**Supporting Information:**

Origin of Long-Term Storage Stability and Nitric Oxide Release Behavior of CarboSil Polymer Doped with S-Nitroso-N-acetyl-D-penicillamine

Yaqi Wo a, Zi Li a, Elizabeth J. Brisbois b, Alessandro Colletta a, Jianfeng Wu c, Terry C. Major b, Chuanwu Xi c, Robert H. Bartlett b, Adam J. Matzger a, Mark E. Meyerhoff a,*

a Department of Chemistry, b Department of Surgery, University of Michigan Medical Center and c Department of Environmental Health Sciences, University of Michigan, Ann Arbor, MI 48109, USA

*Corresponding Author

Dr. Mark E. Meyerhoff
930 N. University Ave.
Ann Arbor, MI 48109
Telephone: (734) 763-5916
E-mail: mmeryho@umich.edu
**EXPERIMENTAL DETAILS:**

**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) and N,N-dimethylacetamide (DMAc) were purchased from Sigma-Aldrich (St. Louis, MO). N-Acetyl-D,L-penicillamine disulfide (NAP disulfide) was purchased from Enzo Life Science, Inc. (New York, NY). Methanol, hydrochloric acid, sulfuric acid, Luria Bertani (LB) broth and LB agar were obtained from Fisher Scientific (Hampton, NH). CarboSil 20 80A was obtained from DSM Biomedical Inc. (Berkeley, CA). Elast-eon 5-325 (E5-325) was from AorTech International plc (Scoresby, Victoria, Australia). Dow Corning RTV 3140 silicone rubber (SR) was a product of Ellsworth Adhesives (Germantown, WI). An Agilent ZORBAX rapid resolution high definition (RRHD) Eclipse Plus C18 column (2.1 x 50mm, 1.8 µm particle size) was purchased from Altmann Analytik GmbH & Co.KG (Munich, Germany). All aqueous solutions were prepared with 18.2 MΩ-deionized water using a Milli-Q filter from EMD Millipore (Billerica, MA). Phosphate buffered 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. S. aureus ATCC 25923 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA).

**SNAP Synthesis:**
SNAP was synthesized as previously reported.\(^1\),\(^2\) In brief, an equimolar ratio of NAP and sodium nitrite was added to a 1:3 mixture of water and methanol containing 2 M H\(_2\)SO\(_4\) and 2 M HCl. The reaction vessel was cooled in an ice bath for 5 h and the green SNAP crystals precipitated. The crystals were collected by vacuum filtration, washed by iced DI water and allowed to air dry for 24 h before being storage at -20 °C. The entire synthesis process was performed in the absence of ambient light.

**Polymer Film Fabrication:**
Polymer films containing different wt% SNAP (1-15 wt%) were prepared by solvent evaporation, based on a modified version of the previously reported method. Briefly, 3 different biomedical grade polymers (CarboSil 20 80A, Elast-eon 5-325 and Dow Corning RTV silicone rubber) were dissolved in THF to prepare the casting solution. For the 10 wt% SNAP films, 200 mg of CarboSil and E5-325 were dissolved in 3 mL of THF and SR was dissolved in 1 mL THF. Then, 22.5 mg of SNAP was added and dissolved into the polymer solution. After 5 min of stirring, the solutions were cast in Teflon rings (d=2.5 cm) on a Teflon surface, and the films were allowed to dry overnight under ambient conditions. Small film disks (d=0.7 cm) were cut from the parent films and weighed individually. These disks were then dip coated twice in non-SNAP containing topcoat solution; i.e. the CarboSil topcoat solution (200 mg polymer in 4 mL THF), the E5-325 topcoat solution (200 mg polymer in 4 mL) or the SR topcoat solution (800 mg polymer in 4 mL THF), respectively. The SNAP-doped polymer films that were subjected to Raman spectroscopy and powder X-ray diffraction (PXRD) studies were not coated with any topcoats in order to more easily study the chemical properties of the SNAP-doped CarboSil matrix directly. All films were dried under vacuum for additional 48 h to remove any residual THF before storage at -20 °C. All SNAP-doped films and SNAP containing solutions were protected from ambient light during preparation. The thickness of the SNAP-doped film layer was ca. 250 µm and the thickness of the two topcoat layers was ca. 45 µm, as measured with a Mitutoyo micrometer.

**Preparation of SNAP-doped CarboSil Catheters**

The SNAP/CarboSil solution was prepared by dissolving SNAP (450 mg) and CarboSil (1800 mg) in THF (18 mL). The CarboSil control solution consisted of CarboSil (2250 mg) in THF (18 mL). The topcoat solution was prepared with SR (1600 mg) in THF (8 mL). For the SNAP-doped CarboSil catheters, layers of SNAP/CarboSil were coated onto the stainless steel mandrels as the active coats and dried overnight before removal. Then, the SNAP/CarboSil catheters were then dipcoated in the SR topcoat solution (which coats both the inside and outside surfaces). The topcoats were prepared with two-minute intervals in between. For the CarboSil control catheters, coatings were applied in similar methods using non-SNAP containing CarboSil polymer solution and the SR top-
coating solution. For the dripflow biofilm experiments (*in vitro*), the final catheters had an i.d. of 2.0 mm and an o.d. of 4.0 mm (SR : CB : SR = 7:30:7). However, because of the size limit of rabbit vein, catheters with 1.1 mm i.d. and 2.2 mm o.d. (SR : CB : SR = 3:10:3) were fabricated for the *in vivo* experiments.

**Polymer Water Uptake:**
Control films (without SNAP) made from the 3 different biomedical grade polymers (CarboSil, E5-325 and SR) were cast as described above. The weight of each film was recorded before immersing them in 1 mL DI water for 48 h at 37 °C and after taking them out and wiping the surface dry. The polymer water uptake was calculated and represented as weight percent: wt% = (W\text{after} – W\text{before})/(W\text{before}) x 100, where W\text{before} and W\text{after} are the weights of the same film before and after soaking.

**Shelf-life Storage Stability Study:**
To simulate the harsh environments that could potentially occur during actual storage and shipping, 10 wt% SNAP-doped CarboSil/E5-325/SR films were prepared and stored in amber vials with desiccant at 37 °C. After various time points over an 8-month period, the total NO release of the films were measured by the NOA to determine the wt% of SNAP remaining in the film, as compared to the initial amount of SNAP. Films were cut into smaller pieces and immersed in a clear glass vial containing 50 mM CuCl\textsubscript{2} and 10 mM L-Cysteine, which led to the rapid catalytic decomposition of SNAP and the release of NO. Meanwhile, a 100 W halogen floodlight (GE model 17986), a broad-spectrum light source, was placed 20 cm away from the sample vessel and utilized to further enhance the NO release rate via a photoinitiated decomposition process. The corresponding NO release measured by a Sievers chemiluminescence Nitric Oxide Analyzer (NOA) 280i (Boulder, CO). The total moles of NO released was integrated and used to calculate the amount of SNAP remaining in the films.

**Ethylene Oxide (EtO) Sterilization:**
Ten wt% SNAP-doped CarboSil/E5-325/SR films were prepared and sent to the University of Michigan hospital sterilization facility for ethylene oxide treatment, which
is a standardized procedure for many devices used in clinical applications. Briefly, the films went through 1 h of preconditioning and humidification process, followed by 2-3 h of EtO gas exposure, both performed at a high temperature (54 °C) and high humidity environment (40-80 %). After the 1-2 h of EtO gas evacuation (54 °C), the films were subjected to 12 h of air washes (54 °C). The amount of SNAP remaining in the films was determined as described above.

**UV-Vis:**
All UV-Vis spectra were recorded in the wavelength range of 250 nm - 650 nm with a UV-Vis spectrophotometer (Lambda 35, Perkin-Elmer, MA) at room temperature. The molar absorptivity of SNAP in PBS at 340 nm was determined as $\varepsilon_{\text{SNAP}} = 1075 \text{ M}^{-1} \text{ cm}^{-1}$. The characteristic absorbance at 340 and 590 nm correlated to the $\pi \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ electronic transitions of the S-NO functional group.$^{2-6}$

**Polarized Optical Microscopy:**
Five wt% SNAP-doped CarboSil films and a blank CarboSil film, both without topcoats, were prepared as described above. Optical images were captured by a Leica DMLP polarization microscope equipped with an N Plan 20× objective under crossed polarizers in combination with a quarter-wave plate, and were then taken with a SPOT Flex Mosaic 15.2 camera using SPOT 5.2 Software from Diagnostic Instruments, Inc.

**Mathematical Derivation of SNAP Solubility:**
Based on the following assumptions that (1) crystalline SNAP is uniformly distributed in CarboSil and that (2) the preferred orientation of SNAP crystals in CarboSil could be eliminated by cutting samples into cubes and rotating the samples during PXRD characterization, the ratio of peak areas (at a chosen $2\theta$ angle) of a specific SNAP PXRD peak over the total area of the obtained pattern would be proportional to the weight percentage of crystalline SNAP in the sample. In fact, by using the area ratio as a quantitative representation, all the other factors that could influence the peak area (e.g., the volume of the sample irradiated by the X-ray source, the exposure time of sample under the X-ray, etc.) can be eliminated. Here, we set the doped SNAP weight
percentage as $x$ and the ratio of the $k^{th}$ orthorhombic SNAP peak area over the SNAP-doped CarboSil pattern total area as $y_k$, and then $y_k$ can be calculated as:

$$y_k = \frac{k^{th} \text{ SNAP peak area}}{\text{total SNAP peaks area} + \text{CarboSil pattern area}} = \frac{a_k(x - x_o)}{a(x - x_o) + b(1 - x)}$$

For a PXRD pattern of a sample with a unit volume and taken with a unit exposure time, $a_k$, $a$ and $b$ correspond to the area of the $k^{th}$ peak in the orthorhombic SNAP pattern, the total area of all orthorhombic SNAP peaks, and the total area of blank CarboSil pattern, respectively. The term $x_o$ is the SNAP solubility in SNAP-CarboSil solid solution system in the percentage representation. As a result, for any SNAP-doped CarboSil sample, $a_k(x - x_o)$ represents the area of the $k^{th}$ orthorhombic SNAP peak in the pattern, $a(x - x_o)$ represents the area of all signals from orthorhombic SNAP, and $b(1 - x)$ represents the area of all signals from CarboSil in this sample. Since $y_k$ is a ratio of areas, which is independent of sample volume and exposure time, etc., the only factor that influences $y_k$ is the SNAP weight percentage $x$ in the prepared CarboSil films. By substituting various $x$ and $y_k$ at chosen $2\theta$ angles (three main SNAP peaks, $2\theta = 9.5, 14.5, 14.9$), $a_k$, $a$, $b$ and $x_o$ can be determined. The $a$ values calculated using three different SNAP peaks were 0.099, 0.082 and 0.102, respectively, and the $b$ values were 12.996, 12.934 and 12.985, respectively. As both values are within the error of tolerance, it suggests that the derived equation and the fitting model are successful quantitative representations of the doped polymer system.

**Raman Mapping:**

Raman mapping characterization in the low wavenumber regions, where SNAP and polymer peaks can be distinguished readily, was conducted on a randomly selected $32 \mu m \times 24 \mu m$ rectangular regions of the SNAP-doped CarboSil film cross section, with $1 \mu m$ steps in both the x and y dimensions to create a fine grid. Raman spectra of all steps were compared with the orthorhombic SNAP spectrum using direct classical least squares (DCLS) analysis.  

**In vitro characterization of SNAP-doped CarboSil catheters against microbial biofilm**
A drip-flow biofilm reactor system (Biosurface Technologies Corp., Bozeman, MT), which mimics the catheter environment and bacteria growth condition (at air-liquid interface) in vivo, was used to test the antibiofilm properties of SNAP/CarboSil catheters against biofilm formed by Staphylococcus aureus ATCC 25923. The catheters were secured in the center of the bottom surface. The biofilm chambers were first inoculated with 10 mL of bacteria culture (diluted with overnight culture, ~1 x 10^6 CFU/mL) for 1 h to allow bacterial cell adhere on the catheter surfaces. Then the biofilm chambers were supplemented with continuous sterile nutrient medium (10% Luria Broth) at the flow rate of 100 mL/h (controlled by a peristaltic pump) for 7 days at 37 °C. Finally, the catheters were aseptically removed and each catheter was cut into two 1 cm pieces, which was used for plate counting and imaging, respectively. For plate counting, the catheter segment was homogenized (OMNI TH, Omni International, Kenesaw, GA) for 30 s in 2 mL of 10 mM sterile PBS (pH 7.4) in order to disintegrate the biofilm to single cell suspension, which was later diluted by 10-fold each time and plated onto LB agar plates. For imaging, the catheter segment was stained with fluorescent dyes by using Live/Dead BacLight Bacterial Viability kit (Invitrogen, Carlsbad, CA) for 20 min in the dark, exactly per the kit’s instructions. Fluorescent images were acquired with an inverted fluorescence microscope (Olympus IX71, Center Valley, PA) equipped with Fluorescence Illumination System (X-Cite 120, EXFO) and filters for SYTO-9 (excitation = 488 nm/emission = 520 nm) and Propidium Iodide (excitation = 535 nm/emission = 617 nm). Images were obtained using an oil immersed 60× objective lens, in which red indicates dead bacteria while green indicates live ones.

**In vivo evaluation of SNAP-doped CarboSil catheters in rabbits**

All animal handling and surgical procedures employed in this research were approved by the University Committee on the Use and Care of Animals in accordance with university and federal regulations. A total of 3 New Zealand white rabbits (Covance, Battle Creek, MI) were used in this study. All rabbits (2.5-3.5 kg) were initially anesthetized with intramuscular injections of 5 mg/kg xylazine injectable (AnaSed® Lloyd Laboratories Shenandoah, Iowa) and 30 mg/kg ketamine hydrochloride (Hospira, Inc. Lake Forest, IL). Maintenance anesthesia was administered via isoflurane gas inhalation at rate of 1.5-3%
by mechanical ventilation via a tracheotomy and using an A.D.S 2000 Ventilator (Engler Engineering Corp. Hialeah, FL). Peek inspiratory pressure was set to 15 cm of H2O) and the ventilator flow rate was 8 L/min. To facilitate the maintenance of blood pressure stability, IV fluids of Lactated Ringer’s were given at a rate of 10 mL/kg/h. In order to monitor blood pressure and to collect intermittent blood samples for analysis during the experiment, the rabbit’s right carotid artery was cannulated using a 16-gauge IV angiocatheter (Jelco®, Johnson & Johnson, Cincinnati, OH). The blood pressure and derived heart rate were monitored by a Series 7000 monitor (Marquette Electronics Milwaukee, WI) while the animal body temperature was monitored with a rectal probe and maintained at 40 °C using a water-jacketed heating blanket. Sample blood gas analysis (arterial blood pH, PCO₂, PO₂, total hemoglobin and methemoglobin) was conducted using an ABL 825 blood-gas analyzer and an OSM3 Hemoximeter (Radiometer Copenhagen, DK). Prior to the placement of catheters, the rabbit left and right external jugular veins were isolated. Five cm lengths of the catheters (one SNAP and one control) were inserted into the veins. The animals were not treated with anticoagulant systemically during the experiments.

During the experiment, the mean arterial pressure (MAP) of the rabbit was maintained at 38 ± 2 mmHg for 7 h by continuous IV fluid maintenance. The heart rate average was 228 ± 4 beats/min and no significant change was noted for the duration of experiments. The blood gas was measured once every hour and the results were all within the normal ranges.

After 7 h of catheter implantation, all animals were first given (400 U/kg) sodium heparin (APP Pharmaceuticals, LLC Schaumburg, IL) systemically to prevent thrombosis during necropsy and were then euthanized with a dose of Fatal Plus (130 mg/kg sodium pentobarbital) (Vortech Pharmaceuticals, Dearborn, MI). The jugular veins were clamped and the catheters were carefully removed from the vein, leaving the thrombus intact on the catheter surface. After rinsing the catheter with saline, any residual thrombus was photographed and quantitated using Image J imaging software provided by the National Institutes of Health (Bethesda, MD).
Blood sampling

Rabbit whole blood samples were collected in non-anticoagulated 1 cc syringes for activated clotting times (ACT) analysis at the beginning of the experiments. The whole blood samples were also collected by 1 cc heparinized syringes (40 U/mL of sodium heparin) for blood gas analysis every hour for 7 h.
Figure. S1. The UV-Vis spectrum of 1mM SNAP dissolved in PBS buffer. The molar absorptivity of SNAP in PBS at 340 nm is $\varepsilon_{\text{SNAP}} = 1075 \, \text{M}^{-1} \, \text{cm}^{-1}$.

Figure. S2. The calibration curves of peak area vs analytes concentration. 1, 5, 10, 20, 40 and 60 µM standard solutions of SNAP, NAP and NAP disulfide were prepared using Miili-Q Millipore purified water (18.2 MΩ) and analyzed by LC-MS. Data are mean ± SEM (n=3).
Figure. S3. Optical image of (a) blank CarboSil and (b) 5 wt% SNAP-doped CarboSil film surface taken under crossed polarizers in combination with a quarter-wave plate. The 5 wt% film clearly shows patterns which suggest the presence of crystalline structures. The scale bars are both 100 μm.

Figure. S4. Raman spectra comparison of SNAP powder (black), blank CarboSil (blue) and 15 wt% SNAP-doped CarboSil (red). The existence of SNAP crystals in SNAP-doped CarboSil is verified by the characteristic peaks of crystalline SNAP between 500-600 cm⁻¹ (see inset).
Figure. S5. The PXRD patterns comparison for grounded SNAP powder, simulated orthorhombic SNAP powder\textsuperscript{10} and simulated monoclinic\textsuperscript{11,12} SNAP powder suggests that the SNAP crystal synthesized is orthorhombic. The consistent difference in peak positions of powdered SNAP and simulated orthorhombic SNAP patterns (20 values of powdered SNAP peaks are always slightly smaller than those of the simulated pattern) can be attributed to the difference in operating temperature. Powdered SNAP samples were tested at room temperature while the simulated patterns were based on single crystal XRD patterns taken at low temperature (-100 °F).
Figure. S6. (a) The orthorhombic SNAP crystal structure shown in 3D representation, suggesting that one SNAP molecule can form 4 intermolecular hydrogen bonds with 4 surrounding SNAP. (b) The elaboration of the number and position of the hydrogen bonds of one SNAP molecule in 2D schematic.

Figure. S7. Correlation of data obtained by powder X-ray diffraction for SNAP in CarboSil. Linear regression lines were fitted using least squares approach. The 3 most prominent orthorhombic SNAP peaks were chosen to do the fitting (2 theta = 9.5 (a), 14.5 (b) and 14.9 (c)) and the calculated SNAP solubility in CarboSil polymer was 3.6 wt%, 3.5 wt% and 3.9 wt%, respectively.
Figure. S8. PXRD pattern comparison between freshly prepared (blue spectra) and 10 d old (red spectra) of (a) 5 wt% and (b) 15 wt% SNAP-doped CarboSil films. The crystal peaks intensity of 5 wt% films (19.8 wt% SNAP remained) decreased significantly while the 15 wt% one has minimal decrease in intensity (83.2 wt% SNAP remained) after storage under ambient light at room temperature for 10 d.
Figure. S9. Long-term (28d) NO release from the 20 wt% SNAP-doped CarboSil catheter for antibiofilm studies (o.d. 4mm). NO release was measured in PBS via chemiluminescence at 37 °C.

Figure. S10 Representative NO surface flux profile from a section of the SNAP/CarboSil rabbit catheter for 7h (o.d. 2.2mm). NO release was measured in PBS via chemiluminescence at 37 °C.
Table. S1. The water uptakes of 3 biomedical grade polymer films. Blank polymer films were soaked in DI water for 48 hours at 37 °C. Water uptake was calculated in the percentage representation, wt % = (W_{after} – W_{before})/(W_{before}) x 100, where W_{before} and W_{after} are the weights of the same film before and after soaking. Data are mean ± SEM (n=3).

| Polymer               | Water Uptake [wt%] |
|-----------------------|---------------------|
| Silicone Rubber       | 1.5 ± 0.5           |
| Elast-eon 5-325       | 0.8 ± 0.2           |
| CarboSil 20 80A       | 0.7 ± 0.2           |

Table. S2. Stability of 10 wt% SNAP-doped CarboSil, SR and E5-325 films after ethylene oxide sterilization process. The SNAP remaining in the films after the sterilization were determined and compared with the initial level. Data are mean ± SEM (n=3).

| 10 wt% SNAP/CarboSil films | % SNAP remaining after EtO sterilization |
|----------------------------|----------------------------------------|
| Silicone Rubber            | 78.7 ± 3.1                             |
| Elast-eon 5-325            | 82.7 ± 3.8                             |
| CarboSil 20 80A            | 91.8 ± 3.2                             |
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