Unexpected Behavior of a Nitric Oxide-Releasing Polymer Dopant: A Study of Novel, Lipophilic Analogues of *S*-Nitroso-*N*-acetylpenicillamine in Solution, Crystalline, and Polymer Phases

Thesis by
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To EJT, CKS, and AEM, for shaping who I am today.

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ABSTRACT

The use of blood contacting medical devices, such as intravenous catheters, intravascular chemical sensors, and vascular grafts, is often associated with risks of blood clotting and bacterial infections. As nitric oxide (NO) displays both anti-thrombotic and antibacterial activity, NO-releasing materials effectively reduce these risks when used in conjunction with biomedical devices. One class of NO-releasing materials, *S*-nitrosothiols (RSNOs), release NO via thermal and light-catalyzed decomposition reactions and have been applied to physiological systems as therapeutic agents. In particular, polymers doped with *S*-nitroso-*N*-acetylpenicillamine (SNAP) have shown great promise for long-term NO release at physiological levels. However, SNAP/polymer formulations suffer from significant leaching of SNAP and the corresponding disulfide decomposition product from the polymer phase into the solution phase, posing a potential biohazard and decreasing the lifetime of localized NO release.

In response to this problem, two novel and lipophilic analogues of SNAP were synthesized and doped into hydrophobic polymer films. The modified RSNOs were hypothesized to have a lower water/octanol partition coefficient, resulting in increased interaction with the polymer. Unexpectedly, the lipophilic RSNOs showed much higher levels of leaching from polymer films when compared to analogous SNAP films, as well as much shorter NO release profiles. To investigate the source of these unexpected results, all three RSNOs were analyzed for their solution-phase and crystalline stability. Interestingly, RSNO lipophilicity appeared to have little effect on solution-phase stability, but had a strong, inverse relationship with crystalline stability.

CHAPTER 1

Introduction

1.1 Nitric Oxide

Since the discovery of nitric oxide (NO) as the endothelium-derived relaxing factor (EDRF) in 1987,[1] considerable research has focused on this radical, polar gas. The molecule is produced along with citrulline in the body from L-arginine and oxygen by enzymes known as NO synthases (NOS), as described in Figure 1.1. The NOS enzymes are categorized by three isoforms, all of dimeric structure: neuronal, inducible, and endothelial (NOS1, NOS2, and NOS3). NOS relies on multiple co-factors and co-enzymes in the production of NO, including calmodulin, tetrahydrobiopterin (BH_4), FAD, FMN, iron protoporphyrin IX, and NADPH.[2]

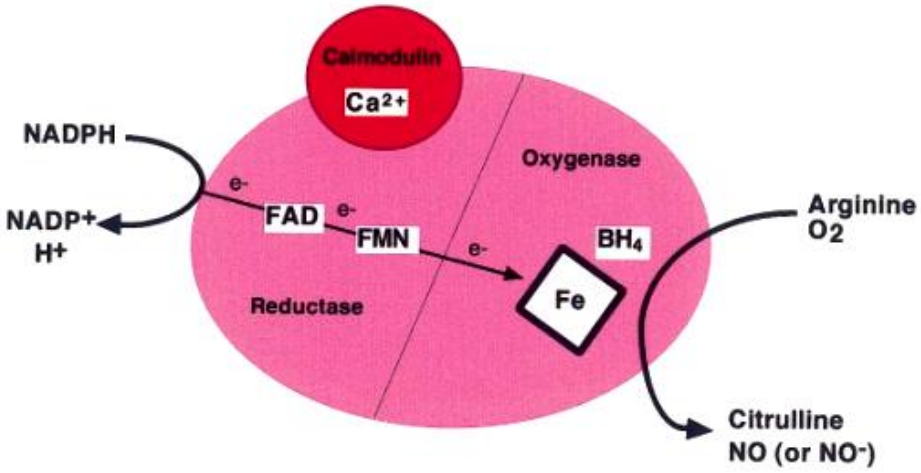


Figure 1.1. Pathway of NO production by NOS enzymes.[2b]

In the body, NO reacts quickly, usually within a 100 μm distance from its production, and usually does not exceed nanomolar concentration.[3,4] The molecule is implicated in many processes in the body, including (but not limited to) oxidative and nitrosative stress, cell-cell signaling, inhibition of viral replication, anti-microbial activity, vasodilation, smooth muscle relaxation, post-translational regulation of proteins, inhibition of platelet aggregation and adhesion, and both angiogenesis and apoptosis.[5] The effect of NO within the body is also highly dependent on concentration and location in the body.[6] For example, healthy endothelial cells produce NO with a flux of 0.5-4.0 $\times 10^{-10}$ mol cm^{-2} min^{-1},[7] sufficient variation to increase or down-regulate cell apoptosis at high and low concentrations, respectively (Figure 1.2).[7]

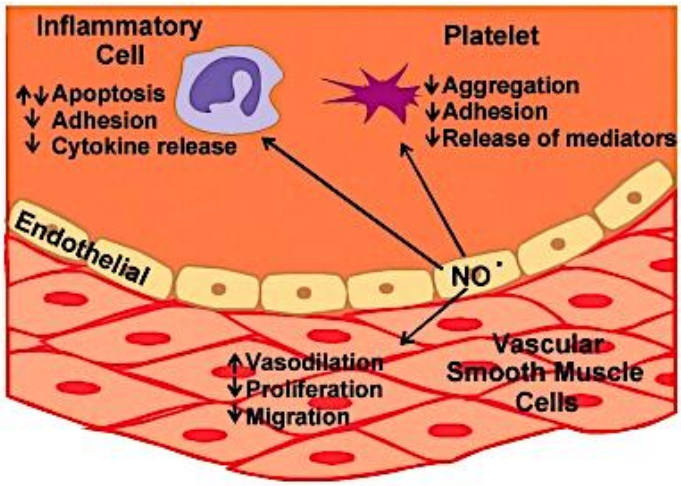


Figure 1.2. Summary of NO's role in the vascular endothelium and cellular activities.[7]

1.2 Biomaterials: Challenges and Previous Solutions

The field of biomaterials includes all materials that interface with biological systems. Commercial medical devices that are made of or coated with polymeric biomaterials include implantable sensors for important biological ions (e.g. K^+, Na^+), glucose, gases (such as oxygen

or carbon dioxide), or metabolites (such as lactate); intravenous and urinary catheters; and artificial prosthetics.[9,10] However, all biomaterials face significant obstacles, principally infection and host response to foreign surfaces. It is estimated that more than 1 million implant-related infections account for medical costs of more than $3 billion annually in the U.S.[1] Moreover, the number of infections will only increase as the number of patients receiving implants grows. For example, from 1996 to 2001, hip and knee joint replacements increased 14%.[12] In spite of modern implant sterilization techniques, patient infection can result from secondary factors, such as the ambient atmosphere, clothing worn by medical professionals, or host-contained bacteria.[13] Infection occurs over two phases: reversible adhesion of bacteria to a biomaterial surface over 1-2 hours after implantation, and irreversible molecular bridging between bacteria and the foreign material between 2-3 hours after implantation (Figure 1.3). Following the second phase of bacterial adhesion, many bacteria can form biofilms coated with polysaccharides that are extremely resistant to antibiotics after about 24 hours.[14]

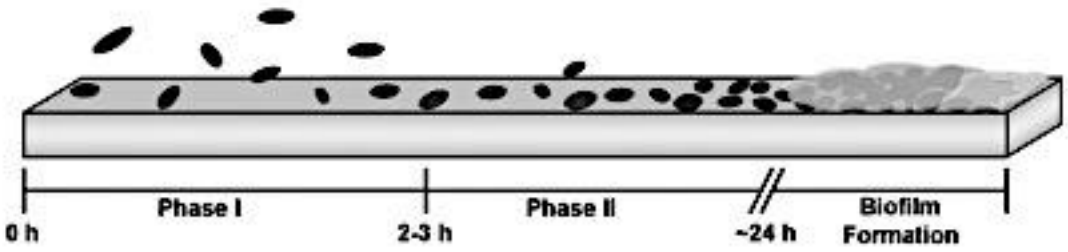


Figure 1.3. Progression of bacterial adhesion and infection of a biomaterial.[10]

Numerous strategies have been developed to reverse the progression of bacterial infection of implanted medical devices. "Passive" coatings, such as hydrophilic polyurethanes[15] or poly(ethylene glycol),[16] make bacteria-substrate interactions unfavorable but are limited in the efficacy and are largely species-dependent. Instead, most research has focused on active release of antibacterial agents. As mentioned above, antibiotic release is effective in reducing bacterial adhesion, but only before biofilm formation, requiring continuous drug release. Long-term release of antibacterial silver cations are effective in reducing bacterial adhesion of some species,[17] but can cause argyria.[18] Antibody-releasing coatings have also shown promise as antibacterial agents for *in vitro* assays, but there are few examples of effective *in vivo* antibody-releasing implants.[10]

Besides infection, physiological response is a significant obstacle to sustained implant function. When a foreign material is introduced to the bloodstream, coagulation is initiated, resulting in clotting that may change the local chemical environment of a sensor or block the flow from a catheter or other device (Figure 1.4).[9]

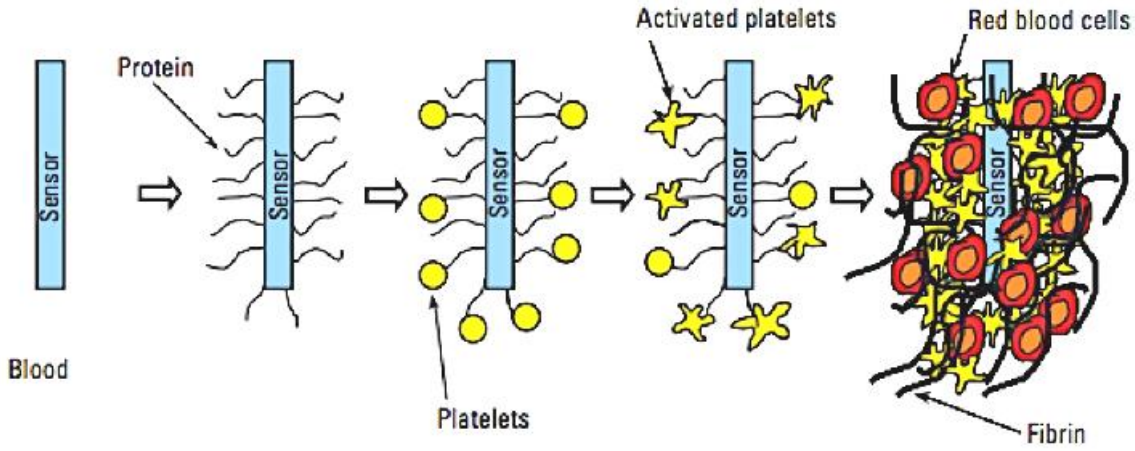


Figure 1.4. Clot formation on an intravascular sensor.[9]

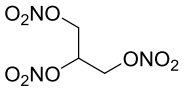
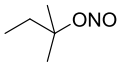
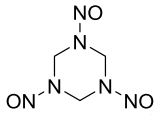
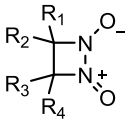
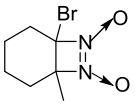
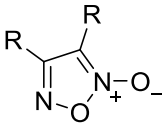
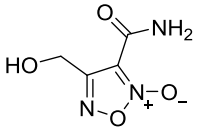
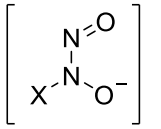
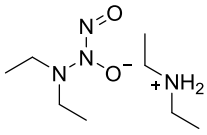
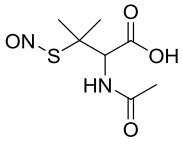
Like bacterial adhesion and biofilm formation, thrombotic response has traditionally been addressed with a range of passive polymer coatings (such as Nafion, polyurethanes, polyethylene glycol, hydrogels, and epoxies) that reduce inflammatory response or reduce adsorption of fibrinogen.[10,19] In addition, immobilized heparin has been used to prevent thrombosis on intravascular devices by inhibiting thrombin and factor Xa, two key inzymes in the coagulation process.[20] However, most of the results from enhanced polymer coatings show only short-term improvement, and coated, implanted catheters and sensors often display significant thrombosis.[9]

Nitric oxide is particularly well suited to combat both of these obstacles in biomaterials, as the molecule is produced endogenously as a potent antimicrobial agent in immune response and prevents platelet adhesion and activation in the body. Therefore, NO-releasing materials which are designed to generate NO at the same level as vascular endothelial cells have the potential to provide a truly a truly thromboresistant and anti-bacterial surface.[21] Additionally, because NO has a very short half-life, NO-releasing materials should avoid any systemic effects in the body as released NO is consumed near the surface of the biomaterial from which it is emitted.

1.3 Nitric Oxide Donors

Due to the applicability of nitric oxide to improving biomaterials, many classes of NO donors have been developed and studied (Table 1.1).[22,23] The largest body of research, however, has been dedicated to nitrogen-bound NONOates and RSNOs due to their ability to spontaneously release NO under physiological conditions without decomposition co-factors and low toxicity.

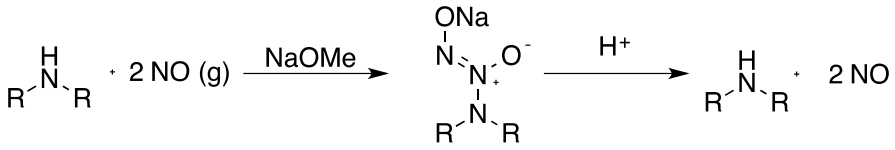
Table 1.1. Major Classes of NO Donors

Entry	NO Donor	General Structure	Example Structure
1	**Organic Nitrates**	R-ONO_2	 Glyceryl Trinitrate
2	**Organic Nitrites**	R-ONO	 Isobutyl Nitrite
3	**Metal-NO Complexes**	M-NO	$Na_2[Fe(CN)_5NO]$ Sodium Nitroprusside
4	***N*-nitrosamines**	R-NNO	 1,3,5-trinitroso-hexahydro-1,3,5-triazine
5	***C*-nitroso Compounds**	R-CNO	 2-Methyl-2-nitrosopropane
6	**Diazetine Dioxides**		 DD2
7	**Furoxans**		 CAS 1609
8	**Diazeniumdiolates**	 X = C, N, O, S	 Diethylamine NONOate
9	***S*-Nitrosothiols**	R-SNO	 *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP)

1.3.1 *N*-Diazeniumdiolates

One of the most widely studied classes of NO donors, nitrogen-bound NONOates were first discovered in 1961, although practical application of the functional group were not realized until at least 1988, when the important biological roles of NO were discovered.[24] NONOates are formed by the reaction of secondary amines with NO gas in the presence of a strong base. Subsequent NO release is driven by protonation of the secondary amine, releasing two equivalents of NO and regenerating the original amine (Scheme 1.1).[25]

Scheme 1.1. NONOate Formation and Decomposition



NONOates are attractive NO donors for the production of two moles of NO per mole of donor, and for the ability to control NO release by variations in pH. NONOates are very stable in basic solution due to the lack of protons to initiate NO release, while NO release can be accelerated by lowering the pH of the surrounding environment. However, NONOates face several drawbacks: NONOates are purely synthetic compounds, raising concerns about their biocompatibility, and are often too instable to be used beyond local NO delivery (i.e. target site delivery).[26b] Significant research has been performed to control NO release kinetics by changing molecular structure around the NONO functional group. As a result, a huge variety of NONOate structures have been synthesized with highly variable NO release kinetics. For example, diazeniumdiolated proline (PROLI/NO) has a short half-life of 2 seconds in physiological temperature and pH, while diazeniumdiolated diethyltriamine (DETA/NO) has a half-life of 20 hours in solution (Figure 1.5).[24]

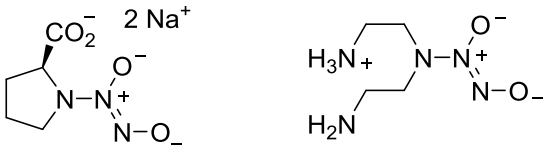


Figure 1.5. Structure of two NONOates with divergent NO release kinetics.

NONOates have been applied to a wide breadth of biological applications for both short- and long-term NO release. NONOates have been doped into polymers as anti-thrombotic or anti-microbial coatings for biomaterials such as vascular grafts,[1] intravascular catheters,[28] sensors,[29] and extracorporeal circuits.[30] The NONOate functionality has been covalently linked to macromolecular scaffolds to yield low-leaching polyurethanes,[21] glucose sensor coatings (as diazeniumdiolated dendrimers),[31] anticoagulant and antiplatelet agents,[32] and xerogels for use in sensors,[33] among other applications.[25,26]

1.3.2 *S*-Nitrosothiols

Whereas diazeniumdiolates are purely exogenous compounds, *S*-nitrosothiols are appealing NO donors in part because of their presence in the body. Characterized by the S-NO functional group, most of the body's NO content is stored in cells by endogenous RSNOs that may be in the μM range or even higher.[4] Several endogenous RSNOs have been identified (Figure 1.6), including *S*-nitrosogultathione (GSNO), *S*-nitrosocysteinyl glycine (CGSNO), *S*-nitrosocysteine (CSNO), and *S*-nitroso-albumin (AlbSNO). These endogenous reservoirs of NO play multiple roles in the body, including regulation of protein function, mediation of nitrosative stress, intracellular signaling, immune and vascular function, and antimicrobial cytotoxicity.[4,34]

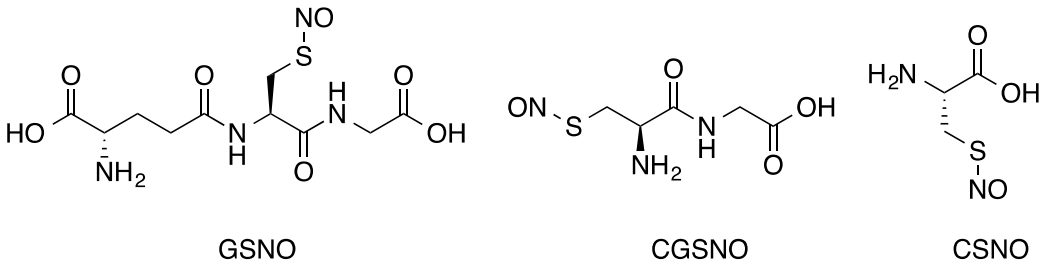
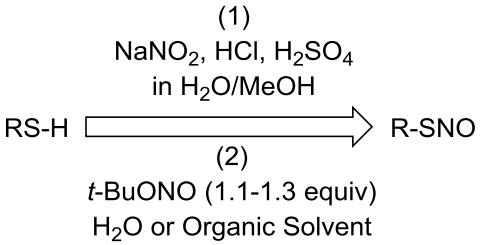


Figure 1.6. Structure of three endogenous RSNOs.

RSNOs can be formed from thiols by various methods. The two most commonly employed synthetic routes proceed by (1) addition of sodium nitrite to an acidified solution of the parent thiol, resulting in formation of the nitrous acid (a nitrosating agent),[35] or (2) reaction of thiols with a slight excess of *tert*-butyl nitrite in aqueous or organic solvent (Scheme 1.2).[36] The use of nitrous acid results in faster quantitative nitrosation of thiols compared to *tert*-butyl nitrite, however *tert*-butyl nitrite is appealing for its compatibility with organic solvents and water-insoluble thiols.[37]

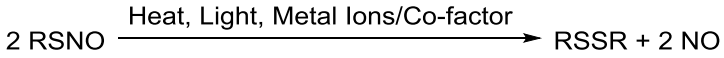
Scheme 1.2. Predominant Nitrosative Conditions for RSNOs



The decomposition of RSNOs has been widely studied to understand the effects of RSNO molecular structure and decomposition co-factors on RSNO stability. RSNOs undergo thermal decomposition at room temperature, photodecomposition by irradiation at 340 or 545 nm, and

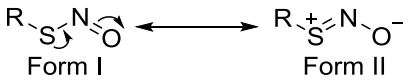
metal-catalyzed decomposition (including Cu^{+}, Fe^{2+}, Hg^{2+}, and Ag^{+}), yielding the disulfide product and releasing NO gas (Scheme 1.3).[22a] Other co-factors have been found to catalyze RSNO decomposition, such as endogenous reductants (e.g. ascorbate),[38] organotelluride,[39] organoselenide,[40] and low concentrations of other thiols.[41]

Scheme 1.3. Decomposition of RSNOs



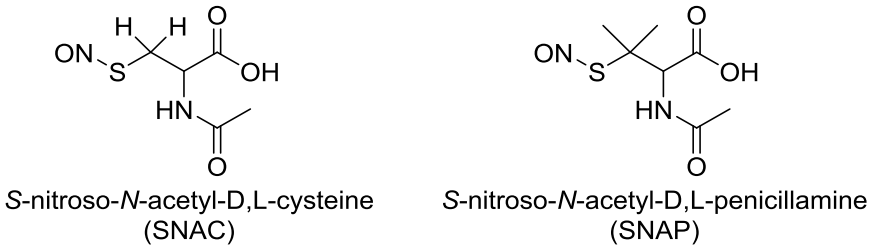
Besides species that catalyze RSNO decomposition, the molecular structure of RSNOs plays a large and often disputed role in determining NO release kinetics. Roy *et al.* postulated that the stability of RSNOs can be rationalized by the prevalence of two resonance structures (Scheme 1.4).[36] Form II provides strength to the S-N bond, preventing homolytic cleavage, the first step of thermal- and photodecomposition. Therefore, RSNOs that support form II more will have higher stability and slower decomposition.

Scheme 1.4. Two Resonance Structures of *S*-nitrosothiols



As an illustration, primary RSNOs such as *S*-nitroso-*N*-acetylcysteine (SNAC) have been reported to have lower thermal stability compared to tertiary RSNOs, such as *S*-nitroso-*N*-acetylpenicillamine (SNAP).[36] Roy *et al.* argues that the difference in stability is a result of the additional electron donation from the two geminal methyl groups in SNAP (Figure 1.7).

However, this reasoning is contradicted by other findings that SNAC displays higher stability than SNAP in acidic media.[42]



S-nitroso-*N*-acetyl-D,L-cysteine (SNAC)

S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP)

Figure 1.7. Structures of SNAC and SNAP.

Additionally, computational calculations of SNAC, SNAP, GSNO, and other RSNOs show that electronic structure does not play a large part in stability, and that the geminal methyl groups of SNAP do not significantly affect the S-NO bond strength compared to SNAC.[43] Instead, steric hindrance was found to have a much larger effect on stability by providing an energy barrier against formation of disulfide products in SNAP and other bulky RSNOs. While the relative stabilities of primary and tertiary RSNOs has been studied thoroughly, the effect of long-range structural modification of RSNOs has little presence in the literature. For instance, SNAC has increased thermal stability compared to CSNO, derived from the acetamido group present in SNAC. It has been postulated that the acetamido group forms a cyclic structure with the carboxyl oxygen that stabilizes the electron-deficient sulfur, but this suggestion remains unsupported by theoretical calculations.[36,44]

Endogenous and synthetic RSNOs have been used in a wide variety of applications, primarily in creating new biomaterials.[26] Blood-contacting devices that generate or release NO effectively reduce thrombotic and inflammatory response *in vivo* and exhibit strong antimicrobial activity.[45] NO-generating materials cause *in situ* production of NO from

endogenous RSNOs by use of immobilized catalysts. Copper(II) has been used to this end in ligand complexes linked to polymers,[46] (nano)particles,[47] and even in metal-organic frameworks (Figure 1.8).[48] Organoselenium and organotellurium compounds have also been used successfully as immobilized catalysts as well.[40a,49] This approach is advantageous due to the virtually inexhaustible supply of RSNOs *in vivo* that can be utilized to generate NO at the device/blood interface.

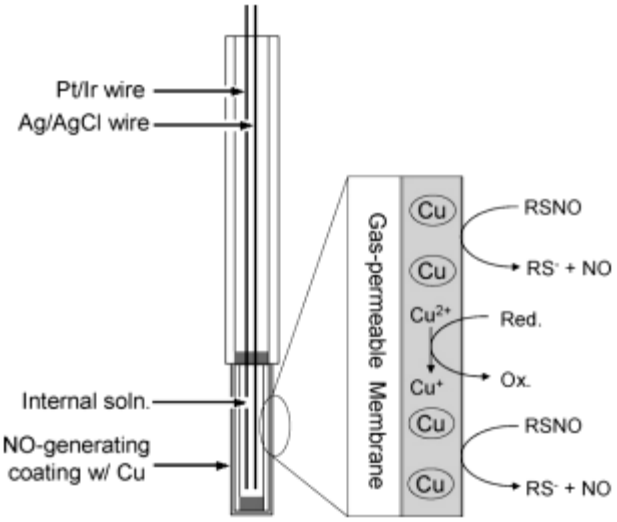


Figure 1.8. Schematic of an intravascular oxygen sensor. NO is generated by an immobilized copper catalyst, which is regenerated by endogenous reducing agents.[47c]

Materials have also been doped or functionalized with RSNOs to achieve NO release for various biomedical applications. As a result, a huge variety of *S*-nitrosothiols have been developed, ranging from poly-nitrosated macromolecules to small molecules.[25] *S*-nitrosothiols have been tethered to polymers,[50] polysaccharides,[51] fumed silica,[52] and dendrimers[53] as NO delivery vehicles at the polymer-blood interface (Figure 1.9). Due to the relative synthetic simplicity, RSNOs have also been incorporated into polymers as small molecule dopants for similar applications.[54,55]

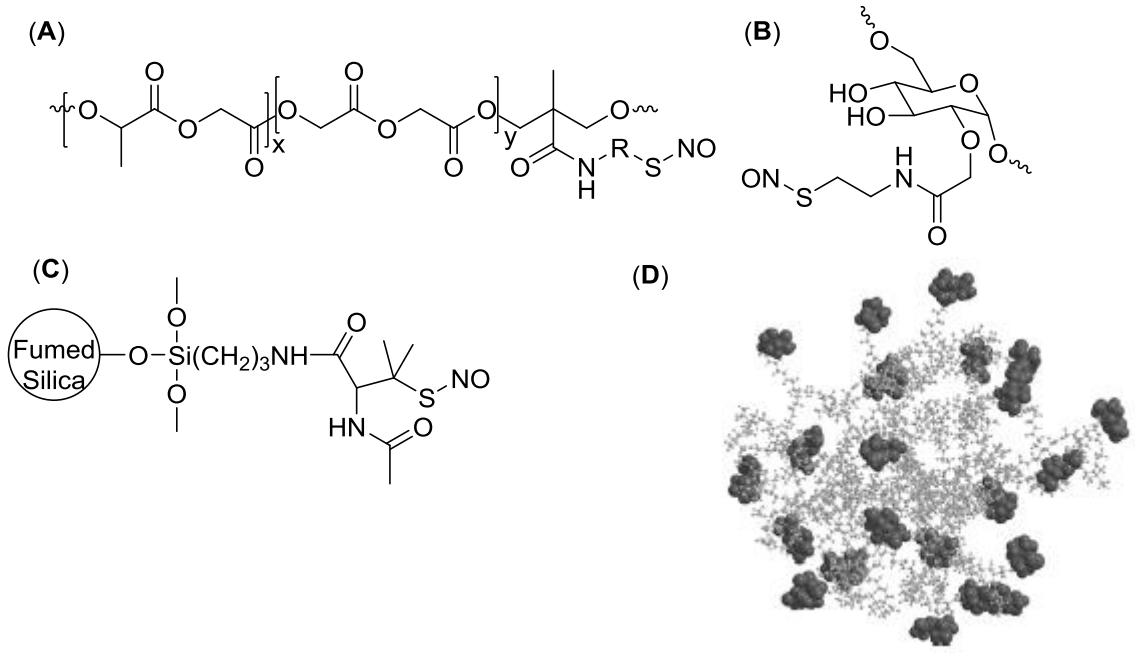


Figure 1.9. Structure of macromolecular *S*-nitrosothiols. (**A**) RSNO-functionalized PLGH polymer,[49] (**B**) *S*-nitrsoated dextran,[50] (**C**) SNAP-FS,[51] and (**D**) G4-SNAP dendrimer with some linked SNAP donors highlighted.[52]

The research presented in this thesis was initiated to build on previous work performed in the Meyerhoff research group on the doping of RSNOs into polymers for application to blood contacting medical devices.[55] Specifically, polymer films doped with SNAP (Figure 1.7) have been shown to release physiologically relevant levels of NO for over a week, reducing thrombotic response in animal studies. However, such materials suffer from significant leaching of SNAP and the corresponding disulfide decomposition product from the polymer phase into the solution phase, which poses a potential biohazard and may decrease the lifetime of localized NO release at the polymer/blood interface. In response to this problem and given the success of lipophilic SNAP analogues as effective vasodilators and inhibitors of platelet adhesion,[56] it was hypothesized that increasing the lipophilicity of a SNAP analogue would decrease leaching from the biomedical polymer into the aqueous solution phase. The modified RSNO would have increased interaction with a hydrophobic polymer, resulting in a higher partition coefficient of

the RSNO between the polymer film and the aqueous solution phase. However, the novel, lipophilic RSNOs that were synthesized in this work displayed unexpected properties when doped into polymers, prompting an expansion of the research to include fundamental studies on the kinetics of NO release from neat, solution phase, and polymer phase RSNOs.

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CHAPTER 2

Materials and Methods

2.1 Materials

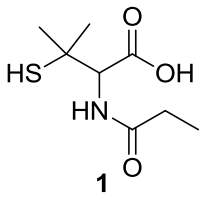
All reagents were obtained from Aldrich and used without further purification unless noted. All aqueous solutions were prepared with 18.2 MΩ deionized water using a Milli-Q filter (Millipore Corp., Billerica, MA). Phosphate buffered saline (PBS), pH 7.4, containing 2.7 mM KCl, 138 mM NaCl, and 10 mM sodium phosphate was used for all experiments with 100 µM EDTA added unless otherwise noted. Elast-eon™ E2As was obtained from AorTech International, plc (Scoresby, Victoria, Australia).

2.2 Synthesis

General Considerations. ^{1}H NMR spectra were recorded on a 400 MHz Varian spectrometer using $CDCl_3$ or CD_3SOCD_3 as an internal standard (δ = 7.26 ppm and 2.50 ppm, respectively). The description of signals include: s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. ^{13}C NMR spectra (proton decoupled) were recorded on a Varian 400 (100 Mhz) spectrometer with $CDCl_3$ or CD_3SOCD_3 as internal standard (δ = 77.0 ppm and 39.5 ppm, respectively).

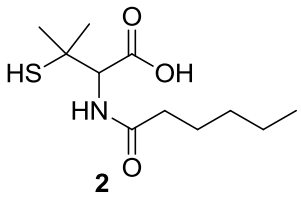
General Procedure for Synthesis of Acylated Penicillamine Derivatives. Penicillamine was acylated according to a modified procedure presented previously.[1] A round bottom was charged with a stirbar, penicillamine (1 equiv), sodium acetate trihydrate (1.35 equiv), and hydrobromic acid (1 equiv) in H_2O (12 mL). After stirring for 15 minutes, the appropriate acylating agent

(1.17 equiv) was added dropwise and subsequently stirred for 2 hours. The solution was acidified to pH 1 with HBr, filtered, and washed with H_2O and boiling hexanes to give the desired acylated product.



1

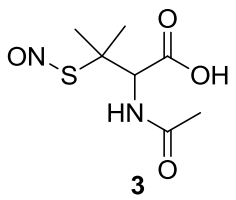
***N*-propionylpenicillamine (1)**. Prepared from penicillamine (4 g, 26.8 mmol) and propionic anhydride (4.03 mL, 31.6 mmol) according to the general procedure, yielding 3.80 g (69 %) of the title compound as a fine white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.48 (d, *J* = 8.8 Hz, 1 H), 4.59 (d, *J* = 8.8 Hz, 2 H), 2.33 (q, *J* = 7.6 Hz, 2 H), 2.11 (s, 1 H), 1.58 (s, 3 H), 1.36 (s, 3 H), 1.16 (t, *J* = 7.6 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0, 172.7, 60.5, 50.6, 46.25, 31.0, 29.6, 9.7.



2

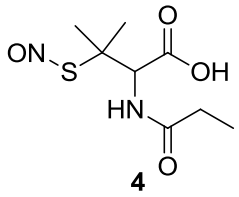
***N*-hexanylpenicillamine (2)**. Prepared from pencillamine (4 g, 26.8 mmol) and hexanoic anhydride (7.16 mL, 31.0 mmol) according to the general procedure, yielding 3.80 g (70 %) of the title compound as a fine white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.47 (d, *J* = 8.8 Hz, 1 H), 4.59 (d, *J* = 8.8 Hz, 1 H), 2.29 (t, *J* = 7.6 Hz, 2 H), 2.10 (s, 1 H), 1.67-1.63 (m, 2 H), 1.58 (s, 3 H), 1.36 (s, 3 H), 1.33-1.29 (m, 4 H), 0.88 (t, *J* = 6.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.3, 172.2, 60.4, 46.0, 36.5, 31.3, 31.0. 29.4, 25.2, 22.3, 13.9.

General Procedure for Synthesis of *S*-Nitrosothiols. *S*-nitrosothiols (RSNOs) were prepared using a modified procedure presented by Brisbois *et al.*[2] A round-bottom flask equipped with a stirbar was charged with the appropriate thiol (1.0 equiv) in methanol (2 mL/mmol thiol), HCl (1 M, 2 mL/mmol thiol), and H_2SO_4 (conc., 0.2 mL/mmol thiol) and cooled to 0 ˚C. $NaNO_2$ (2.5 equiv) was dissolved in a minimum volume of H_2O and added dropwise to the round-bottom flask over 5 minutes. The resulting green suspension was stirred at 0 ˚C overnight under N_2 flow. The remaining solid was washed thoroughly with cold H_2O, cold hexane, and lyophilized over 24 hours to yield the desired *S*-nitrosothiol as a green powder. Purity was confirmed by chemiluminescent assay of the *S*-nitrosothiol in the presence of cysteine and $CuCl_2$. RSNOs were stored at -20 ˚C due to heat and light-induced instability.



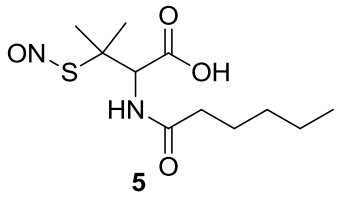
3

***S*-nitroso-*N*-acetylpenicillamine (3)**. Prepared from *N*-acetylpenicillamine (3.28 g, 17.2 mmol) according to the general procedure, yielding 3.23 g (86 %) of the title compound. ^{1}H NMR (400 MHz, CD_3SOCD_3) δ 8.51 (d, J = 9.5 Hz, 1 H), 5.16 (d, J = 9.5 Hz, 1 H), 1.96 (d, J = 12.6, 6 H), 1.87 (s, 3 H); ^{13}C NMR (100 MHz, CD_3SOCD_3) δ 171.2, 170.0, 59.6, 58.8, 26.7, 25.7, 22.7. Molar absorptivity maxima (in M^{-1} cm^{-1}): PBS, ε_{340} = 1000; *N*,*N*-dimethylacetamide (DMAc), ε_{342} = 917.



4

***S*-nitroso-*N*-propionylpenicillamine (4).** Prepared from **1** (2.32 g, 11.30 mmol) according to the general procedure, yielding 2.37 g (90 %) of the title compound. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.32 (d, *J* = 9.2 Hz, 1 H), 5.31 (d, *J* = 9.2 Hz, 1 H), 2.25 (q, *J* = 7.6 Hz), 2.07 (s, 3 H), 1.99 (s, 3H), 1.11 (t, *J* = 7.6 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.5, 171.1, 59.5, 58.5, 28.4, 26.1, 25.0, 9.4. Molar absorptivity maxima (in M^{-1} cm^{-1}): PBS, ε_{340} = 987; DMAc, ε_{342} = 855.



5

***S*-nitroso-*N*-hexanylpenicillamine (5).** Prepared from **2** (2.32 g, 9.38 mmol) according to the general procedure, yielding 2.46 g (95 %) of the title compound. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.30 (d, *J* = 9.2 Hz, 1 H), 5.31 (d, *J* = 9.2 Hz, 1 H), 2.20 (t, *J* = 7.6 Hz, 2 Hz), 2.07 (s, 3 H), 1.99 (s, 3 H), 1.59-1.55 (m, 3 H), 1.30-1.23 (m, 4 H), 0.85 (t, 6.8 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9, 172.4, 60.0, 57.7, 36.5, 31.2, 27.6, 26.2, 25.1, 22.3, 13.8. Molar absorptivity maxima (in M^{-1} cm^{-1}): PBS, ε_{340} = 854; DMAc, ε_{342} = 756.

2.3 Presparation of RSNO-doped Films

Polymer films containing 10 wt% of the test RSNO species were prepared by solvent evaporation.[2] Casting solutions were prepared by dissolving 180 mg of the respective polymer in

THF (3 mL). RSNO (20 mg) was added to the solution and briefly stirred. The film solution was then cast in a Teflon ring (d = 2.5 cm) on a Teflon plate and dried overnight at room temperature. Small disks (d = 0.7 cm) were cut from the parent films and dip coated twice in a topcoat solution (200 mg polymer in 4 mL THF) and dried overnight at room temperature. The weight of each disk was recorded prior to top coating. All films and film solution were protected from light. The final films had an RSNO-doped layer that was ~150 μm thick and a topcoat layer that was ~50 μm thick, as measured using a Mitutoya digital micrometer.

2.4 *In Vitro* Characterization of RSNO-doped Films

UV-Vis Spectra. All UV-Vis spectra were recorded on a Lambda 35, Perkin-Elmer spectrophotometer at room temperature. The S-NO group of RSNOs has characteristic absorbance peaks around 340 and 590 nm, corresponding to $\pi \rightarrow \pi^*$ and $n_N \rightarrow \pi^*$ electronic transitions. The larger peak around 340 nm is directly associated with homolytic cleavage of the S-NO bond and subsequent NO release, so RSNO present in solution was determined using ε_{max} and λ_{max} around 340 nm for the RSNO species being studied.

Leaching of RSNOs from Films. Top coated films were placed in individual vials soaked in PBS (4 mL) at 37 °C. At various time points the UV-Vis spectrum of a 2 mL aliquot of the PBS was taken for determination of RSNO concentration with PBS buffer used as the blank. The aliquots were then immediately returned to the sample vials for the duration of the experiment. PBS buffer was replaced daily. The % RSNO remaining in the film was determined by the difference between the amount of RSNO that had leached into the PBS and the initial amount of RSNO in the film based on mass.

Initial Film RSNO Content. After the 10 wt% RSNO/polymer films were prepared, the UV-Vis spectra of individual films dissolved in DMAc were used to determine the initial mass of RSNO dopant. DMAc was used as the blank.

2.5 Nitric Oxide Release Measurements

General Procedure. Nitric oxide (NO) released from RSNOs (neat, in solution, and in polymer films) was measured using a Sievers Chemiluminescence Nitric Oxide Analyzer (NOA) 280 (Boulder, CO). NO was continuously purged from the buffer and swept from the headspace using an N_2 sweep gas and bubbler into the chemiluminescence detection chamber.

***S*-nitrosothiol Purity Tests.** Injections of an RSNO (25 μL, known concentration ~4 mM) in PBS (no EDTA) were made into a solution of PBS (no EDTA 5 mL) containing $CuCl_2$ (0.3 mM) and cysteine (3 μM) in an amber sample cell at room temperature. The signal from the NOA was allowed to reach baseline before the next injection was made. Purity tests were performed immediately before RSNOs were used for any other tests, and only RSNOs that showed ≥ 95% purity were used in other tests.

Neat *S*-nitrosothiols. Known masses of an RSNO were added to an amber sample cell at room temperature and allowed to reach steady NOA signal.

Solution Phase *S*-nitrosothiols. Solutions of an RSNO at various concentrations (0.5-4 mM) in 2 mL PBS were added to an amber sample cell at room temperature and allowed to reach steady NOA signal. Solution volume was kept constant at 2 mL for all samples.

Polymer Films. Films were placed in an amber sample vessel and immersed in 4 mL PBS at 37 ˚C and allowed to reach steady state NO signal. Films were incubated in PBS under the same conditions as the NOA measurements (amber vial at 37 ˚C) with buffer replaced daily.

2.6 Statistical Analysis and Computational Methods

Data are expressed as mean ± SEM (standard error of the mean) with n ≥ 3. Octanol/water partition coefficient (Log*P*) values were calculated using ChemDraw.

2.7 References

1. Miller, M.R.; Roseberry, M.J.; Mazzei, F.A.; Butler, A.R.; Webb, D.J. Megson, I.L. *Eur. J. Pharmacol.* **2000**, *408*, 335-343.

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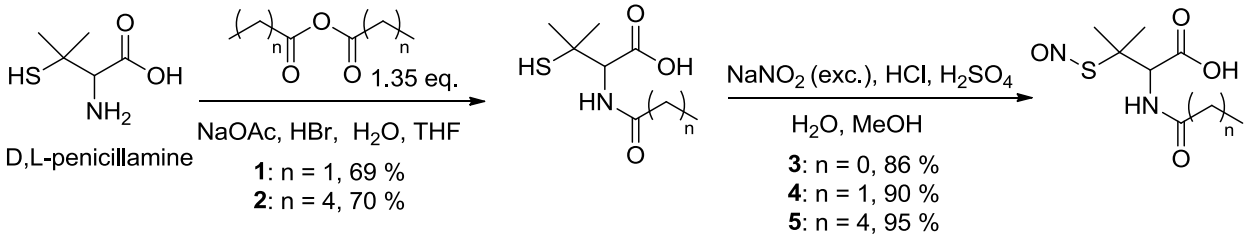
CHAPTER 3

Results, Discussion, and Conclusions

3.1 Synthesis of *S*-Nitrosothiols (RSNOs)

Looking to build on the unprecedented stability of *S*-nitroso-*N*-acetylpenicillamine (SNAP) in the low water uptake polymer, E2As,[1] we first developed a simple, high-yielding synthesis of the desired SNAP analogues. We chose to acylate the amine group of penicillamine, a precursor to SNAP, due to the relative synthetic ease compared to derivatization of the carboxylic group, which requires the use of an activating group such as DCC. Optimized synthesis of *N*-propionylpenicillamine (**1**) and *N*-hexanylpenicillamine (**2**) in moderate yield was accomplished by adding hydrobromic acid and sodium acetate to a mixed solvent system with an anhydride as the acylating agent (Scheme 3.1). No acylation was detected with removal of NaOAc or HBr from the reaction conditions, and replacement of NaOAc with NaOH resulted in production of an inseparable *S*-aceylated byproduct (data not shown). Subsequent nitrosation was accomplished by adding aqueous sodium nitrite to an acidified solution of the thiol, resulting in high yields of the corresponding *S*-nitrosothiols: *S*-nitroso-*N*-acetylpenicillamine (**3**), *S*-nitroso-*N*-propionylpenicillamine (**4**), and *S*-nitroso-*N*-hexanylpenicillamine (**5**). Use of the nitrosating agent *t*-buNO_2 in THF resulted in incomplete nitrosation of thiols (data not shown).

Scheme 3.1. Synthetic Scheme to form RSNOs



Purity of the resulting RSNOs was confirmed by a chemiluminescent assay using copper(II) chloride and cysteine as catalysts (Figure 3.1), as nitrite salt impurities were undetectable in ^{1}H-NMR spectra. RSNOs were not used in further analytical tests unless they were found to be ≥ 95% pure.

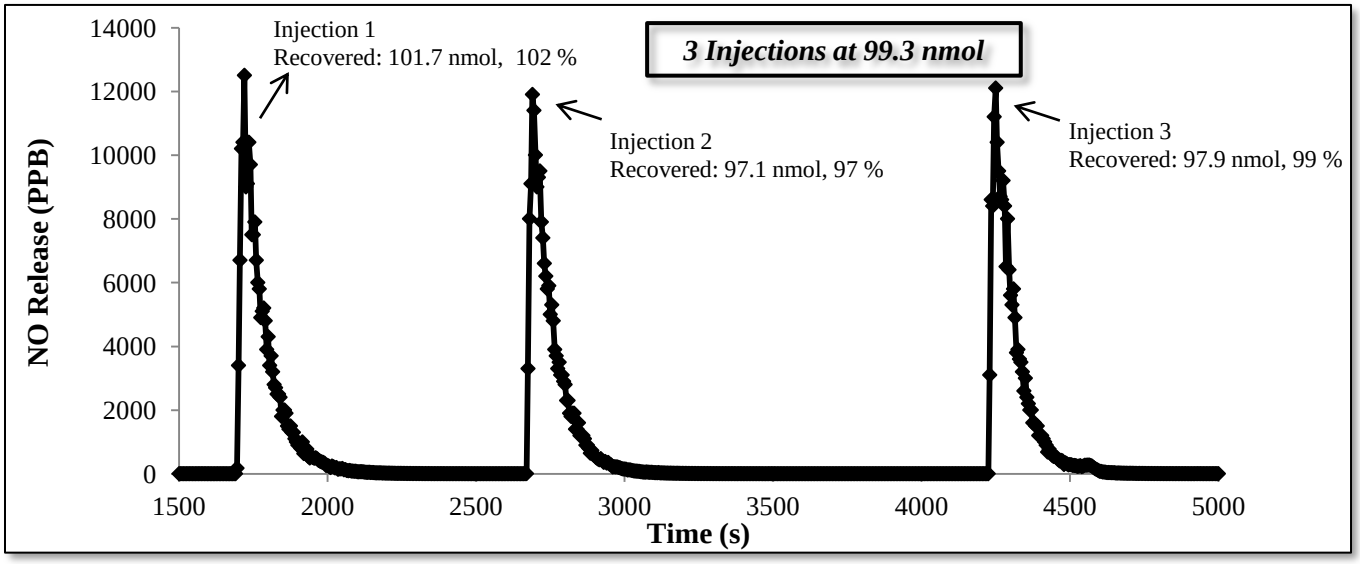
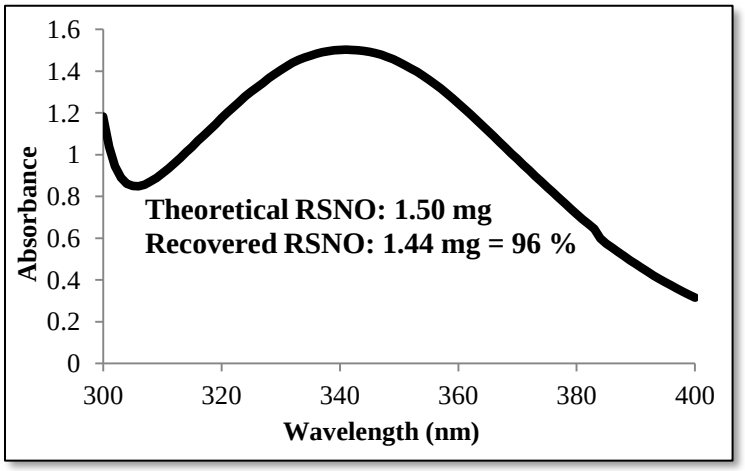


Figure 3.1. Representative plot from chemiluminescent assay of RSNOs. Shown: Assay of **3** with average purity of 99 ± 3 %.

3.2 Characterization of RSNO-Doped E2As Films

Preparation of **3**/E2As and **4**/E2As films proceeded according to previous work performed in the Meyerhoff group.[1] Fabrication of film doped with **5** required

caution, as these films experienced a color change to brown when left in ambient atmosphere or light for longer than a few hours. Brown films did not release significant levels of NO, and may result from a trans-nitrosation reaction between **5** and a catalyst in the polymer. After fabrication of films, we confirmed RSNO film content by UV-Visible spectroscopy. By using the RSNO absorbance maximum at 342 nm in *N*,*N*-dimethylacetamide (DMAC), films for all three RSNOs contained ≥ 95 % of the expected *S*-nitrosothiol content, based on film mass after doping with RSNOs (Figure 3.2, Table 3.1).



RSNO/E2As	RSNO recovery (%)
3	95 ± 1
4	102 ± 2
5	103 ± 4

Left: **Figure 3.2**. Example spectrum of a dissolved 10 wt% **3**/E2As film in DMAc. Absorbance ≤ 300 nm is due to the E2As polymer. Right: **Table 3.1.** RSNO Content of Doped Polymer Films

Next, the relative RSNO leaching and NO release profile of the RSNO-doped E2As films were investigated in phosphate buffered saline (PBS) at physiological pH and temperature. Due to increased lipophilicity of the novel RSNOs (**5** > **4** > **3**), we expected **5** to show the lowest leaching out of the lipophilic polymer into the aqueous phase, followed by **4** and **3**. Surprisingly, the films showed the opposite trend in leaching, with films doped with **4** or **5** having greatly increased leaching compared to films doped with **3** (Figure 3.3).

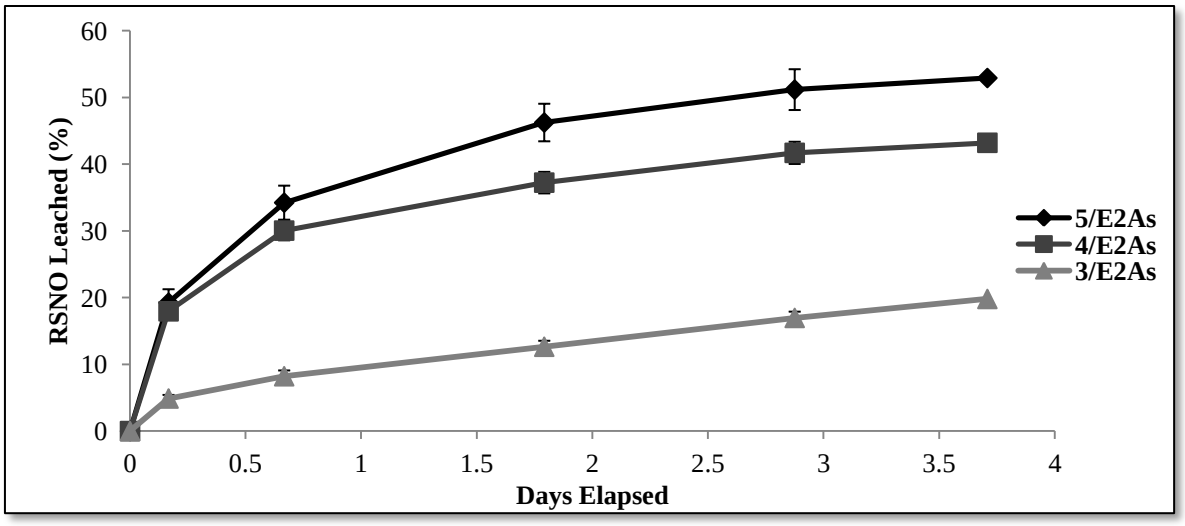


Figure 3.3. Cumulative leaching of RSNOS from doped E2As films immersed in PBS at 37 °C and pH 7.4 as measure by UV-Vis spectroscopy.

The unexpected trend in leaching may indicate that dispersion forces are not the predominant factor in determining RSNO retention in the E2As polymer matrix. If dispersion forces were the primary interaction between RSNOs and the hydrophobic polymer, the longer alkyl chain of **4** and **5** would increase the attraction between the RSNOs and the polymer matrix, resulting in decreased leaching compared to **3**. Instead, the observed trend indicates that the longer alkyl chain of the RSNO decreases favorable interaction of these species with the polymer matrix. This contradicts the previous conjecture by Brisbois *et al.* that the hydrophobicity of **3** gives it a preference to stay in the hydrophobic polymer phase.[1] The observed trend in leaching is not well accounted for by water uptake into the E2As polymer matrix, either. **3** is considerably more soluble in water compared to the lipophilic analogues, so the slight uptake of water into the lipophilic polymer phase would be expected to contribute more to leaching in films doped with **3** than in films doped with **4** or **5**.

There are several possible explanations for why **3** has lower leaching from the E2As polymer matrix compared to its more lipophilic analogues. Besides hydrophobic interactions, which do not correlate with the observed trend in leaching, all three RSNOs can interact with the E2As polyurethane/siloxane-base via H-bonding. In particular, the carboxylic acid and amide groups on the RSNOs can interact with amide groups in the polyurethane units or the hydrogen-accepting oxygen on the siloxane or polyether units of E2As. If the longer alkyl chains next to the amide moiety reduce H-bonding capability due to sterics, then the more lipophilic analogues of **3** may interact less favorably with the polymer matrix, explaining the observed trend in leaching.

In terms of sterics, the amide group of **3** can only adopt one conformation (Figure 3.4). Meanwhile, the extra carbon near the amide group of **4** may freely rotate, adding significant steric hindrance; the longer carbon chain of **5** merely adds to the rotational freedom that is already present, resulting in a more subtle decrease in H-bonding compared to **4**. This trend in rotational freedom may explain why **4** shows much closer leaching levels to **5** than **3**, despite only having one extra carbon compared to **3**.

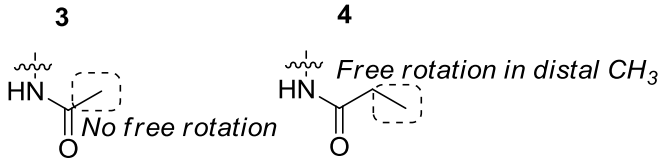


Figure 3.4. Comparison of rotational freedom between RSNOs.

The presence of H-bonding between RSNOs and the polymer matrix could be demonstrated by NMR spectroscopy, where a constant concentration of E2As can be added to solutions of each RSNO. If the amide group or carboxylic acid group of the RSNOs H-bond with

the polymer, the corresponding RSNO proton peak should shift downfield upon addition of the polymer, and the relative H-bond strength of the RSNOs can be indicated by how far the proton peak shifts.

Alternatively, the importance of H-bonding could be investigated by altering the polymer matrix or the H-bonding capability of the incorporated RSNO. For instance, silicone rubber (SR) has a higher density of highly H-bond accepting oxygen atoms compared to the E2As co-polymer, while still maintaining a low water uptake. SR films doped with **3** have also been shown to have nearly identical leaching levels compared to E2As films doped with **3**.[1] Therefore, doping **4** or **5** into SR films may show the relevance of H-bonding. If H-bonding is the predominant force between doped RSNOs and the SR polymer matrix, leaching levels for the lipophilic RSNOs should increase greatly, as the new dopants are expected to have significantly reduced H-bonding capability compared to **3** and will interact less favorably with the SR matrix than previously with the E2As matrix.

The H-bonding capability of doped RSNOs could be modulated by other synthetic methods, as well. For example, if nitrosated penicillamine (PSNO) was doped into a polymer film, it would have higher H-bonding capability compared to **3** due to the free amine group, while still being a tertiary RSNO. If H-bonding is the predominant interaction with the polymer, PSNO may show even lower leaching than **3**, despite having about the same lipophilicity (Table 3.2). Incorporation of *S*-nitrosoglutathione (GSNO) into polymer films could also be tested, as GSNO has multiple H-bonding moieties and is even less lipophilic than PSNO, although GSNO is a primary RSNO (Chapter 1, *vida supra*). Another simple alteration to **3** could be made by derivatizing the carboxylic acid with a methyl ester, which would decrease the H-bonding capability of the RSNO without drastically changing its lipophilicity.

Table 3.2. RSNO Lipophilicity Values.

RSNO	LogP[a]
5	1.98
4	0.73
3	0.08
PSNO	0.14
GSNO	-2.7
SNAP-methyl ester	0.41

[a]Octanol/water partition coefficient.

Besides H-bonding, the unexpected trend in leaching could be explained by the size accommodation of the polymer matrix. **3** may be small enough to fit inside the surrounding polymeric units, resulting in low leaching due to favorable interaction between the RSNO and polymer matrix. However, the additional carbon(s) of **4** and **5** may crowd the surrounding polymeric units, resulting in unfavorable steric interactions that push the RSNOs out of the polymer matrix, causing higher leaching levels. The importance of this effect could be tested by incorporating **3**-**5** into microporous polymers with known pore sizes[2] and comparing their leaching rates.

The long-term NO release profile of RSNO-doped E2As films during immersion at physiological temperature and pH was also tested (Figure 3.5). Films containing **4** and **5** released NO for a very short period, in both cases falling to below 0.5 flux in less than 2 days. Meanwhile, films doped with **3** maintained ≥ 1 flux for more than 4 days, as shown previously.[1]

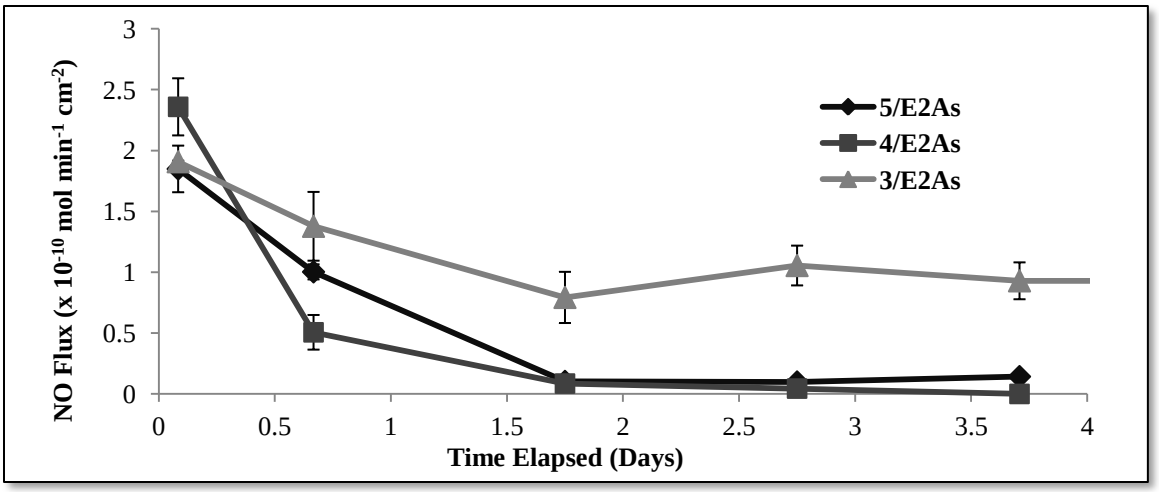


Figure 3.5. NO release behavior of 10 wt% RSNO/E2As films at 37 ˚C in the dark.

The very short NO release profile of the two novel RSNOs is likely due to the leaching of NO donors from the polymer phase. During the NO release tests, the immersion solution (PBS) was replaced daily, so leached RSNOs would be removed from the system before decomposing. Since films doped with **4** or **5** showed much higher leaching than films doped with **3**, the daily replacement of PBS liked reduced the observed NO release from films doped with the lipophilic RSNOs significantly.

However, in addition to the loss of leached RSNOs, the intrinsic stability of the doped RSNOs in solution and in crystalline phase also contributes to the rate of NO release from the polymer films. The intrinsic stability of **3** should play an especially key role, as the RSNO showed very low levels of leaching from the polymer phase. E2As has a water uptake of ~1 wt% during immersion.[1] Therefore, some RSNOs in the polymer matrix and RSNOs that leach into the bulk solution phase (before being removed during solution replacement) will be dissolved and subsequently release NO. Dry RSNOs in the polymer matrix will also slowly decompose depending on their interaction with the polymer matrix and on their interaction with other RSNO molecules in the absence of solvent. Therefore, the kinetics of NO release from solution,

crystalline, and polymer phase RSNOs are of importance in determining the NO release profile from doped polymer films.

3.3 Characterization of RSNOs

To elucidate the relative kinetic stability of the three studied RSNOs, two tests were performed. The solution phase stability of the three RSNOs was investigated by measuring NO release from the three RSNOs in PBS at known concentrations (Figure 3.6). While keeping the total solution volume constant, the RSNOs were diluted from 4 mM to 0.5 mM (Figure 3.7). The three RSNOs displayed very similar NO release that was linear across the concentrations used, indicating similar solution stability that would not contribute to the shorter observed NO release profile for **4** and **5**. In fact, **5** displayed increased solution phase stability compared to **3** and **4**. This result suggests that **5** (or even more lipophilic analogues) may be suitable for solution-phase applications that require long-term solution stability, such as delivery of NO to arteries for vasodilatory effects.[3]

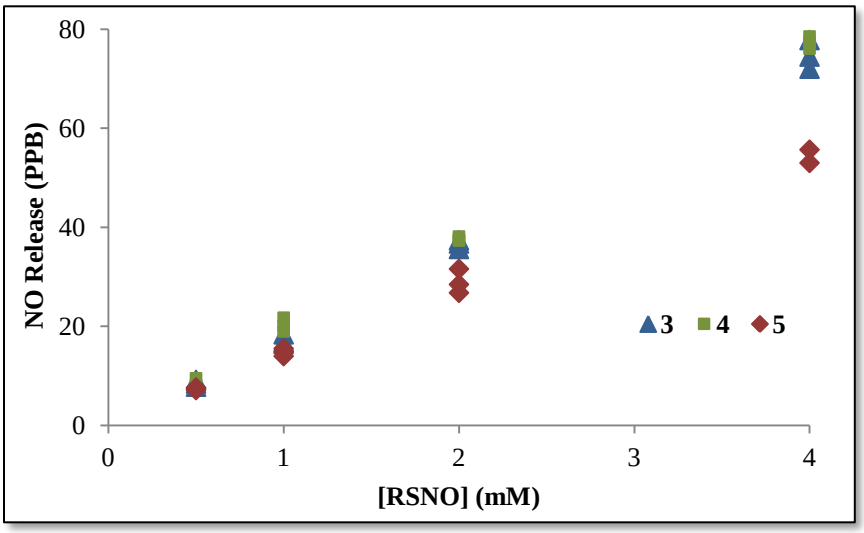


Figure 3.6. NO release from RSNOs dissolved in PBS in the dark at room temperature. For all RSNOs, $R^2 > 0.99$. All release tests were performed in 1 day on the same instrument.

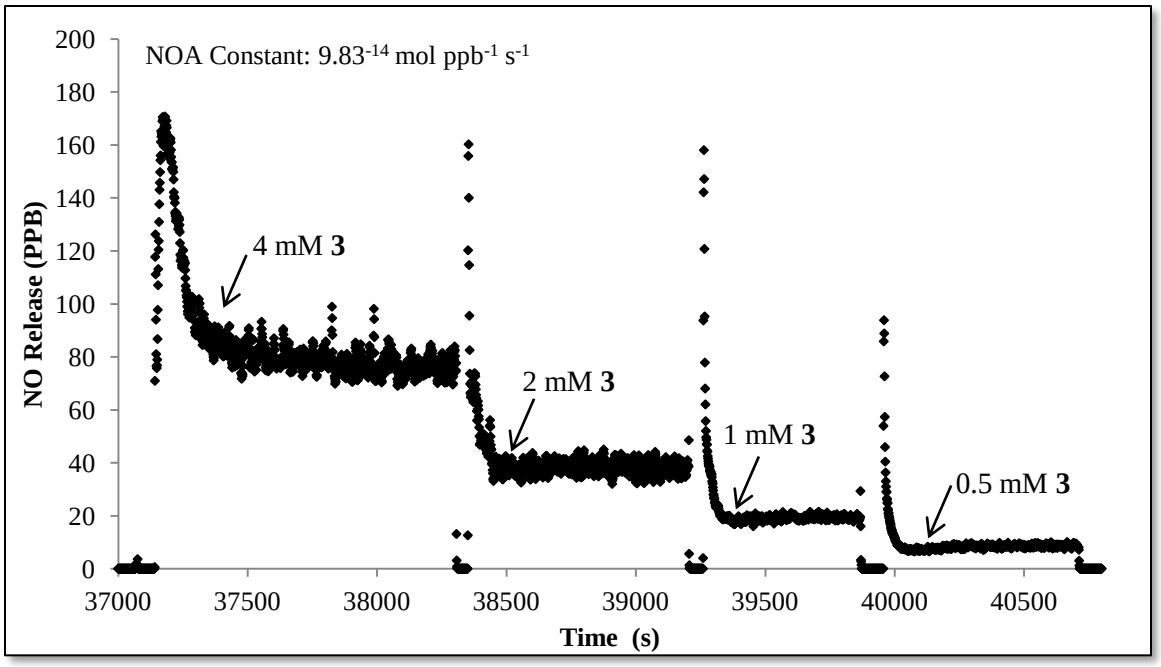


Figure 3.7. Example NO release from **3** in solution phase in the dark at room temperature.

The crystalline/neat phase stability of **3-5** was also studied by observing NO release from known masses of crystalline RSNOs. Interestingly, the three RSNOs displayed highly divergent neat stability (Table 3.3). **3** was extremely stable (Figure 3.8), while **4** released NO more than a magnitude faster based on the total mols of RSNO present; **5** released NO another magnitude faster than **4**.

Table 3.3. NO Release from Neat RSNOs

RSNO	AlogP	Neat Release (mols lost/mol*s)
3	1.98	$1.31 \pm 0.09 * 10^{-9}$
4	0.73	$7.8 \pm 0.1 * 10^{-8}$
5	0.08	$1.02 \pm 0.04 * 10^{-7}$

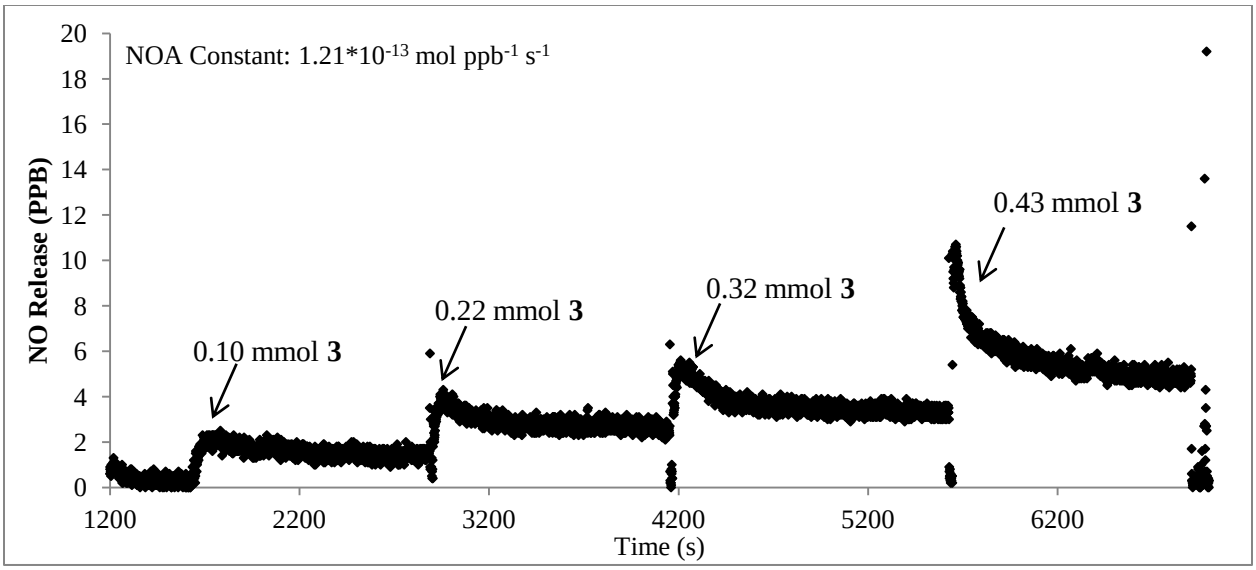


Figure 3.8. Example NO release profile from crystalline **3** in the dark at room temperature.

The very high solid stability for **3** indicates that the RSNO would decompose very little in the polymer matrix in the absence of solution, and probably does not contribute significantly to NO release from **3**/E2As films. Only about 6 μmols of **3** are present in each polymer film, which would have almost no NO release in the absence of water (Figure 3.8). Instead, most of the NO release in films doped with **3** is probably the result of dissolved RSNO.

Even though the relative neat and solution stabilities of **3**, **4**, and **5** may not account for the short NO release profiles for polymers doped with **4** and **5**, the trend in neat stability gives insight into the structure-activity relationship of RSNOs when altering long-range structure (that is, structural moieties located distally from the S-NO group). This relationship has not been significantly studied before in RSNOs, although a similar trend between lipophilicity and stability has been noted in diazeniumdiolates.[4] The inverse relationship between lipophilicity and decomposition rate shown by the three RSNOs investigated in this research (Figure 3.9) could be confirmed by the synthesis of analogues of **3** with even longer alkyl chains, such as

S-nitroso-*N*-oleoylpenicillamine (LogP = 6.67). Furthermore, the derivatization of the carboxylic acid moiety in **3** with a long ester group would increase lipophilicity and lower H-bonding capability of the corresponding RSNO, modulating the solid stability of the NO donor.

In terms of applications, **5** has high enough release in the crystalline phase that when incorporated into a polymer and kept dry, it may release physiologically relevant levels of NO. Such a polymer formulation may be useful as an antibacterial or to help in wound healing.[5] The current obstacle in such an application is that the **5**/E2As polymer formulation undergoes a color change from green to brown when left in ambient atmosphere/light without immersion that is accompanied by elimination of NO release from the films when they are ultimately. **5**/SR film formulations are currently being explored for their dry NO release profile, as preliminary experiments show that the color change does not occur when **5** is doped into these films (data not shown).

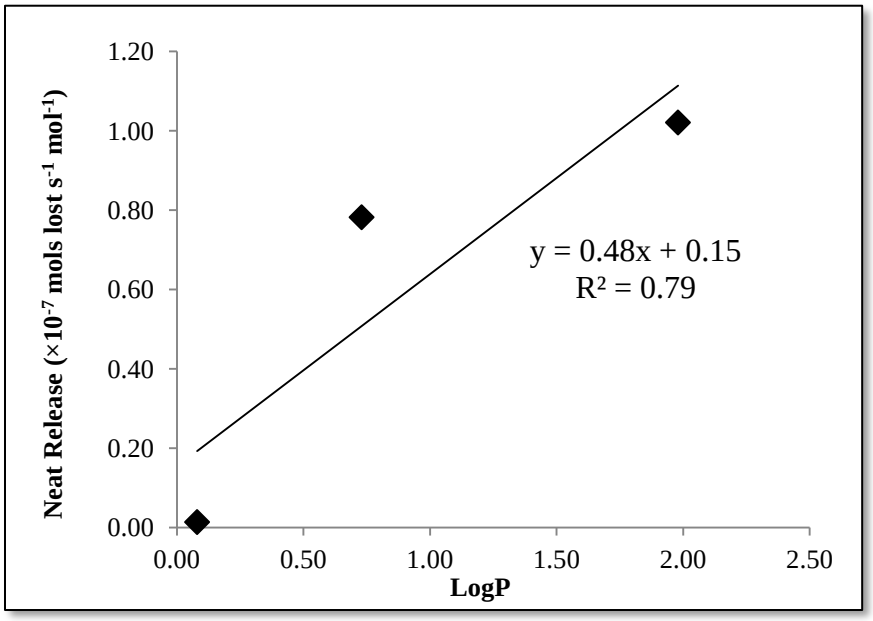


Figure 3.9. Trend in crystalline stability of RSNOs with changing lipophilicity (given by LogP).

Future studies of solution and crystalline phase RSNOs should also be performed using computational methods. In acquiring NO release profiles from **3-5**, the manner in which the longer alkyl chains modulate RSNO stability is mostly conjecture at this point. By using computational models of single or small groups of RSNOs, the predominant interactions between different RSNOs may be elucidated, giving insight to the changes in lipophilicity that result in a small variation of solution stability and very large variation of crystalline stability.

3.4 Conclusions

In this study, two novel RSNOs, *S*-nitroso-*N*-propionylpenicillamine (**4**) and *S*-nitroso-*N*-hexanylpenicillamine (**5**) were compared to a previously studied compound, **5**, through multiple methods. While the lipophilic E2As polymer is an excellent matrix for **3** as a reservoir of NO during immersion under physiological conditions, **4** and **5** unexpectedly display high levels of leaching out of the polymer matrix and show very short NO release profiles under the same conditions. This result shows that simple lipophilic dispersion forces between doped RSNOs and polymeric units are not the primary concern in determining leaching and stability of RSNO/polymer formulations. To gain a more fundamental understanding of the effect of long alkyl chains on RSNO stability, NO release profiles from solution and crystalline phase samples of all three *S*-nitrosothiols were obtained. These data showed that all the RSNOs had similar solution phase stability, but lipophilicity had a very strong inverse correlation with crystalline stability. Further experimental studies to determine the viability of a dry formulation that dopes **5** into silicone rubber and computational studies to investigate the interactions between RSNOs on a molecular level are underway.

3.5 References

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