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Biliverdin-copper complex at physiological pH

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Biliverdin (BV), a product of heme catabolism, is known to interact with transition metals, but the details of such interactions under physiological conditions are scarce. Herein, we examined coordinate/redox interactions of BV with Cu²⁺ in phosphate buffer at pH 7.4, using spectrophotometry, HESI-MS, Raman spectroscopy, 1H NMR, EPR, fluorimetry, and electrochemical methods. BV formed a stable coordination complex with copper in 1:1 stoichiometry. The structure of BV was more planar and energetically stable in the complex. The complex showed strong paramagnetic effects that were attributed to an unpaired delocalized electron. The delocalized electron may come from BV or Cu²⁺, so the complex is formally composed either of BV radical cation and Cu⁺ or of BV radical anion and Cu⁶⁺. The complex underwent oxidation only in the presence of both O₂ and an excess of Cu⁶⁺, or a strong oxidizing agent, and it was resistant to reducing agents. The biological effects of the stable BV metallocomplex containing a delocalized unpaired electron should be further examined, and may provide an answer to the long-standing question of high energy investment in the catabolism of BV, which represents a relatively harmless molecule per se.

Introduction

Heme, iron protoporphyrin IX complex, is released from hemoglobin in senescent and impaired erythrocytes.1 It is further subjected to enzymatic degradation to bilirubin (BV) (1), which is rapidly converted to bilirubin by BV reductase (2).

Heme(Fe³⁺) + O₂ + 4NADPH + 4H⁺ [heme oxygenase] → BV + Fe²⁺ + CO + 4NADP⁺ + 3H₂O (1)

BV + NADPH + H⁺ [biliverdin reductase] → Bilirubin + NADP⁺ (2)

It is still not clear why physiologically harmless BV, which may be easily excreted without conjugation,2 requires energetically expensive two-electron reduction to potentially toxic bilirubin.3 A potential explanation may reside in the coordinate and/or redox interactions of BV with copper and other physiologically-relevant metals.4 However, the interactions of BV with copper ions have not been examined in physiological settings. Previous studies have preformed the synthesis/analysis of BV-copper complexes in organic solvents,5 in aqueous medium at a very high pH,6 or using Tris buffer,7 which shows high affinity for Cu²⁺ and may interfere with the interactions with BV.8 It is important to point out that at physiological pH, BV is present in the dianion form with the interactions with BV.

Results and discussion

UV-Vis spectrophotometry was applied to investigate formation/degradation of complex of BV with copper ions at pH 7.4 (Fig. 1). BV showed bands with λmax at 315 nm (non-restricted open-chain bilatriens), 375 nm (Soret-like band), and 670 nm (transitions in C=C and C≡N systems).10 Different [BV]/[Cu²⁺] concentration ratios were applied to evaluate the stoichiometry (Fig. 1a). At [BV]/[Cu²⁺] = 1, an immediate decrease in the intensity of the λ₆₇₀ absorption band was observed. At [BV]/[Cu²⁺] = 2, the λ₆₇₀ band was absent, whereas the λ₃₁₅ peak was shifted to 400 nm. It is noteworthy that a new band emerged at ~800 nm. The spectrum for [BV]/[Cu²⁺] = 2 corresponded to the sum of experimental spectra for [BV]/[Cu²⁺] = 1 and BV. The signal in the presence

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†Electronic Supplementary Information (ESI) available: [UV-Vis spectra of different BV/copper systems, BV/copper system in DMSO – UV-Vis and 1H NMR spectra, speciation of Cu²⁺ in phosphate buffer, ESI-MS spectrum, scan rate analysis of BV and BV-Cu complex, list of Raman bands for BV.]. See DOI: 10.1039/x0xx00000x

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of an excess of Cu²⁺ ([BV]/[Cu²⁺] = 0.5) was similar to the [BV]/[Cu²⁺] = 1 system. Altogether, this implies that BV interacted with Cu²⁺ in a 1:1 stoichiometry, which was further confirmed by titration (Fig. 1e). The same stoichiometry of BV (analog/copper complexes has been observed in organic solvents. The reaction was over within 5 min and all signals were stable under anaerobic conditions for at least one hour. The [BV]/[Cu²⁺] = 2 system was also stable in the presence of O₂ (Fig. 1b), which implies that the complex is not susceptible to oxidation by O₂ per se. On the other hand, signals for [BV]/[Cu²⁺] = 1 and [BV]/[Cu²⁺] = 0.5 systems showed a decay with time under aerobic settings (Fig. 1c, d). The decrease was more pronounced for the latter. It appears that the complex undergoes oxidation/degradation only in the presence of both O₂ and ‘free’ Cu²⁺, which is present in traces in the [BV]/[Cu²⁺] = 1 system, and in excess in the [BV]/[Cu²⁺] = 0.5 system. In the [BV]/[Cu²⁺] = 2 system, BV sequestered (almost) all available copper thus preventing the oxidation.

Figure 1. Changes in UV-Vis spectra of biliverdin (BV) in the presence of Cu²⁺ in phosphate buffer (50 mM; pH 7.4). (a) Different concentration ratios under anaerobic conditions (Ar atmosphere). Spectra were recorded after 5 min of incubation and remained stable for at least 60 min. (b) The system with [BV]/[Cu²⁺] = 2 molar ratio under aerobic conditions. (c) [BV]/[Cu²⁺] = 1 under aerobic conditions. (d) [BV]/[Cu²⁺] = 0.5 under aerobic conditions. (e) Absorbance titration curve. Spectra were obtained after 5 min incubation period. In all experiments [BV] = 20 μM.

The sensitivity of complexes of Cu²⁺ and porphyrin model molecules to O₂ has been observed previously in organic solvents. However, the involvement of ‘free’ Cu²⁺ has not been taken into consideration. It is important to point out that the complex was not affected by bathocuproine, a copper chelating agent (ESI Fig. S1†), implying that copper cannot be easily removed from the complex. Further, we observed that BV degradation at [BV]/[Cu²⁺] = 1 molar ratio was promoted at higher BV and Cu²⁺ concentrations (ESI Fig. S2†). This is most likely related to a higher amount of ‘free’ copper ions and/or to different Cu²⁺ speciation in phosphate buffer at different [Cu²⁺] (ESI Fig. S3†). The degradation of the complex at high concentrations was prevented by an excess of BV (in the [BV]/[Cu²⁺] = 2 system). Therefore, we applied a [BV]/[Cu²⁺] = 2 molar ratio for methods/measurements that required high concentrations and longer recording periods at room T. It is worth mentioning that the interactions of porphyrins with metals are frequently studied in DMSO, which is a more convenient solvent for some analyses. Pertinent to this, BV showed similar UV-Vis spectra in DMSO and phosphate buffer (ESI Fig. S4†). However, spectral features of the [BV]/[Cu²⁺] = 1 system differed significantly. The 660 nm band was still present, whereas the 380 nm band showed a decrease and developed a “shoulder” at approximately 440 nm. This implied that the interactions of BV with Cu²⁺ in DMSO and in phosphate buffer differ, and pointed out the importance of analysing BV/Cu²⁺ system in the aqueous medium.

HESI-MS analysis confirmed that BV built a complex with copper ions in 1:1 stoichiometry (Fig. 2a). The mass spectrum of BV showed a pseudomolecular ion [M + H]⁺ at m/z 583.07. In the presence of Cu²⁺ in 1:1 molar ratio, a peak at m/z 643.36 emerged, whereas the peak of free BV was absent. This m/z value corresponded to the sum of masses of BV and copper [M + H + Cu - 3H]⁺. HESI-MS of the [BV]/[Cu²⁺] = 0.5 system showed a significantly higher number of detectable fragments compared to other systems (ESI Fig. S5†), further confirming that the complex and BV undergo oxidation/degradation in the presence of O₂ and an excess of Cu²⁺. Next, we applied Raman spectroscopy (Fig. 2b). The Raman spectrum of BV was in good agreement with previous reports (ESI Table S1†). Comparing spectra of BV and BV-Cu complex, the following differences were observed: (i) a new band at 540 cm⁻¹ emerged for the complex; (ii) the band at 844 cm⁻¹ was shifted to 821 cm⁻¹; (iii) the peak at 1303 cm⁻¹ was (almost) absent; (iv) the band at 1333 cm⁻¹ was stronger; (v) the line at 1480 cm⁻¹ was stronger; (vi) it appears that two peaks/shoulders of 1616 cm⁻¹ band emerged/were stronger at ~1580 cm⁻¹ and ~1630 cm⁻¹. The 540 cm⁻¹ band may be attributed to Cu-N bond vibration. The band at 844 cm⁻¹ was attributed to ring (C-C bond) stretching. The shift to lower energies implicates increased stability of BV in the complex. The band at 1303 cm⁻¹ may be attributed to wagging vibration of C-H bond, which are very sensitive to environmental factors. The 1333 cm⁻¹ band has been previously identified as a structure-sensitive band for Cbilibilirubin complex, and has been attributed to CH(CH₃) in-plane vibration. Therefore, changes in the intensities of these two bands imply a more planar structure of BV in the complex. This is in line with the development of the band at 800 nm in UV-Vis spectrum (Fig. 1), which has been related previously to cyclic near-planar configuration of BV-metal complexes. Stronger bands at 1480 cm⁻¹ (stretching of aliphatic C-C bonds), ~1580 cm⁻¹ (stretching of C=C bonds in the ring), and ~1630 cm⁻¹ (stretching of C=C and C=O bonds in the ring), imply higher delocalization of π-electrons and consequently a higher stability of the BV structure. Pertinent to this, it has been proposed that complexes of BV model compounds with Cu²⁺ may show unusual electronic structures that exhibit a significant ligand radical character.16

Figure 3a shows ¹H NMR spectrum of BV in phosphate buffer (prepared with D₂O). Poor resolution of signals, which may originate from aggregation, did not allow reliable assignment. However, this was of little relevance here, since the addition of copper ions led to a very strong effect - the complete loss of almost all lines. The loss of signals represents a result of strong paramagnetic effects that may come from an unpaired e' that is delocalized in px orbitals of the ring/ligand, influencing all protons in the complex. The broadening of NMR signals beyond detection is not surprising considering that radicals are molecules with the longest electron relaxation time among paramagnetic species, which leads to a large
nuclear relaxation rate. It is worth mentioning that $^1$H NMR lines of BV in deuterated DMSO were comparatively well resolved (ESI Fig. S6†), which points to an alteration of the protonation state and/or location, and in particular to different aggregation pattern. In addition, the lines were broadened but not lost (except for NH signals that could not be observed after D$_2$O addition because of chemical exchange) in the presence of even higher Cu$^{2+}$ concentration (ESI Fig. S6†), underpinning the significant difference in BV/Cu$^{2+}$ interactions in the two media. Low-$T$ EPR was applied to further examine paramagnetic properties of the BV-Cu complex. The EPR spectrum of Cu$^{2+}$ ($J = 1/2$; $I = 3/2$) in phosphate buffer shows that Cu$^{2+}$ is weakly coordinated in an axial symmetry with one $g\|$, line and four lines coming from hyperfine coupling along $g\perp$ (Fig. 3b). The addition of BV in equimolar concentration led to the loss of Cu$^{2+}$ signal. In the [BV]/[Cu$^{2+}$] = 0.5 system the double integral of the signal of Cu$^{2+}$ in phosphate buffer was decreased by half, implying that an excess of copper is not bound to BV. The remaining signal in the [BV]/[Cu$^{2+}$] = 1 system was broad, and did not show hyperfine structure. The $g$-value of the isotropic signal of BV-Cu complex was significantly lower than the average $g$-value of Cu$^{2+}$ in the phosphate buffer indicating delocalization of the spin away from the metal nucleus. Similar EPR signals have been reported previously for porphyrin radical cation,19 and for an oxidized copper-porphyrin model molecule complex.20

This speaks in favour of delocalization processes that compete with fluorescence, such as intersystem crossing and spin–orbit coupling.21

Further, redox properties of the complex were examined. BV showed a well-defined anodic peak at $E_{pa2} = 117$ mV (Fig. 4a). Cyclic voltammogram of Cu$^{2+}$ in phosphate buffer showed reversible redox behaviour at a peak potential at approximately -400 mV, with very weak currents. Therefore, the redox activity of BV-Cu systems in cyclic voltammograms was complex-centered. The [BV]/[Cu$^{2+}$] = 2 system showed two additional oxidation peaks at much lower potentials than BV: $E_{pa2} = -91$ mV and $E_{pa3} = -341$ mV. The former potential corresponds to the oxidation of Cu$^{2+}$. No reduction peaks could be observed. Differential pulse voltammetry delivered similar results – the [BV]/[Cu$^{2+}$] = 2 system showed two additional peaks, most likely coming from two 1e$^-$ oxidations (Fig. 4b). It is noteworthy that a similar oxidation pattern has been observed previously for a complex of BV model molecules in organic media.23

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settings. There was a slight consumption of O$_2$ in [BV]/[Cu$^{2+}$] = 1 system during the process (Fig. 4c), which may be explained by traces of ‘free’ copper as discussed above (see Fig. 1 and ESI Fig. S2†). However, in the presence of an excess of copper ([BV]/[Cu$^{2+}$] = 0.5), the consumption of O$_2$ was significant. This implies that ‘free’ Cu$^{2+}$ reacts with the complex and ‘shuttles’ an e$^-$ to O$_2$. The addition of catalase to [BV]/[Cu$^{2+}$] systems after 15 min incubation, resulted in O$_2$ release, implying that hydrogen peroxide is accumulated. The concentration of accumulated H$_2$O$_2$ corresponded to the amount of O$_2$ that was consumed in the [BV]/[Cu$^{2+}$] = 1 system and to approximately half of O$_2$ consumed in the [BV]/[Cu$^{2+}$] = 0.5 system. As an illustration of the redox mechanism, we added Cu$^{2+}$ to the buffer. Cu$^{2+}$ rapidly reduced O$_2$ to produce superoxide radical anion resulting in O$_2$ concentration drop, which was partially reversed by catalase. Further analysis of stoichiometry could not be performed because the system composed of transition metal, reactive oxygen species and organic molecule involves multiple reactions that resulted in fragmentation of BV (see ESI Fig. S5†).

Figure 4. Redox properties of BV/Cu systems in phosphate buffer (50 mM; pH 7.4). (a) Cyclic voltammograms of BV and Cu$^{2+}$ complex at a boron-doped diamond electrode (scan rate 0.1 V/s). Concentrations of BV and Cu$^{2+}$ were 0.4 mM and 0.2 mM, respectively. Oxidation/anodic peak current potentials ($E_p$) are labeled. (b) Differential pulse voltammograms (increment, 0.004 V; pulse width, 0.05 s; sample width, 0.01 s; quiet time, 2 s). (c) Consumption of molecular oxygen in BV solutions following addition of Cu$^{2+}$, or the addition of Cu$^{2+}$ to phosphate buffer. The trace without Cu addition is presented for reference. In all experiments [BV] = 200 μM. Catalase (200 IU) was added 15 min after copper addition, to quantify the accumulation of H$_2$O$_2$ (2 H$_2$O$_2$ → O$_2$ + 2 H$_2$O). Concentrations of H$_2$O$_2$ were as follows: 4 μM (red trace), 18 μM (blue), 45 μM (green), and 75 μM (magenta). (d) UV-Vis spectra of BV with Cu$^{2+}$ and Cu$^{3+}$ under anaerobic and aerobic conditions. The signals are compared to the spectra of analogous BV/Cu$^{2+}$ systems. Cu$^{3+}$ does not show detectable absorbance at the applied concentration. (e) The effects of reducing agent – ascorbate (Asc) on BV-Cu complex. (f) The effects of oxidizing agent – KMnO$_4$ on BV-Cu complex. Both, Asc and KMnO$_4$ did not affect the spectrum of free BV.

Next, the effects of reducing (Cu$^{3+}$ and ascorbate) and oxidizing (KMnO$_4$) agents on BV-Cu complex were examined. The complex was not affected by Cu$^{3+}$ (Fig. 4d). BV was exposed to 1:1 mixture of Cu$^{3+}$ and Cu$^{2+}$ under anaerobic conditions. The spectrum of 20 μM BV with 10 μM Cu$^{2+}$ and 10 μM Cu$^{3+}$ corresponded to the spectrum of an analogous [BV]/[Cu$^{2+}$] = 2 system. Initially present Cu$^{3+}$ did not affect the spectrum, i.e. the system was stable. When exposed to air, Cu$^{3+}$ was oxidized by O$_2$ to produce Cu$^{2+}$ that reacted with free BV, resulting in the production of an additional amount of complex. The complex was not affected by ascorbate as well (Fig. 4f). On the other hand, the complex was degraded by KMnO$_4$.

Conclusions

At physiological pH, BV builds a complex with copper ions in 1:1 stoichiometry. The formation of the complex involves the rearrangement of electronic structure which provides increased energetic stability and strong paramagnetic effects. We believe that a complex with highly localized unpaired e$^-$ and the formal BV$^{2-}$-Cu$^{1+}$ or BV$^{2-}$-Cu$^{3+}$ character best suites the outlined properties. The presented results may shed new light on long-standing issues of BV chemistry and catalysis in biological systems.

Experimental

Chemicals

All chemicals were of analytical grade: BV, buffer components, dimethyl sulfoxide (DMSO), DMSO$_d_6$ (deuterated DMSO, 99.9 % D atom), D$_2$O (99.9% D atom), and urea were purchased from Sigma-Aldrich (St. Louis, MO, USA); Cu$^{2+}$ was from Merck (Kenilworth, NJ, USA); solvents for MS (acetonitrile, formic acid; LC–MS grade) were obtained from Fisher Scientific (Loughborough, UK). All experiments were performed using bidistilled deionized water (18 MΩ) that was obtained by reagent grade water system (Millipore, Billerica, USA). Stock solutions of BV - 20 mM in DMSO for experiments with high final concentrations (NMR, Raman, cyclic voltammetry) or 1 mM in 5 mM NaOH for all other experiments were prepared.
daily and kept on ice in the dark. Phosphate buffer - 50mM KH$_2$PO$_4$ with pH adjusted to 7.4 with KOH, was prepared daily.

**UV-VIS spectroscopy**

UV-VIS absorption spectra were obtained using a 2501 PC Shimadzu spectrophotometer (Kyoto, Japan). Sample volume was 1 mL. Scan time was 50 s. Samples were freshly prepared and immediately scanned at wavelengths from 800 to 200 nm at room T. Changes of spectra were monitored for 60 min. Each system was prepared in light-protected glass and stirred. Aliquots were measured and discarded for each time-point, since we noted that irradiation during the collection of UV-VIS spectra may result in BV degradation.

**HESI-MS spectrometry**

MS analysis was performed using a TSQ Quantum Access Max mass spectrometer equipped with a HESI source, which was used with ion source settings as follows: spray voltage, 3500 V; sheath gas, N$_2$ pressure, 30 AU; ion sweep gas pressure, 3 AU; auxiliary gas (N$_2$) pressure, 10 AU; vaporizer temperature, 450°C; capillary temperature, 380°C; skimmer offset, 0 V. Multiple mass spectrometric data were acquired in positive mode, including full scanning in m/z range from 100 to 1000 (FS) for qualitative analysis and product ion scanning (PIS) mode for the quantitative analysis. Collision-induced fragmentation experiments were performed using Ar as the collision gas, with collision energy set at 5 eV. SRM experiments for quantitative analysis was performed using two MS2 fragments for each compound, which were previously defined as dominant in PIS experiments. Samples were introduced into the mass spectrometer with a syringe pump and continuous flow injection for a period of 5 min at a flow rate of 5.0 μL/min. Analyst version 1.4 of Xcalibur software (Thermo Fisher Scientific, Waltham, MA, USA) was used for data acquisition and processing.

**Raman spectroscopy**

The Raman spectra of sample solutions were recorded on a Thermo DXR Raman microscope (Thermo Fisher Scientific). Sample aliquots of 5 μL were placed on the Raman grade calcium fluoride holder following the adjustment and stabilization of pH of the solution and Raman spectra were recorded. The 532 nm laser excitation line was used, with the following settings: exposure time, 10 s; number of exposures, 10; grating, 900 lines/mm; pinhole, 50 μm; laser power at the sample, 2 MW.

**Fluorescence Spectroscopy**

Fluorescence spectra were acquired using a Fluorolog FL3-221 with a 450 mW Xe lamp (Jobin Yvon Horiba, Paris, France), and FluorEssence 3.5 software (Horiba Scientific, Kyoto, Japan), and the following settings: excitation range, 320–380 nm; emission range, 400–600 nm; increment, 2 nm; slit (band pass), 3 nm for establishing excitation and emission spectra, and 1 nm for acquiring emission spectra at 293 K and 328 K, and emission spectra in the presence of urea (5 M). Emission detector signal was scaled by reference quantum counter signal (S1c/R1c). Relative quantum yield was determined from emission and excitation spectra using FluorEssence 3.5 software. Lifetime was calculated from fluorescence decay profile which was established using excitation nano-led diode (380 nm). The emission was detected at 470 nm and 480 nm for BV and BV/Cu system, respectively. The decay was fitted using 3 exponentials.

**$^1$H NMR Spectroscopy**

$^1$H NMR spectra of BV (0.3 mM) in the absence or in the presence of CuCl$_2$ (0.3 mM) were recorded on a Bruker Avance III 500 spectrometer with TopSpin v3.2 interface, using 5 mm BBO probe-head, at 298 K. All samples (and solutions) were prepared in D$_2$O/DMSO-d$_6$ and placed in 5-mm quartz tubes. Residual HD signal at 4.7 ppm was used as chemical shift reference. Spectra were analyzed in MestReNova 12.0.1 (Mestrelab Research, Santiago de Compostela, Spain).

**EPR Spectroscopy**

Perpendicular-mode EPR spectra at 30 K were recorded on a Bruker Elexys-111 EPR spectrometer operating at X-band (9.616 GHz), using the following conditions: power, 32 dB; modulation amplitude, 0.8 mT; modulation frequency, 100 kHz. Parallel-mode signals were obtained at 25.5 K, using a Bruker EMXplus spectrometer operating at X-band (9.272 GHz) with the dual-mode cavity, and the following settings: power, 16 or 32 dB; modulation amplitude, 1 mT; modulation frequency, 20 kHz. Samples were placed in quartz EPR tubes and quickly frozen in cold isopentane following 5 min incubation period under anaerobic conditions. All spectra were non-saturated, baseline corrected, and showed no signs of freezing-induced sample inhomogeneity. Measurements at room T were conducted on a Varian E104 operating at X-band (9.51 GHz), using the following settings: microwave power, 20 mW; modulation amplitude, 0.2 mT; modulation frequency, 100 kHz. Spectra were obtained under aerobic conditions. The simulation of the room T EPR spectrum was performed in WINEPR SimFonia software (Bruker Analytische Messtechnik GmbH, Darmstadt, Germany), using previously described parameters.

**Oximetry**

Concentration of O$_2$ in samples was measured/monitored using a Clark type oxygen electrode (Hansatech Instruments Ltd., King’s Lynn, UK) operating with Lab Pro interface and Logger Pro 3 software (Vernier, Beaverton, OR, USA). All systems were recorded for 2-5 min before the addition of Cu to establish the stability of baseline and zero rate of O$_2$ change at room T.

**Cyclic voltammetry and differential pulse voltammetry**

The voltammetric measurements were performed using a potentiostat/galvanostat CHI 760b (CH Instruments, Inc, Austin, TX, USA). The electrochemical cell (total volume of 3 mL) was equipped with a boron-doped diamond electrode (surface area 7.07 mm$^2$; Windsor Scientific LTD, UK; declared performances: resistivity, 0.075 Ω cm; boron doping level,
1000 ppm), as the working electrode, an Ag/AgCl (3M KCl) and platinum wire as reference and counter electrode, respectively. All potentials reported in this paper are referred versus this electrode. Scan rate was 0.1 V/s. Differential pulse voltammetry was performed on the same instrument using the following settings: initial E, -1 V; final E, 1 V; increment, 0.004 V; amplitude, 0.05 V; pulse width, 0.05 s; sample width, 0.01 s; quiet time, 2 s. Measurements were initiated immediately after sample preparation and performed at room T.

Conflicts of interest
There are no conflicts to declare.

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Notes and references
Table of content: In physiological settings, biliverdin and Cu\(^{2+}\) build a paramagnetic complex with formal structure: radical cation/Cu\(^{1+}\) or radical anion/Cu\(^{3+}\).