, which provided >85% inhibition. There was nothing found in the literature that explained or proposed the possible interaction between the protein and the cations that would cause them to be more effective than the other agents.

In retrospect, the studies examining myeloperoxidase inhibition probably suffered with a design flaw. It was observed that PSS-glutathione was the only formulation that seemed able to inhibit the activity of the enzyme. The ability of glutathione to directly inactivate hydrogen peroxide essentially interfered with the intended purpose of this assay. However, some solace can be gleaned from the implied ability of PSS-glutathione to act as an antioxidant. Another interesting observation was the apparent increase in MPO activity observed with several of the PSS-formulations. There are several potential explanations that could explain this unusual observation. Firstly, the enhanced enzyme activity observed can be as a result of the PSS binding to the enzyme and stabilizing it. Alternatively, PSS-myeloperoxidase complexes may conduct subtle changes in the myeloperoxidase protein making it efficient at substrate utilization/product synthesis. It should be noted that the serine proteases targeted in this study act primarily as monomers. In contrast, the 150-kDa myeloperoxidase protein is a dimer consisting of two 15-kDa light chains and two variable-weight glycosylated heavy chains bound to a prosthetic heme group. Such a complex structure may make access of the 70 kDa PSS molecule to the active site difficult (assuming that that is the operative mechanism for inhibition).

Molecular modeling of the serine proteases and identification of the various binding sites on the enzymes would better help researchers understand the manner in which they interact with
PSS. It will also be helpful because maybe PSS is not an effective tool in shutting down the enzyme’s activity but knowing the structure of the enzyme may help researchers use a suitable substitute for PSS that can bind to the active site.

Infection is another key feature of the chronic wound that often must be taken into consideration when managing the injury. Bacterial infection contributes to the severity of the wound because in addition to releasing toxins that can damage tissue, it also helps propagate the arrival of the phagocytic neutrophils, the cell responsible for the elevated protease levels. Therefore, it would be in the best interest of the patient to reduce and subsequently eliminate the presence of bacteria in the wound. To that end, the formulations were added to bacterial cultures to determine whether or not they were able to inhibit microbial growth of three bacteria commonly associated with chronic wounds namely *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. It is worthy to note that only two formulations with cations that have a known antimicrobial property were used in these set of experiments – PSS-chlorhexidine, PSS-doxycycline. All of the formulations were able to inhibit microbial growth and their respective MIC\(_{50}\)s were calculated. At least in the case of doxycycline, complexing with PSS did not significantly diminish or increase antimicrobial effectiveness.

Relatively low concentrations (in the microgram range) of the formulations were needed to prevent the growth of the bacteria, which is important because you want to be able to stop the bacterial growth while minimizing potential toxicity. To this end, further experiments were done to see whether the concentration of the formulations used was strong enough to prevent bacterial growth but not damage the cells specifically fibroblasts. The results from these experiments were used to calculate the IC\(_{50}\) and combined with the MIC\(_{50}\) to generate a biocompatibility index of the various formulations. This information can be particularly useful when developing a
treatment because one would be able to figure out the maximum concentration of the drug that
can be used without potentially damaging healthy tissue. It is recognized that other cell types are
also present in wounds and they may differ in their sensitivity to these formulations. Of
particular note is keratinocytes. These cells are responsible for generating the epithelium and
would perhaps have more contact with topical PSS-formulations than any other cell type. Future
studies could also determine biocompatibility for these cells.

In summary, in recognition of the need for improved strategies for treating chronic
wounds, this study examined the potential of a polyanionic molecule to serve as a platform that
could affect multiple targets within a wound. Experiments confirmed PSS’s ability to inhibit the
activity of two serine proteases even when complexed with various cations. Addition of cations
such as doxycycline, tetracycline and silver generated formulations with the additional ability to
act as antimicrobials. Although very tentative, PSS-glutathione may act as an effective
antioxidant. Importantly, none of these formulations demonstrate unacceptable toxicity to cells
likely to be found at a wound site. The multidimensionality of the formulations would make it
easy for one to custom design dressings depending on the severity and complexity of the wound
environment. The fact that PSS already has FDA approval for oral use should also make this a
relatively easy process to take this product from bench to clinic.


Vitae

Necrisha Roach was born on July 25, 1981 in a small town located in the eastern part of the Caribbean island Trinidad. She graduated from St. Augustine Girl’s High School, Curepe, Trinidad in 2000. She received her Bachelor of Business Administration in Finance and Investments from Baruch College, City University of New York in 2006 after which she returned for an additional two years to pursue a study in the natural sciences. During her undergraduate studies, she worked full time at several non-profit organizations and along side Emergency Department physicians at a New York City trauma center. In addition to her secular jobs and volunteer positions, she was also an active member of a research lab at Mt. Sinai School of Medicine, New York. There she participated in experiments involving the study of ion channel kinetics. Prior to starting her graduate career at VCU, she worked as an adjunct professor and taught two introductory biology courses. During her time at VCU, her research was focused on improving the elevated proteolytic environment commonly associated with chronic wounds. She received an award in April 2010 from the National Wound Healing Society for her contribution to the field.