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INHIBITION OF BACTERIAL GROWTH AND PREVENTION OF BACTERIAL ADHESION WITH LOCALIZED NITRIC OXIDE DELIVERY

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INHIBITION OF BACTERIAL GROWTH AND PREVENTION OF
BACTERIAL ADHESION WITH LOCALIZED NITRIC OXIDE
DELIVERY

By

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ABSTRACT

Bacterial infections continue to be a problem at the site of an indwelling medical device, and over the years, various bacterial strains have become more resistant to current antibiotic treatments. Bacterial infection at an indwelling medical device can be dangerous and affect the performance of the medical device which can ultimately lead to the failure of the device due to bacterial resistance to treatment.

Nitric Oxide (NO) has been shown to possess antibacterial properties to prevent and inhibit bacterial growth. NO releasing coatings on indwelling medical devices could provide a reduction in bacterial infections that occur at the device site such as for use in a urinary catheter. This work demonstrated that 1.7×10^{-8} moles of NO delivered over 18 hours prevented the growth and proliferation of *Staphylococcus epidermidis*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and *Escherichia coli* using S-Nitroso-N-acetyl-D-penicillamine linked to polydimethylsiloxane (SNAP-PDMS). It was also demonstrated that this effect is highly localized, with NO affecting bacteria only directly touching the polymer films. This localization should prevent systemic effects commonly observed with oral antibiotics when fabricating devices such as urinary catheters.

CHAPTER 1

INTRODUCTION

Since the 1940s, antimicrobial agents have been used to fight infections [1]. The success of antibiotics over the years has aided in the reduction of illness symptoms and has decreased the number of deaths due to infectious diseases. Over time, bacteria have evolved, developing a resistance to some commonly used antibiotics. These drug resistant bacteria are more difficult to treat, and according to the CDC, "at least 2 million people become infected with bacteria that are resistant to antibiotics and at least 23,000 people die each year as a direct result of these infections" in the United States [1]. Additionally, these antibiotic-resistant illnesses cost $20 billion per year in the United States [1]. The current trend is to develop new antibiotics to treat the antibiotic-resistant bacteria, however the bacteria continue to evolve and adapt, and thereby generating new resistance to the developed drugs. Because of this continuous adaptation cycle, there is a need for a new approach that can counteract the growing population of antibiotic-resistant bacteria.

The ability to treat bacterial infections is crucial for human survival. If antibiotics continue to fail against evolving bacteria, there will likely be an increase in mortality as a result of bacterial infections. One promising approach to battling the antibiotic-resistant bacteria epidemic is the use of nitric oxide (NO). Nitric oxide is a highly reactive, endogenously produced molecule that is capable of killing bacteria. The aim of the work described herein is to demonstrate that NO releasing polymers can produce the appropriate surface flux to inhibit and prevent bacterial growth *in vitro*.

1.1 PROBLEMS WITH INDWELLING MEDICAL DEVICES

The human body is a very complicated machine; it can be predictable as well as unpredictable. When an indwelling medical device is placed within the body, the body cannot discriminate whether the device is there as an aid or is an assault on the homeostasis of the body. Typically, the implantation procedure causes injury and a cascade of tissue responses will occur, such as inflammatory and immune responses [2]. The body can recognize the device as foreign and stimulate foreign body reactions or fibrous encapsulation of the device [2]. Therefore, rejection by the body is one problem with indwelling medical devices.

Another key problem with indwelling medical devices is device associated infection. Infections on medical devices can lead to serious complications such as failure of the device or the need for early removal. The infection can become chronic or untreatable and lead to an assortment of secondary complications as extreme as amputation or death. In the United States, out of the roughly 2 million annual hospital-acquired infections, fifty percent take place at the site of an indwelling medical device [3]. One example of an indwelling medical device is the catheter, specifically urinary catheters, which have been noted to cause urinary tract infections. There are nearly 100 million Foley catheters sold worldwide per year, and approximately 25 million of those are sold in the United States [4]. The most frequent healthcare-associated infection in the United States is catheter-associated urinary tract infection [5]. These catheter-associated urinary tract infections cost at least $600 per episode, and a minimum of $2800 per incident if the infection leads to bacteremia or infection within the blood [5], rendering

the need for interventions that can prevent bacterial infections on indwelling medical devices.

1.1.1 DEFINITION OF BACTERIAL INFECTION

Bacteria are prokaryotic organisms. The majority of bacteria fall into one of two classes: gram-positive or gram-negative. The gram-positive bacteria have a thicker cell wall, also referred to as a peptidoglycan layer. On the contrary, gram-negative bacteria have a thin cell wall (peptidoglycan layer), but have an extra layer surrounding their cell wall referred to as an outer membrane [6]. Figure 1.1 represents a basic comparison of the structural differences between gram-negative and gram-positive bacteria. In the case of gram-positive bacteria, the thicker peptidoglycan layer provides some protection against both the immune response and antibiotics because of the physical barrier whereas the extra layer to gram-negative bacteria also serves as an additional barrier for immune cells and antibiotics to penetrate before being effective. The outer membrane present on gram-negative bacteria is hard to penetrate and in general makes them harder to kill than gram-positive bacteria.

The human body is covered with thousands of bacteria both internally and externally. Several bacteria are considered part of normal body flora or bacteria naturally found on or within the body. Bacteria are also present throughout the environment; there are bacteria on various surfaces, in the air, on food, and within drinks [6]. For the most part, these bacteria do not lead to infection or disease. Complications arise when bacteria

Gram Negative — Outer membrane — Peptidoglycan — Periplasmic space — Cytoplasmic membrane — A
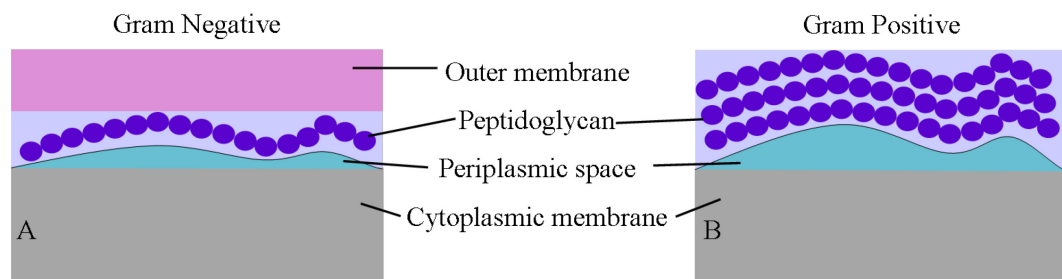
Gram Positive — B

Figure 1.1. Generalized images comparing the structural differences between gram-negative (A) and gram-positive (B) bacteria. Gram-negative bacteria have a thin peptidoglycan layer and an additional layer referred to as the outer membrane. Gram-positive bacteria have a thick peptidoglycan layer and no outer membrane.

invade locations they are not naturally found. For example, when bacteria located on the surface of the skin get pushed within the lower layers of tissue due to a cut or injury, an infection may occur. Most human diseases are caused by endogenous infections, which are defined as when one's normal bacterial flora invade locations they are not normally found [6].

When implanting a medical device, there are a variety of sources for bacterial infection such as the operating room, surgical materials, and bacteria present on the patient's skin or already residing in the body [7]. Indwelling medical devices are subject to infection once bacteria are adhered to the surface of the medical device. The adhered bacteria then spread, grow, and propagate, with certain bacterial strains capable of forming a biofilm on the device [7].

1.1.2 BIOFILM FORMATION

Not all bacteria have the ability to produce a biofilm, but many bacteria can. Depending on the medical device and time within the body, biofilms can arise from a single bacterial strain or multiple bacterial strains [8]. Biofilms form in several stages. The first stage is rapid attachment of bacteria to the device surface. Once attached, bacteria proliferate and attach to one another [7]. The attached bacteria begin secreting an exopolysaccharide matrix that serves to protect the bacteria [3]. Figure 1.2 visually represents the stages of biofilm formation. The biofilm effectively serves as a barrier to retain nutrients and allow the bacteria to thrive, and also as a barrier to components of the body's immune response to infection [3]. The biofilm is essentially a physical barrier
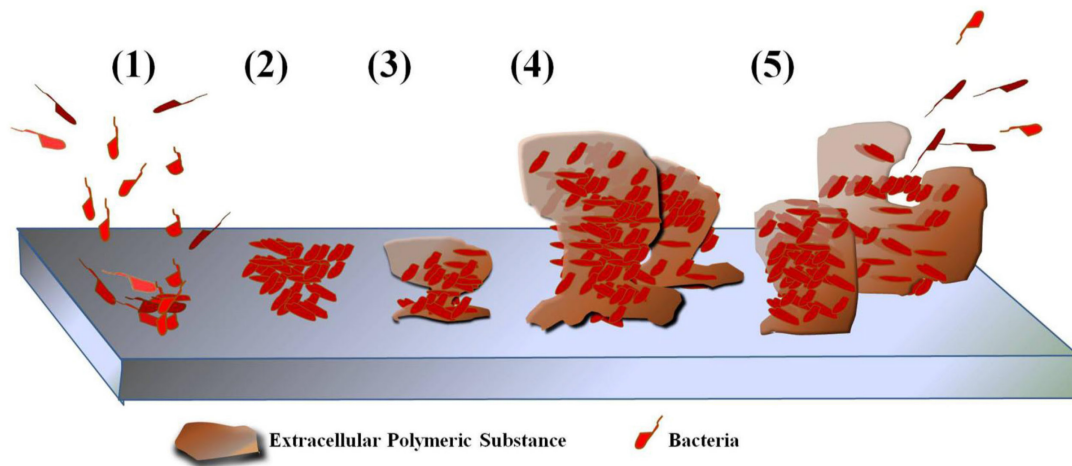
Figure 1.2. Visual representation of bacterial biofilm formation stages: 1) reversible adhesion of bacteria, 2) irreversible adhesion of bacteria, 3) initial production of exopolysaccharide matrix, 4) maturation of biofilm, and 5) dispersion of planktonic bacteria. Printed from Park [9].

between the bacteria and the host. Not only do biofilms prevent immune cells from entering, but they can also prevent antibiotics from reaching the bacteria. Research has shown that it can take up to a thousand times the necessary antibiotic dose to kill bacteria in a biofilm than those not protected by the biofilm [10]. Figure 1.3 represents a scanning electron micrograph of a biofilm present on the luminal surface of an indwelling catheter. The slime-like biofilm protects the gram-positive cocci *Staphylococcus aureus* bacteria from the host response as well as antibiotics.

Knowing the negative effects of bacterial infection on an indwelling medical device, it is important to take steps to prevent an infection. One key step is to prevent biofilm formation, which is most efficiently done by preventing bacteria from initially adhering to the surface of the device.

1.1.3   CURRENT APPROACHES TO PREVENTING DEVICE INFECTION

Due to the complications that arise from bacterial adhesion on medical devices, there has been a significant amount of research focused on preventing bacterial adhesion. Proper sterilization and cleaning is one of the first preventative measures taken to minimize the risk of infection, however, considering the presence of bacteria almost constantly in the environment, such as within the air or on the surrounding surfaces, more needs to be done to prevent infection from bacteria that make it past the standard sterilization protocols.

There are a variety of different approaches to preventing bacterial infection, while the primary treatment for a bacterial infection is to prescribe antibiotics. As previously

Acc.V Spot Magn Det WD ⊢———————————┤ 10 µm
10.0 kV 3.0 2363x   SE 12.6 98-11-62
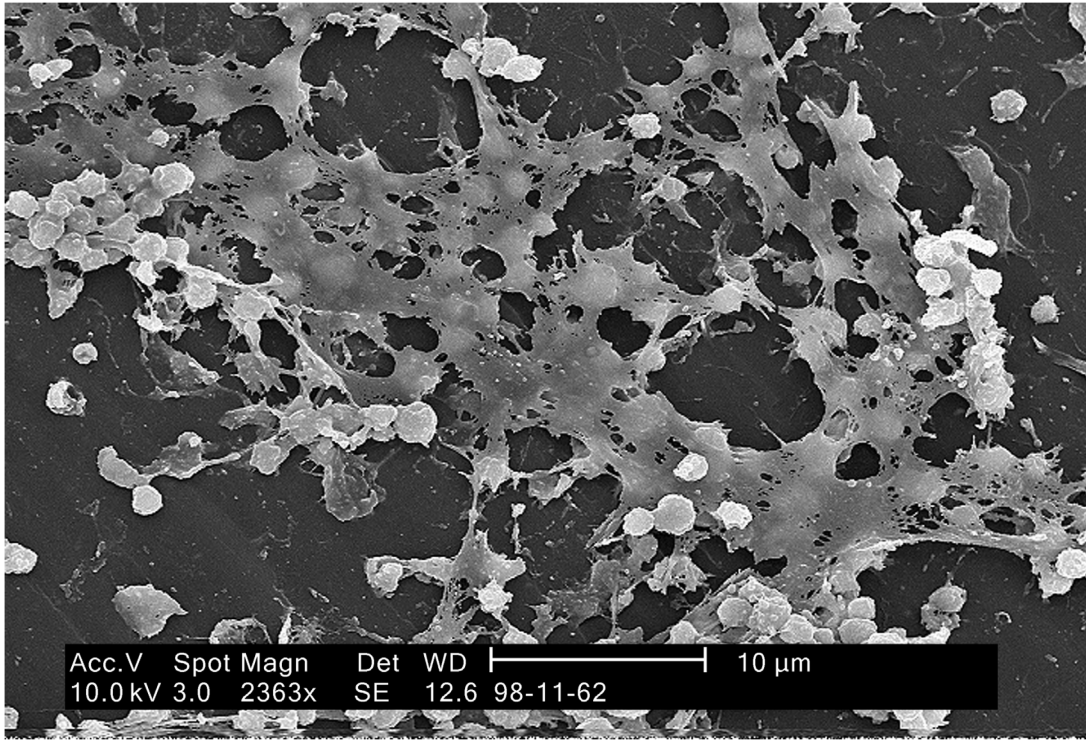
Figure 1.3.   Scanning electron micrograph of *Staphylococcus aureus* biofilm found on the luminal surface of an indwelling catheter. Photograph by Janice Carr, Centers for Disease Control and Prevention, Atlanta, GA USA.

stated, bacteria have the ability to evolve and develop resistance to antibiotics. Because of the growing number of strains of antibiotic-resistant bacteria, intense broad spectrum antibiotics are used to bombard the body, which not only kill the infectious bacteria, but also destroy the body's normal flora or bacteria present but does not cause disease. Thus, there is a continued need for development of new antibiotics. To break the cycle of bacteria becoming resistant to new antibiotics, research has been undertaken to develop different treatments for bacterial infection.

In order to deal with this issue, research has been focused on developing measures that can prevent an infection from occurring through the use of modified or specialized coatings on medical devices. An example of one of these approaches is through the use of passive coatings such as hydrophilic polyurethanes. This type of coating reduces bacterial adhesion, but cannot kill bacteria that manage to adhere. This is due to the fact that hydrophilic surfaces tend to absorb fewer proteins than hydrophobic surfaces, and protein absorption promotes bacterial adhesion. Therefore, limiting protein absorption would reduce bacterial adhesion. [11]. It only takes a low level of healthy bacteria to ultimately lead to device infection [3]. Another approach is active coatings, which release antibacterial agents directly [3]. Examples of active coatings can release "antibiotics, silver ions, bioactive antibodies, and NO" [3]. These types of coatings can reduce adhesion as well as kill bacteria that still adhere. However, there are many downfalls with the use of active agents. Silver ions can lead to permanent skin discoloration and silver-resistant bacteria have occurred in addition to the expense of using silver [10]. Another current approach is the use of povidone iodine, but this antiseptic has presented to be

toxic to fibroblasts along with a significant occurrence of recorded allergic reactions [10]. Each approach has success in one aspect, and failure in another. The ideal preventative measure has not yet been found.

1.2   NITRIC OXIDE AS ANTIBACTERIAL AGENT

Nitric oxide (NO) is essential in many processes throughout the body. Physiologically, NO not only inhibits platelet adhesion and aggregation, but also hinders smooth muscle cell growth and proliferation. It serves as a neurotransmitter and NO is also involved in mediating the inflammatory response toward implanted medical devices. Additionally, NO has been shown to inhibit bacterial growth as well as prevent bacterial adhesion [12]. Because of the vast array of physiological functions of NO, there has been a significant amount of research reported with the focus of either releasing or generating NO. There are numerous applications where controlled NO release is beneficial and the ability to control release is essential for determining the amount of NO needed to prevent bacterial adhesion, and ultimately prevent bacterial infection.

1.2.1   BENEFITS TO NITRIC OXIDE AS ANTIBACTERIAL AGENT

Nitric oxide is "a diatomic free radical produced by macrophages as part of the natural immune response to bacterial infection" [3]. The body utilizes NO to fight infection naturally, so using an NO releasing material to mimic this natural response may be an effective strategy to fight bacterial infections including bacteria that are resistant to antibiotics. The half-life of NO is on the order of seconds in both blood and tissue [13]. The short half-life means treatment with NO is localized and will not systemically affect

areas other than the target for treatment (i.e., the negative side effects of a broad spectrum antibiotic treatment that kills natural flora will not take place). Antibiotics as a treatment for infection are typically not localized because the antibiotics are dissolved in tissues and fluids and circulate throughout the body until they encounter bacteria.

1.2.2   MODE OF ACTION OF NITRIC OXIDE ON BACTERIA

Nitric oxide is a part of the body's immune response against infection specifically by the macrophages. Macrophages also produce other components including acid, glutathione, cysteine, hydrogen peroxide, and superoxide. All of these products contribute to enhancing the antibacterial properties of NO in the presence of an infection [14].

Nitric oxide has many modes of action in preventing growth and proliferation of bacteria. One effect NO has on bacteria is inhibiting bacterial adhesion by altering cell membrane adhesion proteins that mediate cell-substrate interactions [13]. Preventing bacterial adhesion is essential to preventing infection. NO has been shown to stimulate the breakdown of biofilms [13]. Disrupting biofilm formation is crucial because it breaks down the physical barrier protecting bacteria. Once the barrier is broken down, the bacteria are more easily destroyed. One mechanism through which NO destroys bacteria is by destroying the bacterial membrane, which can be achieved by lipid peroxidation by NO-derived peroxynitrite [13]. Other mechanisms include damaging the bacterial DNA beyond repair and inhibiting crucial metalloproteins that are part of the bacterial respirator reactions [13]. Figure 1.4 represents the various antibacterial mechanisms NO
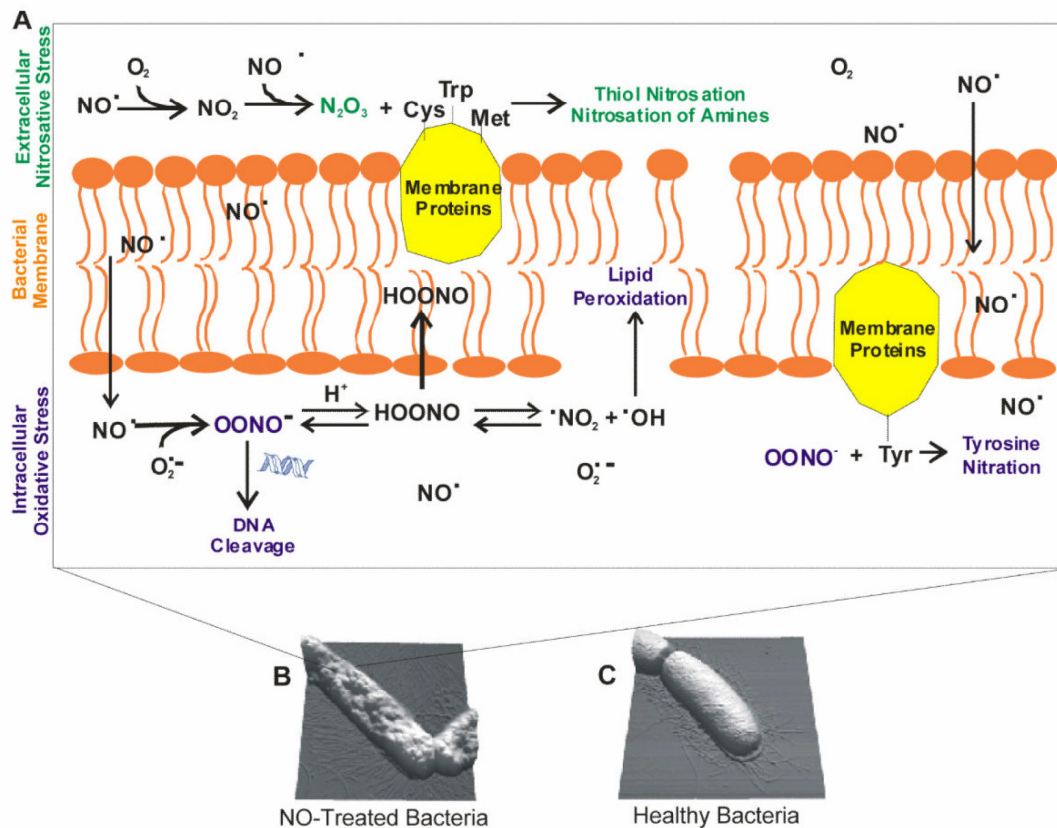
Figure 1.4. Schematic demonstrating the various antibacterial mechanisms of nitric oxide (NO) and its byproducts (A). Atomic force microscopy images show the decreased bacterial viability surfaces (B) after subject to these mechanisms compared to a control surface (C). Printed from Carpenter [15] with permission of The Royal Society of Chemistry.

and its byproducts have on bacteria [15]. Because of NO's natural approach to being a bactericide, it is less likely for bacteria to become resistance to NO like they have become resistant to current antibiotics.

1.2.3 NO RELEASING MATERIAL

Over the years, there has been a great deal of effort focused on the development of NO releasing polymers. One such class of material is the development of polymers that contain S-nitrosothiols (RSNOs), an endogenously produced class of NO donors that are able to release NO under fairly mild conditions. One pathway for which NO can be released from RSNOs is via photoinitiated decomposition [16]. This is important to note because it allows the possibility of having an on/off switch to control NO release when needed through the use of light, assuming other NO release triggers can be avoided, such as metal ion and/or ascorbate mediated decomposition [16]. Frost et al. developed a photoinitiated NO releasing polymer material by covalently linking S-Nitroso-N-acetyl-D-penicillamine to polydimethylsiloxane (SNAP-PDMS) [12]. The RSNOs present give the polymer its green color [12]. Figure 1.5 provides a visual comparison of SNAP-PDMS films to RTV3140 (PDMS) films. It is important to note that the base material for the NO releasing polymer is PDMS, which allows for the polymer to be easily sandwiched between two outer layers of medical grade PDMS. This helps make the polymer more stable and resistant to uncontrolled NO release. Another benefit to having PDMS as a base material is that it is easily adaptable as a coating to various medical devices such as urinary catheters.

SNAP-PDMS   RTV3140

A   B
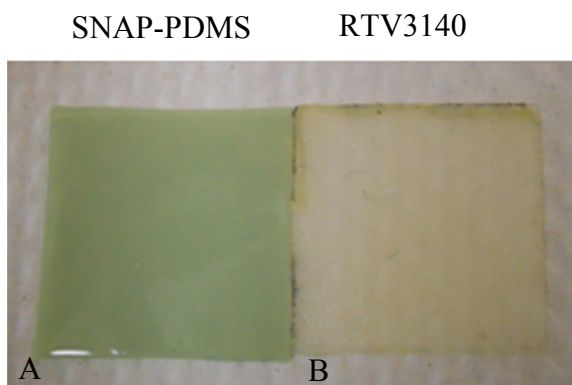
Figure 1.5.   Comparison of SNAP-PDMS (A) to RTV3140 (B) cast and cured films. SNAP-PDMS appears a green color whereas RTV3140 is more transparent and clear. Printed from Frost [12].

Due to the light-controlled NO releasing property of SNAP-PDMS, it is a desirable polymer to use in determining the appropriate surface flux of NO needed to prevent bacterial adhesion. SNAP-PDMS remains relatively inert until it interacts with light, therefore, NO release can be initiated with the use of a light emitting diode (LED) [12]. Different intensities of light and period of exposure affect the NO surface flux. Controlling the surface flux of NO is critical to determine the required NO surface flux needed to inhibit bacterial adhesion. Once the necessary NO surface flux needed to prevent bacterial adhesion is determined, other NO releasing materials can be used to deliver the NO, such as a material that does not need light to cause NO release. One example is a material that utilizes diazeniumdiolate as the NO donor, these types of materials release NO over time depending on the amount of donor blended in the polymer or the overall thickness of polymers containing covalently linked diazeniumdiolate functional groups [17]. These types of materials are important because, as in the example of use in a catheter, it would be difficult to add a light source to initiate the NO release from the polymer when there are other NO donating polymers that release by proton or thermal degradation mechanisms.

1.3 STATEMENT OF PURPOSE

Indwelling medical devices are subject to a variety of complications such as rejection from the body or the development of bacterial infection. There are several precautionary measures in place to limit the chance of a bacterial infection. Some of these preventative measures include proper sterilization of medical tools, hand washing, sterile gloves and apparel, regular operating room cleaning, etc. Given that bacterial infections

at indwelling devices still occur; new approaches need to be taken to further prevent bacterial infection. Research has shown that NO has antibacterial properties and can be harnessed into applications to fight infection. With the use of a light-controlled NO releasing polymer, it is believed that the necessary surface flux of NO needed to prevent bacterial adhesion can be determined. This is important because several indwelling medical devices can benefit from an antibacterial coating.

Depending on the type and location of the indwelling medical device, different types of bacteria are responsible for infection. The main target application envisioned to demonstrate the utility of this approach for coating medical devices is for use in a urinary catheter. In order to be an effective treatment in preventing bacterial infection, bacterial strains that are common culprits of urinary tract infections were chosen: *Staphylococcus epidermidis*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and *Escherichia coli*. Using a method similar to the screening of antibiotics for effectiveness, the antibacterial properties of NO can be quantified. The goal of this research is to determine the necessary NO surface fluxes needed to prevent bacterial growth as well as prevent bacterial adhesion.

To be characterized as an effective antibacterial treatment, a standard of testing was developed to be comparable to current antibacterial treatments. It was shown that with an increase in NO surface flux there is a greater inhibition on bacterial growth. Testing was done to determine the NO surface flux needed to prevent bacterial growth on a bacteria coated surface. Adjustments to the NO surface flux were made with the use of photoinitiated NO release. A second characterization was done to determine the

necessary NO surface flux required to prevent bacterial adhesion when a material was submerged in bacteria rich solution.

The NO releasing polymer was shown to provide localized treatment to bacterial infection, only affecting bacteria on or in very close proximity to the polymer. This is very beneficial in the treatment of bacterial infections specifically at the site of medical devices rather than systemically affecting patients (i.e., disruption of normal gut and intestinal bacteria that results from administration of oral antibiotics).

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CHAPTER 2

BACTERIAL GROWTH INHIBITION WITH NITRIC

OXIDE

As was previously discussed, catheter associated urinary tract infections (UTIs) are the most frequent healthcare-associated infection in the United States [1]. There is a growing increase in antibiotic-resistant bacterial species, and therefore a need for a new approach to minimize, as well as treat, bacterial infections without the use of antibiotics. Current literature indicates that nitric oxide (NO) possesses the ability to treat bacterial infections [2]. With the use of the NO releasing polymer developed by Frost et al., the effectiveness of NO as a viable treatment option against common culprits of infection can be evaluated.

2.1   SELECTION OF BACTERIA

Of the wide array of known bacterial species, four species that possess different characteristics and that are known to be significant sources of healthcare associated infections were selected to serve as the test organisms. Each strain of bacteria had qualities and virulence factors that made them ideal choices for testing growth inhibition due to NO. The four bacterial strains to be evaluated were *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus aureus* (*S. aureus*), methicillin-resistant *Staphylococcus aureus* (MRSA), and *Escherichia coli* (*E. coli*). *S. epidermidis*, *S. aureus*, and MRSA are three of the most common species associated with human diseases [3], and of all the

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community-acquired UTIs, 80% are the result of *E. coli* [3].

2.1.1   GRAM-POSITIVE COCCI

S. epidermidis, *S. aureus*, and MRSA are all gram-positive cocci species of bacteria, and are all opportunistic pathogens. *S. epidermidis* is part of the normal skin flora and has the ability to produce a biofilm. On the other hand, *S. aureus* is part of the normal skin and nasal passage flora. *S. epidermidis* can be referred to as the most recovered staphylococcus because it is extremely sticky and tends to adhere to medical devices such as catheters. This stickiness is due to the ability to produce adhesins to allow attachment to host matrix proteins including collagen and fibronectin, but most importantly the production of adhesins that promote attachment to synthetic medical devices [4]. *S. aureus*, referred to as the golden staphylococcus for its color [3], is becoming more resistant to various antibiotics hence the evolution of MRSA which is a *S. aureus* strain resistant to a once effective broad spectrum antibiotic, methicillin. *S. aureus* continues to grow in antibiotic resistance. One common treatment to MRSA strains is the antibiotic vancomycin, but there are now strains of *S. aureus* referred to as VRSA due to their resistance to vancomycin.

2.1.2   GRAM-NEGATIVE RODS

E. coli is characterized as a gram-negative rod strain of bacteria. Out of the whole *Escherichia* genus, *E. coli* is considered the most common and important [3], and can be found as part of the normal intestinal flora. *E. coli* strains responsible for gastroenteritis have been subdivided into five groups, with each group possessing specialized virulence

factors that include adhesins and exotoxins. The adhesins are used for attaching to areas other than intestines, while the toxins are used to damage host tissue [3]. As previously stated, *E. coli* is responsible for a large majority of UTIs which can be attributed to its virulence factors in addition to convenience based on close proximity to the urethra on the human body, especially for women.

2.2   EXPERIMENTAL SETUP

To begin the evaluation of the use of NO as a preventative and treatment option for bacterial infection, positive and negative control materials were also used for comparison purposes. In total, four materials were used: three different controls and the NO releasing polymer.

2.2.1   SELECTION OF MATERIALS

Two known antimicrobial controls (Ioban2 and levofloxacin) and one positive control (RTV3140 surgical grade silicone rubber) that allow bacterial growth to occur were selected. Ioban2 is a type of antimicrobial film incision drape developed by 3M (St. Paul, MN) that releases the antimicrobial agent iodophor, a known skin antiseptic and bactericide [5]. The purpose of the drape is to create a broad sterile surface surrounding and abutting the wound edges. The second negative control was paper disks saturated with the potent broad spectrum antibiotic, levofloxacin, which is part of the quinolones family. Levofloxacin is an effective antibiotic against all four strains of bacteria being tested. The antibiotic works by inhibiting DNA gyrase and topoisomerase IV, two important enzymes for DNA replication and transcription [6]. The positive control chosen

was RTV3140, a surgical grade silicone rubber. RTV3140 was chosen because not only is it the base material of the NO releasing polymer, but it is also commonly used in medical devices. It possesses no inherent antimicrobial properties.

S-Nitroso-N-acetyl-D-penicillamine linked to polydimethylsiloxane (SNAP-PDMS) is a proprietary polymer developed by Frost et al., and as previously discussed, was chosen for its controlled release of NO. The controlled release of NO is important for determining the effective NO surface flux needed to prevent and treat bacterial adhesion.

2.3   DISK SUSCEPTIBILITY TESTING

Antibiotic susceptibility testing was undertaken to evaluate the effectiveness of NO as an inhibitor of bacterial growth, in addition to comparing results with standard materials being used to prevent bacterial infection.

2.3.1   PREPARATION OF MATERIALS

Specific bacterial strains were chosen based on their use in similar current literature: *S. epidermidis* (ATCC 12228), *S. aureus* (ATCC 29213), MRSA (ATCC 33591), and *E. coli* (ATCC 11775), and were purchased from Microbiologics, Inc. (St. Cloud, MN) in single strain LYFO DISK pellets.

Ioban2 surgical drapes were donated by Dr. Jennifer Bow, while the levofloxacin disks used for antimicrobial susceptibility testing were purchased from Hardy Diagnostics Inc. (Santa Maria, CA). The levofloxacin disks are a 6mm diameter filter paper with a known concentration of 5μg of antibiotic loaded onto them. Ioban2 disks

were punched out of the drape with a 4mm diameter cork borer. Dow Corning RTV3140 was purchased from Ellsworth Adhesives (Germantown, WI). SNAP-PDMS was synthesized in the laboratory [7]. Both the RTV3140 and SNAP-PDMS materials were solution cast into large tri-layer films approximately 30mm in diameter. RTV3140 solution was made with a 1:10 weight percentage RTV3140 coating to toluene which was purchased from Sigma Aldrich (St. Louis, MO). Once the RTV3140 and SNAP-PDMS films were completely cured, smaller disks were cut from the parent film. For the Kirby-Bauer method for disk testing and 10 day testing, the same 4mm diameter cork borer was used to obtain sample disks of the same sizes. For the quantitative streak testing, 8mm diameter disks were punched with a cork borer then a 4mm diameter disk was cut from the center of the 8mm disks.

2.3.2   LIGHT BOARD SETUP

A light board was assembled in order to reproducibly illuminate the SNAP-PDMS to control NO release from the polymer. The system was created to shine light from the bottom to the samples. A black Delrin board (3/8" x 12" x 12") was fitted to hold four 100mm media plates in a square design. Poly(vinyl chloride) (PVC) tubing of 101.6mm (4in) diameter was purchased from the hardware store and cut down to four rings of 60mm (2.36in) height. Each ring was drilled with three 6.35mm (0.25in) diameter holes to create a level plane of 20mm (0.807in) from the top where shelf pegs were inserted to hold the media plates at a level and equal height. Holes were drilled into the Delrin board to fit nuts and bolts in place to hold the PVC rings in the same location between different experiment batches.

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SNAP-PDMS is a light-controlled releasing polymer, so holes were placed in the board to allow the placement of light emitting diodes (LEDs) to stimulate NO release. Each ring placement possessed four locations for LEDs epoxied in a similar square design. The 460nm VAOL-5GSBY LEDs were purchased from Mouser Electronics, Inc. (Mansfield, TX). The distance between the LEDs was 32mm to an adjacent LED to assure no overlap in the focal point of the LEDs. The LEDs were connected in series with an RS-200 series resistance substitution box purchased from IET LABS Inc. (Roslyn Heights, NY) and a 2 amp multi-output power supply purchased from Circuit Specialists (Tempe, AZ).

PVC tubing was used to shield individual plates from neighboring light and affecting one another, as well as to provide a shelf to hold the media plates at the same distance from the LEDs. A consistent height above the LEDs was essential for uniformity, and to keep the focal points of each LED the appropriate size such that they did not overlap with one another. Figure 2.1 provides a visual of the light board setup from the side (A) and a view from the top of the board (B).

2.3.3   STOCK PLATE PREPARATION

Before any experimental testing could proceed, stock plates of the bacteria were created; the same procedure was used for each type of bacteria. To start, Mueller-Hinton 28ml filled 15x100mm media plates, sterile disposable 1ul inoculating loops, and sterile cotton swabs were purchased from Hardy Diagnostics Inc. (Santa Maria, CA). The Mueller-Hinton plates were used in testing to be discussed later.

Figure 2.1. Visual representation of designed light board setup in its entirety from the side (A), and an image from the top of setup (B).

The media plates and LYFO-DISK bacterial pellets were removed from storage in the refrigerator and allowed to acclimate to room temperature. Instructions provided from Microbiologics Inc. were followed. A sterile test tube was filled with 0.5ml of sterile water, and then a single bacterial pellet was aseptically added to the test tube. The pellet was then crushed with a sterile cotton swab and stirred until the solution was homogeneous. Excess liquid was removed along the inside of the test tube before inoculating approximately one third of the media plate. Bacteria were then streaked for isolation using sterile, disposable loops. The inoculated plate was then sealed and placed inverted in the incubator at 37˚C and allowed to grow. The excess solution in the tube was incubated for 18-24 hours before removal and storage in the refrigerator.

2.3.4 KIRBY-BAUER METHOD FOR LIGHT VERSUS DARK TESTING

The Kirby-Bauer disk susceptibility testing method was the chosen foundation for experimental setup. The Kirby-Bauer test is used to determine the sensitivity of bacteria to antimicrobial compounds [8]. In the simplest of terms, a controlled density of bacteria is lawned over a Mueller-Hinton media plate then antibiotic disks are placed on the plate. The antibiotic disks are filter paper with a known drug concentration that will diffuse in the media and affect the bacterial growth. Therefore, it is important to place the antibiotic disks with enough space between one another such that there is no overlap between diffused antibiotic. The plates are then incubated for 18-24 hours, depending on the bacteria being tested. The circular area of inhibition (if present) is measured (diameter) and compared to a standard table to determine if the strain of bacteria is resistant or sensitive to the antibiotic being tested.

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As a control test, light and dark testing was done. The purpose of the light versus dark testing was to determine whether or not there was a change in bacterial growth when placed on the light board setup and exposed to maximum LED light (12V power supply) or no light. The Kirby-Bauer disk susceptibility test protocol was adapted to fit experimental needs by following a similar setup with the exception of no placement of disks on the inoculated plates.

Mueller-Hinton media was the standard media chosen for susceptibility testing. Mueller-Hinton 28ml filled 15x100mm media plates were purchased from Hardy Diagnostics. Bacterial suspensions were created to the equivalent 0.5 McFarland standard. To verify the appropriate cell density UV-VIS spectroscopy was used to determine an absorbance between 0.08 and 0.13 at 625nm wavelength which is $1x10^8$ and $2x10^8$ (CFU/ml). Bacterial solutions were created by adding 2ml of sterile 0.85% saline to a sterile, disposable cuvette, and isolated bacterial colonies from a stock plate were added and stirred until uniform clarity. Depending on the UV-VIS absorbance reading, more bacteria or more saline was added until the desired absorbance occurred. Using a sterile cotton swab, the Mueller-Hinton media plates were inoculated with the bacterial suspension, covering the plate's entirety. The plates were sealed with parafilm and placed inverted in the incubator at 37˚C for 24 hours. For the light testing, the decade box was set to 130ohms and voltage set to 12V. For the two conditions, maximum light (12V) and minimal light (dark), four plates each were tested. Bacterial density colonies were imaged with ViTiny Pro10 Plus Portable Digital Microscope 10x - 200x purchased from Oasis Scientific, Inc. (Greenville, SC) and compared with MATLAB analysis.

To begin the analysis the images were scaled and aligned with one another; the center of each plate was aligned before converting to greyscale. The plates were then sectioned into twelve 12 x 12mm squares; however, given the presence of physical markings on the plate, such as the tick mark, one square was eliminated from each plate as seen in Figure 2.2. The sections were then converted to black and white. Finally, a MATLAB script was written to compare the percentage of black area to white area which was related to the area of the plate covered by bacteria. It was these areas and percentages that were used to compare bacterial density when exposed to light and dark conditions.

2.3.5   KIRBY-BAUER METHOD FOR DISK TESTING

As for the remaining testing, the procedure stated above was followed except after inoculation of the plates, the four materials being tested were placed on the media plate. A template was made to control sample disk placement. The four material disks were placed aseptically on each plate. Each media plate consisted of an Ioban2, levofloxacin, RTV3140, and SNAP-PDMS disk sample. Lastly, the plates were sealed with parafilm and placed inverted in the incubator at 37°C. Pictures were taken at 18 hours, and bacterial growth under each disk sample was compared qualitatively both with and without disks present.

2.3.6   KIRBY-BAUER METHOD FOR 10 DAY TESTING

For the ten day testing, MRSA was lawned over Mueller-Hinton media plates following the Kirby-Bauer method. Instead of the four different material disks being placed, one RTV3140 and one SNAP-PDMS disk were placed on the plate. The disks
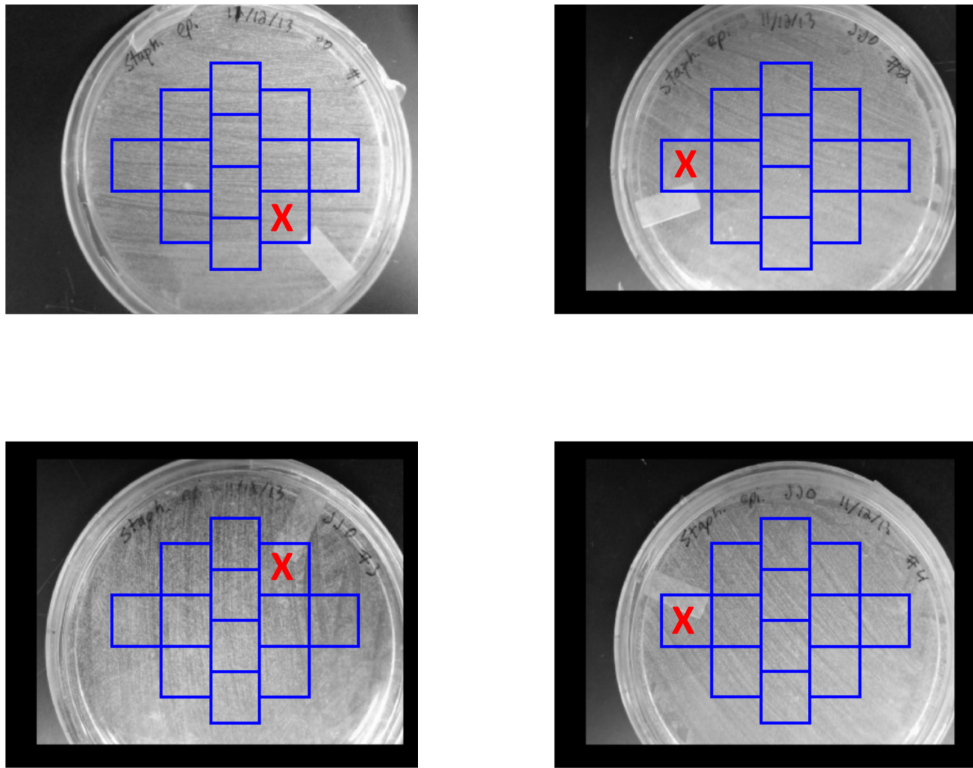
Figure 2.2. MATLAB images of the four *S. epidermidis* dark testing plates. Each plate was sectioned into twelve 12 x 12mm squares with one square eliminated due to physical properties on the plate i.e. the tick mark used for scaling.

were imaged every day for ten days before being removed. The plates were returned to the incubator for an additional day, and the voids of the disks were imaged.

2.3.7 QUANTITATIVE STREAK TESTING

Quantitative streak testing was a combination testing method based on the previously discussed Kirby-Bauer method for disk testing and the urine streak—semi quantitative method to assess bacterial viability on RTV3140 disks compared to SNAP-PDMS disks. The urine streak test is designed to quantify bacterial species responsible for urinary tract infections. This method utilizes a volumetric loop calibrated to hold either 0.001ml or 0.01ml of a sample. The basic procedure for this testing is taking a loopful of urine and streaking it across the diameter of a blood agar plate. Using the same loop, the plate is turned 90° and streaked again in a zigzag pattern to uniformly distribute the bacteria over the entire surface area of the plate. After the plate is allowed an appropriate incubation period, the original cell density (OCD) can be calculated by counting the colony forming units (CFU) and dividing that value by the loop volume (0.001ml or 0.01ml). OCD is reported as colony forming unites per milliliter (CFU/ml) [9].

The four bacterial species used in the previous testing (*S. epidermidis*, *S. aureus*, MRSA, and *E. coli*) were used for this method. Based on the Kirby-Bauer method, bacteria of an appropriate density was lawned onto Mueller-Hinton plates. Using two Mueller-Hinton plates, three polymer samples were placed on each plate: three RTV3140 and three SNAP-PDMS. Each polymer sample consisted of an 8mm diameter ring with a

concentric 4mm diameter disk punched from the center. The plates were incubated at 37°C for 18 hours in dark conditions at which point they were imaged both with and without the center disks present using a ViTiny Pro10 Plus Portable Digital Microscope 10x - 200x purchased from Oasis Scientific, Inc. (Greenville, SC). The removed disks were placed in sterile 2ml Cryo.s Cryogenic Storage Vials purchased from VWR International (Radnor, PA) along with 1ml of prepared tryptic soy broth (TSB) #2 dehydrated culture media purchased from Hardy Diagnostics Inc. (Santa Maria, CA).

The submerged disks were again incubated at 37°C with gentle shaking on a VWR Mini Shaker 15 purchased from VWR International (Radnor, PA) for 90 minutes. Streak plates based on the urine streak method were done from 1:10 dilutions of the TBS used to bathe the center disks in the previous step with sterile TSB of each incubated vial after 5 minutes and 90 minutes of incubation. The streak plates were sealed and incubated at 37°C for 24 hours. Lastly, plates were imaged and the bacterial colonies present on each streak plate were counted and OCD was determined to compare RTV3140 disks to SNAP-PDMS disks.

2.3.8   NITRIC OXIDE RELEASE MEASUREMENTS

A Sievers Nitric Oxide Analyzer (NOA) 280i (GE Instruments, Boulder, CO) was calibrated and used to evaluate the NO release for the SNAP-PDMS materials. Individual SNAP-PDMS disks from the same polymer film batch used in experiments were analyzed to determine the NO surface flux at 37°C with ambient air sweep gas. For the Kirby-Bauer method for disk testing a sample size of N=7 was used. For the quantitative

streak testing, SNAP-PDMS disks were from a different batch and a sample size of N=3 was used for analysis. Total NO released was measured over 18 hours and the average NO surface flux over the final 10 minutes was reported.

2.4   RESULTS

To evaluate the effectiveness of SNAP-PDMS as a treatment and preventative approach to bacterial infection, experimental testing was completed to assess both the localization and effective dose needed to inhibit bacterial growth by comparing different levels of NO release and different known antimicrobial agents.

2.4.1   LIGHT VERSUS DARK TESTING

The first variable tested was to determine if the light itself used to initiate NO release from SNAP-PDMS causes changes in bacterial growth and proliferation. Plates were lawned with *S. epidermidis* and placed in both light and dark conditions for 24 hours. Figure 2.3 shows images of the plates after the 24 hour incubation period. The images were analyzed as described for density of bacteria present. Table 2.1 lists the percentage of plate covered with bacteria by zone along with standard deviations and variances for all eight plates exposed to light or dark conditions. The comparison of bacterial density on the plates incubated in the dark compared to plates incubated with light exposure showed no significant difference between the percentage bacterial coverage (N=8) using one-way ANOVA. Table 2.2 shows the critical F values for a 5% confidence level for the corresponding degrees of freedom as well as the observed F value calculated with MATLAB ANOVA One-Way testing. By comparing the F critical
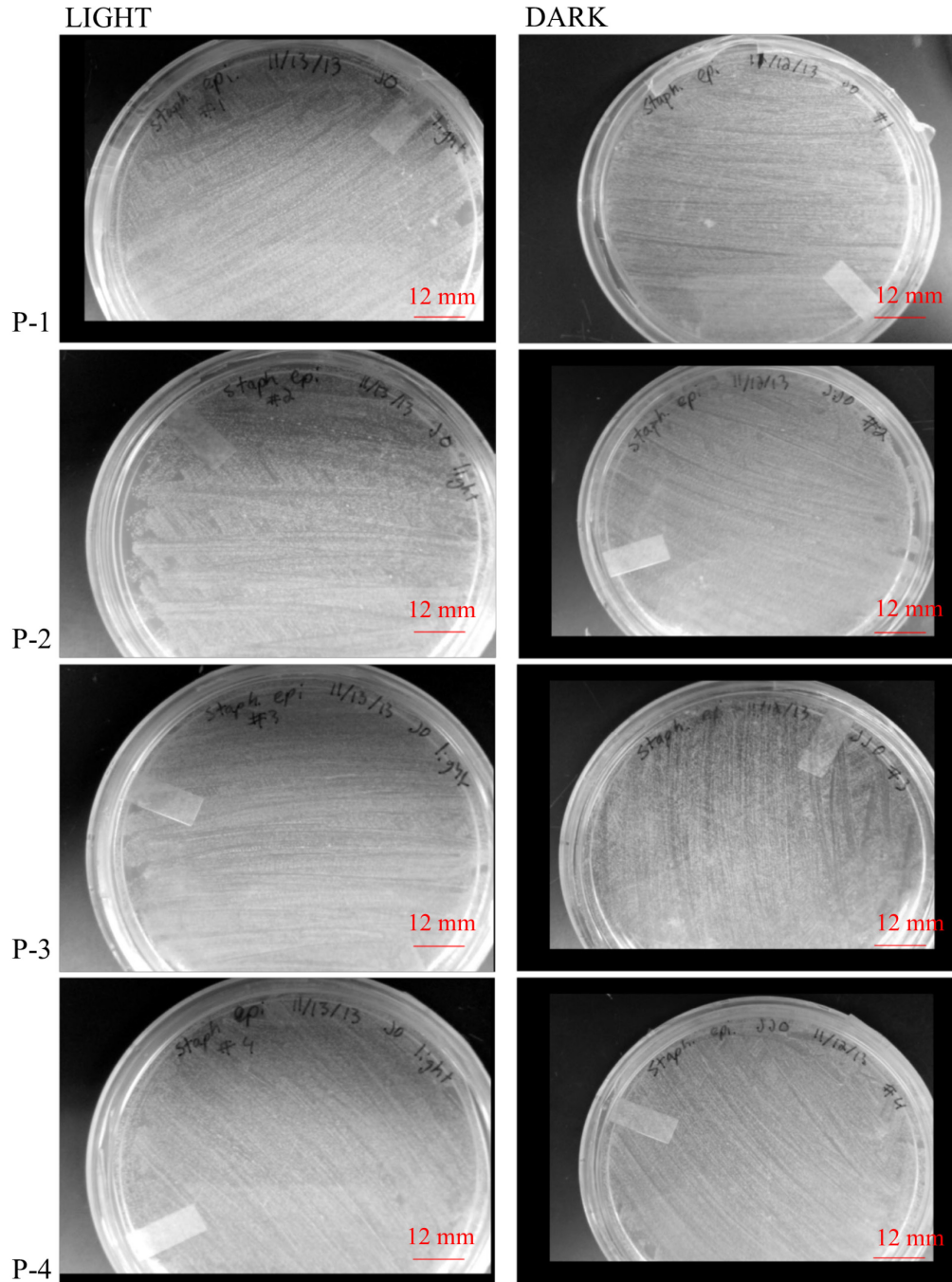
Figure 2.3. MATLAB images of all eight plates from light versus dark *S. epidermidis* after 24 hours of incubation at 37°C. The left column is the four light plates that were exposed to maximum light for the light board setup in order from P-1 to P-4 down the page. The right column is the four dark plates in the same order down the page.

Table 2.1.   Comparison of *S. epidermidis* density based on overall coverage of the media plate with the use of MATLAB output values for percentages for all eight plates in the light versus dark testing along with additional statistical information.

Comparison of Bacterial Density for Light and Dark Testing by Percent Coverage								
% No Bacterial Coverage	Light Plate 1-Plate 4				Dark Plate 1-Plate 4			
Zone 1	44.24	41.90	46.89	42.17	45.63	37.16	44.93	46.94
Zone 2	40.72	43.53	44.91	44.91	41.96	46.63	45.40	45.58
Zone 3	42.90	44.26	41.54	45.54	39.04	37.16	45.58	37.68
Zone 4	47.67	40.35	42.91	43.09	47.68	51.60	46.96	47.15
Zone 5	41.17	44.33	43.04	42.60	47.86	42.46	46.43	38.69
Zone 6	41.57	41.74	42.55	47.36	46.21	46.43	51.91	45.80
Zone 7	45.12	48.01	47.89	42.27	46.71	43.61	46.50	39.19
Zone 8	43.48	38.23	40.61	44.10	38.86	46.95	42.44	43.41
Zone 9	41.56	41.98	41.27	41.13	43.23	45.97	42.78	44.81
Zone 10	40.56	47.93	42.71	42.66	45.37	42.10	39.14	41.12
Zone 11	40.60	46.76	45.15	45.40	49.44	48.22	40.82	39.19
Average % of all Zones	**42.70**	**43.55**	**43.59**	**43.75**	**44.71**	**45.31**	**44.81**	**42.69**
Standard Deviation	**4.64**	**8.86**	**4.96**	**3.18**	**3.37**	**3.64**	**3.30**	**3.43**
Variance	**2.15**	**2.98**	**2.23**	**1.78**	**11.33**	**13.71**	**10.89**	**11.73**

Table 2.2.   Comparison of critical F values at α=0.05 to the observed F value generated from MATLAB ANOVA one way testing.

ANOVA One Way Comparison of F Values at α=0.05		
Critical Values	Observed Value	Prob>F
$F_{7,60}=2.1665$	$F_O=1.02$	$P_O=0.4217$
$F_{7,120}=2.0868$		

and F observed values, there is no statistical difference between all eight sets of data (four light and four dark) because F observed (1.02) is less than F critical (2.0868) as well as P observed (0.4217) is greater than 0.05 confidence level.

2.4.2   DISK SUSCEPTIBILITY TESTING

Figure 2.4 illustrates that all bacterial strains were sensitive to Ioban2 and levofloxacin. Figure 2.5 shows images of all four bacterial strains with RTV3140 and SNAP-PDMS disks present. It was seen that bacteria appear unaffected by RTV3140 whereas there is a slight density change and colony size difference next to the SNAP-PDMS disks. The true evidence to SNAP-PDMS being antimicrobial is detected when the polymer disks are removed, as seen in Figure 2.6. It was then that there was a cloud of bacterial smear under the RTV3140 disks whereas under the SNAP-PDMS disks the media was clear and bacteria free. Lastly, Figure 2.7 demonstrates whether or not MRSA bacteria were able to regrow in the voids left behind from the disks. Ioban2, RTV3140, and SNAP-PDMS had bacterial regrowth while levofloxacin had stunted bacterial regrowth as the streak got closer to the center of where the disk was placed. Streak data was done for the other three bacteria, but not pictured.

2.4.3   TEN DAY RTV3140 AND SNAP-PDMS DISK TESTING

Figure 2.8 represents a series of enlarged photos showing bacterial growth around RTV3140 and SNAP-PDMS disks after one day (24hrs), five days (120hrs), and ten days (240hrs). It is observed that with RTV3140 disks the *S. epidermidis* bacteria grew uninhibited, and the colonies were of uniform size and blended together. On the other
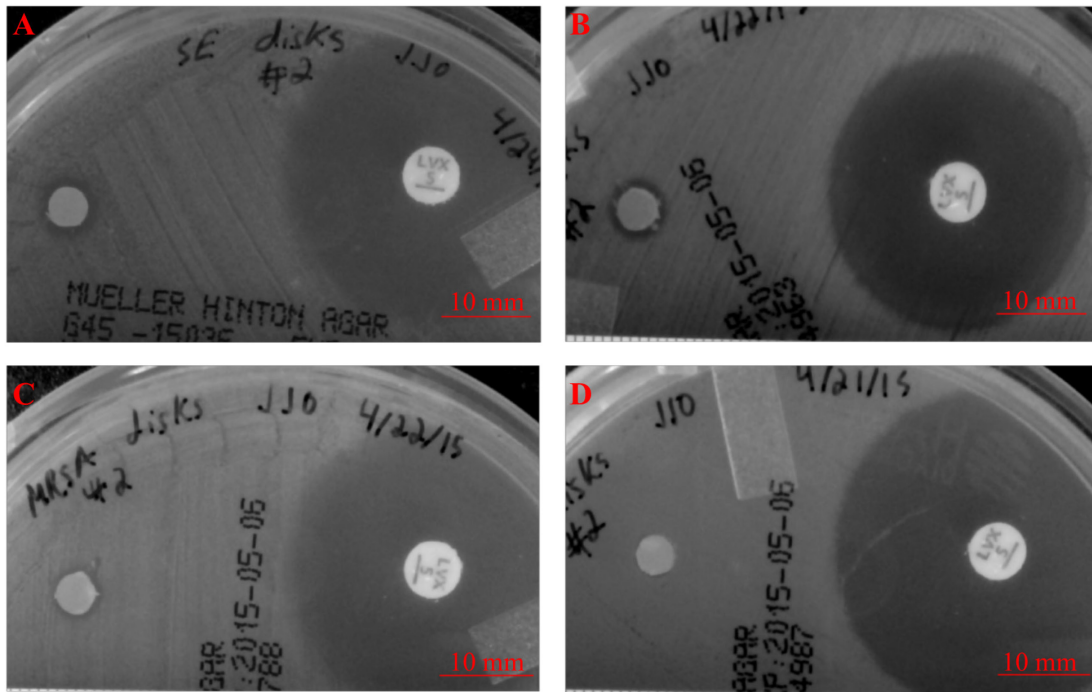
Figure 2.4. Images of Ioban2 (left disks) and levofloxacin (right disks) disks for plate 2 of *S. epidermidis* (A), *S. aureus* (B), MRSA (C), and *E. coli* (D) after 18 hours of incubation at 37°C in dark conditions. Each image was converted to grayscale in MATLAB to improve contrast.
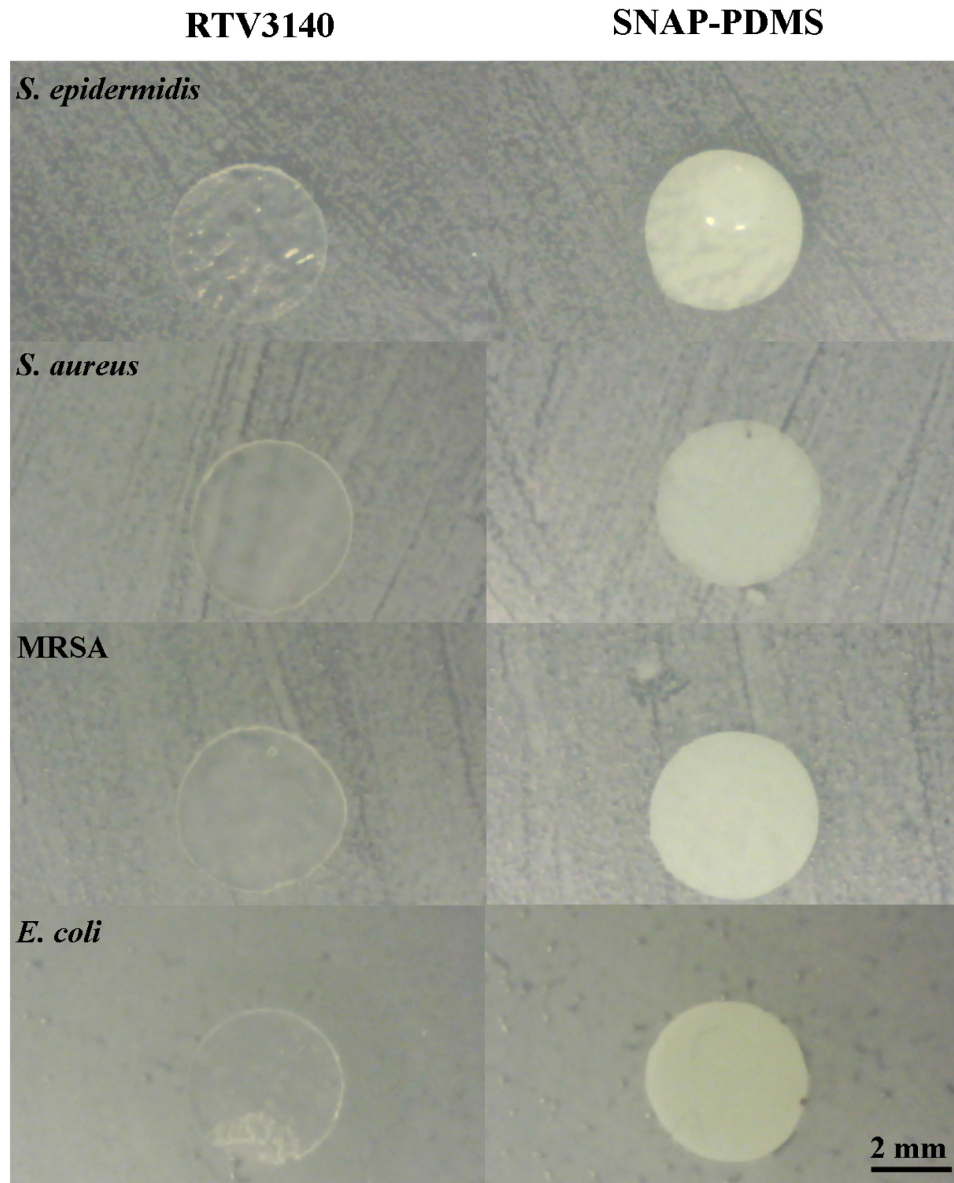
RTV3140   SNAP-PDMS

Figure 2.5.   Images of RTV3140 disks (left) compared to SNAP-PDMS disks (right) for *S. epidermidis*, *S. aureus*, MRSA, and *E. coli* strains at dark conditions after 18 hours of incubation at 37˚C for plate 2.
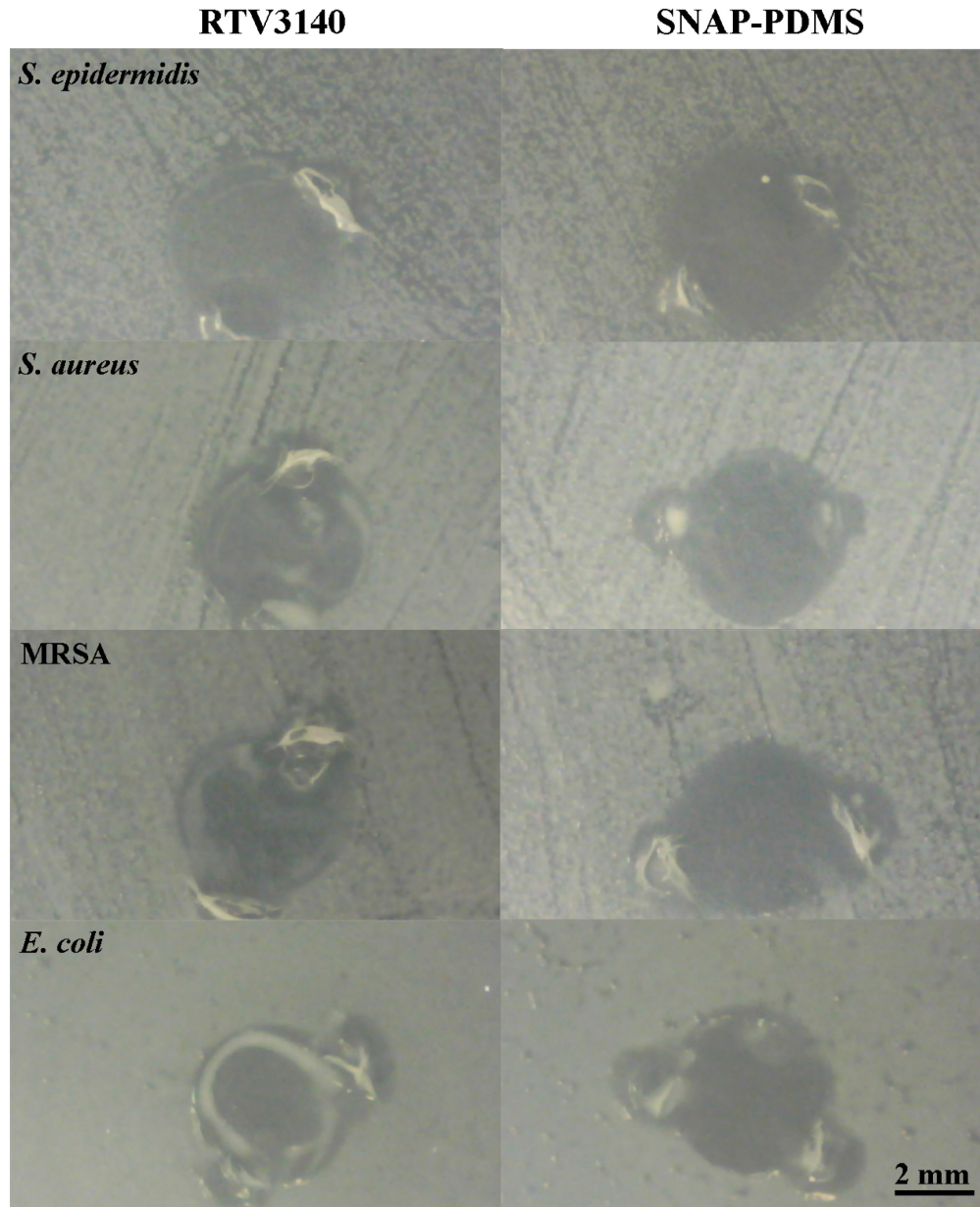
	RTV3140	SNAP-PDMS

Figure 2.6. Images of RTV3140 disks (left) compared to SNAP-PDMS disks (right) for *S. epidermidis*, *S. aureus*, MRSA, and *E. coli* strains at dark conditions after 18 hours of incubation at 37°C for plate 2 after polymer disks were removed.
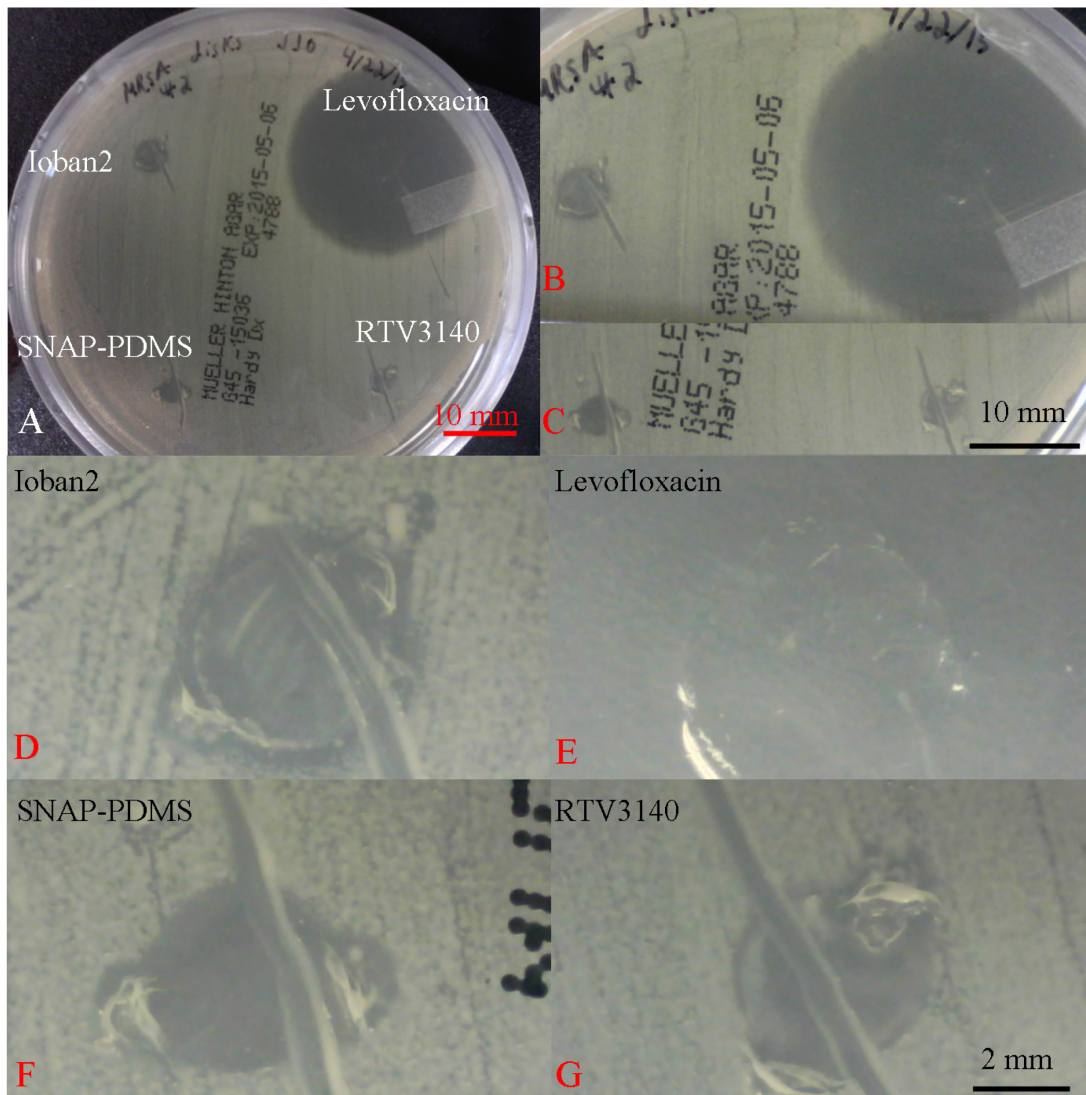
Figure 2.7. Images taken of MRSA plate 2 streaks through disk voids after incubation at 37°C for 24 hours in dark conditions: A) the entire plate, B) Ioban2 (left) and levofloxacin (right), C) SNAP-PDMS (left) and RTV3140 (right), D-G) magnified Ioban2, levofloxacin, SNAP-PDMS, and RTV3140, respectively.
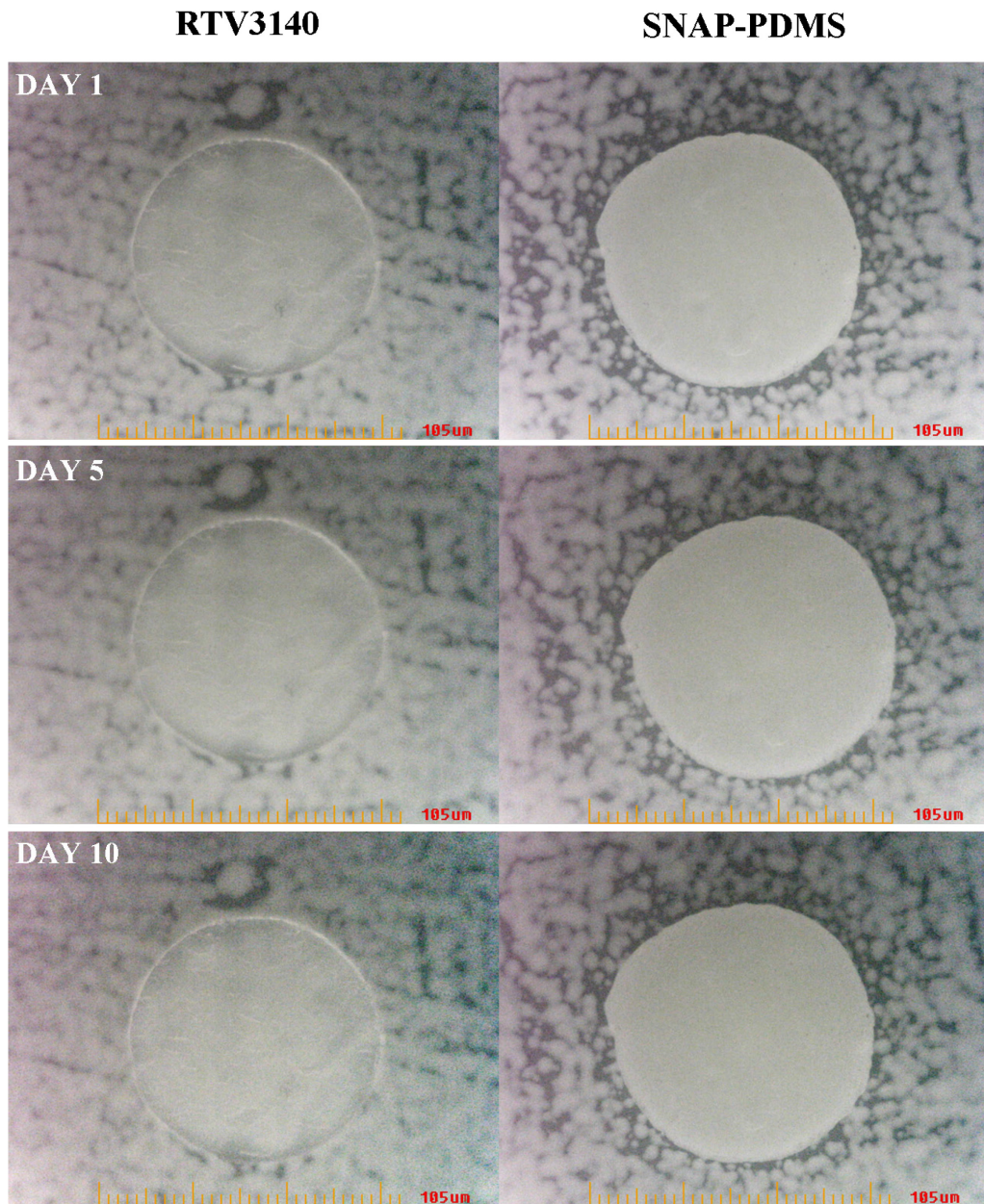
RTV3140   SNAP-PDMS

Figure 2.8.   Images taken of MRSA ten day disk testing comparing RTV3140 (left) to SNAP-PDMS (right) after one, five, and ten days incubation at 37˚C in dark conditions.

hand, bacteria around the SNAP-PDMS disk had smaller bacterial colonies close to the disk and as distance increased away from the polymer the bacteria blended together as seen near the RTV3140 disk. As seen in Figure 2.9, after the ten days of incubation, both polymer disks were removed and voids imaged. It was observed that there are fewer bacteria present under the SNAP-PDMS disk compared to the RTV3140 disk.

2.4.4   QUANTITATIVE STREAK TESTING

Figures 2.10-2.13 are visual representations of magnified images comparing RTV3140 to SNAP-PDMS after the center disks were removed for *S. epidermidis*, *S. aureus*, MRSA, and *E. coli*, respectively. Qualitatively, there are fewer bacteria present under the SNAP-PDMS disks compared to the RTV3140 disks for all four bacterial strains tested. Figure 2.14 and Figure 2.15 are streak plates for *S. epidermidis* after 5 minutes submersion and 90 minutes, respectively. Quantitatively there are no bacteria present for the SNAP-PDMS streaks whereas RTV3130 streaks have a significant amount of bacteria present. Next, Figures 2.16-2.21 reveal the streak results after 5 minutes and 90 minutes for *S. aureus*, MRSA, and *E. coli*, respectively. For all RTV3140 streaks there is an abundance of bacteria whereas for the SNAP-PDMS streaks there are minimal to no bacteria present which validates NO inhibiting bacterial adhesion. Lastly, Table 2.3 compares the OCD (density) for all bacterial strains tested along with averages and standard deviations providing quantitative results revealing the inhibitory effect of NO against bacterial growth.

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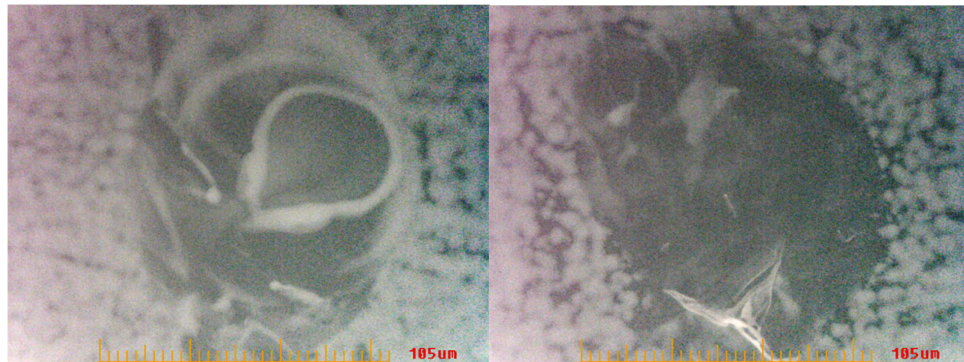
RTV3140   **SNAP-PDMS**

Figure 2.9. Images taken after RTV3140 (left) and SNAP-PDMS (right) disks were removed for ten day MRSA testing.
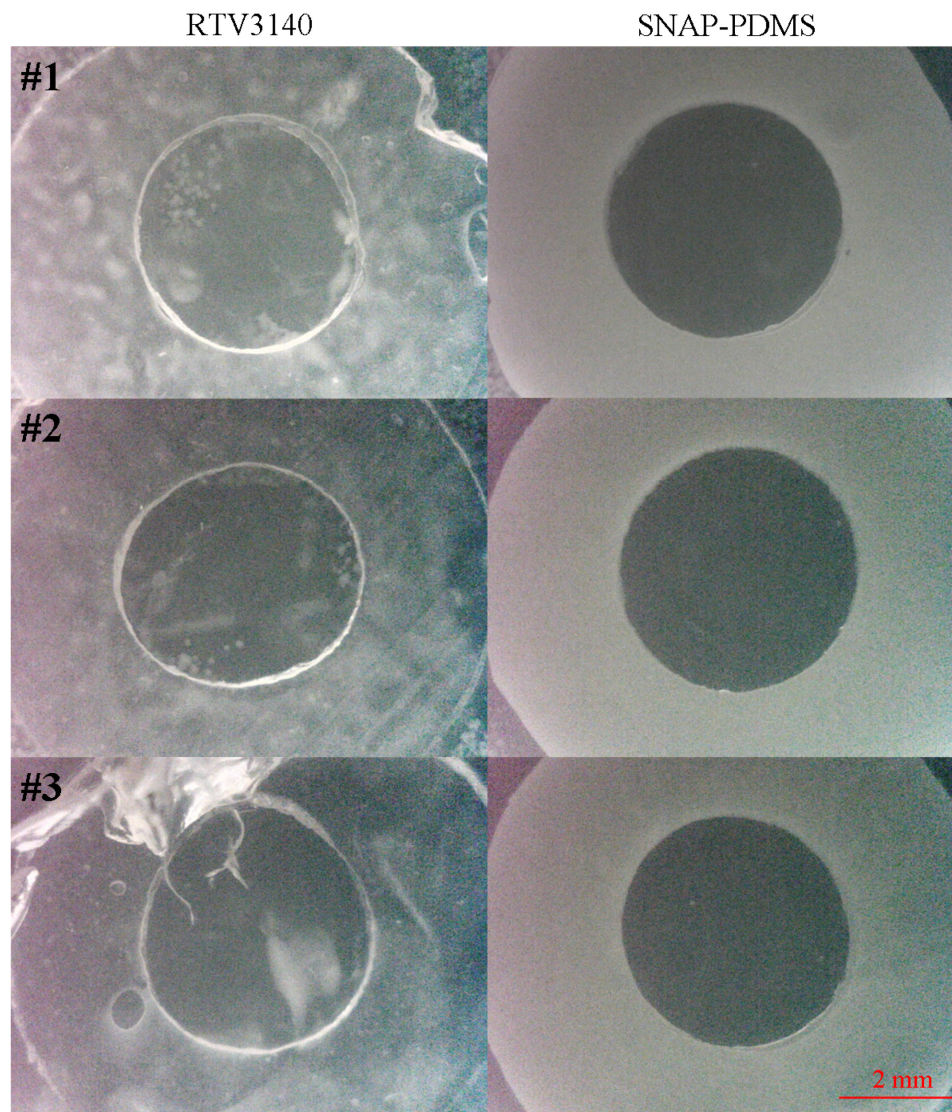
RTV3140   SNAP-PDMS

Figure 2.10. Images taken after disks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) were removed for *S. epidermidis* quantitative testing.
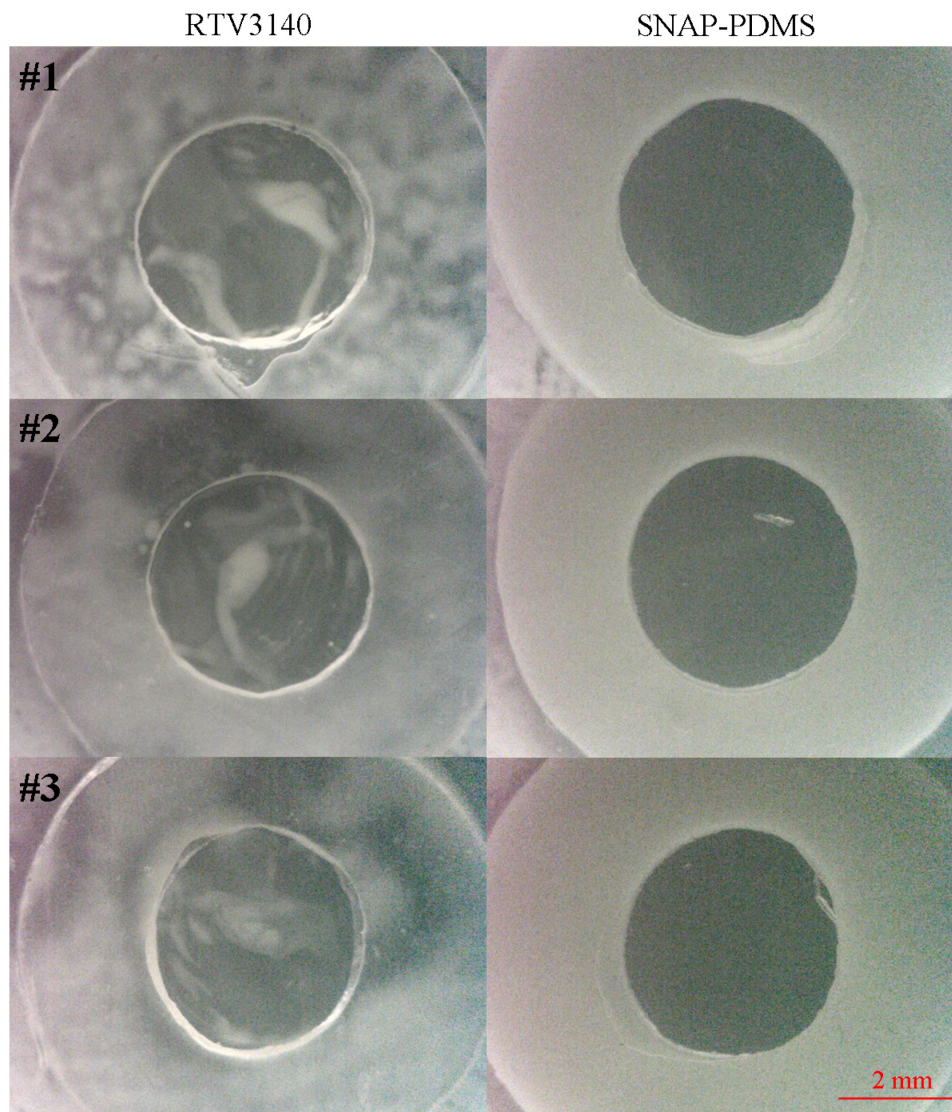
Figure 2.11. Images taken after disks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) were removed for *S. aureus* quantitative testing.

	RTV3140	SNAP-PDMS

#1

#2

#3

2 mm
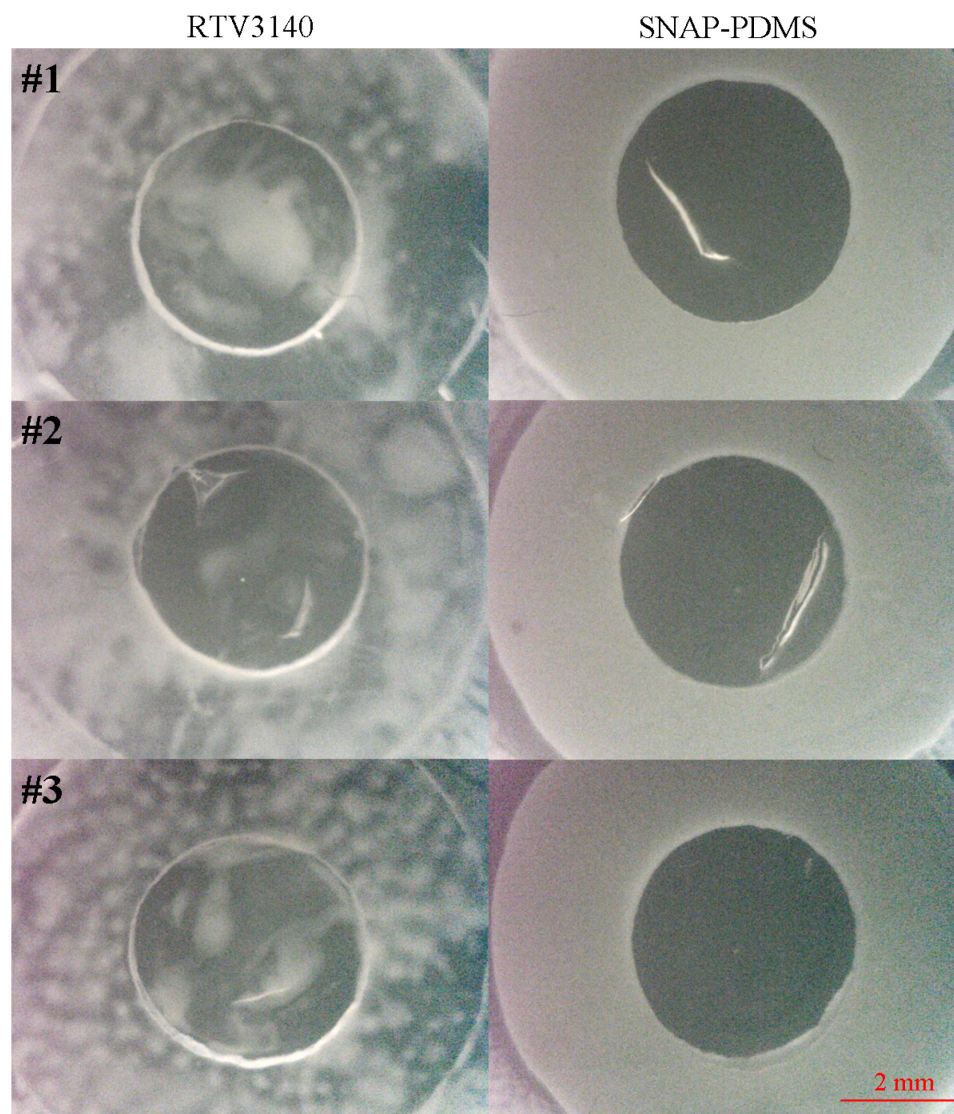
Figure 2.12. Images taken after disks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) were removed for MRSA quantitative testing.
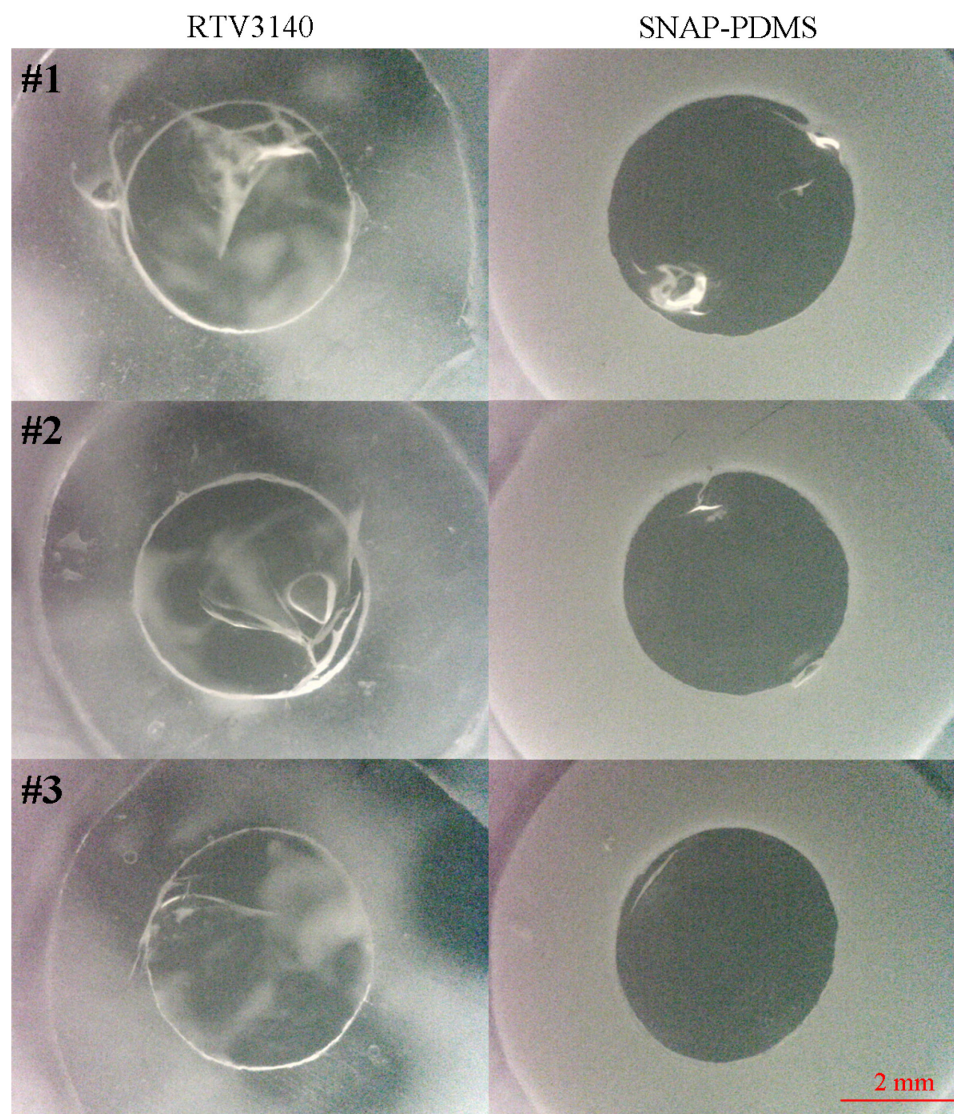
Figure 2.13. Images taken after disks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) were removed for *E. coli* quantitative testing.
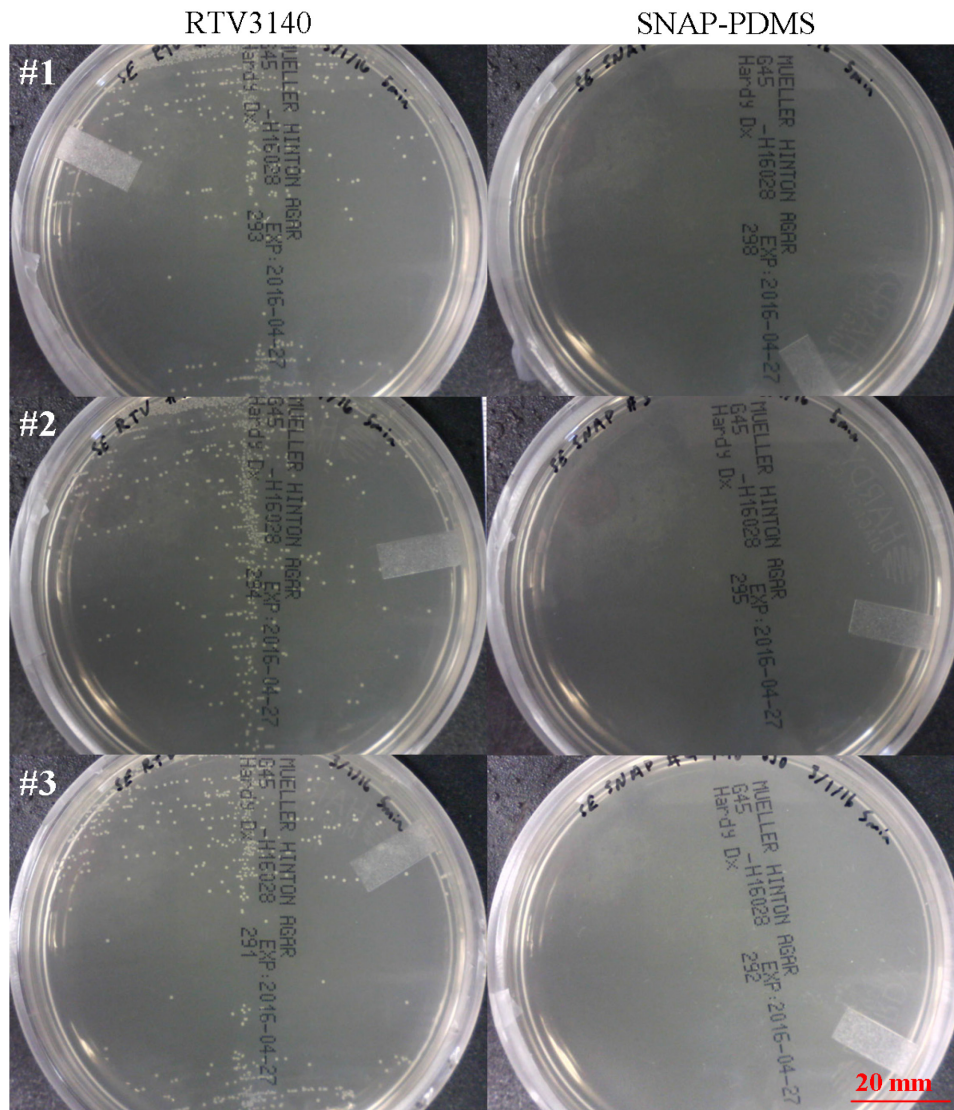
RTV3140   SNAP-PDMS

Figure 2.14. Images taken of 1:10 dilution streaks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) for *S. epidermidis* after 5 minutes.
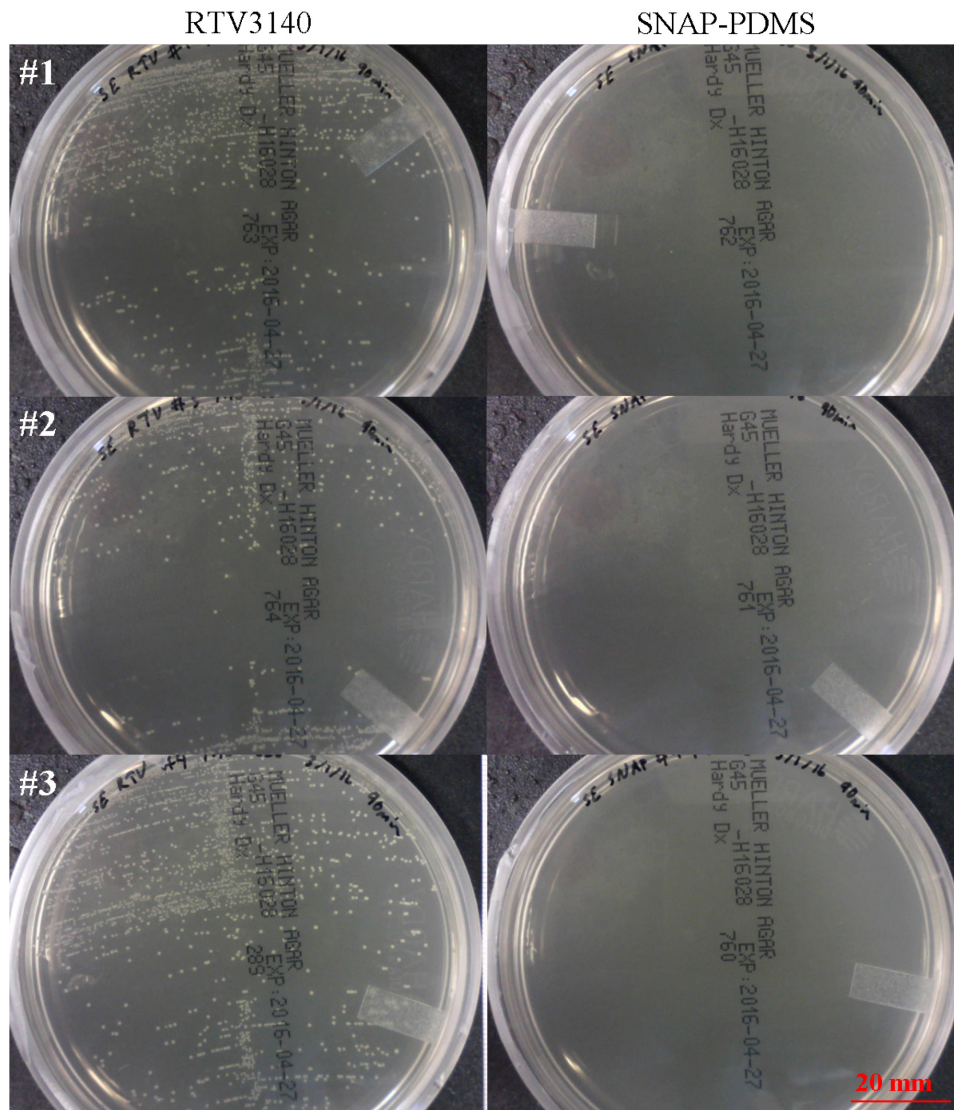
RTV3140   SNAP-PDMS

Figure 2.15. Images taken of 1:10 dilution streaks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) for *S. epidermidis* after 90 minutes.
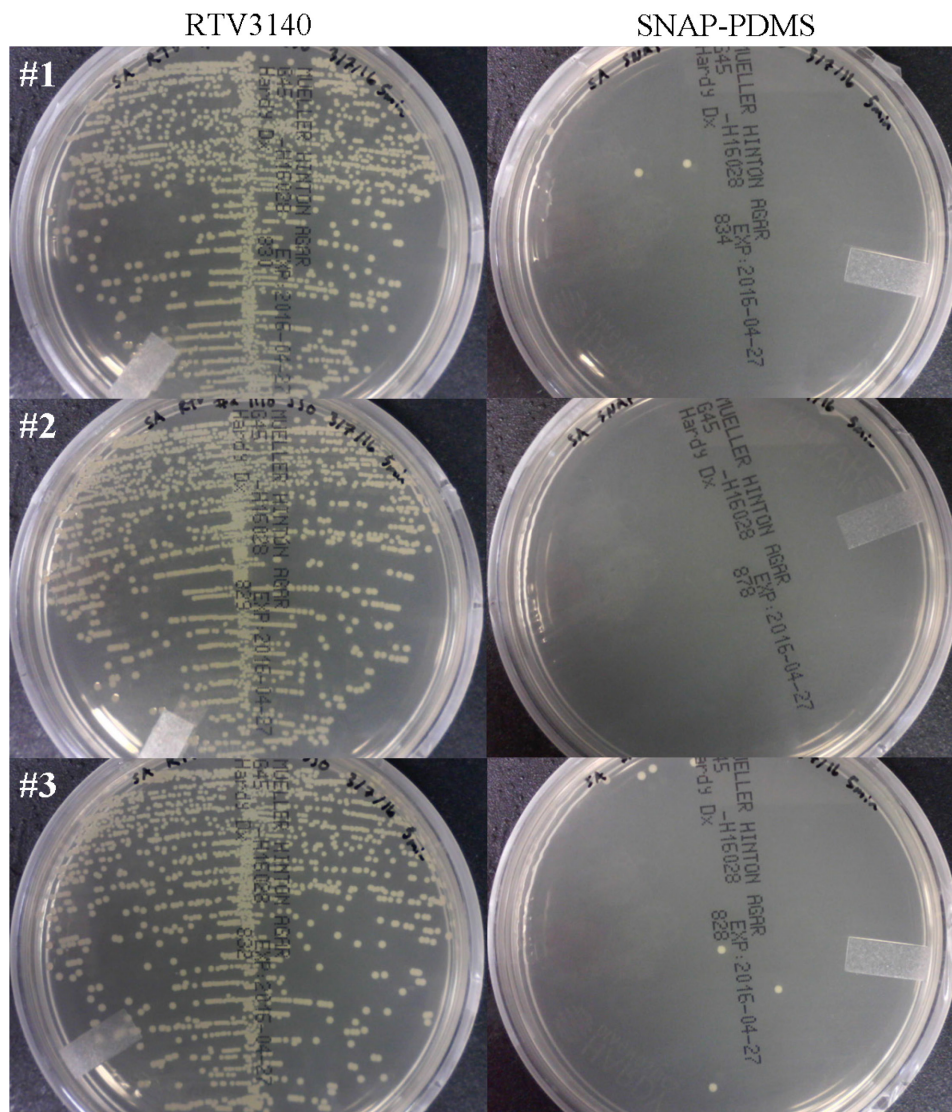
RTV3140   SNAP-PDMS

Figure 2.16.   Images taken of 1:10 dilution streaks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) for *S. aureus* after 5 minutes.
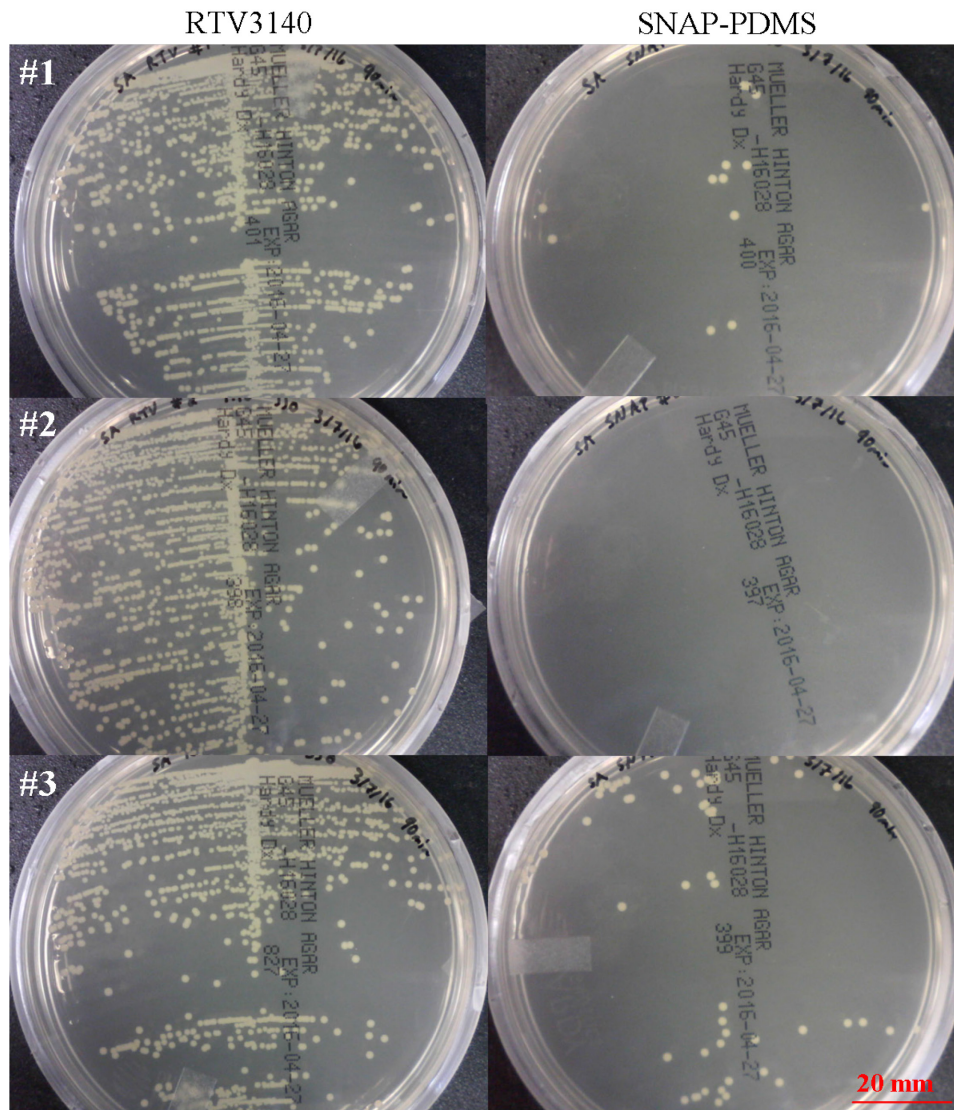
RTV3140   SNAP-PDMS

Figure 2.17.   Images taken of 1:10 dilution streaks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) for *S. aureus* after 90 minutes.
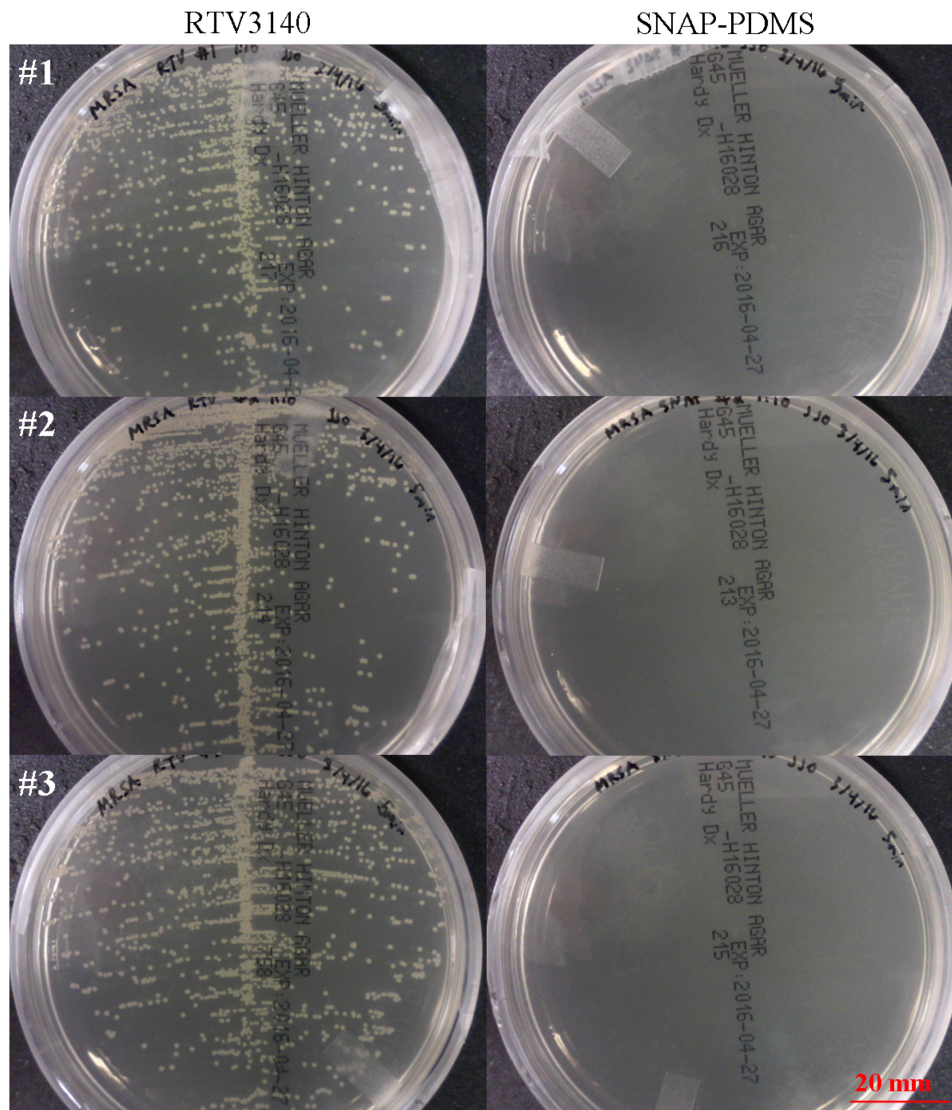
RTV3140   SNAP-PDMS

Figure 2.18. Images taken of 1:10 dilution streaks 1 through 3 for both RTV3140 (left)
and SNAP-PDMS (right) for MRSA after 5 minutes.

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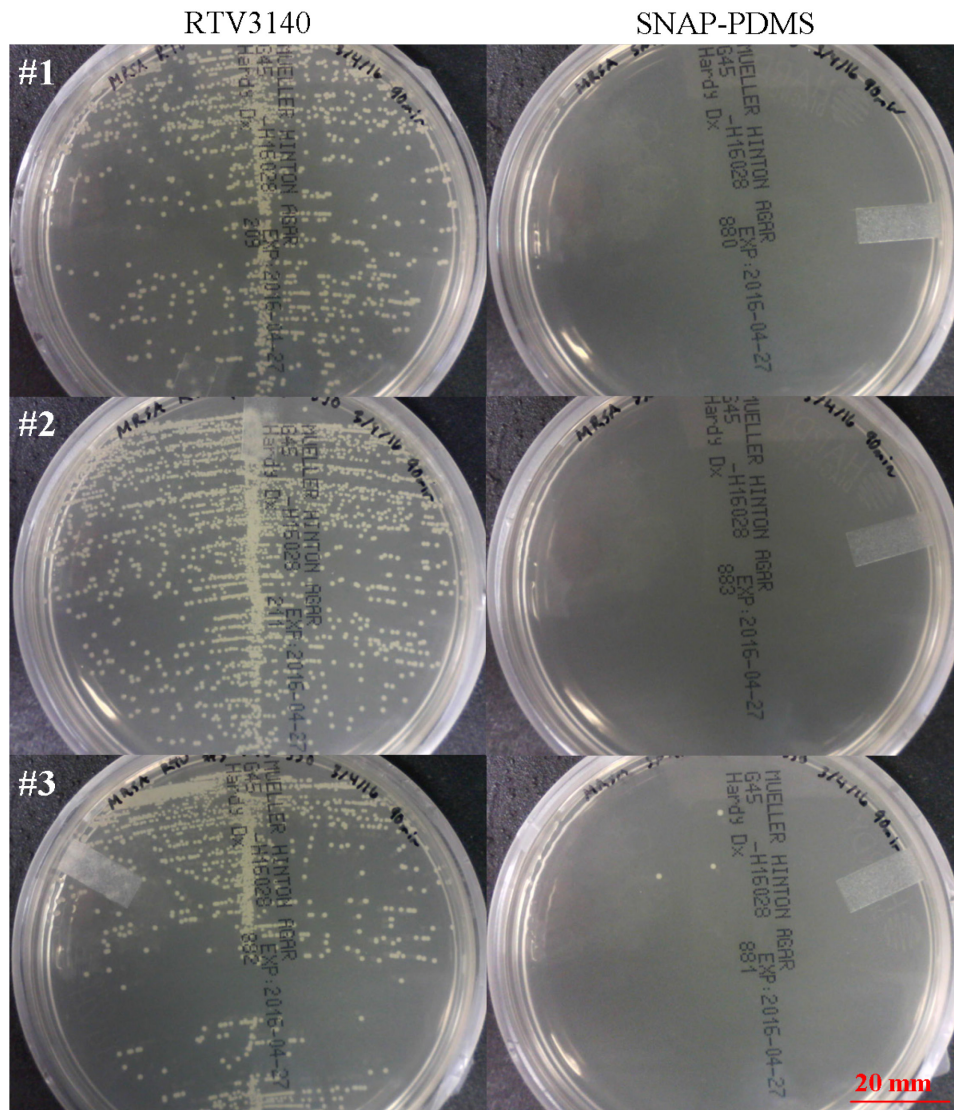
RTV3140   SNAP-PDMS

Figure 2.19.   Images taken of 1:10 dilution streaks 1 through 3 for both RTV3140 (left)
and SNAP-PDMS (right) for MRSA after 90 minutes.
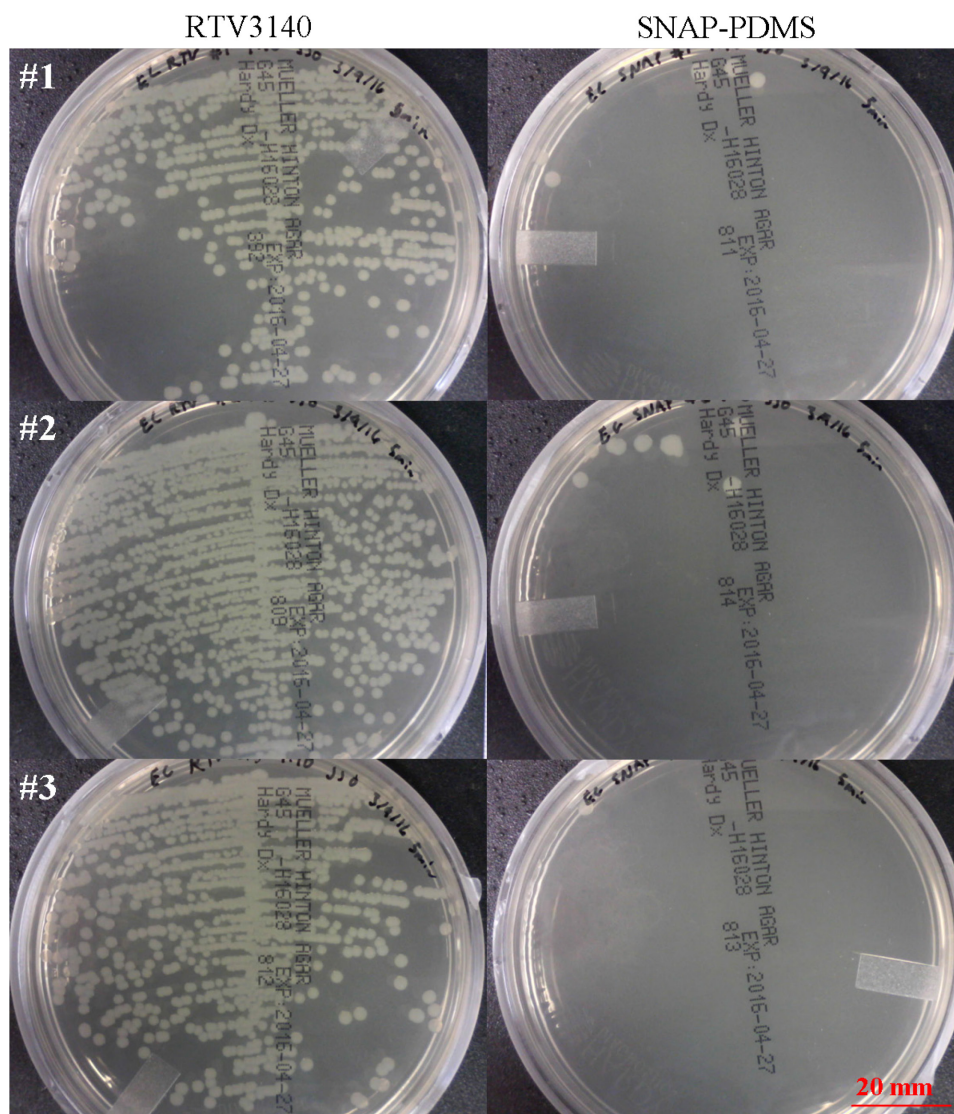
RTV3140   SNAP-PDMS

Figure 2.20. Images taken of 1:10 dilution streaks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) for *E. coli* after 5 minutes.
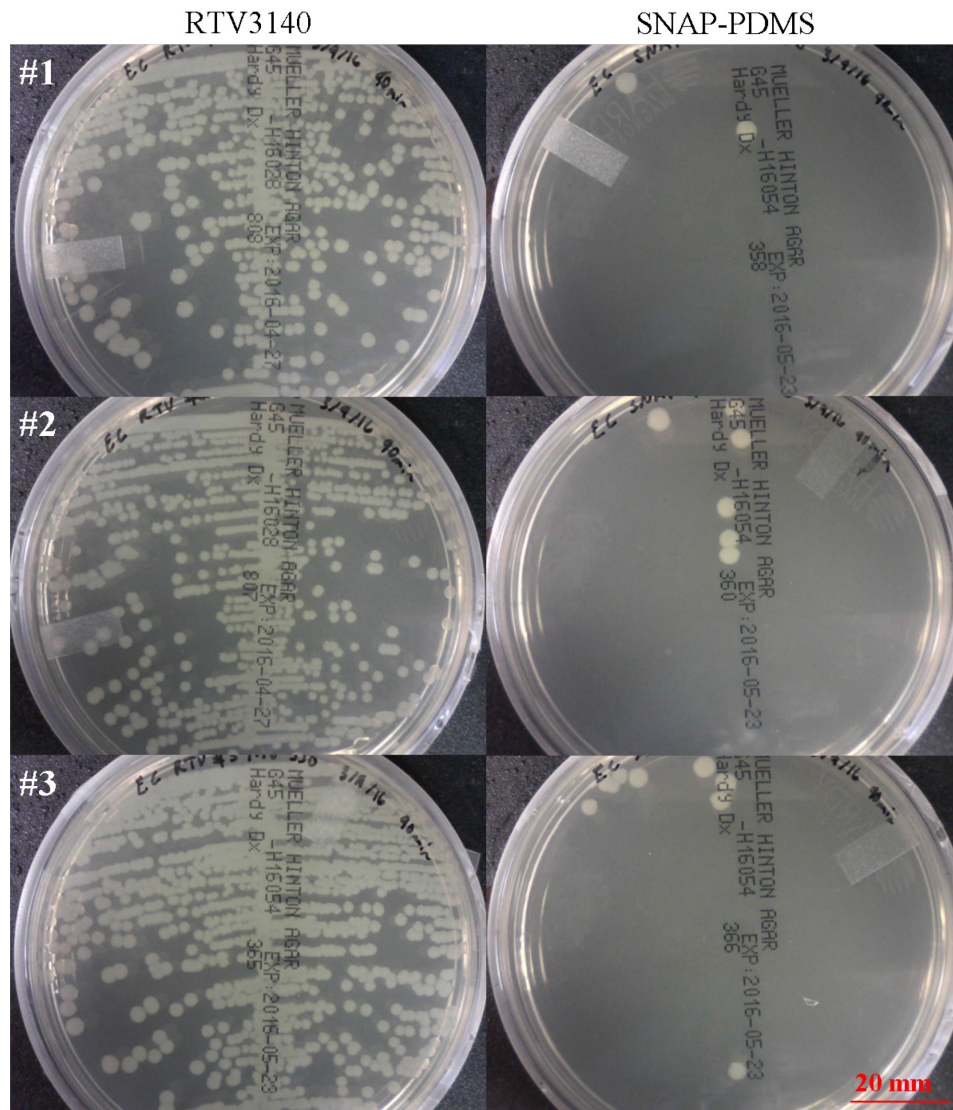
RTV3140   SNAP-PDMS

Figure 2.21.   Images taken of 1:10 dilution streaks 1 through 3 for both RTV3140 (left) and SNAP-PDMS (right) for *E. coli* after 90 minutes.

Table 2.3. Quantitative streak testing results comparing bacterial density present on RTV3140 and SNAP-PDMS disks for all four bacterial strains tested.

Quantitative Comparison of Bacterial Density Adhered to RTV3140 and SNAP-PDMS Disks				
Bacterial Density, x 10^6 CFU/ml				
		Disk #	RTV3140	SNAP-PDMS
S. epidermidis	5 minutes	1	3.50	0.00
		2	3.34	0.00
		3	3.78	0.00
		Average	**3.54 ± 0.223**	**0.00 ± 0.00**
	90 minutes	1	8.06	0.00
		2	5.29	0.00
		3	14.0	0.00
		Average	**9.11 ± 4.44**	**0.00 ± 0.00**
S. aureus	5 minutes	1	13.7	0.030
		2	14.6	0.0
		3	11.1	0.080
		Average	**13.1 ± 1.79**	**0.0367 ± 0.0404**
	90 minutes	1	10.2	0.130
		2	9.60	0.0
		3	10.2	0.600
		Average	**9.97 ± 0.323**	**0.600 ± 0.316**
MRSA	5 minutes	1	7.11	0.00
		2	7.59	0.00
		3	9.91	0.00
		Average	**8.20 ± 1.50**	**0.00 ± 0.00**
	90 minutes	1	8.55	0.00
		2	11.1	0.00
		3	7.7	0.030
		Average	**9.11 ± 1.75**	**0.010 ± 0.017**
E. coli	5 minutes	1	5.88	0.050
		2	11.0	0.080
		3	6.76	0.010
		Average	**7.89 ± 2.75**	**0.0467 ± 0.0351**
	90 minutes	1	6.06	0.020
		2	6.74	0.060
		3	7.41	0.090
		Average	**6.74 ± 0.675**	**0.0567 ± 0.0351**

2.4.5   NITRIC OXIDE RELEASE MEASUREMENTS

For the Kirby-Bauer method for disk testing, Figure 2.22 is NOA results of NO released over 18 hours for Samples 1 through 4, and Figure 2.23 is results of NO surface flux over 18 hours for Samples 1 through 4. Visually, the results overlap and appear to be similar. Table 2.4 compares the NO released and NO surface flux for all samples as well as averages and standard deviations after 18 hours. There was an average total NO release of 3.9 ± 0.69 x 10^{-8} moles and 1.1 ± 0.19 x 10^{-10} moles/(cm^2·min).

Respectively, Figure 2.24 and Figure 2.25 represent NOA results of NO released and NO surface flux over 18 hours for samples analyzed for the quantitative streak testing. Table 2.5 provides comparison of NO released and NO surface flux for each sample along with averages and standard deviations after 18 hours. Results reveal an average total NO release of 7.5 ± 1.3 x 10^{-8} moles and a final average surface flux of 2.2 ± 0.46 x 10^{-10} moles/(cm^2·min).

2.5   DISCUSSION

The qualitative images presented in this work demonstrate that NO is an effective bacterial inhibitor and can be utilized in polymer form (SNAP-PDMS). For all four bacterial strains tested, SNAP-PDMS established a negative effect on bacterial growth.

2.5.1   LIGHT VERSUS DARK TESTING

From a qualitative perspective, the bacterial growth appeared equivalent between the light and dark plates of *S. epidermidis* tested. If there was a drastic effect due to the
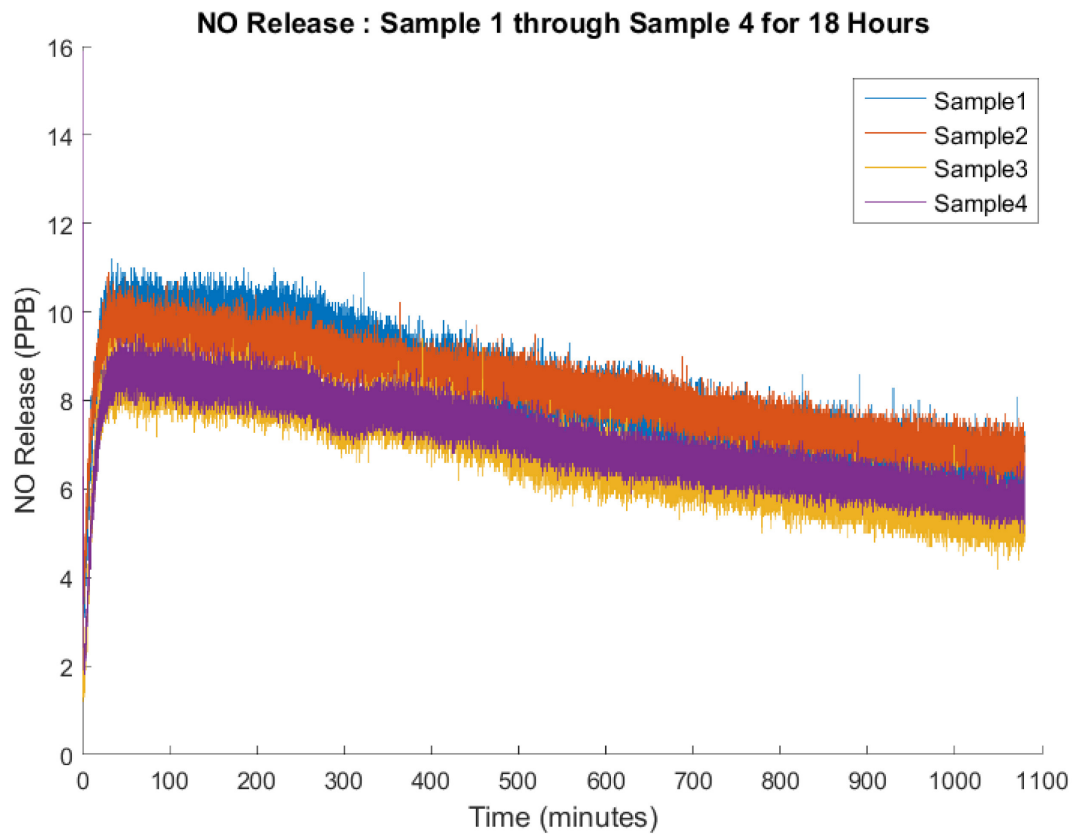
Figure 2.22. Plot representation of nitric oxide release from SNAP-PDMS disks over an 18 hour period for samples 1 through 4 used in disk susceptibility testing.
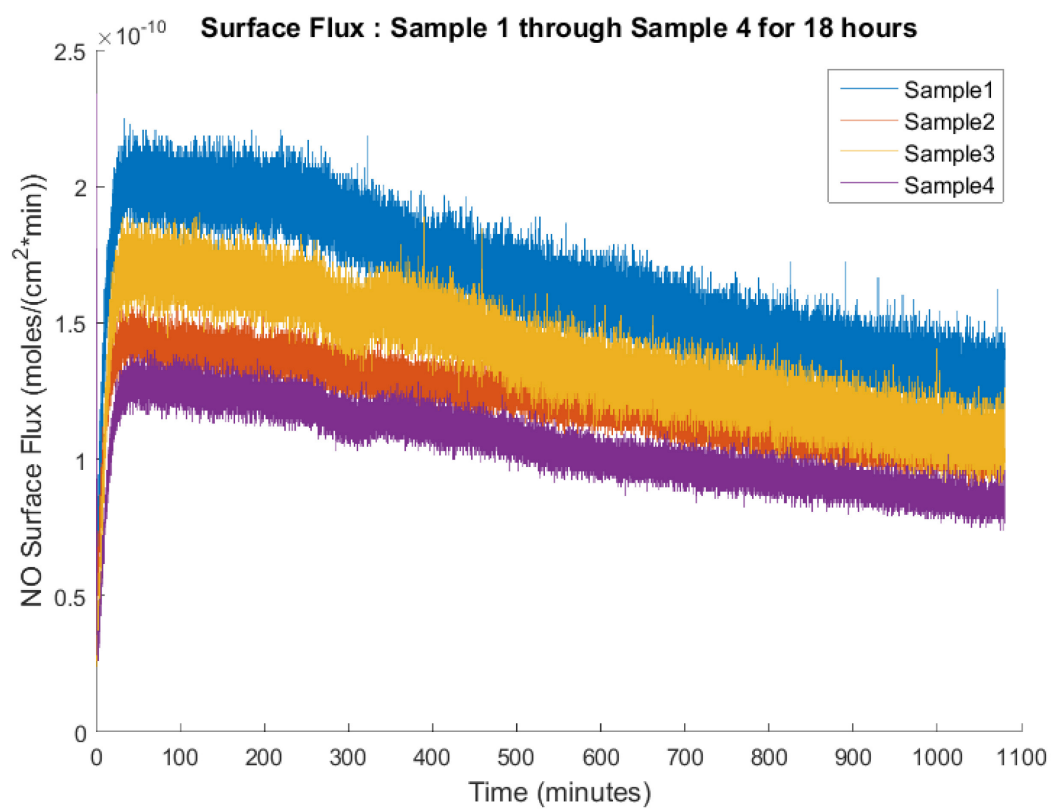
Figure 2.23. Plot representation of nitric oxide surface flux from SNAP-PDMS disks over an 18 hour period for samples 1 through 4 from disk susceptibility testing.

Table 2.4. Comparison of total nitric oxide released and nitric oxide surface flux for all seven samples including average and standard deviation over an 18 hour period for disk susceptibility testing.

NOA Measurements for Disk Susceptibility Testing, T=18 hr		
Sample #	Total NO released, moles	NO Surface Flux, moles/(cm^2·min)
1	4.5×10^{-8}	$1.3 \pm 0.063 \times 10^{-10}$
2	3.3×10^{-8}	$1.0 \pm 0.035 \times 10^{-10}$
3	3.7×10^{-8}	$1.1 \pm 0.057 \times 10^{-10}$
4	2.8×10^{-8}	$0.84 \pm 0.031 \times 10^{-10}$
5	4.1×10^{-8}	$1.2 \pm 0.057 \times 10^{-10}$
6	4.8×10^{-8}	$1.4 \pm 0.060 \times 10^{-10}$
7	4.1×10^{-8}	$1.1 \pm 0.061 \times 10^{-10}$
Average ± Standard Dev.	$\mathbf{3.9 \pm 0.69 \times 10^{-8}}$	$\mathbf{1.1 \pm 0.19 \times 10^{-10}}$

Figure 2.24. Plot representation of nitric oxide release from SNAP-PDMS disks over an 18 hour period for all three samples used in quantitative streak testing.
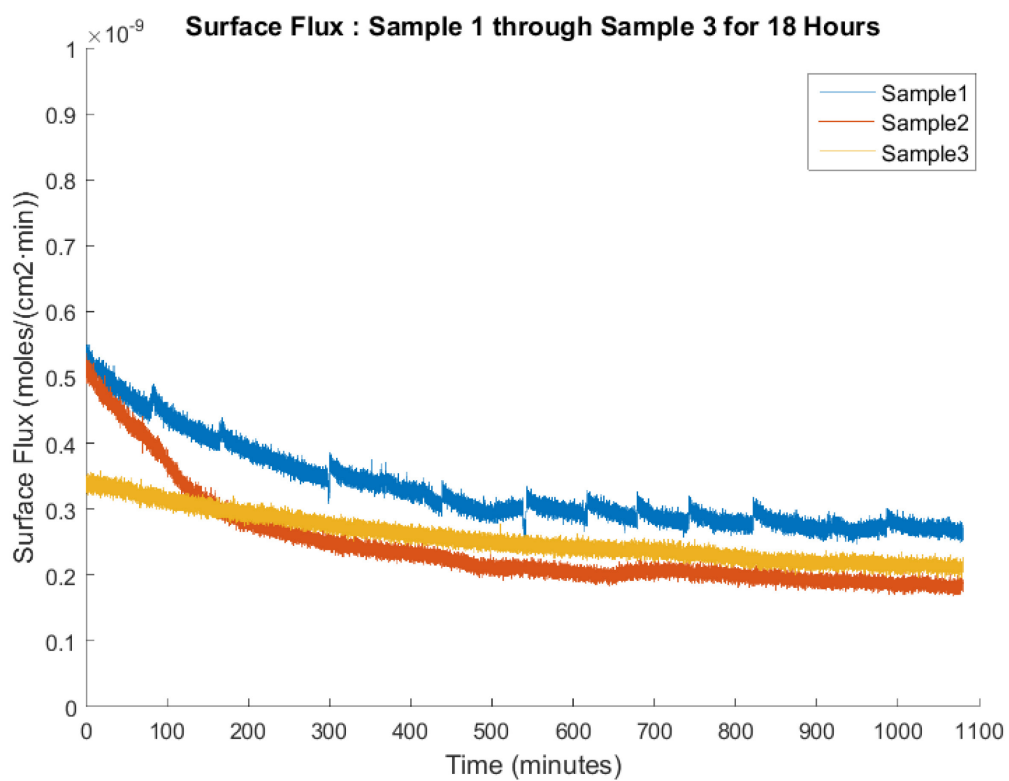
Figure 2.25. Plot representation of nitric oxide surface flux from SNAP-PDMS disks over an 18 hour period for all three samples from quantitative streak testing.

Table 2.5. Comparison of total nitric oxide released and nitric oxide surface flux for all three samples including average and standard deviation over an 18 hour period for quantitative streak testing.

NOA Measurements for Quantitative Streak Testing, T=18 hr		
Sample #	Total NO released, moles	NO Surface Flux, moles/(cm^2·min)
1	9.0×10^{-8}	$2.7 \pm 0.055 \times 10^{-10}$
2	6.6×10^{-8}	$1.8 \pm 0.048 \times 10^{-10}$
3	6.9×10^{-8}	$2.1 \pm 0.049 \times 10^{-10}$
Average ± Standard Dev.	$\mathbf{7.5 \pm 1.3 \times 10^{-8}}$	$\mathbf{2.2 \pm 0.46 \times 10^{-10}}$

LED light, there would be hot spots of either excessive or moderate bacterial density in the focal point areas from each LED. After image analysis, it was confirmed that based on percentage of plate covered, there was no significant difference between the eight plates tested. It was important to validate that the LED light has no effect on bacterial growth to eliminate it as a potential contributing factor while testing of bacterial inhibition from NO released with the light-controlled SNAP-PDMS disks. *S. epidermidis* bacteria was selected for this testing because it was considered the less virulent (weakest) of the four bacterial strains [10], meaning if there was an effect to bacterial growth due to LED the *S. epidermidis* growth would have shown the strongest effect.

2.5.2   DISK SUSCEPTIBILITY TESTING

When comparing the effectiveness of Ioban2 and levofloxacin, both displayed the anticipated inhibitory properties on *S. epidermidis*, *S. aureus*, MRSA, and *E. coli*, which were the bacterial strains used for testing the NO releasing SNAP-PDMS, see Figure 2.4. It was essential to test these controls because they are current approaches used in the medical field to prevent or treat bacterial infections. It can be observed that there is a halo or ring around the Ioban2 disks for each bacteria as well as a much larger zone of inhibition around the levofloxacin disks. Because Ioban2 is not a potent antimicrobial and levofloxacin is a powerful broad spectrum antibiotic, the difference in size of inhibition zones was expected. Although not all images are shown, the effects of the two controls were consistent between the four plates for each strain of bacteria tested.

After assessing and comparing the RTV3140 results to the SNAP-PDMS results,

there is inhibition of bacteria at the SNAP-PDMS disks but no effect from the RTV3140. Although there was some variance in potency between different bacterial strains, all the SNAP-PDMS disks were from the same batch of films and released a similar NO surface flux. As seen in Figure 2.5, there is more inhibition against *S. epidermidis* as compared to *E. coli*. There is a faint halo of a smaller density of *S. epidermidis* around the SNAP-PDMS disk, whereas on the *E. coli* plate, bacteria grow at a uniform density up to the disk edge.

Additionally, Figure 2.6 shows the amount of bacterial growth under the disks after aseptic removal. Due to the softness of the media after incubation and thinness of the polymer disks, tweezer marks are observed in the images from removal. They are the markings opposite one another that disturb the center circular form. When comparing the density of bacteria under the disks, it can be concluded that there are much more bacteria under the RTV3140 disks and minimal to no bacteria under the SNAP-PDMS disks for each bacterial strain evaluated. It is also important to point out that when looking at these images, bacteria can be easily seen under the RTV3140 disks due to their transparency whereas the SNAP-PDMS disks are opaque.

Lastly, bacteria were streaked through each void after removal of the test disk and allowed to grow for 24 hours. Figure 2.7 is representative of one MRSA plate; the other plates for MRSA and the other three bacteria showed similar and consistent results. It was observed that bacteria can easily regrow in the Ioban2, RTV3140, and SNAP-PDMS voids. As for the levofloxacin void, bacteria readily grow, but as the streak gets closer to the center of the inhibition there is a reduction in bacterial regrowth. This is due to the

increase in concentration of antibiotic left in the media as distance gets closer to the center of the zone. This regrowth and the size of the halos observed indicate that bacterial growth is inhibited at the disk itself and a distance away from the disk. The SNAP-PDMS disks clearly demonstrate the effect of NO on bacterial growth and reveal it is a highly localized inhibitory effect.

It is important to note that all bacteria were tested in dark conditions with no exposure to LED light. This is relevant because the surface flux of NO needed to inhibit bacterial growth was found to be 1.1 ± 0.19 x 10^{-10} moles/(cm^2·min) and was much lower than initially predicted. The SNAP-PDMS disks thermally degrade at 37°C to release NO without photo stimulation from an LED. This was an unexpected result. The average surface flux of NO due to thermal degradation (1.1 ± 0.19 x 10^{-10} moles/(cm^2·min)) was much lower than the average surface flux resulting from light initiated release ((3.1 to 4.9) x 10^{-10} moles/(cm^2·min) for 4.5V and 6V, respectively) [7]. The dose required to inhibit bacteria was much lower than initially anticipated. In conclusion, from these experiments, it is confirmed that NO released from SNAP-PDMS disks is a bacterial growth inhibitor. The lack in inhibition zone size is important to note because of NO's short diffusion distance due to its short half-life. This along with the positive bacterial regrowth in the void under the SNAP-PDMS disks validates NO release as a highly localized treatment for bacterial infections.

2.5.3 TEN DAY RTV3140 AND SNAP-PDMS DISK TESTING

Ten day testing was performed to assess SNAP-PDMS effectiveness over a longer

period of time. Only RTV3140 and SNAP-PDMS were utilized for this testing. These experiments revealed a continuous lack of inhibition at the RTV3140 disk and positive inhibition for the SNAP-PDMS disk against MRSA. MRSA was chosen as the testing bacteria due to it becoming increasingly more problematic in the healthcare field, as it continues to become more virulent and resistant to antibiotics.

Figure 2.8 shows that over time the RTV3140 disk has no inhibitory effect on bacteria grown while SNAP-PDMS inhibits bacteria for at least ten day. The inhibition is defined by the individual and smaller colonies observed around the SNAP-PDMS disk whereas MRSA colonies essentially blend together coating the plate up to and under the RTV3140 disk. Once the disks were removed, comparisons were made for the corresponding void spaces (see Figure 2.9). The RTV3140 void is a cloud of smeared bacteria, and disk was difficult to remove because of all the bacteria under the disk. On the other hand, there was minimal bacterial smear under the SNAP-PDMS disk, which is partially due to the pulling in of bacteria as the disk was removed. Although bacterial smear is present under the SNAP-PDMS disk, it was inferred from a qualitative inspection that there are fewer bacteria present than under the RTV3140 disk.

2.5.4 QUANTITATIVE STREAK TESTING

The quantitative streak testing was essential in quantifying the antimicrobial effects SNAP-PDMS has on bacteria compared to the control, RTV3140 for *S. epidermidis*, *S. aureus*, MRSA, and *E. coli*. As stated from the qualitative disk testing, bacteria readily grew up to and under the RTV3140 disks. On the other hand, bacteria

grew up to the edge of the SNAP-PDMS disks, but qualitatively were not present under the disks. Because of the likelihood of bacteria adhering to the edge of the polymer disks during disk removal, disks were designed such that the center of the polymer could be removed, eliminating the possibility of bacterial adherence due to proximity.

Figures 2.10-2.13 are images of the voids after the center disks were removed for *S. epidermidis*, *S. aureus*, MRSA, and *E. coli* comparing RTV3140 (left) to SNAP-PDMS (right). Similar to the previous disk testing and from a qualitative perspective, there appears to be a plethora of bacteria present under the RTV3140 disks for all four bacterial strains. Whereas, there appears to be minimal to no bacteria present under the SNAP-PDMS disks. Figure 2.14 and Figure 2.15 are streak results for *S. epidermidis* after suspension in sterile TSB for 5 minutes and 90 minutes, respectfully. Figure 2.16 through Figure 2.21 are similar images for *S. aureus*, MRSA, and *E. coli*. The purpose of using two time intervals was to test whether there was a difference at initial suspension and after sufficient time to allow bacteria to get into solution. For all bacterial strains tested, there was a significant decrease in bacteria present under the SNAP-PDMS disks compared to RTV3140 disks. Table 2.3 reveals the breakdown for bacterial density under each disk for all four bacteria tested. It is important to note that for *S. epidermidis* 5 minutes and 90 minute streaks as well as MRSA 5 minutes streaks there were no bacteria present. Although there were some bacteria present for the SNAP-PDMS streaks for the 90 minutes MRSA and all *S. aureus* and *E. coli* streaks, there were significantly fewer bacteria than the RTV3140 counterparts. Overall, there was a greater than 1600% reduction in bacterial density for SNAP-PDMS samples. Lastly, Table 2.4 reveals that an

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average total NO release of 7.5±1.3 x 10^{-8} moles and average NO surface flux of 2.2±0.46 x 10^{-10} moles/(cm^2·min) are sufficient doses to inhibit bacterial growth and adhesion.

2.5.5 SUMMARY

Taking into account all of the experimental data collected, it is confirmed that NO is a bacterial growth inhibitor. The NO utilized here was released from SNAP-PDMS. Experimental results confirm that *S. epidermidis*, *S. aureus*, MRSA, and *E. coli* were all inhibited by SNAP-PDMS under dark conditions. Qualitatively, the level of inhibition varied slightly between differing strains of bacteria, but all were inhibited by the SNAP-PDMS disks that had an average total NO release of 3.9±0.69 x 10^{-8} moles and an average surface flux of 1.1±0.19 x 10^{-10} moles/(cm^2·min) at the end of 18 hours. The ten day testing established lasting effectiveness of SNAP-PDMS against MRSA. Quantitatively, an average total NO release of 7.5±1.3 x 10^{-8} moles and average NO surface flux of 2.2±0.46 x 10^{-10} moles/(cm^2·min) at the end of 18 hours does inhibit bacterial growth for all four bacterial strains. There was complete inhibition for *S. epidermidis* and a greater than 1600% reduction for *S. aureus,* MRSA, and *E. coli.*

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CHAPTER 3

LOCALIZED BACTERIAL GROWTH INHIBITION WITH

SNAP-PDMS

As discussed previously, the number of antibiotic-resistant bacteria are increasing at a rapid frequency, and therefore are recognized as a key problem for the treatment of infections [1]. As a result, there is need for a new approach to treating bacterial infections. One of the current approaches for fighting an infection is through the use of oral antibiotics [2]. In general, these antibiotics can be in pill, tablet, or capsule form, and are absorbed into the bloodstream from the small intestine. Once the antibiotic is in the bloodstream, the drug circulates the body and destroys bacteria as it comes in contact with it.

One issue with these types of antibiotics is that the antibiotics cannot distinguish healthy bacteria from infectious bacteria. For example, *Escherichia coli* (*E. coli*) is an opportunistic pathogen meaning the intestines have *E. coli* present as part of the normal body flora, but *E. coli* is not a part of the urinary tract (UT) flora and therefore if present in the UT, is infectious. Additionally, it is believed that the over prescription of antibiotics [3], as well as improper use by patients (i.e. failure to complete the course of the prescription), contributes to the growing antibiotic resistance problem worldwide. In 2009, the United States had approximately $42 billion of antibiotic sales [4]. The overuse and misuse of antibiotics are another reason for developing a new and more localized treatment to fight infection.

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There are several benefits to having a localized treatment for bacterial infection. One benefit is preventing the destruction of normal bacterial flora as well as damage to surrounding healthy tissues. Localized treatment leads to lower dosing and decreased toxicity which in turn reduces side effects and prevents resistance [2]. As discussed in the previous chapters, nitric oxide (NO) has a short half-life which supports its use for a localized treatment to infection. S-Nitroso-N-acetyl-D-penicillamine covalently linked to polydimethylsiloxane (SNAP-PDMS) can be used to show the localized treatment against bacterial growth. Two batches of experiments were completed. The first was through the use of a gradient strip of increased NO concentration, while the second set of experiments were multiple SNAP-PDMS disks at varying distances from one another in order to test cross-talk between disks.

3.1   SELECTION OF BACTERIA

Staphylococcus epidermidis (*S. epidermidis*) was chosen for the following experiments because it readily sticks to indwelling medical devices such as a catheter. Also, *S. epidermidis* is considered the most frequently isolated species responsible for infection [5]. *S. epidermidis* (ATCC 12228) was purchased from Microbiologics, Inc. (St. Cloud, MN) in single strain LYFO DISK pellets.

3.2   PREPARATION OF MATERIALS

SNAP-PDMS was again utilized to reveal the localized release of NO to inhibit bacterial growth in disk and strip form. The following sections describe the synthesis of

the SNAP-PDMS to be used for each experiment.

3.2.1 KIRBY-BAUER METHOD FOR GRADIENT STRIP TESTING

Gradient strips were developed to demonstrate the effect of an increase in NO surface flux to bacterial growth. The surface flux of released NO in the gradient strips consisted of increase concentration of SNAP-PDMS ranging from 0% SNAP-PDMS (RTV3140), 1% SNAP-PDMS, 10% SNAP-PDMS, 100% SNAP-PDMS, and back to 0% SNAP-PDMS (RTV3140) as seen in Figure 3.1. The gradient strips were created by spray coating each range of the silicone rubber strip with different concentrations of SNAP-PDMS and masking regions during spraying to control polymer deposition.

A 2.25 x 5in PDMS film was purchased from McMaster Carr Co. (Elmhurst, IL) and used as the base material. First, 1ml of SNAP-PDMS (0.05 g/ml) was sprayed over the 100% region. Next, a 1ml solution of 0.1ml SNAP-PDMS (0.05g/ml) and 0.9ml of RTV3140 (0.05 g/ml) was sprayed over the 10% and 100% regions. Thirdly, a 1ml solution of 0.01ml SNAP-PDMS and 0.99ml of RTV3140 (0.05 g/ml) was sprayed over the 1%, 10%, and 100% regions. The overlap between regions was necessary to ensure a more uniform release of NO at the boundaries of the different SNAP-PDMS concentrations. Lastly, the entire area including both RTV3140 ends and the SNAP-PDMS regions were top coated with 2ml of RTV3140 (0.05 g/ml). Toluene was used as the solvent for all the above solutions. The strips were allowed to cure overnight in the dark, and then a sterile razor blade was used to cut 0.635cm (0.25in) wide, alternating strips from the bulk polymer. The strips were alternated such that strip one was used for
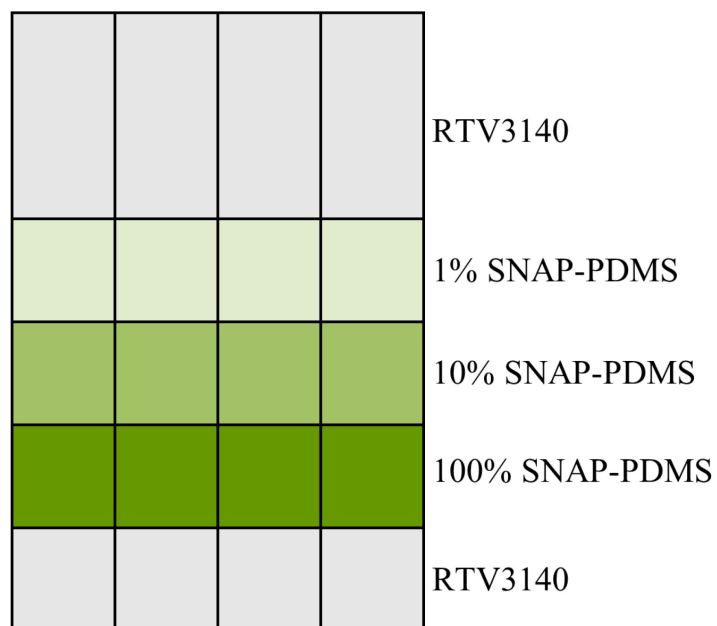
Figure 3.1. Visual representation of the gradient strip design for four strips from left to right showing the increase in SNAP-PDMS concentration down the strip as well as the capping of each strip with RTV3140.

bacterial testing and the following strip was used for analyzing the amount of NO released then the next strip would go to bacterial testing and so on.

3.2.2   KIRBY-BAUER METHOD FOR CROSS-TALK TESTING

As described in section 2.3.1, SNAP-PDMS solution was synthesized and cast into films. Disks were then punched from the cured films using a 4mm diameter cork borer. This testing was done to evaluate the localized effect of NO release on bacterial growth by placing multiple disks at various distances from one another. The goal was to determine at what distance the SNAP-PDMS disks begin to cross-talk with one another while on the same media plate.

3.3   EXPERIMENTAL SETUP

Stock plates of bacteria were created as described in Chapter 2. The Kirby-Bauer disk susceptibility testing method described in the previous chapter was also followed in regards to lawning the appropriate density of *S. epidermidis* onto the media plates.

3.3.1   KIRBY-BAUER METHOD FOR GRADIENT STRIP TESTING

After inoculating the media plates, the gradient strips described above were placed on the plates. Between three inoculated plates, eight gradient strips were applied. The plates were then sealed with parafilm and incubated for 18 hours in dark conditions before being imaged with and without gradient strips present using a ViTiny Pro10 Plus Portable Digital Microscope 10x - 200x purchased from Oasis Scientific, Inc. (Greenville, SC).

3.3.2 KIRBY-BAUER METHOD FOR CROSS-TALK TESTING

For these experiments, three Mueller-Hinton media plates were inoculated with *S. epidermidis* per the Kirby-Bauer method. Three different clusters of seven SNAP-PDMS disks were placed on each plate in a circular pattern; one disk in the middle surrounded by six other disks a uniform radial distance away. The distances chosen were 1.25cm, 1.00cm, and 0.75cm measured from the center of one SNAP-PDMS disk to the center of a directly neighboring SNAP-PDMS disk. Additionally, two #8 zinc plated flat washers were then added to void space on the plate to provide scaling in images. See Figure 3.2 for a template display of plate setup. After SNAP-PDMS disks and washers were added, the plates were sealed and incubated at 37°C for 24 hours before being imaged with and without the SNAP-PDMS disks present. Data is not shown for when disks were placed as close to one another with no overlap (Distance=0.4cm) because there was clear cross-talk and minimal bacterial growth from the outermost edge in.

3.3.3 NITRIC OXIDE RELEASE MEASUREMENTS

Nitric oxide release measurements were taken using a Sievers Nitric Oxide Analyzer (NOA) 280i (GE Instruments, Boulder, CO). For the gradient strip testing, five corresponding strips were analyzed. Each strip was cut into 0.635 x 0.635cm (0.25 x 0.25in) squares at the border between the differing concentrations to determine the total NO released over an 18 hour period for each stair step increase in SNAP-PDMS concentration. Additionally, the surface flux for each stair step increase in SNAP-PDMS concentration was measured by averaging the surface flux over a 10 minute interval after
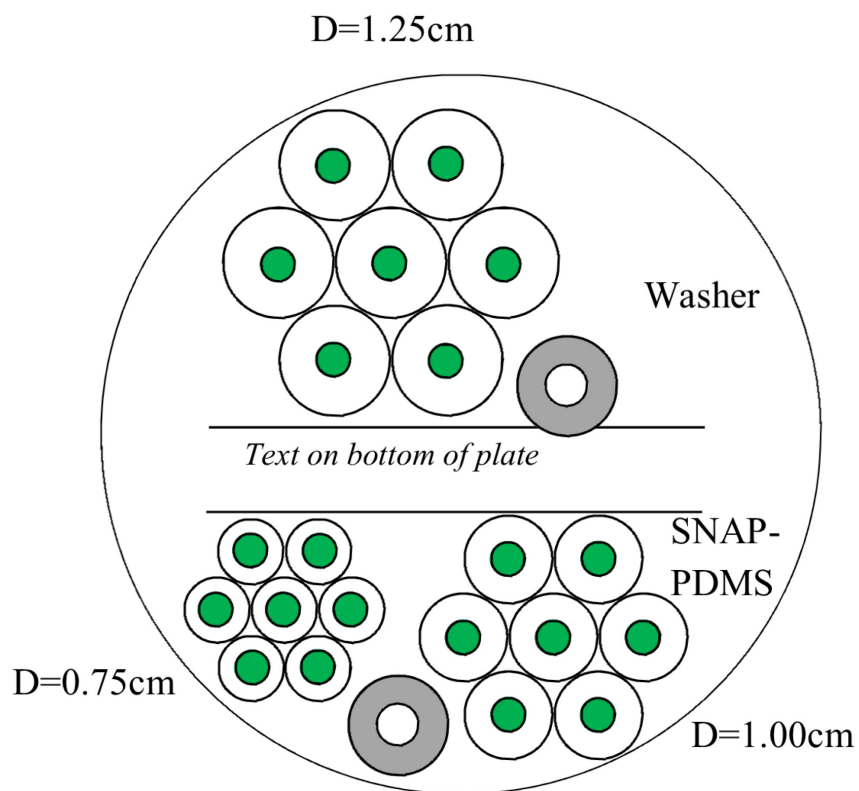
Figure 3.2. Template design used for placing SNAP-PDMS disks and washers on a 100mm media plate for Kirby-Bauer method for cross-talk experiment. D is the center to center distance from one SNAP-PDMS disk to another.

1 hour, 4 hours, 13 hours, and 18 hours of release.

As for the cross-talk experiment, the SNAP-PDMS disks were analyzed similarly to the previous chapter. The total NO released and NO surface flux was measured for three SNAP-PDMS disks over a 24 hour period. The NO surface flux was measured over a 10 minute interval at the 24 hour mark. Both measurements were tabulated for comparison on top of averages and standard deviations calculated.

3.4   RESULTS

The above experiments were completed to indicate the highly localized effect NO has against bacterial growth. SNAP-PDMS was utilized in gradient strip form to show what happens to bacterial growth as there is a stair step increase in SNAP-PDMS concentration. The cross-talk experimental setup was done to test at what distance the SNAP-PDMS disks begin to cross-talk with one another.

3.4.1   KIRBY-BAUER METHOD FOR GRADIENT STRIP TESTING

Figure 3.3 is the qualitative results of two of the eight gradient strips tested against *S. epidermidis*, with and without the gradient strip present. At the point where SNAP-PDMS concentration is highest, there is a void of bacterial growth under the strip. As the gradient strip increases in SNAP-PDMS (left to right), bacterial density decreases before going back up at the far right, which, as described above, is RTV3140.

WITH STRIP **WITHOUT STRIP**
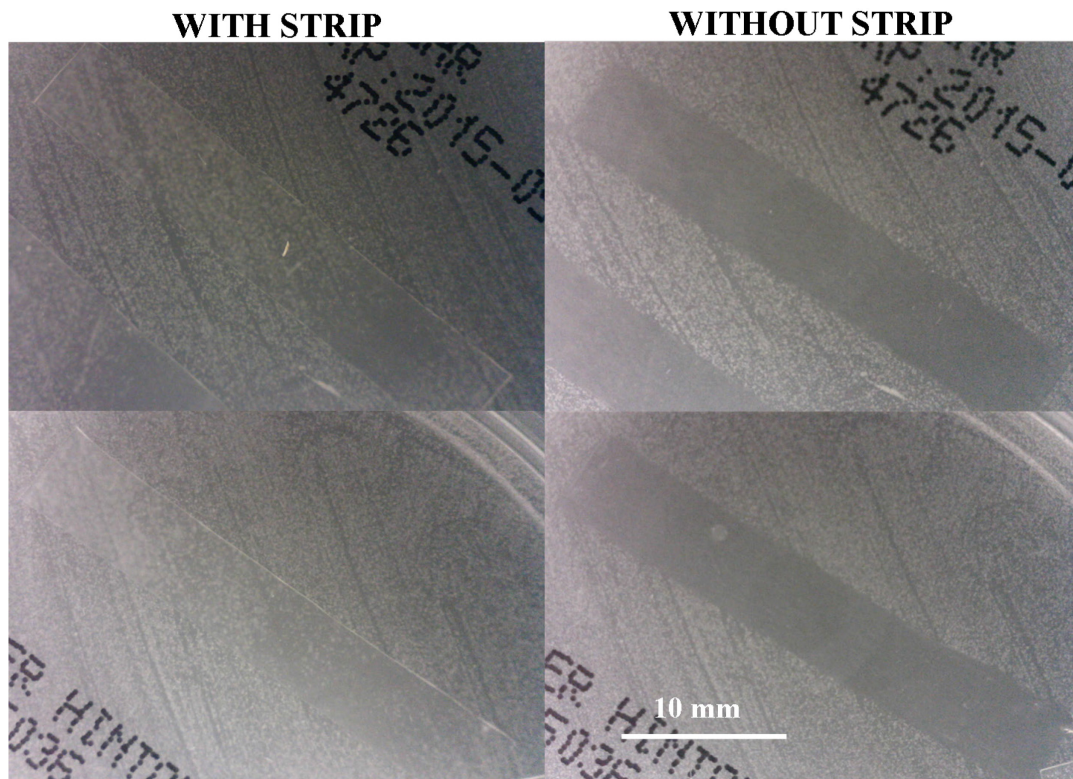
Figure 3.3.   Images taken of *S. epidermidis* entire gradient strips #4 (top) and #3 (bottom) with strip (left) and without strip (right) after 18 hours incubation at 37˚C in dark conditions. SNAP-PDMS increases from left to right along the strip with the end capped with control RTV3140.

3.4.2 KIRBY-BAUER METHOD FOR CROSS-TALK TESTING

Figure 3.4 represents qualitative results of one set of SNAP-PDMS disks used for the cross-talk experiments. At the furthest distance of D=1.25cm, there is no overlap in growth inhibition caused by the SNAP-PDMS disks, contrarily, at the shortest distance of D=0.75cm, there is an overlap in inhibition zones because visually there is a lower density of bacteria between the center SNAP-PDMS disk and the outer disks. Figure 3.5 reveals that once the disks are removed, from a qualitative perspective, there is no bacterial growth under the SNAP-PDMS disks.

3.4.3 NITRIC OXIDE RELEASE MEASUREMENTS

Figure 3.6 is an example plot of NO released with respect to time for one gradient strip for a full 18 hours. It can be seen that the higher the concentration of SNAP, the higher the amount of NO released. Similarly, Figure 3.7 reveals that as SNAP concentration increased along the strip, there is an increase in NO surface flux. Table 3.1 shows total NO released per section of strip for all five strips tested after 18 hours. The strips were most effective at 100% SNAP concentration, and the average total NO released for that portion of the gradient strips was $17\pm6.7 \times 10^{-9}$ moles. Additionally, Table 3.1 compares the NO surface flux for each strip at all three concentrations after 1 hour, 4 hours, 13 hours, and 18 hours, as well as the averages and standard deviations. The average NO surface flux after 1 hour of release was $28\pm9.7 \times 10^{-12}$ moles/(cm^2·min) for the 100% SNAP-PDMS stair step.

D=1.25cm

D=1.00cm

D=0.75cm

10 mm
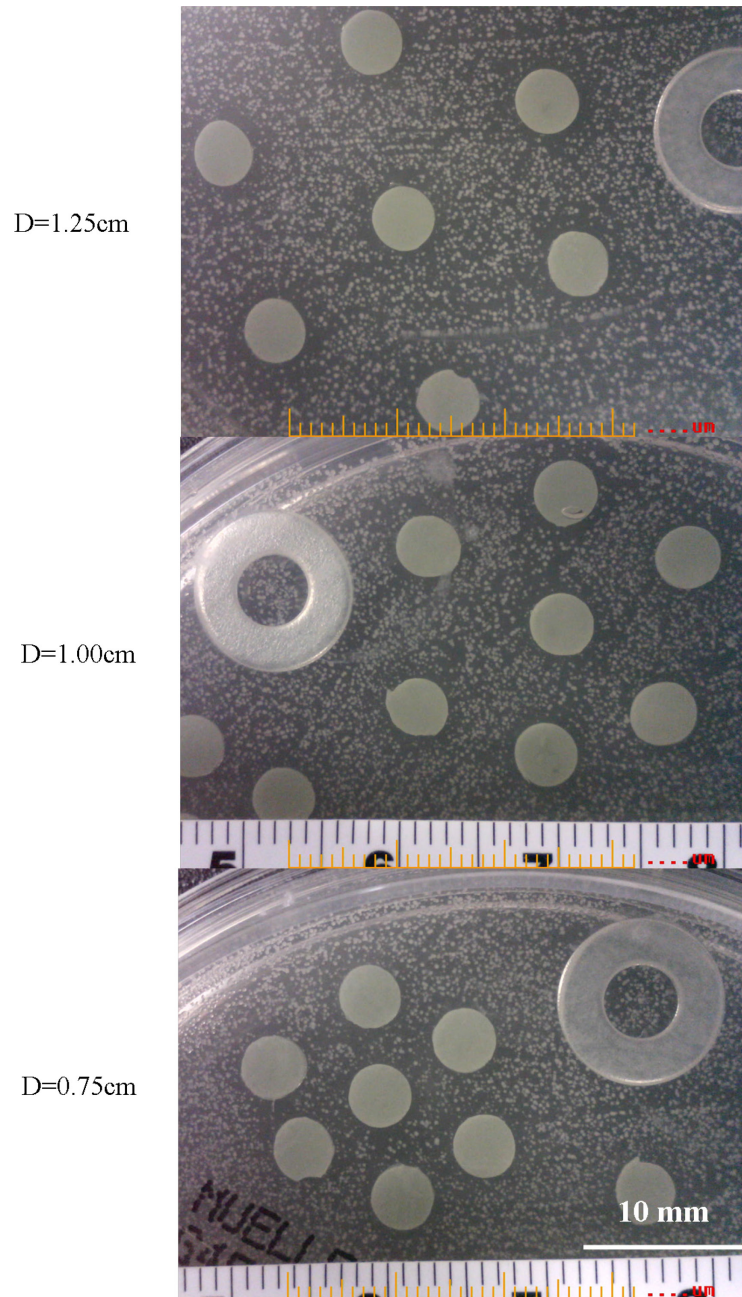
Figure 3.4. Images taken of *S. epidermidis* cross-talk experiment of plate 2 after 24 hours incubation at 37˚C in dark conditions with SNAP-PDMS disks present. SNAP-PDMS disks were placed at various distances from one another: 1.25cm (top), 1.00cm (middle), and 0.75cm (bottom).
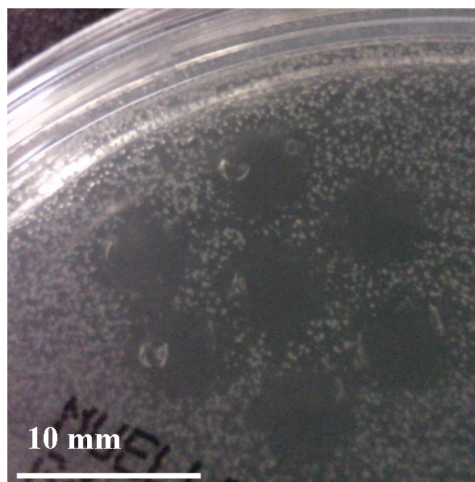
Figure 3.5.   Magnified image taken of *S. epidermidis* cross-talk experiment of plate 1 after 24 hours incubation at 37˚C in dark conditions without SNAP-PDMS disks present. The distance between disks was 0.75cm.
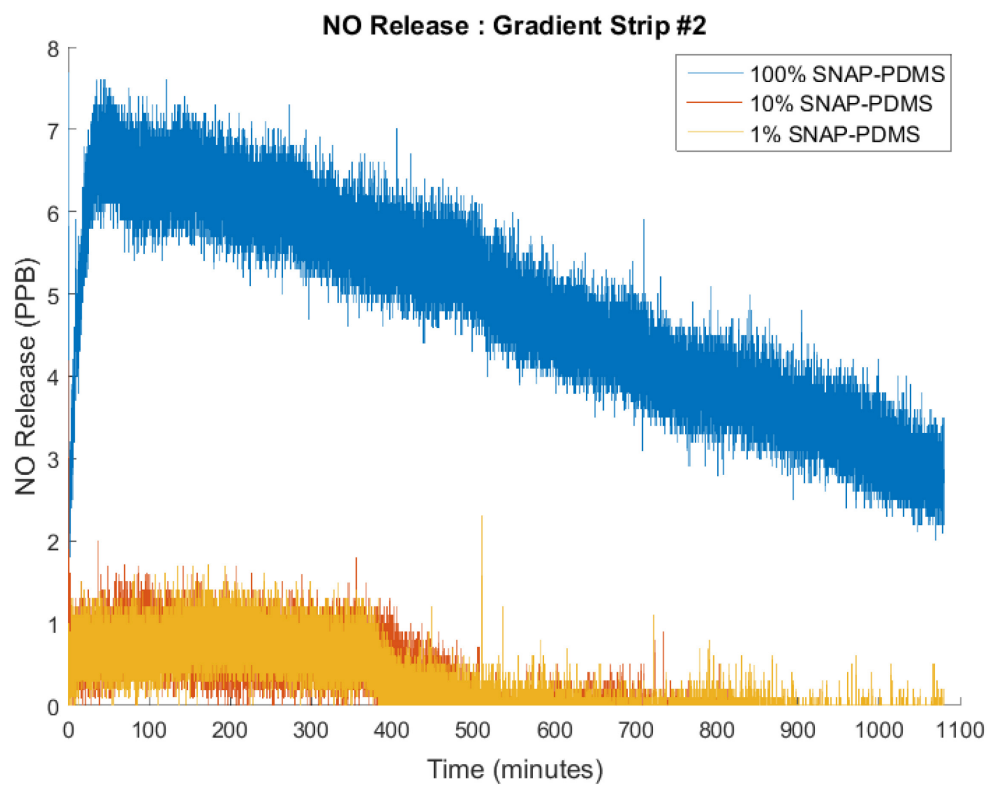
Figure 3.6. Plot representation of nitric oxide release over an 18 hour period for gradient strip #2 comparing the release for all three SNAP concentrations.
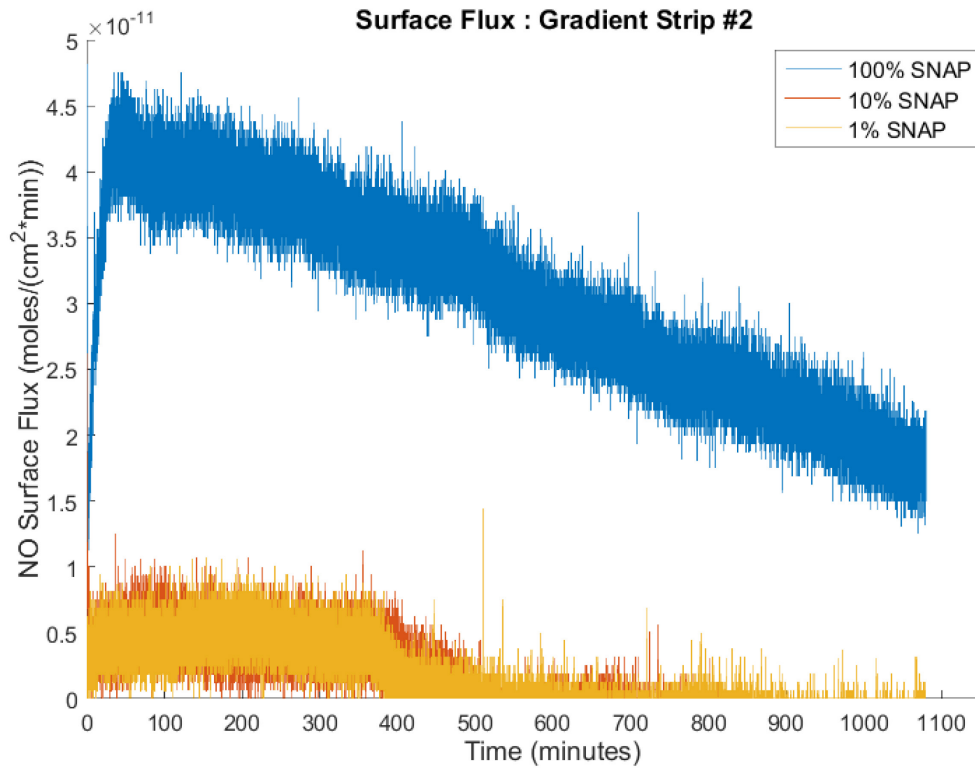
Figure 3.7. Plot representation of nitric oxide surface flux over an 18 hour period for gradient strip #2 comparing the surface flux for all three SNAP concentrations.

Table 3.1. Comparison of total nitric oxide released and nitric oxide surface flux for all five gradient strip samples including averages and standard deviations over an 18 hour period.

Nitric Oxide Analyzer Results for Gradient Strip Samples				
Total Nitric Oxide Released, x 10^{-9} moles				
	Sample	**100% SNAP**	**10% SNAP**	**1% SNAP**
T=18 hours	#1	20	1.4	0.71
	#2	26	1.5	1.5
	#3	16	0.72	1.2
	#4	14	1.9	2.0
	#5	8.2	3.6	3.2
	Average	**17 ± 6.7**	**1.8 ± 1.1**	**1.7 ± 0.95**
Nitric Oxide Surface Flux, x 10^{-12} moles/(cm^2·min)				
	Sample	**100% SNAP**	**10% SNAP**	**1% SNAP**
T=1 hour	#1	31 ± 2.0	0.91 ± 1.1	2.5 ± 0.86
	#2	41 ± 1.8	5.2 ± 1.4	5.2 ± 1.4
	#3	26 ± 1.2	3.7 ± 0.79	2.3 ± 0.68
	#4	27 ± 2.0	10 ± 1.7	2.5 ± 1.4
	#5	14 ± 0.84	4.8 ± 0.69	3.6 ± 0.66
	Average	**28 ± 9.7**	**4.9 ± 3.3**	**3.2 ± 1.2**
T=4 hours	#1	27 ± 2.1	2.9 ± 1.5	0.0069 ± 0.10
	#2	38 ± 1.9	4.3 ± 1.4	4.3 ± 1.4
	#3	23 ± 1.2	1.6 ± 0.74	2.4 ± 0.67
	#4	18 ± 1.8	2.5 ± 1.4	1.5 ± 1.2
	#5	11 ± 0.82	5.0 ± 0.70	3.6 ± 0.71
	Average	**23 ± 10**	**3.3 ± 1.4**	**2.4 ± 1.7**
T=13 hours	#1	17 ± 1.9	0.18 ± 0.51	0 ± 0
	#2	24 ± 1.7	0.0042 ± 0.062	0.078 ± 0.37
	#3	15 ± 1.1	0.00076 ± 0.019	0.82 ± 0.59
	#4	12 ± 1.7	0.22 ± 0.56	1.9 ± 1.3
	#5	7.5 ± 0.80	3.4 ± 0.68	3.7 ± 0.69
	Average	**15 ± 6.2**	**0.76 ± 1.5**	**1.3 ± 1.5**
T=18 hours	#1	13 ± 1.8	0.0010 ± 0.026	0 ± 0
	#2	17 ± 1.5	0 ± 0	0.028 ± 0.18
	#3	9.8 ± 0.98	0 ± 0	0.46 ± 0.48
	#4	14 ± 1.7	0 ± 0	1.7 ± 1.3
	#5	6.1 ± 0.72	2.2 ± 0.69	2.5 ± 0.68
	Average	**12 ± 4.2**	**0.44 ± 0.98**	**0.94 ± 1.1**

Figure 3.8 represents total NO released for the three SNAP-PDMS disks measured, and Figure 3.9 is NO surface flux measurements for all three of the SNAP-PDMS disks. Table 3.2 compares the total NO released and NO surface flux for the three samples in addition to averages and standard deviations. The average total NO released and average surface flux after 24 hours was $2.1\pm0.058 \times 10^{-7}$ moles and $5.0\pm0.38 \times 10^{-10}$ moles/(cm^2·min), respectively.

3.5   DISCUSSION

The results of the gradient strip and cross-talk experiments further validate NO as an effective bacterial growth inhibitor. The two experiments also demonstrate the localized treatment effect of NO against bacterial growth.

3.5.1   KIRBY-BAUER METHOD FOR GRADIENT STRIP TESTING

The most crucial characteristic of the gradient strip testing was to expose the effects of increased NO concentration which is related to the amount of SNAP-PDMS present to determine the minimal dose of NO required to inhibit bacterial growth in a 18 hour period. Both ends of the strip were capped with RTV3140 to show that viable bacteria had the capability to grow readily and uninhibited along the length of the strip. In a stair step fashion, the concentration of SNAP-PDMS increased through the middle of the strips. Figure 3.3 shows that with an increase in SNAP-PDMS, there is an increase in bacterial inhibition. Again, *S. epidermidis* strain was selected for its ease of use and high sensitivity to NO from SNAP-PDMS.
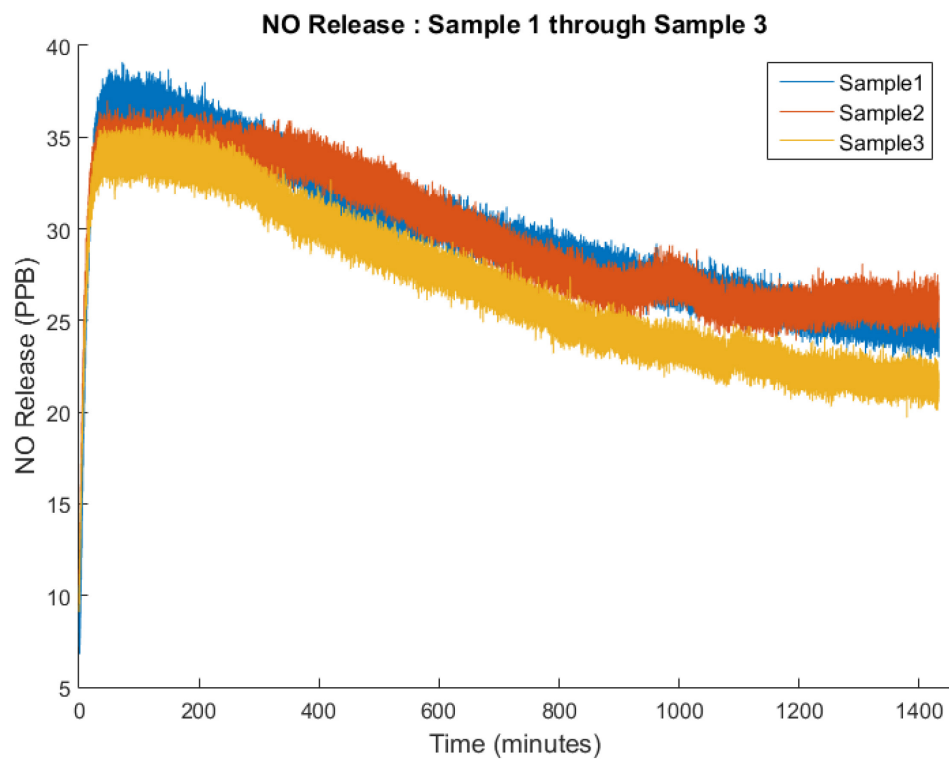
Figure 3.8. Plot representation of nitric oxide release from SNAP-PDMS disks over a 24 hour period for samples 1 through 3 from cross-talk experiment.
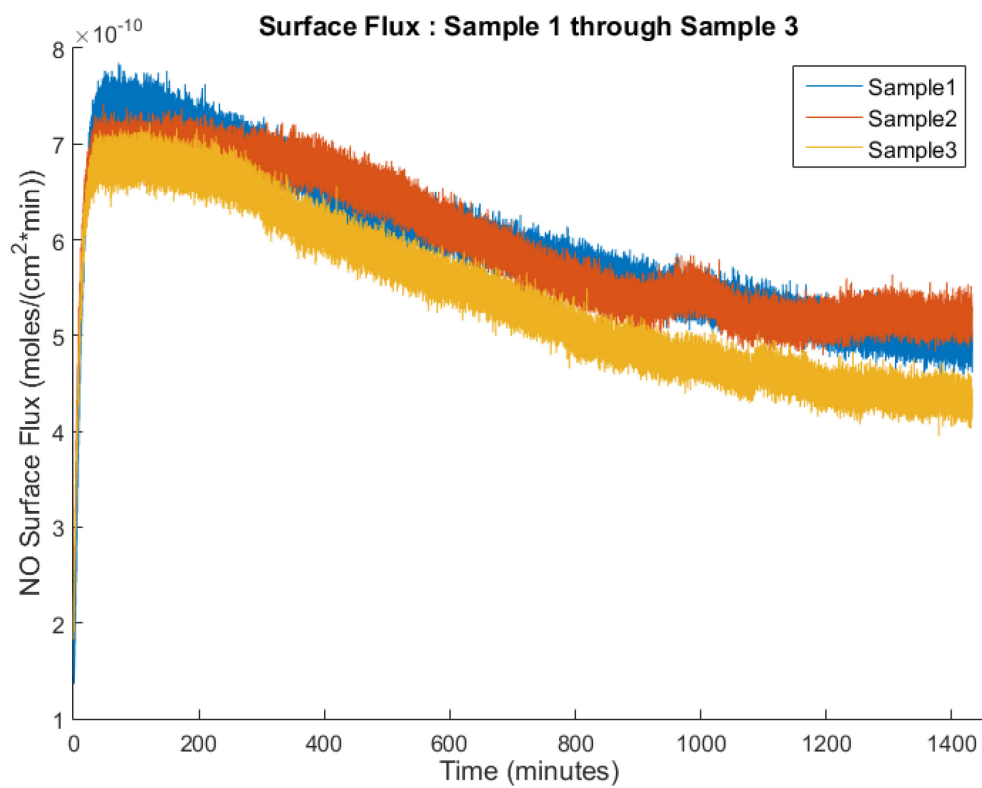
Figure 3.9.   Plot representation of nitric oxide surface flux from SNAP-PDMS disks over a 24 hour period for samples 1 through 3 from cross-talk experiment.

Table 3.2. Comparison of total nitric oxide released and nitric oxide surface flux for all three samples including averages and standard deviations over a 24 hour period.

NOA Measurements for Disk Susceptibility Testing, T=24 hr		
Sample #	Total NO released, moles	NO Surface Flux, moles/(cm^2·min)
1	2.1 x 10^{-7}	5.3 ± 0.010 x 10^{-10}
2	2.1 x 10^{-7}	5.2 ± 0.096 x 10^{-10}
3	2.0 x 10^{-7}	4.6 ± 0.010 x 10^{-10}
Average ± Standard Dev.	**2.1 ± 0.058 x 10^{-7}**	**5.0 ± 0.38 x 10^{-10}**

The gradient strips were important to aid in determining the effective SNAP-PDMS concentration required to inhibit bacteria which was found to be 28 ± 9.7 x 10^{-12} moles/(cm$^2\cdot$min) at 100% SNAP-PDMS after 1 hour. With the use of the NOA, the necessary average total NO release needed to inhibit bacterial growth was 17 ± 6.7 x 10^{-9} moles. A student t-test with a 95% confidence level concluded that there was a statistical difference between both 100% SNAP-PDMS and 10% SNAP-PDMS as well as between 100% SNAP-PDMS and 1% SNAP-PDMS. But, there was no statistical difference between 10% SNAP-PDMS and 1% SNAP-PDMS. The gradient strips also validated the highly localized treatment of SNAP-PDMS against bacteria. After the highest concentration of SNAP-PDMS, the strips were capped by a section of RTV3140 as described above, and bacteria under the RTV3140 sections was uninhibited by the high concentration of SNAP-PDMS next to it.

3.5.2   KIRBY-BAUER METHOD FOR CROSS-TALK TESTING

The purpose of the cross-talk experiment was to determine the distance at which the SNAP-PDMS disks began to cross-talk with one another. This is important to show that the treatment is localized and does not spread affecting other areas. Furthermore, finding the distance at which cross-talk begins to occur can aid in more efficient future testing. For example, future experiments could be done with a greater number of SNAP-PDMS disks per inoculated media plate.

From Figure 3.4 it can be seen that, as the disks go from 1.25cm to 0.75cm there is some cross-talk between disks occurring. At the greatest distance of 1.25cm it can be

seen that there is no cross-talk between the disks as there is no change in bacterial density between disks versus surrounding space from a qualitative perspective. At the nearest distance of 0.75cm, there is some cross-talk. Even though, bacteria still grow between the disks, there are some spaces between disks where bacterial density appears to be less than that of the surrounding density. At the middle distance of 1.00cm, bacteria appear to grow between the disks at a similar density to that of the surrounding space. Taking this into account, it can be concluded that a distance of 1.00cm can be used as the minimum required distance between disks to prevent cross-talk between SNAP-PDMS samples that have an average total NO release of $2.1 \pm 0.058 \times 10^{-7}$ moles and average NO surface flux of $5.0 \pm 0.38 \times 10^{-10}$ moles/(cm$^2 \cdot$min) after 24 hours of release.

As seen with Figure 3.5 qualitatively there is no bacterial growth under the disks. Considering the experiment results in the previous chapter, this confirms NO release as a feasible, localized treatment and preventative measure to bacterial infection from medical devices.

3.5.3 SUMMARY

The gradient strip experiments created a visual representation relating SNAP-PDMS concentration to *S. epidermidis* inhibition, and allowed the determination of the appropriate dose needed. It can be seen that an increase in SNAP-PDMS concentration produces an increase in bacterial growth inhibition. It is important to note that bacteria were uninhibited under RTV3140 when next to an effective concentration of SNAP-PDMS. As for the cross-talk experiment, SNAP-PDMS disks provided a highly localized

effect on bacterial growth. Although, there was some cross-talk as the disks got closer, bacteria were still able to grow between the disks just at a lower density.

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CHAPTER 4

BACTERIAL ADHESION INHIBITION WITH SNAP-PDMS

DISKS IN SOLUTION

Continuing with the previous chapters, bacterial adhesion is of current concern to the medical field. In fact, the number one reason for failure of medical devices is biomaterial-associated infections [1]. This is of extreme concern because it can lead to removal of the device which is not only costly, but can be traumatic to the patient [2]. Adding to concerns, there are approximately one million implant-associated infections each year, and traditional antibiotics are no longer a sufficient treatment in many of those cases [3]. Because of the growing antibiotic resistance problem, one of the simplest ways to prevent infection is by inhibiting bacterial adhesion.

As previously stated, certain bacteria have the ability to form a biofilm which are more difficult to treat. One factor in biofilm development is whether or not the bacteria are allowed to adhere long enough to where they become irreversibly attached [4]. The biofilm makes the infection harder to treat by obstructing treatment from the host as well as traditional antibiotics [2]. Again, one way to prevent implant-associated infection is to prevent bacteria from adhering in the first place which would limit biofilm formation.

S-Nitroso-N-acetyl-D-penicillamine covalently linked to polydimethylsiloxane (SNAP-PDMS) was once again utilized for its antibacterial properties. SNAP-PDMS provides a highly localized release of nitric oxide (NO) which has been shown to inhibit

bacterial growth. Experiments were completed to test whether bacteria adhered to SNAP-PDMS and RTV3140 disks when submerged in various bacterial broth solutions. The solution experiments served to compare whether or not SNAP-PDMS had an inhibitory effect on bacterial adhesion prior to being plated on media plates.

4.1   SELECTION OF BACTERIA

For the broth experiments, three different bacterial strains were tested: *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus aureus* (*S. aureus*), and methicillin-resistant *Staphylococcus aureus* (MRSA). As described in Chapter 2, all three bacterial strains are prevalent causes of infections as well as are becoming harder to treat with traditional antibiotics due to their growing antibiotic resistance. Specifically, the most frequently recovered bacteria are *S. epidermidis*, and *S. aureus* is often considered the most important staphylococcus [5]. Due to *S. aureus* virulence factors, antibiotic-resistant strains have developed, and MRSA is one of the most popular superbugs due to its multidrug resistance [6]. *S. epidermidis* (ATCC 12228), *S. aureus* (ATCC 29213), and MRSA (ATCC 33591) were purchased from Microbiologics, Inc., (St. Cloud, MN) in single strain LYFO DISK pellets.

4.2   PREPARATION OF MATERIALS

Similar to the disk susceptibility and cross-talk experiments, SNAP-PDMS was again used to test whether the localized release of NO had an inhibitory effect on bacterial adhesion. SNAP-PDMS and RTV3140 disks were submerged in bacteria

inoculated tryptic soy broth (TSB) and then plated on Mueller-Hinton plates.

As described in section 2.3.1, SNAP-PDMS and RTV3140 films were synthesized and disks punched out using a 4mm diameter cork borer. This testing was completed to determine whether or not bacteria adhered to the polymer after being submerged in bacterial broth solution. The goal was to investigate the inhibitory effects SNAP-PDMS has on bacterial adhesion.

Mueller-Hinton 28ml filled 15x100mm media plates and tryptic soy broth #2 dehydrated culture media were purchased from Hardy Diagnostics Inc. (Santa Maria, CA). TSB was prepared by dissolving the dehydrated media into deionized water. For every 100ml of water, 3000mg of dehydrated media was added; lastly, the TSB was then sterilized in an autoclave.

4.3   EXPERIMENTAL SETUP

Stock plates of each bacterial strain were created as described in Chapter 2 with Mueller-Hinton media plates. VWR SuperClear 50ml centrifuge tubes, sterile 5ml Cryo.s Cryogenic Storage Vials, and a VWR Mini Shaker 15 were purchased from VWR International (Radnor, PA). 5-0 chromic gut on a RB-1 sutures were donated by Portage Health (Hancock, MI).

4.3.1   TRYPTIC SOY BROTH

The sterile TSB was removed from storage, and 25ml was pipetted into a 50ml

centrifuge tube (one per bacterial strain) where the TSB was then allowed to acclimate to room temperature. For each bacterial strain tested, six vials were used in total: three for RTV3140 and three for SNAP-PDMS. Two disks were placed in each vial; for example, vials #1-3 would have a RTV3140 disk placed at the bottom as well as an additional disk was suspended approximately half way down the vial. Therefore, six RTV3140 disks and six SNAP-PDMS disks were required per bacterial strain evaluated. The disks were suspended by stitching a suture through the polymer disk and then attaching the additional suture on the outside of the vial near the top. This allowed the second disk to be hanging down the middle of the vial. Figure 4.1 is a visual representation for one RTV3140 vial and one SNAP-PDMS vial.

Once the disks were in place, isolated bacterial colonies were added to the TSB filled centrifuge tube from the appropriate stock plates until there were approximately 10^8 colony-forming-units (CFU) per ml. According to literature, the appropriate density can be established through the use of UV-VIS spectroscopy and evaluating absorbance values at a given wavelength. For *S. epidermidis* and *S. aureus* there should be an absorbance of 0.2 at a wavelength of 600nm [7]. After isolated colonies were added to the TSB, the solution was stirred thoroughly until it was a homogeneous solution. Similar to previous experiments, 2ml of the TSB with bacteria was pipetted from the centrifuge tube into a disposable cuvette and put into the UV-VIS. Depending on the UV-VIS reading, the cuvette was emptied back into the centrifuge tube and more bacteria or TSB was added until the desired absorbance was reached.
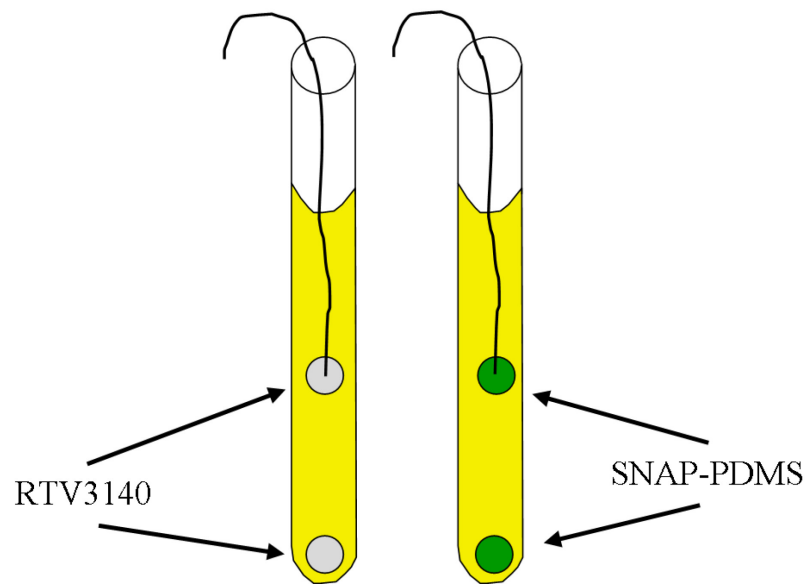
Figure 4.1. Visual representation of TSB experimental setup for one RTV3140 (left) vial and one SNAP-PDMS (right) vial.

Once the appropriate bacterial concentration was achieved, the TSB bacterial solution was divided among all six vials. This was done by pipetting 4ml of the TSB bacterial solution per vial. The vials were loosely capped and placed in a test tube holder which was placed on top of the mini shaker table set to 50rpm inside the incubator at 37˚C for 90 minutes in dark conditions.

4.3.2   PLATED RTV3140 AND SNAP-PDMS DISKS

After the 90 minutes of incubation, the TSB bacterial vials were removed from the incubator. The disks were removed one by one and rinsed before being plated on Mueller-Hinton media plates. To begin, three sterile 150ml beakers were filled with approximately 100ml of sterile water. RTV3140 disks were removed first. Each disk was submerged and removed from one beaker and moved to the next until it was rinsed by all three beakers to remove any loosely adhered bacteria. Sterile tweezers were used between each beaker as well as before plating each disk to minimize contamination from bacteria moving from one beaker to another. After all six RTV3140 disks were rinsed and plated, the same procedure was used for the SNAP-PDMS disks.

At the end of the three rinses, each disk was placed on a Mueller-Hinton media plate. The media plate was sectioned into six wedges, so that one plate could be used for RTV3140 disks and one plate for SNAP-PDMS disks. After all RTV3140 and SNAP-PDMS disks were appropriately plated and sealed with parafilm, the two plates were inverted and placed in the incubator at 37˚C for 18 hours in dark conditions. Additionally, a third Mueller-Hinton plate was sectioned into six wedges. Using a sterile loop per vial,

bacteria were inoculated onto the plate from each TSB bacterial vial to show the presence of bacteria in solution. The plate was again sealed, inverted, and placed in the incubator at the same conditions to the plates with disks.

After 18 hours of time had elapsed, all three plates were removed from the incubator and imaged with a ViTiny Pro10 Plus Portable Digital Microscope 10x - 200x purchased from Oasis Scientific, Inc. (Greenville, SC). Multiple images were taken at various magnifications both with the RTV3140 and SNAP-PDMS disks present as well as after the disks were removed.

4.3.3 NITRIC OXIDE RELEASE MEASUREMENTS

A Sievers Nitric Oxide Analyzer (NOA) 280i (GE Instruments, Boulder, CO) was used for NOA measurements. The SNAP-PDMS disks used in the TSB bacterial solution experiments were from the same batch as the disks used for the Kirby-Bauer disk susceptibility testing done in Chapter 2. Therefore, the data collected from the previous experiment was used to quantify NO released and surface flux for these experiments.

4.4 RESULTS

The TSB experiments were done to compare the bacterial adhesion of RTV3140 disks to SNAP-PDMS disks when suspended in bacterial solutions. From a visual perspective, there was no difference seen between bacterial adhesion from samples placed at the bottom of the vial and those in suspension. As seen in Figure 4.2, RTV3140 disks visually have much more bacteria present around and under the disks as compared

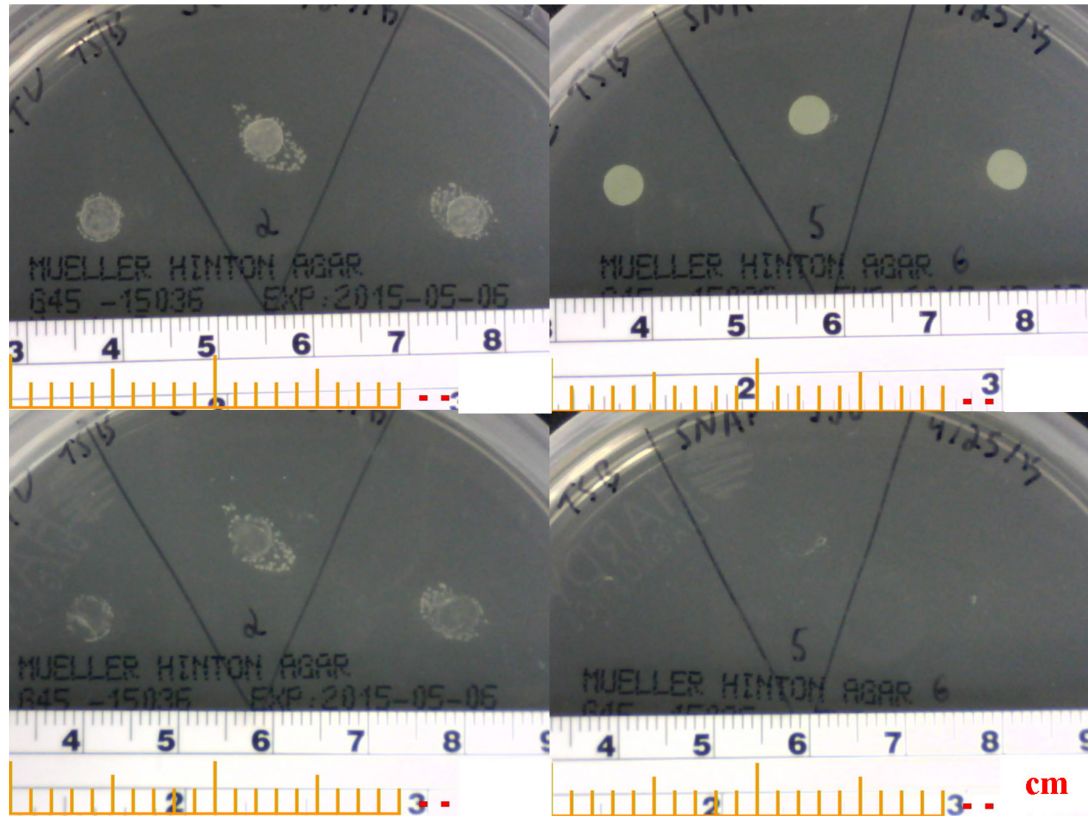**Figure 4.2.** Half plate images taken of *S. epidermidis* tryptic soy broth bacterial adhesion testing. The top row is images with polymer disks present whereas the bottom row is after the disks are removed after 18 hours incubation at 37°C in dark conditions. The left column represents the 3 RTV3140 disks in suspension whereas the right column is the SNAP-PDMS disks on the bottom of the vial.

to the SNAP-PDMS disks for the *S. epidermidis* experiment. Figure 4.3 is one RTV3140 and one SNAP-PDMS disk example which further validates bacterial adhesion inhibition for *S. epidermidis*. Figure 4.4 and Figure 4.5 are single disk representations of *S. aureus* and MRSA, respectively. Although there are some bacteria present around the edge of the disks, qualitatively there are fewer bacteria present beneath the disks. Lastly, Figure 4.6 represents the bacterial streaks made from each vial for *S. aureus*. It can be seen that there were plenty of bacteria within the TSB solution.

Because the SNAP-PDMS disks were punched from the same batch of polymer used for the disk susceptibility testing in Chapter 2, the results of the total NO released and surface flux for the seven disks can be found in Table 2.3. The average NO released was 3.9 ± 0.69 x 10^{-8} moles while the average NO surface flux was 1.1 ± 0.19 x 10^{-10} moles/(cm^2·min).

4.5   DISCUSSION

Presented in this work are qualitative images further proving that NO is an effective bacterial adhesion inhibitor. The SNAP-PDMS used in these experiments produces enough NO to have a negative effect on bacterial adhesion for all three strains of bacteria evaluated.

4.5.1   TRYPTIC SOY BROTH ADHESION TESTING

The purpose of these experiments was to demonstrate the effect NO has on bacterial adhesion, specifically NO released from SNAP-PDMS. RTV3140 was chosen

RTV3140   SNAP-PDMS

Figure 4.3. Images of RTV3140 (left) compared to SNAP-PDMS (right) for *S. epidermidis* for tryptic soy broth bacterial adhesion testing at dark conditions after 18 hours of incubation at 37˚C for vial #2 bottom disks. The top row is with disks present and the bottom is after disks are removed.
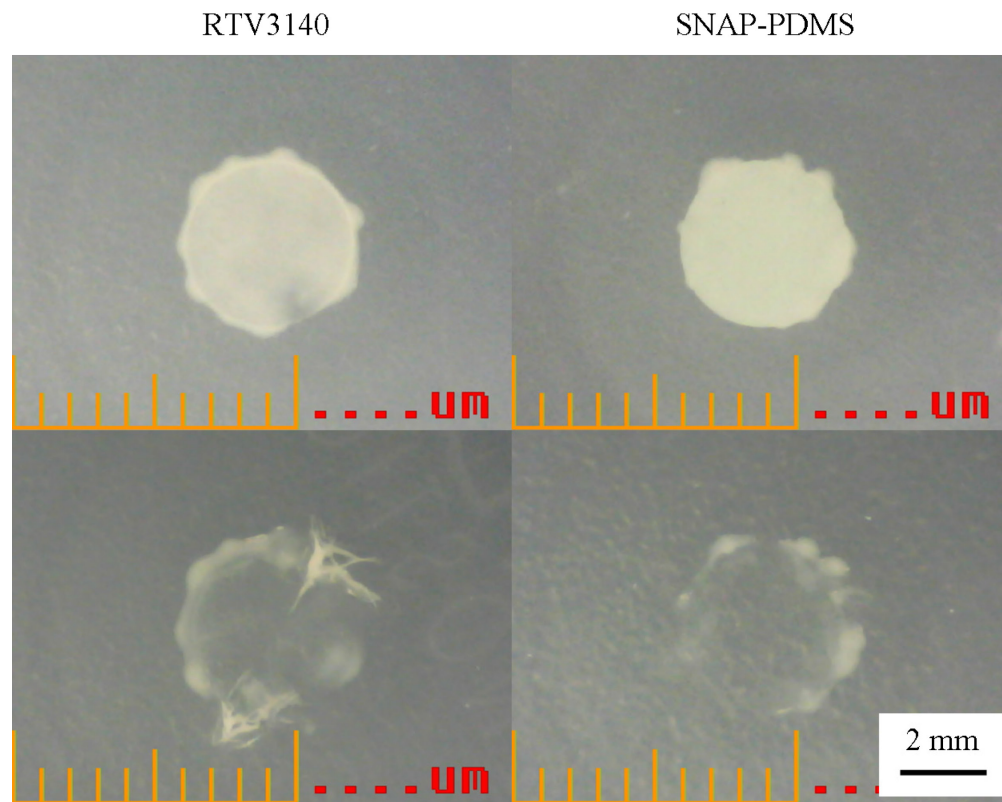
RTV3140   SNAP-PDMS

Figure 4.4. Images of RTV3140 (left) compared to SNAP-PDMS (right) for *S. aureus* for tryptic soy broth bacterial adhesion testing at dark conditions after 18 hours of incubation at 37°C for vial #3 top. The top row is with disks present and the bottom is after disks are removed.
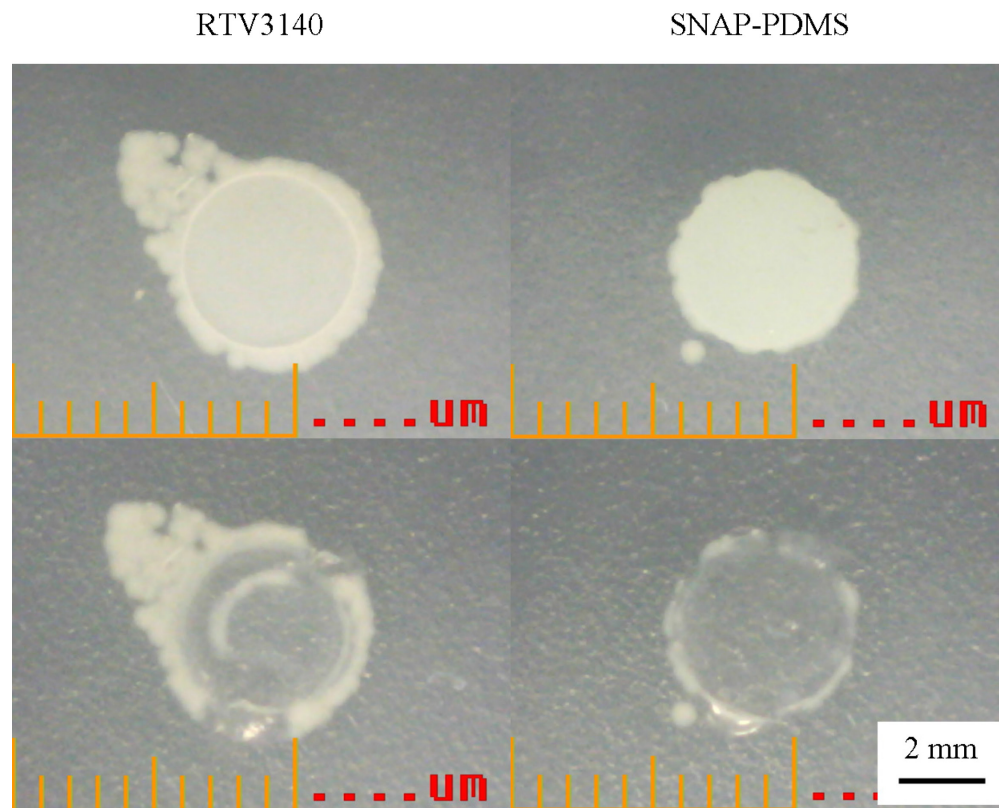
RTV3140   SNAP-PDMS

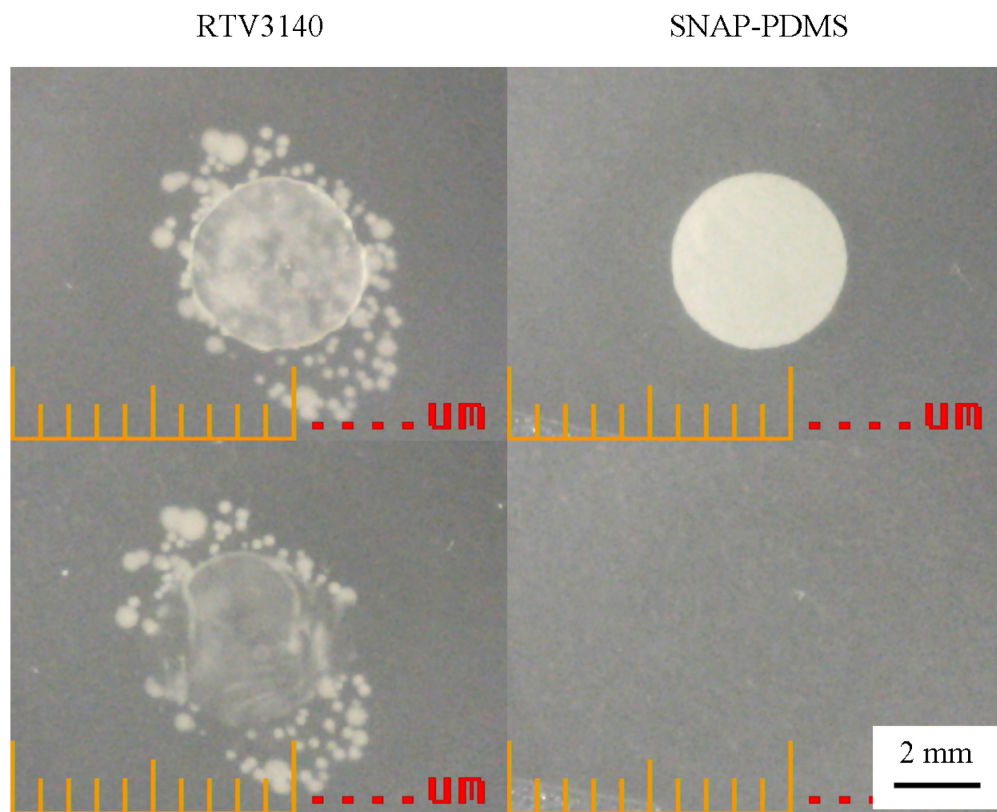**Figure 4.5.** Images of RTV3140 (left) compared to SNAP-PDMS (right) for MRSA for tryptic soy broth bacterial adhesion testing at dark conditions after 18 hours of incubation at 37˚C for vial #2 bottom. The top row is with disks present and the bottom is after disks are removed.
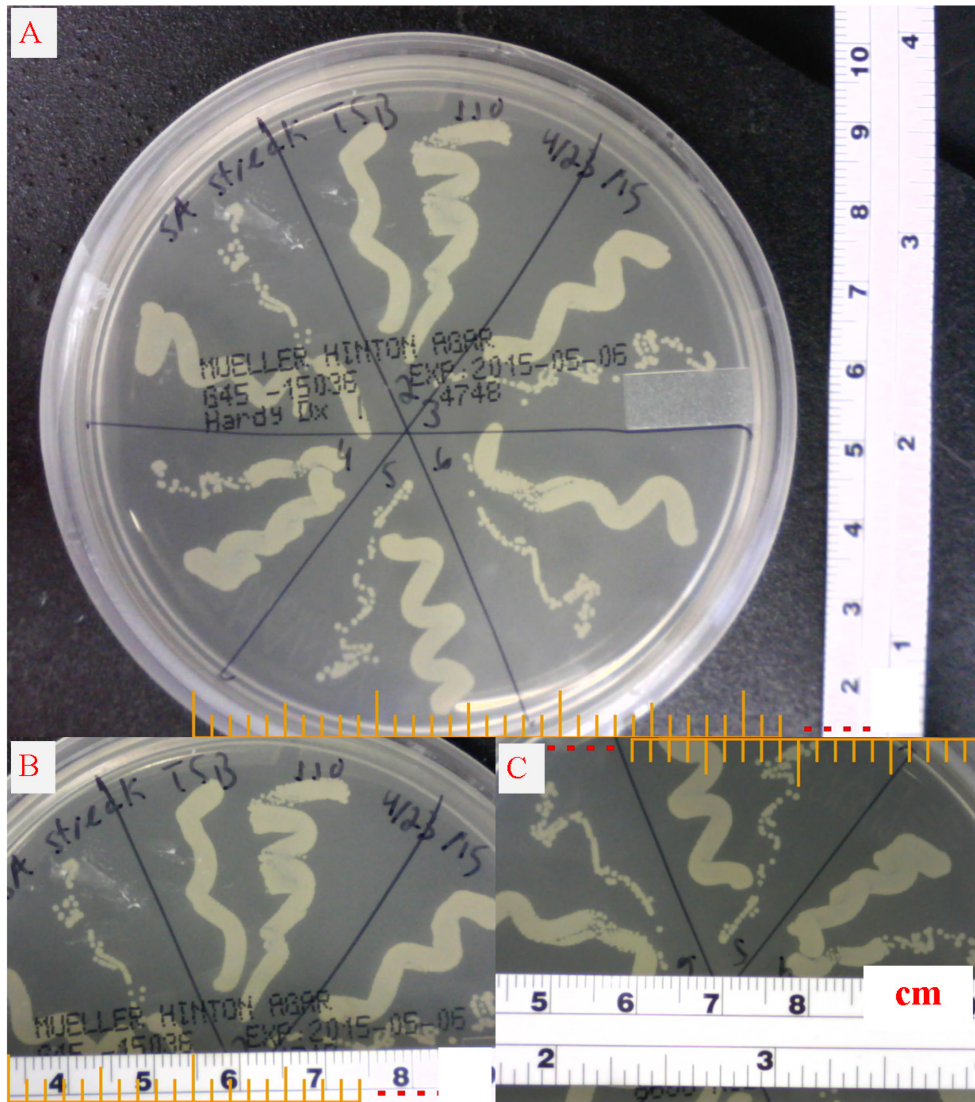
Figure 4.6. Images taken of *S. aureus* bacterial streaks made from tryptic soy broth bacterial solutions after being incubated at 37°C for 18 hours. A) Image of entire media plate showing all six vials bacteria was streaked from, B) Image of half media plate showing vials 1-3, and C) Image of half media plate showing vials 4-6.

as the control because it is surgical grade silicone rubber and readily allows bacteria to adhere to it and provides no antimicrobial properties.

The disks in suspension were qualitatively compared to those placed at the bottom of the vials to evaluate whether gravity was a significant factor to increased bacterial adhesion. The theory was that there would be more bacteria present on the disks placed in the bottom because of gravity causing bacteria to settle on the polymer. However, the gentle stirring caused by the mini shaker allowed the bacteria to float and move around while suspended in the TSB.

Figure 4.2 provides a bigger picture for comparison. It can be seen that qualitatively there are fewer bacteria around and under the SNAP-PDMS disks as compared the RTV3140 disks. Additionally, it is difficult to distinguish where the SNAP-PDMS disks once were because there was minimal to no bacteria present for *S. epidermidis*. Figure 4.3 provides a closer look to one of the six disks evaluated against *S. epidermidis* adhesion. SNAP-PDMS did successfully prevent *S. epidermidis* adhesion as compared to RTV3140 to the point where there is almost no sign of where the disk once was.

Further validating SNAP-PDMS as an effective bacterial adhesion inhibitor can be seen against *S. aureus* and MRSA which can be seen in Figure 4.4 and Figure 4.5, respectively. Although there are still bacteria present around the SNAP-PDMS disks, it can be seen there are fewer bacteria found after the disks are removed. There is some cloudiness present which can be partially due to the pulling in of bacteria from the edge

as SNAP-PDMS disks were removed. From a physical stand point, the RTV3140 disks were much harder to remove for all three strains of bacteria tested because there was an abundance of bacteria under the disk. This was observed by the disks being almost suctioned to the media plate.

Figure 4.6 is the bacterial streaks from the vials after disks were plated for *S. aureus* experiments. This was important to show the presence of bacteria in the TSB solution while disks were submerged and suspended. Both *S. epidermidis* and MRSA had similar results displaying an abundance of bacteria within solution (images not shown).

It is important to note that the disks were only in TSB solution for 90 minutes, and in that short window of time, there were copious levels of bacteria allowed to adhere to RTV3140 disks. Not only did all three bacteria types adhere, but they adhered well enough to not be rinsed off in the three submergences in sterilized water. On the other hand, for all three strains of bacteria (*S. epidermidis*, *S. aureus*, and MRSA) SNAP-PDMS disks with an average NO release of 3.9 ± 0.69 x 10^{-8} moles and average NO surface flux of 1.1 ± 0.19 x 10^{-10} moles/(cm^2·min) after 18 hours was adequate to inhibit bacterial adhesion. It should be noted that the minimal surface flux of NO that is required to inhibit adhesion of bacteria was not determined in this series of experiments. The minimal level of NO required may be less than 1.1 ± 0.19 x 10^{-10} moles/(cm^2·min).

4.5.2   SUMMARY

In conclusion, from a qualitative perspective, it can be seen that SNAP-PDMS disks did reduce bacterial adhesion as compared to the RTV3140 control disks for three

strains of bacteria that are frequent culprits to infection. The SNAP-PDMS disks most suggestively reduced *S. epidermidis* bacterial adhesion, as well as inhibited *S. aureus* and MRSA bacterial adhesion. Literature has shown NO to having an inhibitory effect on bacteria, but these results show that NO released from SNAP-PDMS disks directly provides a reduction in bacterial adherence. Based on NOA measurements, an average of NO release of 3.9 ± 0.69 x 10^{-8} moles and NO surface flux of 1.1 ± 0.19 x 10^{-10} moles/(cm^2·min) is needed to inhibit bacterial adhesion.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

There is an abundance of research demonstrating that the problem of antibiotic-resistant bacteria is growing at an alarming rate. Research continues to develop more efficient and potent antibiotics to fight these virulent strains of bacteria, but there has also been new research into other possible approaches to fighting as well as preventing bacterial infection. As a result, research has been conducted assessing nitric oxide (NO) as a means to prevent bacterial infection. The results of this research have concluded that NO does possess the ability to inhibit bacterial growth, and thus it can be utilized in improving the use of a variety of medical devices such as a urinary catheter.

Currently, the majority of research focuses on an effective versus ineffective treatment against various bacterial strains. However, the success of NO as a means to fight bacterial infection has motivated further research into the necessary dose or surface flux of NO needed to inhibit bacterial growth as well as an assortment of delivery materials. Additionally, whether or not the amount of NO needed to prevent infection is different amongst various strains of bacteria has yet to be determined. Answering some of these questions could improve the success of a several medical devices by preventing their failure due to an untreatable bacterial infection. S-Nitroso-N-acetyl-D-penicillamine covalently linked to polydimethylsiloxane (SNAP-PDMS) can be utilized to determine the NO dosage and surface flux necessary to inhibit bacterial growth because of its controlled NO release due to its photosensitivity.

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The experiments shown in this work have further validated NO at a surface flux of 0.28×10^{-10} moles/(cm^2·min) as an effective treatment for preventing bacterial growth and adhesion specifically for *Staphylococcus epidermidis*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and *Escherichia coli*. SNAP-PDMS has been shown to deliver a highly localized level of NO which through a total NO release of 1.7×10^{-8} moles has an inhibitory effect on bacterial growth. Not only did SNAP-PDMS prevent bacterial growth on media plates and adhesion in solution, it also produced lasting effects on a virulent strain of bacteria over a ten day period.

Future work based on the results presented here can be done to further determine whether it is the total amount of NO delivered or the specific surface flux that is responsible for inhibiting bacterial growth. Additionally, through the use of SNAP-PDMS, it can be studied whether the type of dosage has an effect on bacterial inhibition. For example, whether a high initial NO release for a short period of time is just as effective as a lower initial NO release over a longer period of time.

With SNAP-PDMS determining the necessary dosage and surface flux of NO to impede bacterial growth, it can be utilized for use in a specific medical device to improve success rates. Considering the base material of urinary catheters and SNAP-PDMS, a transition into the development of an NO releasing urinary catheter could provide great success because of the vast amount of catheter-associated urinary tract infections occurring each year. The success of this work validates NO as an extremely localized treatment which is beneficial to the fight against the growing number of antibiotic-

resistant bacteria which can require high doses of potent drugs that can have serious side effects.

APPENDIX

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