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Synthetic Modeling Chemistry of Iron−Sulfur Clusters in Nitric Oxide Signaling

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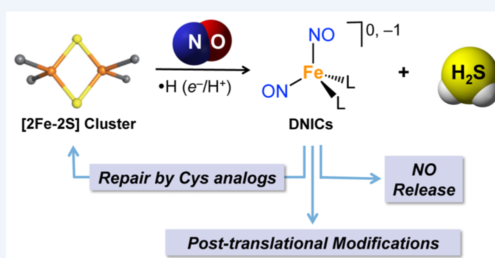
CONSPECTUS: Nitric oxide (NO) is an important signaling molecule that is involved in many physiological and pathological functions. Iron−sulfur proteins are one of the main reaction targets for NO, and the [Fe−S] clusters within these proteins are converted to various iron nitrosyl species upon reaction with NO, of which dinitrosyl iron complexes (DNICs) are the most prevalent. Much progress has been made in identifying the origin of cellular DNIC generation. However, it is not well-understood which other products besides DNICs may form during [Fe−S] cluster degradation nor what effects DNICs and other degradation products can have once they are generated in cells. Even more elusive is an understanding of the manner by which cells cope with unwanted [Fe−S] modifications by NO. This Account describes our synthetic modeling efforts to identify cluster degradation products derived from the [2Fe−2S]/NO reaction in order to establish their chemical reactivity and repair chemistry. Our intent is to use the chemical knowledge that we generate to provide insight into the unknown biological consequences of cluster modification.



Our recent advances in three different areas are described. First, new reaction conditions that lead to the formation of previously unrecognized products during the reaction of [Fe−S] clusters with NO are identified. Hydrogen sulfide (H_2S), a gaseous signaling molecule, can be generated from the reaction between [2Fe−2S] clusters and NO in the presence of acid or formal H• (e^-/H^+) donors. In the presence of acid, a mononitrosyl iron complex (MNIC) can be produced as the major iron-containing product. Second, cysteine analogues can efficiently convert MNICs back to [2Fe−2S] clusters without the need for any other reagents. This reaction is possible for cysteine analogues because of their ability to labilize NO from MNICs and their capacity to undergo C−S bond cleavage, providing the necessary sulfide for [2Fe−2S] cluster formation. Lastly, unique dioxygen reactivity of various types of DNICs has been established. N-bound neutral $\{Fe(NO)_2\}^{10}$ DNICs react with O_2 to generate low-temperature stable peroxynitrite ($ONOO^-$) species, which then carry out nitration chemistry in the presence of phenolic substrates, relevant to tyrosine nitration chemistry. The reaction between S-bound anionic $\{Fe(NO)_2\}^9$ DNICs and O_2 results in the formation of Roussin's red esters (RREs) and thiol oxidation products, chemistry that may be important in biological cysteine oxidation. The N-bound cationic $\{Fe(NO)_2\}^9$ DNICs can spontaneously release NO, and this property can be utilized in developing a new class of NO-donating agents with anti-inflammatory activity.

INTRODUCTION

Nitric oxide (NO) is a ubiquitous and important messenger molecule, the misregulation of which is implicated in numerous pathological conditions, including vascular diseases, cancer, and neurodegeneration.[1] Despite the existence of multiple NO-signaling mechanisms that operate in biological systems, our understanding of NO signaling is still limited. NO synthesis by nitric oxide synthases in conjunction with the heme-based soluble guanylate cyclase/cGMP pathway is the only well-defined NO signaling cascade to date.[1] Our group has been motivated to address the current knowledge gaps by providing chemical insights into the mechanistic underpinnings of NO signaling pathways. We are particularly intrigued by biological reports that have emerged since the 1990s that reveal that NO can modify a number of iron−sulfur proteins.[2−4] The most commonly observed NO-modified products are the monomeric dinitrosyl iron complexes (DNICs) with a characteristic g = 2.03 EPR signal[5,6] (Scheme 1); however, there are reported cases in which multinuclear DNICs are the major product.[7,8] In fact, the presence of DNICs in cells was detected in the 1960s, several decades before the signaling role of NO was discovered.[9−11] Some of the [Fe−S] proteins that generate DNICs are regulatory proteins whose [Fe−S] clusters react with NO to control their transcriptional or translational activity.[12,13] DNICs are also formed in many nonregulatory [Fe−S] proteins under conditions of pathophysiological NO production, which often constitutes protein damage.[14] Furthermore, recent studies revealed that, in addition to [Fe−S] proteins, the chelatable iron pool is another source of iron for DNIC formation.[15] Although the majority of biological DNICs have S-donor ligands, such as cysteinate, DNICs ligated by other O/N-donor residues have also been observed.[5]

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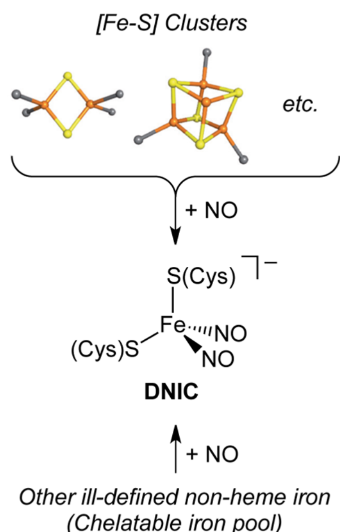
Scheme 1



Significant progress has been made in the identification of DNICs as the products of NO-mediated [Fe−S] cluster degradation, but it is not well understood what other product(s) may form during cluster degradation. Moreover, the effects that DNICs and other degradation products elicit once they are generated in cells also remain unknown. Even more elusive is an understanding of how cells cope with unwanted [Fe−S] modifications by NO. These are highly challenging questions to answer due to the complicated coordination and redox chemistry that arises from the combination of multiple redox-active entities, such as iron, NO, and thiol. Moreover, the unidentified reaction partners can be constituents of the biological milieu, which adds another layer of complication. One of the ways to cope with these daunting challenges is to use discrete synthetic systems in which one can identify the [Fe−S] cluster degradation products with certainty, elucidate their chemical reactivity, and establish the chemistry required to regenerate iron−sulfur clusters from degradation products. Once such information becomes available, there is considerable promise that new paradigms for [Fe−S]-mediated NO signaling can be established. This knowledge can then be used to inform strategies for the prevention, diagnosis, and treatment of diseases associated with NO signaling.

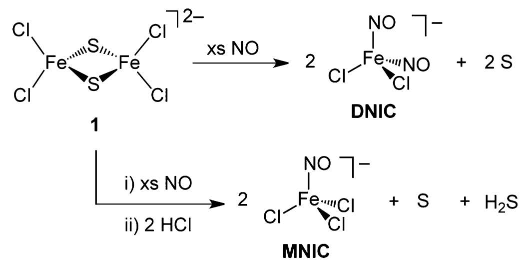
In this Account, we describe our recent efforts to identify unique chemical reactivity associated with [Fe−S] clusters and NO using well-defined synthetic systems. The chemical insight obtained from our studies suggests that iron−sulfur clusters might be intimately involved in redox signaling by providing an intersection for two important gasotransmitters, NO and H_2S, and by generating iron dinitrosyl species that are capable of propagating redox signals through protein post-translational modifications. Our studies also suggest that there might be an efficient way to regenerate [Fe−S] clusters from iron nitrosyls via previously unexplored thiol chemistry. In the following sections, we will use the Enemark−Feltham notation[16] when we describe iron nitrosyl systems. According to this formalism, the metal nitrosyl is treated as a single unit and represented as $\{M(NO)_x\}^n$, where x is the number of nitrosyl ligands, and n is the total number of electrons associated with the metal d and π^*(NO) orbitals.

GENERATION OF HYDROGEN SULFIDE BY THE ACTION OF NITRIC OXIDE AT IRON−SULFUR CLUSTERS

Hydrogen sulfide (H_2S)[17] has been recognized as an important signaling molecule in recent decades. Interestingly, H_2S has biological functions highly related to those of NO, which suggests that crosstalk mechanisms for these gasotransmitters may exist.[18] Studies have demonstrated the cooperative effects of NO and H_2S, including their control of cGMP generation via interaction with their respective target proteins, guanylate cyclase and phosphodiesterase-5,[19] and the production of HNO via direct chemical reaction between NO and H_2S.[20] We hypothesized that [Fe−S] clusters, ubiquitous inside the cell, could be sites of crosstalk between NO and H_2S because (i) [Fe−S] clusters are already known to react with NO and (ii) H_2S is simply the protonated form of the sulfide ion (S^{2-}) that is embedded in [Fe−S] clusters. To explore this hypothesis, we investigated the feasibility of H_2S generation from [2Fe−2S] clusters following nitrosylation.

When we began our studies, it had already been established by researchers such as Lippard[21,22] and Liaw[23,24] that the reaction of NO with [2Fe−2S] clusters results in DNIC formation with elimination of the bridging sulfides as elemental sulfur. Given this knowledge, we directed our initial effort toward understanding what reaction environments might alter the known NO−[Fe−S] cluster reactivity patterns and lead to H_2S generation. We first investigated the influence of acidic environments on the reaction of $[Fe_2S_2Cl_4]^{2-}$ (**1**) with NO (Scheme 2).

Scheme 2. [a]



[a]S represents 1 equiv of elemental sulfur (S_x).

The reaction of $[Fe_2S_2Cl_4]^{2-}$ (**1**) and NO in the absence of acid led to the formation of $\{Fe(NO)_2\}^9$ DNIC and elemental sulfur, as expected from prior studies (Scheme 2). The presence of acid in the reaction medium, however, dramatically altered the cluster degradation chemistry. When HCl was introduced immediately after the addition of NO to **1**, a new type of iron degradation product was generated; the $\{Fe(NO)\}^7$ mononitrosyl iron complex (MNIC) $[Fe(NO)Cl_3]^-$ was formed, and one of the bridging sulfides in **1** was released as H_2S (Scheme 2).[25] Reversing the order in which the reagents were combined or delaying acid addition produced either $[FeCl_4]^-$ or $[Fe(NO)_2Cl_2]^-$, neither of which could be converted to the MNIC. The sulfur-containing reaction products obtained from this sequential addition of NO and HCl to **1** indicated that there was a *net* two-electron oxidation of the bridging sulfides upon nitrosylation, $2\ S^{2-} + 2\ H^+ \rightarrow H_2S + S + 2\ e^-$. This result suggests that the initial binding of 2 equiv of NO to **1** promoted a $2e^-$ oxidation of the bridging sulfide, whereas each of the two ferric ions received one electron to form 2 equiv of

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the $\{Fe(NO)\}^7$ MNIC. In the absence of acid, further addition of 2 more equiv of NO led to another $2e^-$ oxidation to produce elemental sulfur (S_x) and $\{Fe(NO)_2\}^9$ DNICs. The presence of acid appears to provide an alternative second reaction, which ultimately results in the production of MNIC and an equimolar mixture of H_2S and S_x. It is noteworthy that, although MNICs are known to be present in cells,[26,27] they have not been identified in association with [Fe−S] clusters. This study implies that [Fe−S] clusters might be another source of cellular MNICs and such species may be present as intermediates in cluster degradation by NO. More importantly, this study showed for the first time that NO can induce H_2S evolution from [Fe−S] clusters in an acidic environment. As described below, this H_2S formation does not require strong acids, such as HCl. In physiological situations, acidic residues from amino acids (e.g., cysteine, tyrosine, aspartic acid, etc.) would likely play an important role in H_2S (HS^-) formation.

Our efforts soon expanded to a group of thiolate-containing [2Fe−2S] clusters, and we demonstrated that both of the bridging sulfides in prototypical [2Fe−2S] clusters can be released as H_2S upon nitrosylation provided that the environment is capable of providing a formal equivalent of H• (e^-/H^+) from donors such as thiols and phenols.[28] For example, nitrosylation of $[Fe_2S_2(SPh)_4]^{2-}$ (**2**) in the presence of PhSH or ^{t}Bu$_3$PhOH resulted in the formation of H_2S and the DNIC $[Fe(NO)_2(SPh)_2]^-$ with the concomitant generation of PhSSPh or ^{t}Bu$_3$PhO• (Figure 1).[28]

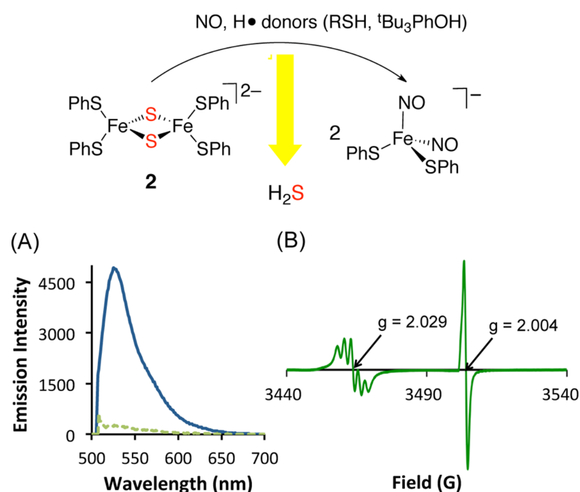

Figure 1. (A) Fluorescence spectra of a turn-on H_2S sensor following incubation with the headspace gas of the **2**/NO reaction in the presence (blue) or absence (green) of HSPh. (B) X-band EPR spectrum of **2**/NO in the presence of ^{t}Bu$_3$PhOH. The formation of $[Fe(NO)_2(SPh)_2]^-$ and ^{t}Bu$_3$PhO• are indicated by their characteristic EPR signals at g = 2.029 ($A_{N(NO)}$ = 2.4 G) and g = 2.004, respectively.

There are several factors that could play a role in H_2S generation from the reaction of [2Fe−2S] clusters and NO. The amount of H_2S produced is largely dependent on the nature of the H• donor and the electronic environment of the [2Fe−2S] cluster. When a series of [2Fe−2S] clusters with *p*-substituted benzenethiolates, $[Fe_2S_2(SPh\text{-}4\text{-}R)_4]^{2-}$ where R = Cl, H, Me, and OMe, were allowed to react with NO in the presence ^{t}Bu$_3$PhOH, varying amounts of H_2S (24−87%) were evolved.[28] The [2Fe−2S] clusters bound by *p*-substituted benzenethiolates with more electron-donating groups produced larger amounts of H_2S. Similarly, when an H• donor with a weaker X−H bond, such as PhSH (BDE(S−H) = 76.9 kcal/mol in DMSO),[29] was added instead of ^{t}Bu$_3$PhOH (BDE(O−H) = 80.6 kJ/mol in DMSO),[29] a larger amount of H_2S was produced (80 vs 55%).[28]

There was no detectable intermediate in the reaction of $[Fe_2S_2(SPh)_4]^{2-}$ (**2**) and NO in the presence of PhSH. We were, however, able to monitor the decay of **2** by UV−vis spectroscopy and observed a biphasic process comprising two consecutive first-order decays with k_1 = 0.168(19) min^{-1} and k_2 = 0.0087(16) min^{-1} at 0 °C.[28] The decay rates in both phases increased significantly when electron donating *p*-substituted benzenethiols, such as 4-MeO-PhSH and 4-Me-PhSH, were used as the H• (e^-/H^+) donor, whereas no changes in the decay rates were observed when PhSH was substituted by PhSD.[28] These results suggest that proton transfer is not involved in the rate-determining step for the nitrosylation of **2** in the presence of PhSH, and that electron transfer from thiol and/or thiolate to iron nitrosyl moieties must be critical in determining the overall reaction rate.

Thiols such as glutathione and mycothiol are typically found inside cells in high concentrations. Given the availability of thiols in the cellular environment, it is conceivable that the NO reactivity of prototypical cysteinate-bound [Fe−S] clusters can be associated with proton-coupled electron transfer chemistry that leads to the formation of H_2S. Therefore, it may be possible that iron−sulfur clusters provide a platform for crosstalk between two important signaling molecules, NO and H_2S, inside the cell.

■ REPAIR OF NITRIC OXIDE-MODIFIED [2FE−2S] CLUSTERS

Nitric oxide reacts with [2Fe−2S] clusters to yield $\{Fe(NO)_2\}^9$ DNICs. In some cases, as shown with SoxR and ferredoxin in *E. coli*, the resulting DNICs are efficiently repaired back to intact [2Fe−2S] clusters in vivo.[3,30] Although de novo protein synthesis would be a valid pathway for reconstruction of any new [2Fe−2S] proteins, the successful repair of ferredoxin [2Fe−2S] clusters even in the presence of a protein synthesis inhibitor suggests that there must be pre-existing repair mechanism(s) in the cells.[30] Several synthetic inorganic research groups have tried to understand the chemistry required for the repair of NO-modified [Fe−S] clusters. The Liaw group developed two pathways to synthesize [2Fe−2S] clusters from $\{Fe(NO)_2\}^9$ DNICs. The first involved photolytic removal of NO from an $\{Fe(NO)_2\}^9$ DNIC in the presence of elemental sulfur.[23] The second route was a multistep conversion that first utilizes Me_2S_3 or $HSCPh_3$ to generate $[Fe_2S_2(NO)_4]^{2-}$, which then reacts with two equivalents of $[Fe(SEt)_4]^-$ to generate $[Fe_2S_2(SEt)_4]^{2-}$ and $[Fe_2(\mu\text{-}SEt)_2(NO)_4]^{2-}$.[24] Although not involving DNICs, Lippard reported that $[Fe_2S_2(S^tBu)_4]^{2-}$ can be synthesized from a mononitrosyl iron complex, $[Fe(NO)(S^tBu)_3]^-$, by photolysis in the presence of S_8.[31]

We have recently initiated our own efforts to identify chemistry that can reverse the [Fe−S] cluster nitrosylation reaction (i.e., formation of [Fe−S] clusters from DNICs). We suspected that appropriate thiols might mediate the reverse reaction even in the absence of an enzyme or photolysis because it is well documented that thiols can remove an NO ligand from metal-nitrosyl species[32] and that transition metal complexes can mediate thiol desulfurization.[33,34] A thiol could therefore act as both an NO acceptor and an S atom donor, permitting a DNIC to be transformed into an [Fe−S] cluster.

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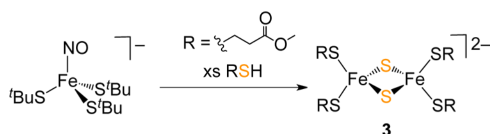
Thiols are also redox-active and abundant in cells. Although our goal of establishing a DNIC-to-[Fe−S] conversion has not yet been fully realized, a significant breakthrough has been made in recent years. Described below is the chemistry that can convert mononitrosyl iron complexes (MNICs) to [2Fe−2S] clusters at room temperature.

To test our hypothesis that thiols can mediate cluster repair, we first examined the reactions of alkylthiolate-bound DNICs or MNICs with various thiols. In most cases, such reactions resulted in thiolate ligand substitution without change in the iron nitrosyl moieties of the starting compounds. This pattern drastically changed, however, when a special group of thiols, cysteine analogues, were used. When an alkylthiolate-bound $\{Fe(NO)\}^7$ MNIC, such as $[Fe(NO)(S^tBu)_3]^-$, was allowed to react with excess 3-methylmercaptopropionate (MMP), a [2Fe−2S] cluster bearing MMP thiolate ligands, $[Fe_2S_2(SCH_2CH_2C(O)OCH_3)_4]^{2-}$ (**3**), formed (Scheme 3).[35]

Scheme 3



Microcrystals of $[(Ph_3P)_2N]_2{\cdot}\mathbf{3}$ are stable at low temperatures (e.g., −40 °C), but in solution, **3** is unstable and rapidly decomposes even at low temperatures. The presence of excess MMP in solution, however, can increase its stability. In spite of the unusual instability of **3** compared to other $[2Fe-2S]^{2+}$ clusters, the bond metrics of **3** obtained by X-ray crystallography (Figure 2) are quite similar to those reported for the

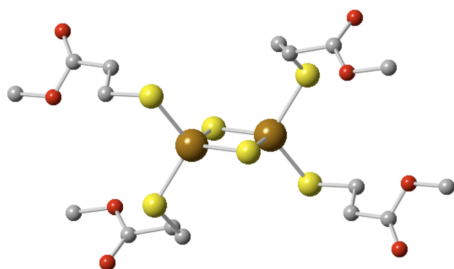
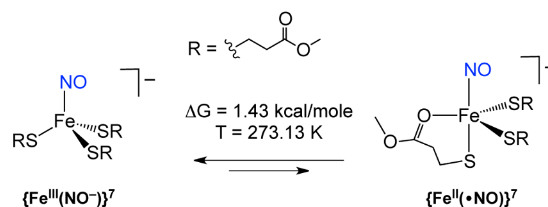


Figure 2. Ball-and-stick depiction of the anion from the crystal structure of $[(Ph_3P)_2N]_2[Fe_2S_2(SCH_2CH_2C(O)OMe)_4]$ (**3**). Hydrogen atoms are omitted for clarity. Fe, orange; S, yellow; O, red; and C, gray.

ethylthiolate-bound cluster, $[Fe_2S_2(SEt)_4]^{2-}$.[35] In acetonitrile, **3** exhibits UV−vis absorption maxima at 330, 425, and 450 nm and displays a quasi-reversible $[2Fe-2S]^{2+/1+}$ redox couple at −870 mV vs Ag/AgNO$_3$, all of which are highly reminiscent of the properties of analogous [2Fe−2S] clusters.[35] Other cysteine analogues, such as *N*-acetylcysteine methyl ester and 4-mercaptobutan-2-one ($HSCH_2CH_2C(O)Me$), were also able to convert MNICs to [2Fe−2S] clusters.[36]

The MNIC-to-[2Fe−2S] cluster conversion that is mediated by a cysteine analogue relies on two very special features of these cysteine analogues. When the original alkyl thiolate ligand of the starting MNIC is substituted by a cysteine analogue, such as MMP, the resulting MNIC is expected to exist in an equilibrium mixture between a four- and five-coordinate isomer (Scheme 4). Electronic structure calculations for these two

Scheme 4



species suggest that the four-coordinate MNIC has a high-spin Fe^{3+} center antiferromagnetically coupled to an NO^- ligand, whereas the five-coordinate isomer is best described as a high-spin ferrous species antiferromagnetically coupled to a neutral •NO ligand.[35] Furthermore, this neutral •NO in the latter isomer is poised for release because of the presence of a labilizing thiolate ligand disposed trans to it about the metal center. This special feature caused by coordination of a cysteine analogue makes the MNIC prone to disproportionation, which produces a stable DNIC and a putative thiolate bridged diferrous species (**4**, Scheme 5). The dimer would subsequently be oxidized by the disulfide that is produced during the conversion of MNIC to DNIC (Scheme 5). The proposed thiolate-bridged species with cysteine analogue **4** is not known, but we found that the known[37] ethylthiolate analogue $[Fe_2(\mu\text{-}SEt)_2(SEt)_4]^{2-}$ is readily oxidized by disulfides such as $(SEt)_2$ or $(SCH_2CH_2C(O)OMe)_2$.[35,36] Interestingly, a [2Fe−2S] cluster can be generated from $[Fe_2(\mu\text{-}SEt)_2(SEt)_4]^{2-}$ by addition of a disulfide derived from a cysteine analogue.[35,36] If a simple alkyl disulfide, such as $(SEt)_2$, was added to $[Fe_2(\mu\text{-}SEt)_2(SEt)_4]^{2-}$, the monomeric ferric tetrathiolate species $[Fe(SEt)_4]^-$ was produced with no indication of the formation of $[Fe_2S_2(SEt)_4]^{2-}$.[36] This result suggests that there must be additional features of cysteine analogues that facilitate the cluster repair chemistry in addition to their chelating potential and NO-labilizing effect in MNICs (vide infra).

Sulfur isotope labeling studies of the reaction between $[Fe(NO)(S^tBu)_3]^-$ and MMP indicated that the bridging sulfides in **3** were exclusively derived from MMP.[35] Complementary to this observation, a stoichiometric amount of the expected MMP fragment, the lanthionine analogue $S(CH_2CH_2C(O)OMe)_2$, was observed as a byproduct.[35] This result indicates that efficient C−S bond cleavage occurred in MMP, providing a source of the bridging sulfides of the [2Fe−2S] cluster. We propose that this C−S bond cleavage is possible for a cysteine analogue because of the appropriate positioning of the acidic α-proton (Scheme 5). Support for our hypothesis came from an experiment with related thiols. An analogue of MMP with one fewer methylene units between the thiol and carbonyl functional group, $HSCH_2C(O)OMe$, no longer has an α-proton properly positioned to promote deprotonation-triggered C−S bond cleavage and, consequently, no cluster formation was observed when it was added to $[Fe(NO)(S^tBu)_3]^-$.[36] In contrast, when another analogue of MMP, $HSCH_2CH_2C(O)Me$, that could provide the acidic α-protons at the same position as MMP, was added to $[Fe(NO)(S^tBu)_3]^-$, the corresponding [2Fe−2S] cluster was formed.[36]

The ability of cysteine analogues to act as an efficient source of S^{2-} when coordinated to ferric ions was further explored as a novel means by which to synthesize [2Fe−2S] clusters. In fact, [2Fe−2S] clusters bearing cysteine analogues can be easily synthesized by the addition of appropriate forms (i.e., thiol or disulfide) of a cysteine analogue to monomeric ferric or ferrous

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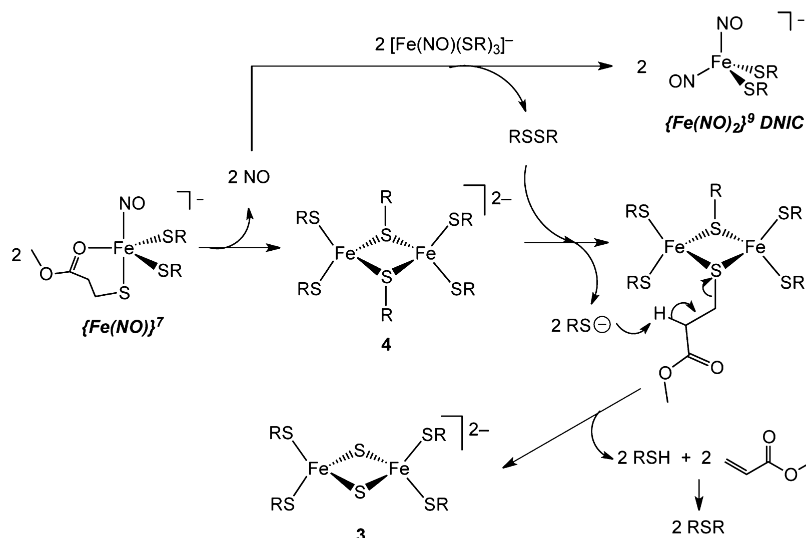
Scheme 5



tetrathiolate compounds.[36] These newly found reactions add to the rich chemistry of iron−sulfur cluster assembly and provide unique insight into how iron tetrathiolate species can self-assemble to form a [2Fe−2S] cluster without the need for other reagents besides a general base. It is intriguing to envision that cysteine inside the cell could carry out such concurrent multifaceted reactions involving disruption of a stable Fe−NO bond, redox transformation, and production of sulfide.

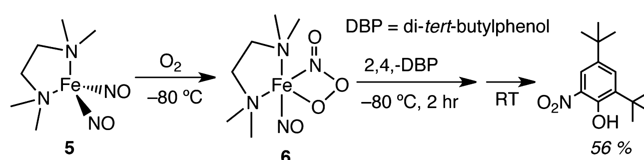
DIVERSE REACTIVITY OF DINITROSYL IRON COMPLEXES (DNICS)

Although the formation of DNICs in large quantities is used as a biomarker for NO toxicity, physiological roles of DNICs have also been suggested. The suggested roles include NO transport and storage and Fe mobilization.[5] An understanding of the chemistry of DNICs that underlies these suggested physiological and/or deleterious functions is, however, lacking. Considerable effort has been expended to synthesize various DNICs in different ligand environments and to gain a fundamental understanding of the physical and chemical properties of such species. Excellent recent reviews on synthetic DNICs are available.[6,38,39] Readers are especially encouraged to read two insightful Accounts describing work with DNICs[6,39] that have been published in this special issue related to *Synthesis Chemistry in Biological Inorganic Chemistry*. Here, we focus on our contributions to unprecedented O_2 chemistry of DNICs in which the chemical and electronic structures of DNICs appear to direct their O_2 reactivity.

Nitration Chemistry of DNICs as Relevant to Protein Tyrosine Nitration

To study the intrinsic reactivity of $\{Fe(NO)_2\}^{10}$ DNICs, we prepared the $\{Fe(NO)_2\}^{10}$ compound $[Fe(NO)_2(TMEDA)]$ (**5**), where TMEDA = *N,N,N′,N′*-tetramethylethylenediamine, and examined its O_2 reactivity (Scheme 6).[40] Bubbling O_2 through a solution of **5** in CH_2Cl_2 at −80 °C yielded an EPR-silent, dark purple species with electronic absorption bands at 460 and 560 nm and with two IR ν_{NO} stretching frequencies at 1589 and 1805 cm^{-1} (Figure 3).[40] These IR and EPR characteristics, in conjunction with EXAFS studies, led us to formulate the purple species as peroxynitrite iron mononitrosyl complex [Fe(TMEDA)(NO)(ONOO)] (**6**, Scheme 6). In

Scheme 6



collaboration with Jason Shearer (University of Nevada, Reno), complex **6** was further probed by iron K-edge X-ray absorption spectroscopy. Comparison of the edge energies of **5** and **6** showed a shift of +1.8(4) eV, consistent with formal oxidation of the iron center in **5** by one electron.[40] The EXAFS region for **6** was best modeled as a five coordinate Fe species with a coordinated bidentate O_2NO^- moiety (Figure 3B; best fit includes: shell #1:2 N scatterers; O_2NO shell: 1 O_2NO scatterer, r_1 = 1.91(1) Å, r_2 = 1.91 Å (restrained); NO shell: 1 NO scatterer, r = 1.67(1) Å).[40]

Peroxynitrite can often be detected via its characteristic oxidation and/or nitration chemistry, especially that of phenolic substrates. When 1 equiv of 2,4-di-*tert*-butylphenol (DBP) is added to **6**, and the reaction mixture is warmed to room temperature, 2,4-di-*tert*-butyl-6-nitrophenol is observed along with the oxidative coupling product (Scheme 6).[40] These reaction products do not form when DBP is added *after* warming the solution of **6** to RT, signifying that intermediate **6** is a crucial species in phenol nitration and oxidation. At room temperature, **6** is too unstable to be observed by UV−vis spectroscopy. However, when O_2 is added to a mixture of **5** and DBP, nitration still occurs (NO_2-DBP = 58%).[40]

We have been unable to crystallize the final product generated from the reaction of **5** and O_2 at RT. However, with $[Fe(dmp)(NO)_2]$, where dmp = 2,9-dimethyl-1,10-phenanthroline, which shows similar nitrating chemistry to **5** under aerobic conditions,[41] we obtained a final room-temperature-stable product that could be characterized by spectroscopic methods and X-ray crystallography. The X-ray structure reveals that the original NO ligands of $[Fe(NO)_2(dmp)]$ are converted to nitrato chelating ligands binding to the oxo-bridged Fe(III) centers (Figure 4).[41] The presence of nitrate, a stable isomer of peroxynitrite, is consistent with the generation of the latter.

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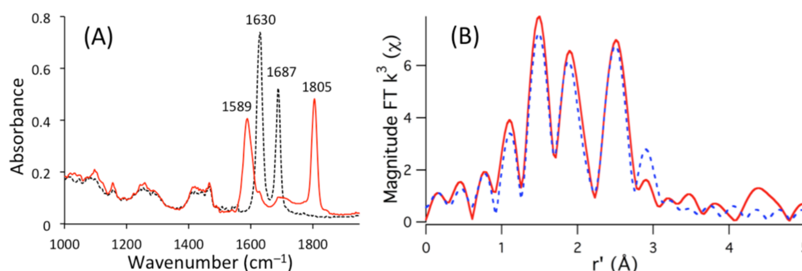


Figure 3. (A) IR spectra of **5** (black dashed) and **6** (red solid) at −80 °C in CH_2Cl_2. (B) Experimental (red solid) and simulated (blue dashed) magnitude FT k^3 EXAFS data for **6**.

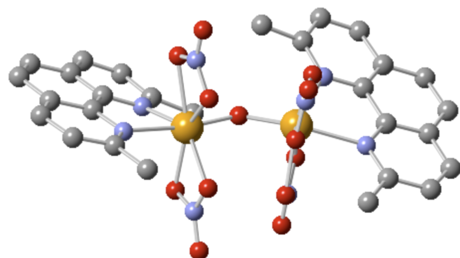


Figure 4. Ball-and-stick depiction of the crystal structure of $[Fe_2O(dmp)_2(NO_3)_2]$. Hydrogen atoms are omitted for clarity. Fe, orange; O, red; N, blue; and C, gray.

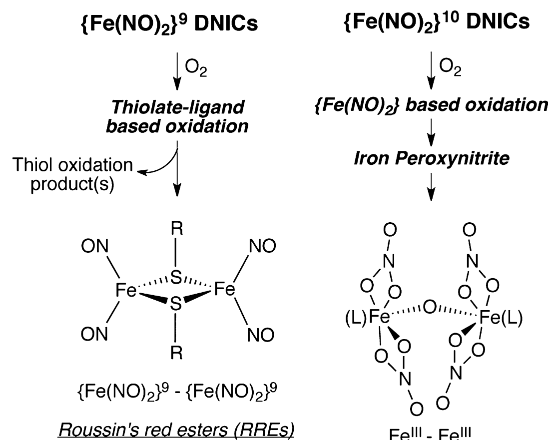
Nitration of biological phenols, such as tyrosine, is an important post-translational modification associated with various pathological conditions, including inflammatory, neuro-degenerative, and cardiovascular diseases, in which free peroxynitrite and nitrite are widely accepted cellular nitrating agents.[42] Our results suggest that cellular N-bound DNICs could be another class of nitrating agent employed by cells to induce protein tyrosine nitration.

Thiol Oxidation Chemistry by DNICs as Relevant to Protein Cysteine Oxidation

In the case of thiolate-bound anionic $\{Fe(NO)_2\}^9$ DNICs, completely different O_2 reactivity was observed. The first apparent difference was that the O_2 reaction with this group of DNICs was rather slow compared to that with N-bound $\{Fe(NO)_2\}^{10}$ DNICs. More importantly, no changes occurred at the iron-nitrosyl unit of the starting $\{Fe(NO)_2\}^9$ DNICs upon O_2 exposure, which was in sharp contrast to the O_2 chemistry of neutral $\{Fe(NO)_2\}^{10}$ DNICs. When solutions of a series of thiolate-bound DNICs, $[Fe(NO)_2(SR)_2]^-$, where R = Et, ^{t}Bu, and Ph, were exposed to O_2 at room temperature, UV−vis characteristics of the compounds known as Roussin's red esters (RREs), $[Fe_2(\mu\text{-}SR)_2(NO)_4]$, slowly developed over a period of hours with well-defined isosbestic points.[43] The identities of the RRE final products were further confirmed by other analytical methods, including IR spectroscopy, with which their characteristic ν_{NO} signals were easily distinguished.[43] Interestingly, an analogous conversion of DNIC to RRE following O_2 exposure was subsequently observed by Vincent and co-workers[44] during their studies of the protein [2Fe−2S] ferredoxin from spinach. This example nicely demonstrates how chemistry observed with synthetic models can be highly relevant to biological systems.

The products derived from thiolate-bound $\{Fe(NO)_2\}^9$ DNICs and O_2 are Roussin's red esters (RREs), which are dinuclear $\{Fe(NO)_2\}^9$ DNICs (Scheme 7), and thiolate oxidation products (e.g., disulfide, sulfonic acid, etc.). This result indicates that oxidation occurs at the sulfur atoms of the thiolate ligands of the starting DNICs as opposed to the iron nitrosyl unit (Scheme 7). The observed differential O_2 reactivity between anionic $\{Fe(NO)_2\}^9$ DNICs and neutral $\{Fe(NO)_2\}^{10}$ DNICs can be explained by their respective electronic structures. Analyses of two representative model complexes, $[Fe(NO)_2(SEt)_2]^-$ and $[Fe(NO)_2(TMEDA)]$, indicate that the SOMO of the anionic $\{Fe(NO)_2\}^9$ DNIC is a thiolate-sulfur-dominated wave function, whereas that of the neutral $\{Fe(NO)_2\}^{10}$ DNIC is an Fe-dominated wave function.[43]

Scheme 7



Controlled generation of reactive oxygen species (ROS) mediates numerous physiological signaling events in which the -SH group of cysteine is one of the major targets of ROS.[45] Our results demonstrate that $\{Fe(NO)_2\}^9$ DNICs are capable of inducing thiol oxidation via their O_2 reactivity. Thus, along with other ROS, cellular $\{Fe(NO)_2\}^9$ DNICs may likely be involved in redox signaling via their ability to mediate cysteine oxidation.

■ SYNTHETIC DNICS AS POTENTIAL THERAPEUTICS

While studying the O_2 reactivity of various DNICs, we learned that the stability of synthetic DNICs was very much influenced by their coordination number and oxidation state. When an $\{Fe(NO)_2\}^{10}$ DNIC, $[Fe(NO)_2(TMEDA)]$ (**5**), was oxidized by an outer-sphere oxidant, such as ferrocenium hexafluorophosphate ($FcPF_6$), cationic $\{Fe(NO)_2\}^9$ DNIC $[Fe(NO)_2(TMEDA)]^+$ was formed. This species was unstable in solution and spontaneously decomposed and released NO to the headspace. This same metastable cationic $\{Fe(NO)_2\}^9$ DNIC could also be prepared from a stable five-coordinate neutral $\{Fe(NO)_2\}^9$ DNIC, $[Fe(TMEDA)(NO)_2I]$ (**7**), via treatment with $AgPF_6$ to remove the labile iodide ligand (Scheme 8).[46]

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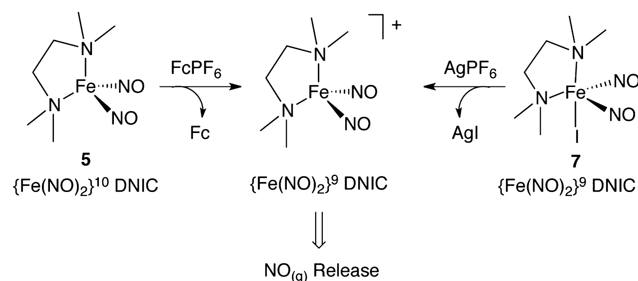
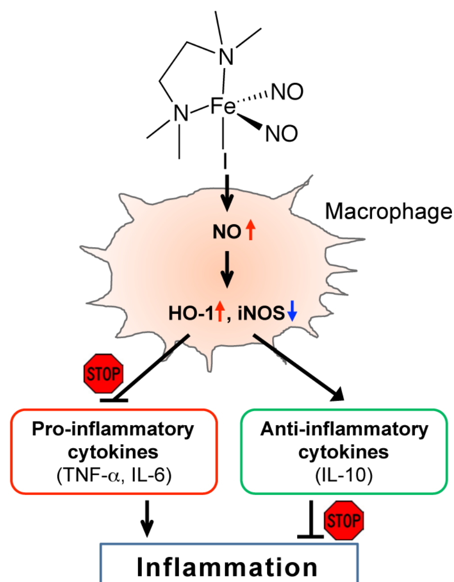
Scheme 8



These differences in DNIC stability inspired us to test the feasibility of developing synthetic DNICs as NO-donating therapeutics. We hypothesized that a neutral five-coordinate $\{Fe(NO)_2\}^9$ DNIC bearing a labile anionic ligand, $[Fe(NO)_2(TMEDA)I]$ (7), could be a reasonable pro-drug candidate that might become active upon entering the cell via loss of the labile ligand, triggered by the decrease in chloride ion concentration inside the cell, as compared to the extracellular space. We predicted that once the labile iodide ligand dissociates from iron, the resulting cationic DNIC, $[Fe(NO)_2(TMDEA)]^+$, would spontaneously release NO. The formation of an active species by the dissociation of a halide ligand upon entering the cell is reminiscent of that which occurs with the anticancer agent cisplatin.[47] In collaboration with Su Wol Chung (Ulsan University, South Korea), we investigated changes in the expression levels of two proteins known to be regulated by NO, heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS), following treatment of cultured mammalian cells with our pro-drug candidate (Scheme 9). When murine RAW264.7 macrophages were treated with

Scheme 9



$[Fe(NO)_2(TMEDA)I]$ (7), upregulation of HO-1 (280%) and downregulation of iNOS (35%) were observed,[46] consistent with the known effects of NO.[46] Because both HO-1 and iNOS are known to be involved in inflammatory processes, we further examined the effects of $[Fe(NO)_2(TMEDA)I]$ (7) in inflammation by assessing the levels of well-known pro-inflammatory cytokines, such as TNF-α and IL-6, and that of anti-inflammatory cytokine IL-10 upon treatment with 7. Downregulation of TNF-α and IL-6 and upregulation of IL-10 were observed (Scheme 9).[46] These results suggest that a synthetic DNIC, such as 7, can become a potent anti-inflammatory agent and offers promise in developing a new class of DNIC-based anti-inflammatory agents. The results also provide new insight into the possible anti-inflammatory roles that cellular N-bound DNICs may play.

SUMMARY AND CONCLUDING REMARKS

Over the past several years, we have identified new reactivity of [2Fe−2S] clusters with NO and their degradation products. The reaction environment plays a significant role in determining the type of nitrosylated iron products that form and the fate of bridging sulfides. In the presence of acid, nitrosylation of [2Fe−2S] clusters leads to the formation of $\{Fe(NO)\}^7$ mononitrosyl iron complexes (MNICs), H_2S, and elemental sulfur. In the presence of proton- and electron-donors, $\{Fe(NO)_2\}^9$ dinitrosyl iron complexes (DNICs) and H_2S are generated from the nitrosylation of [2Fe−2S] clusters. Cysteine analogues can efficiently transform MNICs back to [2Fe−2S] clusters at room temperature without the need for any other reagents. Various types of DNICs have unique dioxygen reactivity. Neutral N-bound $\{Fe(NO)_2\}^{10}$ DNICs react with O_2 to generate iron peroxynitrite species leading to nitration chemistry in the presence of phenolic substrates. In the case of anionic S-bound $\{Fe(NO)_2\}^9$ DNICs, oxygenation occurs at the sulfur atom of the thiolate ligand to yield thiolate oxidation products and dimeric $\{Fe(NO)_2\}^9$ DNICs, the species known as Roussin's red esters (RREs). A metastable cationic $\{Fe(NO)_2\}^9$ DNIC can be prepared via oxidation of a neutral N-bound $\{Fe(NO)_2\}^{10}$ DNIC or by removing a labile anionic ligand from a five-coordinate neutral $\{Fe(NO)_2\}^9$ DNIC. Using these properties, the feasibility of using $[Fe(NO)_2(TMEDA)I]$ (7) as a cellular NO donor agent has been tested, and anti-inflammatory activity of 7 has been observed.

Iron−sulfur clusters are common cofactors necessary for nearly all organisms, and they are one of the main reaction sites of nitric oxide inside the cell. Whether the global modification of iron−sulfur clusters by NO is a simple biomarker for NO toxicity or a trigger for cellular defense mechanisms remains to be seen. We are beginning to understand the complex nature of the reaction chemistry of [Fe−S] clusters, NO, molecular oxygen (O_2), hydrogen sulfide (H_2S), and thiol, all of which are key cellular components. We hope that the chemical knowledge and insight obtained from our synthetic studies will be extrapolated to better understand currently unknown biological consequences of cluster modifications.

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Notes

The authors declare no competing financial interest.

Biographies

Jessica Fitzpatrick received her B.Sc. (Hons) in chemistry from McGill University in 2010 after performing research with Professors Ian Butler and Bruce Arndtsen. She subsequently joined the Chemistry Department of Brown University where she obtained her Ph.D. in 2015 under the supervision of Professor Eunsuk Kim. Her research at

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Brown focused on the reactivity of thiolate-bound iron nitrosyl complexes, particularly the conversion of mononitrosyl iron complexes to iron−sulfur clusters.

Eunsuk Kim is an Associate Professor of Chemistry at Brown University. She received her B.S. from Sangmyung University (1994) and M.S. from Korea University (1996) in South Korea. She came to the United States in 1998, completed her Ph.D. under Professor Kenneth Karlin at Johns Hopkins University (2004), and performed her postdoctoral work with Professors Bruce Demple at the Harvard School of Public Health (2004-2005) and John Essigmann at the Massachusetts Institute of Technology (2005-2008). She has been at Brown University since 2008. Her research interests in bioinorganic chemistry include reactivity of iron−sulfur clusters in redox signaling and synthesis of biomimetic electrocatalysts for CO_2 conversion.

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