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ARTICLE

Nitric Oxide Release Study of a Bio-inspired Copper(I)-nitrito Complex on Chemical and Biological Conditions

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The selective and efficient nitrite reduction process ubiquitous in a biological system. To understand the copper-mediated nitrite reduction, we developed a bio-inspired model system to investigate the mechanism of copper-containing nitrite reductase. Structurally well-characterized copper(I)-nitrate complex with amino functionalized 2-(diphenylphosphino)aniline ligands, [(Ph2PC6H4(o-NH2))2Cu(ONO)], demonstrated the NO release and first protonation of aniline in an acidic environment. To further understand NO releasing ability we also performed pH dependency experiments and confocal imaging method to release NO under physiological buffer condition. Based on titration and spectroscopic studies of complex [(Ph2PC6H4(o-NH2))2Cu(ONO)], we proposed a mechanistic pathway for NO release and first proton transfer site. Besides, DFT calculations predict that the release of NO takes place via aniline in both organic and aqueous media. These results highlight the importance of proton-rich microenvironment around copper(I)-nitrite core to induce the nitrate reduction in a chemical and biological environment.

Introduction

Copper containing nitrite reductase (Cu-NIRs) holds a blue-green type 1 copper center that functions as a transfer of redox electron and type 2 copper center where nitrite binds and could catalyse nitrite reduction of nitric oxide formation.[1-13] Binding modes of nitrite in type 2 Cu(I)/(II) redox process and microenvironment around copper center, which is essential for catalytic copper redox protein designs.[14] In consideration of nitrite binding modes, copper(I)-nitro or -nitrito species both are postulated as the key intermediates for type 2 center of Cu-NIRs. Synthesis and reactivity studies of the Cu-NIRs models such as copper(I)-nitro or -nitrito complexes lead to the detail understanding of the catalytic mechanism. Several Cu(I)-NO_2 complexes possessing a η^1-nitro (N-bound) mode with biomimetic ancillary ligands intensively studied for their reactivity.[15-21] Regarding copper(I)-nitrito (O-bound) complexes, there are few known examples containing η^2-*O*,*O*-coordination.[22-25] Moreover, only three of them have been demonstrated that, the nitrite reduction activity accompany NO releasing under acidic condition.[23-25] Recently, another approach towards these studies emphasized through the electron-deficient β-diketiminate copper(II)-nitrito complexes, showed the activation of nitrite through the oxygen atom transfer pathway or thiol reduction.[26, 27] More recent studies also show that the electrocatalytic and proton coupled electron transfer approach to reduction of nitrite to release NO.[28]

Apart from nitrite binding mode and proton/electron transfer pathways for denitrification, Solomon *et. al*, demonstrated the effect of pH using spectroscopic and DFT studies on the nitrite bound type 2 copper site in wild-type enzyme of *Rhodobacter sphaeroides*. They found at high pH, there is no electron transfer from reduced type 1 to the nitrite bound oxidized type 2 copper, while protonation triggers type 1 → type 2 electron transfer and generation of NO.[11] Indeed, steady state kinetic turnover experiments for nitrite reduction as function of pH indicated the existence of two pKas, where the lower pKa (~5) has been invoked as the protonation equilibrium of an Asp residue and the higher pKa (~7) has been ascribed as the pKa of His residue.[8] To understand the effect of pH and denitrification mechanism in Cu-NIRs, we used biomimetic designing strategy and synthesized copper(I)-nitrito model system. A copper(I)-nitrito complex [($Ph_2PC_6H_4$(*o*-OMe))$_2$Cu(ONO)] (**1**), was reported by our lab, represent as a rare example of complexes exhibiting copper(I)-nitrito binding mode.[24] Complex **1** confined as a hemilabile phosphine-ether type *o*-(diphenylphos-phino)anisole ($Ph_2PC_6H_4$(*o*-OMe)) ligand and could mimic the reactivity of type 2 Cu-NIRs to produce NO gas under acidic condition. We concerned in this regards, how does the adjacent basic groups (e.g., Ph-NH_2) will influence the nitrite reduction ability of copper(I)-nitrito species? To address these concern, we used a 2-(diphenylphos-phino)aniline ($Ph_2PC_6H_4$(*o*-NH_2))[29] as a ligand of choice and compared with known type 2 copper(I)-nitrito model complexes.[23-25] The aniline group of this

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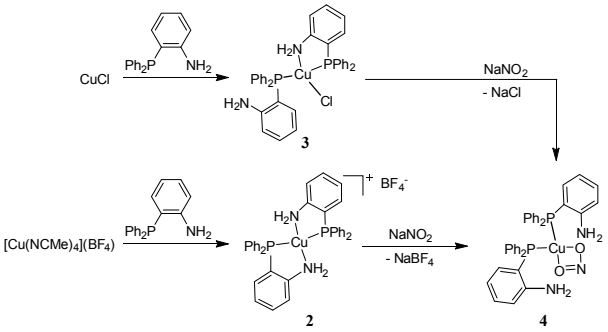
type of bidentate $Ph_2PC_6H_4(o\text{-}NH_2)$ ligand can act as a proton acceptor, which may cause a hydrogen bonding interaction between the aniline hydrogen and copper(I)-nitrito unit. Herein, we describe the synthesis of three copper(I) complexes containing the $Ph_2PC_6H_4(o\text{-}NH_2)$ ligands. Elucidate their reactivity and, furthermore, we explained how copper(I)-nitrito containing 2-(diphenylphosphino) aniline ligands, $[(Ph_2PC_6H_4(o\text{-}NH_2))_2Cu(ONO)]$ (**4**), releases the NO in chemical and biological conditions.

Result and Discussion

The reaction of bidentate P,N-donor ligand $Ph_2PC_6H_4(o\text{-}NH_2)$ with $[Cu(CH_3CN)_4](BF_4)$ (2:1 molar ratio) in CH_2Cl_2 under N_2 gave the copper(I) complex $[Cu(\kappa^2\text{-}Ph_2PC_6H_4(o\text{-}NH_2))_2](BF_4)$ (**2**), which was previously isolated as a colourless solid.[30-33] We also examined the reaction of $Ph_2PC_6H_4(o\text{-}NH_2)$ with CuCl to afford a neutral compound $[CuCl(Ph_2PC_6H_4(o\text{-}NH_2))_2]$ (**3**). Treatment of complexes **2** and **3** with $NaNO_2$ gave a complex $[(Ph_2PC_6H_4(o\text{-}NH_2))_2Cu(ONO)]$ (**4**), which exhibits an asymmetric η^2-*O,O'* bound nitrito ligation (Scheme 1).



Scheme 1 Synthesis of copper(I)-phosphine complexes.

All copper(I)-phosphine complexes **2**-**4** give significant π-π* transitions bands due to the phenyl and aniline groups of phosphine ligands and no absorption band observed in visible region (Fig. S1). In case of complex **3** and **4**, a shoulder peak appeared at 310 nm, assigned as an absorption band for unbound aniline group. Interestingly, complex **2** shows a different absorption peak at 275 nm that suggest the bound aniline groups to the copper center. These observations are consistent with crystallographic data (Fig. S4). The FT-IR spectra of **2**-**4** (KBr) displays the stretching frequencies of ν(N-H) in the range of 3200-3500 cm^{-1}. Complex **4** displays ν_{as} and ν_s stretching of the copper-nitrito at 1265 and 1209 cm^{-1}, respectively (Fig. S2). The ^{15}N sensitive bands of **4** are at 1247 and 1169 cm^{-1} (Fig. S3), also assigned to ν_{as} and ν_s stretches, respectively.[15, 17] The $^{31}P\{^1H\}$ NMR spectra of **2**-**4** exhibit a broad signal indicates the direct coordination of phosphorus atom of the $Ph_2PC_6H_4(o\text{-}NH_2)$ ligand to the copper(I) center. Additionally, the resonance of NH_2 occurred further downfield than the free $Ph_2PC_6H_4(o\text{-}NH_2)$ ligand in ^1H NMR.

The crystallographic results of **2** suggested that copper ion is bound by two chelated $Ph_2PC_6H_4(o\text{-}NH_2)$ ligands in a distorted tetrahedral coordination geometry (Fig. S4), and consistent with literature reports on ClO_4^- or PF_6^- salts.[31, 32] Complex **3** affords a four-coordination environment around the copper ion with axially bound chloride (Fig. S4). Moreover, one of the aniline group of $Ph_2PC_6H_4(o\text{-}NH_2)$ in **3** is dangling while another one is bound, shows a weaker Cu-N bonding interaction [Cu-N =2.251(2) Å]. In case of complex **4**, the structure consists of two $Ph_2PC_6H_4(o\text{-}NH_2)$ ligands with two dangling aniline groups and an O-bound nitrito ligand exhibiting an asymmetric η^2-*O,O'*-bonding mode (Fig. 1). The asymmetric nitrito-*O,O'* coordination (Cu-O) distance in **4** [2.226(3) and 2.257(3) Å] compared with the known copper(I) complexes $[(PPh_3)_2Cu(ONO)]$ [2.191(4) Å],[22] suggests that the Cu(I)-$O_{nitrito}$ interaction in **4** is rather weak. The exclusive bonding mode may be derived from the more sterically hindered phosphine ligand in **4** compared with other phosphine ligands.[22-24]

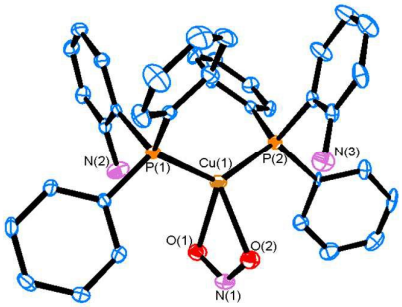


Fig. 1 ORTEP representation of complex **4** (50% ellipsoids, hydrogen atoms are not shown for clarity).

We examined the NO-generating ability of **4** by using a reported GC-TCD technique.[19, 20] To a solution of **4** in CH_2Cl_2, excess of acetic acid was added at room temperature, the color of the mixture solution changed from yellow to green-blue, followed by the evolution of NO gas (~75% v/v; see Fig. S5). Comparing the NO evolution results with those arising from studies of the known copper(I)-nitrito complexes $[(Ph_2PC_6H_4(o\text{-}OMe))_2Cu(ONO)]$ (**1**)[24] and $[(2,6\text{-}(Ph_2P(o\text{-}C_6H_4)CH{=}N)_2C_5H_3N)Cu(ONO)]$[23] demonstrated that complex **4** can also be a functional model for Cu-NIRs.

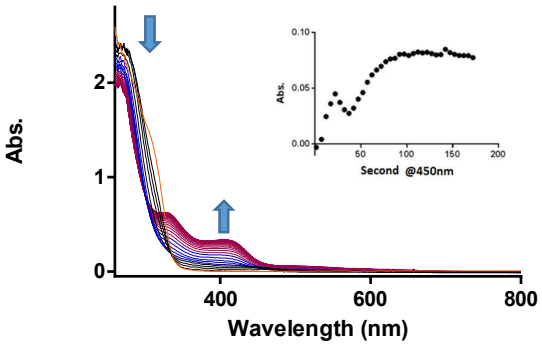


Fig. 2 Spectral changes observed during the anaerobic reaction of **4** with 300 eq. of TFA in CH_2Cl_2 at 273K. Inset: absorbance change over reaction time at 450 nm. (5 seconds interval)

The reaction of **4** with acetic acid is sufficiently slow that intermediates might be detectable, but none were observed. We think the protonation intermediate might be detected if a much stronger acid were used. To examine this, we monitored the reaction of **4** with trifluoroacetic acid (TFA), which is as stronger acid than acetic acid. The reaction of **4** with TFA was monitored by recording the UV-vis absorption spectra at 5-second intervals (Fig. 2). The 310 nm band of **4** was observed to be a shoulder at 273K. Upon addition of excess TFA to a solution of **4** under N_2, a decrease in the peak at 310 nm and an increase in a peak at 266 nm were

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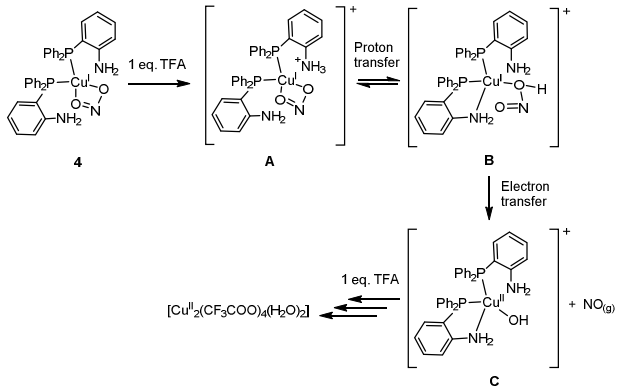
observed. A new intermediate with absorption peaks at 450 nm (less than 40 seconds) immediately disappeared and the absorption band at 325 nm, 410 nm, and 510 nm then rapidly increased. A similar case on the protonation intermediated observed by stronger acid could be found in literature.[13] The spectrum of the final product is similar to the known Cu(II) complex $[Cu(Ph_2PC_6H_4(o\text{-}NH_2))_2]^{2+}$ which was synthesized by following the literature procedure.[30] Efforts on the isolation of final product $[Cu(Ph_2PC_6H_4(o\text{-}NH_2))_2]^{2+}$ was failed, a known Cu(II) complex $[Cu_2(CF_3COO)_4(H_2O)_2]$ was isolated after NO generation reaction mixtures and demonstrated by crystallographic characterization. Complex **4** contains two dangling aniline groups, which may influence the nitrite reduction process. To address this issue, the reactivity study of complexes **1** and **4** were performed (Table S1). Both complexes **1** and **4** follow the first order kinetics at 273-243 K and the rate of reaction almost directly proportional to the concentration of TFA. Analysis of these results using the typical Eyring approach gave the $\Delta H^{\ddagger}$ = 24.1 ± 2.8 kJ mo1^{-1} and $\Delta S^{\ddagger}$ = -196.7 ± 7.4 JK^{-1}mol^{-1} for complex **4**. Comparisons of the activation parameters with known copper(I)-nitrito phosphine complex **1** ($\Delta H^{\ddagger}$ = 29.4 ± 7.6 kJ mo1^{-1} and $\Delta S^{\ddagger}$ = -171.1 ± 9.5 JK^{-1} mol^{-1}) reveals that complex **4** has smaller $\Delta H^{\ddagger}$ and larger $\Delta S^{\ddagger}$ values (Fig S7 and S8). The smaller $\Delta H^{\ddagger}$ value and larger negative $\Delta S^{\ddagger}$ value for reactions of **4** means a nearly complete protonation (association) intermediate in the nitrite reduction process.

The possible protonation sites for complex **4** are either nitrogen atom of aniline or the oxygen atom of nitrite. In order to understand the preferred protonation site, control experiment in complex **3** was chosen as this complex has dangling aniline arm which may shed some light on the protonation mechanism. Therefore, the titration of complex **3** with 0.5~4 eq. of TFA performed anaerobically in CH_2Cl_2 and observed the UV-vis spectral changes (Fig. S9). These results reveals, the protonation of complex **3**, shows a decrease in a peak at 290 nm and no copper(II) species formation observed. Moreover, after consumption of two equivalents TFA by complex **3**, anilines get protonated to become anilinium ion which is supported by the FT-IR and NMR. In FT-IR, N-H vibration peak after protonation shows peaks around 3050 cm^{-1} for the stretching modes of Ph-NH_3^+. Further, the symmetric deformation peak for NH_3^+ observed at 1558 cm^{-1} a weak band in the FTIR spectrum (Fig. S10).[34] Indeed, NMR titrations of complex **3** at room temperature also support the anilinium ion formation (Fig. S11). Hence, the protonation should occur at the nitrogen of aniline. UV-vis titration curve of complex **4** clearly illustrates the consumption of only one equiv of TFA to reach the plateau (Fig. S12). Extended titration experimentation to EPR for complex **4** also shows the one equiv. of TFA gives a maxima signal of EPR (Fig. S13). These phenomena may suggest that one equiv. TFA protonates the amino group of complex **4** and triggers the nitrite reduction to release the $NO_{(g)}$.

Based on UV-vis and EPR titration experimental observations in acidic environments, a plausible mechanism of the nitrite reduction of complex **4** was proposed and depicted in Scheme 2. The titration experiments of **4** with TFA, is one of the supporting evidence for the proposed mechanism. The shift of absorption bands towards the shorter wavelength, support the existence of protonated anilinium intermediate **A**. From intermediate **A**, anilinium proton transferred to nitrite group resulting into formation of intermediate **B** ($CuNO_2H$). Further, intermediate **B** might trigger an electron transfer process by releasing NO gas and become rearranged to form a stable copper(II) species **C**. Finally, a known Cu(II) complex $[Cu_2(CF_3COO)_4(H_2O)_2]$ was isolated after NO generation from reaction mixtures. These findings provide a novel insight for the bio-inspired Cu-NIRs models. The second coordination sphere effects of aniline group on complex **4** would facilitate the elucidation of mechanism in type 2 copper center in enzymes. As well, these results will also help us to understand the interaction and function of amino group (aniline) residues in microenvironment around copper(I)-nitrito core.



Scheme 2 Proposed mechanism for nitrite reduction of complex **4** with TFA.

Density functional theory (DFT) calculations were carried out to provide further insight into the nitrite reduction mechanism of complex **4** on chemical and biological (aqueous) conditions. The activation free energy of the HO–NO bond cleavage (i.e., from **B** to **C** in Scheme 2) was estimated to be 22.4 and 19.6 kcal mol^{-1} in dichloromethane and water, respectively. Our calculations indicate the intermediate **B** as a closed shell singlet state. However, the transition state of HO–NO bond breaking $\mathbf{TS_{BC}}$ displays an open shell singlet state that possesses biradical characteristics. The plots of highest occupied molecular orbital (HOMO) and spin density at $\mathbf{TS_{BC}}$ are shown in Fig. 3. The distribution pattern of HOMO for α and β electrons is dramatically dissimilar; the former mainly localized on the leaving NO, whereas the latter spread over the Cu–P bonds and OH ligand. The spin density plot also clearly shows the bi-radical character of the bond breaking transition state. The natural population analysis reveals that the atomic charge of copper increases from 0.274(CH_2Cl_2)/0.307(H_2O) at **B** to 1.025/0.655 at $\mathbf{TS_{bc}}$. These results suggest that the HO–NO bond cleavage is triggered by the electron transfer from Cu(I) to NO_2H. In comparison with complex **4**, we also calculated the nitrite reduction ability of complex **1**. Interestingly, the activation free energy of HO–NO bond breaking for complex **1** was 17.4 kcal mol^{-1} in both CH_2Cl_2 and aqueous solutions, somewhat lower than that for complex **4**. The moderate activation energies of HO–NO bond cleavage catalysed by Cu(I) in complex **1** and **4** are consistent with the experimental observation that both complexes can release NO in acidic conditions. However, as will be shown later, the complex **4** displays a superior NO releasing ability over complex **1** in neutral and basic buffer environments, which seems to contradict with the calculated activation energies of HO–NO bond cleavages. We thus calculated the free energy changes of protonation of complex **1** and **4** by H_3O^+ in aqueous environment. It turned out that the protonation on amine moiety of **4** was 3.4 kcal mol^{-1} lower than the direct protonation on NO_2^- of **1**. This result suggests that the amine group on the second coordination sphere can act as an absorber to capture proton from neutral and basic environments, which in turn

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renders complex **4** capable of undergoing nitrite reduction and releasing NO in physiological conditions.

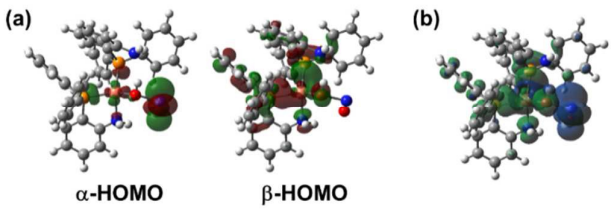

Fig. 3 Representations of plots (a) highest occupied molecular orbitals and (b) spin density for transition state $\mathbf{TS_{bc}}$.

To explore the potential of nitrite reduction and NO releasing ability we extended the experimentations to physiological condition. Complex **1** or **4** were tested under basic buffer condition with different pH values and noted the fluorescence response for NO release (see Fig. 4). In these experiments, FA-OMe (5-amino-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid methyl ester, green fluorescence)[35] used as a fluorescence detector to detect the nitric oxide release from complexes **1** or **4**. In this case, DETA NONOate used as a standard NO releasing source. The normalized fluorescence response (F-F0) is directly proportional to the amount of NO release and the linear correlation between NO release and normalized fluorescence response.[35] To determine the chemical yield of NO, we recorded the normalized fluorescence response of FA-OMe with varying equivalence of DETA NONOate (Fig S14 and S15). The gradual decrease in the fluorescence intensity of DETA NONOate indicates the decrease in the decomposition rate of primary amine of DETA NONOate with increasing pH.[36-38] For complex **1** we didn't observed a significant release of NO at different basic pH values. However, complex **4** shows a similar trend in fluorescence intensity like DETA NONOate which suggest the importance of the amine group microenvironment closed to copper(I)-nitrito center. Hence, the insight of pH dependency experiments explains the influence of aniline group of complex **4** to act as a NO donor source at physiological conditions.

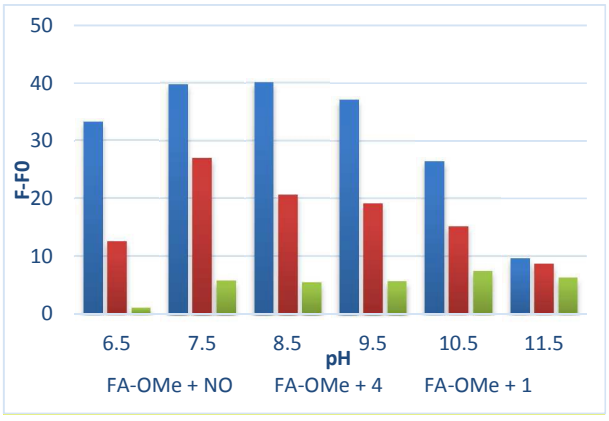

Fig. 4 Normalized fluorescence response of the FA-OMe (1 µM) with DETA NONOate, **1**, and **4** (5 µM) in different pH values (100 mM PBS buffer with 0.54% DMSO) and 25.0 ± 0.1 °C.

To understand the application of NO release in actual biological system, we performed in-vitro treatment of complexes **1** or **4** with macrophages (RAW 264.7 cells) and investigated the NO release studies using confocal microscopy. In this studies also, we used FA-OMe as fluorescent material. In case of complex **1** no green fluorescent signal were observed (Fig. 5a). However, in complex **4** strong green fluorescent signals observed with increase in concertation as shown in Fig. 5(b). These results suggest that microenvironment around copper(I)-nitrito core in complex **4** plays a key role to release NO in pH 7.5 buffer conditions. From the chemical and biological experimental data, we observed that amine protons plays key role in the release of NO at basic or physiological conditions. Usually, the nitrite reduction of copper(I)-nitrito species will perform under acidic condition. As a result, these findings demonstrated that nitrite reduction ability have great impact of adjacent groups of complex **1** and **4** to release the $NO_{(g)}$.

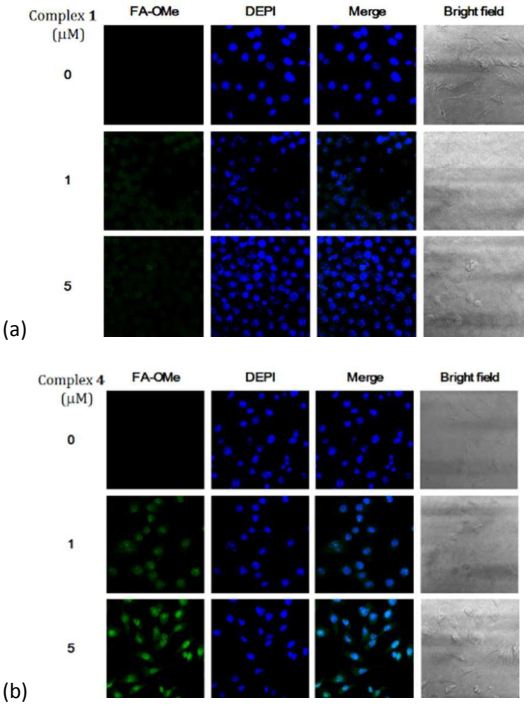

Fig. 5 Confocal imaging of macrophage (RAW 264.7 cells) treated with complexes **1** (a) or **4** (b) in 0, 1, and 5 µM at 37°C for 1 hr. Nuclei were stained with DEPI in blue and FA-OMe were displayed in green fluorescence.

Conclusions

We successfully synthesized a copper(I)-nitrito complex **4** containing $Ph_2PC_6H_4(o\text{-}NH_2)$ ligands as a bio-inspired compound for Cu-NIRs. The titration experiments and DFT studies helped to understand the preferred protonation site and NO release from complex **4**. pH dependency, and confocal imaging results elaborates the role of microenvironment around complexes **1** and **4**, and how aniline groups influenced the copper(I)-nitrito core to induce the nitrite reduction under physiological as well in basic buffer condition. Indeed, computational results also show the aniline microenvironments in complex **4** acts as a proton absorber at chemical and biological (aqueous) conditions. Overall, these results

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will enhance our understanding of nitrite reduction process at copper center of Cu-NIRs.

Experimental Section

General considerations

All manipulations carried out under an atmosphere of purified dinitrogen with standard Schlenk techniques. Chemical reagents purchased from Aldrich Chemical Co. Ltd., Lancaster Chemicals Ltd., or Fluka Ltd and Cayman Chemical. All the reagents were used without further purification, apart from all solvents that were dried over Na (Et_2O) or CaH_2 (CH_2Cl_2, CH_3CN) and then thoroughly degassed before use. The compounds $[Cu(CH_3CN)_4](BF_4)$,[39] $[(Ph_2PC_6H_4(o\text{-}OMe))_2Cu(ONO)]$ (**1**),[24] $Ph_2PC_6H_4(o\text{-}OMe)$,[40] and $Ph_2PC_6H_4(o\text{-}NH_2)$[29] were prepared as described in the literature. Dulbecco's Modified Eagle Medium (DMEM) and sodium pyruvate purchased from Cellgro. Fetal bovine serum (FBS, 10%) purchased from HyClone. Bright-field and fluorescence images recorded on an Olympus IX71 fluorescence microscope equipped with a 100 W mercury lamp, B-2A filters, and a color CCD camera system. IR spectra recorded on a Perkin-Elmer System 2000 FT-IR spectrometer. UV-vis spectra recorded on an Agilent 8453 spectrophotometer. The fluorescence spectra recorded on a Hitachi F-7000 fluorescence spectrophotometer. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were acquired on a JEOL JNM ECS 400 MHz. ESI mass spectra were collected on a Waters ZQ 4000 mass spectrometer. Elemental analyses performed on a Heraeus CHN-OS Rapid Elemental Analyzer. Gas chromatography thermal conductivity detector (GC-TCD) experiments were performed by using a Varian CP-3800 gas chromatography, Porpak Q column (6 ft, 20 mL/min flow rate, 30°C, nitrogen carrier gas), and TCD detector.

$[Cu(\kappa^2\text{-}Ph_2PC_6H_4(o\text{-}NH_2))_2](BF_4)$ (2)

A solution of $[Cu(CH_3CN)_4](BF_4)$ (2.265 g, 0.0072mol) in CH_3CN (30 mL) was added dropwise to a stirring CH_3CN (20 mL) solution of $Ph_2PC_6H_4(o\text{-}NH_2)$ (4 g, 0.0144mol). The reaction mixture was stirred for an hour and concentrated to about 10 mL, then slow diffusion of Et_2O into the solution and allowed to stay at room temperature for one day to obtain colorless crystals of $[Cu(\kappa^2\text{-}Ph_2PC_6H_4(o\text{-}NH_2))_2](BF_4)$ (**2**). Yield: 96% (4.88 g, 0.0069mol). ^{1}H NMR(DMSO-d_6): δ 5.68 (s, 4H,-NH_2), 6.776-7.479 (m, 28H, Ph). $^{31}P\{^1H\}$NMR (DMSO-d_6): δ -12.09(s). UV-vis absorption (CH_2Cl_2, λmax, nm) (ε/$M^{-1}cm^{-1}$) 260 (20531), ESI-MS: 618.11(100%) $[M]^+$.

$[CuCl(Ph_2PC_6H_4(o\text{-}NH_2))_2]$ (3)

A solution of CuCl (0.196g, 1.98 mmol) in CH_3CN (10 mL) was added to a stirringCH_3CN (15 mL) solution of $Ph_2PC_6H_4(o\text{-}NH_2)$ (1.1 g, 3.97 mmol) and stirring for two hours. A white solid precipitate forms immediately. The solvent removed under vacuum. The residue was extracted withCH_2Cl_2, The resulting white powder was recrystallized from CH_2Cl_2/ Et_2O to yield the product as colorless needles of $[CuCl(Ph_2PC_6H_4(o\text{-}NH_2))_2]$ (**3**). Yield: 82.5% (1.07 g, 1.64mmol). ^{1}H NMR (DMSO-d_6): δ 3.32(s, 4H,-NH_2), 6.42-7.39(m, 28H, Ph). $^{31}P\{^1H\}$ NMR (DMSO-d_6): δ -12.612(s). UV-vis absorption (CH_2Cl_2, λmax, nm) (ε/$M^{-1}cm^{-1}$) 303 (14912). ESI-MS: 647.08 (100%) $[M\text{-}Cl]^+$. Anal. Calcd for $C_{36}H_{32}ClCuN_2P_2$: C, 66.16; H, 4.94; N, 4.29. Found: C, 66.12; H, 5.00; N, 4.29.

$[Cu(Ph_2PC_6H_4(o\text{-}NH_2))_2(ONO)]$ (4)

A solution of 2 (0.5 g, 0.709 mmol) in MeOH (20 mL) was added to a stirring MeOH (10 mL) solution of $NaNO_2$ (0.049 g, 0.709 mmol) and stirring for two hours. The solvent removed under vacuum. The residue extracted with CH_2Cl_2 and layer with hexane. The resulting white powder was recrystallized from CH_2Cl_2/ Et_2O to yield the product as colorless needles of $[Cu(Ph_2PC_6H_4(o\text{-}NH_2))_2(ONO)]$ (**4**). Yield: 67.8% (0.319 g, 0.481mmol). ^{1}H NMR (DMSO-d_6): δ 5.50(s, 4H, -NH_2), 6.57-7.55(m, 28H, Ph). $^{31}P\{^1H\}$ NMR (DMSO-d_6): δ -12.93(s). UV-vis absorption (CH_2Cl_2, λmax, nm) (ε/$M^{-1}cm^{-1}$) 309 (15165). ESI-MS: 618.18 (100%) $[M]^+$. Anal. Calcd for $C_{36}H_{32}CuN_3O_2P_2$: C, 65.10; H, 4.86; N, 6.33. Found: C, 64.79; H, 4.86; N, 6.31.

Measurement of NO Generated from 4.

A solution of **4** (4 mg, 0.006 mmol) in CH_2Cl_2 (2 mL) was prepared in a small vial capped with a rubber septum. A solution of acetic acid (10.3 µL) in CH_2Cl_2 (0.1 mL) was then introduced with a syringe at room temperature. The solution changed immediately from colorless to blue. Analysis of the headspace gas by a thermal conductivity detector indicated that NO had been generated (75.43±1.12% for **4**). The NO generation data obtained by three different experiments (see Table S1, Supporting Information). NO concentration performed by calibrating curve response with known concentrations of NO gas mixed with N_2 (10, 20, 30, and 40 ppm of NO in N_2); molar quantities were calculated using the ideal gas equation.

Kinetics. The kinetics studies of nitrite reduction of **4** in CH_2Cl_2 were carried out by monitoring the intensity decrease of the 266 nm band. The absorbance detected by an Agilent 8453 spectrophotometer. The reaction started with the addition of 100 equiv. of TFA, which degassed with N_2 before use.

pH Dependent NO release study.

The different pH values of buffer solution were prepared by 100 mM PBS buffer and adjust pH value by $NaOH_{(aq)}$. The stock solution: FA-OMe was 2.5 mM in DMSO, complexes **4** and **1** were 0.5 mM in DMSO, respectively and DETA NONOate was 0.25 mM in DMSO. The FA-OMe stock solution diluted in different pH value of PBS buffer, and added **4, 1** or NO solution, respectively for 4 hr at room temperature. Then the reaction solution was recorded fluorescence by the Hitachi F-7000 fluorescence spectrophotometer (λex = 460 nm; λem = 515 nm).

Cell Culture and In Vitro Cell Imaging.

Raw 264.7 murine macrophages obtained from the American Type Culture Collection (Manassas, VA). The cells cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, and 1% MEM nonessential amino acids at 37 °C under a humidified 5% CO2 atmosphere. For cell imaging studies, Raw 264.7 murine macrophages passed and plated into poly-D-lysine coated plates containing 2 mL of DMEM and incubated at 37 °C with 5% CO_2. For 5-amino-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl (FA-OMe)[35] localization studies, Raw 264.7 murine macrophages were pre-stimulated with complexes **1** or **4** in 0, 1, and 5 µM at 37°C for 1 h, and then the cells were co-incubated with 10 µM of FA-OMe for 4 h. The cells washed three times with 1 mL of PBS prior to imaging and then bathed in 2 mL of PBS during the imaging procedure.

DFT calculations.

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Geometry optimizations and vibrational frequency calculations were performed by using M06/6-31G* method at gas phase. To obtain more reliable energies, M06/6-311+G** single point energy calculations were carried out at the M06/6-31G* optimized geometries. The solvent effect (dichloromethane and water) taken into account in the energy calculations by SMD continuum solvation model. The free energy corrections were made at the standard conditions of 1 atm and 298.15 K. Numerical integrations were done using ultrafine grids. All calculations were accomplished by Gaussian 09 program.[41]

X-ray crystal structure determinations.

The crystal structures of complexes **2**, **3**, and **4** suitable for single crystal X-ray diffraction analysis were grown by layering a CH_2Cl_2 solution with diethyl ether. All X-ray reflections were measured with Mo Kα radiation (λ = 0.71073 Å) on a Nonius Kappa CCD diffractometer. The data collection was executed using the SMART program.[42] Cell refinement and data reduction were made with the SAINT program.[43] The structure was determined using the SHELXTL/PC program[42] and refined using full-matrix least-squares. All non-hydrogen atoms were refined anisotropically, whereas hydrogen atoms placed at the calculated positions and included in the final stage of refinements with fixed parameters.

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Conflicts of interest

There are no conflicts to declare.

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Table of contents entry:

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