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# Coordination-triggered NO release from a dinitrosyl iron complex leads to anti-inflammatory activity†

Kelsey M. Skodje,[a] Min-Young Kwon,[b] Su Wol Chung*[b] and Eunsuk Kim*[a]

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Dinitrosyl iron complexes (DNICs) are widely considered NO storage and donor molecules in cells. However, what induces an NO release from iron in DNICs and the subsequent biological consequences remain elusive. The chemistry and biology of the NO release activity of DNICs are reported here. Changes in redox status or coordination number of discrete N-bound DNICs, respectively [Fe(TMEDA)$(NO)_2$] (**1**) and [Fe(TMEDA)$(NO)_2$I] (**2**), can generate a metastable $\{Fe(NO)_2\}^9$ DNIC, $[Fe(TMEDA)(NO)_2]^+$, with $\nu_{NO}$ at 1769 and 1835 $cm^{-1}$ and an EPR signal at $g = 2.04$, that spontaneously releases NO in solution. The NO release activity of **2** results in the up- and downregulation of heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS), respectively, in murine RAW 264.7 macrophages. Furthermore, treatment with **2** leads to downregulation of pro-inflammatory cytokines, TNF-α and IL-6, and upregulation of the anti-inflammatory cytokine, IL-10. Taken together, these results demonstrate that the appropriate control of redox and coordination chemistry of DNICs could enable them to become anti-inflammatory agents, suggesting a potential new role for cellular DNICs.

## Introduction

Dinitrosyl iron complexes (DNICs) are naturally occurring iron species formed by reactions between nitric oxide (NO) and cellular non-heme iron species such as iron–sulfur clusters and labile iron pools (LIP).[1] Although the biological fate of DNICs is largely unknown, protein-bound DNICs have been observed during the activation of several regulatory proteins[2] while glutathione-bound DNICs have been suggested to bind to glutathione transferases (GSTs)[3] and be transported *via* multidrug resistance-associated protein 1 (MRP1).[4] The first *in vivo* DNICs were discovered in the 1960s,[5] decades before the importance of NO as a signaling molecule in humans was noted. These DNICs were EPR active, cysteine bound and formulated as $[Fe(NO)_2(SR)_2]^-$, $\{Fe(NO)_2\}^9$ species in the Enemark–Feltham notation,[6] Chart 1. S-bound DNICs of this variety are the most common, but both N- and O-bound ones have been found in nature.[7] Following the discovery of endogenous DNICs, the biological effects of exogenously added DNICs have been investigated, and suggest that DNICs could become a class of drugs useful in regulating diverse physiological functions that are associated with nitric oxide.[1] For example, administration of cysteine- or glutathione-bound DNICs resulted in hypotensive effects in animal studies and clinical trials.[8]

Recognition of the biological importance of DNICs subsequently inspired chemists to study the chemical reactivity and physical properties of synthetic DNICs. These investigations have shown that the ligand environment and redox status of the $\{Fe(NO)_2\}$ unit are important factors that may determine the various forms of NO (*i.e.*, NO, $NO^+$, or $NO^-$) released from DNICs.[9] Despite a great deal of synthetic and reactivity studies, the cellular response to these well characterized DNICs has not been tested. In contrast, the water-soluble DNICs that are known to exert vasodilation[8,10] have not been isolated in a pure form, which presents significant challenges in correlating the chemical properties of DNICs to their corresponding biological effects. Because of this, we have chosen to use a synthetic DNIC whose chemical reactivity can be studied both inside and

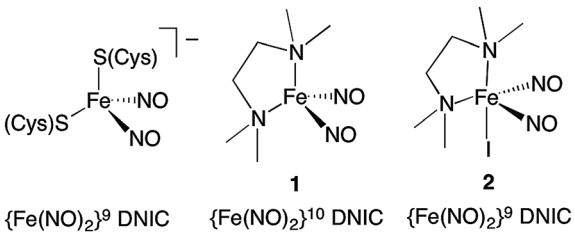


Chart 1 Dinitrosyl iron complexes.

[a]Department of Chemistry, Brown University, 324 Brook Street, Providence, RI 02912, USA. E-mail: eunsuk_kim@brown.edu; Fax: +1 401-863-9046; Tel: +1 401-863-3591

[b]Department of Biological Sciences, University of Ulsan 93 Daehak-ro, Nam-gu, Ulsan 680-749, Korea. E-mail: swchung@ulsan.ac.kr; Fax: +82 52-259-1694; Tel: +82 52-259-2353

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outside of a cell. Herein we report the chemistry and biology of discrete DNICs that can spontaneously release NO with external stimuli such as changes in their redox status or coordination.

## Results and discussion

We chose to investigate a pair of TMEDA based $\{Fe(NO)_2\}^{10}$ and $\{Fe(NO)_2\}^9$ DNICs for this study (**1** and **2**, Chart 1), where TMEDA = *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, because these are known to be stable complexes in the absence of external stimuli, which makes them an ideal system in searching for the factors that trigger NO release. The $\{Fe(NO)_2\}^{10}$ DNIC, $[Fe(TMEDA)(NO)_2]$ (**1**), has two characteristic NO stretching frequencies at 1629 and 1690 $cm^{-1}$ (blue dashed line, Fig. 1A). We observed that oxidation of **1** by one equivalent of ferrocenium hexafluorophosphate ($FcPF_6$) leads to complete loss of its NO ligands by IR spectroscopy. During the reaction, however, it was possible to detect a new intermediate species formed by the reaction of **1** with $FcPF_6$, with $\nu_{NO}$ at 1769 and 1835 $cm^{-1}$ (red solid line, Fig. 1A) before the complete loss of NO. The reversible redox couple of **1** in the cyclic voltammogram ($E_{1/2} = -527$ mV *vs.* $Fc/Fc^+$ in MeCN) suggests that a cationic $[Fe(TMEDA)(NO)_2]^+$ species would be accessible by oxidation. The EPR spectrum of the intermediate displays a strong signal at $g = 2.04$ (10 K, THF), supporting the formation of an $\{Fe(NO)_2\}^9$ species. Thus, a metastable species generated by chemical oxidation of **1** with $FcPF_6$ is proposed to be a cationic $\{Fe(NO)_2\}^9$ DNIC, $[Fe(TMEDA)(NO)_2]^+$, from which NO is released, Scheme 1. The evolution of NO gas during the reaction was further confirmed by employing an NO trapping agent, iron(II) phthalocyanine (PcFe), Scheme 1. When the headspace gas from the reaction mixture of **1** and $FcPF_6$ was transferred to a solution of PcFe, the formation of PcFe–NO was observed in the IR spectrum with the known[11] $\nu_{NO}$ at 1686 $cm^{-1}$. The assignment of $\nu_{NO}$ was further confirmed by employing $^{15}NO$ labeled **1**, from which the expected shift was observed at $\nu_{^{15}NO} = 1654$ $cm^{-1}$ ($\Delta\nu_{NO} = -32$ $cm^{-1}$) for PcFe–$^{15}NO$.

We anticipated that removing the iodide ligand from [Fe(TMEDA)$(NO)_2$I] (**2**), a five-coordinate $\{Fe(NO)_2\}^9$ compound, would generate the same metastable cationic $\{Fe(NO)_2\}^9$ species that leads to NO release. To test this, we added one equivalent of $AgPF_6$ to an acetonitrile solution of **2**. This resulted in precipitation of AgI, loss of $\nu_{NO}$ at 1719 and 1777 $cm^{-1}$ from **2**, and trapping of $NO_{(g)}$ from the headspace by PcFe (Fig. 1B), as expected.

The observed properties of [Fe(TMEDA)$(NO)_2$I] (**2**) subsequently led us to investigate its biological activity towards living cells as a pro-drug candidate for an NO releasing agent. We reasoned that **2** is a stable and inactive molecule that can be easily administered to the cell and then it can be converted to its active form, a cationic four-coordinate $\{Fe(NO)_2\}^9$ species that spontaneously releases NO, through ligand dissociation inside the cell. Because the chloride concentration inside a cell is significantly lower than outside, it is likely that the labile iodide ligand would dissociate upon entering the cell to form a metastable cationic DNIC, leading to NO release. A very similar activity is well known for the anticancer agent cisplatin, $[Pt(NH_3)_2Cl_2]$, which becomes an effective DNA damaging agent by losing its $Cl^-$ ligand once inside the cell.[12] In the case of **2**, physiological effects due to NO release would be expected upon treatment of cells with **2**. Accordingly, we monitored changes in expression levels of heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS) upon treatment with **2**, because NO is known to be a potent inducer of HO-1 in macrophages and vascular cells[13] and a negative feedback regulator of iNOS activity and expression.[14]

The effects of [Fe(TMEDA)$(NO)_2$I] (**2**) on the protein expression levels of HO-1 and iNOS were investigated in a murine

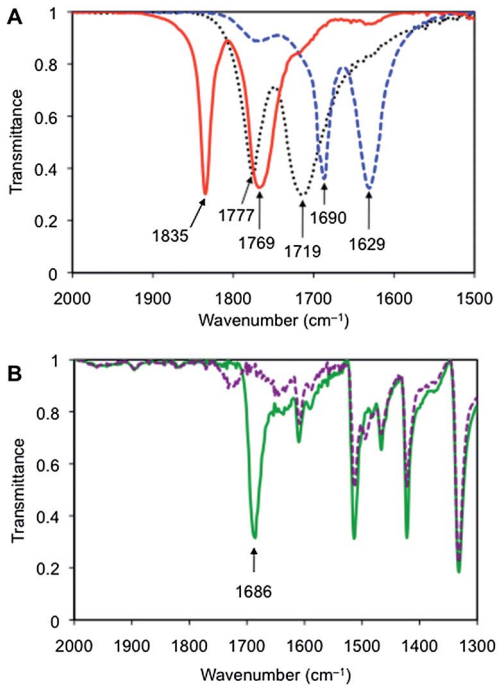
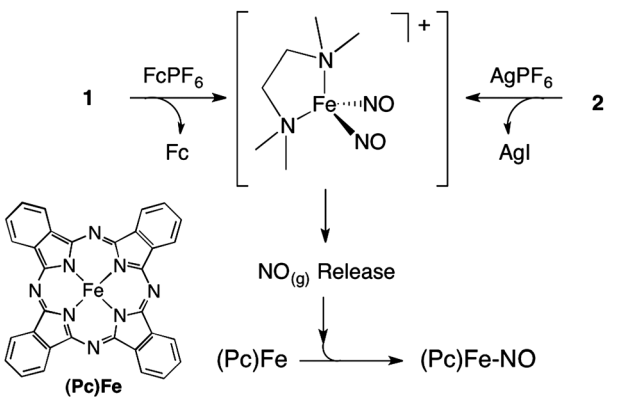



Fig. 1 (A) IR spectra (KBr) of [Fe(TMEDA)$(NO)_2$] (**1**) (blue dashed line), [Fe(TMEDA)$(NO)_2$I] (**2**) (black dotted line), and the metastable intermediate generated from **1**/$FcPF_6$ (red solid line) and (B) IR spectra (KBr) of iron(II) phthalocyanine before (purple dashed line) and after (green solid line) exposure to headspace from the **2**/$AgPF_6$ reaction.




Scheme 1 Oxidation of **1** and removal of iodide from **2** result in the release of NO, which is then captured from the headspace by PcFe.

macrophage cell line. RAW 264.7 cells were treated with **2**, lipopolysaccharide (LPS), or a combination of **2** and LPS in a time- and dose-dependent manner (Fig. 2), where LPS is a bacterial virulence factor with known pro-inflammatory properties.[15] The treatment of **2** resulted in an increase of the HO-1 level in the absence or the presence of LPS (Fig. 2A and B). On the contrary, the LPS-induced iNOS protein levels were markedly decreased by treatment with **2** (Fig. 2A and B). These results are reminiscent of what was observed from cells treated with a well known NO donor, NONOate,[13d,14a–c] implying that **2** likely acts as an NO donor inside the cell. In order to further support a hypothesis that the changes in HO-1 and iNOS levels were due to the NO release from **2**, the effects of a NO-free control compound, $[Fe(TMEDA)Cl_2]_2$ (**3**), on the HO-1 and iNOS expression levels were also examined, in which no change was observed (Fig. 2C).[16]

The effects of $[Fe(TMEDA)(NO)_2I]$ (**2**) on HO-1 and iNOS at the gene expression level were subsequently studied by quantitative real-time RT-PCR in RAW 264.7 cells. Upon treatment of **2** (500 μM) in the presence of LPS (100 ng $mL^{-1}$), mRNA expression of HO-1 was increased by 280.2 ± 7.6% while that of iNOS was decreased by 34.9 ± 2.6% (Fig. 3). In the absence of LPS, however, only the basal expression of HO-1 and iNOS mRNA were observed upon treatment of **2** (Fig. 3), which suggests that **2** may have post-transcriptional regulation effects on HO-1 expression in the absence of LPS.

It is well known that cytoprotective HO-1 plays a critical role in defending the body against oxidant-induced injury during inflammatory processes,[17] whereas the activation of iNOS can lead to organ destruction in inflammatory diseases.[18] Therefore, one can expect that $[Fe(TMEDA)(NO)_2I]$ (**2**) would have regulatory effects on inflammation in macrophages. In order to examine such effects, the levels of well known pro-inflammatory cytokines such as TNF-α and IL-6, and an anti-inflammatory cytokine, IL-10, were assessed upon treatment of **2**. RAW 264.7 cells were stimulated with **2** (500 μM), LPS (100 ng $mL^{-1}$), or a combination of **2** and LPS for 6 hours, after which supernatants were harvested and measured for TNF-α, IL-6, and IL-10 by ELISA. The results (Fig. 4) show that the LPS-induced pro-inflammatory cytokines (TNF-α and IL-6) were downregulated in the presence of **2**, whereas the production of anti-inflammatory cytokine, IL-10, was upregulated by **2**. These data strongly suggest that **2** leads to an anti-inflammatory response in macrophages.

Nitric oxide (NO) is a signalling molecule involved in cardiovascular function, neural signalling, immunodefence, and apoptosis.[19] Loss of endogenous NO has harmful effects that include vasoconstriction, greater smooth cell proliferation, and increased platelet and inflammatory cell activity and adherence at sites of endothelial damage.[19,20] Because of the great physiological importance of NO and the associated medical needs, synthetic NO donors have emerged as a class of

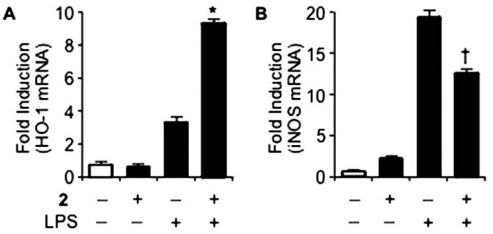



Fig. 3 Levels of mRNA for HO-1 (A) and iNOS (B) in RAW 264.7 cells assessed by quantitative real-time RT-PCR. Total RNA was extracted after administration of **2** (500 μM), LPS (100 ng $mL^{-1}$), or a combination of LPS + **2** at 12 hours. Expression levels of HO-1 and iNOS mRNA are divided by expression of the control gene, β-actin, and shown as a fold induction of the vehicle (DMSO). The asterisk indicates significant upregulation of HO-1 mRNA expression by the treatment of LPS with **2** *vs.* LPS alone ($P < 0.05$). The dagger indicates significant downregulation of iNOS mRNA expression by the treatment of LPS with **2** *vs.* LPS alone ($P < 0.05$). For all of the real-time PCR experiments, values are presented as mean ± SD, $n = 3$.

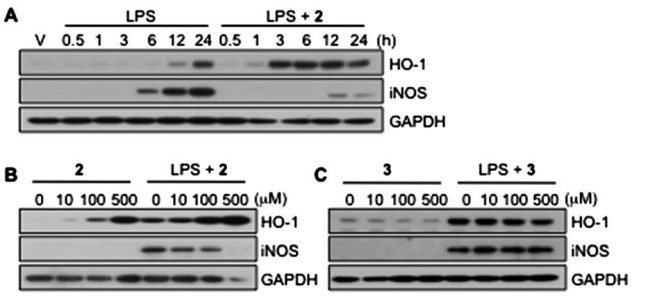



Fig. 2 The time- and dose-dependent expression of HO-1 and iNOS protein levels in RAW 264.7 cells assessed by Western blot analyses. Dimethyl sulfoxide (DMSO) was used as a vehicle to solubilise the test compounds. (A) Time course of HO-1 and iNOS levels after administration of lipopolysaccharide (LPS) (100 ng $mL^{-1}$) or a combination of LPS (100 ng $mL^{-1}$) and $[Fe(TMEDA)(NO)_2I]$ (**2**) (500 μM). Lane V shows protein levels in a vehicle-treated cell control; (B) dose-dependent HO-1 and iNOS levels after exposure to varying concentrations of **2** (0, 10, 100, and 500 μM) in the absence or the presence of LPS (100 ng $mL^{-1}$) at 12 hours and (C) dose-dependent HO-1 and iNOS levels after exposure to varying concentrations of $[Fe(TMEDA)Cl_2]_2$ (**3**) (0, 10, 100, and 500 μM) in the absence or the presence of LPS (100 ng $mL^{-1}$) at 12 hours. The housekeeping protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used as a loading control. These experiments were performed three independent times.

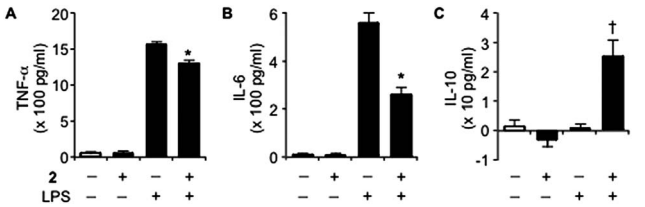



Fig. 4 The effects of $[Fe(TMEDA)(NO)_2I]$ (**2**) on cytokine production measured by ELISA. RAW 264.7 cells were stimulated with **2** (500 μM) LPS (100 ng $mL^{-1}$), or a combination of LPS and **2** for 6 hours. Dimethyl sulfoxide (DMSO) was used as a vehicle for **2**. Supernatants were then analyzed for TNF-α (A), IL-6 (B), and IL-10 (C). The asterisks indicate significant downregulation of TNF-α and IL-6 production by the treatment of LPS with **2** *vs.* LPS alone ($P < 0.05$). The dagger indicates significant upregulation of IL-10 by the treatment of LPS with **2** *vs.* LPS alone ($P < 0.05$). Values are mean ± SD, $n = 6$. Results are representative for three independent experiments.

drug useful in regulating these functions.[20] The present study shows that controlling the coordination properties of DNICs can be a useful strategy to create a new class of therapeutic NO donors. A neutral five-coordinate $\{Fe(NO)_2\}^9$ DNIC possessing a labile anionic ligand can be used as a pro-drug that becomes active upon entering the cell by forming a cationic DNIC that readily releases NO. One may expect that the NO releasing ability of DNICs can be systematically tailored *via* coordination chemistry. Possible fine-tuning of these compounds would involve changing the electronics and bite angle of the chelates to modulate the reaction rate, and using hydrophilic substituents on the periphery of the chelate to increase the solubility of the compounds in an aqueous medium. Some such efforts are currently being made in our laboratory.

## Conclusions

We have demonstrated previously unknown NO donor activity of two synthetic DNICs, $[Fe(TMEDA)(NO)_2]$ (**1**) and $[Fe(TMEDA)(NO)_2I]$ (**2**), as well as anti-inflammatory activity of a DNIC for the first time. Chemical oxidation of **1** or removal of the iodide ligand from **2** lead to the formation of a putative 4-coordinate $\{Fe(NO)_2\}^9$ DNIC that spontaneously releases NO in solution. When complex **2** is administered to cells, it becomes a potent regulator for HO-1 and iNOS expression in macrophages and causes anti-inflammatory effects by downregulating pro-inflammatory cytokines (TNF-$\alpha$ and IL-6) and upregulating an anti-inflammatory cytokine (IL-10) in macrophages. Taken together, these results show that DNICs offer promise in developing a new class of anti-inflammatory agents. Furthermore, the current finding regarding the biological effects of **2** sheds light on the possibility of a new physiological role for naturally occurring DNICs. It is conceivable that cellular DNICs may act as anti-inflammatory agents as part of the defense mechanism against oxidative–nitrosative stress.

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