REDUCING INFECTION OF MEDICAL DEVICES VIA SOLVENT SWELLING-IMPREGNATION OF *S*-NITROSO-*N*-ACETYLPENICILLAMINE

by

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(Under the Direction of Hitesh Handa)

ABSTRACT

Healthcare-associated infections are a major hindrance in the healthcare industry, increasing hospital costs, hospital stay, and mortality rates. Two of the most common HAIs are catheter-associated urinary tract infections (CAUTIs) and ventilator-associated pneumonia (VAP), related to urinary catheters and endotracheal tubes, respectively. Nitric oxide (NO), an endogenous gas released from the endothelium, is proven to be a strong bactericidal agent. Nitric oxide can be incorporated into biomaterials using NO donors for localized release by a variety of methods. These studies will focus on solvent swelling to incorporate the NO donor *S*-nitroso-*N*-acetylpenicillamine. The studies herein examine the effects of (i) solvent swelling to create a synergistic combination of NO with liquid-infused properties for the reduction of CAUTIs and (ii) solvent swelling with endotracheal tubes for the use of NO to reduce the occurrence of VAP.

INDEX WORDS: Nitric oxide, healthcare-associated infections, antimicrobial, catheter-associated urinary tract infections, ventilator associated pneumonia

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CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

1.1 Healthcare-Associated Infections and Biofilm Formation

The use of biomedical devices in a clinical setting are inevitable in order to administer treatments. The rate at which these devices are used will only rise as increased hospitalization rates along with an aging population become more prevalent. Infections are often associated with indwelling devices used in medical procedures such as catheters or endotracheal tubes. Since the 1970s, healthcare-associated infections (HAIs) have been a major threat to the healthcare industry. The threat of HAIs is demonstrated by the Centers for Disease Control and Prevention (CDC) reporting that on any given day, about one in 25 hospital patients has at least one healthcare-associated infection.[1,2] Therefore, prevention and elimination of HAIs has become a priority for the Department of Health and Human Services.

Healthcare-associated infections ultimately lead to increased hospital costs, length of stay for patients, and mortality rates. In 2011, the CDC reported that 722,000 HAIs occurred in US hospitals and about 75,000 patients with HAIs died during their hospitalization.[2] Additionally, more than half of the HAIs occurred outside of the intensive care unit (ICU), demonstrating that all types of patients within the hospital are susceptible.[1] The major HAIs include central line-associated bloodstream infections (CLABSI), surgical site infections (SSI), catheter-associated urinary tract infections

(CAUTI), and ventilator-associated pneumonia (VAP). Central line-associated bloodstream infection is an infection that occurs when bacteria enter the bloodstream through a central line catheter that is placed in the patient's vein. These infections result in thousands of deaths each year and billions of added costs to the healthcare system.[1-3] Catheter-associated urinary tract infection is an infection involving any part of the urinary system resulting from urinary catheter implantation to drain the urine. Urinary tract infections are the most common type of HAI reported with 75% of the infections associated with a urinary catheter.[1,4] Lastly, VAP is a lung infection that develops in a patient who is on a ventilator to help them breathe by providing oxygen through an endotracheal tube.[1,5] As described above, it is clear that each of these infections are associated with the introduction of a medical device into the body. The contamination of the medical devices most likely occurs by the inoculation of microorganisms from the patient's skin or mucous membranes during implantation, the hands of the surgical or clinical staff treating the patient, contaminated disinfectants, other patients in the hospital, or distant local infections.[3,6] The patient characteristics, microorganisms involved in the infection, and the type of medical device all factor into the morbidity and mortality of HAIs, but medical devices significantly contribute to the increasing problem of nosocomial infections.[3] Once the bacteria comes into contact with the device and is introduced to the surrounding medium, the formation of a biofilm on the surface of the device becomes likely, increasing the risk of HAIs and decreasing the chance of eradication. This attributes to the fact that among all microbial and chronic infections, 65% and 80%, respectively, are associated with biofilm formation.[7-9]

Naturally, most bacteria species alternate between planktonic (free-floating) and sessile (stationary) states depending on the current environmental stimuli, such as the availability of nutrients.[10] The transition to a sessile state from planktonic frequently occurs in response to nutrient limitation and is hypothesized to be the developmental switch leading to biofilm formation on a surface.[10] Due to bacterial cells having a strong preference for life on surfaces rather than in planktonic suspension, free-floating bacteria can become attached to foreign surfaces in the surrounding medium within minutes.[4,11] Upon attachment, the bacterial cells secrete exopolysaccharides that secure their attachment to the surface and encase the cells in extracellular polymeric substance (EPS).[4,11] The production of the EPS allows the underlying cells in the emerging biofilm community to mature and protects them from environmental factors including the ability for the underlying biofilm to evade host defenses and withstand antibiotics or antiseptics.[4,11,12] Additionally, aggregated cells can become detached from the biofilm, or roll along a surface remaining protected from the EPS.[11] The detached bacteria can move to other areas on the surface or even other areas in the body, propagating other biofilm communities, and increasing the risk of infection.[4] **Figure 1.1** demonstrates the biofilm formation process as planktonic bacteria mature into a biofilm community. The primary host defense against infection is the immunity that is provided by neutrophils, macrophages, and dendritic cells. However, some bacteria have the ability to thwart neutrophils and macrophages by inhibiting chemotaxis, negating opsonization, and thwarting phagocytosis.[13]

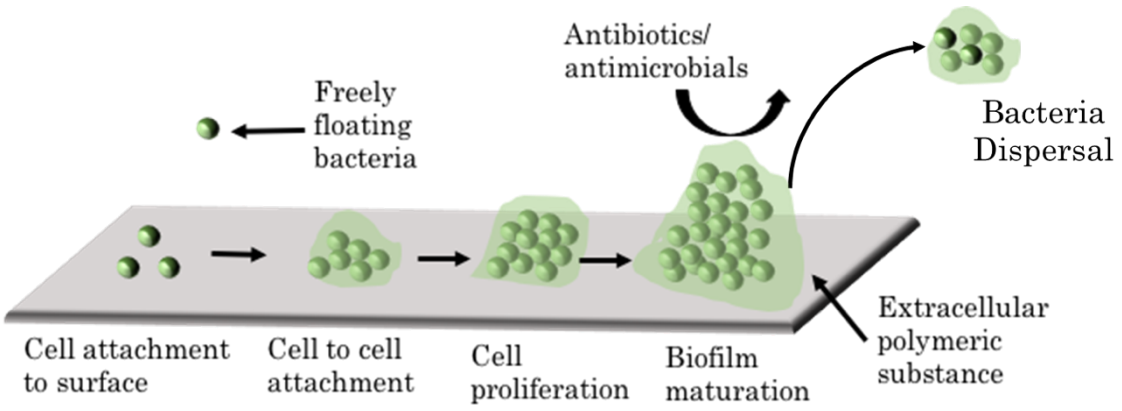

Figure 1.1: Conceptualization of biofilm formation on foreign surface. Free floating bacteria adhere to the foreign surface, and then proliferate, leading to biofilm maturation and development against antimicrobials due to protection from the extracellular polymeric substance. Aggregated cells can become detached and remain in protected biofilm state.

Biofilms are commonly regulated by inter- and intraspecies quorum-sensing mechanisms, allowing the cells to exchange genetic elements.[10] The genotypic response of quorum-sensing is of clinical significance in the development and spread of antibiotic resistance traits in nosocomial pathogens.[10] Three characteristics of biofilms attribute to their high resistance characteristics: 1) slow penetration of antibiotics due to the complex EPS network, often trapping the antimicrobials as they attempt to infiltrate the matrix;[10] 2) formation of a resistant phenotypes resulting in persister cells that remain in a transient dormant state and have the ability to cause recurrent infections;[9] and 3) an altered environment of physiologic heterogeneity within the biofilm that is composed of different anaerobic niches, different nutrient or oxygen concentration gradients, and the local accumulation of inhibitive waste and by-products.[10] The inadequate exposure of antimicrobial agents at all relevant sites of infection is one of the fundamental factors underlying the failure of therapies to treat the infection and presumably limits the efficacy of some agents in longstanding biofilm-associated infections.[10] Even at

concentrations 1000 times the traditional therapeutic dose, antibiotics have been shown to have no effect in treating biofilms.[14] The presence of biofilms is dangerous because biofilm infections can linger for months, years, or even a lifetime. These infections are often traced to popular species of opportunistic pathogens, such as *Pseudomonas aeruginosa* or *Staphylococcus epidermidis*, that persist because they are adept at forming biofilms to protect them.[12] These characteristics of biofilms are why it is important to eliminate the chance of infection from the insertion of the medical device, especially with HAIs that are often caused by antibiotic resistant bacteria. Overall, HAIs and the development of biofilms on indwelling foreign surfaces are a threat to the healthcare industry. Consequently, there have been numerous attempts in prevention of HAIs with modifications to the various medical devices that aid in causing these infections.

1.2 Current Preventative Methods

As HAIs have become a major issue and economic drawback to the healthcare system, there have been many preventative methods employed in clinical and research settings to devise measures to prevent and eliminate them. Overall, the replacement or removal of an infected device, administration of antibiotics, improvement to the mechanical design, and the use of antimicrobials incorporated into the devices are all preventative and treatment methods currently considered to prevent HAIs. Often, when a device becomes infected, replacement or removal of the device is necessary, resulting in additional distress and discomfort to the patient and increased cost to the patient and hospital.[15] The removal or replacement of the device is usually the last resort when

antibiotic therapies do not treat the infected patient, and is not an ideal solution for addressing the issue related to HAIs.

Much like other industrial products, the design of medical devices has evolved to address the changing demands of the industry related to patient comfort and prevention of infection. To help prevent infections, many medical devices have gone through mechanical design changes. These changes include the addition of skin cuffs and/or changing the material of the device.[13,16,17] The material is important in preventing the initial adhesion of organisms that can lead to biofilm formation. The characteristics that influence this adhesion are surface roughness, surface free energy, and the physical composition of the material.[6,16] Surface roughness is important to consider as increasing the roughness increases the available attachment area by a factor of 2-3 and surfaces with higher surface free energy are more prone to bacterial adherence.[6,16] Medical device design alternatives have only had marginal success and are proven successful only for short-term applications.[13,15] Although medical devices often differ in design and use characteristics, specific factors determine the overall susceptibility of a device to microbial contamination and biofilm formation such as duration of use, the types of organisms to which the device is exposed, flow rate and composition of the medium in or around the device, and device material construction.[18] These types of design modifications still have a high risk lingering infection, which potentially leads to complete removal of the device and increasing the overall discomfort of the patient. Since replacement or removal of an infected indwelling medical device and design changes are not ideal solutions on their own, they are often combined with systemic antibiotics.[15]

Antibiotics are compounds isolated from one living organism to kill or inhibit the growth of other organisms.[4] Antibiotics have been extensively researched due to their antibacterial, antifungal, antiviral, antiparasitic, and anticancer activity.[4,19-21] They are classified based on the cellular component or system they affect, in addition to whether they induce cell death or solely inhibit cell growth.[22] Most antibiotics are bactericidal in four ways including the inhibition of DNA synthesis, RNA synthesis, cell wall synthesis, and/or protein synthesis.[4,22,23] **Figure 1.2** provides a schematic representing the 4 main bactericidal mechanisms of antibiotics.

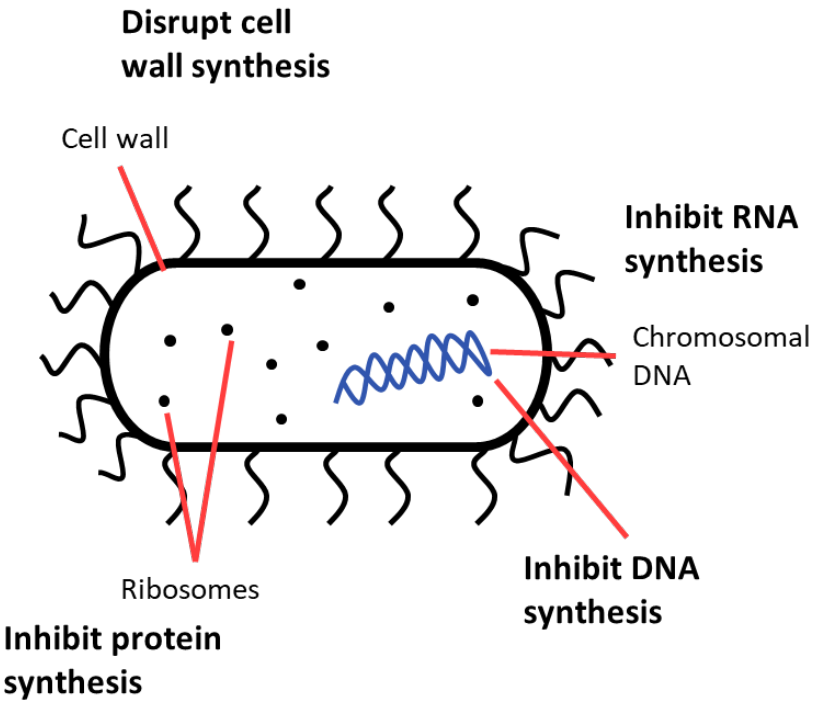

Figure 1.2: Bactericidal mechanisms of antibiotics. Antibiotics enter the bacteria cell and can disrupt cell wall synthesis, inhibit RNA synthesis, inhibit DNA synthesis, and/or inhibit protein synthesis.

The inhibition of DNA replication is typically accomplished by the quinolone antibiotic class (e.g., levofloxacin, gemifloxacin), interfering with the maintenance of chromosomal

topology, trapping the enzymes during the DNA cleavage stage and preventing DNA strand rejoining.[22] The inhibition of RNA synthesis is accomplished by rifamycins. The inhibition of RNA synthesis has a catastrophic effect on prokaryotic nucleic acid metabolism. Rifamycins are effective at inducing cell death as they inhibit DNA-dependent transcription that transcribes the RNA polymerase enzyme.[22] Furthermore, much of the bacterium's ability to survive environmental conditions comes from the cell wall, composed of peptidoglycan layers. The antibiotics that interfere with cell wall synthesis are β-lactams (including penicillins, carbapenems and cephalosporins) and glycopeptides (e.g., vancomycin).[22] These antibiotics change the cell shape and size, induce cellular stress response and result in cell lysis due to cell wall synthesis inhibitors.[22] Drugs that inhibit protein synthesis are among the broadest classes of antibiotics, including macrolide (erythromycin), lincosamide (clindamycin), streptogramin (dalfopristin/quinupristin), amphenicol (chloramphenicol) and oxazolidinone (linezolid).[22] These antibiotics work by physically blocking either the initiation of protein translation, or inhibiting the reaction that elongates the peptide chain.[22]

Once an infection in the patient has been identified, conventional systemic antibiotics, via an oral pill or intravenously, are often administered as the first line of defense. When treating HAIs with antibiotics, prolonged and high-dosage of antibiotics are the typical basis for being successful. For example, antibiotic treatment of bacterial endocarditis using antibiotic levels at least tenfold above the minimal bactericidal concentration was shown to be more successful,[24] but even with 8 weeks of treatment, few patients were cured by antimicrobial therapy alone.[15,25] The issues with systemic

administration of antibiotics are systemic toxicity, renal and liver complications, as well as poor penetration of the bacterial cell.[23] Therefore, the most direct approach for improving the efficacy of conventional antibiotics is to deliver the antibiotics locally by antibiotic coatings and antibiotic impregnation.[26] Antibiotics have been impregnated into a variety of medical devices and have demonstrated to decrease the rate of infection.[27,28] These devices include urinary catheters[28] and central venous catheters.[27] Furthermore, a variety of antibiotic-releasing coatings have been developed with the goal of inhibiting the formation of biofilms on the devices with and without drugs. The coatings are typically fabricated by the encapsulation of various antibiotics within the device polymer matrix to prevent the adhesion of viable bacteria.[23,26,29,30] These coatings incorporate high-affinity moieties such as cyclodextrin (CD) to encapsulate the antibiotic.[23,26] Cyclodextrins are cyclic oligosaccharides with a hydrophilic exterior and a hydrophobic interior that enable the encapsulation of hydrophobic drugs for a longer release.[23] However, the effectiveness of local release is heavily dependent on the rate and manner in which the drug is released, and the material used.[23,26,29] Therefore, if the entire drug is released before the infection is eliminated, the infection persists and antimicrobial resistance becomes a major concern.[23,26]

Unfortunately, in biofilm-based infections, antibiotics only suppress symptoms of the infection by killing the planktonic bacteria that detach from the biofilm population through the mechanisms described above, but fail to eradicate bacteria cells that are still embedded in the biofilm.[12] Preventative use of antibiotics can reduce the incidence of biofilms and infections, however the most prevalent issue associated with antibiotics is the problem of bacterial resistance, often rendering these antibiotics useless after repeated

application.[4,12] Since biofilms require higher doses of antibiotics as a treatment, antibiotic resistance has become a more challenging problem and common infections are caused by the resistant bacteria leading to an increase in resistance rate. According to the World Health Organization, antimicrobial resistance occurs when microbes change upon exposure to antimicrobial drugs. This resistance has led to the development of organisms that are resistant to many antimicrobial therapies, compounding the problem of nosocomial infections. Therefore, a treatment for infections that does not promote the development of resistant bacterial strains is in high demand.

Lastly, metal ions are a popular preventative method for HAIs as well. Metal ions can be incorporated into the polymer surface of medical devices as an antimicrobial agent typically in the form of nanoparticles. Two important parameters that affect the resulting antimicrobial effectiveness of nanoparticles are the type of the materials used for preparing the nanoparticles and the particle size.[31] Transition metal ions like silver (Ag), gold (Au), zinc (Zn), and copper (Cu) are commonly used due to their robust antimicrobial and anti-biofilm properties compared to other metallic ions.[6] Silver is the only metal ion that is currently FDA approved and is used in commercial medical devices such as urinary and vascular catheters. Metal oxide nanoparticles have also gained interest as antimicrobial agents because of their extremely high surface area and unusual crystal morphology increasing the number of potential reactive sites.[6] Commonly used metal oxides are zinc oxide (ZnO), copper oxide (CuO), and iron oxide for their antimicrobial properties.[6,31]

Metal ions, whether through the use of pure metal ions, metal oxides, or in combination, act through similar bactericidal mechanisms. While the exact mechanisms

for the bactericidal effects of metal ions are still being investigated, there are two main proposed possibilities: (1) through free metal ion toxicity arising from the dissolution of metals from the surface of the nanoparticles, and (2) oxidative stress via the generation of reactive oxygen species (ROS) on surfaces of the nanoparticles, damaging proteins involved in adhesion and biofilm formation.[6,31] **Figure 1.3** demonstrates the various bactericidal mechanisms of metal ions.

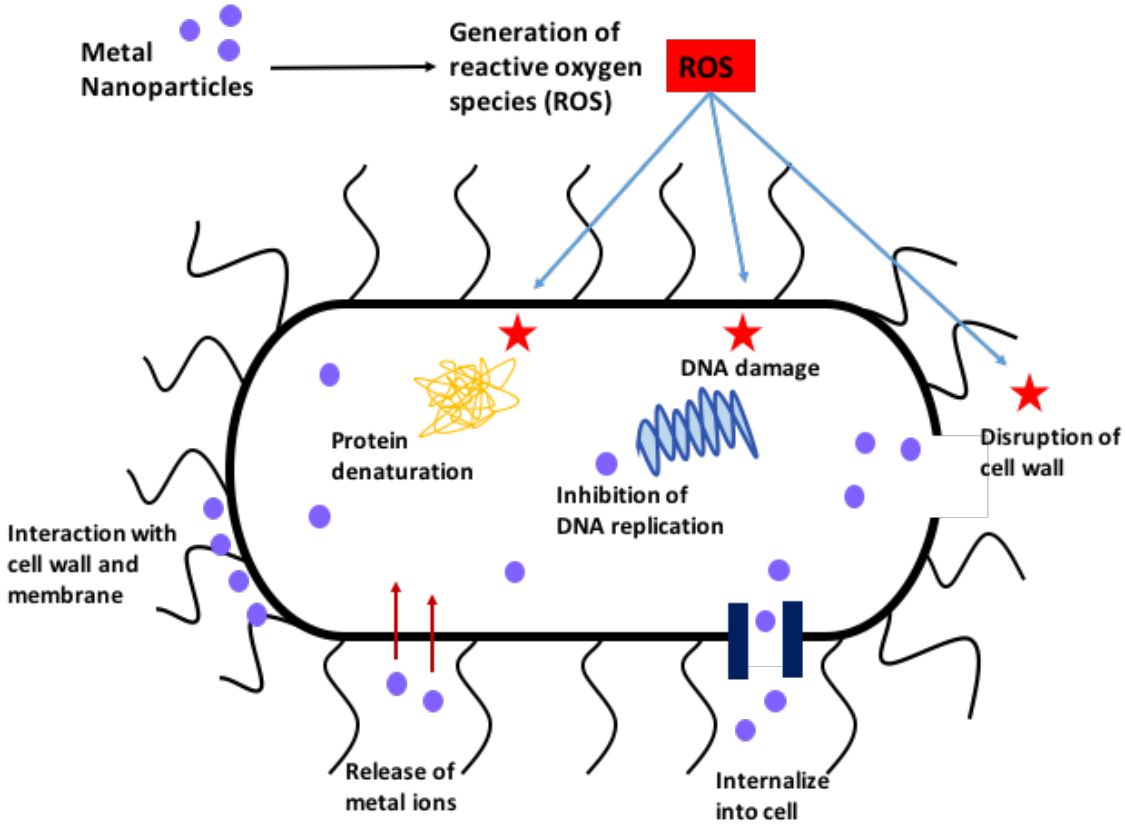

Figure 1.3: Various bactericidal mechanisms of metal ions via nanoparticles.

The metal ion that acts through a separate pathway to inhibit bacterial function is gold (Au). The bactericidal activity of Au is due to the attachment and transportation of the ions through the bacterial membrane which then directly modifies and inhibits tRNA binding to ribosomes.[31] The morphological and physicochemical characteristics of metal ions also have an effect on their antimicrobial activity. Small nanoparticles have the

strongest antimicrobial effect, since they can easily cross the bacteria cell membrane. The positive surface charge also facilitates binding to the negatively charged surface of bacteria through electrostatic attraction, ultimately disrupting the cell wall and membrane.[6,31] The metals ions as nanoparticles can be incorporated into biomaterials and medical devices through impregnation and coatings for their respective application. Silver nanoparticle coatings on medical devices have been shown to inhibit biofilm formation by preventing the initial bacterial adhesion on surfaces with a 3.5 logarithmic reduction in the biofilm development for *P. aeruginosa* and *Acinetobacter baumannii*.[32,33] The impregnation of silver nanoparticles has also proven to be an effective strategy for the reduction of bacterial adhesion and formation of biofilms of *Escherichia coli* and *S. epidermidis*.[34,35] Coatings of ZnO and CuO have also demonstrated to be effective against biofilm formation of *Streptococcus mutans*, reducing formation by 85% for ZnO and 70% for CuO.[36] Catheters with a surface coating of iron oxide improved its resistance against *S. aureus* and *P. aeruginosa* biofilm development.[37]

Although metal ions have shown to have promising and effective antimicrobial effects on preventing bacterial adhesion and biofilm formation, there are disadvantages to using them. A major disadvantage is the accumulation of metal nanoparticles in tissues and organs throughout the body, such as the liver, spleen, brain, and skin, causing long-term damage to the surrounding cells.[6,23] Specifically, silver metal ions deactivate protein anions, inhibiting vital functional pathways for certain cell phenotypes. Also, silver containing medical devices are not economically feasible for large scale production and the possibility of photo-oxidation of the Ag^{+} ions can occur during storage, which can hinder its antimicrobial abilities. Metal ions involving Zn, Cu, and Au are only effective

at large ionic concentrations which is not ideal because the nanoparticles need to release metal ions at an effective concentration that is simultaneously high enough to inhibit bacteria growth but also low enough to prevent downstream tissue toxicities.[23] While metal ions are considered effective as an antimicrobial strategy, the development of antimicrobials that are more biocompatible and non-cytotoxic is necessary to help reduce and prevent HAIs.

1.3 Nitric Oxide-Releasing Materials

Nitric oxide (NO) is an endogenous gaseous signaling molecule released in various parts of the body with numerous biological roles. Nitric oxide is synthesized from the reaction of L-arginine and the appropriate nitric oxide synthase enzyme and oxygen to generate L-citrulline and NO.[38,39] Its continuous release from the surface of endothelial cells effectively promotes vasodilation through smooth muscle cell relaxation and prevents platelet adhesion.[40-45] The release mechanism is also supportive in wound healing, as NO has been shown to promote angiogenesis through the upregulation of vascular endothelial growth factor (VEGF).[46]

Nitric oxide production is part of an effective host response to foreign pathogens as significant increases in systemic NO production has been seen in humans and experimental animals that have infections.[47] Production from macrophages is stimulated by proinflammatory cytokines as well as by microbial products such as lipopolysaccharide located on the outer membrane of Gram-negative bacteria and lipoteichoic acid in the cell wall of Gram-positive bacteria.[47] Aside from the nitrosative and oxidative action NO exhibits on its own, NO can react with oxygen or reactive

oxygen intermediates (e.g. superoxide and hydrogen peroxide) to form oxidative/nitrosative species including peroxynitrite (OONO-) and reactive nitrogen intermediates (RNI).[41,47] Peroxynitrite is primarily utilized from macrophages and affects microorganisms through direct oxidation, lipid peroxidation, protein nitration, and inactivation of enzymes, all due to its ability to pass through membranes.[47] RNIs have been shown to modify DNA, proteins, and lipids; mechanisms through which NO is bactericidal.[47] The reactive species ultimately disrupt vital cellular functions and structures of the bacteria demonstrating the broad antimicrobial efficacy of NO.[41] **Figure 1.4** provides a schematic detailing the biosynthesis of NO and antibacterial mechanisms.

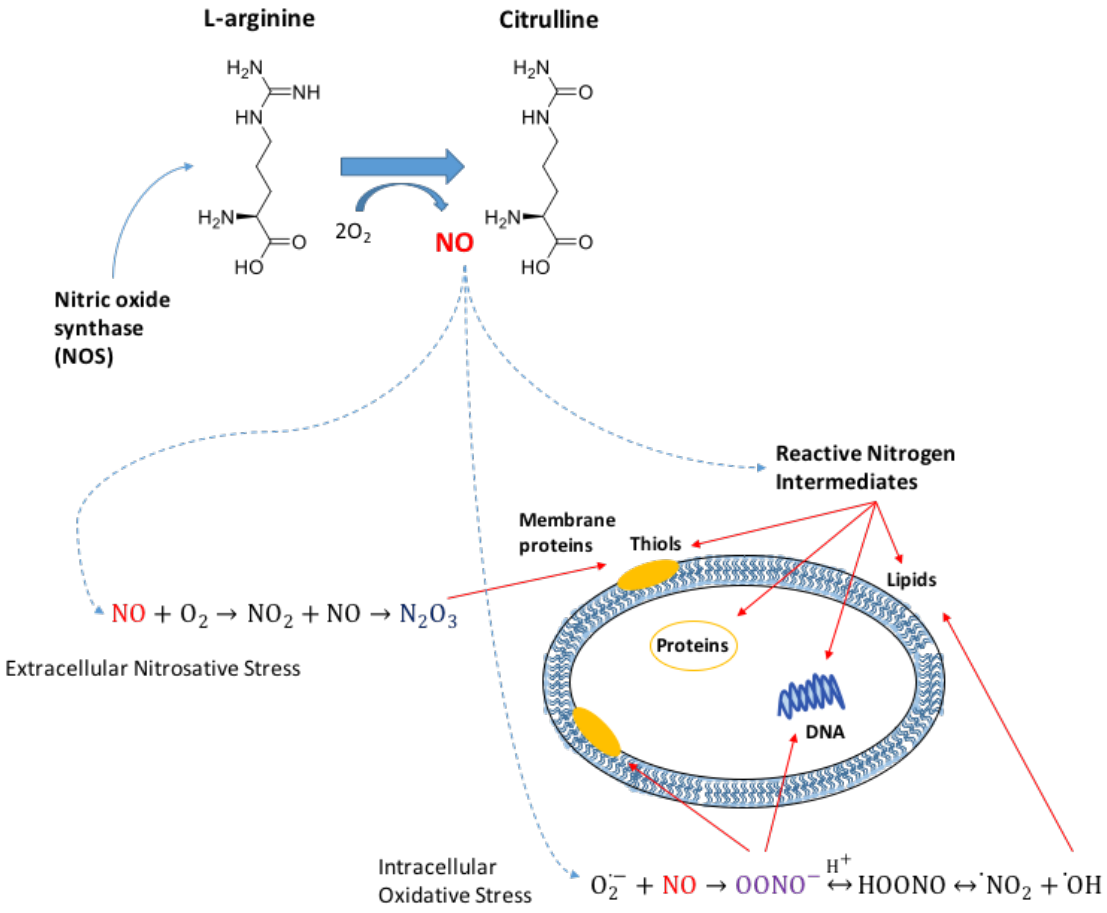

Figure 1.4: Biosynthesis of NO from L-arginine and the various bactericidal mechanisms of NO.

Nitric oxide is capable of acting on bacteria in either a planktonic state or encapsulated within a biofilm, which gives it an advantage over traditional therapeutic treatments where the penetration of immune cells and antibiotics are significantly reduced due to the biofilm's protective EPS matrix.[41,47] Even extremely low levels of NO have demonstrated in the past to be able to disperse biofilms into a planktonic state, making them more vulnerable to antibiotic therapies.[48,49] The success of many indwelling medical devices is hindered by infections due to biofilm formation, therefore the incorporation of an antimicrobial into medical devices that is able to inhibit and disperse biofilms is highly desired. The mechanisms by which NO influences bacterial cell viability along with its ability to eradicate biofilms indicate that NO-releasing materials as antimicrobials are an optimal solution for addressing antibiotic resistant infections.[41]

As gaseous NO has been successful for select medical applications, such as topical uses for dermatological and inhaled for pulmonary treatments, NO donors have been developed to mimic the endogenous release to benefit a wider range of applications.[41] Many classes of NO donors exist, however the most popular donors to facilitate NO storage and controllable release are N-diazeniumdiolates (NONOates)[42,50-52] and S-nitrosothiols (RSNOs) due to their ability to spontaneously release NO in physiological media.[40,41,52-54] Nitric oxide donors can be incorporated by physically blending the donor with the polymer solution,[43,54] immobilization of the donor onto the polymer,[50,55,56] and impregnation of the donor within the polymer.[57,58] The major advantage of using donors is that the type of donor and the concentration can be fine-tuned based on the application to obtain the desired NO reservoir and release kinetics for localized release.[40] Additionally, different stimuli can be applied to initiate donor

decomposition, and the release of the NO molecule, depending on the donor type.[40] NONOates decompose and release NO as a function of pH while RSNOs decompose by multiple triggers such as exposure to heat, visible light, and metal ions.[40,52] The ability for these NO donors to release NO under physiological conditions contributes to their advantageous qualities in a clinical setting.

The incorporation of NO donors into various biomaterials and medical devices has been shown to have broad-spectrum antimicrobial properties, effective at killing both Gram-negative and Gram-positive bacteria.[42,43,47,59,60] The efficacy of NO-releasing materials has demonstrated bactericidal properties *in vitro* using a variety of materials.[53,54,57,61] For example, NO-releasing silicone rubber tubing exhibited a 97% and 82% reduction against *S. aureus* and *P. aeruginosa,* respectively, over 7 day period[57] Additionally, NO-releasing CarboSil® demonstrated a 5 logarithmic unit reduction of *S. aureus* biofilms on the surfaces compared to the control.[61] Nitric oxide-releasing materials have also exhibited the ability to significantly reduce biofilm formation *in vivo*.[42,43] The implantation of NO-releasing Elast-eon™ E2As catheters in a 7 day sheep animal model resulted in a 90% reduction in bacterial adhesion compared to the controls.[43] Due to the potent bactericidal activity, NO-based antimicrobials have become a popular area of research to address the desperate and constant need for better antimicrobials that do not give into bacteria resistance.

The overarching goal of developing bactericidal materials to prevent HAIs is to completely eradicate all pathogens at the location of infection while inhibiting the development of bacteria resistance. Due to bacterial communities protecting themselves from antibiotics and host immune defenses by forming biofilms, the future of

antimicrobial agents is dependent on treatments that can properly penetrate the protective EPS of the biofilm and eradicate the underlying bacteria cells. The copious mechanisms by which NO influences bacterial cell and biofilm activity provide promising applications for using NO as an antimicrobial agent. Therefore, in this thesis I will explore the bactericidal effects from the incorporation of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) into urinary catheters and endotracheal tubes using the solvent swelling technique. In Chapter 2, the fabrication of nitric oxide-releasing endotracheal tubes (NORel-ETT) to produce an active release of NO from the surface of the ETT is shown to significantly reduce bacteria cell proliferation. The impregnation of SNAP successfully provides a 7 day NO release from the NORel-ETTs without altering the tensile strength of the commercially available endotracheal tubes. As a result, the application of using these modified ETTs in a clinical setting offers a promising application to prevent the risk of VAP. In Chapter 3, the fabrication of nitric oxide-releasing silicone Foley urinary catheters in combination with liquid-infused properties (LINORel-UC) represents a synergistic approach to prevent the proliferation of bacteria but also prevent biofilm formation on the surface of the urinary catheter. This combination incorporates the active release of NO with the liquid-infusion of silicone oil to prevent the adhesion of bacteria through the formation of a hydrophobic interface. The LINORel-UCs are shown to effectively prevent the biofilm formation of pathogens commonly associated with CAUTIs over a 24-hour and 7-day period while providing 60 days of NO release. The fabricated urinary catheter would be able to significantly aid in preventing the risk of CAUTIs when compared to the current commercially used catheters. Overall, the fabrication of NORel-ETTs and LINORel-UCs demonstrates using

nitric oxide as an effective antimicrobial to combat the occurrence of HAIs, decreasing mortality and morbidity rates, hospital costs, and length of stay.

CHAPTER 2

S-NITROSO-*N*-ACETYLPENICILLAMINE (SNAP) IMPREGNATED ENDOTRACHEAL TUBES FOR PREVENTION OF VENTILATOR ASSOCIATED PNEUMONIA[1]

[1] Katie H. Homeyer, Priyadarshini Singha, Marcus J. Goudie, Hitesh Handa. To be submitted to *Biomaterials Science*.

2.1 Introduction

Endotracheal intubation is a common hospital procedure implemented if the patient is not able to breathe oxygen on their own. In a normal, non-intubated respiratory tract, the region above the vocal cords is normally heavily colonized by bacteria due to the proximity of the oral and nasal cavity while the lower respiratory tract is sterile.[62] The major defense mechanisms in keeping the lower respiratory tract sterile include anatomic airway barriers, cough reflexes, mucus, and mucociliary clearance.[62] Mucociliary clearance clears the airways of its own secreted mucus, together with substances trapped in it, with coughing serving as a back-up system to mucociliary clearance.[63] The placement of an endotracheal tube hinders the normal defense mechanisms especially the cough reflex, compromising the mucociliary clearance and providing a conduit for the microorganisms to invade the normally sterile lower respiratory tract.[64]

Ventilator-associated pneumonia (VAP) is pneumonia that occurs after patients have been intubated via an endotracheal tube (ETT) and received mechanical ventilation.[65] Endotracheal intubation increases the chances of VAP 6- to 20-fold,[66] with the risk of contraction being the highest during the first 24 hours of the hospital stay, increasing by 3% per day during the first 5 days of ventilation, and by 2% during days 5 and 10.[66-68] Accounting for 86% of nosocomial pneumonia cases, VAP is one of the most common hospital-acquired infections (HAIs), increasing mortality rates, hospital stay, and hospital costs.[65,68,69] In order to contract VAP, microorganisms must reach the sterile lower respiratory tract and adhere to the mucosa to begin colonization.[62] The main perpetrators of VAP include *Pseudomonas aeruginosa*, *Acinetobacter spp.,* and *Stenotrophomonas maltophilia.*[65] Using the ETT as a vehicle, these microorganisms can

gain access through a variety of mechanisms, mainly by the aspiration of microbe-laden secretions from the oropharynx directly, or from stomach reflux into the oropharynx.[62,64,65] With the indwelling endotracheal tube, these mechanisms make the development of VAP very likely due to the availability of a foreign surface promoting microbial colonization and biofilm formation. Thus making the endotracheal tube a vehicle for colonization and the patient more susceptible to infections.

Due to the fact that VAP accounts for 60% of all deaths that occur from HAIs,[68] and considering the role endotracheal tubes play in increasing the chance for infection in the lower respiratory tract, researchers have strived to develop effective means to prevent VAP. Over the years, there have been design changes to the ETT in order to improve the antimicrobial efforts without the need of additional antiseptics. These changes include adding a cuff to the end of the ETT for better sealing to prevent macro-aspiration, or including a suctioning channel to remove subglottic secretion.[69] However, both of these additions have proven to be non-effective in preventing the spread of bacteria to the lower trachea and have the potential to damage the trachea.[69,70] Additional attempts in prevention have included various surface modifications to endotracheal tubes such as silver-coated,[71] and gentamicin-containing hydrogel coated endotracheal tubes [72]. Currently, silver-coated endotracheal tubes are the only commercially approved antimicrobial endotracheal tubes in the US. However, there are limiting factors to using silver. Silver-coated ETTs have been shown to be effective in *in vivo* studies and in clinical trials, but they were not shown to reduce secondary outcomes such as mortality rates, and length of ICU stay.[66,73] Although, it is the cost of silver that is a major drawback as it is 10-20 fold more expensive compared to commercial endotracheal

tubes.[73-75] Furthermore, the risk with using antibiotics, either as a hydrogel antibiotic-releasing surface coating or a treatment to the infection, is the development of antibiotic resistant bacteria. Despite these attempts to prevent bacterial adhesion and decrease the chance of VAP, these modifications have major limitations in being economically feasible and still provide the risk of infection. Additionally, it has been previously reported that a major limiting factor in the use of surface treated ETTs is that there is still accumulation of secretions within the ETT lumen.[69] This accumulation covers the surface, blocking the specific antimicrobial treatment that is on the surface from reaching either the planktonic bacteria or biofilms present, all the while the risk of VAP persists.

An alternative approach to improving the antimicrobial activity of materials that have high infection rates, like ETTs, is to mimic one of the defense mechanisms of the human body. Nitric oxide (NO) is an endogenous gas that is released from the natural endothelium and is proven to be a strong antimicrobial agent.[42,43,59] Nitric oxide has a number of other biological function such as vasodilation, thrombosis, and wound healing.[42,43,53,76] More specifically, NO that is released in the sinus cavities and by neutrophils and macrophages functions as an effective natural antiseptic agent.[43,77] Endogenous NO up-regulates ciliary motility, an important host defense mechanism, therefore inducing alveolar macrophage activity.[78] NO also has the possibility to act as a signaling molecule to reduce mucus secretions in the trachea in conjunction with acting as an antimicrobial agent.[79] Nitric oxide has been shown to have broad-spectrum antimicrobial properties, effective at killing both Gram-negative and Gram-positive bacteria.[42,43,59,60] The development of NO donors such as *S*-nitrosothiols (RSNOs) have the potential to release NO exogenously by incorporating these donors into various

polymers, mimicking the endogenous release. *S*-nitroso-*N*-acetylpenicillamine (SNAP) is a synthetic RSNO donor that has been extensively studied its antimicrobial properties.[43,53,80] Exposure to heat, light, and catalysis (e.g. using metal ions like Cu^{+}) are the main catalysts for RSNOs species to initiate NO release from the donor.[53,58] Common methods to incorporate NO donors such as SNAP include swelling the polymer with a solvent swelling solution,[58,81] immobilization of the donor onto the polymer itself,[50,55] or blending the NO donor with the polymer in solution.[43,54] The efficacy of NO-releasing materials as a bactericidal agent has been demonstrated *in vitro* with SNAP blended CarboSil® against *Staphylococcus aureus* over 24 h period.[82] Incorporating NO into an ETT has the potential to decrease the buildup of the secretion on the ETT lumen, and increase alveolar macrophage activity, but more importantly, simultaneously provide an active release of NO to inhibit bacteria proliferation and the risk of infection. Therefore, the introduction of NO into ETTs would be beneficial in inhibiting biofilm formation and contraction of VAP in intubated patients.

In this work, the development of nitric oxide-releasing endotracheal tubes (NORel-ETTs) is shown to significantly reduce bacteria cell proliferation via the active surface release of NO to address the prevention of bacterial adhesion and VAP more effectively. Using a solvent swelling method, the NO donor, SNAP, was incorporated into the polymer of the ETT. The impregnation of SNAP demonstrated an effective NO release over a 7 d period while preserving the natural mechanical properties of commercial ETTs. The NORel-ETT was effective in significantly reducing the adhesion of *P. aeruginosa*, pathogens that are frequently found to result in VAP over a 24 h period. The proposed NO-releasing endotracheal tubes effectively provides an active release of a

bactericidal agent commonly found in the natural body resulting in an effective method to prevent the risk of VAP that has become a common occurrence in hospitals, increasing costs and mortality rates.

2.2 Materials and Methods

Materials

N-Acetyl-*D*-penicillamine (NAP), sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, ethylenediaminetetraacetic acid (EDTA), tetrahydrofuran (THF), phosphate buffered saline (PBS), trioctyl trimellitate (TOTM), N, N-Dimethylacetamide (DMAc) and sulfuric acid were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, hydrochloric acid, and sulfuric acid were obtained from Fisher Scientific (Pittsburgh, PA). Acetone was purchased from VWR International (Radnor, PA). Endotracheal tubes, 5-mm, were purchased from Dynarex Corporation (Orangeburg, NY). Phosphate buffered saline (PBS), pH 7.4, containing 138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, 100 μM EDTA was used for all experiments. *Pseudomonas aeruginosa* (ATCC 27853) was originally obtained from American Type Culture Collection (Manassas, VA, ATCC). Luria broth (LB) and Luria Agar (LA) were bought from Fischer BioReagents (Fair Lawn, NJ). Phosphate buffered saline (PBS) was purchased from Sigma Aldrich (St. Louis, MO).

SNAP Synthesis

The NO donor, SNAP, was synthesized using an amended version of a previously reported method.[83] Concisely, an equimolar ratio of NAP and sodium nitrite was dissolved in a 1:1 mixture of water and methanol containing 2 M HCl and 2 M H_2SO_4. After stirring, the reaction vessel was cooled over 6 h using an ice bath to form a crystal precipitate. Upon crystal formation, the crystals were collected by vacuum filtration, washed with DI water, and allowed to dry under ambient conditions. The entirety of the synthesis and the crystals were protected from light at all times.

Preparation of SNAP impregnated ETTs

NORel-ETTs were fabricated using a solvent swelling method. The SNAP swelling solution was prepared by dissolving SNAP in a mixture of acetone and THF, at a ratio of 95:5, respectively, by volume. Acetone successfully swells the ETT by a factor of 2 and the inclusion of THF in the swelling solution allows for a higher loading infiltration of SNAP into the polymer matrix. Various amounts of plasticizer concentrations were used to minimize any changes in the mechanical properties during the swelling process; TOTM was added at a concentration of 1%, 1.5%, and 2% of solution. Due to the the high molecular weight of TOTM, it is less extractable and non-cytotoxic making it safe to use in biomedical applications.[84,85] As later described, a concentration of 2% TOTM of solution proved to have no significant changes in the ultimate tensile strength or Young's modulus when compared to the commercial ETT and was added to the swelling solution for the fabrication of the NORel-ETTs used in the remainder of the methods in order to preserve the original plasticity of the material. Three

concentrations of SNAP were initially used to determine the optimal concentration for the desired release kinetics without altering the properties of the material. The concentrations considered were 20, 40 and 60 mg mL^{-1}. For each solvent swelling solution variation, the commercial ETT was submerged in the swelling solution for 5 hours to allow for the proper infiltration of solution into the polymer. The samples were removed from solution and dried for 24 hours to ensure the removal of the solvents. The samples were then placed in a 20 mL vial with DI water and sonicated for 5 minutes in a Fisher Scientific 1.9 L sonicating bath to remove any residual SNAP crystals that may have adhered to the surface of the samples. Throughout the entire fabrication process, the samples were shielded from the light.

Total SNAP loaded percentage in fabricated NORel-ETTs

To determine the total amount of SNAP loaded in the ETT after swelling for each concentration, UV-Vis spectroscopy was performed. The NORel-ETT was swelled and dried as described above, then massed before being dissolved in 2 mL of DMAc. UV absorbance values were recorded for each SNAP concentration at a wavelength of 340 nm (n=3), corresponding to the local maxima of the SNAP molecule at 340 nm.[86] The absorbance value was compared to a previously created calibration curve, with consideration of the mass of the sample, to determine the percentage of SNAP present within each sample.

Tensile testing of the NORel-ETTs

Commercial ETTs were dissolved in THF then casted into films. These films were swelled with different variations of the swelling solution described above in order to observe the effect of plasticizer concentration on the mechanical properties when compared to the commercial ETT, as well as the addition of various swelling concentration of SNAP. The variations are as follows: THF and acetone swelling solution without TOTM, with 1%, 1.5%, and 2% TOTM, and with SNAP concentrations of 20, 40, and 60 mg mL^{-1}. The original ETT films and fabricated films were cut according to IPC-TM-650 standards with a 6:1 length/width ratio (n=3).[59] Tensile testing was performed by carefully securing the samples in place on the jaws of an Instron material testing machine. The samples were tested at a constant extension rate of cross head speed of 2 mm s^{-1} at room temperature (23°C). The stress and stain relationship was derived from the test and the ultimate tensile strength was then determined for all samples and averaged for each variation.

Nitric oxide release measurements

The active release of nitric oxide from the fabricated NORel-ETT was measured using a Sievers chemiluminescence Nitric Oxide Analyzer (NOA) model 280i (Boulder, CO). The NORel-ETT was cut for a total surface area of 1 cm^2 (n=3). The samples were threaded with silk surgical suture to be suspended above 3 mL of PBS buffer with 100 mM EDTA in an amber glass vial at 37°C. The samples are suspended above the solution rather than submerged in order to create a humid environment mimicking the lower trachea and respiratory tract. Once the sample is placed in the vial, nitrogen is bubbled

through the PBS buffer and the purged nitric oxide is continuously swept from the vial into the chemiluminescence detection chamber. The NO release measured from the NORel-ETT is normalized using the surface area and the flux unit ($\times 10^{-10}$ mol cm^{-2} min^{-1}). After each measurement, the samples were kept on the surgical suture and suspended in a vial above 5 mL PBS with EDTA in an incubator at 37°C to maintain physiological conditions.

Storage stability of NORel-ETT

To determine the ability of the NORel-ETT to be stored under various conditions, the sections of NORel-ETT tubing (40 mg mL^{-1} concentration) were stored under temperatures of 37°C, 23°C, 4°C, and -20°C with desiccant for 3 months. After being stored for 2 weeks, 1 month, and 3 months, segments (n=3) of the samples were dissolved in DMAc, then using UV-Vis spectroscopy, the absorbance valve was recorded at 340 nm for each condition and compared to a predetermined calibration curve. The percent SNAP remaining was calculated and compared to the initial NORel-ETT. The NO release kinetics of the samples stored for 3 months at 23°C were measured and compared to newly made NORel-ETT samples with a SNAP concentration of 40 mg mL^{-1}. All samples were stored at the respective temperatures in dark conditions.

Bacteria adhesion analysis: 24-hour test with *Pseudomonas aeruginosa*

The fabricated NO-releasing endotracheal tubes were tested for bacterial adhesion inhibition by comparing them to the control tubes. The samples were 0.5 cm^2 in surface area and data analysis was adjusted for this surface area. Inhibition of bacteria adhesion

was studied using a modified American Society for Testing and Materials protocol (E2180) 24h of exposure to *P. aeruginosa*.[82,87] To obtain the required pathogenic culture, a colony of *P. aeruginosa* was inoculated and grown overnight in LB media to a CFU/mL of 10^6-10^8. This overnight culture was washed twice in PBS by centrifuging at 2500 rpm for 7.5 min. This centrifuge wash was done to get rid of nutrient waste and unused media from the overnight culture. The resulting bacteria pellet obtained from centrifugation was then resuspended with PBS to get an OD adjusted culture used for the bacteria adhesion study.

Once the suspension was ready, the samples were incubated with the bacteria culture in a shaker incubator (140 rpm, 37°C). After 24h of exposure to the bacteria, samples were gently rinsed with sterile PBS to get rid of any unattached bacteria and then homogenized to release all the attached bacteria into the sterile PBS. A consistent volume of sterile PBS was used for each sample. This process also helps in homogenously mixing any biofilm bacteria formed during the 24h study. The bacterial solutions attained were then serially diluted and plated. The plates were incubated in 37°C for 18h and "CFU of bacteria/cm^2 of sample" measurements were then calculated from them using the following formula:

$$\frac{CFU\ of\ bacteria}{cm^2 of\ sample} = \frac{CFU\ x\ dilution\ factor\ x\ suspension\ in\ solution\ /\ suspension\ plated}{surface\ area\ of\ sample}$$

Statistical Analysis

Data for the 24 h bacteria adhesion analysis is expressed as mean ± standard deviation (SD). Data for all other experiments is expressed as mean ± standard error of the mean (SEM). The results between the data for the control ETT and NORel-ETT were

analyzed by comparison of means using two tailed Student's *t*-test. Values of $p < 0.05$ were considered statistically significant for all tests.

2.3 Results and Discussion

Total SNAP loaded percentage

In order to determine the amount of SNAP loaded into the ETT for each concentration after swelling, the UV-Vis spectra was recorded and the amount of SNAP was calculated (**Figure 2.1**). The 60 mg mL^{-1} concentration had the highest percentage, 19.52 ± 0.39 wt%, as expected. The concentration of 20 mg mL^{-1} was the lowest with 7.97 ± 0.90 wt%, and the 40 mg mL^{-1} concentration ETT was 14.21 ± 0.82 wt% of SNAP. Wo et al. found the solubility of SNAP in CarboSil® polymer to be ca. 3.4-4.0 wt%, with higher concentrations leading to crystal formation within the matrix of the polymer,[61] which is why increasing the level of SNAP does not always result in an increase in the NO release. The formation of SNAP crystals within the polymer matrix lead to extended release profiles as compare to typical burst release rates. However, an initial burst release is commonly seen in these materials, stemming from the portion of integrated SNAP that is soluble within the polymer matrix. Therefore, since the SNAP loading percentages for the NORel-ETT are higher than the solubility of SNAP in the polymer matrix, it is likely that there is SNAP crystal formation with in the ETT polymer matrix. The formation of SNAP crystals is important for the NO release kinetics as it provides a longer release period and a longer shelf life in its crystalline form.[53,61] The

different concentrations of SNAP will be referred to by their wt% for the remainder of the studies.

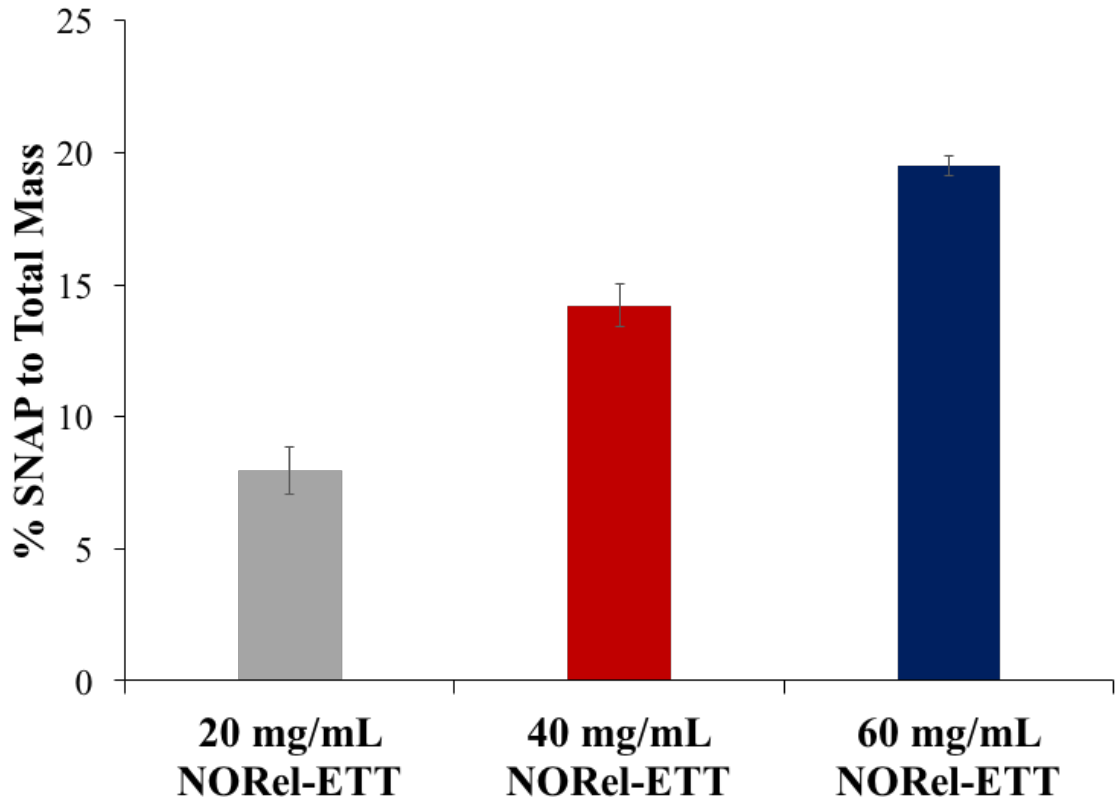

Figure 2.1: Percent of SNAP loaded compared to total mass of endotracheal tube after solvent swelling fabrication process for each concentration: 20 mg mL^{-1}, 40 mg mL^{-1}, 60 mg mL^{-1}. The amount of SNAP loaded was measured using UV-vis Spectroscopy.

Mechanical Testing Analysis

The altering of polymers with additives can affect the mechanical properties of the base polymer, including the tensile strength. Due to ETTs being composed of poly(vinyl chloride) (PVC), plasticizers are often added to create the proper plasticity for the application of the material. PVC is characteristically a rigid and brittle polymer, thus the performance is modified by adding plasticizers to obtain the desired flexibility and durability for the particular application.[85] Trioctyl trimellitate (TOTM), is one of the main commercial plasticizers used in PVC products and due to its high molecular weight, it is

less extractable and non-cytotoxic making it safe to use in biomedical applications.[84,85] Therefore, the swelling solution composed of the swelling solvents with added TOTM to preserve the plasticity of the original ETT since the TOTM diffuses out of the PVC during swelling. The mechanical strength of the ETTs was studied by measuring the ultimate tensile strength (UTS) and the Young's modulus. The addition of plasticizer (TOTM) was seen to lower the UTS of the ETTs. The UTS of the ETTs approached the UTS of the commercial ETT as the amount of TOTM increased (**Figure 2.2A)**. The commercial ETT (control) was found to have an UTS of 4.218 ± 0.62 N. The ETT swelled with the swelling solution with no plasticizer added had an UTS of 6.41 ± 0.55 N. Adding 1% of TOTM to solution decreased the UTS to 5.5529 ± 0.19 N, while adding 1.5% TOTM had an UTS 5.11 ± 0.38, and 2% had an UTS of 4.42 ± 0.24 N. The UTS was significantly higher for the ETT swelled with the swelling solution but without TOTM added compared to the control ETT ($p=0.045$), while each of the samples with added plasticizer were not statistically significant from the control ETT. Therefore, the addition of TOTM did not alter the UTS of the ETT. Finally, the addition of 2% TOTM was chosen for the swelling solution used in the remainder of the studies as the UTS resembled the control.

The addition of SNAP was seen to slightly increase the UTS as the amount of SNAP increased (**Figure 2.2B**). NORel-ETTs with 8 wt% SNAP had an UTS of 3.8 ± 0.15 N. The UTS of NORel-ETTs between 8 and 14.2 wt% was not found to be statistically significant ($p=0.46$) with 14.2 wt% NORel-ETT having an UTS of 3.98 ± 0.04 N. As the SNAP concentration increased to 19.5 wt%, the UTS for 19.5 wt% NORel-ETT increased to 4.47 ± 0.14 N. The UTS of NORel-ETTs between 14.2 and

19.5 wt% was not statistically significant (p=0.08), however the UTS for NORel-ETTs between 8 and 19.5 wt% was statistically significant (p=0.038).

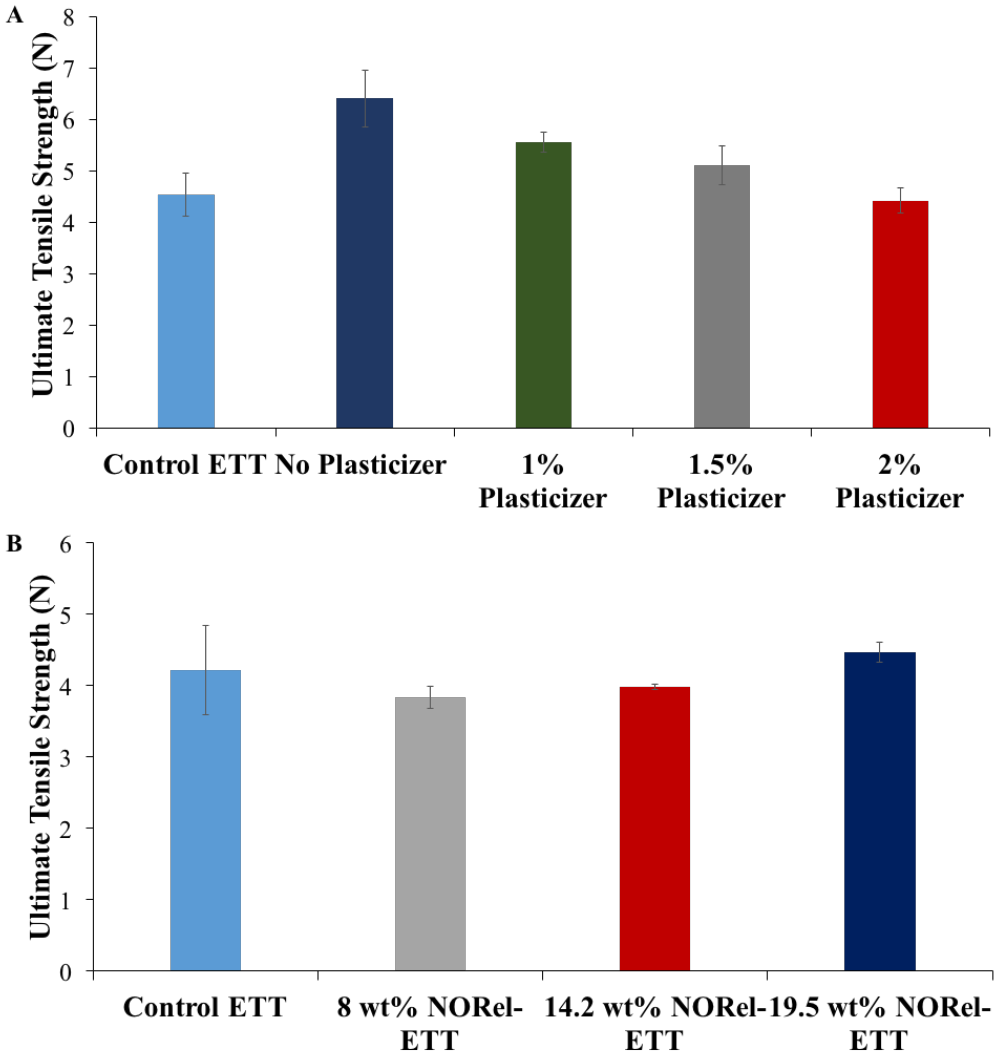

Figure 2.2: A) Ultimate tensile strength of ETTs with varying amounts of plasticizer added to swelling solution (0%, 1%, 1.5%, 2% added) at break point. **(B)** Ultimate tensile strength of ETTs with different concentrations of SNAP added to swelling solution containing 2% TOTM in solution (20 mg mL^{-1}, 40 mg mL^{-1}, 60 mg mL^{-1} added) at break point. Both measured by Instron tensile strength instrument.

Each of the SNAP concentrations were not statistically significant when compared to the control ETT. Consequently, the addition of SNAP in either of these concentrations is not shown to statistically effect the tensile strength of the commercial ETT. In previous

studies, the addition of SNAP to Elast-eon^{TM} E2As above 10 wt% was seen to decrease the UTS drastically when compared to lower weight percentages of SNAP.[53,59] This discrepancy could be due to the material examined, Elast-eon^{TM} E2As or chitosan versus PVC, or the crystal structure of SNAP within the polymer, or the addition of plasticizer during fabrication.

Nitric oxide release measurements

Nitric oxide release was measured for the NORel-ETTs using a Sievers chemiluminescence Nitric Oxide Analyzer (**Figure 2.3**). Since the risk of VAP is crucial during the first 5 days of endotracheal intubation, the NO release was measured over a 7 d period for each concentration. Release kinetics were examined as a function of SNAP swelling concentration. The 8 wt% NORel-ETTs initially released 9.51 ± 1.08 × 10^{-10} mol cm^{-2} min^{-1}, however only lasted till day 3 with a release of 0.12 ± 0.03 × 10^{-10} mol cm^{-2} min^{-1}, running out before day 7. The 14.2 wt% NORel-ETT lasted the entirety of the testing period with an initial release of 16.63 ± 0.75 × 10^{-10} mol cm^{-2} min^{-1} and a final release of 0.69 ± 0.19 × 10^{-10} mol cm^{-2} min^{-1}. Lastly, the 19.5 wt% NORel-ETT measured to have an initial release of 34.45 ± 6.77 × 10^{-10} mol cm^{-2} min^{-1} and a final release of 1.18 ± 0.20 × 10^{-10} mol cm^{-2} min^{-1}. Higher concentrations of SNAP have been previously reported to have a 30 d release,[81] however, due to the solubility of SNAP within the solvents used in this method, the maximum concentration is 60 mg mL^{-1}. The 8 wt% NORel-ETT did not have an NO release past a 3 d period, making this concentration of SNAP not ideal for *in vivo* use as it could potentially be ineffective at inhibit bacteria proliferation and biofilm formation, therefore not helping to reduce the risk of VAP.

While the 19.5 wt% NORel-ETT lasted the entirety of the testing period, this concentration is also not desirable due to the large initial burst release on day 0. This burst release is typical of NO-releasing materials, an undesirable characteristic for medical devices as high NO fluxes have been shown to be cytotoxic.[88,89] The 14.2 wt% NORel-ETT not only lasted the entire testing period but also had a relatively stable release, only gradually decreasing in NO release over the testing period. Considering that the first 5 days is crucial in preventing the bacteria colonization and biofilm formation that can lead to VAP,[66] the 14.2 wt% NORel-ETT showed the most desirable release kinetics of the three concentrations tested, therefore, this concentration will be used in the storage stability study as well as the antimicrobial characterization of the material.

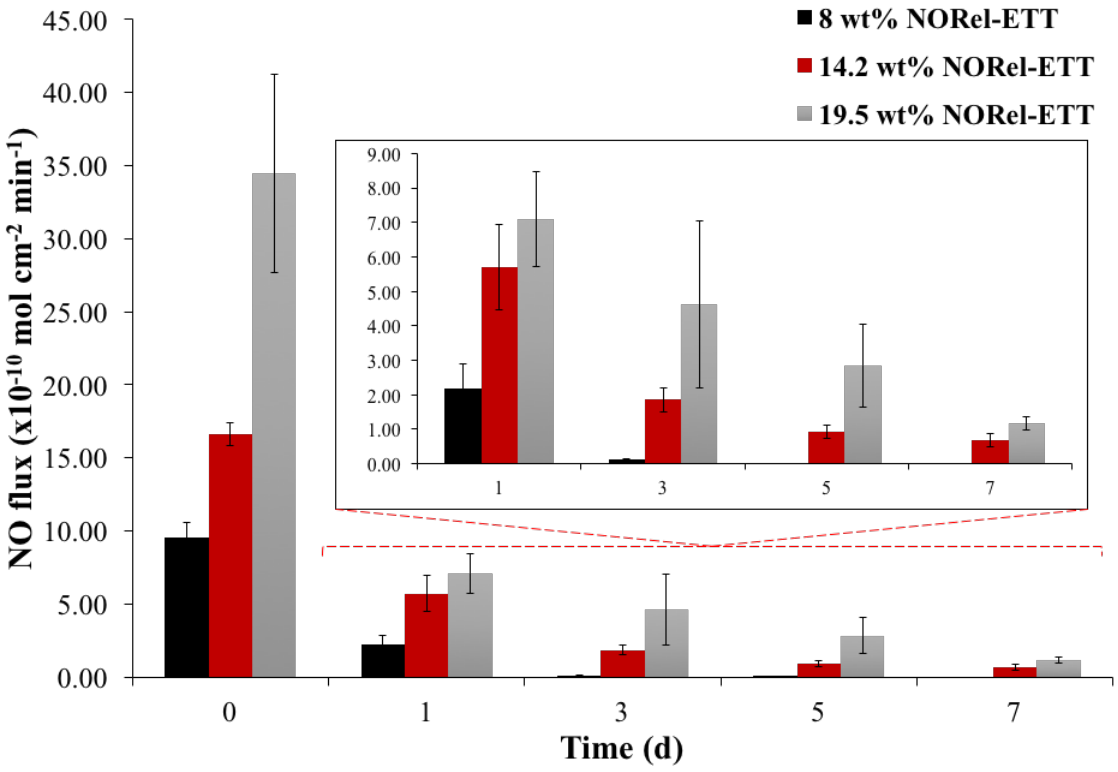

Figure 2.3: Average nitric oxide release measurements from freshly fabricated 8 wt%, 14.2 wt%, and 19.5 wt% NORel-ETT over 7 d period (n=3). NO release measured from ETT samples suspended above PBS at 37°C using a Sievers Chemiluminescence Nitric Oxide Analyzer.

Storage stability of NORel-ETTs

Many NO donors, including SNAP, are sensitive to heat exposure, making their ability to store difficult. In order for NO-releasing materials to be feasible in the market, they need to be able to be stored without a large loss to the NO-releasing properties. Therefore, these materials need to be assessed as to their ability to be stored under various environments. NORel-ETTs with 14.2 wt% SNAP were stored at a variety of temperatures to observe the possibility of storage under various conditions (**Figure 2.4A**). Room temperature (23°C) is the most realistic storage condition and under this temperature the NORel-ETTs retained 91% SNAP after one month and 90.75% after 3 months. Naturally, the amount of SNAP remaining over time decreased as the storage temperature increased as seen when the NORel-ETTs were stored under 37°C. After one month only 69.23% SNAP remained and 61.56% after 3 months. The best storage conditions for the NORel-ETTs is at -20°C with 97.36% remaining after one month and 96.01% after 3 months. The NORel-ETTs were able to successfully retain majority of the SNAP during the three-month storage period under varying temperatures. It is important to have a high retention of SNAP throughout the storage period as well as a maintained release profile from the NORel-ETTs when they are ready to be used. The NO release from the NORel-ETTs with 14.2 wt% SNAP stored at 23°C was measured after 3 months of storage and compared to the release of freshly prepared NORel-ETTs (**Figure 2.4B**). After 3 months of storage the 14.2 wt% NORel-ETT initially released $16.34 \pm 0.012 \times 10^{-10}$ mol cm^{-2} min^{-1} with a final release after 7 days of $1.52 \pm 0.65 \times 10^{-10}$ mol cm^{-2} min^{-1}. The release rates for the stored ETTs are very comparable to those of the freshly prepared samples.

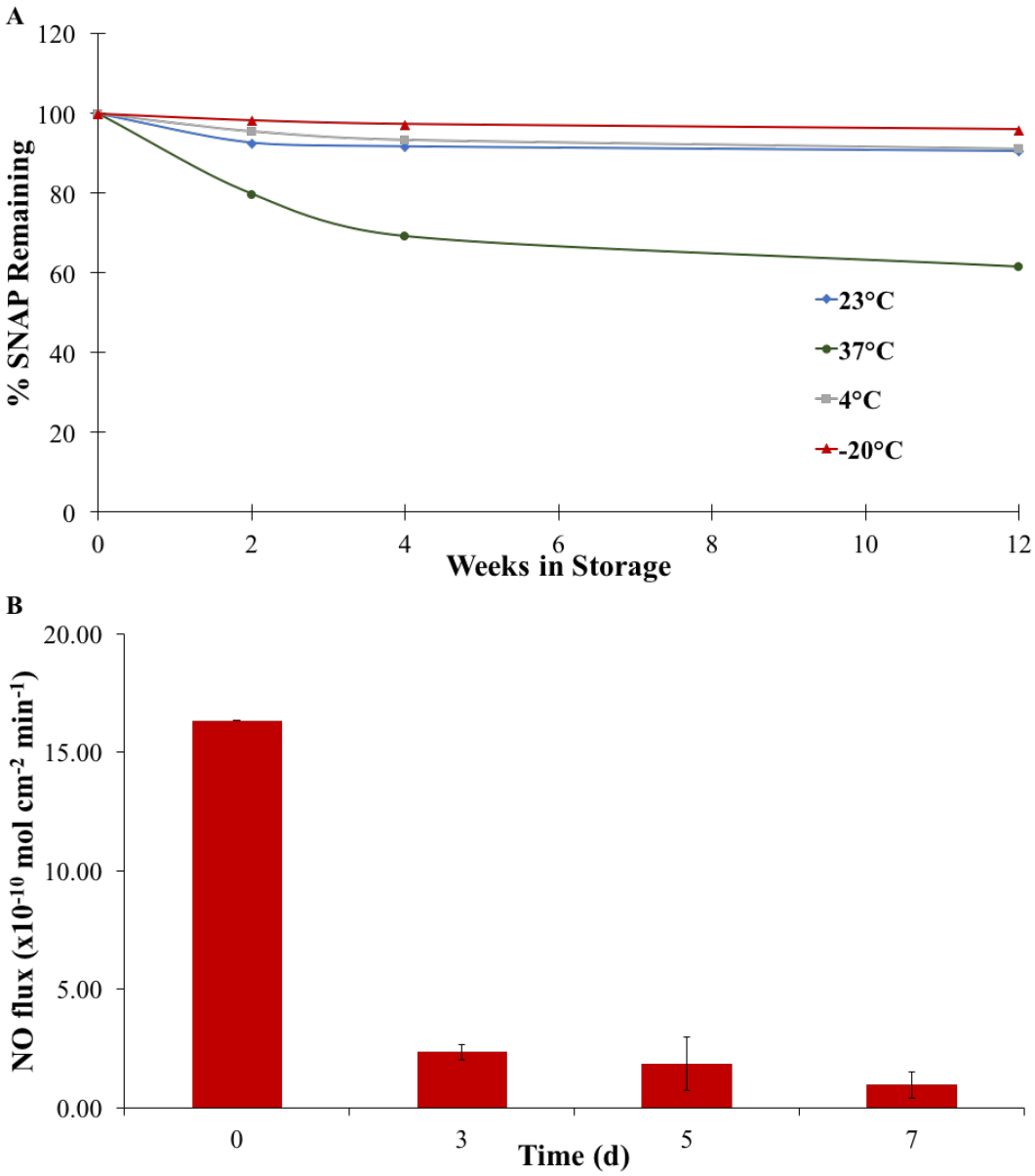

Figure 2.4: A) Storage stability of SNAP in NORel-ETTs. The percent SNAP remaining was measured for NORel-ETTs stored under different storage conditions (-20°C, 4°C, 23°C, 37°C). Error bars are excluded since they are on the order of data point size. **B)** Average nitric oxide release measurements from 14.2 wt% NORel-ETT after 3-month storage at 23°C over 7 d period (n=3).

Typically, the higher release of NO on day 0 is attributed to the soluble SNAP in the polymer, which is unstable and rapidly releases NO, and during storage, the soluble SNAP will decompose with the crystalline SNAP remaining.[53] However, after 3 months of storage the NORel-ETT exhibited a high release on day 0. This irregularity may be

because it takes more than 3 months for the soluble SNAP to completely degrade as previous storage stability studies on SNAP have been performed over 6 months.[53] Aside from storage at 37°C, at most 9.25% SNAP was lost during the 3 month storage period, observed by the UV-vis results. The 7-day release after 3 months of storage confirmed the SNAP crystal formation in the polymer matrix during fabrication which is important for a controlled NO release and storing the NORel-ETTs.

Antimicrobial efficacy of NO-releasing endotracheal tubes

Despite prevention methods like silver ion containing endotracheal tubes, improved antimicrobial therapy, and supportive care, VAP remains a constant complication that has resulted in higher morbidity and mortality. Since VAP develops within 48 hours of infection, it is important to ward off and kill bacteria during the first few hours of intubation. The ETT provides a surface for bacteria to adhere to and grow into biofilms. Free-floating (or planktonic) bacteria can come across the ETT surface and within minutes become attached. These free-floating bacteria are widely present in the microflora of the patient's skin or respiratory tract and find an easy way to the surface of the endotracheal tubes. The attached bacteria then produce slimy, extracellular polymeric substances (EPS) that cover the tubes and form the conditioning film for the stationary, attached bacteria. Extracellular polymeric substance production allows the emerging biofilm community to develop a complex, three-dimensional structure, a biofilm that protects the bacteria from antibiotics. Hence biofilms have become a major hurdle for healthcare-associated infections which validate the reason why we need to develop more

antimicrobial infused tubes which can reduce the incidence of healthcare associate infections and therefore reduce healthcare costs significantly.

In this study, the antimicrobial efficacy of the fabricated endotracheal tubes was examined against the common VAP pathogen *Pseudomonas aeruginosa* in a 24 h model. The total viable *P. aeruginosa* adhered on the ETT samples' surface was determined after 24 h at 37°C. Plate count for 24 h *P. aeruginosa* showed that the viable bacteria attached on the surface of the NORel-ETT samples was 92.72 ± 0.97% less than on ETT controls (**Figure 2.5**; n=4; p=0.01). This corresponded to a ca. 1.5 log reduction in viable bacteria growth. This reduction is also comparable to the reduction of *E. coli* found in another study where silicone-modified antimicrobial polyethylene endotracheal tubes were tested,[90] suggesting that the NO release does have significant antibacterial activity in *P. aeruginosa*. The reduction in *P. aeruginosa* attachment is also comparable to other NO-releasing, SNAP swollen materials.[57]

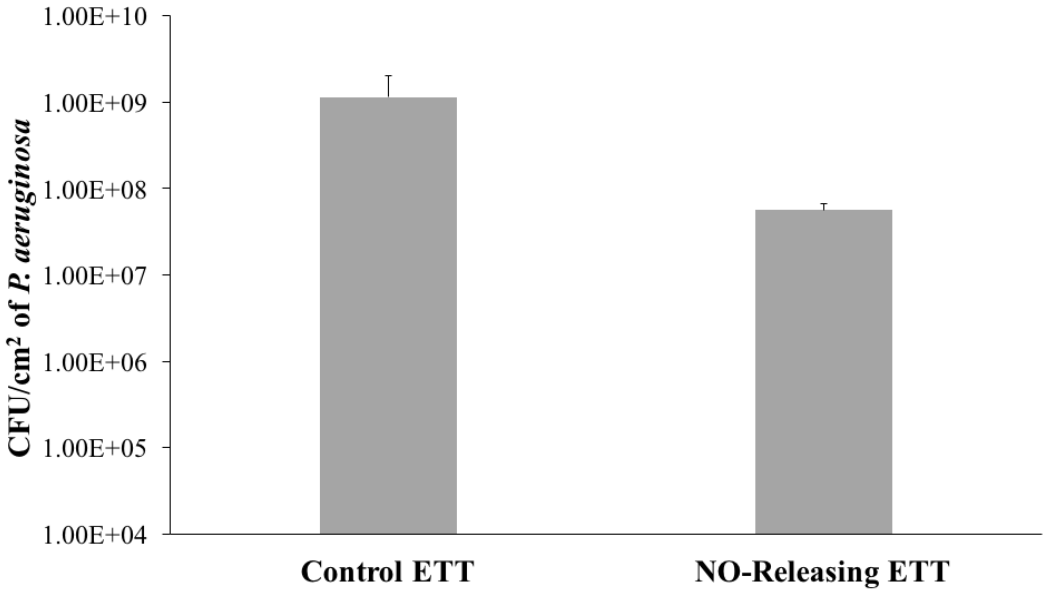

Figure 2.5: *P. aeruginosa* bacteria adhesion per cm^2 for control commercial endotracheal tube, and NORel-ETT over 24-hour period (n=3). Data represent mean ± SD.

Hence, from the biofilm adhesion study, it can be concluded that the NO-releasing endotracheal tube was able to significantly reduce the attachment of viable bacteria to its surface and thus prove to be a major development in the field of antimicrobial endotracheal tubes. However, despite the promising results, in the future *in vivo* studies need to be performed to demonstrate the long-term antimicrobial efficacy of these endotracheal tubes.

2.4 Conclusions

In this study, the incorporation of SNAP to create nitric oxide-releasing endotracheal tubes was investigated, including the effects of SNAP on the mechanical, physical, and bactericidal properties of endotracheal tubes. The storage stability of this material was also considered for clinical feasibility. The endotracheal tube was successfully loaded with SNAP using the solvent swelling method and the addition of SNAP did not alter the mechanical properties as the ultimate tensile strength was not significantly different from the original ETT from the original endotracheal tube. The NORel-ETTs successfully released NO over a 7 d period. Future long-term NO release studies need to be performed to determine the full potential of the release period for the NORel-ETTs. The present study also demonstrated the ability of NORel-ETTs to be stored for three months at room temperature while retaining 90.75 ± 1.21% of initial the SNAP concentration and at harsh conditions (37 °C) retaining 61.56 ± 1.48%. The 3-month storage period did not alter the release kinetics of the NORel-ETTs as it was very similar to freshly made materials, demonstrating that these materials are feasible for the implantation in clinical settings. Furthermore, the incorporation of an active release of

NO into commercially available endotracheal tubes effectively reduced 92.72 ± 0.97% of viable bacteria attached to the surface of NORel-ETTs when compared to the control ETTs. Future long-term bacteria studies and *in vivo* studies need to be performed to validate the long-term antimicrobial efficacy of NORel-ETTs. Overall, the impregnation of SNAP in endotracheal tubes to create NORel-ETTs serves as an inexpensive and effective approach to dramatically reduce the frequency of VAP.

CHAPTER 3

LIQUID-INFUSED NITRIC OXIDE-RELEASING SILICONE FOLEY URINARY CATHETERS FOR PREVENTION OF CATHETER-ASSOCIATED URINARY TRACT INFECTIONS[2]

[2] Katie H. Homeyer, Marcus J. Goudie, Priyadarshini Singha, Hitesh Handa. Submitted to *ACS Biomaterials Science and Engineering*, 10/24/18.

3.1 Introduction

In healthy individuals, the urinary tract is a sterile environment, and implanting a urinary catheter creates an ideal milieu for bacteria to remain in the urinary tract, making it one of the most susceptible medical devices for infection.[91,92] However, urinary catheterization has become a common medical procedure to enable the drainage and removal of urine for various medical purposes, such as post surgeries, or for urinary incontinence. The two main types of urinary catheters are short-term use and long-term use catheters. Short-term use refers to when the catheter is only used for a few weeks and long-term use catheters are typically used for multiple months.[4] Of patients undergoing short-term catheterization, catheter associated urinary tract infections (CAUTIs) occur in 10-15% of patients while virtually all patients undergoing long-term catheterization will become infected, making prolonged catheterization an imperative risk factor.[4,91,93,94] Extended catheterization can lead to biofilm formation, wherein the attached bacteria produce extracellular polymeric substances that colonize the surface, and allows the biofilm community to develop, gaining nutrients from the gentle flow of warm urine through the catheter.[4,11] The most common bacteria associated with CAUTIs are *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Enterococcus faecalis,* and *Klebsiella pneumoniae.*[4,20] Once a biofilm is formed on the surface, the infection becomes harder to treat due to the formation of the extracellular polymeric substance that protects the bacteria cells, inhibiting antibiotics and other bactericidal agents to penetrate the substance and eradicate the biofilm.[4] This allows free-floating (planktonic) bacteria and pathogens within the urine to adhere to the urinary

catheter as a vehicle for colonization, making the patient more susceptible to infections.[4,92] Thus, CAUTIs are one of the most common hospital acquired infections, accounting for 80% of all nosocomial infections worldwide with more than 1 million cases in the United States per year.[4,20,95] These alarming statistics illustrate the necessity to develop effective means to eradicate the potential for CAUTIs.

Typically, catheters are routinely replaced to prevent the formation of infection, or the patient is given antibiotics, both undesirable solutions as to not promote antibiotic resistance and cause further discomfort to the patient.[4,11] If CATUIs are left untreated, the severity of the situation increases to the potential of causing infection in the kidneys and bloodstream.[96] Currently, silver eluting urinary catheters are used as a treatment for eradicating CAUTIs without antibiotics.[91,97-100] Silver has been vastly researched and commercialized to be an effective antimicrobial agent. However, it is still yet to be confirmed if silver eluting urinary catheters significantly reduce CAUTIs clinically.[74,75]

Nitric oxide-releasing (NORel) materials have been readily implemented in various research areas since it was discovered as an endogenous gas released from the endothelium and proven to be a strong bactericidal agent.[40,42,43,53,54,57,81] The endogenous production can be mimicked and NO can be produced exogenously by incorporating various NO donors into a polymeric material via physically blending the NO donor within the polymer,[43,54] swelling the polymer with a swelling solution,[58,81] or immobilization onto the polymer itself.[50,55] Common donors include N-diazeniumdiolates[42,50,51] and S-nitrosothiols (RSNOs).[40,54] Nitric oxide donors allow for several advantages, including storage of NO within the polymer until the initiation of release, ability to take advantage of the tunable concentration, and adjusting the desired

release kinetics of localized delivery for varying applications.[40] For RSNOs, NO release initiation commences upon exposure to heat, catalyst (e.g. copper ions, Cu^{2+}), visible light, and often interactions with moisture.[38,61] The efficacy of NO-releasing materials has demonstrated bactericidal properties *in vitro* using a number of materials, such as Elast-eon™ E2As, tygon, and silicone rubber.[40,53,54,57,61,81] While NO-releasing materials have proven to significantly reduce the presence of bacteria *in vivo,*[42,43] decreasing the risk of biofilm formation is very desirable to prevent the chance of infection from the beginning. NO-releasing materials only inhibit the proliferation of bacteria, lacking the ability to prevent bacterial adhesion. With this, the development of an antibiofouling NO-releasing material can provide vast improvements to the bactericidal properties of silicone Foley urinary catheters.

Liquid-infused materials have been gaining momentum on the research front due to the fact that they create a low-adhesion interface between the material and the contacting liquid. The low-adhesion interface is created by the infiltration of the infused liquid into the polymer network where the liquid takes advantage of the capillary forces and the chemical affinity present between the infused liquid and the underlying polymer.[101] The slippery liquid-infused porous surfaces (SLIPS) idea originates from the thick liquid mucus lining of the gastrointestinal tract.[101] The mucus layer guards the vulnerable tissue in the tract from colonization of bacteria. Similarly, the *Nepenthes* pitcher plant utilizes a liquid water layer to create a low friction surface to prevent the attachment of insects.[102,103] These two ideas pave the way for a biocompatible, antibiofouling surface coating. SLIPS has been proven effective when a biocompatible lubricating liquid was infiltrated into a polytetrafluoroethylene (PTFE)-based system that

was able to prevent up to 96-99% of common bacterial biofilms from attaching over a 7 d period solely on the mobility of the slippery interface.[104] However, there is still the inquiry on the ability of bacteria or other microorganisms to breach the lubricating layer to establish "beachheads", colonizing on the underlying polymeric material.[105] SLIPS cannot influence the behaviors of planktonic microorganisms, this method only prevents the adhesion.[105] Therefore, this approach could be further optimized with an antimicrobial agent.

Herein, we have combined the bactericidal properties of NO-releasing polymers and SLIPS as a synergistic approach to not only prevent the adhesion of pathogenic microorganisms and the formation of biofilms, but also prevent the growth and proliferation of those organisms in the surrounding environment. The approach of incorporating NO release with the low fouling capabilities of liquid-infused materials has been previously proven to effectively prevent biofilm formation.[57] Therefore, this method is incorporated here to be further optimized in commercial urinary catheters. We use a dual swelling method, first swelling the silicone Foley catheter with solvent containing the NO donor SNAP, followed by swelling the NORel urinary catheter with silicone oil. The fabricated catheters provide the desired traits of liquid-infused materials and NO-releasing materials, while controlling the undesired burst release kinetics typical of NO-releasing materials. We demonstrate that liquid-infused nitric oxide-releasing (LINORel) urinary catheters effectively prevent the biofilm formation of pathogens commonly associated with CAUTIs over a 24 h and 7 d period. The proposed urinary catheter successfully combines the advantages of liquid-infused materials with the active release

of an antibacterial agent, a novel method in aiding to prevent the risk of CATUIs that has become one of the major hospital acquired infections.

3.2 Materials and Methods

Materials

N-Acetyl-*D*-penicillamine (NAP), sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, ethylenediaminetetraacetic acid (EDTA), tetrahydrofuran (THF), phosphate buffered saline (PBS), and sulfuric acid were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, hydrochloric acid, silicone oil, and sulfuric acid were obtained from Fisher Scientific (Pittsburgh, PA). Silicone Foley Urinary Catheters (UC), size 18 Fr, were purchased from Medline Industries (Sauget, IL). Phosphate buffered saline (PBS), pH 7.4, containing 138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, 100 μM EDTA was used for all experiments. *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 27853) were originally obtained from American Type Culture Collection (Manassas, VA, ATCC). Luria broth (LB) and Luria Agar (LA) were purchased from Fischer BioReagents (Fair Lawn, NJ).

SNAP Synthesis Procedure

SNAP was synthesized using a previously described method.[83] In short, an equimolar ratio of NAP and sodium nitrite was dissolved in a 1:1 mixture of water and methanol containing 2 M HCl and 2 M H_2SO_4. After dissolving, the reaction vessel was

cooled in an ice bath in order to precipitate the green SNAP crystals. The crystals were collected via vacuum filtration, rinsed with DI water, and dried under ambient conditions. The reaction mixture and the subsequent crystals were protected from light during the entirety of the experiment.

Preparation of the SNAP Impregnated Urinary Catheters and the Liquid-Infused NO-releasing Urinary Catheters

The SNAP swelling solution was prepared by dissolving SNAP in THF at a concentration of 125 mg mL^{-1}, previously found to be the highest concentration of SNAP possible, due to solubility in THF.[81] The silicone Foley catheter was cut into 1 cm long segments and submerged in the SNAP swelling solution for 24 h. The catheter segment was removed from the solution and dried for 24 h, allowing ample time for the THF to evaporate. Upon the completion of drying, the samples were placed in a 20 mL vial with DI water and sonicated for 5 minutes in a Fisher Scientific 1.9 L sonicating bath in order to remove any residual SNAP crystals adhered to the surface of the catheter segment. During the entire preparation period, the samples were kept in the dark. Using the method described above, the NORel-UC samples were prepared. The liquid-infused urinary catheters (LI-UC) and LINORel-UC were prepared by taking a 1 cm segment of the silicone Foley catheter and the NORel-UC and immersing each in silicone oil for 72 h.

Silicone Oil Swelling Characterization

The swelling and deswelling characteristics of the LI-UC and LINORel-UC were examined. For swelling, 1 cm segments of the silicone Foley catheter and the SNAP-

impregnated catheter were massed and submerged in silicone oil for a total of 72 h. These characteristics were defined by the swelling ratio, SR.

$$SR = \frac{M_i}{M_o}$$

The SR is defined as the ratio of the mass of the infused urinary catheter containing both the mass of the oil and of the polymer (M_o) and the initial mass of the polymer (M_i), prior to swelling. At various time points, the mass of each segment was measured to determine the respective SR. The samples were kept at room temperature during swelling. The deswelling of the oil was observed by incubating the LI-UC and LINORel-UC in PBS with EDTA at 37°C and measuring the SR at various time points for a total of 14 d.

Nitric Oxide Release Measurements from NORel-UC and LINORel-UC

The nitric oxide release from the prepared urinary catheters was measured using a Sievers chemiluminescence Nitric Oxide Analyzer (NOA), model 280i (Boulder, CO). For the measurements, 1 cm sections of the NORel-UC or LINORel-UC (n=3) was placed in 4 mL of PBS buffer with 100 mM EDTA in an amber glass vial at 37°C. Upon placing the sample in the amber vial, the nitric oxide release from the surface of the catheter was purged from the surrounding buffer by a constant flow of bubbled nitrogen which was then measured by the chemiluminescence detection chamber. After each measurement, the buffer was refreshed so that it did not become saturated with neither SNAP nor silicone oil. In between NO release measurements, the NORel-UC and LINORel-UC segments were kept in 4 mL of PBS with EDTA in an incubator at 37°C in the dark in order to mimic physiological conditions.

SNAP Leaching from NORel-UC and LINORel-UC

The total amount of SNAP leached from the prepared catheters during the first 24 h was measured. Segments (1 cm, n=3) of the NORel-UC and LINORel-UC were submerged in 2 mL of PBS with EDTA and kept in an incubator at 37°C. At each time point, 1 mL of the buffer was measured for the concentration of SNAP using a Thermo Scientific Genesys 10S UV-Vis Spectrophotometer. The buffer was returned back to the original sample container after each measurement to maintain the total incubation volume throughout the experiment. Absorbance for SNAP molecule has local maxima at 340 nm and 590 nm. Throughout the duration of this experiment, 340 nm was used.[54,86,106] The absorbance of the solution was recorded, and the concentration of SNAP in the solution was obtained from a predetermined calibration curve. Pure PBS with EDTA was used as the blank for the entire experiment. The samples were protected from light for the duration of the study.

The total amount of SNAP leached during the oil swelling study was also examined to confirm that significant amounts of SNAP were not lost during the 72 h swelling period. 1 cm SNAP impregnated urinary catheter segments were massed and submerged in 5 mL of silicone oil. The absorbance spectrum of the silicone oil was measured at various time points over the 72 h swelling period. Due to the fact that pure silicone oil had an absorbance of 0.0 when compared to PBS with EDTA buffer as the blank at 340 nm, the same calibration curve was used to measure the amount of SNAP within the silicone oil swelling solution.

Bacteria adhesion analysis: 24-hour and 7-day exposure

Inhibition of bacteria adhesion on the fabricated catheters was studied using models for both 24 h and 7 d. To obtain the required pathogenic cultures, *S. aureus* and *P. aeruginosa* were grown overnight in LB media to a CFU mL^{-1} of 10^6-10^8. This culture was then washed twice in PBS by centrifuging at 4400 rpm for 7.5 m. This was done to get rid of waste and unused media from the overnight culture. The bacteria pellet was then resuspended with PBS to obtain the suspension culture used for the bacteria adhesion study.

For the 24h study, UC samples (UC, LI-UC, NORel-UC, and LINORel-UC; n=3) were incubated with the bacteria suspension culture in a shaker incubator (200 rpm, 37°C). At the end of 24 h, the samples were washed with sterile PBS to get rid of any loose bacteria and then homogenized using a homogenizer. The samples were homogenized for 1 min each to remove the attached bacteria into a consistent volume of sterile PBS for each sample. This process also helped in homogenizing any bacterial biofilm formed in the 24 h study. The bacterial solutions obtained were then serially diluted and plated on LA media plates. The plates were incubated in 37°C for 24 h and CFU cm^{-2} measurements were done from them.

For the 7d study, a drip flow bioreactor model (Biosurface Technologies, DFR) was used to study bacteria biofilm inhibition. A modified form of the ASTM E2647-13 protocol was used for the experiment. An overnight culture of the bacteria was grown up to 10^6-10^8 CFU ml^{-1}. This culture was further processed like mentioned previously and resuspended in sterile PBS. The samples to be tested were placed in the chambers of the sterile DFR and incubated with the bacteria solution for 1 h. This 1 h incubation was

done to allow for the bacteria to settle on the catheters. After an hour of incubation, nutrient media was allowed to flow through the chambers at a rate of 0.8 mL m^{-1}. This flow rate was used to mimic the conditions of urine flow in a urinary catheter and allow for low shear conditions. At the end of 7 d, catheters were homogenized and the obtained bacteria was serially diluted. The serial dilutions were plated and counted after 18 h of incubation in 37°C.

Statistical Analysis

Data for the 24 h and 7 d bacteria adhesion analysis is expressed as mean ± standard deviation (SD) and for all other experiments is expressed as mean ± standard error of the mean (SEM). The results between the data for the UC, LI-UC, NORel-UC, and LINORel-UC were analyzed by comparison of means using Student's *t*-test. Values of $p < 0.05$ were considered statistically significant for all tests.

3.3 Results and Discussion

Liquid-infused Characterization of the Urinary Catheter

In order to evaluate whether the impregnation of SNAP into the silicone Foley catheter alters the liquid-infused properties when the LINORel-UC is swelled with oil, the swelling and deswelling ratios were observed before and after the incorporation of SNAP into the urinary catheter. The immersion of the silicone Foley catheter in silicone oil expands and swells the polymer due to the polymer chains extending to maximize polymer-solvent interactions.[101] The swelling ratio was observed over 72 hours. The

presence of SNAP within the tubing increased the swelling capacity from 1.44 ± 0.003 for the LI-UC, to 1.54 ± 0.005 for the LINORel-UC, confirming that SNAP does not negatively affect the swelling capabilities of the silicone Foley catheter (**Figure 3.1**).

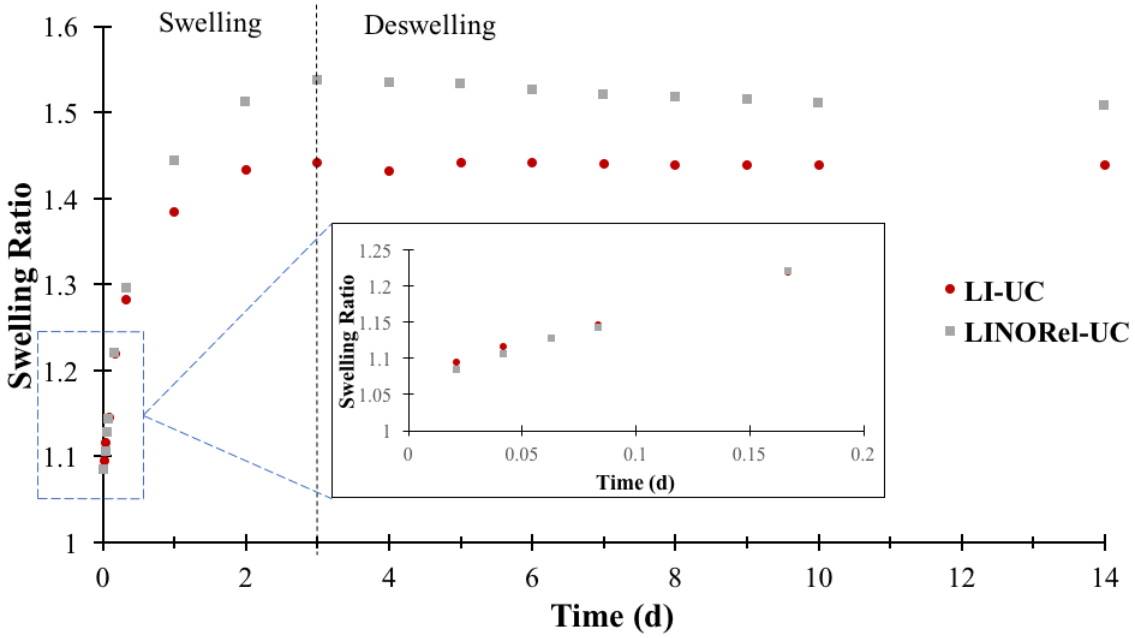

Figure 3.1: Swelling characteristics over 72-hour period and deswelling characteristics over the remaining 14-day period of the urinary catheter in silicone oil for the LI-UC and LINORel-UC (n=3). Error bars are excluded since they are on the order of data point size.

The larger swelling ratio for the LINORel-UC is hypothesized to be due to unfavorable interactions between the polymer matrix and the crystalline SNAP that is present after the solvent swelling process.[57] These interactions allow for further infusion of oil into the NORel-UC rather than what occurs within the commercial UC. The theory that the amount of infused SNAP increases the swelling ratio is also demonstrated by Goudie *et al.* where the presence of SNAP at a concentration of 25 mg mL^{-1} increased the swelling ratio for silicone rubber (SR) from 1.53 ± 0.003 for the LI-SR, to 1.59 ± 0.005 LINORel-SR.[57] The presented data herein supports the theory that increasing the amount of infused SNAP increases the differences in the swelling ratio between the control UC and NORel-

UC especially since the difference in swelling ratios for the higher concentration of SNAP reported here is larger than the difference reported by Goudie *et al*. Both of the prepared catheters were able to maintain these swelling ratios for over 14 d at 37°C. When compared to previous studies, the swelling ratio is lower with an increase in maximum swelling time.[57,101] This increase in swelling time corresponds to the decrease in the diffusion of oil from the polymer matrix, resulting in a lower swelling ratio for the urinary catheters. Because of this, the lubricating surface of the LI-UC and LINORel-UC may be affected.

The sliding angle was not observed for the prepared urinary catheters as previous reports have shown that the sliding angle for liquid-infused materials were significantly reduced, with a sliding angle of 2.1°, compared to the control material that had a sliding angle of 40.1°.[101] Additionally, the incorporation of SNAP into the polymer has been shown to have negligible effects on the sliding angle as the LINORel material had a similar sliding angle when compared to the LI material.[57] Therefore, we have substantial evidence that the liquid-infused properties stand in the presence of SNAP.

Quantification of Leaching of SNAP from NO-Releasing Urinary Catheters

Leaching of the NO donor from the polymer can have disadvantageous effects on the longevity of the release characteristics and could result in a non-localized release. Previous work has reported SNAP to be marginally hydrophobic, meaning it would prefer to stay within the polymer, although some SNAP is likely to diffuse into the surrounding solution.[61,107] Thus, in order to ensure that a minimal amount of SNAP was leached from the catheter surface, the absorbance of the PBS with EDTA buffer and the

silicone oil swelling solution containing the SNAP impregnated urinary catheter was measured. Using UV-vis spectroscopy, the amount of SNAP leached from the prepared urinary catheter samples during both the first 24 h submerged in PBS and the 24 h oil swelling was determined (**Figure 3.2**). Within the first 24 h, 0.80 ± 0.077 % of SNAP was shown to leach out of the NORel-UC and 0.49 ± 0.0061 % of SNAP leached from the LINORel-UC, meaning that there is a high amount of SNAP retention within the polymer of the fabricated urinary catheters. The total amount of SNAP leached from the LINORel-UC was reduced by ca. 38% than that of the NORel-UC from PBS incubation and reduced by ca. 88% from oil incubation.

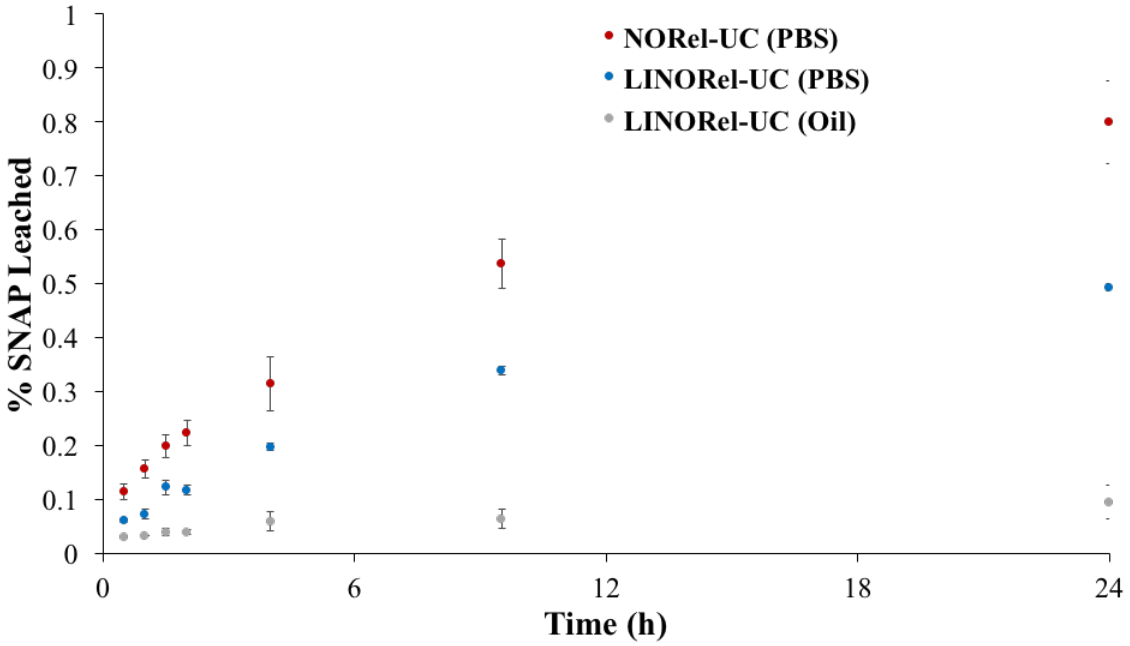

Figure 3.2: Leaching characteristics of SNAP from NORel-UC and LINORel-UC during first 24- hour period of soaking in PBS at 37°C and the 24-hour leaching characteristics of SNAP from LINORel-UC during the 72-hour silicone oil swelling period under ambient conditions using UV-vis spectroscopy (n=3). Data represent mean ± SEM.

The presence of the silicone oil in the NORel-UC successfully inhibited the additional leaching of SNAP since the lubricating layer hinders the surrounding fluid from reaching

the surface. During the 72 h oil swelling period, the amount of SNAP leached did not significantly increase after 9.5 h ($p>0.05$), demonstrating that swelling the UC segments in oil does not have an effect on the amount of SNAP present in the UC sample during the required swelling period. The solubility of SNAP in oil is found to be 0.4 μg mL^{-1}, adding to why the NORel-UC takes a longer amount of time to swell with silicone oil.[57]

Nitric Oxide Release Measurements *in Vitro*

Nitric oxide release was examined for both the NORel-UC and LINORel-UC using a Sievers chemiluminescence Nitric Oxide Analyzer (**Figure 3.3A**). Over a 60 d period, it was observed that the initial NO release for the NORel-UC was $3.59 \pm 0.13 \times 10^{-10}$ mol cm^{-2} min^{-1} with a final NO release of $0.10 \pm 0.04 \times 10^{-10}$ mol cm^{-2} min^{-1}, while the LINORel-UC initially released $0.4 \pm 0.04 \times 10^{-10}$ mol cm^{-2} min^{-1} and $0.41 \pm 0.05 \times 10^{-10}$ mol cm^{-2} min^{-1} at the end of the 60 d period. The percentage of total SNAP released from the both the NORel-UC and LINORel-UC over the 60 d NO release period is shown in **Figure 3.3B**. The amount of SNAP released is attributed to both leaching of the SNAP molecule and the degradation of SNAP to NAP that occurs over the release period. After the 60 d period, only 47.03% of the total SNAP loaded was released for the NORel-UC, and 46.82% for the LINORel-UC. There is still the capacity for the fabricated urinary catheters to release NO as there is about 52.97% and 53.18% of the SNAP remaining in the catheters, respectively. The amount of SNAP released from the NORel-UC and the LINORel-UC is very similar when compared after day 50, however, majority of the SNAP released for the NORel-UC is attributed to the burst release that occurs at the beginning of the testing period.

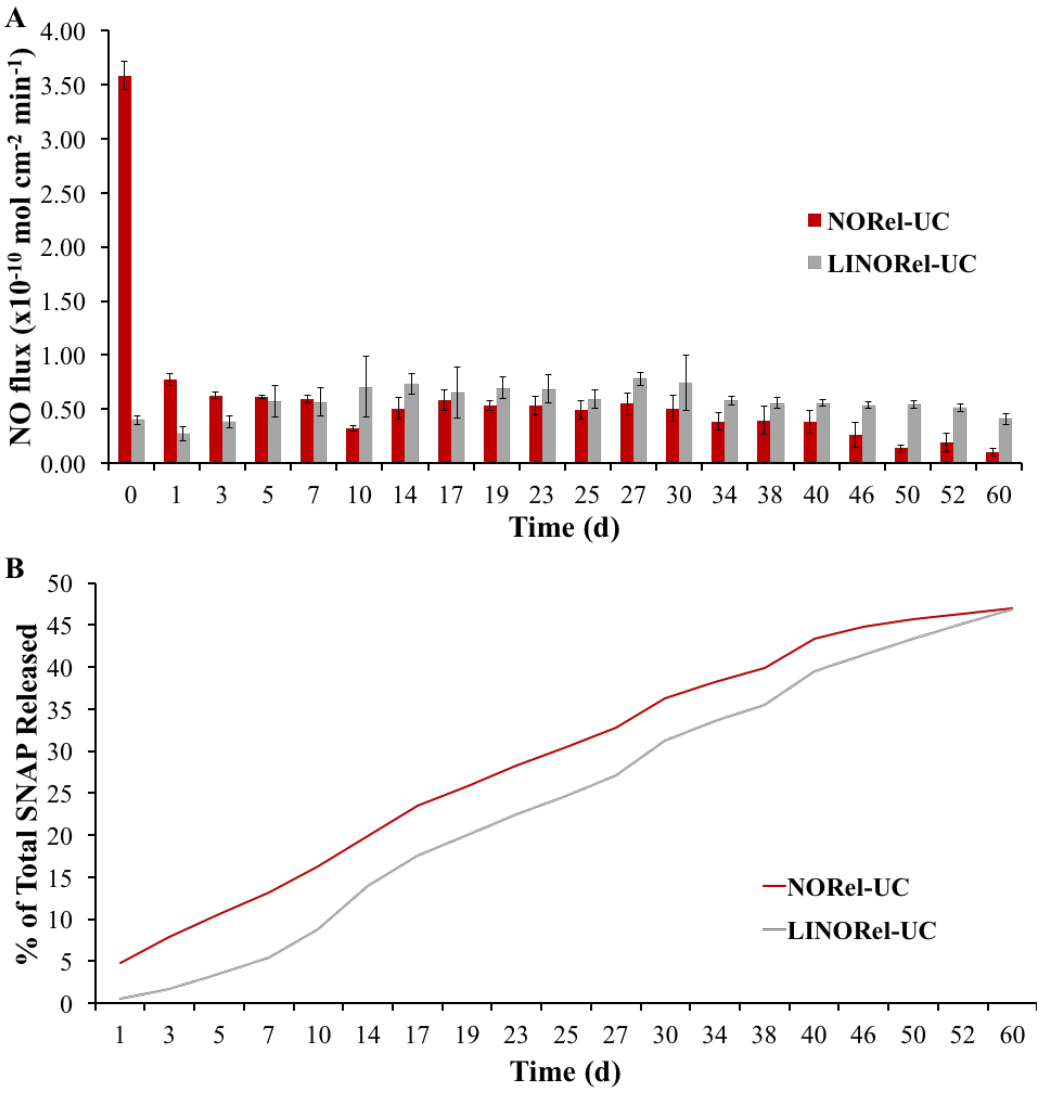

Figure 3.3: (A) Average nitric oxide release measurements from NORel-UC and LINORel-UC over a 60-day period (n=3). NO release measured from catheter samples submerged in PBS at 37°C using a Sievers Chemiluminescence Nitric Oxide Analyzer. Data represent mean ± SEM. **(B)** Percentage of total SNAP released from NORel-UC and LINORel-UC over 60-day period under physiological conditions resulting from the leaching and degradation of the SNAP molecule.

The NORel-UC showed an initial burst release of NO, resulting in a typical release profile consistent with other previously reported materials.[54,57,58,81] Many NO-releasing polymers exhibit this large burst release, which can affect the cytotoxicity levels and the

overall efficacy of the material, so limiting this burst release phenomenon is desirable. The incorporation of the silicone oil works to prevent the large initial burst release of NO when the material first comes into contact with increased temperature. The presence of silicone oil also contributes to a more uniform and constant NO release over the anticipated release period, as shown in **Figure 3.3A**. Both of these desirable characteristics can be attributed to the fact that the slippery surface created from the infusion of silicone oil prevents total hydration of the urinary catheter due to a slow uptake up water, allowing for a more even and controlled release.

Biofilm Formation Inhibition in a 24-hour and 7-day Drip Flow Bioreactor Model

Biofilms are a key hindrance faced by urinary catheter during both short- and long-term operations. Free-floating (or planktonic) bacteria can come across a surface submerged in the urine and within minutes become attached. These free-floating bacteria are widely present in the microflora of the patient's skin or urinary tract and find an easy way to the surface of the urinary catheters.[4,108] The attached bacteria then produce slimy, extracellular polymeric substances (EPS) that cover the catheter and form the conditioning film for the stationary, attached bacteria. Extracellular polymeric substance production allows the emerging biofilm community to develop a complex, three-dimensional structure that is influenced by a variety of environmental factors. This structure, now called the biofilm, protects the bacteria from antibiotics and hence biofilms have become a major hurdle for healthcare-associated infections.[109]

In our study, we examined the fabricated catheters' ability to inhibit bacterial infection by testing them in 24 h and 7 d models with the NO-releasing urinary catheters.

It is important to note here that even though NO-releasing materials do kill bacteria, they are not effective in preventing biofilm formation. Hence, through the antimicrobial analysis we expect to see a greater decrease in biofilm formation and bacteria adhesion in the test catheters due to the presence of infused silicone oil in addition to NO. The bacteria used are commonly found uropathogens – *Staphylococcus aureus* and *Pseudomonas aeruginosa.*[110] *S. aureus* is a Gram-positive pathogen and *P. aeruginosa* is a Gram-negative bacteria. Both have been commonly studied for antimicrobial resistance purpose. Previously, antibacterial analyses of NO-releasing urinary catheters have been mainly done with *Escherichia coli*, *Staphylococcus epidermidis* and *Proteus mirabilis.*[60,81,111,112]

The total viable *P. aeruginosa* adhered on the catheter samples' surface was determined after 24 h at 37°C. Plate count for 24 h *P. aeruginosa* biofilms showed that the viable bacteria attached on the surface of the LINORel-UC samples was 98.678 ± 0.214% less than on UC controls (**Figure 3.4**; n=3). This corresponded to a ca. 2 log reduction in viable bacteria growth. There was also a reduction of 72.12 ± 4.51% and 64.09 ± 5.81% on LINORel-UC when compared to NORel and LI-UC, respectively. Similarly, total viable *S. aureus* adhered on the catheter samples' surface was determined after 24 h exposure at 37°C. Plate count for 24 h *S. aureus* biofilms showed that the viable bacteria attached on the surface of LINORel-UC samples was 99.958 ± 0.004 less than on UC controls (**Figure 3.4**; n=3). This corresponded to a ca. 3.5 log reduction in viable bacteria adhesion. In addition to the reduction compared to UC control samples, the LINORel samples also displayed a 94.08 ± 0.60% and 71.43 ± 2.86% reduction in *S.*

aureus adhesion when compared to LI-UC and NORel-UC, respectively (**Figure 3.4**; n=3).

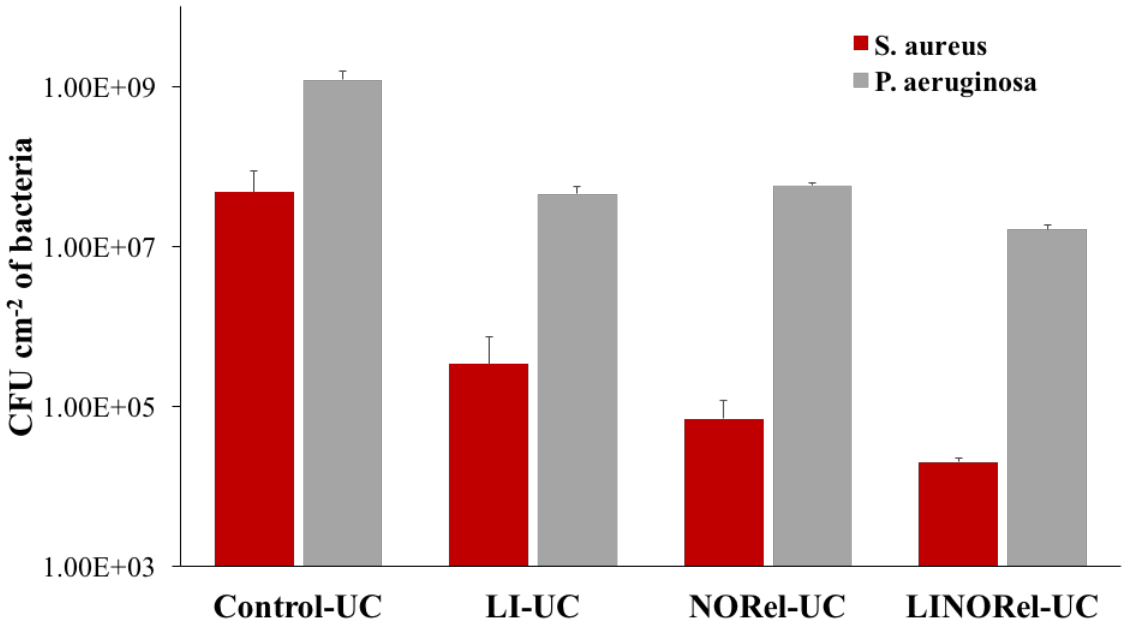

Figure 3.4: *S. aureus* and *P. aeruginosa* bacteria adhesion per cm^2 for control commercial urinary catheter, NORel-UC, LI-UC and LINORel-UC over 24-hour period (n=3). Data represent mean ± SD.

For the 7 d study, we employed a drip flow bioreactor model to analyze the efficacy of the catheters against *S. aureus*. Drip flow bioreactor models have been used previously to study biofilm formation for developing antimicrobial materials that release nitric oxide.[113,114] However, in these studies nitric oxide was released by the material only at the end of the drip flow biofilm growth by electrochemical mechanisms and not over the entirety of the experiment. Considering the ability of biofilms to grow well in a drip flow system, compared to a CDC high-shear bioreactor,[115] the antimicrobial activity of nitric oxide release would have to be continuously significant for the entirety of the study. As seen in **Figure 3.5** (n=3), total viable bacteria count adhered to NORel-UC and LI-UC was reduced by 93.52 ± 1.48% and 76.38 ± 14.15% when compared to UC

controls. However, this reduction was increased for LINORel-UC at 98.49 ± 2.06%. As hypothesized earlier, this increase in reduction of adhered bacteria is possibly due to the synergistic combination of silicone oil with NO-releasing surfaces. While the oil acts as a passive method to prevent any stagnant formation of bacteria colonies, NO is the active bactericidal agent. It is important to note here that a reduction of 93.62 ± 8.72% was seen on LINORel-UC compared to NORel-UC. As mentioned in the previous section, the continuous release of NO overtime also contributes to an even release and prevents bacterial colonization even with a 7-day period of exposure to common nosocomial pathogens in physiological implantation conditions.

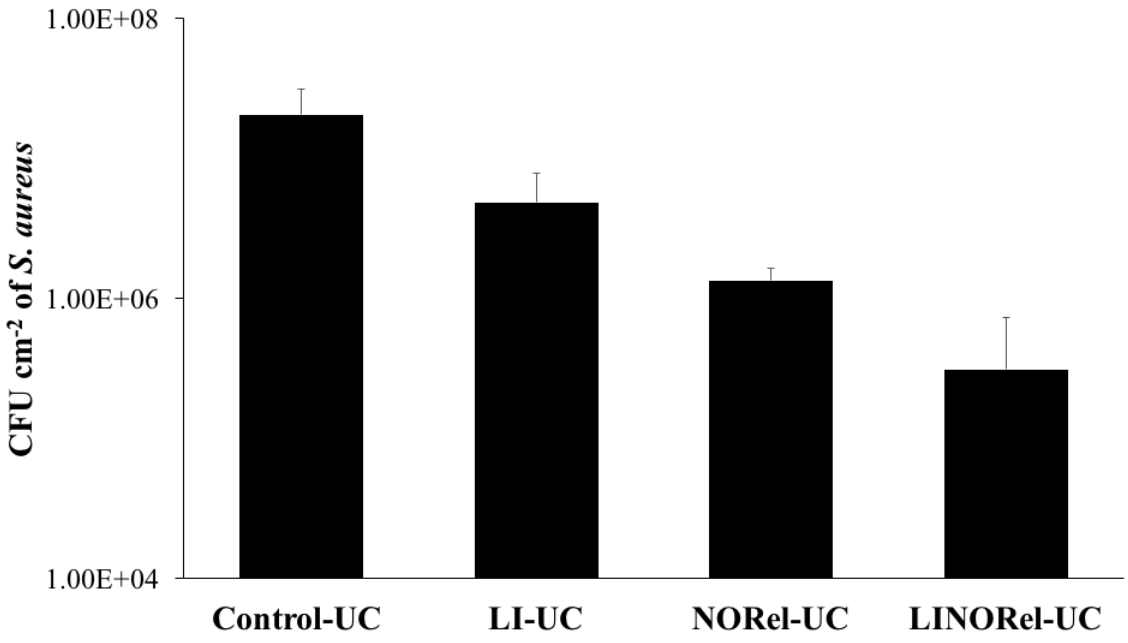

Figure 3.5: Bacteria adhesion for control commercial urinary catheter, NORel-UC, LI-UC and LINORel-UC after 7-day exposure to *S. aureus* in a drip flow bioreactor study (n=3). Data represent mean ± SD.

3.4 Conclusion

Here, the antifouling advantages of liquid-infused materials was incorporated with the active release of an antibacterial agent in a silicone Foley catheter, through the two

stage swelling of silicone oil and the NO donor SNAP. The presence of SNAP in the silicone Foley catheter proved to have no significant effects on the slippery properties of the surface. However, to our advantage, the implementation of silicone oil successfully aided in controlling not only the initial NO burst release, typical of NO-releasing materials, but provided an overall controlled and consistent release over a 60 d period, due to the lubricating layer hindering the total hydration of the urinary catheter surface. There was a high retention of SNAP within the LINORel-UC, only leaching 0.031 ± 0.0004 mg SNAP mg^{-1} tubing, while the NORel-UC leached 0.050 ± 0.0049 mg SNAP mg^{-1} tubing. The NORel-UC exhibited an initial NO-release of 3.59 ± 0.13 × 10^{-10} mol cm^{-2} min^{-1} and a final release of 0.10 ± 0.04 × 10^{-10} mol cm^{-2} min^{-1} over the 60 d period, while the LINORel-UC NO-release had a more consistent release between 0.4 ± 0.04 × 10^{-10} mol cm^{-2} min^{-1} and 0.41 ± 0.05 × 10^{-10} mol cm^{-2} min^{-1} over the 60 d period. The LINORel-UC exhibited a steady NO release, prolonging the desired properties of the liquid-infused nitric oxide releasing urinary catheter. Bacterial adhesion and biofilm formation was examined over a 7-day period in a drip flow bioreactor environment, finding 98.49 ± 2.06% reduction for Gram-positive *Staphylococcus aureus* for the LINORel-UC. Overall, the results suggest that the synergistic combination of the active release of NO and the passive release of silicone oil to prevent biofilm formation on silicone Foley catheters potentially provide a promising application in reducing the risk of CAUTIs.

CHAPTER 4

CONCLUSIONS & FUTURE RECOMMENDATIONS

4.1 Conclusions

This thesis research has focused on innovative methods to achieve the prevention of healthcare-associated infections. Using solvent swelling to impregnate endotracheal tubes and urinary catheters with the NO donor SNAP, bacterial adhesion on the surface of the fabricated devices was significantly reduced. This proves the feasibility of NO-releasing devices in potentially preventing HAIs.

In Chapter 2, the incorporation of SNAP to create nitric oxide-releasing endotracheal tubes was investigated. Specifically, the effects of SNAP on the mechanical, physical, and bactericidal properties of endotracheal tubes to prevent VAP. The impregnation of SNAP to fabricate the NORel-ETTs did not alter the ultimate tensile strength compared to commercially available ETTs and successfully released NO for 7 days. The storage stability was also considered for the clinical feasibility. The NORel-ETTs were stored at room temperature for 3 months and retained 90% of the initial SNAP concentration with no alteration to the NO release kinetics, demonstrating the feasibility of NORel-ETTs. Additionally, the incorporation of NO into the ETTs reduced 92% of viable bacteria. The active release of NO from the surface as an antimicrobial demonstrates an effective method to prevent the contraction of VAP from the use of endotracheal tubes.

In Chapter 3, the incorporation of SNAP in combination with silicone oil to create liquid-infused nitric oxide-releasing urinary catheters was investigated. Silicone oil successfully infiltrated the urinary catheter and the addition of SNAP had no effects on the slippery surface properties. The implementation advantageously controlled the NO release kinetics over a 60-day period, inhibiting the large initial burst release and providing a controlled release throughout the testing period. The LINORel-UCs also prevented biofilm formation on the surface over a 7-day period by a 98.5% reduction for Gram-positive *Staphylococcus aureus*. The results suggest that the synergistic combination of NO and silicone oil effectively prevents biofilm formation on silicone Foley catheters, potentially providing a promising application to reduce the risk of CAUTIs in hospitals.

4.2 Future Recommendations

Although SNAP impregnated medical devices have encouraging properties, as reported thus far in this thesis work, there are some future characterizations that would improve the understanding of NO-releasing devices and their ability as antimicrobials. The ability of the NORel-ETTs to release NO over longer periods of time needs to be evaluated in order to prevent VAP in patients who require an ETT longer than 7 days. The future recommendation for both the NORel-ETTs and LINORel-UCs is to further characterize the antibacterial properties with longer bacteria studies such as over 14 days in a bioreactor. Additionally, it is recommended to evaluate these devices *in vivo* using animal models. *In vivo* evaluation is important is further evaluate the properties and ability to inhibit bacterial adhesion and biofilm formation on the surfaces of these devices

in models that mimic the human body prior to clinical studies. Lastly, combining NO-releasing materials with surface modifications and/or other antimicrobials such as antibiotics and metal ions could lead to promising results in preventing bacteria adhesion and biofilm formation to ultimately inhibit HAIs.

Overall, there are many opportunities to explore with respect to using and optimizing NORel-ETTs and LINORel-UCs developed in this thesis research. The NO release from these materials has the potential to improve the antimicrobial properties of a wide variety of medical device by reducing infection-related complications. Clinical application of NO-releasing medical devices will have a positive impact on patients, especially in terms of saving lives and reducing medical costs associated with HAIs.

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