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Potent, Broad-Spectrum Antimicrobial Effects of *S*-Nitroso-*N*-acetylpenicillamine-Impregnated Nitric Oxide-Releasing Latex Urinary Catheters

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Abstract

Although numerous prevention and intervention techniques have been developed to counteract catheter-associated urinary tract infections (CAUTIs), urinary catheters remain one of the most common sources of hospital-acquired infections. Nitric oxide (NO), a gaseous free radical responsible for regulating many physiological functions in the body, has gained immense popularity due to its potent, broad-spectrum antimicrobial activity, which is capable of combating medical device-associated infections. In this work, a straightforward solvent-swelling method was used to load the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) into commercial latex catheters (SNAP-UCs) for the first time. The effects of swelling catheters with different concentrations of SNAP solutions (25–125 mg/mL SNAP in tetrahydrofuran (THF)) were studied by measuring the NO release kinetics, SNAP loading, and SNAP leaching. SNAP-UCs impregnated with a 50 mg/mL SNAP-THF solution were found to maximize the amount of SNAP loaded into the latex (0.115 ± 0.009 mg SNAP/mg catheter) and showed physiological levels of NO release ($>2 \times 10^{-10}$ mol min^{-1} cm^{-2}) over 7 days and minimal SNAP leaching (<2%). SNAP-UCs showed impressive in vitro contact-based and diffusible antimicrobial efficacy

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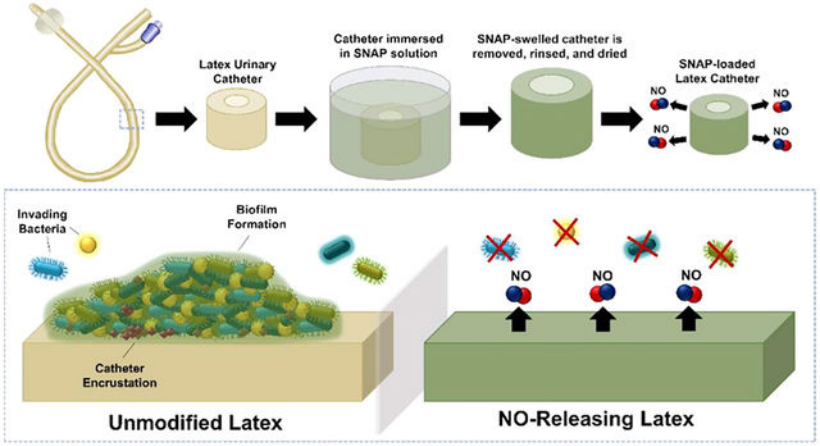
against three CAUTI-associated pathogens, reducing the viability of adhered and planktonic *Escherichia coli*, *Proteus mirabilis*, and *Staphylococcus aureus* by ~98.0 to 99.1% (adhered) and 86.3–96.3% (planktonic) compared to control latex catheters. In vitro cytotoxicity against 3T3 mouse fibroblasts using a CCK-8 assay showed that SNAP-UCs were noncytotoxic (>90% viability). In summary, SNAP-UCs show stable, noncytotoxic NO release characteristics capable of potent, broad-spectrum antimicrobial activity, demonstrating great potential for reducing the devastating effects associated with CAUTIs.

Graphical Abstract



Keywords

antimicrobial; nitric oxide; controlled release; medical device; urinary catheter

1. INTRODUCTION

Urinary tract infections are the most common type of nosocomial infection, composing approximately one-third of all infections that take place in acute-care hospitals.[1] In a surveillance study examining nearly 200,000 intensive care unit (ICU) patients, the National Nosocomial Infections Surveillance System (NNIS) found that 95% of urinary tract infections (UTIs) were associated with urinary catheters.[2] For catheterized patients, the risk of developing a UTI increases 3–7% per day, and for patients that require catheterization for ~7 days, the prevalence of catheter-associated urinary tract infections (CAUTIs) is approximately 25%, limiting long-term catheterization applications.[3–5] The National Opinion Research Center at the University of Chicago (NORC) has estimated that the average additional cost related to CAUTIs is nearly $14,000 per case and approximated that such infections lead to 36 excess deaths per 1000 in-hospital CAUTI events.[6] Hospital and healthcare prevention programs devised by the Centers for Disease Control and Prevention (CDC) have found some success in reducing the number of infections related to healthcare-acquired infections including CAUTIs in non-ICU settings, but the infections in ICU settings remained unchanged.[7] Due to the devastating consequences associated with CAUTIs, once an infection is detected, a combination of antimicrobial therapies and catheter

removal is often required.[8] However, the emergence of antibiotic resistance coupled with the complicated, often polymicrobial composition of CAUTIs has made the eradication of biofilms and treatment of infection difficult.[9] Microbial biofilms readily form on the surfaces of devices, significantly increasing antibiotic and host immune resistance.[10] The shortage of novel antibiotics, inappropriate overuse, and decreased drug efficacy shortly after market introduction have led to a demand for new antimicrobial and infection mitigation strategies.

In addition to infection, indwelling urinary catheters are also plagued by encrustation. The presence of urease-producing bacteria such as *Proteus mirabilis (P. mirabilis*) catalyzes the hydrolysis of urea, generating ammonia and shifting the pH of the urine, causing magnesium and calcium phosphate to crystallize on the catheter surface.[11] Catheter encrustation prevents urine drainage, potentially leading to pyelonephritis, septicemia, and endotoxic shock.[12] Encrustation has been largely linked to CAUTI development, and therefore, methods to prevent the colonization of such bacteria are needed.[13]

Despite frequent complications, urinary catheters remain the most common indwelling medical device, used in approximately 25% of the hospitalized patients in the United States.[14,15] Like the original foley catheter, the majority of urinary catheters manufactured currently are latex-based due to easy processing and low cost.[14] However, latex-based urinary catheters have been associated with a number of complications, including increased risk of infection and catheter encrustation.[16] In response, silicone-based catheters were introduced and were found to have some improvement in decreasing the risk of encrustation, infection, and urethritis.[16,17] However, over half a century later, urinary catheters are still associated with high incidences of infection. Beginning at the turn of the century, several antimicrobial surface strategies for urinary catheters have been developed to reduce the frequency of CAUTIs.[16,18] Silver-coated catheters, one of the only FDA-approved antimicrobial urinary catheters on the market, have become increasingly popular due to silver's well-established antibacterial activity.[19] However, clinical trials comparing silver-coated catheters with control catheters have generated inconsistent results.[19] Antibiotic-eluting catheters have shown some improvements in antimicrobial efficacy but suffer from antibiotic resistance and quick depletion, depreciating the bactericidal effects for long-term indwelling applications.[18,20] Recently, researchers have developed bio-inspired antimicrobial surface strategies to better combat hospital-acquired infections, and in that trend, nitric oxide (NO)-based polymer surfaces have shown great promise.

Nitric oxide is an endogenous free radical that is responsible for many vital functions in the body. In addition to its roles in the cardiovascular and nervous systems, NO is a potent antimicrobial utilized by the immune system through two dose-dependent means: (1) at low concentrations (<1 μM), NO supports the immune cell generation and activity; (2) at high concentrations (>1 μM), NO causes nitrosative and oxidative damage, resulting in protein alterations, DNA deamination and oxidative modifications, and lipid peroxidation.[21–23] To combat device-associated infection, NO-releasing materials have been synthesized by incorporating NO donors including S-nitrosothiols (RSNOs) and *N*-diazeniumdiaolates through blending, covalent immobilization, or solvent-swelling impregnation methods.[24] A unique feature of the solvent-swelling method is that it provides a straightforward

means to load NO donors into already commercially available materials.[25] *S*-Nitroso-*N*-acetyl-penicillamine (SNAP), a well-characterized, stable RSNO, has been successfully impregnated into different medical-grade polymers, including silicone,[24–28] polyvinyl chloride,[29,30] and CarboSil 2080A,[31] but has yet to be impregnated or incorporated into latex materials.

In this study, commercial latex urinary catheters were loaded with the NO donor SNAP via a simple solvent-swelling impregnation method for the first time. The effect of different concentrations of SNAP swelling solutions (25–125 mg/mL) was studied by measuring the NO release kinetics, SNAP loading, and SNAP leaching. The sterilization stability of the materials was measured after ethylene oxide treatment and autoclaving. In addition, an in-depth antimicrobial study was performed through evaluating the diffusible and contact-based antimicrobial efficacies of the optimized materials in vitro against planktonic and adhered *Escherichia coli* (*E. coli*), *Proteus mirabilis* (*P. mirabilis*), and *Staphylococcus aureus (S. aureus)*. The cytotoxicity of the materials was assessed using a 24 h CCK-8 assay against 3T3 mouse fibroblasts. The resulting NO-releasing latex catheter shows great promise in reducing the risk of infection associated with indwelling urinary catheters.

2. MATERIALS AND METHODS

2.1. Materials.

N-Acetyl-D-penicillamine (NAP), sodium nitrite ($NaNO_2$), ethylenediaminetetraacetic acid (EDTA), tetrahydrofuran (THF), LB agar, CLED agar, and a Cell Counting Kit-8 (CCK-8) were purchased from Sigma Aldrich (St. Louis, MO). Phosphate buffered saline (PBS, pH 7.4) was prepared containing 138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, and 100 μM EDTA in deionized (DI) water. Hydrochloric acid, sulfuric acid, methanol, and LB broth were purchased from Thermo Fisher (Waltham, MA). 3T3 mouse fibroblast cells (ATCC 1658), *S. aureus* (ATCC 6538), *E. coli* (ATCC 25922), and *P. mirabilis* (ATCC 29906) were all obtained from American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM) and trypsin–EDTA were obtained from Corning (Corning, NY). Penicillin–Streptomycin (Pen-Strep) and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY). Cross-sections of Bard latex catheters cut into 0.5 cm long segments were used.

2.2. SNAP Synthesis.

SNAP was prepared using a previously established protocol.[32] Briefly, equimolar amounts of NAP (5 g) and $NaNO_2$ (1 M in DI water) were completely dissolved in a methanol (100 mL) and DI water (30 mL) solution containing 20 mL of 12 M HCl and 5 mL of 18 M H_2SO_4. The solution was kept on an ice bath in the dark overnight to allow SNAP crystals to form. The precipitate was collected via vacuum filtration and dried for 24 h in a desiccator.

2.3. Preparation of SNAP-Impregnated Latex Urinary Catheters (SNAP-UCs).

SNAP-impregnated latex urinary catheters (SNAP-UCs) were prepared based on a modified solvent-swelling method previously established.[24] Briefly, different concentrations of SNAP (25, 50, 100, and 125 mg/mL SNAP) were dissolved in THF. Latex urinary catheters were

immersed in the SNAP-THF solutions and allowed to swell for 24 h in the dark under ambient conditions. The SNAP-swelled latex catheters were then removed and allowed to dry for 24 h to allow any excess THF to evaporate. After the THF evaporated, the catheters were quickly rinsed twice in methanol for 5 s to remove any additional SNAP crystals dried on the surface of the catheter and allowed to dry in the dark.

2.4. NO Release Analysis.

The NO release kinetics of the SNAP-UCs were measured using Sievers chemiluminescence nitric oxide analyzers (NOA, model 280i) over 7 days. The samples were immersed in an amber reaction vessel that contained 2 mL of PBS with EDTA and maintained at 37 °C using a water bath. Nitrogen bubbler and sweep gas purged NO released from the sample into the reaction cell of the NOA, where supplied ozone reacted with NO to produce excited-state nitrogen dioxide (NO_2*), which emits a photon as it goes to the ground state. After accounting for the known calibration constant (mol PPB^{-1} s^{-1}) and the surface area of the samples, the NO release rate ($\times 10^{-10}$ mol cm^{-2} min^{-1}) of each sample was calculated. The nitrogen supply gas was maintained at 200 mL/min. The samples remained immersed in a PBS solution throughout the duration of the study. Each day, the NO release was sampled for approximately 1 hour, which was averaged and represented in the graph as the NO flux for that day. Care was taken to ensure that measurements were made at day intervals (e.g., day 1 is the NO release at 24 h, day 3 is the NO release at 72 h, etc.). $N = 4$ samples were measured for each sample type.

2.5. SNAP Loading and Leaching Measurements.

SNAP loading and leaching were measured according to previously established methods using UV–vis spectrometry.[24] First, total SNAP loading was measured by immersing the SNAP-UCs ($n = 4$) in 5 mL of THF for 24 h in the dark to extract any SNAP impregnated in the latex into solution. The optical densities (OD) of the THF samples were measured at 336 nm, which corresponds to the absorbance maxima of the S-NO group in SNAP.[33,34] A calibration curve consisting of known SNAP concentrations dissolved in THF was used to calculate the total amount of SNAP loaded per mg of catheter.

SNAP leaching was similarly measured by immersing the SNAP-UCs in 1 mL of PBS with EDTA and periodically measuring the OD at 336 nm over a 24 h period using UV–vis. The samples were kept at 37 °C in between measurements. A calibration curve of known SNAP concentrations was used to measure the amount of SNAP leached per cm^2 of the latex catheter.

2.6. Scanning Electron Microscopy (SEM).

SEM analysis was performed using a Thermo Fisher Scientific (FEI) Teneo at an accelerating voltage of 5.00 kV. Gold palladium was sputter coated on each sample at a thickness of 10 nm type using a Leica sputter coater prior to imaging.

2.7. Sterilization Stability Assessment.

To measure the sterilization stability of the SNAP-UCs, the prepared samples were sterilized using ethylene oxide and autoclaving methods. For ethylene oxide sterilization, samples

enclosed in a sterilization pouch were exposed to ethylene oxide for 24 h in an Anprolene AN74i sterilizer. For autoclaving sterilization, samples enclosed in a sterilization pouch were autoclaved for 30 min at 121 °C at 15 psi. After sterilization, the samples were removed and added to 5 mL of THF each for 24 h to calculate the amount of SNAP remaining in the catheter ($n = 4$). The absorbance of the THF at 336 nm was measured to determine the total SNAP loaded and compared to the total SNAP loaded in unsterilized samples.

2.8. Antimicrobial Contact-Based and Diffusible Inhibitory Effects of SNAP-UCs.

To measure the diffusible and contact-based antimicrobial activity of the SNAP-UCs, the viability of both planktonic (in solution) and adhered bacteria was measured after 24 h of exposure to both control and test catheters ($n = 4$). First, each strain (*E. coli*, *P. mirabilis*, and *S. aureus*) was separately inoculated in LB broth at 37 °C until reaching ~10^8 colony forming units (CFUs) per mL validated by optical density measurements at 600 nm. The bacterial solution was then centrifuged at 2500×*g* for 7.5 min, resuspended in sterile PBS, and centrifuged again at 2500×*g* for 7.5 min. After again resuspending in sterile PBS, this bacterial solution was dispensed across a 24 well plate, each well containing a sample immersed in 1 mL of bacterial solution. The samples were exposed to bacteria for 24 h at 37 °C at 150 rpm. After incubation, two viability measurements were conducted to assess the antimicrobial efficacy of the samples. First, to assess the contact-based antibacterial activity of the samples, the viability of adhered bacteria was measured by gently rinsing each sample with sterile PBS and homogenizing for 60 s in sterile PBS to transfer any adhered bacteria from the catheters to the solution. Each solution was subsequently serially diluted and plated on LB agar (*S. aureus* and *E. coli*) or CLED agar (*P. mirabilis*). After 24 h incubation at 37 °C, the number of CFUs was counted. To assess the diffusible microbial inhibition of the materials, the viability of planktonic bacteria in the surrounding solution/environment was measured by directly taking the bacterial solution that the samples were incubated in, serially diluting, and plating. Similarly, after 24 h incubation at 37 °C, the number of CFUs was counted. Reduction in bacterial viability for both adhered and planktonic bacteria was calculated by the following equation (where $C = \frac{\text{CFU}}{\text{cm}^2}$). Each experiment was duplicated to ensure reproducibility.

$$\%\text{Reduction in bacterial viability} = \frac{C_{\text{control}} - C_{\text{test}}}{C_{\text{control}}} \times 100$$

2.9. Cytotoxicity Assay.

The cytotoxicity of the SNAP-UCs toward 3T3 fibroblast cells (ATCC 1658) was tested using the cell counting kit-8 (CCK-8) assay according to the manufacturer's protocol (Sigma Aldrich). All samples were sterilized by UV irradiation on both sides for 1 h. Catheter extracts were obtained by immersing the samples in DMEM (1 mg/mL) and incubating at 37 °C, for 24 h ($n = 5$). 3T3 fibroblast cells were cultured in 75 cm^2 flasks containing DMEM supplemented by 10% FBS and 1% penicillin–streptomycin. After reaching a confluency of 80–90%, cells were harvested using 0.18% trypsin and a suspension was prepared with a

density of 50,000 cells/mL. Then, 100 μL of the cell suspension was added to each well of a 96-well plate and incubated at 37 °C for 24 h. After the formation of a cell monolayer, the medium in each well was replaced with the obtained extracts and allowed to interact with cells for 24 h. Following exposure to the leachates, the viability of the cells was evaluated by adding 10 μL of CCK-8 dye to each well, incubating at 37 °C for 2 h, and measuring the absorbance at 450 nm. Wells containing cells exposed to DMEM with no extract were considered as control. The relative cell viability was calculated using the following equation:

$$\text{cell viability (\%)} = \frac{(\text{OD}_{450} - \text{OD}_{650})\ \text{Treated Cells}}{(\text{OD}_{450} - \text{OD}_{650})\ \text{Unreated Cells}} \times 100$$

2.10. Statistical Analysis.

All data measurements are reported in mean ± standard deviation. To measure significance of multiple groups, a one-way ANOVA with Tukey's post hoc analysis was performed. p-values <0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Characterization of SNAP-UCs.

3.1.1. SNAP Loading Measurements.—Urinary catheters routinely fail due to infection, biofilm formation, and encrustation, potentially resulting in pyelonephritis, septicemia, endotoxic shock, and death.[12] Capitalizing on the potent, broad-spectrum antimicrobial activity of NO, researchers have begun developing NO-releasing materials to extinguish the risk of infection associated with indwelling medical devices.[23] One straightforward method of fabricating NO-releasing materials that has recently gained momentum is the impregnation of the NO donor SNAP via solvent swelling. However, although this method has been previously optimized to impregnate silicone or polyurethane-based polymers with SNAP,[25] it has yet to be tested on latex-based materials. To optimize the SNAP swelling process, different concentrations of SNAP (25, 50, 100, and 125 mg/mL) were dissolved in THF and then utilized to swell and impregnate the latex catheter samples with SNAP. THF was used as the choice solvent due to its excellent SNAP solubility and ability to swell latex without dissolving the latex material. As shown in Figure 1A, the amount of SNAP loaded into the latex material maximized using a 50 mg/mL solution, resulting in 0.114 ± 0.009 mg SNAP/mg latex. Swelling catheters in 100 and 125 mg/mL SNAP-THF resulted in 0.109 ± 0.006 mg SNAP/mg latex and 0.115 ± 0.009 mg SNAP/mg latex, respectively. Therefore, swelling catheters in increased concentrations of SNAP beyond 50 mg/mL did not increase the amount of SNAP loaded in the latex ($p > 0.05$). However, swelling latex catheters in 25 mg/mL resulted in 0.043 ± 0.005 mg SNAP/mg latex, which is approximately half of the SNAP loaded compared to the 50 mg/mL SNAP-swelled catheters. In summary, the optimized swelling solution using SNAP and THF for latex materials was found to be the 50 mg/mL SNAP-THF solution likely due to the solubility limit of SNAP in the latex polymer matrix.

3.1.2. SEM Analysis of SNAP-UCs.—Significant changes in the surface morphology of medical devices can facilitate bacterial adhesion and colonization. Surface imperfections

can increase the surface area or create crevices that bacteria can enter to evade the host environment.[35] Therefore, when predicting the antimicrobial efficacy of a material, surface morphology is an important parameter to understand. To analyze the effect of the swelling and deswelling process on the latex catheters, SEM images were taken before (Figure 1B) and after (Figure 1C) swelling the catheters in a 50 mg/mL solution of THF. As shown, no change in surface morphology is visible between catheters with and without SNAP. Therefore, based on surface morphology alone, no disadvantage is expected from the SNAP-UCs.

3.1.3. NO Release Kinetics of SNAP-UCs.—To understand the effect of swelling latex catheters with different concentrations of SNAP (25–125 mg/mL), the NO release from the samples was measured over 7 days. As shown in Figure 2, catheters impregnated with 50, 100, and 125 mg/mL SNAP solutions exhibited NO release rates $>2 \times 10^{-10}$ cm^{-2} min^{-1} for at least 7 days. Interestingly, for the first 3 days, no significant difference was found between any of the sample types ($p > 0.05$). However, after day 3, latex catheters swelled with 25 mg/mL SNAP solutions did not exhibit any NO release, likely due to lower SNAP loading. Although materials with low levels of NO release ($<0.1 \times 10^{-10}$ cm^{-2} min^{-1}) have shown some antimicrobial effects,[36] previous studies evaluating elevated NO release patterns have shown substantially improved antimicrobial efficacy against both Gram-positive and Gram-negative pathogens compared to their low NO-releasing counterparts.[32,37] When targeting invading microbes, immune cells (e.g., neutrophils) produce high concentrations of NO capable of DNA damage, lipid peroxidation, and disruption of protein function.[23] Therefore, materials that release NO at high concentrations can disrupt bacterial viability subsequent colonization, reducing the risk of infection.

3.1.4. SNAP Leaching.—Leaching of NO donors from NO-releasing materials can diminish the NO reservoir stored in the material, significantly limiting the lifetime of the device. Moreover, the accumulation of NO donors as a product of leaching can lead to cytotoxic effects toward mammalian cells. Therefore, NO-releasing platforms that exhibit minimal NO donor leaching are necessary to ensure the longevity and cytocompatibility of indwelling medical devices. To examine the amount of SNAP leached from the SNAP-UCs, samples were incubated in PBS kept at 37 °C. The amount of SNAP leached was measured periodically over 24 h (Figure 3). No significant difference was found between any of the samples over the 24 h period regardless of the solvent-swelling system used to prepare the materials (25–125 mg/mL SNAP-THF) ($p > 0.05$). Irrespective of the sample type, <3% of the original SNAP loaded had leached from the sample after 24 h (25 mg/mL–2.3 ± 0.8%; 50 mg/mL–1.1 ± 0.4%; 100 mg/mL–1.3 ± 0.6%; and 125 mg/mL–0.8 ± 0.4%). Previous reports of SNAP-impregnated NO-releasing platforms exhibiting non-cytotoxic, extended NO release characteristics had similar low leaching profiles.[24,25,27] Therefore, from the information gathered from the SNAP loading measurements (50 mg/mL SNAP-THF maxed SNAP loading), NO release studies (elevated NO release for 7 days), and SNAP leaching data (minimal SNAP leaching), all SNAP-UC catheters were prepared using a 50 mg/mL SNAP-THF solution for the duration of this study.

3.1.5. Sterilization Stability.—An important metric for ensuring the reproducibility of medical devices is evaluating the effect of sterilization techniques. To evaluate the stability of the SNAP-UCs, two common sterilization methods were executed: ethylene oxide (EO) sterilization (24 h) and autoclaving (30 min). Similar to the SNAP loading measurements, the total SNAP content (mg SNAP/mg catheter) was determined using UV–vis spectroscopy and compared to the SNAP content of nonsterilized SNAP-UCs (Figure 4). Comparable to previous studies[34,38,39] that found that EO sterilization had minimal effect on NO-releasing materials, no statistical difference was found between the total SNAP content of EO-sterilized SNAP-UCs and the nonsterilized SNAP-UCs (102.8 ± 12.8% SNAP remaining, $p > 0.05$). However, after autoclaving, the SNAP content was significantly affected (2.5 ± 0.4%, $p < 0.001$ compared to both nonsterilized and EO-sterilized samples). This can be attributed to the rapid thermal decomposition of SNAP at elevated temperatures present during autoclaving. A previous study evaluating different sterilization techniques on SNAP-doped polymeric films also found that autoclaving rapidly diminished the amount of SNAP loaded.[34] Therefore, EO sterilization was found to be an appropriate method of sterilizing SNAP-UCs.

3.2. Evaluation of Contact-Based and Diffusible Antibacterial Activity of SNAP-UCs.

Despite frequent intervention and improved healthcare habits, UTIs compose 40% of all nosocomial infections, and the majority of these infections are associated with catheter use.[40] The majority of CAUTIs are thought to be derived from bacteria originating from the patient's perineal flora or introduced from hospital personnel either extraluminally during catheter insertion or intraluminally through a break in the drainage line or contamination in the urine bag.[41–43] Once exposed to the foreign surface, bacteria readily attach to the catheter and form protective exopolysaccharides, allowing for host defense evasion and biofilm development.[42] To combat infection, antimicrobial surface modifications (e.g., silver-coated; antibiotic-eluting) have been added to commercial urinary catheters. However, the efficacy of silver-coated catheters both in vitro and clinically is mixed,[20,44–47] and antibiotic resistance remains a serious concern of antibiotic-eluting medical devices.[18] Therefore, the demand for a potent, broad-spectrum antimicrobial surface modification for indwelling urinary catheter use remains. To reduce bacterial colonization of both Gram-negative and Gram-positive pathogens, NO-releasing platforms have been synthesized and evaluated both in vitro and in vivo, showing significant promise.[48–50] In this study, the NO donor SNAP was impregnated in latex-based commercial urinary catheters, a commonly used material for urinary catheters, through a straightforward solvent-swelling method to reduce the viability of invading pathogens. To evaluate the contact-based antimicrobial efficacy and diffusible inhibition of bacterial growth, the viability of *E. coli*, *P. mirabilis*, and *S. aureus* bacteria attached to the surface of the samples and in the surrounding solution was evaluated after 24 h of exposure (Figure 5).

E. coli is the most common pathogen associated with CAUTIs, isolated from half of hospital-acquired UTIs.[40,42] After 24 h exposure to *E. coli* at 37 °C, SNAP-UCs were found to significantly reduce the viability of *E. coli* adhered to the catheter surface (98.9 ± 0.9% reduction, $p < 0.001$) and planktonic *E. coli* in the surrounding solution (81.6 ± 9.9% reduction, $p < 0.05$) compared to control latex catheters. Although silver-coated

catheters, the current gold-standard for antimicrobial urinary catheters, reduced the viability of adhered *E. coli* 74.0 ± 14.0% compared to control latex catheters, the silver-coated catheters failed to significantly decrease the viability of *E. coli* in solution, reducing the viability by only 20.3 ± 22.8% ($p > 0.05$). Silver-coated catheters have largely been shown to be ineffective against Gram-negative bacilli including *E. coli*.[51] In fact, previous studies comparing the diffusive inhibitory effects[51,52] and contact-based antimicrobial effects[20,52] of antimicrobial catheters toward adhered *E. coli* have found that silver-coated catheters failed to have any significant effect. However, NO-releasing platforms have shown substantial antimicrobial efficacy against Gram-negative bacteria commonly found with hospital-acquired infections including *E. coli*, *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Klebsiella pneumoniae*.[53,54] Moreover, compared to commercial silver-coated latex urinary catheters, SNAP-UCs reduced the viability of adhered *E. coli* by 96.9 ± 3.6% and the viability of planktonic *E. coli* by 76.9 ± 12.5%.

Particular uropathogens, including *P. mirabilis*, produce urease enzymes that hydrolyze urea, using it as a nitrogen source.[42] Ammonia generated from this reaction shifts the pH of the urine, causing the accumulation of calcium and magnesium that result in crystalline biofilm formation, urethra and bladder trauma, urine retention, and septicemia.[46] *Proteus* species also exhibit unique swarming behavior, allowing for the spread of infection through coordinated migration from the catheter surface to the urothelium in the bladder.[55] Therefore, antimicrobial measures to combat *P. mirabilis* are imperative to prevent these events from occurring. SNAP-UCs significantly reduced the viability of adhered *P. mirabilis* by 99.1 ± 0.1% ($p < 0.001$) and the viability of planktonic *P. mirabilis* by 96.3 ± 1.4% ($p < 0.001$) compared to control latex catheters. However, similar to the antimicrobial trends shown against *E. coli*, although they are moderately able to reduce the viability of adhered *P. mirabilis* (82.3 ± 7.0% reduction, $p < 0.01$), silver-coated catheters failed to have a significant diffusible antimicrobial impact against *P. mirabilis* in the surrounding solution ($p > 0.05$). Morris and Stickler found that after exposing *P. mirabilis*-infected human urine to different commercial urinary catheters, silver-coated latex catheters occluded from encrustation the fastest, suggesting that silver coatings fail to prevent the colonization of *P. mirabilis*.[46] Similarly, Morgan et al. found that indwelling silver-coated urinary catheters were unsuccessful in preventing encrustation and concluded that in order to prevent infection, catheters must contain antimicrobials that are able to diffuse out from the surface.[56] In this study, SNAP-UCs resulted in significantly improved antimicrobial efficacy against *P. mirabilis* over silver-coated catheters. *P. mirabilis* are especially problematic for urinary infections due to the ability to catalyze the hydrolysis of urea, generating ammonia and shifting the pH of the urine, causing magnesium and calcium phosphate to crystallize on the catheter surface.[11] Compared to the silver-coated catheters, SNAP-UCs reduced the viability of adhered *P. mirabilis* by 95.4 ± 0.6% ($p < 0.01$) and the viability of planktonic *P. mirabilis* in solution by 96.5 ± 1.4% ($p < 0.001$), showing great promise for eliminating *P. mirabilis*-related CAUTIs and subsequent encrustation complications.

An increasing number of Gram-positive *cocci* strains (*S. aureus*, *Staphylococcus saprophyticus*, *Enterococcus* spp., and *Streptococcus* spp.) have been detected in UTIs in recent years, identified in approximately 20% of these infections.[57,58] Moreover, among patients with *S. aureus* bacteremia, patients with concomitant *S. aureus* bacteriuria have

been linked to severe clinical outcomes including ICU admission and mortality compared to patients without bacteriuria.[59,60] In this study, SNAP-UCs reduced the viability of adhered *S. aureus* by 98.0 ± 1.9% ($p < 0.01$) and the viability of planktonic *S. aureus* by 92.3 ± 6.3% ($p < 0.001$) compared to control latex catheters, as opposed to silver-coated catheters that were only able to reduce the viability of adhered *S. aureus* by only 16.8 ± 14.1% ($p > 0.05$) and the viability of planktonic *S. aureus* by 46.9 ± 19.2% ($p > 0.05$) compared to control latex catheters. However, SNAP-UCs performed significantly better than silver-coated catheters in reducing the viability of adhered *S. aureus* (97.3 ± 2.5% reduction, $p < 0.01$) and planktonic *S. aureus* in solution (85.5 ± 11.8% reduction, $p < 0.01$). To date, bacteria demonstrating resistance to exogenous sources of NO have yet to be demonstrated.[23] Although *S. aureus* can evade host immune response through the induction of homolactic fermentation[61] and increased expression of protective enzymes (e.g., flavohemoglobin) capable of detoxifying NO,[62] the protective strategies are ineffective against potent, high levels of NO provided by NO donor drugs.[23] Different NO-releasing platforms, including materials,[63] nanoparticles,[64,65] macro-molecular polymers,[66] and ointments,[67] have shown significant antimicrobial and antibiofilm efficacy against antibiotic-resistant strains of *S. aureus*, supporting the case for an NO-releasing urinary catheter.

A major advantage of NO-releasing surfaces is that NO exhibits multiple antimicrobial mechanisms against bacterial cells, which makes the development of resistance difficult (Figure 6). In fact, a previous study assessing bacterial resistance against exogenous NO through spontaneous and serial passage mutagenesis studies showed that no significant increase in MIC values was observed compared to parent *S. aureus*, MRSA, *Staphylococcus epidermidis*, *E. coli*, or *P. aeruginosa*.[68] NO can easily diffuse across bacterial membranes, causing nitrosative and oxidative damage and resulting in protein alterations, DNA deamination and oxidative modifications, and lipid peroxidation.[23] NO-releasing materials exhibiting extended NO release have still shown moderate reductions in decreased adhered bacterial viability when NO release rates are low ($<0.1 \times 10^{-10}$ mol cm^{-2} min^{-1}) as a result of long incubation periods.[36] Moreover, likely due to the high levels of NO release exhibited in the first 24 h, these SNAP-latex catheters were able to reduce not only the viability of bacteria adhered to the surface but also the viability of the pathogens in the solution. Therefore, NO-releasing materials are advantageous not only in their broad-spectrum antimicrobial capabilities but also from the potent, multimechanistic bactericidal nature NO, making the development of antimicrobial resistance less likely and creating a promising platform for preventing and combating CAUTIs.

3.3. In Vitro Cytotoxicity of SNAP-UCs.

In addition to having strong antibacterial properties, urinary catheters must be biocompatible and not elicit any toxicity or adverse host response when used in the human body. Evaluation of the in vitro cytotoxicity is one of the most important initial steps toward the biocompatibility confirmation of the medical devices. In this study, 3T3 fibroblast cells were exposed to the leachate products of the tubing samples and a CCK-8 colorimetric assay was performed to investigate any possible cytotoxic responses. Many studies regarding the development of urinary catheters have used this cell line to evaluate the cytocompatibility of their materials.[69,70] The intensity of the formazan dye produced during the CCK-8 assay

by the activities of dehydrogenases in cells can be measured at 450 nm and is directly proportional to the numbers of living cells.

According to the obtained results (Figure 7), none of the samples were cytotoxic and they all maintained more than 90% cell viability. Although the viability of cells exposed to latex leachates was slightly lower compared to cells without leachate exposure (93.3 ± 1.8% relative cell viability, $p = 0.0158$), no significant difference was found between the viability of any SNAP-UCs and control cells ($p > 0.05$). This can be due to the regulating effect of NO on cell proliferation and protection, which has been demonstrated before in many studies.[24,71] Latex urinary catheters have historically shown some degree of cytotoxicity both in vitro and in vivo.[72–74] The results from this study show promising cytocompatibility from SNAP-UCs and should be further studied.

4. CONCLUSIONS

Indwelling urinary catheter use is routinely complicated from bacterial colonization, resulting in infection and encrustation. In this work, the NO donor SNAP was loaded into commercial latex urinary catheters via a straightforward solvent-swelling technique to reduce the viability of adhered and planktonic bacteria. The solvent-swelling method was optimized by evaluating the SNAP loading, NO release, and SNAP leaching of latex catheters swelled with solutions containing different concentrations of SNAP. As a result, SNAP-UCs impregnated with a 50 mg/mL SNAP-THF solution maximized the amount of SNAP loaded into the material (0.115 ± 0.009 mg SNAP/mg catheter), exhibited high NO release ($>2 \times 10^{-10}$ mol min^{-1} cm^{-2}) for 7 days, and had inconsequential SNAP leaching (<2%). Optimized SNAP-UCs demonstrated impressive contact-based and diffusible antimicrobial efficacy against three common CAUTI-associated pathogens, reducing the viability of adhered and planktonic *E. coli*, *P. mirabilis*, and *S. aureus* by 98.0–99.1% (adhered) and 86.3–96.3% (planktonic) compared to control latex catheters and outperforming commercial silver-coated catheters. After ethylene oxide sterilization, the SNAP loading was not significantly affected, showing great translational promise. Finally, the SNAP-UCs were found to be noncytotoxic (>90% cell viability retained) in vitro against 3T3 mouse fibroblasts using a CCK-8 assay. The resulting SNAP-UCs provide an exciting potent, broad-spectrum antimicrobial platform capable of reducing the risk of infections associated with urinary catheters and other latex-based medical devices.

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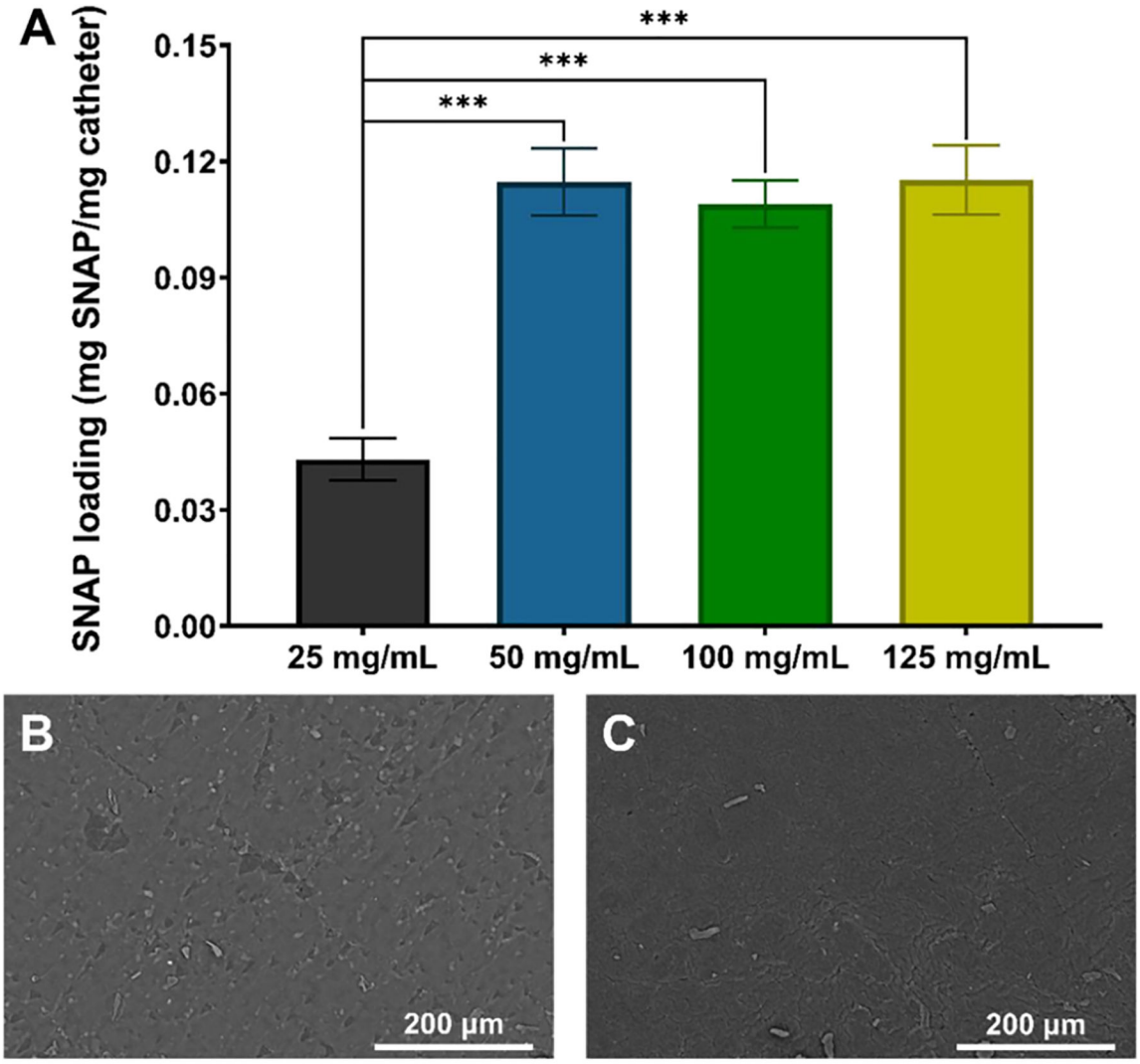

Figure 1.

(A) Total SNAP loading (mg) per mg of latex after 24 h of swelling in 25, 50, 100, and 125 mg/mL SNAP-THF solutions. (*** represents *p*-values < 0.001). SEM images of (B) unmodified control latex catheters and (C) 50 mg/mL SNAP-UCs demonstrate no visible change in surface morphology after the swelling–deswelling process.

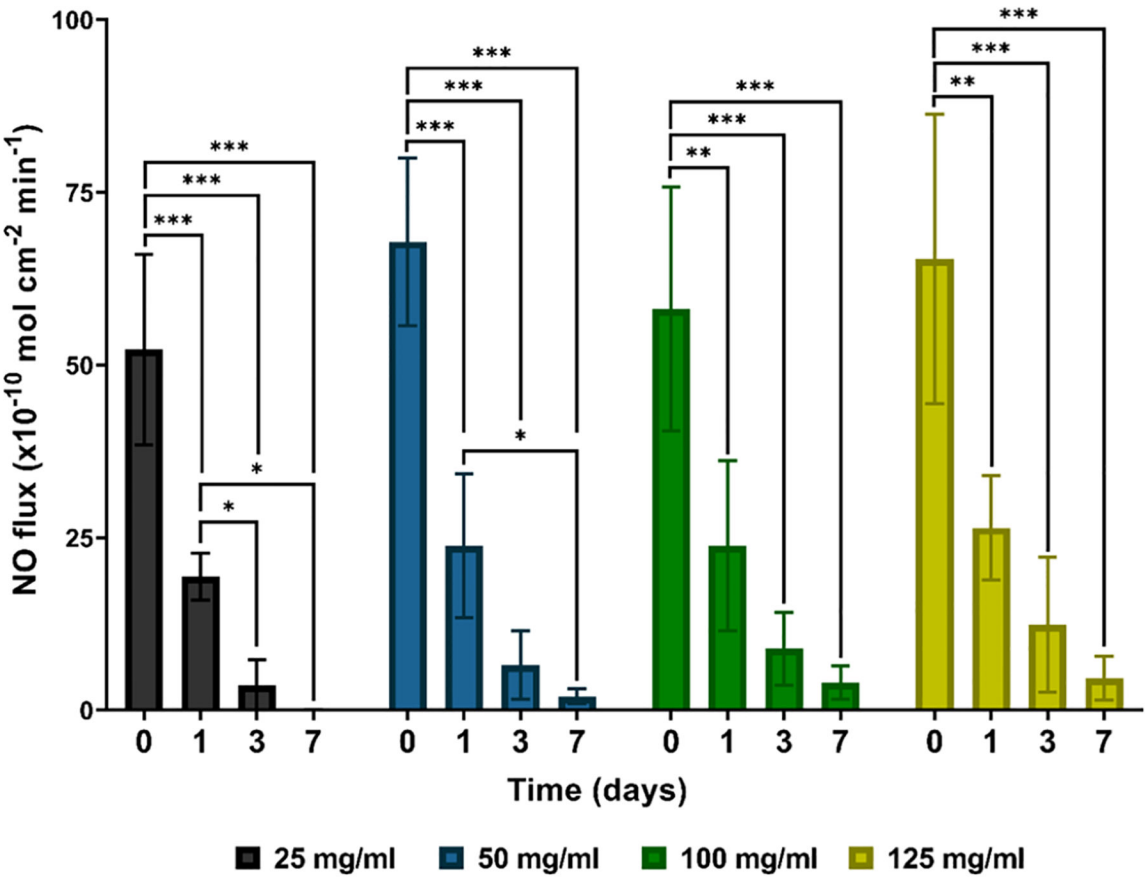

Figure 2.

NO release measurements of 25, 50, 100, and 125 mg/mL SNAP-UCs over 7 days at 37 °C. The degree of statistical significance is indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

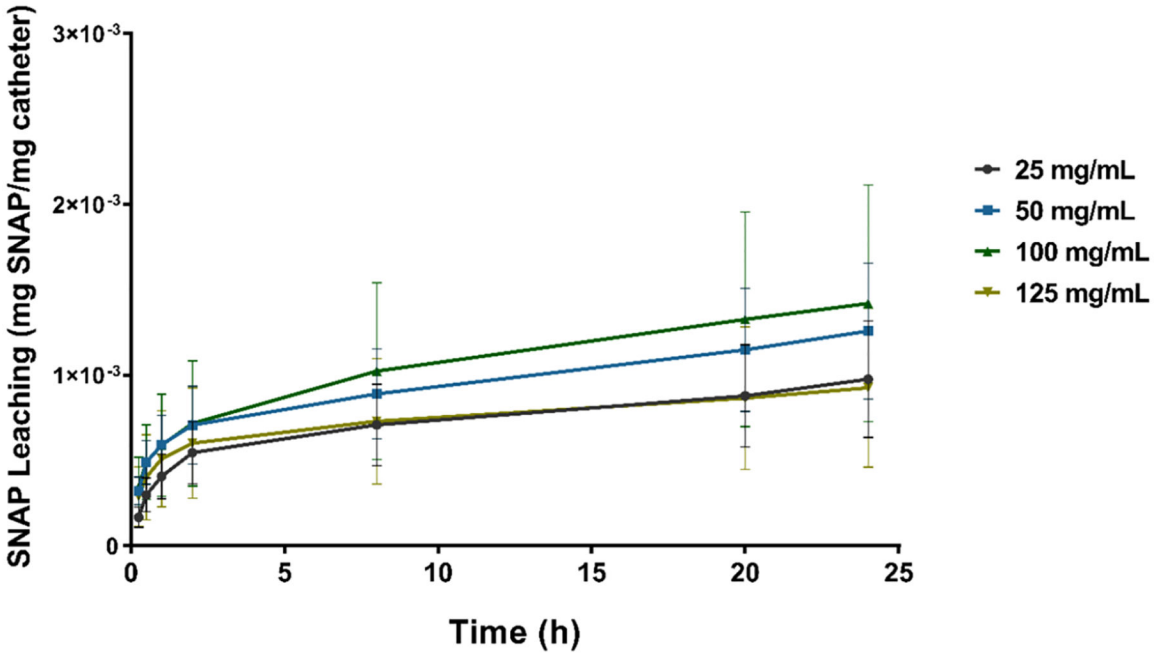

Figure 3.
SNAP leaching measurements (mg SNAP per mg catheter) of 25, 50, 100, and 125 mg/mL SNAP-UCs using UV–vis spectroscopy. Samples were kept in PBS maintained at 37 °C over the 24 h period.

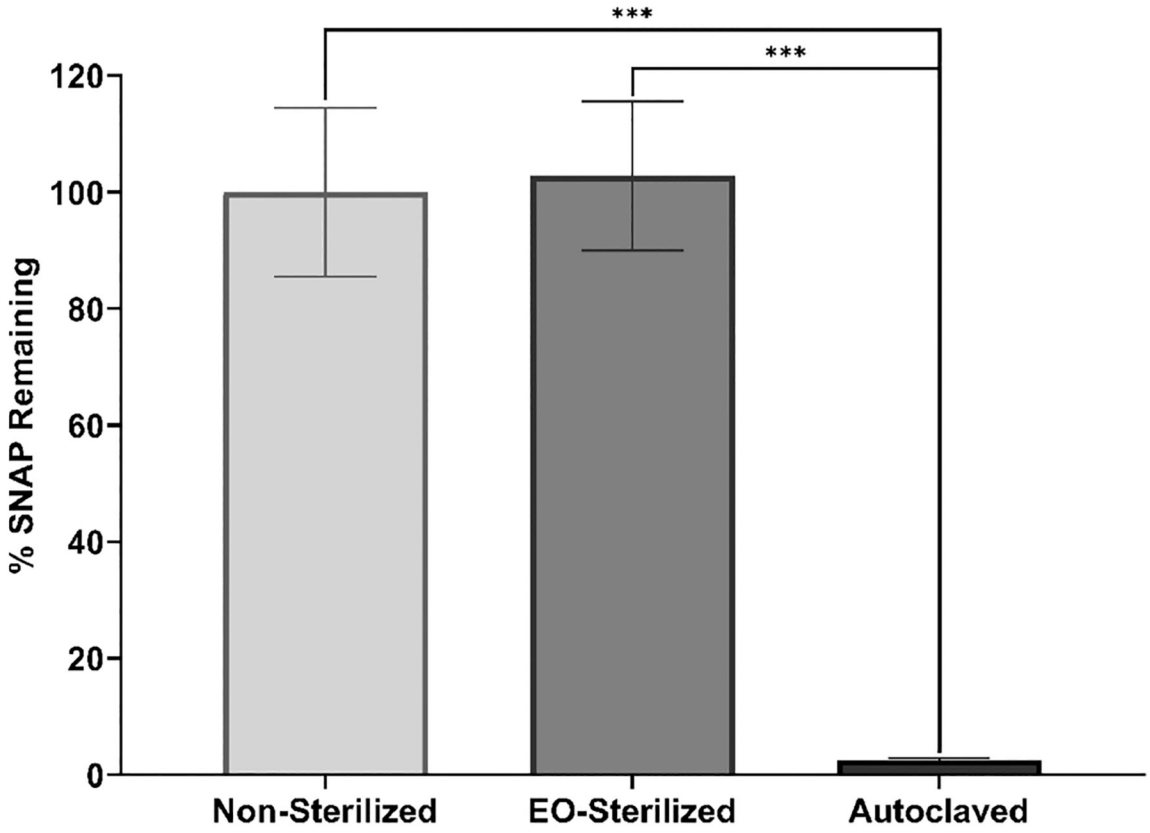

Figure 4.

Sterilization stability (% SNAP remaining) after ethylene oxide (EO) sterilization (24 h) and autoclaving (30 min) compared to nonsterilized catheters. The degree of statistical significance is indicated by *** ($p < 0.001$).

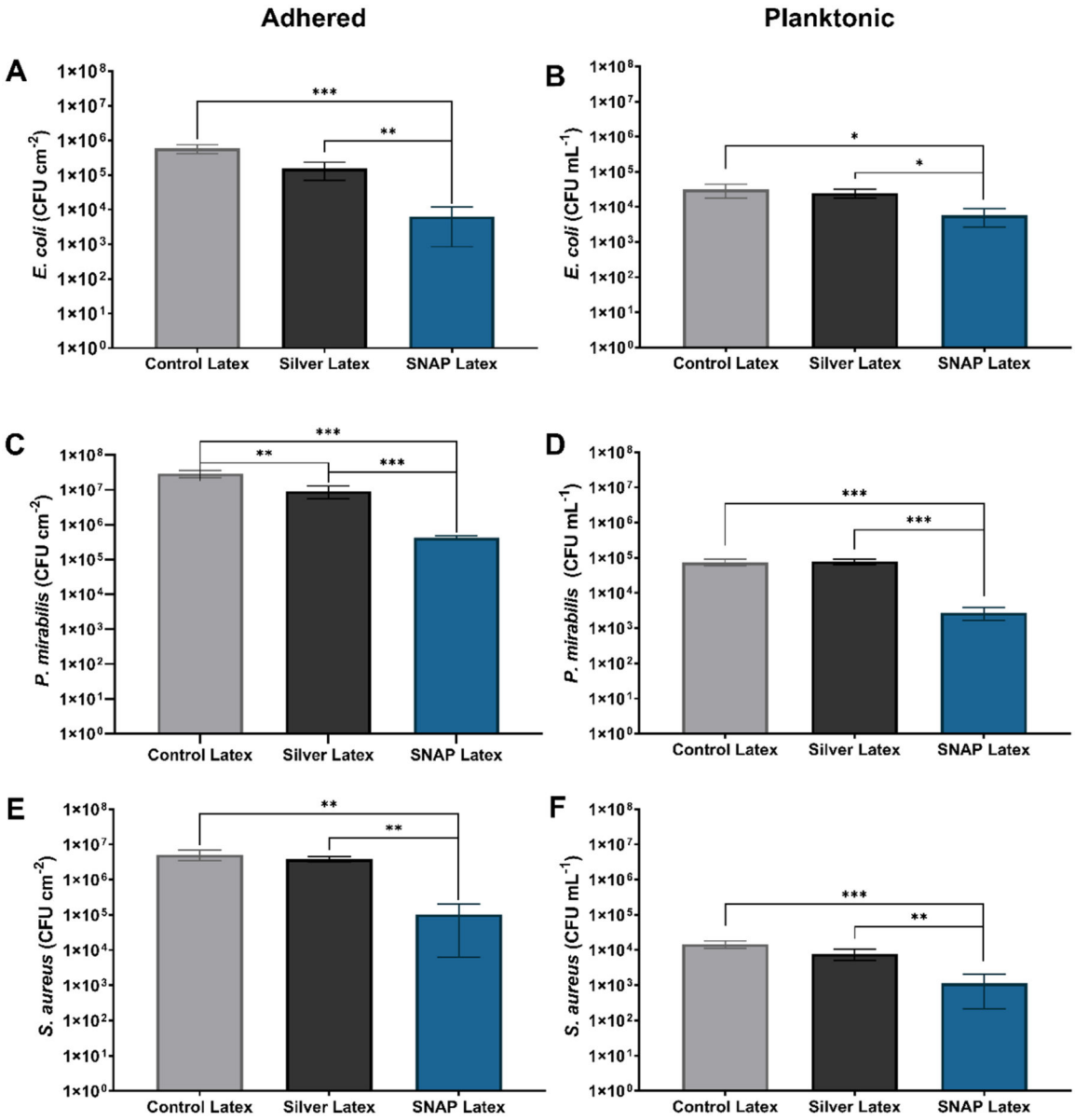

Figure 5.

(A, C, E) Contact-based antimicrobial efficacy of SNAP-UCs compared to commercial latex and silver-coated latex urinary catheters. The viability of *E. coli*, *P. mirabilis*, and *S. aureus* attached to the surface was evaluated after 24 h of exposure. Similarly, the (B, D, F) diffusible inhibition of planktonic bacteria was evaluated by determining the number of viable bacteria in solution after 24 h of exposure. The degree of statistical significance is indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

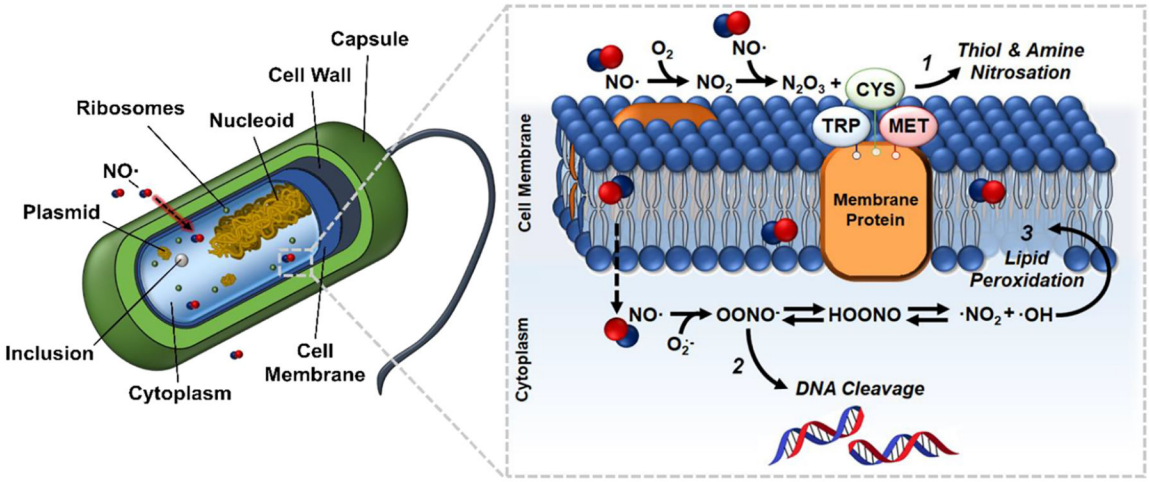

Figure 6.

NO readily diffuses across the bacterial membrane, resulting in multiple antimicrobial reactions, including (1) thiol and amine nitrosation, (2) DNA cleavage, and (3) lipid peroxidation.

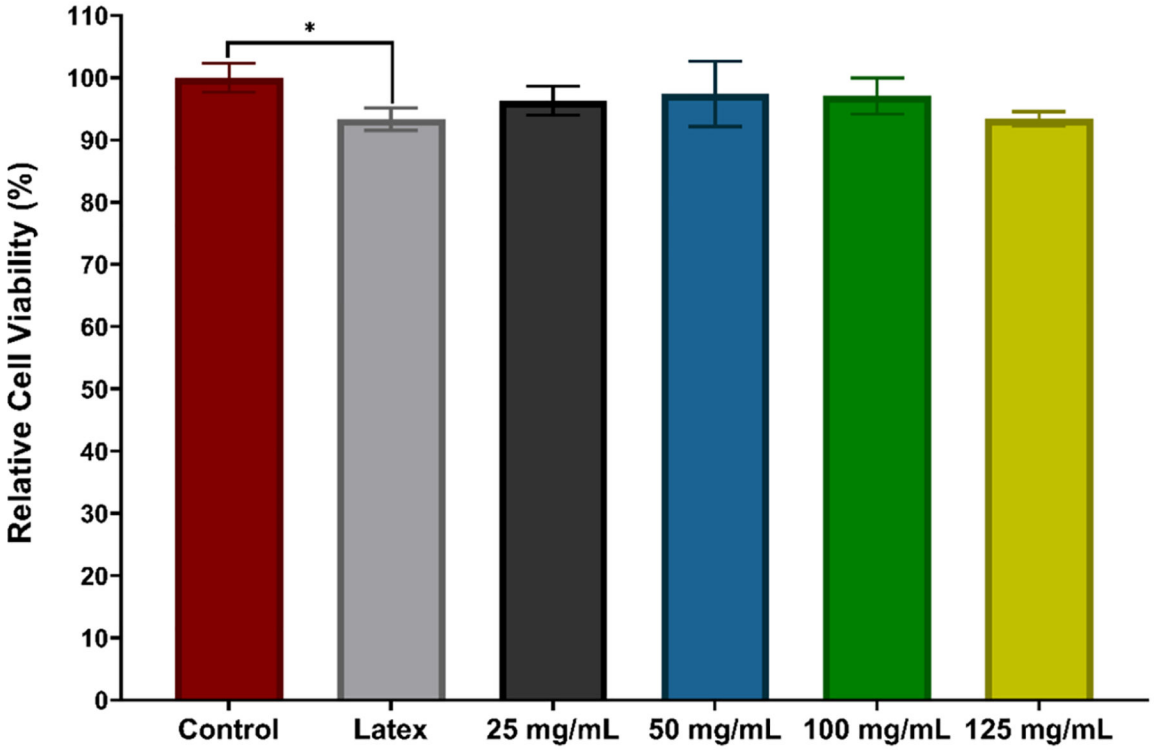

Figure 7.

In vitro cytotoxicity measurements of samples against 3T3 mouse fibroblasts using a CCK-8 assay. The degree of statistical significance is indicated by * ($p < 0.05$).