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Nitric Oxide Releasing Tygon Materials: Studies in Donor Leaching and Localized Nitric Oxide Release at a Polymer-Buffer Interface

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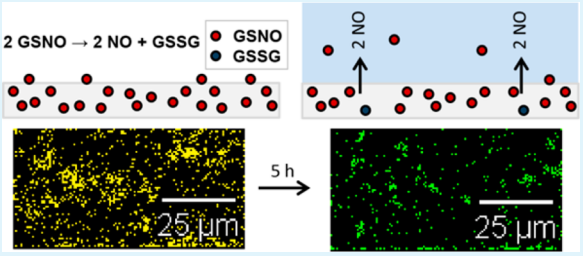
Supporting Information

ABSTRACT: Tygon is a proprietary plasticized poly(vinyl chloride) polymer that is used widely in bioapplications, specifically as extracorporeal circuits. To overcome issues with blood clot formation and infection associated with the failure of these medical devices upon blood contact, we consider a Tygon coating with the ability to release the natural anticlotting and antibiotic agent, nitric oxide (NO), under simulated physiological conditions. These coatings are prepared by incorporating 20 w/w% *S*-nitrosoglutathione (GSNO) donor into a Tygon matrix. These films release NO on the order of 0.64 ± 0.5 × 10^{-10} mol NO cm^{-2} min^{-1}, which mimics the lower end of natural endothelium NO flux. We use a combination of assays to quantify the amount of GSNO that is found intact at different time points throughout the film soak, as well as monitor the total thiol content in the soaking solution due to any analyte that has leached from the polymer film. We find that a burst of GSNO is released from the material surface within 5 min to 1 h of soaking, which only represents 0.25% of the total GSNO contained in the film. After 1 h of film soak, no additional GSNO is detected in the soaking solution. By further considering the total thiol content in solution relative to the intact GSNO, we demonstrate that the amount of GSNO leached from the material into the buffer soaking solution does not contribute significantly to the total NO released from the GSNO-incorporated Tygon film (<10% total NO). Further surface analysis using SEM-EDS traces the elemental S on the material surface, demonstrating that within 5 min −1 h soaking time, 90% of the surface S is removed from the material. Surface wettability and roughness measurements indicate no changes between the GSNO-incorporated films pre- to postsoak that will be significant toward the adsorption of biological components, such as proteins, relative to the presoaked donor-incorporated film. Overall, we demonstrate that, for a 20 w/w% GSNO-incorporated Tygon film, relatively minimal GSNO leaching is experienced, and the lost GSNO is from the material surface. Varying the donor concentration from 5 to 30 w/w% GSNO within the film does not result in significantly different NO release profiles. Additionally, the steady NO flux associated with the system is predominantly due to localized release from the material, and not donor lost to soaking solution. The surface properties of these materials generally imply that they are useful for blood-contacting applications.



KEYWORDS: *S-nitrosoglutathione, Tygon, nitric oxide, localized release, donor leaching, extracorporeal circuit, surface analysis*

INTRODUCTION

Extracorporeal circuits (ECCs) are critical to a number of medical procedures that involve blood transport, including blood oxygenation, transfusion, and hemodialysis. Tygon is a proprietary blend of plasticized poly(vinyl chloride) commonly used in ECC applications. There are many drawbacks associated with the use of polymers in ECCs due to a combination of mechanical failures and adverse physiological responses. The most serious complications typically involve blood clot formation in the ECC device, patient infection, and bleeding out due to the use of systemic anticoagulants. When the material is exposed to blood, platelet activation and adhesion are immediately initiated, leading to blood clot formation during the blood transfer process within the Tygon tubing. Additionally, serious infection can result at the implant site, which could lead to sepsis. Clotting of the device results in serious complications, including gross thromboembolic aggregation and even death; therefore, systemic anticoagulants, such as heparin, are usually administered to the patient to prevent clotting. However, the habitual use of systemic anticoagulants can result in a number of dangerous effects downstream from the site of the ECC device, including platelet consumption and bleeding out at alternate sites of injury. A 2012 report from the Extracorporeal Life Support Organization reveals some alarming statistics regarding the use of ECC devices.[1] Of the patients (16 yrs+) who received ECC treatment, 7.4% were reported to experience mechanical failure due to clotting in the device, and those who experienced clotting had a 33% chance

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of surviving to discharge or transfer. This leads to an overall expected mortality rate of 5% due to clotting incidents. The report also revealed a 12% mortality rate for hemorrhaging at the site of cannulation and a 12% mortality rate due to infection. Of the hundreds to thousands of patients who receive ECC related treatments annually, this is a staggering failure rate. To overcome the issues associated with blood-clotting and infection in ECCs as well as to prevent the use of systemic anticoagulants that increase the risk of hemorrhaging, different approaches must be taken to develop a more biocompatible ECC material.

One major approach to ECC development is to design materials that are capable of releasing therapeutics at the site of blood contact that would serve to control the initial bioresponse to the material. Nitric oxide (NO) is a promising therapeutic agent for these applications and has previously been shown to retain ECC functionality.[2,3] Nitric oxide is released from endothelial cells that line blood vessels to serve as an antiplatelet agent to control coagulation,[4] and recent research indicates that NO also has an effect on the adsorption of blood-clotting proteins to a material surface.[5] In addition to NO's role as a natural anticoagulant, it also serves other critical physiological functions, such as preventing inflammation[6] and infection,[7] which is critical to the performance of ECCs. Overall, creating Tygon coatings capable of releasing NO in a controllable fashion is a promising approach for ECC development. In general, NO-releasing materials have gained much interest in a variety of bioapplications, including cardiovascular materials (stents, grafts) and biosensors.[8−12]

Nitric oxide donors allow for the storage of NO within a material until initiation of release. There are several classes of NO donors which can be preformed and isolated as stable small molecules with further incorporation into a material matrix. The most popular donor-incorporated polymer systems have utilized *N*-diazeniumdiolates[3,13−17] or *S*-nitrosothiols (RSNOs)[18−21] to facilitate NO storage and release. The major advantage to a donor-incorporated system is that the concentration and type of donor can be tuned to obtain the NO reservoir and release kinetics desired for a given application. Additionally, depending upon the donor type, different stimuli can be employed to initiate donor decomposition (i.e., *S*-nitrosothiol decomposition is triggered by heat, light, or metal-ion presence[22]). The major drawback to these systems is the prevalence of significant donor leaching from the material into the soaking solution.[14,18,20,21] For instance, *N*-diazeniumdiolate donors have been found to leach from hydrophobic polymer films and attempts at top coating methods and covalent attachment of the donor have been employed to overcome this.[14] Additional attempts have been made to create more lipophilic donors that will not leach as significantly into the aqueous soaking phase;[16,23,24] however, donor leaching still remains a problem without time intensive methods to apply multiple top coats.[5] If the donor leaches significantly into the soaking fluid, this will result in nonlocalized NO release as the therapeutic NO action will occur downstream from the implant site. In order to help control thrombus proliferation and prevent infection, NO release needs to be localized at the material-biology interface. Additionally, some NO donors, namely *N*-diazeniumdiolates, have potentially toxic decomposition products (i.e., *N*-nitrosamines) that are not intended for release into the bloodstream.[14,23] Essentially, to perform appropriately, the NO donor must stay fixed in the material matrix, releasing the intended flux of NO for ECC applications. The target NO release is that of the natural endothelium ($0.5-4 \times 10^{-10}$ mol NO cm^{-2} min^{-1}).[25] To overcome issues with donor leaching, the field has largely steered toward polymers that contain donor groups that are covalently attached to the backbone, resulting in the desired localized NO release.[8,12,13,26] However, these materials require significant polymer modification, and the NO loading efficiencies are variable depending upon the polymer functionality,[27] making it difficult to tailor the exact reservoir of NO contained within the polymer. Because of the ease of material preparation and stringent control over the NO reservoirs, donor-incorporated systems remain a viable approach toward the development of NO-releasing materials.

Despite prior demonstration of significant donor leaching for different polymer systems, it is important to note that no studies have been performed to investigate how tuning the material properties (i.e., polymer type, donor concentration) will overcome leaching while maintaining the requisite NO profiles. There have been studies performed to consider the impact of the polymer type and *S*-nitrosothiol donor concentration on the NO release profiles, but these systems have been found to still result in significant donor leaching from the system.[18] It is additionally important to determine if, despite donor leaching, the majority of the NO released from the system is occurring in the material phase, yielding a truly localized effect, or if the NO is releasing predominantly in the solution phase. The goal of this paper, therefore, was to take a model *S*-nitrosothiol-incorporated Tygon system and determine (a) if significant donor leaching occurs and (b) if leached donor in the solution phase (i.e., buffer) contributes significantly to the overall NO release. The second piece is key toward understanding whether the NO release occurs in a localized or downstream fashion relative to the material surface. The model donor in these studies was *S*-nitrosoglutathione (GSNO). The benefit of using GSNO is that any leaching of the donor will not produce harmful byproducts as GSNO and its parent thiol, glutathione, are readily available in the body.[28] Additionally, the NO release and donor leaching studies are considered for a 5 h duration, which is relevant to ECC systems,[29] more specifically hemodialysis, and a 4−6 h time duration has been considered an acceptable window of time to study biology-material interactions for such applications.[2,3,23,30−32] Overall, we establish the fate of the NO donor that is incorporated into a Tygon polymer system and determine the location of NO release relative to the material-buffer interface. In addition to understanding the behavior of the GSNO in the system, we perform surface analyses to determine surface wettability and roughness, which inform the use of this system for blood-contacting bioapplications in general.

EXPERIMENTAL SECTION

Materials. Glutathione (GSH, 98%, reduced, Acros Organics, Fair Lawn, NJ, USA), sodium nitrite ($NaNO_2$, ACS grade, EMD Chemicals Inc., Gibbstown, NJ, USA), glutathione disulfide (GSSG, oxidized glutathione, Sigma-Aldrich, St. Louis, MO, USA), hydrochloric acid (HCl, 36−38%, BDH Aristar, Seastar Chemicals Inc., Sidney, BC, Canada), sodium borohydride ($NaBH_4$, MP Biomedicals, Solon, OH, USA), sodium hydroxide (NaOH, ACS grade, Fisher Scientific, Fair Lawn, NJ, USA), DTNB (Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid), Sigma-Aldrich, St. Louis, MO, USA), and tetrahydrofuran (THF, LC grade, Macron Chemicals, Phillipsburg, NJ, USA) were all used as received. All solutions of GSH, GSNO, and GSSG were prepared using phosphate buffered saline (PBS, prepared from

OmniPur tablet with Millipore treated water (18.2 MΩ) and pH adjusted to 7.4). The model polymer in all studies was Tygon (Formula R-3603, Saint-Gobain Performance Plastics, Akron, OH, USA).

Methods. In brief, GSNO-incorporated Tygon films were prepared in the bottom of polypropylene test tubes. The films were then exposed to simulated physiological conditions (1 mL PBS, pH 7.4, 37 °C) for a 5 h period to simulate hemodialysis conditions. The soaking solution of PBS was analyzed for intact GSNO at different time points during the soaking period to inform any donor leaching from the material phase. Additionally, a modified Ellman's assay was developed to measure total thiol (GSH) in the soaking solution to determine if any solution-phase GSNO decomposed during the soaking period. NO measurements were performed over the 5 h period and NO fluxes were subsequently determined. Surface analysis was also performed on the films pre- and postsoak to understand the interfacial behavior of the film-buffer system.

S-Nitrosoglutathione Synthesis. The model NO donor for these studies was *S*-nitrosoglutathione (GSNO), which is formed upon nitrosation of the thiol residue of glutathione. The GSNO was synthesized according to a previously published method.[33] In brief, 5 mmol of glutathione was added to ice-cold water (8 mL), followed by dissolution upon addition of acid (2 M HCl, 2.5 mL). To initiate nitrosation, sodium nitrite was added (5 mmol) resulting in the formation of nitrous acid, which serves as the nitrosating agent. The solution was kept in an ice bath and stirred for 40 min, followed by addition of acetone (10 mL) to crash out the GSNO product. The final solid product was filtered and washed with water to remove excess nitrite. The product was rinsed with acetone and dried on filter paper under vacuum for 1.5 h in the dark before transferring the solid to an amber vial (EnviroWare, EPA-certified copper-free, Fisher Scientific, Fair Lawn, NJ, USA) equipped with a septum-containing lid for subsequent drying under vacuum. The isolated GSNO product was stored in the freezer to prevent donor decomposition.

Tygon Film Preparation. Tygon was dissolved in THF to yield a 0.1 g mL^{-1} polymer solution. The bulk of the analysis was performed on 20 w/w% GSNO-incorporated films, which were prepared by adding GSNO to the Tygon solution at 20 w/w% of the total Tygon in solution. An aliquot of polymer solution (200 μL) was added to the bottom of a polypropylene test tube (7.3 cm length, 10.5 mm inner diameter, 11.5 mm outer diameter). The polymer films were cured by drying overnight under a cover. For additional GSNO concentration studies, 1, 5, 10, and 30 w/w% GSNO films were prepared in the same fashion, only varying the amount of GSNO added to the polymer solution.

Nitric Oxide Analysis. NO measurements were performed using Sievers 280i Nitric Oxide Analyzers (NOAs, GE Analytical Instruments, Boulder, CO, USA). Baseline measurements were collected on the empty NOA cell for 2 min, prior to addition of 1 mL of PBS (37 °C) per test tube containing the GSNO-incorporated Tygon film. The test tube was inserted into the NOA cell, with the N_2 bubbler adjusted near the top of the soaking solution to prevent disruption of the polymer film. The airtight, deoxygenated cell was then lowered into a 37 °C water bath and real-time NO measurements were collected in 5 s intervals for a 5 h soaking duration, after which the PBS soaking solutions were collected for subsequent analysis. During the soaking of the GSNO-incorporated film, the system was exposed to ambient light. Therefore, the NO release from the system is due to heat- and light-initiated decomposition of the GSNO donor. Additionally, the Millipore filtered water used in our lab has been analyzed by OES-MS for elemental analysis of any copper content and contains roughly 0.3 μg L^{-1}. Even at these small concentrations, it is likely that there is some copper-mediated decomposition of the GSNO occurring because of copper in the PBS that is prepared with Millipore filtered water. The GSNO-incorporated Tygon films were also recovered for surface analysis.

Time-Dependent Film Soaks. In addition to the 5 h total soaking period, it was critical to understand the behavior of the system at different points during the analysis period. As such, additional films were prepared for analysis in a 37 °C water bath. At different time intervals (5 min, 1 h, 3 h, 5 h), the PBS soaking solution was collected from the system for GSNO and total GSH analysis. The films were also recovered for surface analysis.

S-Nitrosoglutathione Assay. To monitor the presence of GSNO in the soaking solution due to donor leaching from the Tygon material, we developed a GSNO assay. The PBS soaking solutions from either the NOA or timed-soak experiments were analyzed immediately after collection. Characterization of the GSNO product in PBS was performed by measuring the absorbance at 336 nm, the λ_{max} associated with the SNO moiety of the GSNO. A calibration curve was prepared using a set of GSNO standards (5−100 μM) prepared in PBS. Using the Beer's law plot, the concentration of intact GSNO could be determined in the soaking solution. All measurements involving GSNO quantification were performed by dispensing 300 μL per well of a 96-well UV transparent plate (Thermo Electron Corporation) followed by absorbance readings performed on a Biotek Synergy 2 plate reader (Winooski, VT, USA).

Total Thiol Assay. To analyze the total thiol content in the PBS soaking solution after film soak and GSNO quantification, the samples were subject to an Ellman's assay, a colorimetric assay for free thiol detection.[34,35] The assay was modified to contain a sodium borohydride reduction step that served to decompose any GSNO in solution, as well as cleave any GSSG disulfide[36] that is a byproduct of GSNO decomposition. Samples (50 μL) in PBS were aliquoted into a 96-well plate setup (Corning assay plate, polystyrene) and exposed to sodium borohydride (0.2 M $NaBH_4$ in 0.002 M NaOH, 50 μL) at pH 8−9 to facilitate the GSNO decomposition and GSSG reduction. After a 2 h treatment period, the excess borohydride was quenched with acid (0.2 M HCl, 50 μL) for 10 min (pH 3−4). Addition of NaOH (0.025 M, 50 μL) adjusted the pH to 8, where 50 μL of DTNB (10 mM) was added to produce a yellow-colored chromophore. The absorbance at 414 nm corresponding to the chromophore is directly proportional to the amount of free thiol in solution. GSH and GSNO standards were prepared from 5 to 100 μM and GSSG standards were prepared at half the concentration, 2.5−50 μM (1 mol GSSG: 2 mol GSH after reduction), to determine reduction efficiency.

Surface Analysis. Surface analysis of the GSNO-incorporated Tygon films pre- and postsoak was performed using water contact angle goniometry, optical profilometry, and SEM-EDS. Sample preparation for all methods involved removing the curved film from the polypropylene test tube and trimming the edges to lay the film flat on double-sided tape.

To assess the wettability of the films, water contact angle was obtained by placing a 2 μL ultrapure water droplet on the surface of the film with subsequent imaging using a Krüss Drop Shape Analysis System DSA 10 (Hamburg, Germany). The angle was obtained from the image of the droplet using the circle fitting parameter on Drop Shape Analysis 1.50 software.

Using optical profilometry, the average surface roughness of the film (Ra) was established to determine the effect of incorporating the GSNO into the Tygon system as well as subsequent film soaking. Three 2 × 2 stitch scans at 0.424 × 0.318 mm were performed on each film (control Tygon and pre- or postsoaked GSNO-incorporated films) using a Zemetrics ZeScope Optical Profilometer (Tucson, AZ, USA) at 20 × magnification. The Ra values were assessed and averaged over all replicate samples to obtain an average Ra roughness value.

SEM-EDS surface analysis was further performed to determine the S content on the surface of the GSNO-incorporated Tygon films pre- and postsoak. The film surfaces were imaged with a JEOL JSM-6500F scanning electron microscope (SEM, Peabody, MA, USA) coupled to a Thermo Electron energy dispersive spectrometer (EDS). Scans were collected at 100, 1000, and 5000 × magnification with a 15.0 kV accelerating voltage. EDS scans allowed for atomic weight percent of S to be determined across the sample surface.

Statistical Analysis. All error bars are reported as the standard deviation for each experiment with an $n \geq 3$ for all trials. The % relative error values reported were determined by dividing the standard deviation by the average. The Q-test was used to determine any outliers in the data sets with a confidence level of 99%. The

Student's *t* test was used to assess any significant difference among sets of data at the indicated confidence level (99%). To determine comparability within data sets for each method, a pooled *t* test was performed to determine whether there was any significant difference between sample populations at the 99% confidence level.

RESULTS AND DISCUSSION

NO-Releasing Tygon Films. Upon exposure of a 20 w/w% GSNO-incorporated Tygon film to simulated physiological conditions (1 mL PBS, pH 7.4, 37 °C), the film exhibited a steady release of NO due to heat and light-initiated GSNO decomposition as well as minor copper content in the buffer solution over a 5 h soaking period (Figure 1). Assuming the

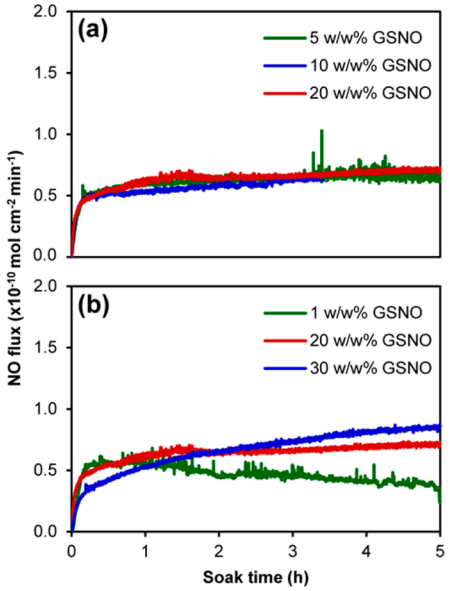

Figure 1. Real-time NO release profiles as measured by chemiluminescence for (a) middle-range w/w% films (5, 10) and (b) upper- and lower-range films (1, 30 w/w%) compared to the 20 w/w% GSNO-incorporated Tygon film exposed to 1 mL PBS (pH 7.4) at 37 °C. Each profile represents $n \geq 3$ with an average flux error of 7, 15, 14, 12, and 20% for the 1, 5, 10, 20, and 30 w/w% films, respectively.

majority of the NO is being released from the surface of the material, the average NO flux for the 0.25−5 h range was found to be 0.64 ± 0.05 × 10^{-10} mol NO cm^{-2} min^{-1} for the half-sphere shaped films with an estimated surface area of 1.25 cm^2. This NO flux marks the lower end of natural endothelial release, which has been reported to range from 0.5 to 4 × 10^{-10} mol NO cm^{-2} min^{-1}.[25] Since these films release NO on the order of the natural endothelium, this demonstrates promise to regulate platelets and clotting proteins at the material surface during blood contact. A previous publication demonstrated that a threshold NO flux greater than that of the natural endothelium (13.65 × 10^{-10} mol NO cm^{-2} min^{-1}) is needed in order to completely preserve platelet function and prevent blood clot formation in an ECC model.[3] In these same studies, the lowest levels of 2.33 × 10^{-10} mol NO cm^{-2} min^{-1} demonstrated activated clotting times that were closer to non NO-releasing control surfaces compared to the higher NO flux materials; however, lower flux levels were still found to preserve platelet function while preventing platelet activation and adhesion. Another study considered varying concentrations of *N*-diazeniumdiolate donor in a poly(vinyl chloride) polymer matrix and found, for films exhibiting fluxes ranging from 0.93 to 7.05 × 10^{-10} mol NO cm^{-2} min^{-1}, a significant decrease in platelet adhesion for all samples compared to the control, demonstrating improved performance with increasing NO flux.[24] Additional investigations of NO-releasing scaffolds have demonstrated a reduction in platelet[37−39] and bacterial[37,39,40] adhesion while preventing clot formation[41] for fluxes below the 13.65 × 10^{-10} mol NO cm^{-2} min^{-1} threshold, as well as a reduction in platelet adhesion for NO fluxes as low as 0.25 × 10^{-10} mol NO cm^{-2} min^{-1},[42] resulting in improved blood compatibility. A comprehensive review of the NO fluxes reported to have an effect on platelets strongly suggests that the NO flux plus the surface properties contribute to the blood response; thus, a one dosage fits all approach to NO-releasing surfaces is not feasible. Overall, the NO flux demonstrated by the 20 w/w% GSNO-incorporated Tygon films in these studies are on the lower end of the natural endothelial release, but are still promising toward controlling initial bioresponses over the 5 h window necessary for critical care applications since the surface properties were maintained.

On the basis of the amount of GSNO incorporated into the material, the theoretical NO reservoir that is available for release over the lifetime of the film is 12 μmol. Over the 5 h analysis period, the films released 0.024 ± 0.005 μmol of NO (Table 1), representing only ∼0.2% of the total NO reservoir.

Table 1. Data summary Corresponding to NO Release from Tygon Films Containing Various w/w% GSNO[a]

w/w% GSNO	NO flux (× 10^{-10} mol cm^{-2} min^{-1})[b]	total NO (nmol)[c]	theoretical NO_{total} (μmol)	% NO release[d]
1	0.47 ± 0.06	17.3 ± 0.7	0.6	2.91 ± 0.12
5	0.63 ± 0.04	23.1 ± 2.8	3.0	0.78 ± 0.09
10	0.59 ± 0.05	21.8 ± 1.5	5.9	0.37 ± 0.03
20	0.64 ± 0.05	23.9 ± 5.1	11.9	0.20 ± 0.04
30	0.68 ± 0.13	24.7 ± 4.4	17.8	0.14 ± 0.02
20 (with top coat)	0.32 ± 0.10	11.5 ± 1.6	11.9	0.10 ± 0.01

[a]Films were exposed to buffer at 37 °C for a 5 h duration. [b]NO flux is reported as the average ± standard deviation for the time duration 0.25−5 h. [c]Total NO release is reported as the average ± standard deviation for the entire time duration (0−5 h). [d]The % NO release is reported relative to a total theoretical NO reservoir based upon w/w% GSNO.

These films also have the potential to release NO over a prolonged period of time. For example, the 20 w/w% GSNO-incorporated Tygon films released NO over an extended 24 h duration under a PBS soak at 37 °C. The NO flux profile is shown in the Supporting Information (Figure S-1), where an average flux of 0.82 ± 0.08 × 10^{-10} mol NO cm^{-2} min^{-1} was maintained. One of the films was further allowed to soak for 1 week (see Figure S-2 in the Supporting Information), where an average flux of 1.2 × 10^{-10} mol NO cm^{-2} min^{-1} was exhibited. The average NO flux associated with the 20 w/w% GSNO films slightly increased with increasing soaking time over a week, where a steadier flux was exhibited within the first day of soaking. Of further note, the films that were soaked for 24 h released a total payload of 0.15 ± 0.02 μmol, representing 1.23 ± 0.17% recovery based upon the 12 μmol GSNO reservoir. The total NO payload was increased by an order of magnitude (1.5 μmol) upon further soak for a 1 week duration. Even after

1 week of soaking, the film only released 12.3% of the available NO, indicating that these materials could be used for applications that require prolonged NO release.

It is critical toward the application of these materials in an ECC setting to demonstrate that the NO being released from the system is truly localized at the material-buffer interface. Since donor-incorporated systems have been demonstrated to leach significant amounts of donor, it is a serious consideration whether or not any donor leached into the soaking solution will significantly contribute to the detected NO. If solution-phase donor decomposition is primarily responsible for the detected NO, this will result in a nonlocalized effect of NO at the material-biology interface. Because NO is a free radical with a short half-life, it is critical that the NO be released as close to the material surface as possible where the therapeutic effect is desired. To determine the extent of donor leaching and the potential for solution-phase donor to contribute to NO release, we performed subsequent GSNO and total thiol assays.

GSNO Leaching Studies. Leaching studies were performed by monitoring the presence of intact GSNO in the soaking solution for 20 w/w% GSNO-incorporated Tygon films exposed to PBS at different time intervals over 5 h. The GSNO concentrations associated with PBS soaking solutions were determined by using the calibration curve presented in the Supporting Information (Figure S-3). The limit of detection using this assay was 5 μM GSNO in PBS. It is important to note that any GSNO present below this 5 μM detection limit can be significant to the biological application. The GSNO concentration in soaking solution was determined at 5 min, 1 h, 3 h, and 5 h intervals (Figure 2). A burst of GSNO was released

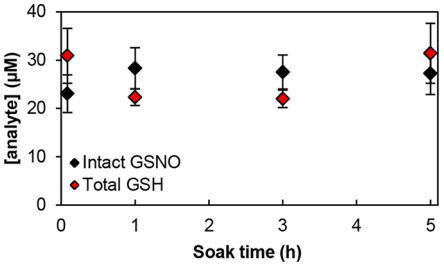

Figure 2. Intact GSNO and total GSH concentrations in PBS soaking solution as a function of time for 20 w/w% GSNO-incorporated Tygon film soaks. Donor-incorporated films were exposed to 1 mL PBS (pH 7.4) at 37 °C for a fixed interval of time (5 min, 1 h, 3 h, 5 h). Each point represents the average of 9 films.

from the film surface between 5 min (23.1 ± 3.9 μM) and 1 h (28.3 ± 4.2 μM) of film soak time (Table 2). Soaking the films past this point up to 5 h demonstrated no additional GSNO detected in solution (27.2 ± 4.3 μM at 5 h; not statistically different from 1 h at 99% CL). Since no additional GSNO is found intact in the soaking solution past 1 h, this indicates that either the amount of leached GSNO is removed quickly from the film upon soaking, or, the leached GSNO in solution could be increasing over soaking time with the solution-phase GSNO decomposing to yield NO. To determine if the GSNO in solution is giving rise to significant amounts of NO, and if so, the total amount of GSNO leached into solution, a total thiol assay was performed.

Table 2. Data Summary for GSNO Leaching and Total Thiol Assays Corresponding to 20 w/w% GSNO-Incorporated Tygon Films Exposed to Buffer at 37 °C

	20 w/w% GSNO[a]	
soaking time (h)	[GSNO] (μM)	[GSH_{total}] (μM)
0.08	23.1 ± 3.9	30.9 ± 5.7
1	28.3 ± 4.2	22.3 ± 1.7
3	27.5 ± 3.5	22.0 ± 1.8
5	27.2 ± 4.3	31.4 ± 6.2

[a]Data represent n = 9.

Total Thiol Content in Soaking Solution. To determine if any of the leached GSNO in solution was giving rise to NO release upon decomposition in PBS, the Ellman's assay for free thiol quantification was modified to include a borohydride reduction step. Essentially, any GSNO that leaches into the soaking solution and decomposes will yield NO and GSSG according to eq 1.

$$2GSNO \rightarrow 2NO + GSSG \quad (1)$$

Because the intact GSNO concentration, [GSNO], is known at the end of the soaking solution from the GSNO assay, a method that can quantitatively decompose any remaining GSNO as well as cleave any GSSG that was formed during the soaking period will yield total thiol content, [GSH_{total}]. If [GSH_{total}] = [GSNO] then there is no excess thiol due to GSSG in the soaking solution at the end of the soaking period and the only source of thiol in the soaking solution is due to the intact GSNO that was detected at the end of the soaking period. In this case, the leached GSNO would not significantly decompose during the soaking period. However, if [GSH_{total}] > [GSNO] at the end of the soaking period, excess thiol is present because of GSSG from solution-phase GSNO decomposition, indicating that the solution-phase GSNO is contributing to the system's released NO.

To demonstrate that the 2 h borohydride treatment effectively and quantitatively decomposed GSNO and reduced GSSG, were prepared GSNO and GSH standards from 5 to 100 μM and the GSSG standards were prepared at half the concentration (2.5−50 μM) because 1 mol GSSG will reduce to 2 mol GSH. Figure S-4 in the Supporting Information demonstrates that the calibration curves for all three analytes overlap, indicating that the borohydride effectively yielded GSH for all samples to result in colorimetric detection of the total thiol content in the soaking solution. When comparing the raw absorbance values from the assay, the GSNO decomposition and GSSG reduction are >95% efficient relative to the GSH standards.

To determine the availability for NO release of the 28 μM GSNO that was found intact in the soaking solution from the 20 w/w% GSNO-incorporated Tygon film, we performed the total thiol assay on the recovered PBS solutions. Figure 2 shows that, at each time point, the [GSH_{total}] values match up quantitatively with the [GSNO] values (values do not differ statistically at a 99% CL, Table 2). This indicates that the GSNO in the soaking solution did not significantly give rise to any NO release because the total GSH content is due to intact GSNO at the end of the soaking period. If the leached GSNO was a significant source of NO, the total GSH content would be higher than the GSNO due to the presence of GSSG that would form upon decomposition of the solution-phase GSNO.

An additional control experiment was performed where a 28 μM GSNO solution was prepared in PBS and added to a blank polypropylene test tube within an NOA cell under the same conditions as the Tygon film analysis. Minimal NO release was

found over a 5 h window compared to the 20 w/w% GSNO-incorporated Tygon film (Figure 3). Comparing the total NO

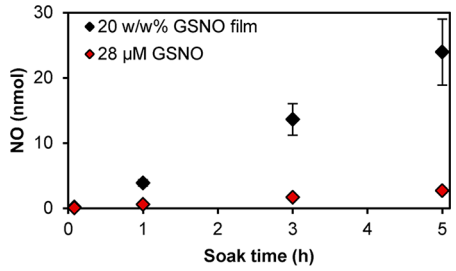

Figure 3. Total amount of released NO at various time points (5 min, 1 h, 3 h, 5 h) over a 5 h analysis duration for 20 w/w% GSNO-incorporated Tygon films exposed to 1 mL PBS or 28 μM GSNO in PBS (each at 37 °C). The data represent an n = 3 for the 28 μM GSNO analysis and an n = 6 for the 20 w/w% GSNO film analysis.

recovery values of the 20 w/w% GSNO-incorporated film with the 28 μM GSNO leachate at the 5 h point, the 28 μM GSNO control yielded 2.7 ± 0.4 nmol NO, only 11% of the 23.9 ± 5.1 nmol recovered from the NO-releasing Tygon film system. The amount of GSNO that decomposed to yield the NO that was recovered from the 28 μM GSNO solution was not significant enough to result in a detectable change in absorbance after 5 h using the GSNO assay. The amount of GSNO (28 μM) that is detected intact in the soaking solution after the 20 w/w% GSNO-Tygon film soak will give rise to detectable NO. However, the decomposition of the solution-phase GSNO only accounts for ∼10% of the total NO that is released from the total GSNO-Tygon/PBS system (not distinguishable within the LOD of the GSNO assay). The GSNO found in the solution phase (0.028 μmol) accounts for only 0.2% of the total GSNO that was blended into the Tygon film (12 μmol GSNO incorporated per film). Because such a small amount of the total GSNO in the system is leaching into the soaking solution, the majority of the GSNO remains in the polymer phase. Subsequently, the majority of the NO that is released from the system is due to the bulk GSNO that is present in the film.

Overall, the GSNO leaching studies coupled to the total thiol assay analysis indicate that a quick burst of GSNO is released into the soaking solution after only 5 min −1 h of film soak, followed by no more significant donor leaching for the remainder of the soaking period, with >80% of the donor leaching within only 5 min. Compared to the theoretical amount of GSNO present in the entire Tygon film (12 μmol), the amount of donor leached into the soaking solution (0.028 μmol) accounts for only ∼0.25% of the total GSNO that is available in the system. The amount of NO that is released by the leached, solution-phase GSNO (28 μM) is quite minimal in comparison to the total amount of NO that is released by the GSNO-incorporated Tygon system (13%) and it is not within the sensitivity of the assays employed to monitor changes in the total thiol content or GSNO concentration in soaking solution.

One approach that has been employed in the field of NO-releasing materials is the use of a polymer top coat to prevent the donor at the surface of the film from leaching into the soaking solution.[2,14,17] We recently reported that top coating methods can be an effective way of accomplishing this.[5] For the Tygon system reported herein, we briefly investigated the effect of a top coat layer to prevent donor leaching for this system. This was accomplished by adding an additional 200 μL aliquot of blank Tygon polymer solution over top of the cured 20 w/w % GSNO-incorporated Tygon base film. The NO flux profile (Figure 4) demonstrated a slowed release over the 5 h duration

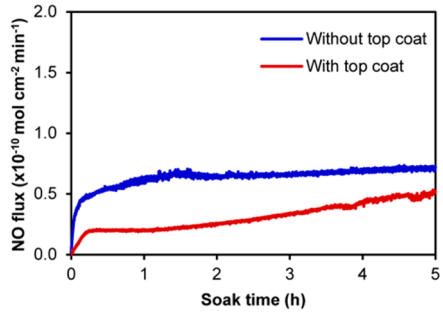

Figure 4. Real-time NO release profiles as measured by chemiluminescence for a 20 w/w% GSNO-incorporated Tygon film with or without a top coat, exposed to 1 mL PBS (pH 7.4) at 37 °C. Each profile represents n = 3 with an average flux error of 12% for each profile.

(0.32 ± 0.10 × 10^{-10} mol NO cm^{-2} min^{-1}) compared to the film that was not top coated (0.64 ± 0.05 × 10^{-10} mol NO cm^{-2} min^{-1}), where the NO flux and total NO recovery was about half that of the non top-coated films (Table 1). Top coat layers have been previously demonstrated to slow the release of NO from donor-incorporated polymer systems.[2] A GSNO assay performed on the recovered soaking solution from the top coated film quantified 10.8 ± 1.7 μM GSNO (Table 3),

Table 3. Data Summary for Intact GSNO and Total Thiol (GSH) in the PBS Soaking Solution Recovered from Various w/w% GSNO-Incorporated Tygon Films Exposed to Buffer at 37 °C for a 5 h Duration

w/w% GSNO	[GSNO] (μM)	[GSH] (μM)
1	2.4 ± 0.1	3.4 ± 0.9
5	5.1 ± 0.4	3.7 ± 0.2
10	9.5 ± 0.7	8.3 ± 0.7
20	27.2 ± 4.3	31.4 ± 6.2
30	31.1 ± 5.4	41.8 ± 6.1
20 (with top coat)	10.8 ± 1.7	13.1 ± 3.8

indicating that some of the donor did leach from the film over the 5 h soaking period. This is a decrease in concentration compared to the nontop coated films (27.2 ± 4.3 μM after 5 h); however, the addition of the top coat did not completely prevent donor leaching, likely due to mixing of the donor-containing polymer layer and the blank top coat layer. An initial attempt at a top coat, therefore, was not an effective method for preventing donor leaching into the buffer soaking solution.

Monitoring Surface Sulfur Content. Using SEM coupled to energy-dispersive X-ray spectroscopy (EDS), the S content on the surface of the 20 w/w% GSNO-incorporated Tygon films was determined before and throughout the soaking period. By measuring the surface S as a function of soaking time, this informed the behavior of the GSNO on the surface of the film. The SEM images of the 20 w/w% GSNO-incorporated Tygon films pre- and postsoak are shown in Figure 5 (1000 × magnification) along with the corresponding EDS maps that track the phase containing S. Further, SEM-EDS data can be found in the Supporting Information, including the blank Tygon pre- and postsoak (see sections

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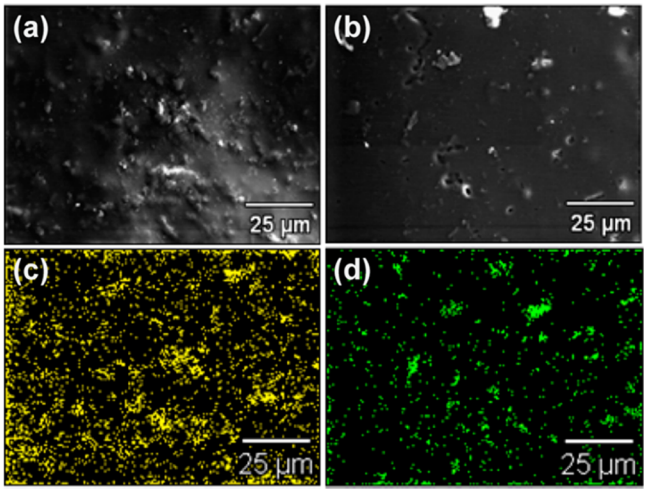

Figure 5. Representative SEM images of the 20 w/w% GSNO-incorporated Tygon films at 1000 × magnification both (a) before and (b) after a 5 h soak (PBS, 37 °C). The corresponding EDS maps are shown for the S-containing phase for the (c) pre- and (d) postsoak samples.

S4–S5 in the Supporting Information), as well as the SEM and EDS analysis of the S-containing phase as a function of time for the 20 w/w% films (see sections S6–S7 in the Supporting Information). The SEM-EDS analysis reveals an obvious phase-separation corresponding to the GSNO donor that is present on the surface of the Tygon film. The surface elemental composition of S for the donor-incorporated film presoak was 0.61 ± 0.02%, and, as shown in Figure 6, 90% of this surface S

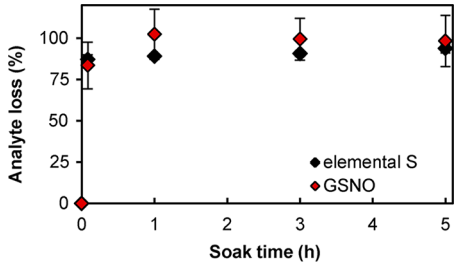

Figure 6. For a 20 w/w% GSNO-incorporated Tygon film system, the % decrease in the amount of elemental surface S or the % of total leached GSNO into soaking solution is represented. Each point represents an $n = 9$ for the GSNO values, while the elemental S data was taken as the atom% based upon SEM imaging (140 μm × 110 μm area imaged per sample).

is removed upon 5 min to 1 h soaking period. This matches up, within error, with the values associated with the % of leached GSNO as a function of time (values at each time point are statistically the same at 99% CL). By tracking the surface S in conjunction with the % leached GSNO from the GSNO assay, we see that the GSNO that is found in solution is due to GSNO that is present on the surface of the film, which is removed almost completely within a 5 min exposure of the polymer surface to the soaking buffer. It can be additionally noted that the surface analysis of the blank Tygon film showed no surface S neither pre- nor postsoak, indicating that the S on the surface of the GSNO-incorporated films is due to the presence of donor on the material surface.

Varying Donor Concentration in the Film. The amount of donor that is incorporated into the Tygon matrix will dictate the amount of NO that is ultimately available for release over the lifetime of the material. As such, it is important to understand how varying the donor concentration will impact the NO release properties as well as the leaching of the GSNO from the system. To investigate this, the amount of donor was varied to include 1, 5, 10, and 30 w/w% GSNO-incorporated films. The average NO fluxes for the films containing 5, 10, and 30 w/w% GSNO were statistically the same as the 20 w/w% GSNO films within error (Table 1, Figure 1). It was not until the concentration of GSNO was dropped to 1 w/w% that there was a decrease observed in the NO flux ($0.47 \pm 0.06 \times 10^{-10}$ mol NO cm^{-2} min^{-1}). When considering the amount of GSNO that was incorporated into each film, the % of NO released based upon that available reservoir was <1% for the 5–30 w/w % films. The kinetics of NO release were the same for these films because the amount of GSNO that decomposed within the film to yield NO is such a small percentage of the total GSNO reservoir.

Previous studies involving GSNO-blended polymer films have indicated a difference in the NO release profile depending upon the amount of GSNO in the system.[18] However, for these hydrophilic polymer systems, the majority (90%) of the NO reservoir was released within a 24 h time period at physiological temperature, where the kinetics of donor decomposition in the polymer system were found to be significantly slowed compared to solution-phase GSNO decomposition. Overall, this cage effect was found to result in slower donor decomposition kinetics due to the presence of a hydrophilic polymer, which has also been demonstrated for other polymer systems.[43,44] Further studies on this previous polymer system indicated that changes in the pore size and morphology of the polymer resulted in differences in GSNO leaching from the polymer matrix.[21] Considering all of these findings, we can see that the Tygon matrix presented herein is such that bulk of the GSNO will remain in the polymer film. It can therefore be determined that the morphology of the material is such that the GSNO is severely diffusion limited compared to these other studies that employ higher water uptake polymers, which would result in an even more increased cage effect. This cage effect is clearly reflected in the NO release kinetics for these GSNO-incorporated Tygon films, where the NO release is severely delayed, with only 1% of the NO reservoir being released for the 20 w/w% samples over a 24 h period. Additional slowing of the GSNO in the Tygon matrix could be due to the stabilization of GSNO within acidic microdomains of the polymer film. It is established that the thermal degradation of poly(vinyl chloride) below 600 °C will result in HCl as the major product,[45,46] which could yield acidic microdomains within the Tygon matrix. Since RSNOs have been shown to be stabilized under acidic conditions,[47] the acidic microdomains could slow down the GSNO decomposition. This microdomain phenomenon and its influence on NO release rates has been demonstrated previously for *N*-diazeniumdiolate materials.[16] The microdomain influence on NO release also strongly suggests that the GSNO is likely remaining entrapped in the material phase. Overall, because the donor decomposition is delayed in the Tygon matrix, such a small percentage of the available GSNO actually decomposes, resulting in similar NO release kinetics for the 5–30 w/w% samples.

To consider the effect of donor concentration within the film on the amount of GSNO leachate, a GSNO assay was performed on the soaking solutions for the 1, 5, 10, and 30 w/w % GSNO-incorporated Tygon films. The amount of GSNO

found intact in the soaking solution is statistically the same as that of the total GSH concentration (Figure 7, Table 3),

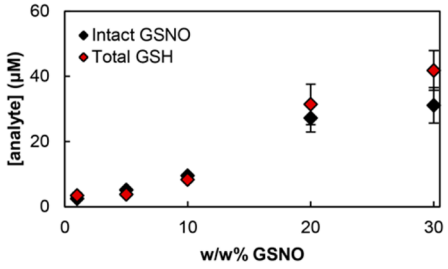

Figure 7. GSNO and GSH concentrations in the soaking buffer after a 5 h film soak (pH 7.4, 37 °C) as a function of w/w% GSNO incorporated into the Tygon film ($n \geq 3$).

indicating once again that the GSNO that leaches into the solution does not contribute to the bulk of the detected NO in the system. There is a dependence on the w/w% of GSNO incorporated into the material matrix and the amount of GSNO donor that is found intact in the PBS solution at the end of the 5 h soaking period. A higher w/w% will result in more surface GSNO which is available to leach into the soaking solution.

An in-depth look at the 10 w/w% GSNO-incorporated samples (see Figure S-14 in the Supporting Information) shows that, in a similar fashion as the 20 w/w% system, the amount of GSNO that leaches from the 10 w/w% donor-incorporated films is nearly completely removed within a 5 min soak period. Additionally, a total thiol assay on the soaking solutions reveals that the [GSH_{total}] values are statistically the same (99% CL) as the [GSNO] values for the 10 w/w% samples (see Table S-2 in the Supporting Information) at different time points across the 5 h soaking duration. This once again demonstrates that the GSNO in solution is not giving rise to the bulk of the NO detected in the system. Overall, by cutting the donor concentration in half, we find that the NO release profile is maintained, with a flux that mimics the lower end of the natural endothelium, but the amount of surface GSNO that is available to leach from the system is significantly reduced.

Surface Properties Pre- and Postsoak. To generally assess the impact of the GSNO-incorporated Tygon film coatings on the biocompatibility of the surface, we made surface wettability and roughness measurements. Each of these surface properties has been found to impact the blood-compatibility of materials in general.[48−51]

The surface wettability was assessed by water contact angle goniometry. The contact angle was established for blank Tygon and 20 w/w% GSNO-incorporated Tygon films pre- and postsoak (Table 4). The blank Tygon film exhibited a contact angle of 102.9 ± 2.9° presoak and 102.5 ± 1.8° postsoak, compared to 97.1 ± 2.6° for the 20 w/w% GSNO-incorporated Tygon surface presoak. The blank Tygon films exhibited the same contact angle before and after 5 h of film soak, indicating no effect on the Tygon surface wettability due to exposure to PBS at 37 °C. Additionally, the contact angle values for the blank Tygon and 20 w/w% GSNO-incorporated Tygon films presoak were not statistically different, indicating that the addition of GSNO did not significantly impact the wettability of the Tygon surface. Upon soaking the GSNO-incorporated films, the water contact angle remained the same (98.4 ± 1.8°). Overall, the water contact angle for all samples ranged from 95 to 103° which indicates generally hydrophobic surfaces. The

Table 4. Summary of Surface Properties Corresponding to Blank and 20 w/w% GSNO-Incorporated Tygon Films before and after Exposure to Buffer at 37 °C

sample	soaking time (h)	water contact angle (deg)	Ra (nm)[a]
blank Tygon	presoak	102.9 ± 2.9	10.4 ± 1.1
	5	102.5 ± 1.8	12.9 ± 2.0
20 w/w% GSNO Tygon	presoak	97.1 ± 2.6	49.8 ± 8.0
	0.08	97.1 ± 2.0	44.6 ± 15.8
	1	95.6 ± 5.1	37.3 ± 5.9
	3	97.1 ± 3.6	35.0 ± 6.1
	5	98.4 ± 1.8	29.9 ± 5.8

[a]Average surface roughness determined from optical profilometry.

small difference in the range of the contact angle values means that the surfaces are of comparable relative hydrophobicity regardless of GSNO incorporation and subsequent exposure to PBS.

When considering the SEM images of the GSNO-incorporated surfaces pre- and postsoak (Figure 5), it is difficult to assess whether or not there was a significant change in the surface features after soaking. In order to account for the topography of the surfaces, optical profilometry was employed to determine the average surface roughness (Ra). Representative surface roughness maps are shown in the Supporting Information (Section S9). The surface roughness of the blank Tygon film (10.4 ± 1.1 nm) did not vary within statistical error after 5 h of film soak (12.9 ± 2.0 nm) indicating no effect of the soaking process on the roughness of the Tygon surface. Compared to the blank Tygon films, the surface roughness increased after the incorporation of 20 w/w% GSNO (49.8 ± 8.0 nm). After exposure of the GSNO-incorporated Tygon films to soaking solution for different time durations, it appears that there is a trend of decreasing surface roughness with increasing soak time (Table 4); however, there is no statistical difference between any of the values at 99% CL. Overall, larger error bars associated with the Ra value due to variance in the surface upon GSNO incorporation does not result in a clear trend for the surface roughness with increasing film soak time. It is clear from the profilometry analysis that the incorporation of the GSNO into the Tygon matrix definitely increases the surface roughness. However, because of variability in the surface morphology, no statistical difference is seen for the donor-incorporated films upon increasing exposure to PBS. The surface roughness of the Tygon film increases by >20 nm when GSNO is incorporated, therefore potentially impacting the adsorption of biological components compared to the blank Tygon surface. For instance, it has been previously established that a surface roughness change of ∼20 nm or greater will fall within the range of surface variability that will impact the interaction of the material with proteins.[52] We mention this to point out that the incorporation of the GSNO into the Tygon film will likely impact the adsorption of key biological components when compared to the blank Tygon film. Overall, the incorporation of 20 w/w% GSNO into a Tygon film will not affect the surface wettability significantly, but does lead to an increase in surface roughness. The surface roughness values, however, do not change significantly for the GSNO-incorporated films over the course of the soaking period within the range of impacting biological factors, such as proteins.

It was mentioned earlier that the 20 w/w% GSNO formulation underwent analysis for 1 week to assess the ability

of the films to release NO for a prolonged period of time. The Tygon film stayed intact for this duration of NO release, and a representative image of the surface is shown in Figure S-11 of the Supporting Information. Additional surface analysis indicated a water contact angle of 101.5 ± 0.6° and an average surface roughness (Ra) of 21.9 ± 10.6 nm. These values are not statistically different at the 99% confidence level from the values corresponding to the 5 h soaked samples, indicating that these surface properties are not changing with a significantly prolonged soaking period.

CONCLUSIONS

For a model Tygon system, we have demonstrated that the addition of 20 w/w% GSNO into the polymer matrix will result in an NO-releasing film. The GSNO on the surface of the film will leach into the soaking buffer nearly completely within 5 min of exposure time, after which point the GSNO concentration remains steady (1−5 h). The total amount of leached donor represents <0.25% of the total GSNO reservoir that remains contained within the Tygon matrix. Analysis of the S content on the surface of the films further indicates that the surface-bound GSNO will be removed from the film almost immediately. Using a total thiol assay, we find that the total thiol content in the soaking solution is due only to intact GSNO at the end of the soaking period, thus demonstrating that the GSNO in the soaking solution does not significantly decompose. The amount of NO recovered because of the solution-phase GSNO decomposition is ∼10% of the total NO that is released by the system. Overall, the bulk of the recovered NO is due to GSNO decomposition within the film, resulting in the localized NO delivery that is desired from these systems for bioapplications involving blood-contacting extracorporeal circuitry. The NO flux associated with this Tygon system is $0.64 \pm 0.05 \times 10^{-10}$ mol NO cm^{-2} min^{-1}, which mimics the lower end of natural endothelium NO release. The NO release and minimal donor leaching over the 5 h time frame are relevant to ECC applications. Further, the surface properties of the system indicate hydrophobic films with minimal variation in surface roughness upon soaking, making them strong candidates as biomaterials. Upon varying the amount of GSNO in the system from 5 to 30 w/w%, the NO release properties remain the same, but a smaller amount of GSNO on the surface is available to leach into the soaking solution for the lower w/w% films.

Overall, toward the ability to develop Tygon surfaces with enhanced biocompatibility to overcome blood-clotting and infection-related complications, the incorporation of GSNO appears quite promising. Donor leaching is considered the prevalent problem with donor-incorporated systems, but the only donor that is leached from this system is initially surface-bound GSNO, and is quickly removed upon soaking (∼5 min). To prevent this surface GSNO from being swept into the blood, which could lead to downstream effects, the GSNO-Tygon coated tubing need only be prerinsed prior to application. More specifically, the GSNO at the surface of the material will be removed nearly completely within 5 min, and the NO flux will reach a steady state at the lower end of the endothelial release range within roughly 15 min of soaking time. Therefore, treating the material coating with a presoak step will effectively eliminate surface bound donor as well as reach a constant NO flux. During the subsequent 5 h time period associated with a typical hemodialysis procedure, these coatings will release NO on the order of the natural endothelium and maintain critical surface properties, such as wettability and roughness relative to the nonsoaked sample. The ability to maintain predominantly localized NO release associated with these coatings is critical to their application as blood-contacting devices.

ASSOCIATED CONTENT

Supporting Information

Prolonged NO release (24 h and 1 week) for 20 w/w% GSNO Tygon films, calibration curves for the GSNO and GSH assays, SEM and EDS analysis of blank and 20 w/w% GSNO-incorporated Tygon films, analysis of 10 w/w% GSNO-incorporated Tygon films as a function of soaking time, and optical profilometry surface roughness maps. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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