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Kinetics of *S*-Nitrosation Processes in Aqueous Polymer Solution for Controlled Nitric Oxide Loading: Toward Tunable Biomaterials

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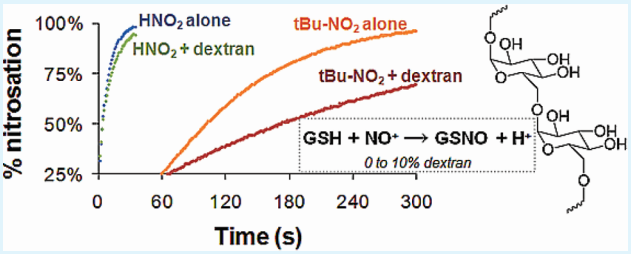
Supporting Information

ABSTRACT: An understanding of the nitrosation processes that dictate *S*-nitrosothiol formation in the presence of a polymer is crucial toward the controlled synthesis of nitric oxide (NO)-releasing materials, an important class of biomaterials that mimic the natural function of cells. Herein, the kinetics of *S*-nitrosoglutathione (GSNO) formation in the presence of dextran under a variety of nitrosation conditions, including the nitrosating agent and the dextran concentration, are reported. When comparing nitrous acid and *t*-butyl nitrite as the nitrosating agent, the use of nitrous acid results in 100% nitrosation of the thiol sites within less than a minute and *t*-butyl nitrite requires more than 5 min to reach completion. This trend establishes nitrous acid as a highly efficient nitrosating agent. In the presence of increasing dextran concentration from 0 w/v% to 10 w/v%, the extent of nitrosation decreases by approximately 5% and 30% using nitrous acid and *t*-butyl nitrite, respectively. With sufficient reaction time, either reagent leads to 100% nitrosation. This indicates that *t*-butyl nitrite is the preferred reagent for fine-tuned NO loading of thiol sites as the extent of reaction is greatly impacted by the polymer concentration. Taken together, these studies provide valuable insights regarding the ability to tailor NO storage within biomaterials for use in a wide range of clinical applications.



KEYWORDS: *S-nitrosation, nitrosating agent, glutathione, dextran, nitric oxide loading, biomaterials*

INTRODUCTION

Nitric oxide (NO) is an important bioregulatory agent and has been implicated in a variety of physiologically relevant processes.[1−7] In addition to the enzymatic production of NO in cells, NO is selectively transported and released in vivo via *S*-nitrosothiols (RSNOs).[8−12] *S*-nitrosothiols constitute a major class of endogenous and exogenous NO donors and are synthesized upon *S*-nitrosation of thiol moieties. As such, RSNOs have attracted widespread attention as vehicles to incorporate long-term NO storage in biomaterials used for blood- and tissue-contacting devices.[13,14] *S*-nitrosothiol moieties decompose through a variety of decomposition mechanisms,[15−17] resulting in NO release from the material surface. These materials can exert control over biological functions that occur at the material surface, leading to selective cell proliferation accompanied by minimized platelet activation and adhesion,[18,19] vasodilation,[20] and antibacterial effects.[21] Therefore, the ability to fine-tune NO loading through the use of NO donors is crucial to develop materials with tailorable reservoirs of NO for a variety of biomedical applications including site-specific delivery for tumor apoptosis, biodegradable tissue scaffolds, extracorporeal circuitry, and cardiovascular implants.

Previous work demonstrates the incorporation of RSNO moieties within various materials, including polymers,[20−24] hydrogels,[18,19,25] xerogels,[26] dendrimers[27] and fumed silica filler particles.[28,29] Of further importance for application is the ability to process NO-releasing materials in usable forms, such as electrospun fibers.[30] Despite achieving NO loading within the materials, no control has been exhibited over this loading process. Further, the kinetics of nitrosation in polymer systems have not been reported. If control over NO loading is to be achieved toward a requisite NO storage capacity for a specific treatment, fundamental studies investigating the factors affecting RSNO formation kinetics are a necessity. For certain applications, the maximum amount of NO loaded onto the material is desired to ensure the highest therapeutic dosage of NO. In such cases, the most efficient nitrosation would be ideal in order to load all of the thiol sites. For other applications, of greater importance is the ability to exert fine-tuned control over the nitrosation process. If only a fraction of the thiol sites present require NO loading for material applications where a lower dosage of NO is required, the nitrosation process needs to occur more slowly under conditions where the reaction can be halted at the desired end point.

To achieve controllable derivatization of thiol sites, we investigate the rate of RSNO formation under different synthetic conditions with emphasis on the kinetics of *S*-

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nitrosation in the presence of a polymer. In this work, we present the kinetics of *S*-nitrosothiol formation in a polymer solution and not within a precast solid material. From an applications viewpoint, many materials are often solvent-cast or dip-coated onto medical device substrates, thus the formation of the RSNO in a solution environment is practical. Further, due to the nitrosation reactions involving aqueous (nitrous acid) or liquid (*t*-butyl nitrite) nitrosating agents, it is more feasible to load NO within a polymer solution. The resulting RSNO−polymer can then be cast from solution into its desired forms (thin-film coatings, electrospun nanofibers, etc.). Despite the ultimate application involving nitrosation of thiol sites covalently attached to a polymer, we consider the formation of a small molecule donor in a polymer solution. This system offers better control over factors under consideration as the amount of thiol sites in the system is easily altered by the amount added to solution. In the covalently bound thiol instance, the concentration of thiol in the system would be more difficult to regulate. Additionally, the system described herein allows the amount of thiol to remain constant while altering the amount of polymer added. The general trends for efficiency and tunability of NO loading for the small molecule case can then be extended to the thiol pendant to a polymer situation. The reaction variables that allow the most efficient loading of thiol sites, as well as those that permit fine-tuned control over the nitrosation process, are described. The reaction conditions under investigation include a comparison of nitrosating agents as well the effect on the rate of *S*-nitrosation because of different concentrations of a water-soluble polymer present during the reaction. During the solution-phase nitrosation, the nitrosating agent and polymer concentration can be altered to control the nitrosation kinetics as we have described, even for a case where a thiol is covalently attached to the polymer. The ability to fine-tune NO loading in such systems is crucial toward the development of tailored NO-releasing biomaterials.

EXPERIMENTAL SECTION

Materials. Glutathione (GSH, Acros Organics, 98%, reduced), sodium nitrite ($NaNO_2$, Alfa Aesar, 99.999%), and hydrochloric acid (BDH Aristar, 36−38%), were all used as received, unless otherwise noted. For all kinetics studies, *t*-butyl nitrite (*t*-BuONO) was obtained from Acros Organics (90% pure) and dextran (MW ≈ 40 000) was from TCI America. For the pH studies described, *t*-butyl nitrite (90%) was from Aldrich and dextran (MW ≈ 40 000) was from Sigma. All solutions were prepared using Millipore treated water (18.2 MΩ). During all solution preparation and reagent addition, the % purity of each reagent was considered.

Methods. Kinetic runs were performed using a Thermo Evolution 300 UV−vis spectrophotometer equipped with a Smart Peltier Thermostatted Single Cell Holder with stir and temperature control capabilities. The temperature of the solution contained within the cuvette was monitored using a temperature probe accessory (Pt100 platinum resistance thermometer installed in a Teflon cuvette stopper) connected to the Peltier device.

Glutathione solutions were prepared in EnviroWare amber, EPA-certified vials to minimize any metal ion contaminants in contact with the thiol solutions. Solutions were prepared freshly and constantly purged with N_2 gas prior to analysis to deoxygenate the solution, preventing thiol oxidation to disulfide in the presence of O_2. Solutions were purged in a 25 °C water bath to minimize temperature equilibration time upon transfer of the solution to the cuvette within the 25 °C Peltier spectrophotometer accessory. The temperature of the reaction cell remained at 25.0 ± 0.4 °C throughout the course of all spectrophotometric experiments.

Nitrosation with HNO_2. The concentration of GSH was held constant as the limiting reagent (6.5×10^{-3} M) with the concentration of acid and nitrite in 4× molar excess (25×10^{-3} M). Two mL of GSH in acid solution were transferred to a quartz cuvette within the UV−vis spectrophotometer. The temperature was monitored by the temperature probe submerged in the solution. At the start of the kinetic run, 100 μL of sodium nitrite solution (prepared in Millipore water) were injected into the cuvette which contained a stir bar. The absorbance at 545 nm was monitored for 4 min with the temperature probe in place to monitor the temperature during the analysis period. Despite the sodium nitrite solution being at room temperature, the addition of the 100 μL aliquot did not change the temperature of the overall solution, as indicated by replacement of the temperature probe immediately after injection.

Nitrosation with t-Butyl Nitrite. Two millilters of glutathione solution (6.5×10^{-3} M, prepared in water) were transferred to a quartz cuvette. The start of the kinetic run was marked by injection of a molar excess of *t*-butyl nitrite (6.75 μL to yield 25×10^{-3} M reagent, 90% purity accounted for) into the cuvette via a Hamilton 25 μL airtight syringe. The temperature probe was employed to determine the initial temperature of the thiol solution. To minimize *t*-butyl nitrite evaporation over the course of the nitrosation period, the temperature probe was replaced with a cuvette lid. The absorbance at 545 nm was monitored for 15 min, and the final temperature of the reaction solution was determined by the temperature probe. The initial and final temperature values were taken to be the temperature range of the reaction.

pH Measurements. To determine pH effects on the kinetics of nitrosation, we determined pH values using a Mettler Toledo InLabRoutine Pro pH probe (KCl electrolyte, calibrated at 4, 7, and 10). For nitrous acid nitrosation measurements, glutathione and dextran polymer were dissolved in an HCl solution. Upon injection of 150 μL of $NaNO_2$ solution into 3 mL of GSH/dextran solution, the final concentrations were 6.5×10^{-3} M GSH, 25×10^{-3} M HCl and 25×10^{-3} M $NaNO_2$. The pH measurement was recorded prior to the NO_2^- injection and then 4 min after the injection to mimic the final pH of the solution at the end of the kinetics run. For the *t*-butyl nitrite nitrosation measurements, glutathione and dextran were dissolved in water and, upon injection of 10.1 μL (using a 100 μL Hamilton airtight syringe) of the nitrosating agent into 3 mL of solution, the final concentrations were 6.5×10^{-3} M GSH and 25×10^{-3} M *t*-butyl nitrite. The pH measurement was recorded before *t*-butyl nitrite injection, the vial was then capped and the pH value recorded 15 min after injection.

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 in figure captions were determined by dividing the standard deviation by the average for the *y* axis values. The Student's *t* test was used to assess any significant difference among sets of data at the indicated confidence level (99% +).

RESULTS AND DISCUSSION

Model System. Because of its water solubility, simplicity as a homopolymer and current use in a range of drug delivery applications,[31] dextran was chosen as the model polymer investigated in these studies (structure shown in Figure 1). Glutathione was used as the thiol substrate for two experimental design reasons: (1) the nitrosated product, *S*-nitrosoglutathione (GSNO), has been well-characterized, and (2) GSNO is among the most stable of reported RSNO species.[32] *S*-Nitrosoglutathione exhibits two UV−visible absorption bands (λ_{max} = 335 nm, ε_{max} = 922 M^{-1} cm^{-1}; λ_{max} = 545 nm, ε_{max} = 15.9 M^{-1} cm^{-1}).[33] The 545 nm peak was monitored to measure the formation of the GSNO product in these studies instead of the 335 nm peak, despite a lower extinction coefficient, because of a lack of interfering species (NO_2^-, HNO_2) at 545 nm.[34] The stability of GSNO is important as the nitrosothiol moiety decomposition will not

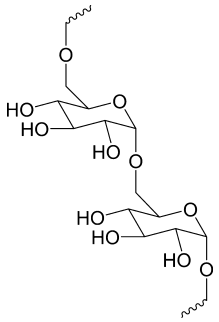

Figure 1. Structure of dextran polymer.

significantly compete with the rate of its formation. We investigated two commonly reported nitrosating agents when used in a fixed molar excess relative to glutathione: nitrous acid (HNO_2) and *t*-butyl nitrite. The use of both HNO_2[34−38] and *t*-butyl nitrite[39,40] for *S*-nitrosation under aqueous conditions has been reported previously, establishing each as a viable nitrosating reagent for these studies. Since the HNO_2 and *t*-butyl nitrite nitrosating agents are aqueous and liquid, respectively, we report RSNO formation in a polymer solution rather than within a cured solid substrate.

Nitrosation under Aqueous Conditions. The kinetics of GSNO formation using nitrous acid or *t*-butyl nitrite as the nitrosating agent under aqueous conditions serve as a point of comparison for the polymer work presented later. Upon nitrosation of GSH, a nitrosonium cation (NO^+) replaces the proton at the thiol site, as indicated in Figure 2.

Glutathione S-Nitrosation with HNO_2 for More Efficient NO Loading. Previous reports of nitrosation kinetics have commonly used a stop-flow apparatus to accomplish in situ formation of HNO_2 prior to addition to the thiol solution.[34,35,37] Here, we employ a simplified method whereby an aliquot of sodium nitrite solution is injected directly into the reaction cell containing a stirring solution of the thiol. Due to the acidic conditions employed (pH <3, before and after reaction), the HNO_2 species forms rapidly upon nitrite injection into the acidic thiol solution (pK_a = 3.15)[41] and is available for reaction at the thiol site. Under these highly acidic conditions, the nitrosonium ion (NO^+) is the species that reacts with the thiol site.[34,42] Because the mechanism of nitrosation remains the same as for the stop-flow apparatus previously described, this method also allows for an appropriate kinetics investigation of HNO_2 nitrosation. For the HNO_2 nitrosation of thiols described herein, a first-order dependence on the thiol has been reported.[34,35]

We experimentally monitor the formation of *S*-nitrosoglutathione, however, we report the kinetics in terms of the disappearance of the thiol reactant. Theoretically, the disappearance of the thiol could be attributed to either the formation of the *S*-nitrosated product or possible disulfide formation. By measuring the formation of the *S*-nitrosothiol and not the disappearance of the thiol, we consider only the reaction of the thiol to form the product of interest. We report the kinetics in terms of the disappearance of the reactant to offer insight into how quickly a thiol within a materials system will react under each set of conditions. However, because we have monitored the formation of the *S*-nitrosated product and then calculated the thiol concentration, disulfide formation is not a concern in the data analysis. Further, the final concentration of the *S*-nitrosated product that is formed in all cases suggests that the starting amount of thiol is completely converted to product and that minimal disulfide is formed (the experimentally determined maximum product concentration matches the theoretical amount of thiol added). The concentration of thiol at time *t*, $[GSH]_t$, was calculated from the GSNO concentration at that time, $[GSNO]_t$, as indicated by eq 1. The absorbance was followed at 545 nm (ε_{max} = 15.9 M^{-1} cm^{-1}), where the Beer−Lambert law holds for the −SNO moiety.[33]

$$[GSH]_t = [GSH]_0 - [GSNO]_t \quad (1)$$

The initial concentration of thiol, $[GSH]_0$, was experimentally determined by considering the maximum GSNO concentration, $[GSNO]_{max}$, reached. The $[GSNO]_{max}$ value is determined from the maximum absorbance point from the absorbance (545 nm) versus time plot (Figure 3 inset). This $[GSNO]_{max}$ directly correlates to the GSH initial concentration in a 1:1 molar ratio (eq 2).

$$[GSNO]_{max} = [GSH]_0 \quad (2)$$

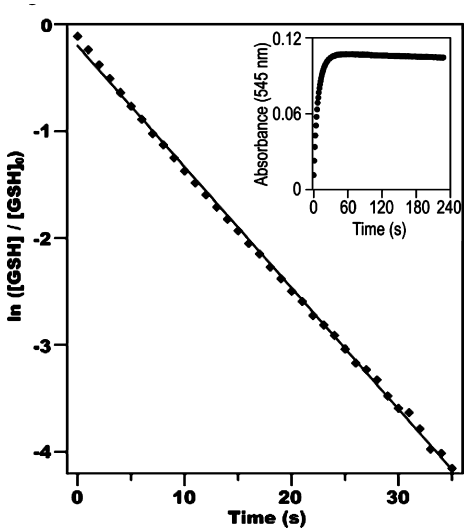

Figure 3. First-order plot of $\ln([GSH]/[GSH]_0)$ as a function of time for the HNO_2 nitrosation of glutathione in aqueous conditions (0% dextran), R^2 = 0.999, *n* = 10, 6.1% relative error, and *T* = 25.0 ± 0.4 °C. The inset shows the representative plot of the absorbance changes at 545 nm due to SNO moiety formation (6.76 ± 0.13 × 10^{-3} M GSH, 0.025 M $HCl/NaNO_2$), *n* = 10, 2.1% relative error).

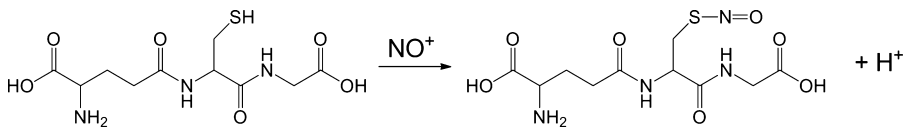

Figure 2. Nitrosation of the thiol site on glutathione results in formation of the −SNO moiety.

Table 1. Data Summary for Nitrous Acid Nitrosation of GSH in the Indicated w/v% Dextran Solution[a]

[GSH] (× 10^{-3} M)[b]	w/v% dextran	% nitrosation[c]	k_{obs} (s^{-1})	$t_{1/2}$ (s)	R^2 value[d]
6.76 ± 0.13	0	98.3 ± 0.6	0.113 ± 0.007	6.2 ± 0.4	0.999
6.59 ± 0.09	2.4	97.5 ± 0.5	0.102 ± 0.002	6.8 ± 0.1	0.997
6.44 ± 0.10	4.8	96.5 ± 1.3	0.094 ± 0.011	7.5 ± 0.9	0.997
6.39 ± 0.13	9.6	94.4 ± 2.1	0.078 ± 0.010	9.0 ± 1.3	0.993

[a] T = 25.0 ± 0.4 °C, $[NO_2^-]_0 = [H^+]_0$ = 0.025 M. [b] Experimentally determined reagent concentration. [c] Extent of nitrosation after a 35 s nitrosation time period. [d] R^2 is indicated for the $\ln([GSH]_t/[GSH]_0)$ vs time.

Because of the excess of nitrosating agent, HNO_2, the rate law becomes time dependent on only the thiol concentration (eq 3), where k_{obs} is the experimentally determined rate constant.

$$\text{rate} = k_{obs}[GSH]^{\alpha} \quad (3)$$

When the reaction is first order with respect to the thiol (α = 1), the integrated rate law results in a linear plot for $\ln([GSH]_t/[GSH]_0)$ as a function of time (eq 4). The rate constant is then determined from the slope (k_{obs} = -slope) of the plot. The half-life of the reagent for a first order kinetic process, or the time required for the thiol concentration to reach one-half its initial value, is calculated using eq 5. These values (k_{obs}, $t_{1/2}$) are presented as a point of comparison for the kinetics of nitrosation under different reaction conditions.

$$\ln([GSH]_t/[GSH]_0) = -k_{obs}t \quad (4)$$

$$t_{1/2} = \ln(2)/k_{obs} \quad (5)$$

The linear relationship between $\ln([GSH]_t/[GSH]_0)$ and time shown in Figure 3 demonstrates first order behavior with respect to glutathione. For this GSH-limiting reaction, 98.3 ± 0.6% nitrosation was achieved within 35 s. Having confirmed first-order kinetics with respect to glutathione, we determined the rate constant, k_{obs}, and half-life of the reagent, $t_{1/2}$, to be 0.113 ± 0.007 s^{-1} and 6.2 ± 0.4 s, respectively (shown in Table 1). Table 2 shows the pH values before initiation of the *S*-nitrosation process (1.69 ± 0.03) and 4 min after the reaction (1.73 ± 0.02). The pH values were determined to be statistically of the same population at the 95% confidence level. This indicates no significant change in the pH after the nitrosation process is complete.

Table 2. pH Value for Either Set of Reaction Conditions (nitrous acid and *t*-butyl nitrite) for a Variety of Dextran Concentrations, before and after Completion of the *S*-Nitrosation Process

		pH value	
nitrosating agent[a]	w/v% dextran	before[b]	after[c]
HNO_2	0	1.69 ± 0.03	1.73 ± 0.02
	2.4	1.68 ± 0.01	1.74 ± 0.01
	4.8	1.69 ± 0.01	1.74 ± 0.01
	9.6	1.68 ± 0.03	1.74 ± 0.01
t-BuONO	0	3.05 ± 0.03	2.71 ± 0.15
	2.5	3.05 ± 0.02	2.73 ± 0.15
	5	3.05 ± 0.02	2.86 ± 0.12
	10	3.09 ± 0.02	2.82 ± 0.18

[a] Final concentrations of 6.5 × 10^{-3} M GSH and 25 × 10^{-3} M nitrosating agent. [b] For HNO_2, the pH was measured prior to $NaNO_2$ injection; for *t*-BuONO, the pH was measured prior to *t*-BuONO injection. [c] The pH was measured after 4 min of reaction time for HNO_2 and 15 min for *t*-BuONO.

Glutathione S-Nitrosation with t-Butyl Nitrite for More Controlled NO Loading. Previous reports have established that nitrosation of a thiol via an alkyl nitrite reagent (RONO) is first order in thiol and nitrosating agent with the rate law described in eq 6.[40]

$$\text{rate} = k[RSH][RONO] \quad (6)$$

As such, we explored the limiting thiol case where a fixed [GSH] was reacted with *t*-butyl nitrite upon addition of a fixed volume of nitrosating agent. Like the nitrous acid case, the molar excess of nitrosating agent results in a rate law that is dependent upon thiol concentration only (see eq 3).

The concentration of glutathione at a given time t, $[GSH]_t$, was determined from the GSNO concentration at that time, $[GSNO]_t$, using eq 1. The initial thiol concentration, $[GSH]_0$, was experimentally determined based upon the $[GSNO]_{max}$, where 100% nitrosation was assumed based upon the concentration of the limiting reagent, as previously described in the nitrous acid case. A representative absorbance versus time plot for the process is shown in the inset of Figure 4.

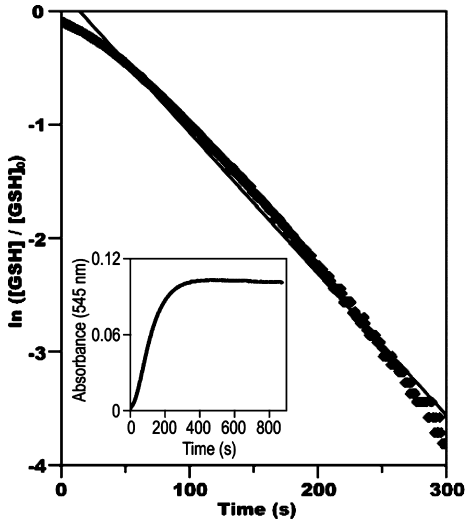

Figure 4. First-order plot of $\ln([GSH]/[GSH]_0)$ as a function of time for the *t*-butyl nitrite nitrosation of glutathione in aqueous conditions (0% dextran), R^2 = 0.992, n = 3, 7.3% relative error, and T = 25.0 ± 0.4 °C. The inset shows the representative plot of the absorbance changes at 545 nm due to SNO moiety formation (6.50 ± 0.13 × 10^{-3} M GSH, 0.025 M *t*-butyl nitrite), n = 3, 3.1% relative error.

Figure 4 shows $\ln([GSH]_t/[GSH]_0)$ as a function of time for *t*-butyl nitrite where the linearity confirms first order kinetics in thiol for *t*-butyl nitrite nitrosation; Table 3 shows the kinetics data associated with 0% dextran solution. The initial behavior is observed to deviate slightly from the first order linear behavior, indicating a slower reaction of the nitrosating agent with the thiol initially. This is attributed to the time required for the *t*-

Table 3. Data Summary for *t*-Butyl Nitrite Nitrosation of GSH in the Indicated w/v% Dextran Solution[a]

[GSH] (× 10^{-3} M)[b]	w/v% dextran	% nitrosation[c]	k_{obs} (× 10^{-2} s^{-1})	$t_{1/2}$ (s)	R^2 value[d]
6.50 ± 0.10	0	97.7 ± 0.5	1.24 ± 0.08	55.9 ± 3.9	0.992
6.39 ± 0.04	2.5	96.7 ± 0.6	1.10 ± 0.05	62.9 ± 2.9	0.995
6.52 ± 0.10	5	87.1 ± 1.9	0.68 ± 0.05	102.8 ± 7.8	0.991
6.27 ± 0.10	10	69.6 ± 7.8	0.38 ± 0.10	189.6 ± 45.2	0.998

[a] T = 25.0 ± 0.4 °C, $[t\text{-BuONO}]_0$ = 0.025 M. [b] Experimentally determined reagent concentration. [c] Extent of nitrosation after a 300 s nitrosation time period. [d] R^2 is indicated for the $\ln([GSH]_t/[GSH]_0)$ vs time.

butyl nitrite to disperse in solution, either due to limited aqueous solubility of the reagent or insufficient stirring in the reaction cell. For thiol-limiting *t*-butyl nitrite nitrosation, 100% nitrosation was accomplished within about 5 min. Table 2 includes the pH measurements for 0% polymer case, with an initial pH of 3.05 ± 0.03 and a final pH of 2.71 ± 0.15 after 15 min of reaction. This slight drop in pH is not significant as the solution is still acidic enough for the reaction to proceed toward further *S*-nitrosation.

Nitrosation in the Presence of Dextran Polymer Solution. With the kinetics of nitrosation of glutathione via nitrous acid or *t*-butyl nitrite established under aqueous conditions, we investigated the effect of polymer concentration on the reaction kinetics. Toward the application of NO loading onto thiol sites pendant to a polymer, the effect of the polymer presence on the *S*-nitrosation process is crucial. The general trends of efficiency and tunability involving the nitrosating agent and the polymer for the small molecule RSNO donor formation can be applied to a polymer containing a pendant thiol.

Glutathione S-Nitrosation with HNO_2 in Dextran Solution for More Efficient NO Loading. To assess the effect of polymer presence on the *S*-nitrosation of glutathione, we conducted the thiol-limiting nitrosation via HNO_2 in the presence of increasing dextran concentrations (2.4, 4.8, 9.6 w/v%). The plots of $\ln([GSH]_t/[GSH]_0)$ as a function of time for all concentrations indicate first order kinetics with respect to glutathione due to their linearity, as in the case of no polymer. The rate constant (k_{obs}) and half-life ($t_{1/2}$) values are shown in Table 1 and Figure 5a shows the linear dependence of the half-life on the increasing polymer concentration. Subsequently, the % nitrosation achieved within 35 s linearly decreases with increasing polymer concentration, as shown in Figure 5b. The significant slowing of the reaction kinetics with increasing polymer concentration suggests an encounter-controlled mechanism, as a higher polymer content serves to hinder the collision of the HNO_2 reagent with the thiol. Of further note is the fact that the pH values before and after *S*-nitrosation do not change with increasing polymer concentration. This indicates that the presence of the polymer is not affecting the mechanism of nitrosation, which is expected to be dependent upon the protonation of the HNO_2 species.[43] Instead, this pH data confirms that the presence of the polymer can prevent the collision of the nitrosating agent with the thiol site.

As shown in Table 1, the experimentally determined $[GSH]_0$ varies with increasing polymer concentration. To determine whether the source of the rate constant decrease arises from increasing polymer concentration or varying $[GSH]_0$, the rate constants were normalized by $[GSH]_0$. A linear decrease of normalized k with increasing polymer concentration was still observed (see the Supporting Information, Figure S1), establishing that the decrease in the rate constant is due to the increase in polymer concentration.

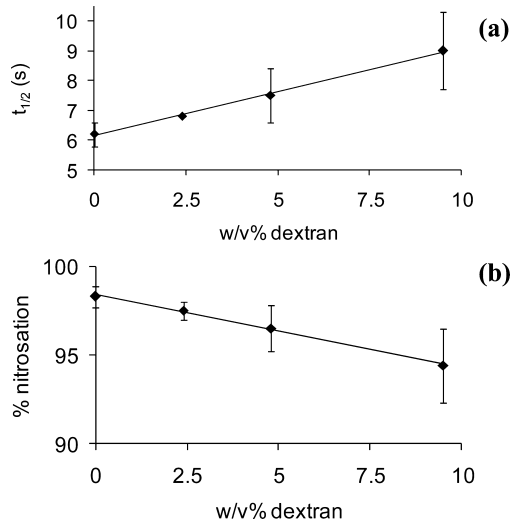

Figure 5. Dependence of (a) thiol half-life, $t_{1/2}$, and (b) % nitrosation on the w/v% dextran present in solution during nitrosation with HNO_2 nitrosating agent. Slopes are 0.30 s/% dextran (R^2 = 0.997) and −0.42% nitrosation/% dextran (R^2 = 0.996), respectively.

Glutathione S-Nitrosation with t-Butyl Nitrite in Dextran Solution for More Controlled NO Loading. Nitrosation via *t*-butyl nitrite in the presence of dextran (2.5, 5, 10 w/v%) confirmed first order kinetics with respect to the thiol regardless of polymer concentration as established by the linear behavior of the $\ln([GSH]_t/[GSH]_0)$ vs time plots. Table 3 summarizes the kinetics of *t*-butyl nitrite nitrosation for solutions of increasing dextran concentration and Figure 6 shows the dependence of the thiol half-life and % nitrosation over 300 s as a function of w/v% dextran in solution. Larger $t_{1/2}$ values for GSH and a lesser extent of nitrosation in dextran solutions with higher % polymer once again provide evidence that the rate of nitrosation depends on polymer concentration. Similar to the case of 0% dextran, the *t*-butyl nitrite reagent results in slower nitrosation kinetics than the nitrous acid reagent in the presence of dextran for each polymer concentration. However, the percent nitrosation is more significantly inhibited over the indicated time frame for *t*-butyl nitrite than for nitrous acid due to increasing polymer concentration as indicated by a larger slope value for *t*-butyl nitrite's % nitrosation vs time plot (see Figures 5 and 6). As in the case of HNO_2, an increase in the dextran concentration did not yield significantly different (95% confidence level) pH values before and after the S-nitrosation process. This once again suggests that the polymer does not affect the pH of the reaction solution; however, the polymer presence serves to delay the nitrosating agent from meeting the thiol.

Comparison of HNO_2 and *t*-Butyl Nitrite Reagents. *Efficiency of Nitrosation.* We investigated the effect of polymer presence on the efficiency of *S*-nitrosation of GSH as a function

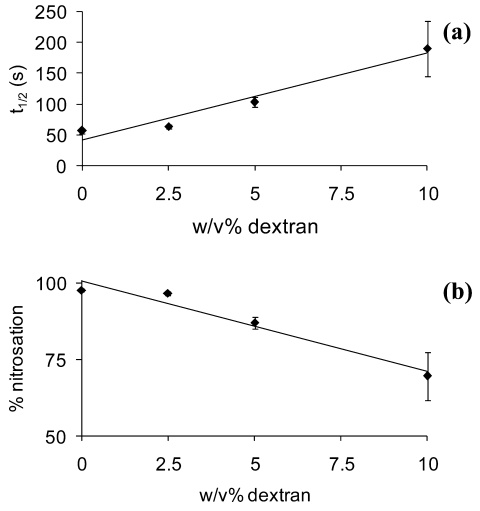

Figure 6. Dependence of (a) the half-life, $t_{1/2}$, and (b) % nitrosation on the w/v% dextran present in solution during nitrosation with *t*-butyl nitrite nitrosating agent. Slopes are 14.0 s/% dextran (R^2 = 0.952) and −3.0% nitrosation/% dextran (R^2 = 0.952), respectively.

of the nitrosating agent: nitrous acid and *t*-butyl nitrite. We found that for the case of limiting thiol with the same molar excess of nitrosating agent, the process was slower for *t*-butyl nitrite. Excess nitrous acid will result in the most efficient nitrosation, as evidenced by nearly 100% nitrosation within 35 s, compared to 300 s for *t*-butyl nitrite. Additionally, the half-life of GSH for the HNO_2 reagent (6.2 ± 0.4 s) is an order of magnitude smaller than the half-life of GSH using *t*-butyl nitrite (55.9 ± 3.9 s). The differences in the efficiency of reaction in either case could be due to effects that limit the diffusion of the nitrosating reagent to the thiol site or pH effects.

It has been previously established that nitrosation occurs through an encounter-controlled process.[35,38] When considering the two nitrosating agents in the same molar excess of thiol, the *t*-butyl nitrite agent is bulkier than the nitrous acid. Therefore, it might be expected that the nitrous acid will interact more readily with the thiol site where reaction toward RSNO formation can proceed. When considering the structures of *t*-butyl nitrite and nitrous acid, the differences in reactivity could be due to electronic or steric effects. Previous reports suggest that, for either nitrosating agent, the nitrosation process is dependent upon the protonation of the O bearing the *t*-butyl or proton side group.[43] Considering that the inductive effect of the *t*-butyl nitrite would result in a more negatively charged O bound to the *t*-butyl group than the O bound to the proton in nitrous acid, the rate of nitrosation would be expected to occur more quickly for *t*-butyl nitrite due to a more efficient protonation of the O bound to the *t*-butyl group under acidic conditions. However, our data indicate a quicker nitrosation with nitrous acid, suggesting that the *t*-butyl group hinders the protonation of the O bound to it. As a result, the formation of the NO^+ nitrosating species is limited. Other steric hindrance contributions can be considered that support an encounter-controlled nitrosation process due to a lesser availability of the *t*-butyl nitrite compared to the nitrous acid. In addition to the bulkier *t*-butyl side group preventing interaction of the thiol site and the −ONO moiety of the nitrosating agent for NO^+ transfer to occur, the results could reflect a slowed diffusion of the *t*-butyl nitrite reagent to the thiol site, *t*-butyl nitrite's limited solubility in water due to the hydrocarbon side group, or *t*-butyl nitrite's relatively high volatility (bp 61−63 °C).[44] Either of these factors will slow the encounter-controlled process, and thus nitrosation will occur at a slower rate for *t*-butyl nitrite. To minimize the evaporation of *t*-butyl nitrite, the cuvette was capped during analysis.

In addition to steric effects that could slow the nitrosation process involving *t*-butyl nitrite, the kinetics results can also be explained due to the differences in pH of each system. Despite the same concentrations of thiol and nitrosating reagent, the HNO_2 system consistently has a pH of 1.7, whereas the *t*-BuONO system has a pH of ∼3 (see Table 2). If the process is dependent upon protonation of the nitrosating agent, the system with the lower pH will result in a more efficient NO^+ production at the thiol site, resulting in more efficient nitrosation for the HNO_2 system.

In the presence of 9.6 w/v% dextran, the nitrous acid nitrosation reached 94.4 ± 2.1% nitrosation within 35 s, only a 4% decrease from the 0 w/v% dextran case. This indicates that, despite relatively high polymer concentrations, nitrous acid will essentially drive the system toward 100% nitrosation very quickly, within less than a minute. Therefore, these results demonstrate the effectiveness of HNO_2 to maximize nitrosation of thiol groups pendant to a polymer. A greater effect on the half-life and % nitrosation due to increasing polymer concentration was exhibited by the *t*-butyl nitrite, making it less efficient over a fixed time frame in the presence of a polymer. For materials applications that require higher amounts of NO delivery, the most efficient *S*-nitrosation would be desired to maximize the NO reservoir. In such a case, nitrous acid offers the most efficient NO loading despite the presence of a polymer.

Control over Nitrosation. A decrease of nearly 30% nitrosation was achieved for the *t*-butyl nitrite reagent when increasing the polymer concentration from 0 to 10 w/v% for a 5 min analysis period, indicating a more significant effect on the kinetics than seen with HNO_2. This larger impact of the polymer concentration on the reaction rate indicates that the use of *t*-butyl nitrite as the nitrosating agent can exert finer control over NO loading. For instance, if a polymer has a fixed number of thiol sites and only a fraction of those sites need to be nitrosated to result in the desired amount of NO incorporated into the material, the use of nitrous acid will essentially drive all thiol sites toward nitrosation. However, *t*-butyl nitrite would be a more viable reagent as, for a fixed polymer concentration, the extent of reaction can essentially be controlled to reach the desired % nitrosation based upon the required NO reservoir for the material application, especially for applications in which excess NO could cause local toxicity. Due to *t*-butyl nitrite's low boiling point, once the reaction has spanned the desired time period, vacuum applied to the system would remove the reagent, effectively halting the nitrosation process.

In addition to the selectivity just described, another benefit of *t*-butyl nitrite involving removal of the nitrosating agent due to the reagent's low boiling point is that residual nitrite in the product due to the nitrosating agent in minimized. This is important as residual nitrite in the material will contribute to the measurement of NO under RSNO reduction conditions due to nitrite reduction in the presence of copper. For example, NO is released from RSNOs upon exposure to copper metal ion and appropriate reducing agents.[32,45] Residual nitrite in the system is also reduced to NO under similar conditions and will thus contribute to the total NO measured using a direct NO

measurement technique (see the Supporting Information, Section 2).

Alkyl nitrites have been demonstrated as useful agents under a variety of solvent conditions such as aqueous pH 6−13,[39] alcohol solvent,[46] and organic solvent,[47] in addition to acidic aqueous conditions,[48] making this class of agents more versatile than nitrous acid. The synthesis of NO-releasing materials under aqueous conditions would limit the nitrosation of hydrophobic polymers in organic solvent solution. Further, polymers that degrade via hydrolysis cannot be nitrosated under aqueous HNO_2 conditions without compromising the integrity of the polymer, therefore *t*-butyl nitrite nitrosation under organic solvent conditions have been recently employed.[24] The ability of *t*-butyl nitrite to nitrosate under a variety of solvent conditions is a significant benefit to synthesize materials under versatile conditions and has been recently demonstrated for polymer-RSNO synthesis under organic solvent conditions.[24]

Despite *t*-butyl nitrite's benefits, slightly poorer linearity is exhibited for the % nitrosation as a function of polymer concentration for the *t*-butyl nitrite system, indicating that the % nitrosation may be less predictable than for HNO_2. This could reflect the slight insolubility of *t*-butyl nitrite in water as well as its low boiling point. If the concentration of *t*-butyl nitrite in reaction solution varies more significantly than that of nitrous acid because of these physical properties, a less predictable extent of nitrosation when using *t*-butyl nitrite may result.

Nitrous acid allows fast and complete nitrosation while slower nitrosation with *t*-butyl nitrite leads to better control of the extent of nitrosation and permits removal of excess reagent after the reaction. Figure 7 summarizes the efficiency and fine-tuned control associated with *S*-nitrosation for either reagent under 0 and 10 w/v% polymer conditions. The use of HNO_2 results in nearly 100% nitrosation much more quickly than with *t*-butyl nitrite for the 0% polymer cases. For the 0% and 10% dextran comparison, the % nitrosation over time profiles do not change nearly as significantly for the HNO_2 case as compared to the *t*-butyl nitrite case. These trends involving the use of nitrosating agent and polymer concentration to control the NO loading process can be expanded to systems involving thiol groups covalently attached to polymers. This improves the ability to exert control over the NO loading of polymer materials for particular biomedical device applications.

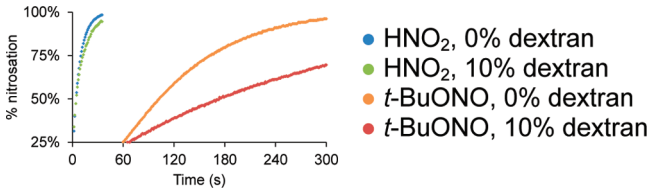

Figure 7. Percent nitrosation over time is dependent upon the nitrosating agent and the presence of dextran in solution.

CONCLUSIONS

We have demonstrated that, in the presence of a polymer solution, the most efficient reaction conditions for glutathione *S*-nitrosation involve the use of nitrous acid with near-complete nitrosation achieved within 35 s. This process is less significantly affected by polymer concentration than for the *t*-butyl nitrite reagent. However, to exert tunable control over the amount of NO loaded in the presence of a polymer, the *t*-butyl nitrite reagent is most viable as the use of this reagent has a larger impact on the nitrosation kinetics with nearly a 30% decrease in the extent of nitrosation with 10 w/v% dextran present. Regardless of the nitrosating agent, first-order kinetics were established with respect to thiol. The experiments described herein involve the nitrosation of a small molecule thiol in a polymer solution, yet similar trends can be expected for the use of these nitrosating agents for the nitrosation of thiol sites pendant to polymer backbones. Taken together, reaction conditions can now be exploited for optimal performance of NO loading onto or in the presence of polymer materials for a range of clinical applications, such as the development of materials for wound-healing, antithrombogenic or antimicrobial properties, where different doses of NO are required.

ASSOCIATED CONTENT

Supporting Information

The rate constants for the limiting thiol nitrous acid nitrosation normalized by $[GSH]_0$ as a function of w/v% dextran in solution and the reduction of nitrite in solution upon addition of Cu^{2+}/GSH. 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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