SUPPORTING INFORMATION

Insight into the Relevance of Dinitrosyl Iron Complex (DNIC) Formation in the Absence of Thiols in Aqueous Media

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Content:

Experimental section

Materials and Methods

The chemicals sodium hydroxide (≥ 99.0%), acetic acid, HEPES (≥ 99.0%), sodium acetate (≥ 99.0%), Chelex® 100, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (97%), sodium phosphate dibasic (≥ 99.0%), sodium dihydrogen phosphate monohydrate (≥ 99.0%), L-glutathione (reduced) (≥ 98.0%), L-ascorbic acid (99.7-100.5%), diethylamine NONOate (≥ 97%), and iron(II) sulfate heptahydrate (> 99.0%) were purchased from Sigma Aldrich. All solutions and buffers were prepared using Milli-Q® water (Millipore).

The data are expressed as the mean ± S.D. of values determined in at least three independent experiments.

UV-vis Spectroscopy

The UV-vis spectra were acquired in a Shimadzu UV-2550 spectrophotometer using a screw cap septum quartz cuvette and argon-degassed solutions at 25±0.1 °C.

EPR Spectroscopy

The EPR spectra were acquired in a Bruker EMX spectrometer equipped with a high-sensitivity cavity. The experiments were performed at room temperature using a flat cell. The EPR instrument operated at 9.80 GHz microwave frequency, 100 G range, 4 G amplitude modulation, 100 kHz frequency modulation, 81.9 ms time constant, 20 mW microwave power, and 2 scans. WinEPR software was used for data processing. The concentration of the paramagnetic species was calculated by double integration of the EPR signal compared to standard curves of known concentrations of tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl).[1]

Nitric oxide gas purification and solution preparation

Nitric oxide gas from the tank (99.5%, Messer) was passed through two washing flasks containing 8 M NaOH solution and one washing flask containing 1 M NaOH previously degassed with argon. This procedure was performed to remove $NO_2^{\bullet}$ and N_2O_3 species.[2] Next, the gas was conducted via cannula to a balloon with a septum containing argon-degassed buffer solution and a needle to release the pressure. The samples were saturated with NO gas for 1 min per mL of solution. The saturated NO solutions (1.86 $mM.atm^{-1}$ at 25 °C)[3] at pH 5.0 (200 mM acetate buffer) or 7.4 (200 mM HEPES buffer) were always manipulated using gas-tight syringes and inert atmosphere techniques.

Preparation of diethylamine NONOate stock solution

Stock solutions of DEA/NO were freshly prepared in argon-degassed 0.01 M NaOH solution. The DEA/NO concentration was determined spectrophotometrically at 250 nm (ε = 6500 M^{-1} cm^{-1}).[4]

Preparation of Fe(II) and ligands (GSH, HPO_4^{2-} and $AscH^-$) stock solutions

All solutions were prepared under an argon atmosphere using balloons with a septum and needle to release the pressure. The Fe(II) solutions were prepared in Milli-Q® water, while GSH, HPO_4^{2-} and $AscH^-$ solutions were prepared in HEPES buffer (200 mM, pH 7.4) being the pH adjusted to 7.4 when necessary.

Fe(II) and NO in aqueous media: UV-vis and EPR analysis

Solutions pH 5.0 (200 mM acetate buffer) containing just Fe(II) and NO were analyzed by UV-vis following the procedure previously described in the literature.[5] Briefly, NO gas was bubbled for 90 seconds into a screw cap septum quartz cuvette containing 1 mL of argon-degassed 3 mM Fe(II) solution. Immediately after, a UV-vis spectrum was acquired. Next, this same solution was argon degassed for 10 s, and a new UV-vis spectrum was recorded. This last procedure was repeated twice.

For the EPR analysis, an aliquot of Fe(II) stock solution was added to a screw cap septum vial containing 1.86 mM of NO solution (pH 5.0, 200 mM acetate buffer or pH 7.4, 200 mM HEPES buffer) to obtain a final concentration of 3 mM. Next, the solution was transferred to a flat cell for EPR analysis. All the procedure performed after the mixture (solution transference to flat cell, EPR tunning, and spectrum scanning) was timed out and considered in the kinetics data processing. The observed rate constants (k_{obs}) were calculated by fitting the kinetic data to a single exponential function using OriginPro 9.7 software.

Fe(II), NO and ligand (GSH, HPO_4^{2-} or $AscH^-$) in aqueous media: UV-vis and EPR analysis

Aliquots of Fe(II) and ligands from stock solutions were added to a screw cap septum vial containing previously degassed buffer solution (200 mM HEPES buffer, pH 7.4) to obtain a final concentration of 0.18 mM of Fe(II) and different concentrations of each ligand (1.8-162 mM of GSH; 0.4-63 mM of HPO_4^{2-}; 0.9-900 mM of $AscH^-$). Next, NO saturated solution was added to this vial (final concentration of 0.93 mM). The solutions were analyzed 5 min after the mixture by both EPR and UV-vis. Then, the equilibrium constants ($K_L^{M/B-DNIC}$) for each system were calculated by fitting the data obtained from UV-vis or EPR by log[ligand] to a sigmoidal function using OriginPro 9.7 software.

Evaluation of DNIC formation using DEA/NO as NO-donor

Aliquots of Fe(II) and GSH or HPO_4^{2-} stock solutions were added to a screw cap septum vial containing previously degassed pH 7.4 buffer solution (200 mM HEPES) to obtain the final concentrations of 5 μM of Fe(II) and 5 mM of GSH or HPO_4^{2-}. Next, an aliquot of DEA/NO stock solution was added to this vial (final concentration of 20 μM, generating a NO flux of about 20 nM s^{-1}). Then, the solution was transferred to a flat cell for EPR analysis. All the procedure performed after the mixture (solution transference to flat cell, EPR tunning, and spectrum scanning) was timed out and considered in the kinetics data processing. The observed rate constants (k_{obs}) were calculated by fitting the kinetic data to a single exponential function using OriginPro 9.7 software.

Evaluation of NO bond reversibility in DNICs

The reversibility of NO bond in DNICs was evaluated by comparing the EPR spectrum area 5 min after the mixture of components (0.18 mM of Fe(II), 0.93 mM of NO, and 1.8 mM of ligand) with the EPR spectrum area after this same solution being argon degassed for 5 min. For solutions containing just 3 mM of Fe(II) and 1.86 mM of NO, after being argon degassed, this solution was also re-exposed to NO (5 min).

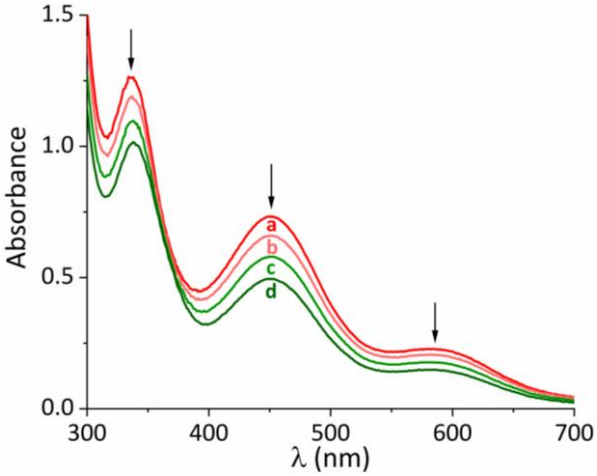

Figure S1 Absorption spectral changes recorded for the reaction between a) 3 mM of Fe(II) exposed to an excess of NO; b) a + argon degassed (10 s); c) b + argon degassed (10 s) ; d) c + argon degassed (10 s) at pH 5.0 (200 mM acetate buffer) and 25 °C.

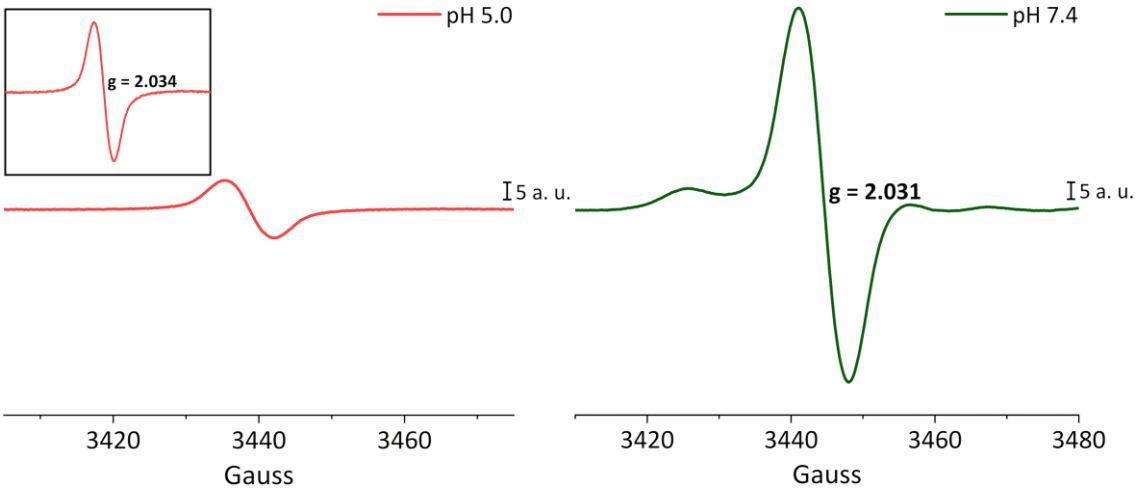

Figure S2 EPR spectra recorded for 3 mM of Fe(II) and 1.86 mM of NO. *Left:* at pH 5.0 (200 mM acetate buffer) and 23 min after mixture. *Right:* at pH 7.4 (200 mM HEPES buffer) and 5 min after mixture. The time that the spectra were acquired after mixture corresponds to the maximum EPR signal achieved in each condition.

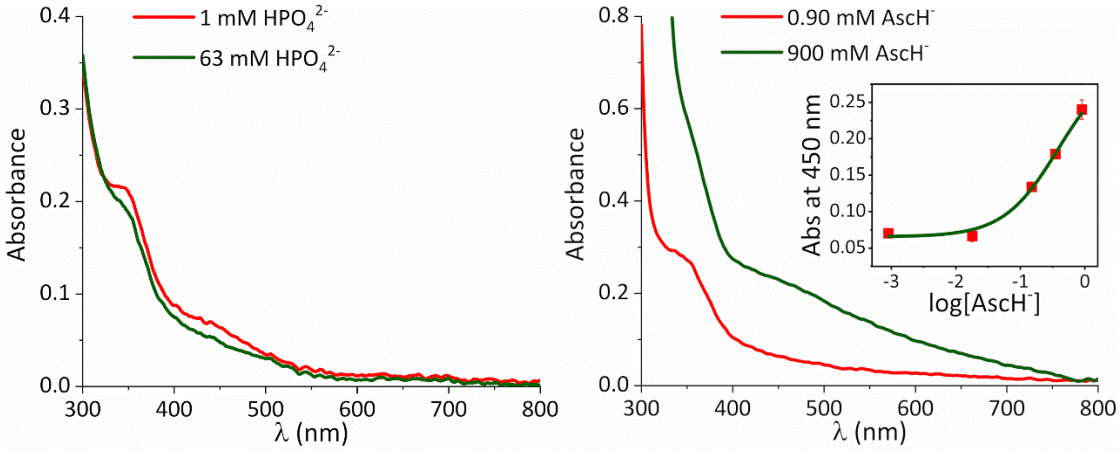

Figure S3 Absorption spectra of solutions containing 0.18 mM of Fe(II), 0.93 mM of NO, and *Left:* 1 and 63 mM of HPO_4^{2-} or *Right:* 0.90 and 900 mM of $AscH^-$ at pH 7.4 (200 mM HEPES buffer) and 25 °C. Inset: Plot of absorbance at 450 nm *versus* log[$AscH^-$] fitted to a sigmoidal function.

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