DEVELOPMENT OF *S*-NITROSO-*N*-ACETYLPENICILLAMINE (SNAP) IMPREGNATED MEDICAL GRADE POLYVINYL CHLORIDE FOR ANTIMICROBIAL MEDICAL DEVICE INTERFACES

by

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ABSTRACT

In the clinical setting, polyvinyl chloride (PVC) accounts for 25% of all polymers used in medical device applications. However, medical devices fabricated with PVC, such as endotracheal tubes, extracorporeal circuits (ECCs), or intravenous catheters suffer from thrombosis and infection. Mortality associated with hospital associated infections (HAIs) exceed 100,000 deaths each year. One method to overcome these challenges is to develop bioactive polymers with nitric oxide (NO) release. Nitric oxide exhibits many physiological roles including, antibacterial, antithrombic, anti-inflammatory activity. In this study, Tygon® PVC tubing was impregnated with a NO donor molecule, *S*-nitroso-*N*-acetylpenicillamine (SNAP), via a simple solvent-swelling-impregnation method, where polymer samples were submerged in a SNAP impregnation-solvent (methanol, acetone, plasticizer). An additional topcoat of a biocompatible CarboSil 2080A (CB) was applied to reduce SNAP leaching. The SNAP-PVC-CB were characterized for NO release using chemiluminescence, leaching with UV-Vis spectroscopy, surface characterization with scanning electron microscopy, tensile strength analysis, stability during storage and sterilization, and antimicrobial properties *in vitro*. The SNAP-PVC-CB exhibited NO flux of 4.29 ± 0.80 x 10^{-10} mol cm^{-2} min^{-1} over the initial 24 h under physiological conditions and continued to release physiological levels of NO for up 14 d (incubated in PBS at 37 °C). The addition of CB-topcoat reduced the total SNAP leaching by 86% during incubation. Mechanical properties and surface topography remained similar to original PVC after SNAP-impregnation and application of CB-topcoat. After ethylene oxide sterilization and 1-month storage, SNAP-PVC-CB demonstrated excellent

SNAP stability (ca. 90% SNAP remaining). In a 24 h antibacterial assay, SNAP-PVC reduce viable bacteria colonization (ca. 1 log reduction) of *S. aureus* and *E. coli* compared to PVC controls. This novel method for SNAP-impregnation of medical grade plasticized PVC holds great potential for improving the biocompatibility of post-fabricated PVC medical devices.

This work is dedicated to my family, whose love and support has been a bright light in my life.

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CHAPTER ONE: INTRODUCTION

Hospital associated infections (HAIs) are a major concern for patient safety. In 2002, the Centers for Disease Control and Prevention reported that among 1.7 million patients in United States hospitals, whom developed a HAI, 99,000 resulted in death [1]. The pathogenesis of disease associated with HAIs is caused by ESKAPE Pathogens, referring to *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp*. [2]. Medical devices provide an opportunistic environment for the colonization of infectious bacteria. The use of indwelling medical devices, which are necessary to provide patient care, account for 86-96% of HAIs such as catheter associated urinary tract infection (CAUTI), central line associated bloodstream infections (CLABSI), and ventilator associated pneumonia (VAP) [3, 4].

The progression of bacterial colonization (**Fig.1**) begins with cellular attachment which develop stronger adhesion by anchoring pili, lipopolysaccharides, teichoic acid, and exopolysaccharides to the device surface [5, 6]. The bacteria assemble and excrete an extracellular polymeric substance (EPS), known as a biofilm, which provides structure and protection for unhindered reproduction [7]. The EPS is a hydrated substance (98% water) which consists of intricate water channels which deliver nutrients to bacteria within the matrix [8]. As the population grows, the interaction between bacteria increases as they compete for resources. The increased cell density heightens the quorum sensing and can activate the alterations in gene expression leading to phenotypic variation [9-11].

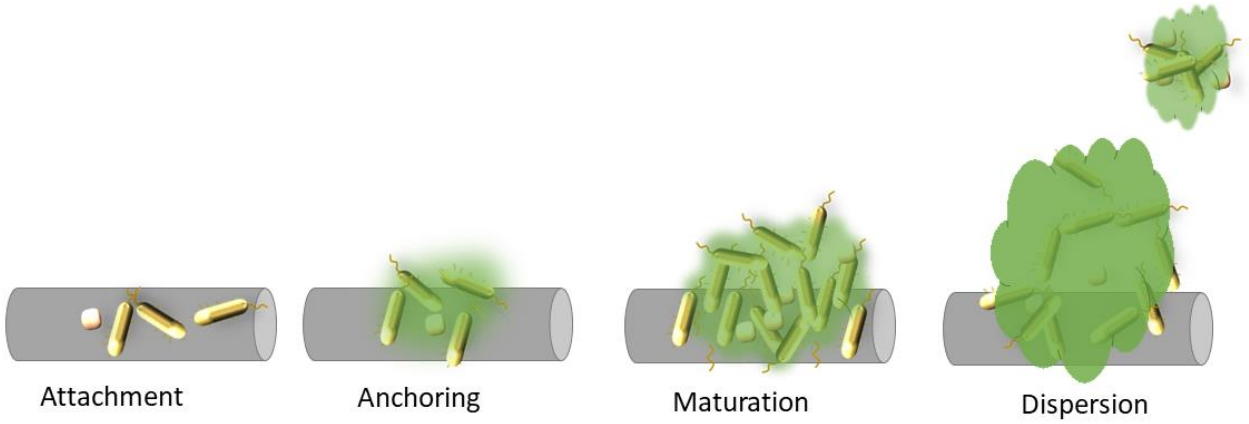

Figure 1. Pathogenesis of bacterial colonization of the surface of a medical device.

Due to the bacterial variations and complexity of the biofilm environment, antibiotics often require 10-5,000 times the concentration to treat their planktonic counterparts [12-14]. Eventually, the bacteria population reaches a critical capacity within the biofilm, dispersion is initiated to recolonize a new environment, and the evolutionary process is repeated. Research has shown, that due to fierce competition amongst bacteria within the biofilm, the dispersed bacteria are phenotypic variants from their initial colonies [15]. The virulent bacteria can exhibit antibiotic resistance and are increasingly infectious toward hosts with weakened immune systems such as patients receiving urgent care [16-19]. The high infection rates associated with biofilm formation on medical devices and the development of antibiotic resistant microbes are a major concern amongst healthcare professionals [20, 21]. O'neill et al. estimated that antimicrobial resistance will cause 10 million deaths per year, by the year 2050 [22].The alarming increase in development of antimicrobial resistance further illustrates the importance for

the development of biocompatible materials that can effectively prevent bacterial colonization and microbial resistance.

Polyvinyl chloride (PVC) is a leading polymer used for the fabrication of medical devices, totaling ca. 700 million lbs each year, accounting for 25% of the medical polymer market [23, 24]. Extensive use of PVC is due to its chemical stability, low cost, transparency, facile surface functionalization, and wide range of mechanical properties upon addition of plasticizers [23-26]. High temperature extrusion, exceeding 190 °C, is widely used for the fabrication of medical devices such as extracorporeal circuits, endotracheal tubes, infusion and drainage tubing, catheters, and blood and bodily fluid storage [26, 27]. Despite the widespread use of medical grade PVC, it does not possess immunological functions like natural tissue to prevent bacterial infections (e.g. antimicrobial peptides) [28]. To solve these issues, a number of passive and active approaches have been investigated to improve the biocompatibility of PVC by physical and chemical mechanisms.

Bacterial adhesion on the surface of a medical device is a complex process which is dependent on surface chemistry, surface charge, topography, and environment [29, 30]. Particularly, passive approaches exhibit control over hydrophobicity and topography by introduction of polymer brushes, self-assembled monolayers, zwitterions, and nano/micro structures which display unfavorable sites on a material surface to prevent bacterial adhesion [29-34]. Active approaches introduce surface immobilization or incorporation of antimicrobial agents which kill bacterial upon contact including antibiotics, nanoparticles, photo activators, quaternary ammonium compounds, antimicrobial

peptides, quorum-sensing interfering molecules, and nitric oxide [30, 35-44]. However, challenges remain with both passive and active approaches such as limited effectiveness, suspect to antimicrobial resistance, and cytotoxicity [29, 43, 45-47].

One promising approach toward improving the biocompatibility of medical devices is the development of nitric oxide (NO) releasing polymers. Nitric oxide is an endogenous free radical, gas molecule that is synthesized by the human body, via nitric oxide synthase enzymes that covert L-arginine into citrulline and NO [48, 49]. Nitric oxide participates in a number of physiological roles, including, attenuation of inflammation and platelet activation [50-53]. The normal endothelium releases NO within the flux range of 0.5 – 4 x 10^{-10} mol cm^{-2} min^{-1} to maintain hemostasis by preventing platelet activation at the blood-endothelium interface [54]. In addition, macrophages and neutrophils express inducible nitric oxide synthase (iNOS) which can produce > 1 μM NO as a biocidal agent and promote biofilm dispersion [48, 49, 55-59]. The radical behavior of NO allows for a multitude of biocidal mechanisms such as, damage to DNA, alteration of vital enzymes and proteins, and induction of lipid peroxidation [48]. Due to the array of antimicrobial mechanisms and short half-life, NO provides broad-spectrum antimicrobial activity [48, 60]. Due to the fact that NO is a highly reactive gas, NO releasing compounds (organic nitrates or nitrate esters, nitrite, metal-NO complexes, *N*-diazeniumdiolates (NONOates), and *S*-nitrosothiols (RSNO)), have been synthesized in order to enhance the stability of NO and allow for addition into polymers for controlled delivery to improve biocompatibility [43, 61-63]. Polymers fabricated with NONOates and RSNOs are the most commonly investigated NO releasing materials. The stimulation of NO release kinetics from these two classes of NO donor are quite different, such that NONOates require proton and

thermal mechanisms, while RSNOs are stimulated by heat, light, and metal ions [43, 64]. Thus, a NO donor can be utilized for specific medical applications. *S*-nitroso-*N*-acetylpenicillamine (SNAP, **Fig.2**) is a well characterized RSNO and due to its low cost, stability, and NO release under physiological conditions (37 °C), it has been incorporated into polymers to create NO releasing medical devices surfaces [63, 65-68].

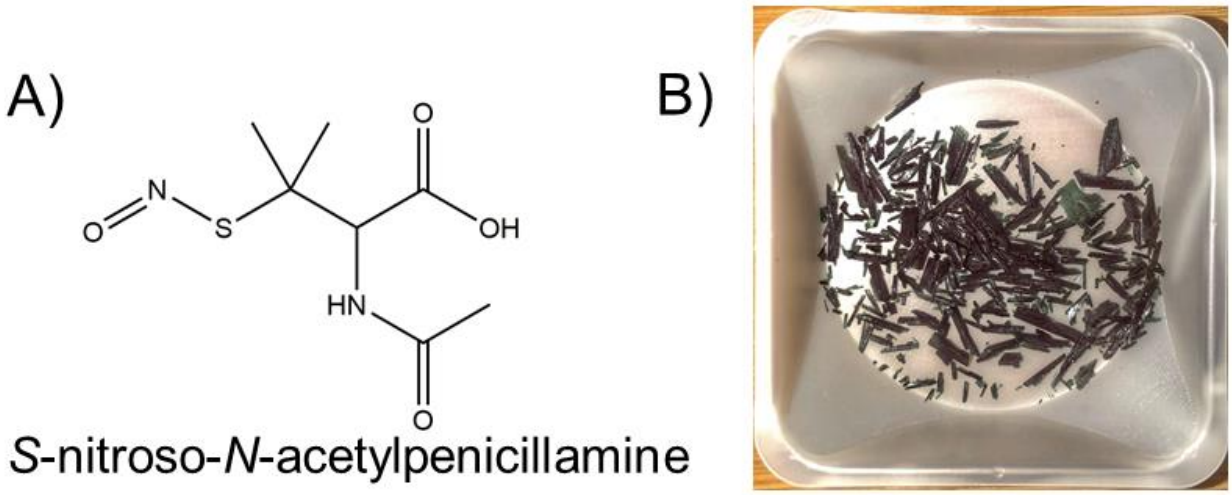

Figure 2. Chemical structure (A) and crystals (B) of *S*-nitroso-*N*-acetylpenicillamine (SNAP).

A key challenge of NO releasing polymers is the leaching of NO donors which may result in nonlocalized NO-mediated responses [69, 70]. The covalent linkage of the NO donor to the polymer has shown significant reduction in leaching, but they suffer from challenges such as time intensive preparation and variability among NO loading efficiencies [71, 72]. Physical dispersion of NO donors can be employed by non-covalent incorporation within a polymer to tailor NO release for specific applications, and additional polymer topcoats have been employed to minimize leaching [73-75]. While the leaching of NO donors is a concern, the cytotoxicity of SNAP and SNAP-polymers is negligible [76, 77]. The parent molecule of SNAP is the FDA approved drug (N-acetyl-D-penicillamine)

[43] and the concentration of leaching is considerably low after application of a polymer topcoat [73-75].

Nitric oxide-releasing medical grade polymers such as silicone [78-85], silicone-based polyurethanes (CarboSil 2080A, Elast-eon) [63, 65-67, 86-92], poly(lactic-*co*-glycolic acid) [93, 94], and polytetrafluoroethylene [95], have been investigated for a variety of clinical applications including hemocompatibility and antibacterial activity [43, 48, 96-108]. Recent literature demonstrated the incorporation of NO donors, *N*--diazeniumdiolates, *S*-nitroso-glutathione, and covalent linkage of SNAP to PVC and further characterized these materials for their clinical potential [53, 69, 70, 72, 73, 75, 109, 110]. While these methods have exhibited significant antithrombic and antibacterial activity, the complexities of fabrication hinder the translation of these materials to the clinical setting. However, commercial PVC devices are manufactured using high temperature extrusion process which can be detrimental to the NO release chemistry [64].

Recent work has demonstrated a new approach of a solvent-swelling-impregnation technique to impregnate SNAP into post-fabricated silicone and CarboSil 2080A (CB) devices [66, 111]. Solvent-swelling methods to impregnate polymers with SNAP provide an advantage compared to previous methods of casting SNAP-polymer mixtures. The solvent-swelling impregnation method utilizes solvents that are able to penetrate into the polymer matrix and allow molecules (i.e. SNAP) to diffuse into the bulk and become trapped after solvent evaporation. When SNAP is impregnated into a polymer beyond the solubility limit, a SNAP-polymer crystal composite is formed, which exhibits enhanced stability and prolonged NO release of the material [65, 66]. The

mechanism governing the enhanced stability of SNAP in polymer matrices is complex, but Wo et al. hypothesized the crystalline SNAP is stabilized by intramolecular hydrogen bonding which slows the rate of NO release, while SNAP soluble within the polymer matrix readily releases NO. The stabilization effect significantly prolongs the NO release under physiological conditions and enabled excellent storage stability where 82% and 88.5 % of initial SNAP content remained in E2As polymers after 2 and 8 month, respectively, stored at 37 °C [67, 87]. Similar results were observed in CB, silicone, and E5-325 88.5%, 86.8% and 68.3% SANP retained, respectively after 8 months storage at 37 °C [63].

The solvent-swelling-impregnation technique is advantageous because it allows for direct incorporation of NO donors into post-fabricated polymeric medical devices. A new method is needed to impregnate PVC with SNAP that can provide sufficient SNAP solubility in the solvent, rapid swelling and drying, maintain plasticizer content in final polymer, and prevent dissolution of PVC matrix. The previously reported SNAP-impregnation methods are not feasible because either PVC is not compatible with the solvent (tetrahydrofuran) or causes plasticizer leaching (methanol and 2-butanone) [66, 79]. The plasticizer concentration directly effects the mechanical properties of PVC, thus control of the plasticizer in the impregnation-solvent is paramount to maintain the mechanical properties during SNAP-impregnation. Herein, a novel solvent-swelling-impregnation method for preparation of SNAP-impregnated PVC (SNAP-PVC) technologies to reduce the colonization of bacteria on PVC medical devices is presented. The material fabrication, SNAP leaching behavior, NO release kinetics, mechanical properties, surface morphology, and storage stability are studied. Finally, the *in vitro*

antibacterial activity of SNAP-PVC with CB-topcoat (SNAP-PVC-CB) is evaluated against two potent HAI causing pathogens*, Staphylococcus aureus* and *Escherichia coli*.

CHAPTER TWO: EXPERIMENTAL DETAILS

2.1 Materials

N-Acetyl-*D*-penicillamine (NAP), sodium nitrite, *L*-cysteine, sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, copper (II) chloride, ethylenediaminetetraacetic acid (EDTA), Tetrahydrofuran (THF), *N*,*N* dimethylacetamide (DMAc), and Bis(2-ethylhexyl) phthalate (DEHP) were purchased from Sigma-Aldrich (St. Louis, MO). Methanol (MeOH), acetone (Ace), hydrochloric acid, sulfuric acid, were products of Fisher Scientific (Hampton, NH). Fisherbrand™ Tygon S3™ E-3603 Flexible Tubing was purchased from Fisher Scientific (Pittsburgh, PA). CarboSil 2080A (CB) was obtained from DSM Biomedical Inc. (Berkeley, CA). All aqueous solutions were prepared using deionized water. Phosphate buffer saline (PBS), pH 7.4, containing 138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, and 100 μM EDTA was used for all *in vitro* experiments.

2.2 Methods

2.2.1 SNAP Synthesis

S-nitroso-*N*-acetylpenicillamine was prepared using a modified version of a previously reported method [112]. In brief, equimolar rations of NAP and sodium nitrite were added to a 2:3 mixture of water and methanol containing 1 M H_2SO_4 and 1 M HCl. The mixture was stirred for 10 minutes then cooled on ice for 8 h to precipitate SNAP crystals. Air was gently blown over the reaction vessel while cooling. The crystals were

collects via suction filtration, washed with ice cold DI water, the dried in vacuum desiccator overnight. The reaction was protected from light at all times.

2.2.2 Method for SNAP-Impregnation of PVC

Initial studies were conducted using Tygon ® PVC (E-3603) with an I.D. of 6.35 mm, O.D. of 9.53 mm and wall thickness of 1.59 mm. Samples of PVC were cut into 2000 mg pieces. Next, an impregnation-solvent of methanol, acetone, and *Bis*(2-ethylhexyl) phthalate plasticizer (MeOH:Ace:DEHP) at molar ratios of 20:60:20 was prepared at a final volume of 20 mL. Two different concentrations of SNAP (40 mg/mL and 80 mg/mL) were dissolved in the MeOH:Ace:DEHP impregnation-solvent to achieve different wt % of SNAP in PVC. The PVC was then soaked in the MeOH:Ace:DEHP impregnation-solvent containing SNAP for a duration of 4 h and 20 h in the dark. The SNAP-PVC was left to dry overnight, under ambient conditions and in the dark. Next, the tubing was dried in a vacuum desiccator for 24 h in the dark. The SNAP-PVC was cleaned with methanol to remove SNAP crystals on the surface. The tubing was then cut to achieve 1 cm x 1 cm square samples for subsequent analysis. Samples were topcoated with CarboSil 2080A (CB) to achieve a smoother surface and prevent excessive SNAP leaching. First, 1200 mg CB was dissolved THF (15 mL), overnight. Next, SNAP-PVC was briefly dip coated 3 times in the CB solution. The CB coated SNAP-PVC (SNAP-PVC-CB) was dried under ambient conditions in the dark followed by 24 h in vacuum desiccator. A uniform coat of CB, approximately 200 µm thick, was applied, as measured by a Digimatic micrometer (MDC-1 PX) from Mitutoyo America. The samples were then stored in the freezer (-20 °C) until testing.

2.3 Characterization of SNAP-PVC-CB

2.3.1 Determination of Wt % via UV-Vis Spectral Analysis

Samples of PVC impregnated with 20:60:20 MeOH:Ace:DEHP containing SNAP (40 and 80 mg/mL) was dissolved in DMAC and the UV-vis spectra was analyzed to determine the wt % SNAP loaded into each sample as described by Equation (2.1):

$$\left(\frac{mg\ SNAP}{mg\ PVC} * 100 = wt\ \%\ SNAP\right) \quad \text{..... (2.1)}$$

Each spectrum was recorded within the wavelength region of 650 – 300 nm using a Cary 60 UV-vis spectrophotometer (Agilent Technologies) at room temperature. The molar absorptivity of SNAP in DMAC was determined as 1008 M^{-1} cm^{-1} at 340 nm. The characteristic absorbance bands of SNAP are 340 and 590 nm which correspond to the $\pi \rightarrow \pi^*$ and $n_N \rightarrow \pi^*$ electronic transitions of the S-NO functional group [105, 113].

2.3.2 Determination of SNAP Leaching from SNAP-Impregnated PVC

The amount of SNAP leached from SNAP-PVC-CB and SNAP-PVC was determined by a UV-Vis spectrophotometer. A known mass of SNAP-PVC-CB and SNAP-PVC were incubated in 10 mM PBS, pH 7.4, with 100 μM EDTA at 37 °C for 7 d. The soaking solution was analyzed for SNAP concentration each day over a 7 d period. The molar absorptivity of SNAP in 10 mM PBS, pH 7.4, with 100 μM EDTA at 37 °C was determined as 1072 M^{-1} cm^{-1} at 340 nm. The amount of SNAP leaching as detected by UV-Vis was normalized by the mass of SNAP-impregnated samples before topcoating. The soaking buffer was replenished with fresh buffer after each measurement and stored at 37 °C.

2.3.3 Nitric Oxide Release Profile of SNAP-PVC-CB

Nitric oxide release of the SNAP-PVC-CB was analyzed by a Zysense chemiluminescence Nitric Oxide Analyzer (NOA) 280i (Frederick, CO). An amber NOA sample cell was filled with 5 mL of 10 mM PBS, pH 7.4, with 100 μM EDTA and incubated at 37 ˚C to mimic physiological conditions. The NOA cell was purged via N_2 bubbling at a flow rate of 200 mL min^{-1}. A baseline measurement of the sample cell was recorded for at least 5 minutes then the polymer samples of known dimensions was added to NOA cell. Real-time NO detection was recorded at 1 s intervals. Samples were stored in PBS buffer at 37 ˚C until subsequent analysis.

2.3.4 Characterization of Mechanical Properties

Comparison of the effect of solvent-swelling-impregnation on PVC was investigated using three different samples that were prepared as previously described. In brief, original PVC, PVC swelled with 20:60:20 MeOH:Ace:DEHP without SNAP (PVC-Solvent), and PVC impregnated with 80 mg/mL SNAP (SNAP-PVC). An active area for each sample was cut to dimensions of 55 mm length, 4 mm width, and 0.4 mm thickness. Samples were subjected to tensile strength measurements by an Instron tensile tester at 23 °C. Samples were clamped in the place then were pulled at a constant rate of 10 mm/min. The recorded tensile strength (MPa) and % elongation was compared for each of the sample materials.

2.3.5 Characterization of Surface Morphology

Scanning electron microscopy (SEM) images were recorded using a JEOL JSM-6480 Scanning Electron Microscope at a magnification of 100x at 5.0 kV. Samples were mounted onto stubs using double sided carbon tape and gold sputtered for 180 s before imaging.

2.3.6 Determination of SNAP Stability During Storage and Sterilization

Samples of SNAP-PVC-CB were stored with desiccant at –20 °C, 23 °C and 37 °C as representative storage conditions. Samples were kept in the dark at all times. The stability of the samples was determined by measuring the amount of SNAP remaining in the samples after 1 week, 2 weeks, and 4 weeks storage. Samples of SNAP-PVC-CB after time were dissolved in DMAc and the SNAP UV-Vis spectra was recorded to measure SNAP concentration after time (C_t) and compared to initial SNAP content ($C_{initial}$) in samples at week 0 to determine the % SNAP remaining (C_{final}) as described by Equation (2.2):

$$\left(C = \%\, wt\ SNAP,\ \frac{C_t}{C_{initial}} * 100 = C_{final}\right) \quad (2.2)$$

Samples of SNAP-PVC-CB were sterilized by ethylene oxide (EO) at 54 °C and 40-80 % humidity. During the ethylene oxide sterilization, samples were first conditioned for 1 h, treated with EO gas for 2-3 h, then aerated for 14 h. Sterilization was performed at the University of Michigan Hospital Central Sterile Processing Department.

2.4 *In Vitro* Analysis of Inhibition of Bacterial Adhesion on Polymer Surface

The NO releasing effect of SNAP-PVC-CB in terms of bacteria adhesion was assessed to determine viable bacterial counts on polymer surface. Gram-positive *S. aureus* (ATCC 6538) and gram-negative *E. coli* (ATCC 11303) bacteria are the most common cause of hospital acquired infections (HAIs). Hence, these strains were used as the model organisms to test the efficiency the antibacterial adhesion of SNAP-PVC-CB.

2.4.1 Preparation of Bacterial Culture

Luria Broth (LB) media was prepared according to the manufacturer's directions. Prior to using, broth was sterilized in an autoclave. A single, isolated colony of bacteria was inoculated in the LB media and suspension was incubated for 15 hours at 37 °C, rotating speed of 120 rpm in shaker incubator. After overnight culture, the suspension was centrifuged at 2500 rpm for 7 min and the supernatant was discarded. The cells were washed with sterile phosphate buffer saline (PBS, pH 7.4), centrifuged at 2500 rpm for 7 min. The supernatant was discarded, and fresh PBS was added to resuspend the bacterial cells. The OD_{600} of the cell suspension in PBS was measured at a wavelength of 600 nm using a Cary 60 UV-vis spectrophotometer (Agilent Technologies). Fresh PBS was used as a blank and OD of bacteria was adjusted to the CFU in the range of 10^6 – 10^8 CFU/mL.

2.4.2 Bactericidal Effect Assay

Samples of PVC-CB were used as the control to compare the difference in number of viable bacteria on SNAP-PVC-CB. Samples were cleaned with 70% ethanol and sterilized using UV light prior to bacterial exposure. A previous report determined exposure of SNAP-polymers to UV light was not detrimental to SNAP stability [87]. All the samples were exposed to 2 mL of bacteria (10^6 –10^8 CFU/ mL) at 37 °C, 120 rpm for 24 h in a shaker incubator. After 24 h, samples were mildly rinsed with 2 mL of sterile PBS to remove any loosely bound bacteria on the polymer surface and then transferred into fresh PBS. The samples were then homogenized for 60 s and vortexed for 30 s to collect the bacteria adhered on the samples. *S. aureus* and *E. coli* were plated in the solid LB agar medium after preparing serial dilutions in the range of 10^{-1} – 10^{-5}. The LB agar plates with the plated bacteria culture were incubated at 37 °C for 24 h. After 24 h, the CFUs were counted considering the dilution factor and the number of viable bacteria on SNAP-PVC-CB were compared to the control samples. All the experiments were done using three replicates.

2.5 Statistical Analysis

All experiments were conducted in at least triplicate. Data are all expressed as mean ± SEM (standard error of the mean). Comparison of means using Student's *t*- test was utilized to analyze the statistical differences between sample groups. Values of $p < 0.05$ were considered statistically significant for all tests.

CHAPTER THREE: RESULTS

3.1 Impregnation Methods to Fabricate *S*-nitroso-*N*-acetylpenicillamine-PVC-CB

The development of a novel SNAP impregnation method for medical grade PVC will significantly reduce the risk of HAIs associated with the abundant applications of PVC medical devices. Since, plasticizer content in PVC directly affect mechanical properties of the device, it was critical to fabricate an impregnation-solvent that maintained the plasticizer proportion. Various solvents for solvent-swelling-impregnation were investigated with the following factors considered: 1) SNAP solubility, 2) ability to promote significant swelling and deswelling of PVC for rapid fabrication, and 3) maintain the desired plasticizer content before and after the impregnation. A solvent mixture of SNAP with methanol (MeOH) to provide sufficient dissolution of SNAP, acetone (Ace) to promote PVC swelling, and *Bis*(2-ethylhexyl) phthalate (DEHP) to maintain original mechanical properties, was investigated. The common plasticizer, DEHP, was used solely as a model to develop the impregnation method. It was found that the impregnation-solvent of ratio 20:60:20 MeOH:Ace:DEHP provided optimal mechanical properties during SNAP impregnation and was used for all further fabrication. The PVC was soaked in the impregnation-solvent containing SNAP (40 and 80 mg/mL) for 4 and 20 h (**Fig. 3**). The wt % SNAP impregnated into PVC was quantitated via UV-vis as previously described using Equation 2.1. The increase of SNAP content (40 mg/mL to 80 mg/mL) resulted in an increase of SNAP impregnation, 4.08 ± 0.08 and 5.48 ± 0.05 wt % SNAP, respectively (**Fig. 4**). Control of the SNAP concentration in the impregnation-solvent can be tuned to acquire a desired wt % SNAP for a specific application.

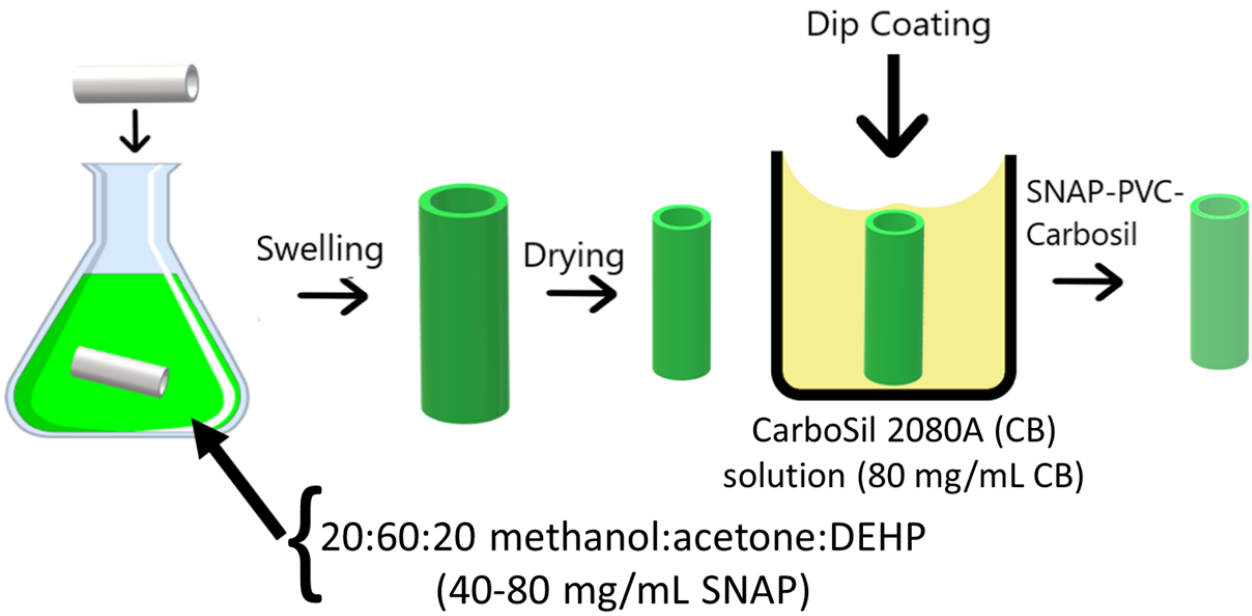

Figure 3. Schematic of the solvent-swelling impregnation of SNAP into the plasticized polyvinyl chloride (PVC) tubing and the addition of CarboSil 2080A (CB) topcoat.

These results coincide with previous reports that demonstrated an increase of SNAP concentration in impregnation-solvent resulted in higher wt % SNAP impregnation [66]. Upon investigation of the SNAP-impregnation duration, increasing the swelling duration to 20 h of 40 and 80 mg/ml SNAP resulted in 4.38 ± 0.05 and 5.60 ± 0.04 wt %, respectively. Therefore, the swelling for 4 h or 20 h had no significant difference in SNAP loading (**Fig. 4**). The swelling duration of 4 h in 20:60:20 (MeOH;Ace:DEHP) with SNAP was chosen for further investigation, due to the decreased swelling duration and optimal SNAP loading. A CB-topcoat was applied to the appropriate samples to ensure a uniform surface topography and prevent leaching of SNAP. The topcoat was applied by dipping (3 times) the SNAP-PVC in an 80 mg/mL CB solution then drying for 1 h. A thickness of ca. 200 µm was confirmed by a digital micrometer [114]. .

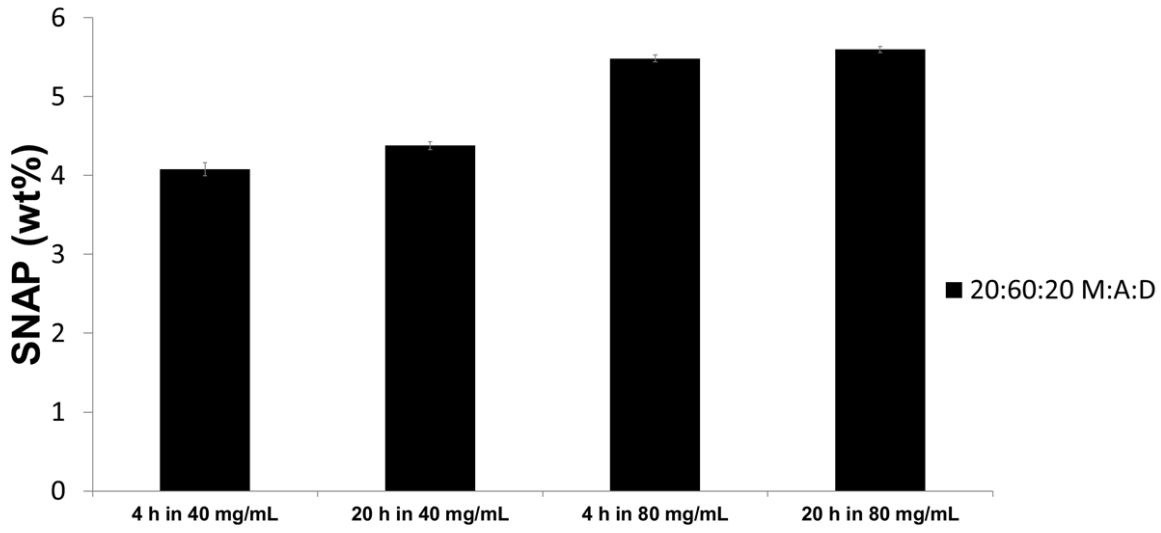

Figure 4. Weight percentage (wt %) of SNAP impregnated into Tygon® PVC after 4 and 20 h solvent-impregnation using a 20:60:20 methanol:acetone:DEHP (MeOH:Ace:DEHP) containing 40 and 80 mg/mL SNAP. Data represents the mean ± SEM (n ≥ 3).

3.2 Determination of SNAP Leaching from PVC Matrix

CarboSil 2080A (CB), a triblock copolymer of polyurethane, poly(dimethylsiloxane) and polycarbonate, exhibits excellent hemocompatibility compared to other polymer systems, which makes it a strong candidate as a topcoat polymer [63, 115]. In order to investigate the effects of a CB-topcoat in terms of SNAP leaching, the SNAP-PVC and SNAP-PVC-CB was immersed in PBS buffer soaking solution to mimic the physiological environment. The amount of SNAP leached from each sample was quantified every 24 h over 7 d, by measuring the absorbance corresponding to SNAP (characteristic absorbance maxima at 340 nm) in PBS buffer soaking solution. The results exhibited a significant reduction in leaching among SNAP-PVC-CB samples compared to SNAP-PVC (**Fig. 5**). After 24 h soaking in PBS buffer solution at 37 °C, the SNAP-PVC-CB leached 1.22 ± 0.01 µg SNAP/mg sample compared to 1.39 ± 0.03 µg SNAP/mg sample. The %

SNAP leached after 24 h compared to total SNAP loaded was 2.1% and 2.5% total SNAP content from coated and uncoated SNAP-PVC, respectively. The reduction of SNAP leaching was more apparent after 48 h soak, when SNAP-PVC-CB and SNAP-PVC leached an additional 0.16 ± 0.27 (0.4% total SNAP content) and 0.89 ± 0.01 (1.6% total SNAP content) µg SNAP/mg sample, respectively. This data demonstrated that most SNAP leaching occurred during the first 24 h. After 7 d, the cumulative SNAP leaching observed in the SNAP-PVC-CB and SNAP-PVC was 1.66 ± 0.02 µg SNAP/mg sample (3.1%) and 4.17 ± 0.03 µg SNAP/mg sample (7.6%), respectively, which correlated to an 86% reduction of SNAP leaching compared to SNAP-PVC.

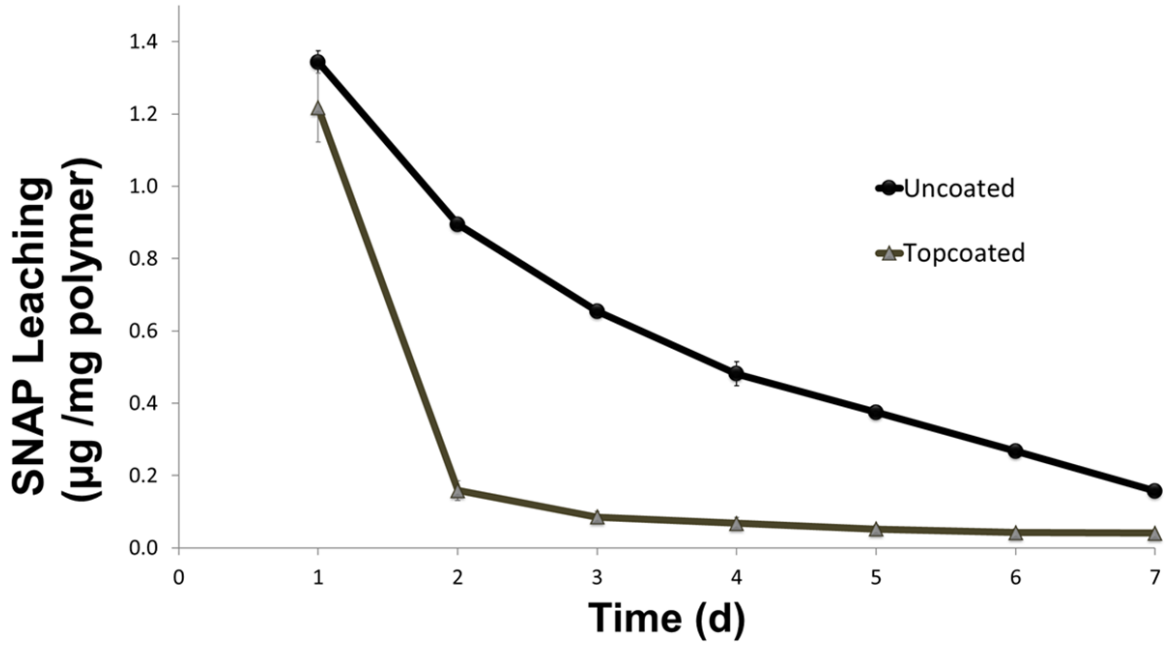

Figure 5. The leaching of SNAP from polymer samples (µg SNAP/mg sample) as measured by UV-vis under physiological conditions (pH 7.4, 10 mM PBS with 100 µM EDTA buffer at 37 °C) over a period of 7 d. Data represents the mean ± SEM (n ≥ 3).

3.3 Real Time Nitric Oxide Release Measurements of SNAP-PVC-CB

The NO release profile was investigated for two formulations of SNAP-PVC-CB prepared in the impregnation-solvent containing SNAP (40 and 80 mg/mL). Due to the reduction of SNAP leaching observed in SNAP-PVC-CB, the NO release of only SNAP-PVC-CB was characterized. The characterization of NO release was measured by a Zysense chemiluminescence NO analyzer, which is considered the gold standard methodology for NO detection due to its response time (< 1 s) and low detection limit (0.5 ppb) [63, 65-67, 78]. The NO release from SNAP-PVC-CB was continuously measured over a 24 h period (**Fig. 6**). The continuous 24 h NO release profile of each formulation of SNAP-PVC-CB (40 and 80 mg/mL SNAP) exhibited an average NO flux of 1.75 ± 0.10 x 10^{-10} mol cm^{-2} min^{-1} and 4.29 ± 0.80 x10^{-10} mol cm^{-2} min^{-1} under physiological conditions (pH 7.4 PBS buffer at 37 °C), respectively. Varying the concentration of SNAP during SNAP impregnation can effectively tune the NO release levels of the polymer while maintaining physiologically relevant levels of NO release over 24 h. Long term NO release was continued with by 80 mg/mL SNAP-PVC-CB due to the significantly greater NO release.

In order to establish the life-time of NO release, SNAP-PVC-CB prepared with 80 mg/mL SNAP was measured until the NO reservoir was depleted (**Fig. 7**). The high NO flux of 4.06 ± 0.87 x 10^{-10} mol cm^{-2} min^{-1} was observed on day zero**,** which may correlate to greatest SNAP leaching (**Fig. 5**). The NO flux reaches a minimum after 1 d showing NO flux of 0.72 ± 0.14 x 10^{-10} mol cm^{-2} min^{-1}, due to the slow diffusion of water into the polymer matrix and solvation of SNAP (**Fig. 7**). After 1 d, the NO release gradually increases over time, until it reaches a second maximum NO flux of 2.51 ± 0.63 x 10^{-10} mol

cm^{-2} min^{-1} on day seven. Hereafter, the NO flux gradually decreases to a NO flux of 0.70 ± 0.14 x 10^{-10} mol cm^{-2} min^{-1} as seen on Day 14. After 16 d, the NO reservoir is depleted. Overall, the NO release of the SNAP-PVC-CB in these studies remained within physiological levels throughout the 14 d. Wo et al. reported a similar NO release profile, seen in SNAP-CB, where NO release decreased on Day 1, reached a second maximum after 7 d, then decreased until complete NO depletion [66].

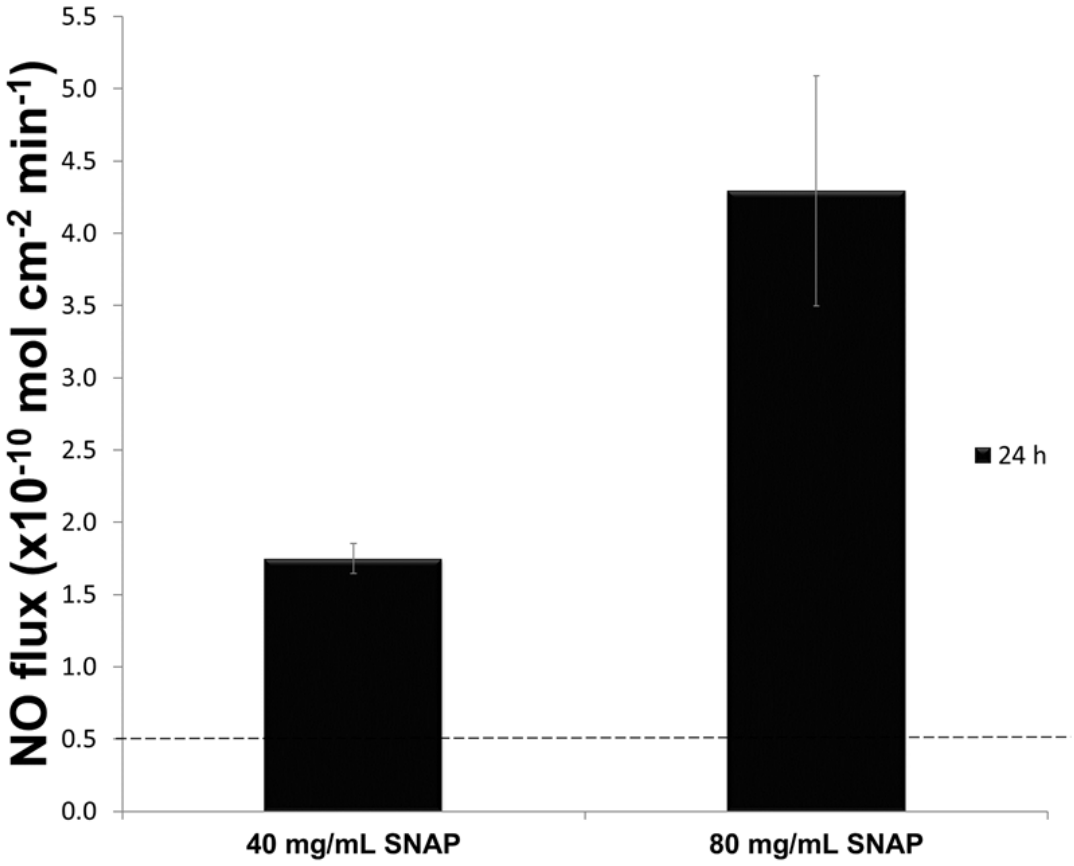

Figure 6. Release of NO from SNAP-PVC-CB (40 and 80 mg/mL SNAP) under physiological conditions (in pH 7.4, 10 mM PBS with 100 µM EDTA buffer at 37 °C) for 24 h as measured by chemiluminescence. Data Represents the mean ± SEM (n ≥ 3). Dashed line represents lower range of normal endothelium NO release.

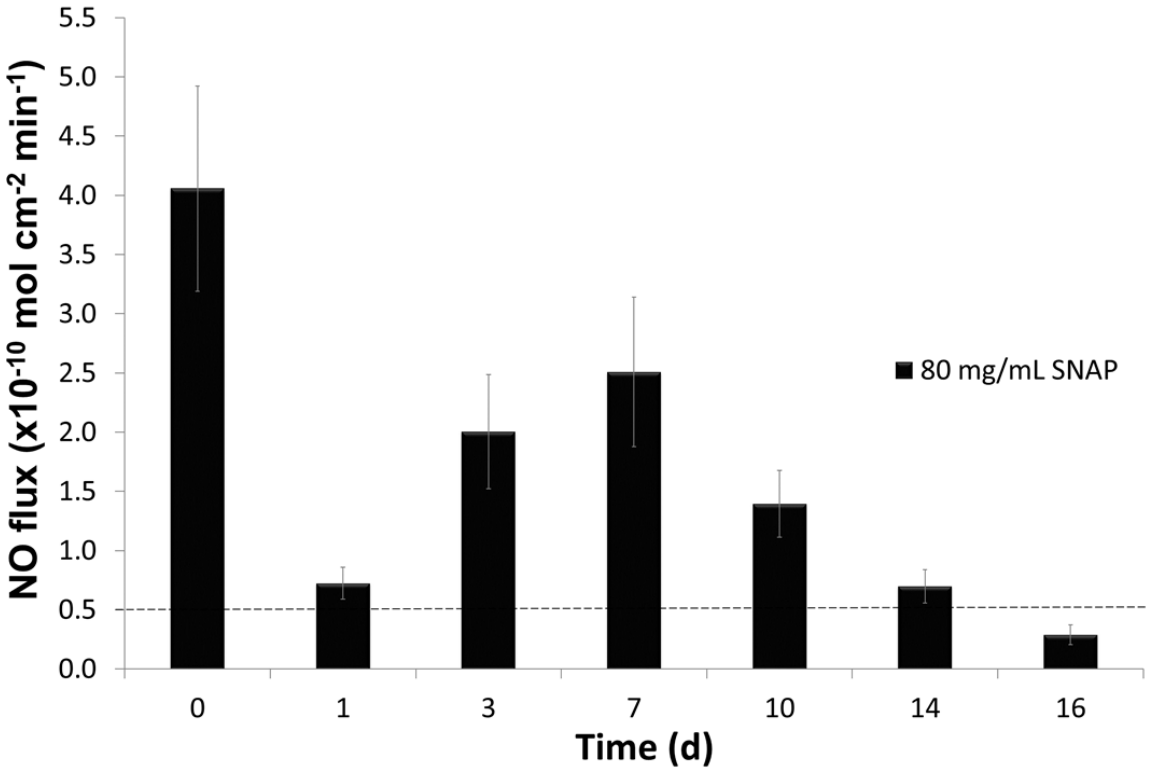

Figure 7. Release of NO from SNAP-PVC-CB (80 mg/mL SNAP) under physiological conditions (in pH 7.4, 10 mM PBS buffer with 100 µM EDTA buffer at 37 °C) for 16 days as measured by chemiluminescence. Data represents the mean ± SEM (n ≥ 3). Dashed line represents lower range of normal endothelium NO release.

3.4 Determination of Mechanical and Surface Properties

It is well known that the mechanical properties of a device are critical for device function. The addition of therapeutics or other additives into polymer matrices may significantly alter the mechanical properties, such as tensile strength. In order to maintain proper device function, an appropriate formulation of impregnation-solvent is critical for maintaining the original mechanical properties of PVC. The ultimate tensile strength (UTS) and % elongation of original PVC, PVC-solvent (without SNAP), and SNAP-PVC (80 mg/mL) were evaluated for changes to mechanical properties. The original PVC was found to have an UTS of 13.4 ± 1.6 MPa, PVC-solvent had an UTS of 12.4 ± 1.1 MPa,

and SNAP-PVC had an UTS of 11.8 ± 1.0 MPa (**Fig. 8A**). The % elongation of original PVC, PVC-solvent, and SNAP-PVC were 473.6 ± 42.6 %, 518.9 ± 21.2 %, and 458.0 ± 31.5 %, respectively (**Fig. 8B**). The results demonstrated that the use of 20:60:20 MeOH:Ace:DEHP impregnation-solvent with SNAP (80 mg/mL) provided the optimal concentration of plasticizer and exhibited no significant change to the mechanical properties compared to original PVC.

The surface properties of medical devices are also key factors in device performance. The surface of each sample was imaged using SEM to observe the topographical changes that occur after SNAP-impregnation and application of topcoat (**Fig. 9**). The original PVC exhibits a uniformly smooth surface**.** After impregnation with SNAP, no significant changes to the surface characteristics were observed**.** Similarly, after impregnation with SNAP and the addition of the CB-topcoat, the samples exhibit a uniformly smooth surface**.** The addition of the CB-topcoat was key to reduce SNAP leaching, prolong NO release, and maintain a smooth surface topography after SNAP impregnation, which are all important factors for the clinical efficacy of the device.

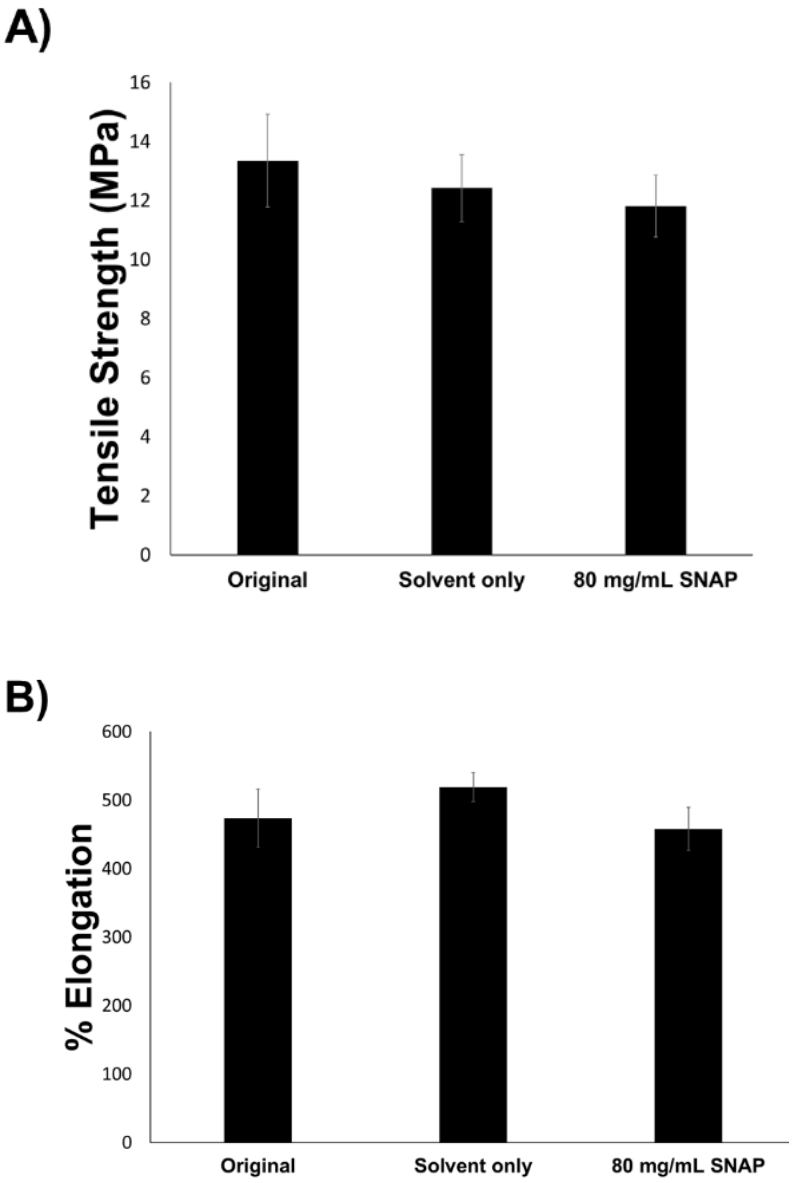

Figure 8. Ultimate Tensile Strength (A) and % elongation (B) of PVC after 4 h solvent-swelling impregnation (80 mg/mL SNAP) as measured by an Instron tensile strength analyzer. Data represents the mean ± SEM (n = 4).

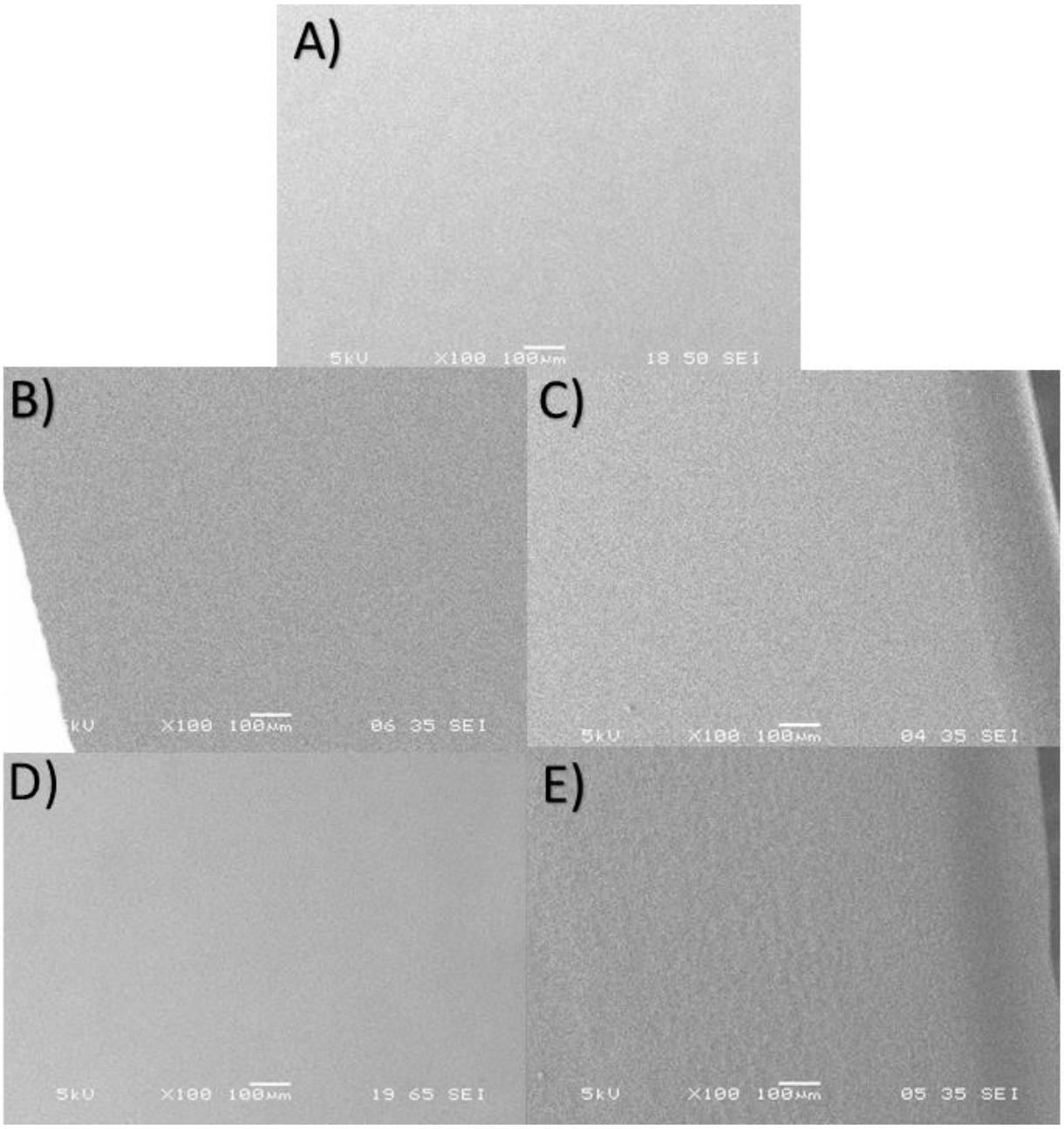

Figure 9. Representative scanning electron microscopy images of original PVC (A), PVC-Solvent (B), PVC-CB (C) SNAP-PVC (D) and SNAP-PVC-CB (E). Images demonstrate that the process of SNAP impregnation and addition of CB-topcoat do not significantly alter the surface topography and a smooth surface is maintained compared to original PVC.

3.5 Stability of SNAP-PVC-CB During EO Sterilization and Storage

Device integrity during sterilization and storage are key components of establishing clinical efficacy. Sterilization of medical devices is a necessary clinical practice to lower the burden of HAIs [116]. Ethylene oxide sterilization (EO) is a common sterilization method for heat and moisture sensitive materials and was used as a model sterilization method for SNAP-PVC-CB [116]. In brief, sensitive medical devices are exposed to EO gas for 16 h at 30-60 °C, which react with bacteria and readily denature proteins and DNA leading to cell death [117]. After undergoing EO sterilization, the SNAP-PVC-CB was dissolved in DMAc and SNAP content was measured using UV-vis as described in the methods. The SNAP-PVC-CB retained 92.6 ± 3.1 % initial SNAP content, as described by Equation 2.2. (**Fig. 10A**).

To evaluate the stability of SNAP-PVC-CB during potential shipping or storage conditions, SNAP-PVC-CB were packaged with desiccant to emulate dry conditions and stored at various temperature in the dark for up to 1 month, as described in the methods. The percent SNAP remaining in each sample was determined by dissolving the samples in DMAc and quantitating the SNAP concentration using UV-Vis spectroscopy at different storage durations as described by Equation 2.2. The SNAP-PVC-CB stored at –20 °C maintained 93.3 ± 1.7 % of initial SNAP content, compared to 92.8 ± 2.6 % when stored at 23 °C and 82.0 ± 3.1 % when stored at 37 °C for 1 month (**Fig. 10B**).

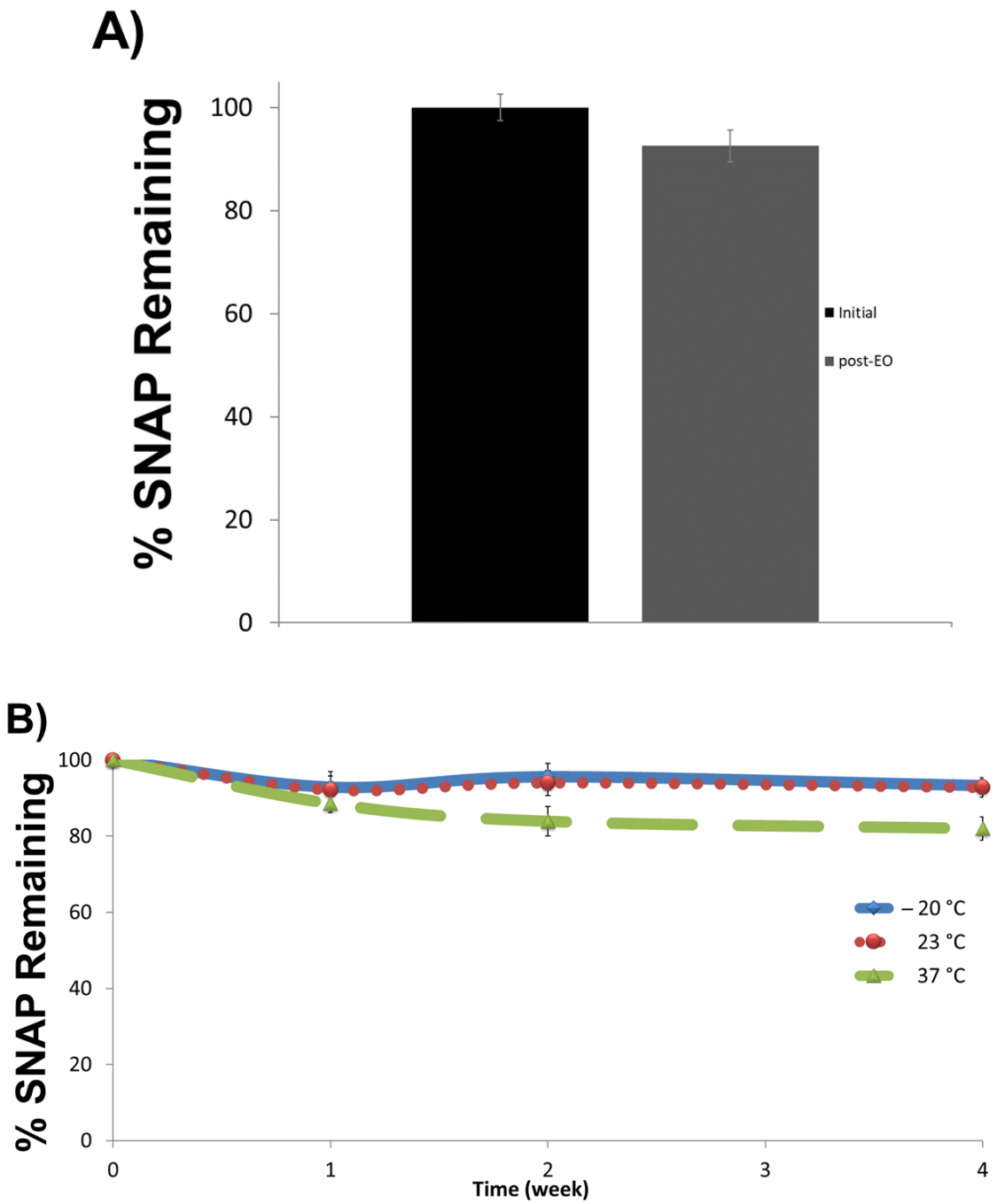

Figure 10. Evaluation of % SNAP remaining after exposure to ethylene oxide sterilization (A) and 1-month storage (B) determined by dissolving SNAP-PVC-CB in DMAc and recording the UV-Vis spectra. Data represents the mean ± SEM (n ≥3).

3.6 Antibacterial Activity of SNAP-PVC-CB

Bacterial colonization on the surface of medical devices causes ca. 86-96% of HAIs [4]. *E. coli* and *S. aureus* are among the highly infectious group known as ESKAPE pathogens, which cause for the majority of HAIs [2]. In order to demonstrate the clinical efficacy of SNAP-PVC-CB, the antibacterial properties were determined by evaluation of *E. coli* and *S. aureus* colonization on the material surface after 24 h exposure to NO and compared with a control PVC-CB. The bacterial colonies were counted and expressed as CFU/cm^2 (**Fig. 11**). A ca. 1 log reduction of *S. aureus* (97.5% reduction) and *E. coli* (92.8% reduction) colonization on SNAP-PVC-CB as compared to CB-control was observed. The reduction of *S. aureus* and *E. coli* demonstrated that the NO release from SNAP-PVC-CB provided significant antibacterial activity. These results are similar to previous reports which characterized SNAP-CB and SNAP-E2A polymers which release similar levels of NO over a 24 h antibacterial assay [89, 118]. The reduction of bacterial colonization on SNAP-PVC-CB after 24 h showed promising results and future long-term antibacterial activity may be evaluated over the entire 14 d NO release period of the SNAP-PVC-CB polymers to demonstrate potential clinical antimicrobial applications of PVC devices.

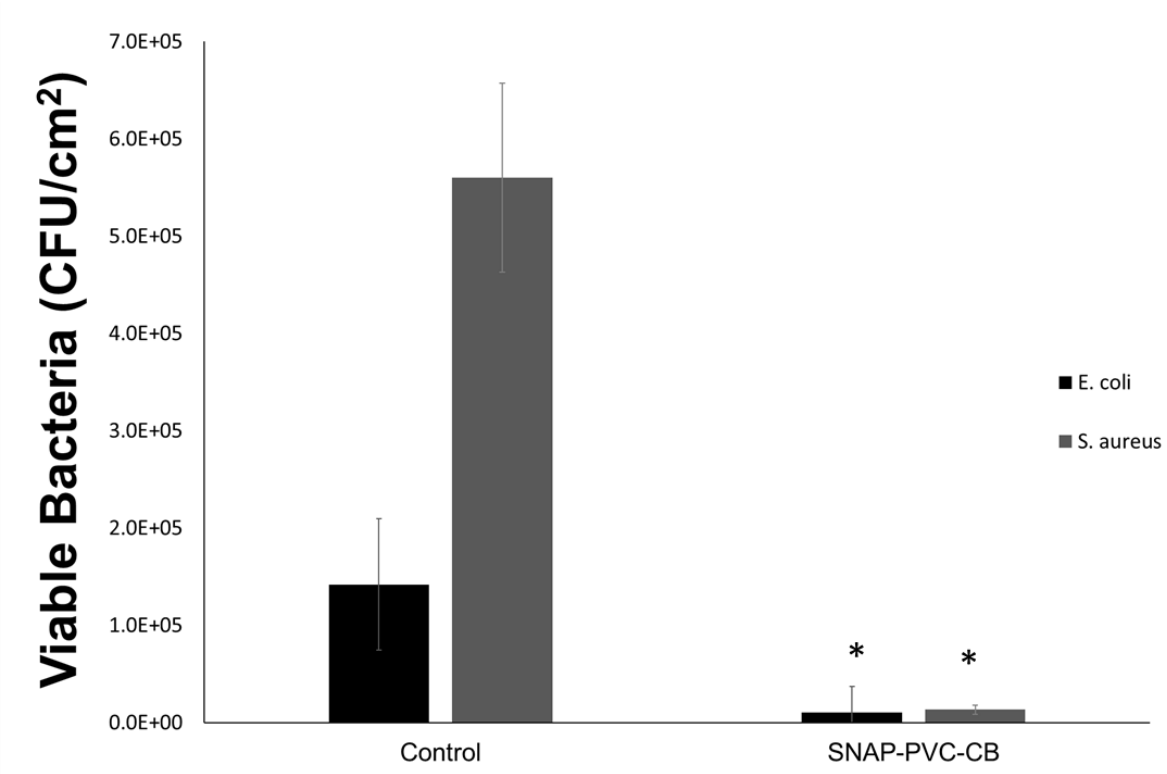

Figure 11. Bacterial adhesion data showing the CFU per cm^2 of S. aureus and E. coli after 24 h of incubation with SNAP-PVC-CB and control polymers. Data represents the mean ± SEM (n = 3).

CHAPTER FOUR: DISCCUSION

4.1 Evaluation of SNAP Impregnation of PVC

. The solvent-swelling impregnation method presented in this work provided a solution to impregnate SNAP into medical grade PVC while maintaining the plasticizer content. The swelling solvent mixture of 20:60:20 methanol: acetone: DEHP provided a high solubility of SNAP, rapid swelling and drying, and control over the mechanical properties, respectively. Rapid impregnation was confirmed by varying the duration of swelling from 4 to 20 h which did not exhibit a significant increase in SNAP loading. Swelling PVC for 4 h with 40 and 80 mg/mL SNAP solution resulted in 4.08 ± 0.08 and 5.48 ± 0.05 wt % SNAP, respectively (**Fig. 4)**. Control over SNAP loading was achieved by varying the SNAP concentration in solution (40 and 80 mg/mL). These findings are consistent with previously reported SNAP impregnated CB (2.5 – 15.7 wt% loaded with 5.5 – 120 mg/mL SNAP) which controlled the SNAP loading by varying SNAP concentration in the impregnation solvent [66]. Additionally, SNAP impregnation of silicone was successful to create NO releasing silicone devices, with ca. 1 wt % SNAP content [79]. SNAP is highly soluble in methanol so increasing the methanol content may allow for increased SNAP loading, but alteration of the mixture ratios may affect the mechanical properties and time of swelling/drying [66]. Overall, this solvent-swelling impregnation mixture provided rapid SNAP impregnation into plasticized PVC.

4.2 Evaluation of SNAP Leaching Behavior

Leaching of SNAP from PVC can cause nonlocalized NO release and have unwanted effects. A dramatic reduction of leaching can significantly improve the lifetime

of NO release and mitigate potential downstream side effects that can be induced by NO such as hypotension [119, 120]. The addition of hydrophobic polymeric topcoats (CB, silicone, E2As, PVC) with low water uptake can act as a barrier between the PVC-solution interface, which impose resistance against diffusion of SNAP out of the PVC matrix [63, 66, 67, 75]. In this work, quantitation of SNAP leaching in PBS buffer over 7 d at 37 °C from SNAP-PVC and SNAP-PVC with CB topcoat (SNAP-PVC-CB), was measured by UV-vis spectroscopy at 340 nm. The SNAP-PVC leached 2.1% SNAP content over 24 h. After 7 d, the cumulative SNAP leaching observed 1.66 ± 0.02 µg SNAP/mg sample (3.1%) and 4.17 ± 0.03 µg SNAP/mg sample (7.6%), from SNAP-PVC-CB and SNAP-PVC, respectively, which correlated to an 86% reduction of SNAP leaching compared to SNAP-PVC **(Fig. 5)**. The SNAP leaching is considerably low compared to total SNAP loading. The addition of the CB topcoat significantly reduced SNAP leaching from PVC. These findings are consistent with previous work, which demonstrated that the addition of a topcoat to SNAP-E2As and SNAP-CB polymer composites significantly suppressed SNAP leaching from the polymer matrix to nanomolar concentrations [63, 66, 67]. Joslin et al. focused on the characterization of leaching behavior of GSNO-PVC. Less than 0.25% GSNO leached from the GSNO-PVC with PVC topcoat over 24 h, which was attributed to GSNO localized near the surface and was quickly washed away. The findings demonstrated that NO release from GSNO-PVC was localized within the bulk to the PVC [75].

4.3 Evaluation of Real-time NO Release Profiles

The normal endothelium is reported to release NO at a flux range of 0.5 – 4 x 10^{-10} mol cm^{-2} min^{-1}, thus fabrication of NO releasing materials within this range will best mimic endogenous NO release [54]. Several reports have demonstrated that NO release at physiological levels prevent platelet activation, clot formation, and exhibit antimicrobial properties [43, 65, 78, 79, 92]. It is important that the NO release profile of SNAP-PVC-CB exhibits physiological levels of NO release under physiological conditions. The data demonstrated physiological levels of NO release for up to 14 d (**Fig. 7**). Day 0 released 4.06 ± 0.87 x 10^{-10} mol cm^{-2} min^{-1}. The NO flux reaches a minimum after 1 d showing NO flux of 0.72 ± 0.14 x 10^{-10} mol cm^{-2} min^{-1}, due to the slow diffusion of water into the polymer matrix and solvation of SNAP. After 1 d, the NO release reaches a second maximum NO flux of 2.51 ± 0.63 x 10^{-10} mol cm^{-2} min^{-1} on Day 7. Hereafter, the NO flux gradually decreases to a NO flux of 0.70 ± 0.14 x 10^{-10} mol cm^{-2} min^{-1} as seen on Day 14.

In terms of SNAP-polymers, the NO release profile of SNAP-PVC best resembles SNAP-CB presented by Wo et al. SNAP-CB had largest NO release on Day 0 (~ 4.0 x 10^{-10} mol cm^{-2} min^{-1}), NO release decreased to lowest flux on Day 2 (~ 1 x 10^{-10} mol cm^{-2} min^{-1}), gradually increased to second maximum NO flux on Day 8, then gradually decreases to depletion after 14 days [63]. Wo et al. hypothesized that exceeding the SNAP solubility in polymer matrices is the key factor for enhanced SNAP storage stability and extended NO release. Concentrations exceeding 3.4 – 4.0 wt% SNAP in CB were needed to induce SNAP crystallization [63]. Concentrations of SNAP, which exceed this solubility limit in a polymer system, form a polymer crystal composite that exhibits greater stability via intramolecular hydrogen bonding among SNAP molecules [63]. In addition,

viscous polymer matrices further stabilize the NO donor by providing a higher propensity for NO recombination by restricting the diffusion of NO through the polymer, known as cage recombination effect [67, 104, 105, 107, 121]. A representative schematic (**Fig. 12.)** demonstrates the NO release behavior of SNAP-PVC in relation to the SNAP-crystal composite theory [63, 66].

Based on this phenomenon, the high levels of NO release seen on Day 0 can be attributed to initial leaching of SNAP and readily soluble SNAP in the PVC matrix. The SNAP nearest the surface region is more likely to be dissolved due to the increased presence of water content compared to water content within the bulk of PVC and readily releases NO [63, 66]. As this soluble SNAP reservoir is depleted, NO generation begins to slow to its lowest observed NO flux until water can penetrate deeper into the material and promote dissolution of crystalized SNAP. This phenomenon is exemplified by a dramatic reduction of NO flux ($0.72 \pm 0.14 \times 10^{-10}$ mol cm^{-2} min^{-1}) seen on Day one (**Fig 7**). After Day one, the SNAP gradually dissolves over time and NO flux increases until it reaches a second maximum on Day seven. Hereafter, the SNAP can be considered to be mostly dissolved, then NO release gradually decreases until the NO reservoir is depleted and NO flux falls below the 0.5×10^{-10} mol cm^{-2} min^{-1} minimum value on Day 16.

In comparison, other NO releasing PVC and SNAP-polymers have been reported however, these methods do not address the issues concerning manufacturing of NO-polymers, extended NO release, or antibacterial activity, [53, 65, 72, 73, 75, 81, 89, 110, 122], excluding the SNAP impregnation methods reported by Brisbois et al and Wo et al

[66, 79]. A brief comparison of various SNAP-polymers and NO releasing-PVC are presented in Table 1.

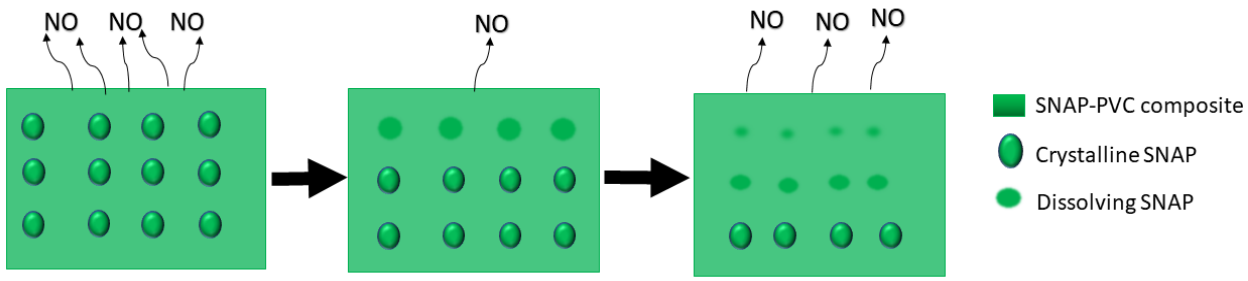

Figure 12. Representation of SNAP-crystal composite theory owing toward the extended NO release behavior and enhanced SNAP stability during storage.

Table 1. Nitric oxide releasing PVC and SNAP-polymers

NO-polymer	NO Flux (x 10^{-10} mol cm^{-2} min^{-1}) Day 0	Long-term NO release test	Clinical Significance	Method of Fabrication	Reference
SNAP-PVC	4 flux	14 d	1 log reduction of *E. coli* and *S. aureus*	Solvent impregnation	Present Work
SNAP-silicone	1 flux	4 h	67% reduction of thrombus formation	Solvent impregnation	[78]
SNAP-CB	4 flux	14 d	2 log reduction of *S. Epidermis* and *P. aeruginosa*	Solvent impregnation	[66]
SNAP-PVC	8 flux	40 d	Light mediated NO release allows for controlled range of 1 – 35 NO flux with prolonged NO release	Covalent functionalization	[72]
SNAP-silicone	9 flux	125 d	Reduced *S. aureus* colonization by 4, 3, and 2 log after 3, 14, and 28 d, respectively	Covalent functionalization	[81]
SNAP-CB	10 flux	30 d	2 log reduction of *P. mirabalis* and *P. aeruginosa*	Film casting	[65]
SNAP-CB- SP60D60	2 flux	24 h	1 log reduction of *S. aureus*	Film casting	[89]
GSNO-PVC	0.6 flux	5 h	GSNO leaching < 0.25 %	Film casting	[75]
H_2O(v) Plasma treated GSNO-PVC	1 flux	3 d	8 log reduction of *S. aureus*	Film casting	[110]
NONOate-PVC (Norel-b)	15 flux	6 h	Platelet activation < 50 %	Film casting	[73]
NONOate-PVC (diazeniumdiolated dibutylhexanediamine)	14 flux	5 h	Platelet activation < 50 %	Film casting	[53]
NONOate-PVC (diazeniumdiolated dibutylhexanediamine)	20 flux	14 d	Platelet activation < 20 % Thrombus formation reduced by 77 %	Film casting	[122]

4.4 Evaluation of Mechanical Properties

It is important to evaluate the mechanical properties of SNAP-PVC to evaluate the effect the SNAP (80 mg/mL SNAP) impregnation method (20:60:20 methanol:acetone:DEHP) had on the mechanical properties compared to original medical grade PVC. In the case of plasticized PVC, a plasticizer (DEHP) was used to maintain the mechanical properties of SNAP-PVC-CB similar to original PVC. Using a tensile strength analyzer to measure tensile strength and % elongation, the tensile strength measurements confirmed the UTS of original PVC (13.4 ± 1.6 MPa) and SNAP-PVC (11.8 ± 1.0 MPa) (**Fig. 8A**). The % elongation of original PVC and SNAP-PVC were 473.6 ± 42.6 % and 458.0 ± 31.5 %, respectively (**Fig. 8B**). These results demonstrate the SNAP impregnation of PVC had no significant effect on the mechanical properties. The wt% SNAP loaded can significantly affect the mechanical properties. Goudie et al. demonstrated that varying SNAP loading (5 –15 wt%) significantly decreased the tensile strength of E2As polyurethane [87]. Thus, the formulation of SNAP impregnation solution (80 mg/mL SNAP 20:60:20 (methanol:acetone:DEHP) was optimal to maintain the mechanical properties when PVC was impregnated with ~6 wt% SNAP. Optimization of the SNAP impregnation solvent would be needed to perform this method on other medical grade PVC as NO release and mechanical properties may differ amongst clinical applications.

4.5 Evaluation of Surface Properties

It has been widely reported that characteristics such as roughness wettability, and surface chemistry can lead to higher amounts of protein adsorption, which facilitate

bacterial adhesion and hinder biocompatibility [30, 123-127]. Therefore, it is imperative toward maintaining device performance that SNAP-impregnation has no significant changes to the surface topography. Scanning electron microscopy images recorded at 100x magnification showed no observable changes to the surface of SNAP-PVC compared to PVC (**Fig. 9**). These results are consistent with a previous report that showed SEM images to demonstrate the SNAP impregnation of silicone had no significant changes to the surface of the device [79]. Additionally, the surface properties of SNAP-E2As polymer was analyzed with atomic force microscopy and contact angle measurements. The results indicated that incorporation of SNAP into E2As polymer had no effect on surface roughness or contact angle of SNAP-E2As (11.65 ± 1.08 nm and 110.7 ± 0.47 °) compared to control (11.04 ± 1.97 nm and 111.7 ± 3.30 °) [87].

4.6 Evaluation of SNAP Stability After Ethylene Oxide Sterilization

The stability of SNAP-PVC-CB under sterilization is paramount for demonstrating their clinical viability. Hospitals often have ethylene oxide sterilization facilities to sterilize heat and moisture sensitive devices. Ethylene oxide sterilization was chosen for this work because it is a useful technique for sterilizing SNAP-PVC-CB since it is both heat and moisture sensitive. The wt% SNAP remaining in SNAP-PVC was determined after expose to EO gas for 16 h at 54 °C. The data demonstrated that SNAP integrity was maintain after ethylene oxide sterilization (92.6 ± 3.1 % initial SNAP content) (**Fig.10A**). The stability of the SNAP-PVC-CB can be attributed to the enhance stability of SNAP crystals (as depicted in **Fig. 12**). The intramolecular hydrogen bonding exhibited by SNAP crystals provide enhance stability which prevented NO release during EO

sterilization. These results are similar to two reports that performed EO sterilization on SNAP-E2As and demonstrated SNAP stability was maintained (88.8 ± 1.7 % and 89.4 ± 2.0% initial SNAP content) [87, 128]. In addition, SNAP-CB retained 91.8 ± 3.2 %, SNAP-E5-325 retained 82.7 ± 3.8 %, and SNAP-silicone retained 78.7 ± 3.1 % [63]. The compilation of reports demonstrates that PVC, E2As, and CB are excellent materials for enhancement of SNAP stability.

4.7 Evaluation of Storage Stability

In addition to sterilization, it is important to demonstrate the stability of SNAP-PVC-CB under potential storage conditions to demonstrate that this material does not need special storage conditions to be clinically viable. The SNAP-PVC-CB was stored under dry conditions at various temperature (–20 °C, 23 °C, and 37 °C) for 1 month and the wt% SNAP remaining was measure using UV-Vis spectrometry. When stored at –20 °C, 23 °C, and 37 °C, SNAP-PVC-CB maintained 93.3 ± 1.7 %, 92.8 ± 2.6 %, and 82.0 ± 3.1 % of initial SNAP content (**Fig. 10B**). Similar reports of enhanced SNAP stability during storage were seen in SNAP-E2As, SNAP-CB, SNAP-silicone and SNAP-E5-325 [63, 67, 87]. Brisbois et al. reported SNAP-E2As remained stable after 2 months storage at –20 °C, 23 °C, and 37 °C (96 %, 89 %, and 82.0 % [67]. Goudie et al. further confirmed that SNAP-E2As stored for 1 – 6 months at 23 °C retained 88.4 ± 2.7 % and 87.1 ± 2.8% SNAP content, respectively [87]. However, SNAP-E2As needed higher SNAP concentrations (≥ 10.0 wt%) to exhibit the enhanced SNAP stability [87]. Wo et al. reported SNAP-CB, SNAP-silicone, and SNAP-E5-325 retained 88.5 ± 4.3 %, 86.8 ± 4.9 %, and 68.3 ± 3.2 % SNAP content, respectively, after 8 months of storage at 37 ° C. The

SNAP-crystal composite theory (depicted in **Fig. 12**) was demonstrated by enhanced stability of SNAP observed in PVC. The results of this work and other studies demonstrate that incorporating SNAP into polymer matrices such as PVC, silicone, CB, E2As and E5-325 polyurethane provide a significant enhancement to SNAP stability which allow for long-term storage of SNAP-polymers.

4.8 Evaluation of Antibacterial Activity

The natural response to infection is the activation of neutrophils and macrophages which phagocytose bacteria and release nitric oxide as a killing mechanism [48, 49, 55-59]. The natural antibacterial activity of NO holds great potential for the fabrication of antibacterial materials with improved biocompatibility. The antibacterial activity of SNAP-PVC-CB was evaluated by measuring the bacterial colonization of *S. aureus* (gram-positive) and *E. coli* (gram-negative) on the surface of the material after incubation for 24 h at 37 °C. Bacterial colonization was significantly reduced on SNAP-PVC-CB compared to PVC (1 log reduction) (**Fig. 11**). The adhesion of *S. aureus onto* SNAP-PVC-CB was reduced by 97.5% and *E. coli* was reduced by 92.8%. The broad-spectrum antibacterial activity is advantageous because SNAP-PVC-CB can be applied to a variety of clinical applications to prevent HAIs caused by multiple pathogens. Numerous reports have demonstrated the significant antibacterial activity (1-8 log reduction) of NO releasing polymers which exhibit varying NO release rates on Day 0 ($2 – 15 \times 10^{-10}$ mol cm^{-2} min^{-1}), addition of antifouling coatings such as zwitterions, hydrophilic topcoats, or surface chemistry modifications [39, 43, 63, 66, 79, 88, 89, 110, 128-130]. The impregnation of SNAP into post-fabricated PVC devices addressed the shortcomings of previous literature

by overcoming the challenges presented by traditional manufacturing, which would destroy SNAP, and provided similar antibacterial efficacy without large bursts of NO release or need for additional surface modifications.

CHAPTER FIVE: CONCLUSION

In this study, a novel solvent-swelling-impregnation method was developed to impregnate plasticized PVC with the NO donor molecule SNAP. The impregnation solvent consisted of a mixture of methanol, acetone, and DEHP plasticizer in a ratio of 20:60:20, respectively. A topcoat of CB was applied and reduced the amount of SNAP leaching of SNAP-PVC-CB compared to SNAP-PVC. No significant effects on surface topography or mechanical properties were observed after SNAP-impregnation and application of CB-topcoat. The wt % SNAP and NO flux from SNAP-PVC-CB could be effectively tuned by changing the concentration of SNAP in the impregnation-solvent and demonstrated sustained NO release within physiological levels for at least 14 d. Evaluation of SNAP stability during sterilization and 1-month storage stability demonstrated excellent retention of SNAP within the PVC matrix, which is beneficial for future clinical applications. The SNAP-PVC-CB showed significant reduction (ca. 1 log reduction) of *S. aureus* and *E. coli* after 24 h, which may prevent the onset of HAIs as related to PVC based medical devices. This novel SNAP-impregnation method can overcome the manufacturing challenges presented by typical high temperature extrusion processing and provide the benefit of NO release to the vast variety of post-fabricated PVC medical devices (catheters, endotracheal tubes, ECC, etc.) to improve biocompatibility, provide antimicrobial activity, and help improve patient care.

5.1 Future Directions

Upon continuation of this work, several important material properties and clinical viability must be evaluated. Fabrication with plasticizers other than DEHP, such as dioctyl sebacate, may have effects of SNAP crystallization and NO release kinetics [122]. Evaluation of this would elucidate the versatility of the SNAP-impregnation of various PVC commercial medical devices, such as extracorporeal circuitry, endotracheal tubes, catheters, drainage/infusion tubing, blood bags, etc. This information may provide control over extended NO release and further enhance the storage stability of SNAP-PVC. Evaluation of SNAP-PVC via powder X-ray diffraction and Ramen spectroscopy can be used to determine the solubility of SNAP in plasticized PVC and optical microscopy can be used to confirm the formation of SNAP crystals in the PVC matrix similar to previous SNAP-crystal composites [63, 65]. This information would confirm the existence of SNAP crystals in PVC and can be correlated with the enhance stability during sterilization, storage, and long-term NO release seen in SNAP-polymers [63, 66, 67, 79, 87]. Additionally, the data would provide insight into the factors affecting crystallization and indicate which fabrication factors are critical to provide significant improvement to NO release properties. Long-term antibacterial testing using the CDC bioreactor to evaluate antibiofilm activity as well as Live Dead staining to evaluate if the NO release of SNAP-PVC is biocidal or biostatic [63, 65, 128, 129]. The evaluation of hemocompatibility properties such as antithrombic activity, hemolysis assay, and platelet aggregation assay will provide critical data to demonstrate the efficacy of *in vivo* evaluations of SNAP-PVC medical devices in an animal model and ultimately clinical applications [43, 63, 66, 67, 79, 129].

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