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Paper

The preparation and characterization of nitric oxide releasing silicone rubber materials impregnated with *S*-nitroso-*tert*-dodecyl mercaptan

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Recently, considerable research efforts have focused on increasing the biocompatibility and bactericidal activity of biomedical polymeric devices (e.g., catheters, etc.) through incorporation of nitric oxide (NO) releasing molecules. NO is an important endogenous molecule that is well known for enhancing blood flow via its vasodilatory activity, but it also exhibits potent antithrombotic and antimicrobial properties. In this work, we demonstrate that silicone rubber tubing can be impregnated with a tertiary *S*-nitrosothiol (RSNO), *S*-nitroso-*tert*-dodecylmercaptan, via a simple solvent swelling method. We further characterize the NO release and RSNO leaching from the tubing over time via use of chemiluminescence and UV/Vis spectroscopy, respectively. The tubing is shown to maintain an NO flux above the physiological levels released by endothelial cells, 0.5-4.0 x 10^{-10} molcm^{-2}min^{-1}, for more than 3 weeks while stored at 37 °C and exhibit minimal leaching. Finally, the RSNO impregnated tubing exhibits significant antimicrobial activity over a 21 d period (vs. controls) during incubation in a CDC bioreactor after inoculation of media with *S. aureus* bacteria. The use of such lipophilic RSNO impregnated silicone rubber tubing could dramatically reduce the risk of catheter-related infections, which are a common problem associated with placement of intravascular or urinary catheters.

1. Introduction

Silicone rubber (SR) has become one of the most common polymers used to prepare biomedical devices since it was first introduced to the medical field in the 1940's. Its low compression set, robust mechanical properties, chemical and temperature resistance, as well as intrinsic flexibility for melding and extrusion processes have allowed its use for numerous health care applications including shunts, implants, medical adhesives, and catheters. Silicone rubbers have demonstrated greater biocompatibility and biodurability for certain applications compared to other common polymeric materials. Polyurethanes lack stability over as broad a temperature range compared to polydimethylsiloxanes, which potentially limits sterilization techniques or storage conditions. Exposure to organic solvents such as acetone and isopropyl alcohol, which are commonly found in adhesives and disinfectants, can solubilize polyurethanes and lead to surface cracking. Unlike polyurethanes, silicone rubber catheters are resistant to hydrolysis due to their high crosslinking. Silicone rubber's lower compression set provides increased flexibility and resistance to deformation. PVC commonly requires plasticizers, which are known to leach from the materials during use. Silicone rubber's stability also lends itself to sterilization by ethylene oxide (EtO), a process often used to sterilize biomedical devices prior to medical procedures.[1-5]

Despite these characteristics, introducing any foreign material such as an intravenous or urinary catheter within a patient can cause health complications including urinary tract or bloodstream bacterial infections.[2] Although progress has been made in preventing catheter-related bloodstream infections (CRBIs), such complications remain prevalent and are estimated to cost between 670 million and 2.68 billion dollars annually in the United States alone.[6] Urinary tract infections (UTIs) are the most common, with catheter-associated infections accounting for approximately 75% of occurrences.[7] Microbial biofilms commonly form on the surfaces of biomedical devices. Bacteria release extracellular polymeric substances that form a hydrated matrix. Bacteria within a biofilm community undergo significant phenotypic changes while in the matrix. Bacteria present within a biofilm demonstrate antibiotic resistance due to adaptive stress responses and poor antibiotic penetration, as well as protection from the host's immune system.[8-10] *Staphylococcus aureus* is the most common cause of nosocomial bloodstream infections, specifically those associated with biofilm formation on indwelling biomedical devices.[11] It can cause a range of illnesses, from skin infections to life-threatening diseases like bacteremia or sepsis. Given this and the ever-growing concern of antibiotic-resistant bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA), developing effective ways to combat infections by this organism is crucial.[6-11]

Many methods have been studied to improve device biocompatibility such as surface modification, passive or bioactive coatings, and silver incorporation, each with their own advantages and disadvantages.[12-15] Indeed, Ag^+ eluting urinary catheters have

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not solved the problem given that The Healthcare Infection Control Practices Advisory Committee (HICPAC) states that silver-alloy coated catheters lead to similar infection rates compared to the conventional silicone ones. Further, they have stated that routine irrigation of the bladder with conventional antimicrobials (antibiotics) is not recommended as it increases antimicrobial resistance.[9,10] Recent work from our group has aimed to increase the biocompatibility and antimicrobial activity of medical grade polymers through the incorporation of nitric oxide releasing molecules.[16-19]

Nitric oxide (NO) is an endogenous molecule that exhibits a diverse range of benefits including its antimicrobial, antithrombotic, and vasodilatory properties—all of which have use in improving the biocompatibility of medical devices. NO releasing polymers can provide treatment at localized sites, thus minimizing side effects and complications experienced with use of systemic or local antibiotics (e.g., drug interactions) and the ever growing concern of antibiotic resistant bacteria.[17-19] Due to its propensity to readily oxidize, NO should be released from biomedical devices in controlled amounts, for specific durations, and at discrete locations. Regev-Shoshani *et al.* successfully demonstrated that Foley catheters impregnated with NO gas could prevent *E. coli* colonization and biofilm formation; however, they acknowledge the model does not immediately translate to clinical environments in which catheters are exposed to high urine flow.[20] Catheters exposed to dynamic urine flow for 24 h showed diminished bactericidal effects in the surrounding solution.[20] Although antimicrobial activity was demonstrated for 24 h following 1 week of storage,[20] retaining NO within polymers would be difficult for extended periods of storage without pressurized containers. Given NO's reactivity and the difficulties associated with the controlled delivery of this gas, various classes of NO donor molecules have been studied for potential use as an NO reservoir within polymers including diazeniumdiolates, *S*-nitrosothiols (RSNOs), metal nitrosyl compounds, and other nitrogen oxides.

Some RSNOs are physiological NO donors, with endogenous species including *S*-nitrosocysteine (CysNO), *S*-nitrosoglutathione (GSNO) and *S*-nitrosoalbumin present within the human blood stream. Given these donors' inherent biocompatibility, they have been thoroughly studied as a means to create controllable NO delivery from materials; however, each has limitations including low stability towards thermal and photolytic decomposition. These traits make device preparation and storage difficult. Further, high water solubility enhances extraction out of the polymeric materials when in contact with blood or urine. Nonetheless, various exogenous NO donors have been incorporated into biomedical polymers in attempt to utilize NO's effects, one of the most promising RSNOs being *S*-nitroso-*N*-acetyl penicillamine (SNAP).[21-23] Indeed, our group recently demonstrated SNAP's ability to release NO over long durations (>3 weeks) and decrease thrombus formation at localized treatment sites.[17] Additionally, Colletta *et al.* recently reported that silicone rubber Foley catheters impregnated with SNAP were able to decrease *Staphylococcus epidermidis* and *Proteus mirabilis* levels for 2 weeks relative to controls. One limitation, however, is that an appreciable amount of SNAP, and likely its dimer, leach from the polymers.[18] Leaching decreases NO's efficacy at the localized polymer site and can cause NO's effects at unintended locations within the body.

Since scientists discovered NO to be the endothelial derived relaxing factor in 1987, researchers have continued to synthesize and develop a library of numerous NO donors, each bearing characteristics potentially suitable for unique medical applications.[21,22] Lipophilic alkyl RSNOs have previously been synthesized, however, they have not seriously been considered as potential medical sources of NO until recently.[24-26] Currently, *S*-nitroso-*tert*-dodecyl mercaptan (SNTDM) is the most promising alkyl RSNO we have studied to date. Giles *et al* demonstrated SNTDM's potential for photoactivated vasorelaxation, however, to the best of our knowledge this species has not been studied within polymeric environments.[25] SNTDM's highly lipophilic character (CLogP=5.31) should increase retention within silicone rubber due to its hydrophobic nature.[27] As an RSNO's stability is largely dependent upon the substitution at the site of the nitroso functionality, we suspected that SNTDM could provide a long-term NO release.[28,29]

Typically, to add NO release to a polymer tubing, it would be desirable to incorporate the NO releasing agent into the extrusion process. However, RSNOs cannot withstand the elevated temperature employed for extrusion.[18] To overcome this limitation we have adapted our recently reported solvent swelling method to impregnate SR tubing with SNTDM, a process which can be conducted at room temperature. Several solvents have previously been reported to effectively swell silicone rubber with no harm to its properties.[30] It will be shown here that silicone rubber tubing impregnated with SNTDM demonstrates significant NO release duration, reasonable storage stability, minimal leaching when in contact with an aqueous phase, as well as substantial antimicrobial activity toward *S. aureus*.

2. Materials and Methods

2.1 Materials

tert-Dodecyl mercaptan (TDM), *tert*-butyl nitrite ($tBuNO_2$), chloroform ($CHCl_3$), diethyl ether (Et_2O), dimethyl sulfoxide (DMSO), sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, ethylenediaminetetraacetic acid (EDTA), copper (II) chloride, cysteine, and magnesium sulfate were purchased from Sigma Aldrich (St. Louis, MO). Dow Corning RTV 3140 Silicone Rubber (SR) was purchased from Ellsworth Adhesives (Germantown, WI). Standard silicone tubing (1.58 mm I.D, 3.18 mm O.D.) was purchased from Helix-Medical (Carpinteria, CA). All aqueous solutions were prepared with deionized water from a MilliQ system (18 MΩ cm^{-1}; Millipore Corp., Billerica, MA). Phosphate buffered saline (PBS) containing 10 mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl, and 100 μM EDTA (pH 7.4) was used as the buffer for all *in vitro* experiments. Luria Bertani (LB) broth and LB agar were obtained from Fisher Scientific Inc. (Pittsburgh, PA). *Staphylococcus aureus* ATCC 45330 was obtained from the American Type Culture Collection.

2.2 Synthesis of *S*-nitroso-*tert*-dodecyl mercaptan (SNTDM)

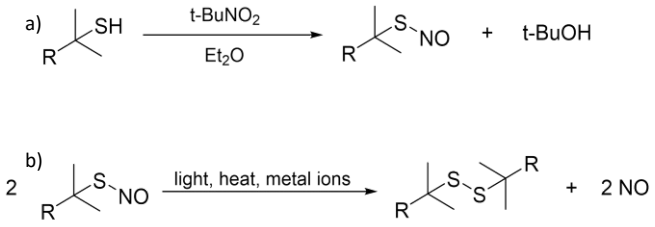

Fig. 1 (a) Nitrosation of *tert*-dodecyl mercaptan (TDM) to yield *S*-nitroso-*tert*-dodecyl mercaptan (SNTDM). (b) Decomposition can be catalysed by light, heat, or metal ions such as Cu (I) to yield the disulfide and 2 equivalents of nitric oxide (NO). R=C_9H_{19} isomer.

SNTDM was synthesized using a modified version of previously reported methods.[24,25] *tert*-Dodecyl mercaptan was dissolved in anhydrous diethyl ether, before adding 1.1 equivalents of *tert*-butyl nitrite. After 45 min of vigorous stirring, the Et_2O solution was washed with an excess of DI water and then dried with $MgSO_4$. Et_2O, residual $tBuNO_2$, and the *tert*-butanol by-product were removed via rotoevaporation to yield a green/red liquid (Fig. 1). SNTDM could be quantified via its absorbance ($CHCl_3$, ε_{341}=596 $M^{-1}cm^{-1}$, DMSO ε_{340}=606 $M^{-1}cm^{-1}$) using a Lambda 35 UV-Vis spectrophotometer (Perkin-Elmer, MA). Following synthesis, SNTDM was immediately used for experiments or kept in a -20 °C freezer to be used soon thereafter. Light exposure was minimized during all experiments.

Stimulated SNTDM decomposition: di-*tert*-dodecyl disulfide synthesis (DTDD)

A 100W halogen floodlight (GE model 17986) was used as a broad spectrum light source to facilitate photo-decomposition of the SNTDM doped materials. The technique was employed for various experiments requiring quick conversion to the disulfide and measuring the initial amount of RSNO present. The rate of decomposition was at times also stimulated by addition of a 50 µM $CuSO_4$/cysteine solution. DMSO was required as a co-solvent to ensure SNTDM solubility.

2.3 Characterization techniques

UV-Vis Spectroscopy. Following SNTDM's synthesis, determination of the molar absorptivity at 341 nm in $CHCl_3$ and 340 nm in DMSO allowed the concentration to be measured during subsequent experiments. Pure SNTDM was decomposed via photo-irradiation to form DTDD and the molar absorptivity determined to be ε_{275}=418 $M^{-1}cm^{-1}$ in $CHCl_3$ (see Figs. 1 & 2). Experiments were conducted in triplicate.

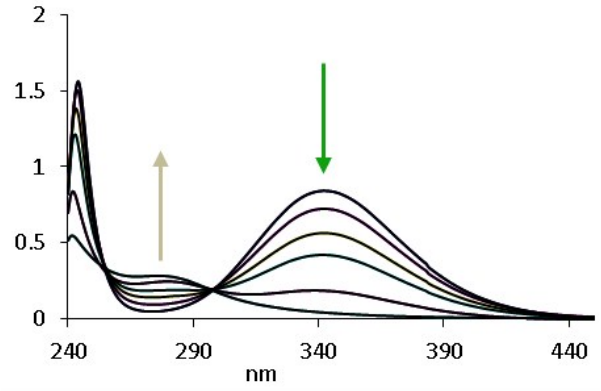

Fig. 2 Spectra displaying the inverse relationship between SNTDM (341 nm) and DTDD (275 nm) absorbances during decomposition.

Chemiluminesence NO release measurements. Samples were placed in a glass sample cell and NO release was measured by a Sievers chemiluminescence Nitric Oxide Analyzer (NOA) 280 (Boulder, CO). Conditions varied depending on the experiment. Clear or amber cells were used to control light exposure, and a water bath provided variable temperatures. If required, the RSNO/SR sample was submerged in PBS (pH=7.4, 100 µM EDTA). NO was purged from the buffer and/or headspace into the detection chamber using N_2 sweep-gas. Measurements were obtained in triplicate.

2.4 Preparation of SNTDM impregnated silicone rubber tubing

Swelling. Segments of commercial silicone rubber tubing were submerged in vials containing a SNTDM/$CHCl_3$ solution. After stirring/soaking in the dark, the pieces were removed, briefly rinsed with a lower solubility solvent, dried with a Kim-wipe, placed in clean vials and allowed to return to the original length/diameter in the dark before being further drying in a vacuum oven in the dark to remove residual $CHCl_3$ (see Fig. 1S in the ESI†). SR segments were 0.5 cm in length unless otherwise specified.

Loading efficiency. Tubing segments were impregnated using the swelling method described above. After the tubes had dried, the wt% of impregnated SNTDM was determined by extracting the contents of a weighed piece of SR into $CHCl_3$ and measuring SNTDM's absorbance at 341 nm and/or accounting for the total released NO via NOA measurements during stimulated decomposition (by photolysis).

2.5 Characterization of SNDTM/silicone rubber

Long term NO release. Samples were tested on an NOA periodically to determine their average NO release/flux. Between measurements, the tubings were soaked in PBS, and stored in a dark 37.5 °C oven to simulate physiological conditions. Prior to each measurement, the soaking solutions were replaced with fresh PBS and saved to test for leaching.[17] All measurements were conducted in triplicate.

Leaching measurements. PBS soaking solutions were extracted into $CHCl_3$, and the amount of leached RSNO and RSSR was determined via UV-Vis spectroscopy.[30] SNTDM and DTDD's lipophilic character (CLogP=5.31 and 11.38, respectively) assured that they could be quantitatively accounted for in the organic phase after extraction into $CHCl_3$.[27] All measurements were conducted in triplicate.

Storage stability studies. SNTDM impregnated SR tubing storage stability was determined in various environments including natural and ambient light, a RT/dark cupboard, 4 °C refrigerator, and in a -20 °C freezer. The RSNO levels were monitored periodically via

UV/Vis spectroscopy. Stability testing for polymeric environments was achieved by first extracting the RSNO/RSSR contents into $CHCl_3$. Experiments were conducted in triplicate.

2.6 Effects of ethylene oxide (EtO) sterilization

Three cm length silicone rubber tubing segments (1.58 mm I.D, 3.18 mm O.D.) were impregnated using the technique described above and submitted to the University of Michigan Hospital sterilization facility. During the procedure, the segments were sequentially exposed to 40-80% humidity for 1 h, EtO gas for 2-3 h (40-80% humidity) followed by exhaust/aeration for 14 h. A temperature of 54 ^{o}C was maintained during the entire procedure. The SNTDM content of the tubings was measured using UV-Vis before and after sterilization. The NO flux levels were also measured following the procedure.[31]

2.7 Antimicrobial and biofilm study.

Three cm SR tubing pieces were impregnated using the above swelling technique, and the open ends were sealed with RTV silicone. The SR pieces were attached to the coupon holders of a CDC biofilm reactor (Biosurface Technologies, Bozeman, MT) using rubber bands.[32] The bioreactor was filled with 10% LB broth and injected with 4 ml of overnight grown bacteria (*S. aureus*) culture. Fresh 10% LB broth was continuously supplied with a flow rate of 100 mL/h via a peristaltic pump while maintaining stirring in the bioreactor over the course of the experiment. All equipment was autoclaved prior to use and the media reservoir replaced as needed. The bioreactor was kept in a dark 37 ^{o}C oven. After 7, 14, and 21 d, the SR tubing segments were removed and the portions not touching the rubber bands cut into 2 pieces. One piece was vortexed in 2mL of 10 mM PBS buffer (pH=7.4) to homogenize any biofilm and form a single cell bacteria suspension for plating. The PBS solution was serially diluted, plated on LB agar plate and incubated overnight at 37 ^{o}C. The other was stained with Live/Dead BacLight Bacterial Viability Kit (Life technologies, Grand Island, NY) to obtain images via fluorescence microscopy (Olympus IX71, Center Valley, PA) using Fluorescence Illumination System (X-Cite 120, EXFO), filters for SYTO-9 (ex. 488 nm/em. 520 nm), and propidium iodide (ex. 535 nm/em. 617 nm). Experiments were conducted in triplicate.[16,33]

2.8 Statistical Analysis

Data for all experiments are reported as mean ± SEM (standard error of the mean). Statistical significance between the control and SNTDM impregnated SR catheters was determined using a student's t-test. Values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1 Nitrosation and characterization of SNTDM

Nitrosation of thiols has found considerable success using acidified nitrite. t-$BuNO_2$ is commonly employed for the nitrosation of thiols that are not soluble in aqueous conditions, as it readily dissolves in organic solvents, forming a homogeneous solution with the thiol starting material. Yields are typically quantitative and require minimal purification. Following nitrosation, the byproduct, *tert*-butanol along with residual $tBuNO_2$ can be removed by room temperature rotoevaporation. Their relatively low boiling points do not require higher temperatures that would jeopardize RSNO purity and yields. These conditions proved useful for synthesizing SNTDM; however, Et_2O was used as the solvent rather than the more common DMSO. Et_2O's low boiling point facilitated removal, which was crucial as neat SNTDM was required for subsequent experiments.[24,25,34,36]

Given RSNOs' inherent thermal and photolytic instability coupled with the often lengthy preparation times for experiments in this research, being able to determine SNTDM's concentration at various time points was paramount. The most common way to measure RSNO concentrations is by detection of their n_0->π^* transition (320-360 nm). Despite this, different molar absorption coefficients are frequently reported for the same RSNOs, as determining them is difficult due to decomposition during experiments. For example, extinction coefficients for GSNO's absorbance at 335 nm have been reported as 586, 767, and 922 $M^{-1}cm^{-1}$.[34-40]

Consequently, when determining the molar absorptivity, maximum RSNO purity is essential. The extent of nitrosation was initially determined using chemiluminescence, the known gold standard for quantitative NO (and thus RSNO) measurements.[39] Immediately following purification, a SNTDM/DMSO solution was injected into an NOA cell containing a $CuCl_2$, cysteine, water/DMSO solution to facilitate NO release. ~99.8 +/- 3.5 % of SNTDM was accounted for via integration of NO release curves. This agreed well with UV/Vis measurements that were obtained concurrently and showed minimal DTDD to be present following synthesis.

Under thermal or photolytic decomposition, RSNOs dimerize to the corresponding disulfide. By starting with pure SNTDM, and shining a 100 W broad spectrum light to induce decomposition, we are able to quantitatively convert SNTDM to DTDD (2:1 stoichiometry).[17] This inverse relationship can readily be monitored by UV-Vis spectroscopy as seen in Figure 2. Consequently, SNTDM and DTDD concentrations can be rapidly determined which facilitates the included research.

RSNO Impregnation of Silicone Tubing

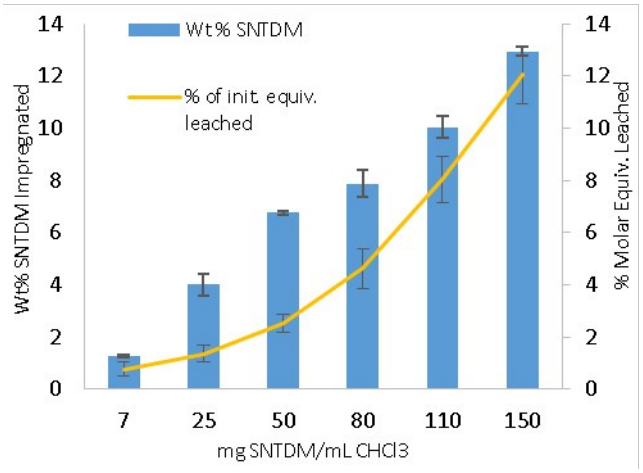

Fig. 3 The soaking solutions' SNTDM concentration affects the amount of impregnation, which in turn relates to leaching. The reported leaching levels are the final cumulative concentrations corresponding to the SNTDM/SR tubing's NO release lifetime.

SNTDM has previously shown potential for use as a medicinal NO donor. Giles *et al*. utilized SNTDM's photoactivity to controllably induce vasorelaxation and also induce cell death in A549 lung carcinoma cells. However, to date, SNTDM has not yet been studied for use as a NO donor in polymeric materials.[25,26] After determining suitable methods for RSNO/RSSR characterization, we developed conditions to efficiently impregnate SNTDM into silicone rubber, as it is one of the most common biomedical polymers and shares a nonpolar character with SNTDM/DTDD, thus increasing retention. Silicone rubber tubing was impregnated with SNTDM via a swelling technique using a $CHCl_3$ solution. This yielded a translucent green polymer with no visible phase separation (See Fig. 2S in ES†). $CHCl_3$ is known to be compatible with PDMS and swell the polymer by a 1.39 length ratio.[2,30] The tested tubing swelled by the same ratio, and returned to their original length following solvent evaporation (Fig. 1S in ESI†). There was no noticeable change to the silicone rubbers' mechanical properties following SNTDM impregnation, but more detailed quantitative testing will be required before any definitive conclusions can be made. Previously, Bayston *et al.* impregnated SR with conventional drugs such as rifampicin, trimethoprim, and spiramycin using similar swelling conditions with no detriment to the polymers' mechanical properties.[41] SNTDM's homogeneous impregnation can be observed by the green color that is visible throughout the length and cross section of the tubing (See Fig. 2S in ES†). This technique provides an effective method for high RSNO loading that is attractive for industrial use.[18,30,43-45]

Impregnation contrasts with dip-coating, another common method, which results in the NO donor being largely concentrated near the polymer's surface.[17] This may yield instability due to the RSNO molecules' close proximity to higher dielectric solution phase which facilitates decomposition and potentially decreases any polymeric "shielding" to light.[28] Increased RSNO concentration closer to the solution/polymer interface likely encourages leaching as well. Dip-coated catheters frequently require top-coats to increase stability and minimize leaching,[17] a process that increases the catheters' diameters, potentially making them less suitable for certain applications. With a CLogP of 5.31, SNTDM's lipophilic nature lends itself to swelling in hydrophobic polymers, whereas more polar RSNOs like *S*-nitroso-glutathione and *S*-nitroso-*N*-acetyl-penicillamine have less affinity for hydrophobic polymers such as silicone rubber.[17]

Different amounts of SNTDM were impregnated by altering the concentration of SNTDM in the soaking solution. Various soaking solutions with differing concentrations (ranging from 7 - 150 mg SNTDM/mL $CHCl_3$) were examined (Fig. 3). SR tubing soaked in a 50 mg/mL solution resulted in 6.8 wt% impregnation. These samples provide a NO flux above physiological levels for more than 3 weeks and exhibit very low leaching.

Tubing with 1.3 and 4.0 wt% SNTDM levels leach SNTDM and the disulphide product at only slightly lower rates than the 6.8 wt% loaded SR tubing (Fig. 3) and exhibit shorter NO release duration. Therefore, SR tubings impregnated with 6.8 wt% SNTDM were used for the majority of subsequent experiments, as they combined low leaching levels with a longer NO release duration.

Fig. 4 shows the NO release profile for various SR tubing with different wt% SNTDM loading during the initial days following preparation and soaking at physiological pH and temperature. All the SNTDM impregnated SR tubings reach a fairly steady-state NO flux after ~10 min. This is in contrast with SR impregnated with SNAP, which requires more than 30 min to achieve a constant flux.[18] The ease of varying the RSNO's concentration within the polymer potentially allows its use for a diverse range of applications.

Long-term NO Release From SR Tubing

Within blood vessels, the levels of NO produced by endothelial cells never reach the surface of IV catheters, etc. owing to the rapid consumption of NO by oxyhemoglobin. Consequently, longer term use of polymeric devices is often associated with increased thrombus and biofilm formation, leading to a greater risk of an embolism and infection.[16,17] By liberating NO at their own surfaces, these risks associated with catheter devices can be reduced, allowing their extended use.[17,18] As shown in Fig. 5, the SR tubing impregnated with 6.8 wt% SNTDM provides an NO flux exceeding or comparable to physiological levels for more 26 d under physiological conditions. RSNOs commonly exhibit a "burst release" of NO, as seen in Figure 5.

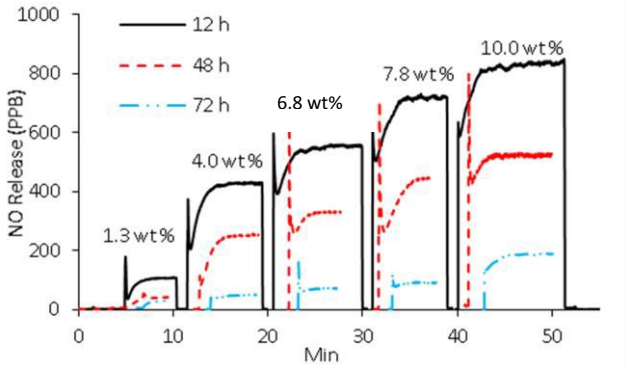

Fig. 4 Initial NO release in parts per billion (ppb) for SR tubing impregnated with various wt% SNTDM (S.A. 0.42 cm2). SNTDM/SR adopts a steady-state flux quickly, taking less than 10 min.

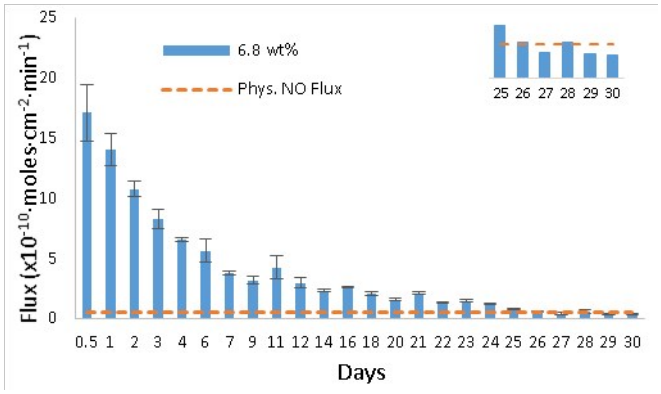

Fig. 5 Long term NO flux for the 6.8wt % loaded SR tubing stored under physiological conditions (37 °C, PBS + EDTA buffer, in dark).

In fact, SNAP impregnated silicone Foley catheters were recently reported to have a 4-fold greater NO flux on day 1 than on day 2 (in contrast with SNTDM's behaviour which was less than a 2-fold difference).[11] This phenomenon doesn't pose a toxicity concern for intravascular catheters since excess NO is rapidly consumed by the surrounding oxyhemoglobin in blood. Therefore, an intravascular catheter's therapeutic window is maximized, accounting for the entire duration of NO release (at least 26 days). Further, NO is not foreign to the urothelium, as it is naturally produced and released by urothelial and other neighbouring cells;[46] however, to the best of our knowledge, the normal NO flux has not been measured from urothelial cells. Consequently, it is not possible to make a direct conclusion regarding the effects of the burst release on the urothelium. The effects of long-term exposure to high concentrations of SNAP (2.5-5 mM) has been shown to decrease transepithelial resistance; however, following a washout of SNAP the effects were reversed.[46] In future work, It will be necessary and interesting to determine how the levels and duration of NO release affect the urothelial function and integrity.

Leaching Tests:

Many NO donors have been studied within polymers, but leaching remains a significant problem for non-covalently bound molecules.[17] Leaching decreases the effectiveness of NO releasing polymers for localized treatments because the NO donors not retained in the polymer will release NO at undesired locations and times. Seabra *et al.* improved GSNO's lifetime by dispersing it throughout PVA and PVPD blended films. Unfortunately, 90% of the GSNO was released during the first 24 h when the material was exposed to physiological conditions.[47] In the case of RSNOs, potentially leached species include the original RSNO and the corresponding disulfide, formed within the polymer or in the soaking solution. Shining a broad spectrum halogen light on the soaking solutions can assure complete conversion of any RSNO to RSSR. This allowed for the detection of any RSSR to account for all leached species. In comparing the DTDD content with the initial amount of SNTDM impregnated, approximately 2.5% of the impregnated RSNO leached from the SR tubing during the 30 d NO release measurements (Fig. 5). Measurements following one day of soaking indicated ~2% had been released. The larger initial release is likely due to the effect of higher water content within the SR polymer phase at the outermost surfaces of the material.[16,42] Leaching during subsequent days was quite minimal, thus requiring the daily soaking solutions to be combined before extraction and concentration in $CHCl_3$ in order to quantitate the levels of the thiol dimer. The 2.5% leach rate determined for SNTDM from SR over 30 d is much lower than the leaching of SNAP or other RSNO's reported in similar experiments.[17,18] SNTDM's highly lipophilic nature likely accounts for its retention, particularly within a lipophilic polymer like silicone rubber which has a 1.2 +/- 0.3 wt% water uptake in the bulk of the polymer phase.[3]

Storage Stability

SNTDM's storage stability was tested under various conditions including as a neat material, in solution, and impregnated within silicone rubber. Tertiary alkyl substitution should render SNTDM more stable than other less substituted RSNOs such as *S*-nitroso-cysteine or GSNO, NO donors whose biomedical use has been limited by low stability.[28,48] Giles and Kumari *et al.* recently reported on SNTDM's substantial photoactivity.[25,26] Previous research using SNAP has found its NO release profile to essentially not be affected by laboratory light (fluorescent).[17] In contrast, the SNTDM in SR samples show a 7-fold increase in flux upon turning on laboratory lights. Additionally, shining light from a 100 W broad spectrum halogen lamp at close proximity results in a ~60 fold increase of NO flux, relative to a dark room (see Fig. 6). After removing light sources the NO flux returns to the original baseline level.

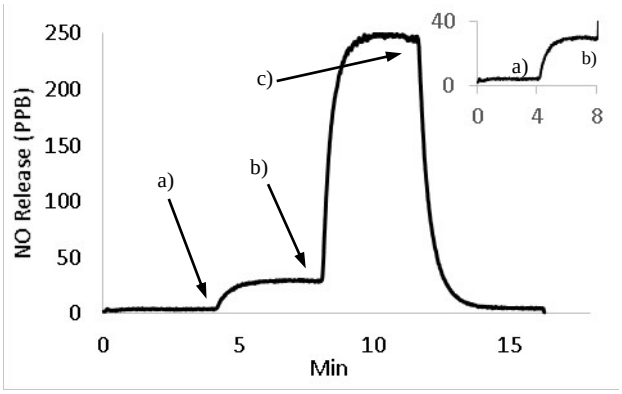

Fig. 6 SR tubing impregnated with 1.3 wt% SNTDM (S.A. 0.42 cm^2) at 22.5 °C in the dark, with a) fluorescent light, with b) fluorescent light and a broad spectrum halogen lamp, and again, in the c) dark.

General storage stability in the absence of light is another important property of any potential NO release medical device. By incorporating SNTDM into SR tubing, its lifetime can be extended relative to the neat and solution phase samples (see Fig. 3s in ESI†), however, not to the extent to allow the SNTDM-doped SR tubing to be stored in ambient conditions. This is a limitation of the presented system, since SNAP has shown significant stability under ambient conditions. Recently, our group reported ~87% of the SNAP impregnated in SR to still be present after 8 months of storage in the dark.[11] While SNAP impregnated SR provides superior storage stability, one must also keep in mind that the higher levels of RSNO may not be beneficial since a larger portion of them will leach from the polymer. For clinical applications, SNTDM impregnated SR devices could be stored in foil packages containing desiccant at reduced temperatures to extend their lifetime.

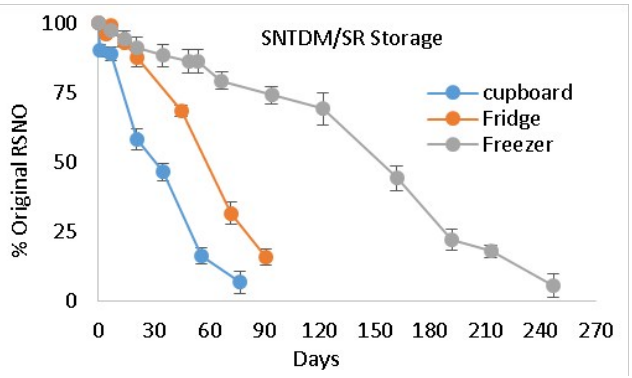

Fig. 7 SNTDM impregnated SR tubing stored in various environments

In contrast with its considerable photoactivity, SNTDM's tertiary substitution provides substantial thermal stability, likely due to the steric hindrance at the site of dimerization. This makes storage in dark environments viable (see Fig. 7). SNTDM/SR storage in the freezer provided the highest stability with 75% of the initial SNTDM remaining even after 3 months. As expected, SNTDM had a shorter lifetime during refrigerator and cupboard storage.

SR's stabilizing effect was more significant regarding photo induced decomposition likely because the disulfide product increased the polymer's opacity and impeded the penetration of incoming light. SNTDM/SR, neat, and $CHCl_3$ samples were stored in a freezer for the duration of their lifetimes. Within error, no stabilizing effects were observed by SR, nor were there statistical differences between the neat and solution phases (see Fig. 8).

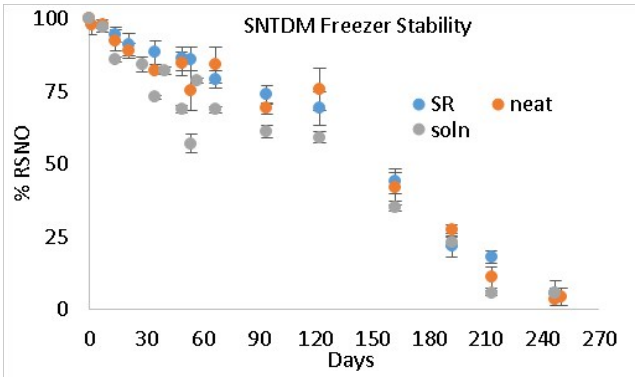

Fig. 8 Freezer storage for SNTDM in various phases.

EtO Sterilization

Given RSNOs' thermal or photo-induced decomposition, biomedical devices incorporating RSNOs are susceptible to sterilization procedures requiring high heat. To evaluate the SNTDM impregnated SR tubing's behaviour under applicable conditions, we submitted SR tubing segments impregnated with several different wt% levels of SNTDM to the University of Michigan hospital's sterilization facility to undergo EtO sterilization. Following the sterilization procedure, the NO flux levels from the samples were measured at different time points (See Fig. 4S in ESI†). The samples were stored in the dark while submerged in PBS containing EDTA between measurements so as to mimic physiological conditions. All tubing segments exhibited NO fluxes above physiological levels for several days, with even the 4 wt% tubings lasting 5 d. The 6.8 wt% tubings lasted ~6 d, while the higher concentrations released NO above physiological flux for 8 d. Although care was taken to minimize light exposure, the samples were not sterilized immediately following their submission, and several days elapsed before the procedure was performed. Consequently, the SR tubing's NO release duration would likely be extended were this not the case.

Despite the reduced SNTDM levels following EtO sterilization, recent work within our group has discovered that even lower levels of NO flux (e.g., 0.3 x 10^{-10} mol $cm^{-2}min^{-1}$) are able to produce substantial antimicrobial effects on silicone rubber surfaces against *Pseudomonas aeruginosa.* Hence, it is anticipated that the EtO sterilized SNTDM impregnated catheters will still have considerable antimicrobial activity despite loss of active SNTDM during this sterilization process. Other sterilization techniques are also possible and remain to be explored, including methods which do not require elevated temperatures such as gamma and glutaraldehyde sterilizations.

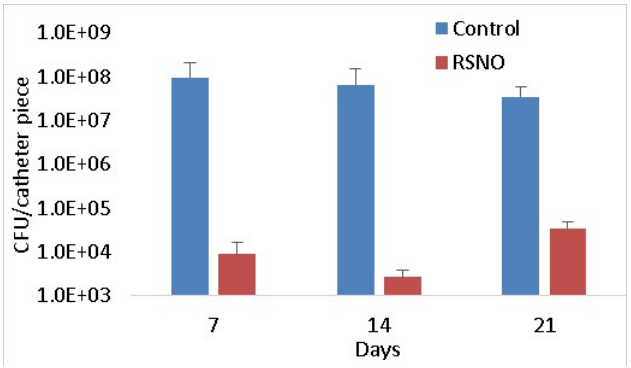

Fig. 9 *S. aureus* levels determined from the SR tubing pieces' homogenized solution cell counts. n=3 for each day. ($P < 0.01$)

Anti-biofilm Activity

Although biomedical devices are essential for medical care, microbial infections remain a serious concern. Bacteria bear attached to implanted medical devices. Biofilm formation often coincides with this colonization and complicates medical procedures by further increasing the risk of infection. Biofilm formation can decrease the effectiveness of antibiotics and hinder opsonophagocytosis, thus leading to chronic infections.[49]

For bloodstream catheters, if the devices emit NO at levels similar to endothelial cells, there is zero risk of any toxicity to tissue

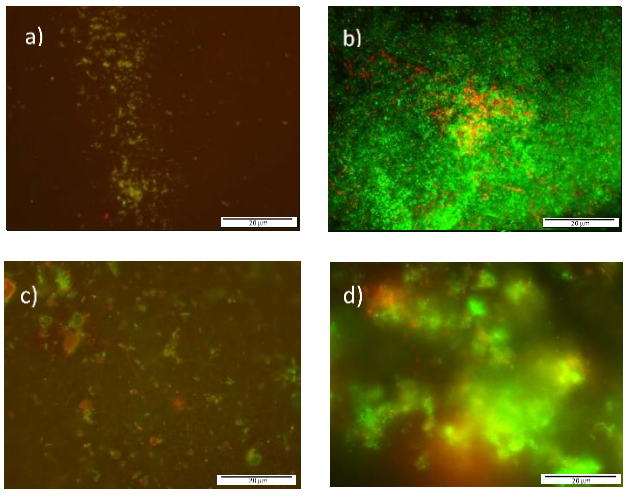

Fig. 10 (a) SNTDM impregnated and (b) control silicone rubber tubing images after 7 days demonstrating NO's effect at reducing *S. aureus* levels. (c) SNTDM and (d) control tubing images from day 21 indicating difference in biofilm formation.

cells, since the liberated NO is immediately scavenged by excess oxyhemoglobin in the blood. Therefore, we tested our SNTDM/SR system against *Staphylococcus aureus*, a microbe responsible for a range of infections, using a CDC biofilm reactor to determine whether NO release from the surface of the tubing was effective in minimizing bacterial growth and biofilm formation.[49] The bioreactor emulates *in vivo* biofilm forming conditions. Tubing segments were suspended in flowing media containing *S. aureus* and incubated at 37 ^{o}C. Periodically, the SR tubing pieces were vortexed in PBS solutions to remove and homogenize bacteria/biofilms. The solutions were serially diluted before plating them on agar. Colonies were incubated overnight and then counted. After 1 week, the NO releasing tubings had 4 orders of magnitude less *S. aureus* relative to controls (see Fig. 9). After 14 days, the control bacteria levels had remained constant, within error, while the SNTDM/SR tubing had a further reduction in amount of live bacteria. Even after 3 weeks, the SNTDM doped tubings had 3 orders magnitude less live bacteria on their surface than the controls, indicating the NO had killed or inhibited growth of 99.9% of the bacteria (See Fig. 9). Portions of the tubings were analysed with fluorescence microscopy at the same time, and the image results further supported the SNTDM/SR's antimicrobial properties (see Fig. 10), including the ability to reduce biofilm formation on the surface of the NO release tubing. The catheters' outer surfaces were examined because they are in more direct contact with the bacterial culture when in the bioreactor. Since the NO flux from the catheters' inner and outer surfaces are equivalent, it is expected that the same level of antimicrobial activity will be observed on each surface.

When tubing pieces were removed for cell counting and biofilm testing, pieces were also tested via NOA measurements to compare NO flux before and after the CDC experiments (see Fig. 5S in ESI†). Before the CDC experiment, a set of tubing pieces had comparable NO fluxes with the oven samples stored at the same temperature. This also held true for the samples measured on days 14 and 21. By the end of the 3rd week, the tubings in the CDC were releasing NO below physiological levels (0.33 +/- 0.12 vs. 0.5). Despite this, they still exhibited a 99.9% reduction in live *S. aureus* bacteria on their surfaces, thus indicating their effective window of treatment can potentially extend further than originally expected.

4. Conclusions

In summary, *S*-nitroso-*tert*-dodecyl mercaptan has been examined for its use as a long-term NO donor within silicone rubber tubing. A solvent swelling/impregnation method has proven useful for impregnating SNTDM at ranges from 1.3–12.9 wt% within the tubing. When impregnated with 6.8 wt% SNTDM, the tubings exhibit long term NO release, lasting more than 3 weeks above the normal physiological flux that occurs at the endothelium/blood interface. SNTDM's lipophilicity combined with silicone rubber's hydrophobicity provides a low leaching system in which >97% of the original molecule is retained within the polymer after 3 weeks of soaking. Given SNTDM's tertiary substitution, the impregnated SR tubings exhibit reasonable stability during storage in a freezer, retaining 75% SNTDM after 3 months. Due to SNTDM's thermal stability, 7.9 and 10 wt% impregnated SR tubings are able to release NO for 8 d above physiological flux levels following EtO sterilization. This level could likely be increased following further optimization. During a 3 week incubation in a CDC bioreactor, the impregnated tubings reduced surface levels of *S. aureus* by 4 orders of magnitude during the first 2 weeks and 3 orders after the third week. These results confirm the potential for SNTDM impregnated SR materials to improve the biocompatibilty of medical devices such as urinary and intravascular catheters for at least 3 weeks. Current efforts are focused on exploring the effect of incorporating SNTDM in other biomedical grade polymers, especially with respect to photoactivated NO release applications (e.g., to create a source for inhaled NO gas, etc.). In addition, efforts to improve the shelf life of this new NO release polymer using additives to help stabilize the NO donor are underway. Lastly, a proprietary polyurethane from Braintree Scientific, Inc. (Braintree, MA) was discovered very recently to have substantial stabilizing effects regarding both photolytic and thermal NO release from SNTDM impregnated into this material. Therefore, SNTDM's behavior in alternate biomedical polymer matrices warrants considerable investigation.

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