J. Braz. Chem. Soc., Vol. 20, No. 7, 1302-1312, 2009.
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0103 - 5053 $6.00+0.00

Article

Oxidative DNA Damage Induced by S(IV) in the Presence of Cu(II) and Cu(I) Complexes

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O dano ao DNA induzido por S(IV) na presença de alguns complexos de Cu(II) em soluções saturadas com ar foi investigado. A adição de S(IV) a uma solução saturada com ar contendo $Cu^{II}GGA$ (GGA = glicilglicil-L-alanina), $Cu^{II}G_3$ (G_3 = triglicina) ou $Cu^{II}G_4$ (G_4 = tetraglicina) e traços de Ni(II) origina a formação rápida do respectivo complexo de Cu(III), com o simultâneo consumo de oxigênio e a oxidação de S(IV). $SO_3^{\bullet-}$ e $HO^{\bullet}$ foram detectados por experimentos de EPR-spin trapping. As quebras das fitas de DNA foram atribuídas aos radicais de óxido de enxofre formados. Na redução de Cu(II)/BCA (BCA = 4,4' dicarboxi-2-2'-biquinolina) por S(IV), com a formação do complexo $Cu^{I}BCA$, há a possível formação de um radical centrado em carbono do BCA ou um radical peróxido ($ROO^{\bullet}$), capazes de oxidar as bases de DNA. A intensidade do dano ao DNA na presença desses complexos de Cu(II) e S(IV) (10-300 μmol L^{-1}) seguiu a ordem: $Cu^{II}BCA \sim Cu^{II}G_4 \sim$ Cu(II) (adicionado como $Cu(NO_3)_2$) > $Cu^{II}G_3 \sim Cu^{II}GGA$. Especialmente para o $Cu^{II}BCA$, o dano ocorreu mesmo em concentrações baixas de S(IV) (0,1 μmol L^{-1}). Para os complexos de Cu(II) com glicilglicilhistidina, glicilhistidilglicina, glicilhistidillisina e glicilgliciltirosilarginina a formação de Cu(III) e o dano do DNA não foram observados.

The DNA damage induced by S(IV) in the presence of some Cu(II) complexes in air saturated solution was investigated. The addition of S(IV) to an air saturated solution containing $Cu^{II}GGA$ (GGA = glycylglycyl-L-alanine), $Cu^{II}G_3$ (G_3 = triglycine) or $Cu^{II}G_4$ (G_4 = tetraglycine) and Ni(II) traces, causes rapid formation of the respective Cu(III) complex, with simultaneous O_2 uptake and S(IV) oxidation. $SO_3^{\bullet-}$ and $HO^{\bullet}$ were detected by EPR-spin trapping experiments. The DNA strand breaks were attributed to the oxysulfur radicals formed. In the reduction of Cu(II)/BCA (BCA = 4,4' dicarboxy-2-2'-biquinoline) by S(IV), with $Cu^{I}BCA$ complex formation, there is the possible formation of carbon centered radical of BCA or peroxyl radical ($ROO^{\bullet}$) capable of oxidizing DNA bases. The intensity of DNA damage in the presence of these Cu(II) complexes and S(IV) (10-300 μmol L^{-1}) followed the order: $Cu^{II}BCA \sim Cu^{II}G_4 \sim$ Cu(II) (added as $Cu(NO_3)_2$) > $Cu^{II}G_3 \sim Cu^{II}GGA$. Specifically for $Cu^{II}BCA$ the damage occurred even at lower S(IV) concentration (0.1 μmol L^{-1}). For the Cu(II) complexes with glycylglycylhistidine, glycylhistidylglycine, glycylhistidyllysine and glycylglycyltyrosylarginine the Cu(III) formation and the DNA damage was not observed.

Keywords: DNA damage, copper complexes, Cu(III), Cu(I), sulfite

Introduction

Copper peptide complexes, with low reduction potentials and high stability in aqueous solution, are of special interest in biological redox processes[1] due to the probable participation of Cu(III) in the activity of some enzymes and as an intermediate in the enzymatic DNA cleavage mediated by metalloproteins.

Most of the studies on DNA damage involving Cu(II) complexes were carried out in the presence of hydrogen peroxide and ascorbic acid.[2-6] In these studies the generation of reactive oxygen species in a Fenton type mechanism was proposed, where Cu(II) is reduced to Cu(I) which reacts with H_2O_2 to generate $HO^{\bullet}$. However, the intermediate that causes DNA cleavage has not been identified.

Nowadays it is widely known that S(IV) (SO_2, HSO_3^- and SO_3^{2-}) autoxidation is catalyzed by transition metal

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ions, such as Cu(II), Ni(II), Mn(II) and Co(II), where free oxy sulfur radicals ($SO_3^{\bullet-}$, $SO_4^{\bullet-}$ or $SO_5^{\bullet-}$) and $HO^{\bullet}$ are formed as intermediates.[7-18] These radicals might cause DNA damage, as already described in our previous work.[19-22]

The literature reports only few studies on DNA damage mediated by Cu(II) (added as a free ion or complex) in the presence of S(IV) and dissolved oxygen.[19-24] Previously, we showed that the oxidation of S(IV) in air saturated solution ($[O_2]$ = 0.25 mmol L^{-1}, pH 7), in the presence of the $Cu^{II}G_4$ (G_4 = tetraglycine) complex, occurs with $Cu^{III}G_4$ formation and oxygen consumption.[13,14,16,17] In addition, we verified that $Cu^{II}G_4$ complexes interact with DNA producing strand breaks in significant yields in the presence of S(IV) without or with a second metal ion (traces of Ni(II)).[22] $Cu^{II}G_4$ alone induced little or no DNA damage in the absence of S(IV).[19,21] The oxidation of 2´deoxyguanosine to 8-oxodGuo and both single and double-strand breaks in DNA were observed under the same conditions.[19,21]

In the present work, the DNA damage induced by S(IV) in the presence of some Cu(II) peptide complexes in air saturated solution was investigated. Cu(II) peptide complexes can be oxidized to Cu(III) species, as demonstrated by Margerum and co-workers.[25-27] The peptides: glycylglycylhistidine (GGH), glycylhistidyllysine (GHK), glycylglycyltyrosylarginine (GGYR), glycylhistidylglycine (GHG), glycylglycyl-L-alanine (GGA), triglycine (G_3) and tetraglycine (G_4) were selected for this study in order to evaluate the probable role of trivalent copper in the DNA damage mechanism.

Due to the different protonation degree of the coordinated ligand, in this study the representations $Cu^{II}L$ and $Cu^{III}L$ refer to all complexes species present in solution (at pH 7.5) formed with the metal ions.[28,29]

In addition, DNA damage was also investigated in the presence of Cu(II) and BCA (4,4' dicarboxy-2-2'-biquinoline), a specific chelator for Cu(I). It was observed that Cu(II) is reduced by S(IV) to form the Cu(I)/BCA complex.[30]

Experimental

Reagents

All reagents were of analytical grade. All solutions were prepared by using deionized water purified with a Milli-Q Plus Water System (Millipore).

5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), Chelex 100 chelating resin, the peptides (GGH, GHK, GGYR, GHG, GGA, G_3 and G_4), 4,4' dicarboxy-2-2'-biquinoline disodium salt (Na_2BCA), ethidium bromide, Ficoll type 400, bromophenol blue and the reagents used for gel electrophoresis were obtained from Sigma.

Supercoiled pUC-19 DNA and electrophoresis grade agarose were purchased from MBI Fermentas.

Stock solutions of S(IV) (0.010 mol L^{-1}, Merck) were fresh prepared by dissolving $Na_2S_2O_5$ salt in water previously purged with nitrogen. Deionised water was flushed with nitrogen for at least half an hour to remove dissolved oxygen. To prepare diluted solutions of S(IV), small volumes of the stock solutions were added to air saturated water.

Cu(II) and Ni(II) (0.2 mol L^{-1}) stock solutions were prepared from the direct reaction of Cu (wire, 99.99%) and Ni (powder, 99.99%) with double distilled nitric acid followed by standardization with EDTA by a conventional procedure.[31]

In the experiments fresh Cu(II) complex solutions were prepared by dissolving the appropriate peptide in water (pH 7.5) followed by the addition of Cu(II) solution (solutions were prepared to have 10% excess of peptide to restrain any $Cu(OH)_2$ precipitation). In some experiments, aliquots of Ni(II) solution was added to Cu(II) complex solution in order to study the synergistic effect. The final pH was adjusted with 0.1 mol L^{-1} NaOH or 0.1 mol L^{-1} $HClO_4$ solutions. The ionic strength, *I*, was kept at 0.05 mol L^{-1} with $NaClO_4$ only for the spectrophotometric studies.

0.2 mol L^{-1} BCA solution was prepared by dissolution of Na_2BCA in water. Cu^{II}BCA solutions was prepared few minutes before the experiments by mixing $Cu(NO_3)_2$ and BCA solutions, such as the final working solution was 3 mmol L^{-1} BCA and 1 mmol L^{-1} Cu(II). As Cu^{II}BCA precipitates after a few minutes, the solution must be freshly prepared followed by the fast addition of S(IV). Diluted solution of Cu^{II}BCA was employed in gel electrophoresis experiments (see Figure 2).

Air saturated solutions were employed in all the experiments for which the dissolved oxygen concentration can be considered to be 0.25 mmol L^{-1}. A pH meter Metrohm 713 with a glass electrode (filled with sat. NaCl) was used in the pH measurements.

Spectrophotometric measurements

Equals volumes of S(IV) and metal ion complex solutions were mixed. The concentrations of each reagent just after the mixture are indicated in the Figure 1.

The kinetic runs were followed at the wavelength of maximum absorption of each Cu(III) complex by using a HP8453A diode array spectrophotometer coupled to a Pro-K.2000 Stopped-Flow Mixing Accessory (Applied Photophysics).

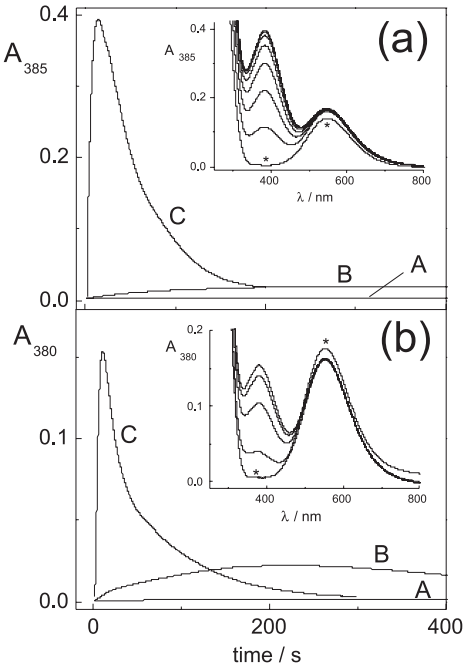


Figure 1. Absorbance changes after mixture of air saturated solutions of (a) $Cu^{II}GGA$ or (b) $Cu^{II}G_3$ and S(IV). $[Cu(II)]_{Total}$ = 1.0 mmol L^{-1}; [Peptide] = 1.1 mmol L^{-1}; I = 0.1 mol L^{-1} ($NaClO_4$); pH = 7.5; [S(IV)]: (A) Zero, (B) 100 μmol L^{-1}, (C) 100 μmol L^{-1} + Ni(II) 10 μmol L^{-1}. T = 25.0 °C. Inset figure: Sucessive spectra after addition of 100 μmol L^{-1} S(IV) to air saturated solutions containing (a) $Cu^{II}GGA$ 1.0 mmol L^{-1} / Ni(II) 10 μmol L^{-1} (every 2 s) or (b) $Cu^{II}G_3$ 1.0 mmol L^{-1} / Ni(II) 10 μmol L^{-1} (every 1 s); the spectra (*) are before the addition of S(IV).

Gel electrophoresis experiments

The DNA strand break efficiencies induced by the copper complexes, in the presence or absence of S(IV), were determined by mixing the Cu(II) complex solution with 100 ng of pUC 19 plasmid DNA followed by the addition of S(IV) in a total volume of 50 μL. The final concentrations after mixing are indicated in Figures 2 and 3.

The separation of the different conformations of pUC 19 plasmid DNA (supercoiled, open circular and linear) was performed by gel electrophoresis, using 0.8% agarose/1.8 μmol L^{-1} ethidium bromide in a horizontal gel electrophoresis chamber at 30 mA for 120 min in 90 mmol L^{-1} tris-borate /2 mmol L^{-1} EDTA buffer (pH 8.0). The bands were visualized under UV light and quantified with the ImageMaster VDS densitometer (Pharmacia Biotech, San Francisco, CA, USA).

EPR experiments

Direct EPR of copper(II) complexes

EPR spectra were recorded in a Bruker EMX EPR (Electron Paramagnetic Resonance) spectrometer equipped with a standard cavity, operating at X-band frequency, using standard Wilmad quartz tubes, at −196 ºC or at

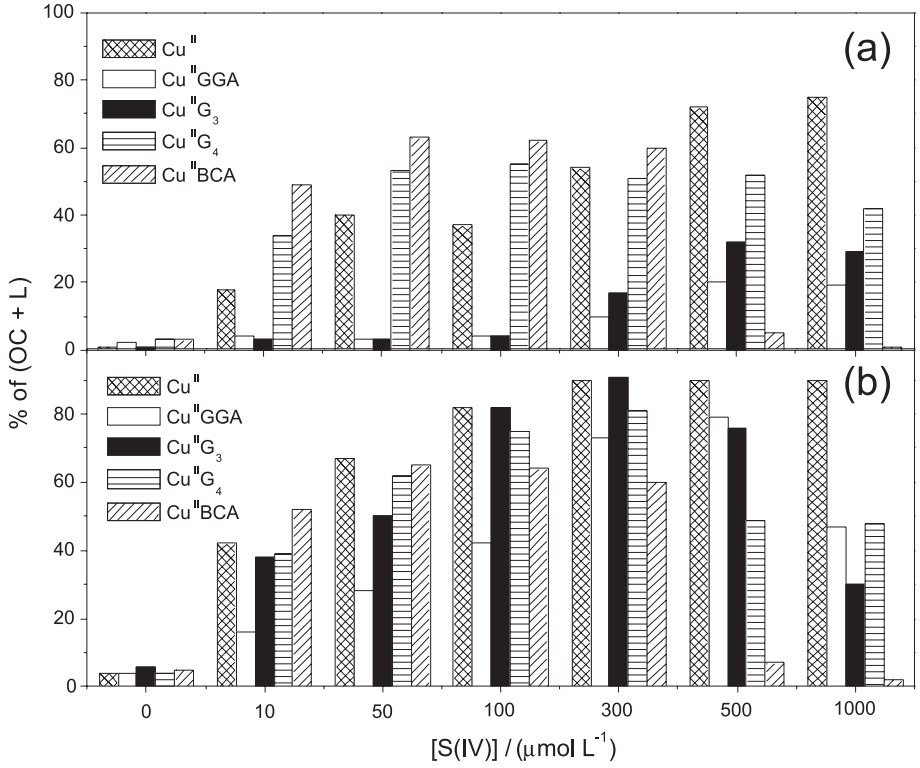
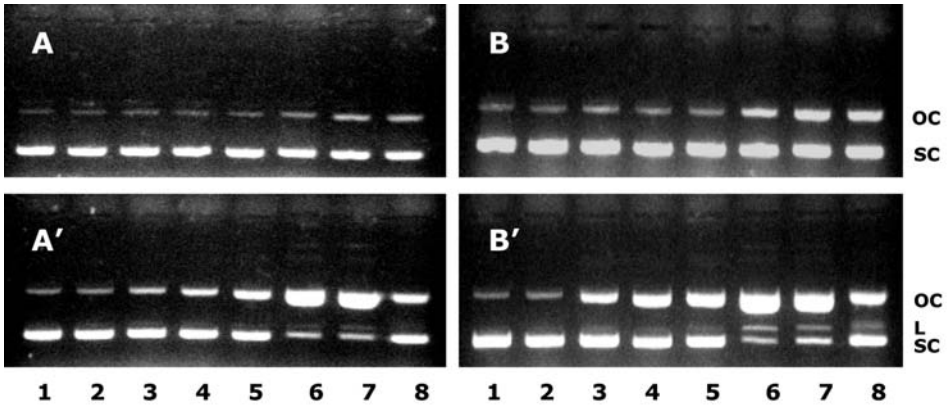


Figure 2. Total percentages of the amount of damaged DNA (OC + L) upon DNA treatment with 50 μmol L^{-1} Cu(II) complexes and different concentrations of S(IV). (a) No incubation (T = 25 ºC) and (b) incubation for 2 h (T = 37 ºC). [pUC19] = 5 μg mL^{-1}, pH 7.2, air saturated solutions. The data points represent the average of three trials (limit of error between 2 and 13%).



[S(IV)] / (μmol L^{-1})	% total DNA strand breaks			
	$Cu^{II}GGA$		$Cu^{II}GGA$ + Ni(II)	
	No incubation A	Incubation A'	No incubation B	Incubation B'
Lane 2: zero	2.0 ± 0.1	4 ± 0.3	2.0 ± 0.4	3.0 ± 0.2
Lane 3: 10	4.0 ± 0.3	16 ± 2	6.0 ± 0.3	39 ± 4
Lane 4: 50	3.0 ± 0.2	28 ± 3	5.0 ± 0.3	50 ± 3
Lane 5:100	4.0 ± 0.2	42 ± 4	8.0 ± 0.2	52 ± 4
Lane 6: 300	10 ± 1	73 ± 3	27 ± 3	79 ± 2
Lane 7: 500	20 ± 1	79 ± 4	35 ± 4	78 ± 3
Lane 8: 1000	19 ± 2	47 ± 2	37 ± 3	49 ± 3

Figure 3. Formation of DNA strand breaks upon exposure of plasmid pUC 19 to $Cu^{II}GGA$ and different concentrations of S(IV) with (A) no incubation (T = 25 °C) and (A') incubation for 2 h (T = 37 °C). (B) and (B'): results obtained without and with incubation respectively in medium containing 50 μmol L^{-1} $Cu^{II}GGA$ / 0.5 μmol L^{-1} Ni(II) and different concentrations of S(IV). [pUC19] = 5 μg mL^{-1}; pH 7.5; air saturated solutions. % DNA strand breaks represent the sum of OC and L forms. The percentages are the average of three trials. Lane 1: DNA alone.

room temperature using a flat quartz cell. DPPH (α,α´-diphenyl-β-picrylhydrazyl) was used as frequency calibrant (g = 2.0036) with samples in frozen aqueous solution, at −196 °C. Usual conditions used in these measurements were 10 G modulation amplitude and 50 mW power (spectra at room temperature) or 20 mW power (at −196 °C).

EPR spin trapping experiments

EPR spectra were recorded at room temperature (22 ± 2 °C) on a Bruker EMX EPR spectrometer equipped with a standard cavity, operating at X-band frequency, using standard flat quartz cell. Instrumental conditions were usually 2.00 × 10^4 gain, 1 G modulation amplitude and resolution of 1024 points. The magnetic field was calibrated with 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPOL, *g* = 2.0056).

In a typical spin trapping experiment, a solution of copper complex was previously mixed with DMPO (2,2'-dimethyl-pyrroline-N-oxide, from Aldrich) followed by the addition of S(IV) solution, and 200 μL of the mixture was transferred to a flat quartz cell and the EPR spectra recorded during the time. The concentrations of the final solutions are indicated in Figures 4 and 5.

The working solutions (at pH 7.5) were treated with Chelex 100 to remove metal ion contaminants. DMPO was vacuum distilled previously to use.[32] The stock aqueous solution of DMPO 2 mol L^{-1} was maintained at 6 °C.

Results and Discussion

The DNA damage was investigated in the presence of Cu(II) complexes (with GHK, GGYR, GGH, GHG, GGA, G_3, G_4, and BCA), S(IV) and dissolved oxygen. The ability of each complex to cleave DNA was verified by gel electrophoresis experiments, as will be further discussed. DNA strand breaks occurred with high efficiency only in the presence of free Cu(II), $Cu^{II}GGA$, $Cu^{II}G_3$, $Cu^{II}G_4$ or $Cu^{II}BCA$ and dissolved oxygen after the addition of S(IV). Therefore, to gain a better understanding of the reactions involved, most of the EPR and UV-Vis experiments were carried out with these complexes.

Spectrophotometric studies of the oxidation of Cu(II) complexes in the presence of S(IV) and dissolved oxygen

The addition of S(IV) (a reducing agent) to an air saturated solution containing $Cu^{II}GGA$, $Cu^{II}G_3$ or $Cu^{II}G_4$

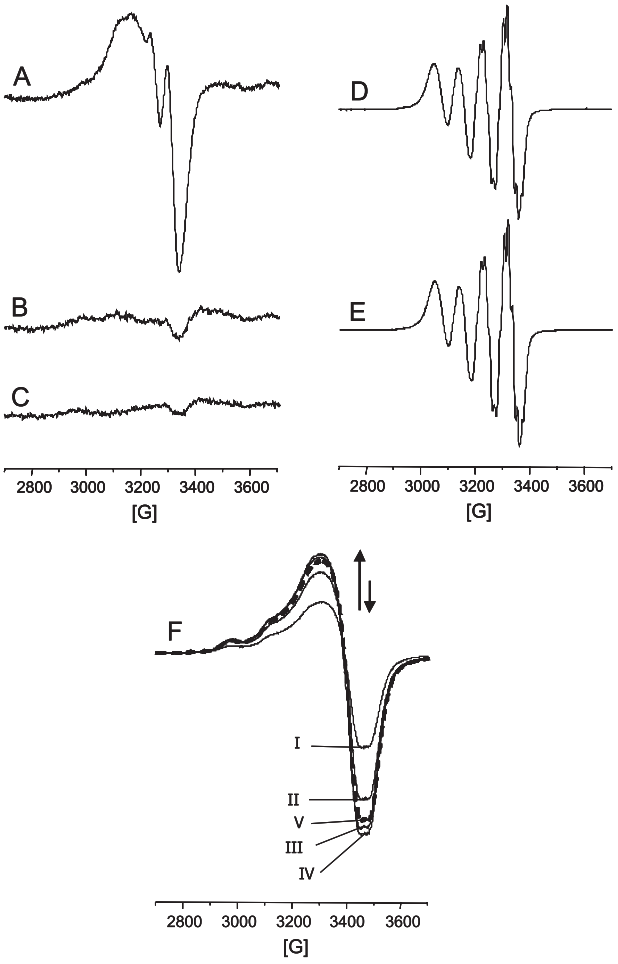


Figure 4. EPR spectra at room temperature of (A) Cu^{II}BCA, (B) Cu^{II}BCA immediately after the addition of 1 mmol L^{-1} S(IV), (C) the same of (B), after 2h, (D) Cu^{II}GGA and (E) Cu^{II}GGA after 2 min of the addition of S(IV) 0.5 mmol L^{-1}. (F) EPR spectra at −196 ºC (in liquid nitrogen) of the complex Cu^{II}GGA (containing 50 μmol L^{-1} Ni(II)) (I), and after the addition of S(IV): 10 s (II), 1 min (III), 3 min (IV) and 6 min (V). $[Cu(II)]_T$ = 1 mmol L^{-1} and [BCA] = 3 mmol L^{-1} for A, B and C; $[Cu(II)]_T$ = 5 mmol L^{-1} and [GGA] = 5.5mmol L^{-1} or $[G_3]$ = 6.5 mmol L^{-1} for D, E and F; pH 7.5; T = (22±1) °C. Spectra parameters: Power 50 mW (A, B, C, D and E) and 20 mW (F); Modulation amplitude 10 G; Time constant 40.96 ms; Gain 5.0 × 10^4 (A, B and C), 1.0 × 10^4 (D and E) and 4.5 × 10^3 (F).

and Ni(II) traces, originates in the rapid formation of the respective Cu(III) complexes, with simultaneous O_2 uptake and S(IV) oxidation.

According to Anast and Margerum,[27] the oxidation of $Cu^{II}G_4$ to $Cu^{III}G_4$ in aqueous medium, by dissolved oxygen, is strongly accelerated in the presence of S(IV) with simultaneous oxidation of Cu(II) and S(IV). However, our studies[12-14] showed that in fact, this reaction is very slow in the presence of S(IV). The $Cu^{III}G_4$ formation is efficient and fast only in the presence of S(IV) and trace concentrations of nickel(II) ion, present as impurity in the copper (II) salts ($CuClO_4$, Sigma) reagents. These studies were carried out at pH 9.

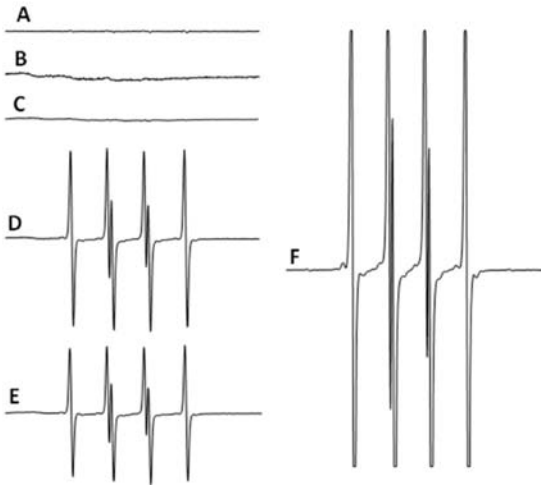


Figure 5. EPR spectra of DMPO radical adducts recorded 1 min after mixing 100 mmol L^{-1} DMPO (pH 7.5) with: (A) S(IV), (B) Cu^{II}GGA, (C) $Cu^{II}G_3$, (D) S(IV) + Cu^{II}GGA, (E) S(IV) + $Cu^{II}G_3$, and (F) S(IV) + Cu(II). $[Cu(II)]_T$ = 0.5 mmol L^{-1}; [GGA] or $[G_3]$ = 0.55 mmol L^{-1}, [S(IV)] = 0.5 mmol L^{-1}. Spectra conditions: power 20 mW; modulation amplitude 1 G; time constant 81.92 ms; Gain 2.0 × 10^4.

In our previous work[12-14] the oxidation of $Cu^{II}G_4$ induced by S(IV) at pH 7, in the presence and absence of Ni(II), was described. The oxidation of 1 mmol L^{-1} $Cu^{II}G_4$, after addition of S(IV), is relatively slow with an induction period, characteristic of autocatalytic reactions. In the presence of 10 μmol L^{-1} Ni(II), the induction period decreases with a slight increase in the rate and effectiveness of $Cu^{III}G_4$ formation, which could be followed by its characteristic absorbance peak at 365 nm.[13,14,26,27] The effectiveness of the $Cu^{III}G_4$ formation and the synergistic effect of Ni(II) were more pronounced at pH 9, which can be explained by the different reactivity of $Cu^{II}G_4$ complexes due to the different protonation degrees of the coordinated ligand.[28,33]

In the present work, similar experiments were carried out focusing on the oxidation of Cu(II) complexes with GGA, G_3, GGH, GHG, GHK and GGYR.

The spectral changing of an air saturated solution of Cu^{II}GGA 1.0 mmol L^{-1} and Ni(II) 10 μmol L^{-1} at pH 7.5 after S(IV) addition are shown in Figure 1a inset, with solution colors changing from purple to yellow. The two new peaks at 250 (not shown) and 385 nm are attributed to Cu^{III}GGA formation. The absorbance changes at 385 nm (Figure 1a), can be followed since the absorbance of Ni^{II}GGA 10 μmol L^{-1} does not interfere.[20] The oxidation of Cu^{II}GGA is not efficient in the absence of traces of Ni(II) ion (Figure 1a, B). However. the synergistic effect of this second metal ion can be seen by the fast Cu^{III}GGA formation (Figure 1a, C), followed by its decomposition with probable ligand oxidation by Cu(III). The absorbance of Cu^{II}GGA (555 nm), of the final solution, is about the same, showing that only a small percentage of the initial Cu^{II}GGA was consumed.

The same behavior was observed for the oxidation of $Cu^{II}G_3$ under similar experimental conditions (Figure 1b). The existence of $Cu^{III}G_3$ was previously confirmed by electrochemical experiments.[34]

Under the same experimental conditions the addition of S(IV) to air saturated solutions of Cu^{II}GHG, Cu^{II}GHK, Cu^{II}GGYR and Cu^{II}GGH (in the absence or presence of Ni(II) traces) led to no spectral changes showing that the respective Cu(III) complexes, if formed, were not at detectable amounts.

In order to better evaluate the oxidation of Cu^{II}GGA and $Cu^{II}G_3$ complexes the S(IV) (reducing agent) was replaced by HSO_5^- (strong oxidant) (data not shown). In the presence of Ni(II) and HSO_5^- the oxidation of Cu^{II}GGA is more efficent than with S(IV), with the appearance of the same absorption peak at 385 nm. After the decomposition of these unstable intermediates , the peak at 555 nm (Cu^{II}GGA) shifts to 575 nm, problably due to the formation of a new complex Cu^{II}GGA**'** (where GGA**'** is an oxidized form of the ligand).

On the other hand, the addition of S(IV) to a solution containing Cu(II) and BCA, the involved mechanism is very different. Cu(II) is reduced, in less than 1 minute, to the stable Cu^{I}BCA complex, which has two absorption peaks 558 nm (weak) and 330 nm (intense).[35]

Agarose gel electrophoresis experiments. DNA damage

Agarose gel electrophoresis was carried out using pUC-19 plasmid DNA in the presence of air-saturated solutions of copper(II) complexes (with GGH, GHK, GGA, GHG, GGYR, G_3, G_4 and BCA) and S(IV) to provide evidence of DNA strand breaks. The effect of incubation time and the synergistic effect of Ni(II) were also evaluated.

The ability of each copper complex to cleave DNA was verified through the conversion of the supercoiled form of pUC-19 plasmid DNA (SC, native conformation) to open circular (OC) and linear (L) forms. DNA damage was quantified based on the ratio of the total amount of OC and L forms produced (normalized with respect to the background produced by DNA alone) to the total amount of DNA present. We observed that the order of addition of the reagents is very important in the extension of the damage. The higher ratio of (OC + L) **:** (SC) percentage was observed when the order of addition of the reagents was: DNA + Cu^{II}complexes (or $Cu(NO_3)_2$) + S(IV).

In the present study, no DNA damage was observed in the presence of S(IV) alone (1-2000 μmol L^{-1}) even after incubation with DNA for 2 h.

When the experiments were carried without incubation, Cu(II) ion (1-500 μmol L^{-1}), added as $Cu(NO_3)_2$, little or no damage occurred in the absence or presence of S(IV) (lower than 10 μmol L^{-1}). However, in the presence of higher concentrations of S(IV) (10-500 μmol L^{-1}) the formation of the OC form was observed. With incubation for 2 h (37 °C) the DNA strand breaks were more efficient and the linear form was also observed. SC form became quantitatively converted to OC and L forms when S(IV) was used in the range 300-1000 μmol L^{-1} (Figure 2b). Therefore, the extent of DNA damage depends on the incubation and S(IV) and Cu(II) concentrations. In addition, an optimum condition to induce 100% DNA damage may be 300 μmol L^{-1} S(IV) and 50 μmol L^{-1} Cu(II) (Figure 2b, with incubation).

Figure 2a shows that considerable DNA damage (only with formation of OC form) also occurs when the experiments were carried out without incubation in the presence of Cu(II) complexes over the entire S(IV) concentration range. The intensity of DNA damage in the presence of the different Cu(II) complexes and for S(IV) (10-300 μmol L^{-1}) followed the order: Cu^{II}BCA ~ $Cu^{II}G_4$ ~ Cu(II) > $Cu^{II}G_3$ ~ Cu^{II}GGA. Specifically for Cu^{II}BCA the damage occurred even at a lower S(IV) concentration (0.1 μmol L^{-1}, data not shown).

By comparing Figure 2a and b, one can conclude that the damage in the presence of Cu^{II}BCA did not depend on the incubation time (for 2 h at 37 °C), which indicates that the species responsible for the DNA damage is produced at the initial stage of the reduction reaction of Cu^{II}BCA by S(IV). It is important to mention that no DNA damage was observed when the order of addition of the reagents was Cu^{II}BCA + S(IV) + DNA, such as Cu^{I}BCA was formed before DNA addition, which shows that Cu^{I}BCA alone does not induce DNA damage.

DNA damage increased with incubation time in the presence of S(IV) and Cu(II), $Cu^{II}G_4$, especially with $Cu^{II}G_3$ and Cu^{II}GGA. For the Cu(II) peptide complexes it can be related to the rate of the oxidation of the Cu(II) complex induced by S(IV) in the presence of dissolved oxygen, as shown by the spectrophotometric data (Figure 1) and previous work.[12,16,19,21,34]

Due to the synergistic effect of Ni(II) on the oxidation of $Cu^{II}G_4$ in the presence of S(IV) and dissolved oxygen,[12,16,17,21] some experiments were carried out to evaluate the DNA strand breakage as shown in Figure 3.

After the treatment of DNA with 50 μmol L^{-1} Cu^{II}GGA and different concentrations of S(IV), in the presence and absence of 0.5 μmol L^{-1} Ni(II), the results showed that DNA strand breakage increased with S(IV) concentration (up to 500 μmol L^{-1}) and incubation time. In the presence of traces of Ni(II) the increment was higher especially at lower S(IV) concentrations (10-100 μmol L^{-1}).

EPR experiments

Direct EPR of copper complexes

In order to verify the variation of the oxidation state of copper(II) complexes in the presence of S(IV) and dissolved oxygen, direct EPR studies were performed at room temperature.

The EPR spectra of the complex Cu^{II}BCA is represented in Figure 4A-C. The EPR signal characteristic of Cu(II) d^9 paramagnetic environment (Figure 4A, before S(IV) addition) disappeared after S(IV) addition (Figure 4B), remaining as a stable copper(I) species (diamagnetic) for 2 h. These results are in agreement with the spectrophotometric ones.

Regarding the Cu^{II}GGA complex, there is no report on the formation of Cu(III) species in the literature. EPR spectra of Cu^{II}GGA solution before (Figure 4D) and after (Figure 4E) S(IV) addition at room temperature showed no changes, indicating that Cu(II) with GGA remains relatively stable after S(IV) addition, in the presence or absence of Ni(II).

In order to verify slight changes in the intensity of Cu(II) signal (in CuGGA complex) during the S(IV) reaction, low temperature EPR spectra were performed with time (Figure 4F). A contineous increase in the EPR Cu(II) signal up to 3 min, with a decay after 5 min was observed. This EPR spectrum of Cu^{II}GGA showed, even after the addition of S(IV), a characteristic profile of an axial environment around copper(II) ion[36] with EPR hyperfine parameter $g_{//} > g_{\perp}$. No change in the oxidation state of Cu^{II}GGA was verified. The increase in the intensity of the EPR signal in the first minutes of reaction indicated an increase of Cu(II) moiety in solution, however, the signal shape did not change, signifying that a similar environment around the copper(II) (in the complex that originates such increase) was maintained during the reaction. The increase in the intensity of EPR signal was accompanied by a color change of the solution from purple to yellow, as described in the spectrophotometric studies (Figure 1a), indicating that the species that produces such an increase is the same as that absorbing at 385 nm. The reduction of the EPR signal (after 5 min), also accompanied by a color change from yellow to purple, indicates that the concentration of Cu(II) complex decreases probably due to either return to its initial form (Cu^{II}GGA) or decomposition via ligand oxidation. The same experiments, carried out in the absence of Ni(II), showed similar results.

All facts appear to indicate that the copper GGA complexes are initially present mainly as Cu^{II}GGA and may be some small amounts as Cu^{III}GGA and/or Cu^{I}GGA, formed by oxidation of Cu^{II}GGA by dissolved oxygen (equation 4) or disproportionation of this complex (equation 3), respectively. After the S(IV) addition, a small amount of Cu^{III}GGA should be initially reduced (equation 5), by this way increasing the total amount of the Cu(II) complex, witch may be also formed by the oxidation of the initial Cu^{I}GGA if it exists.

Very similar results were obtained using the $Cu^{II}G_3$ complex (data not shown).

EPR spin trapping

To verify the formation of free radicals during the oxidation of S(IV) in the presence of dissolved oxygen and Cu^{II}BCA, Cu^{II}GGA or $Cu^{II}G_3$, EPR spin trapping experiments were carried out using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) which forms the DMPO/$SO_3^{\bullet -}$ [37] and DMPO/$HO^{\bullet}$ [38] spin adducts in the presence of sulfite and hydroxyl radicals, respectively.

Air saturated solutions containing only 0.5 mmol L^{-1} S(IV) or Cu^{II}GGA or $Cu^{II}G_3$ generated no EPR signal with DMPO (Figure 5A, B and C, respectively).

The EPR spectra obtained from S(IV) oxidation in the presence of Cu^{II}GGA or $Cu^{II}G_3$ are shown in Figure 5D and E, respectively. Both spectra showed the presence of the DMPO/$SO_3^{\bullet -}$ spin adduct, with a_N = 14.6 G and a_H = 16.0 G.[19,37] For both complexes, the signal intensity reached saturation after 6 minutes of reaction followed by a slow decay, indicating that the spin adduct is not stable over longer time under these experimental conditions. In the presence of $Cu^{II}G_3$ (0.5 mmol L^{-1}) and Ni(II) (5 μmol L^{-1}), sulfite radical generation was more efficient than with Cu^{II}GGA (0.5 mmol L^{-1}) and Ni(II) (5 μmol L^{-1}) (data not shown).

Copper(II) nitrate was used instead of copper(II) peptide to compare the sulfite radical generation (Figure 5F). The DMPO/$SO_3^{\bullet -}$ spin adduct signal was more intense than with copper peptide complexes (Figure 5F), showing that free copper(II) ions enhanced the sulfite radical production in the medium.

The addition of DMPO to the Cu^{II}BCA solution (in the absence of S(IV)) generated the radical adduct DMPO/$HO^{\bullet}$ (data not shown), suggesting that DMPO was oxidized to its radical $DMPO^{\bullet +}$ which could undergo addition of water to yield DMPO/$HO^{\bullet}$ (equations 1 and 2),[39] with $a_N = a_H$ = 14.9.

$$Cu^{II}BCA + DMPO \rightarrow Cu^{I}BCA + DMPO^{\bullet +} \quad (1)$$

$$DMPO^{\bullet +} + H_2O \rightarrow DMPO/HO^{\bullet} + H^{+} \quad (2)$$

The Cu^{II}BCA complex in the presence of S(IV) and DMPO also did not generate sulfite radicals, but the radical adducts DMPO/$HO^{\bullet}$ (*via* equations 1 and 2) and DMPOX (5,5-dimethylpyrrolidone-(2)-oxy-(1), with a_N = 7.1 G and

a_H = 4.2 G) were detected (data not shown). So, in this case, a strong oxidant capable of oxidizing DMPO (1.87 V *vs.* NHE) must be formed.[40] In addition, there is a possible formation of a carbon centered radical of BCA not detected in this case. According to Rosen and Rauckman,[41] the DMPOX signal is an indirect evidence for peroxyl radical ($ROO^{\bullet}$) formation, which is trapped by DMPO.

Taken together, these results proved that the mechanism of free radical generation and consequently DNA damage caused by sulfite oxidation depends on the type of copper(II) complex used, as will be further discussed.

Mechanism of copper peptides oxidation in the presence of S(IV) and dissolved oxygen

The mechanism involved in the oxidation of the copper peptides in the presence of S(IV) and dissolved oxygen, must be similar to that proposed in our previous studies, involving the redox cycling of the metal ion.[7-21] However, depending on the Cu(II) complex there are some differences related to the products formed (reactive intermediates that could damage the DNA).

Reactions 3-6 (Scheme 1) show the main steps that can be involved in the $Cu^{II}L$ (L = G_3, G_4 or GGA) oxidation in the absence of S(IV). $Cu^{III}L$ can be produced by disproportion of $Cu^{II}L$ to $Cu^{I}L$ and $Cu^{III}L$ (equation 3)[12,27] or by the slow oxidation of $Cu^{II}L$ by oxygen (equation 4) to produce H_2O_2, which can subsequently oxidize $Cu^{II}L$ to $Cu^{III}L$ (equation 5). The $HO^{\bullet}$ produced from the slow $Cu^{II}G_4$ oxidation, in absence of S(IV), can also oxidize $Cu^{II}G_4$ to $Cu^{III}G_4$ (equation 6) at high enough levels to initiate the process. Reactions 3 and 4 are more probable to occur for Cu^{II}GGA and $Cu^{II}G_3$ complexes since $HO^{\bullet}$ radical formation was not observed by EPR. However, reactions 3-6 can occur in the case of $Cu^{II}G_4$ since in the absence of S(IV), $HO^{\bullet}$ was detected.[19,21]

According to the proposed mechanism, in the presence of S(IV), some initial $Cu^{III}L$ is necessary at zero time to initiate the process (the formation of sulfite radical, equation 7)). It could be produced by reactions 3-6 or by low Fe(III) concentration (10^{-8}-10^{-7} mol L^{-1}), present as impurity.[42] Thus, the $Cu^{III}L$ formed reacts with sulfite to generate $SO_3^{\bullet-}$ radical (equation 7). In the autocatalytic process, $Cu^{II}L$ is oxidize by $SO_5^{\bullet-}$ (equation 11), produced by oxidation of $SO_3^{\bullet-}$ by dissolved oxygen (equation 10); HSO_5^- and $SO_4^{\bullet-}$ can also oxidize $Cu^{II}L$ in subsequent steps (equations 13-15). $Cu^{III}L$ can then be reduced by SO_3^{2-} (equation 7) to continue the chain reaction. This redox cycling is active as long as sulfite and oxygen are present in solution to generate the $SO_5^{\bullet-}$, HSO_5^- and $SO_4^{\bullet-}$ species. The $Cu^{III}L$ complex can also decompose to $Cu^{II}L'$ (equation 16, where L' is the ligand in the oxidized form).

Scheme 1. Mechanism of $Cu^{II}L$ (L = G_3, G_4 or GGA) oxidation in the presence of S(IV) and dissolved oxygen. Synergistic effect of Ni(II).[12-14,19,21,34]

Initiation in the absence of S(IV)	
$Cu^{II}L \rightleftharpoons Cu^{III}L + Cu^{I}L$ (L= G_4 ref.12, 27)	(3)
$2Cu^{II}L + O_2 + 2H^+ \rightarrow 2Cu^{III}L + H_2O_2$	(4)
$Cu^{II}L + H_2O_2 \rightarrow Cu^{III}L + HO^{\bullet} + OH^-$ (L = G_4, ref. 16, 19, 21)	(5)
$Cu^{II}L + HO^{\bullet} \rightarrow Cu^{III}L + OH^-$ (L = G_4, ref. 16, 19, 21)	(6)
Initiation in the presence of S(IV) and added Ni(II)	
$Cu^{III}L + SO_3^{2-} \rightarrow Cu^{II}L + SO_3^{\bullet-}$	(7)
$2Ni^{II}L + O_2 + 2H^+ \rightarrow 2Ni^{III}L + H_2O_2$ (faster than equation 4)	(8)
$Ni^{III}L + SO_3^{2-} \rightarrow Ni^{II}L + SO_3^{\bullet-}$ (faster than equation 7)	(9)
Autocatalysis	
$SO_3^{\bullet-} + O_2 \rightarrow SO_5^{\bullet-}$	(10)
$Cu^{II}L + SO_5^{\bullet-} \rightarrow Cu^{III}L + SO_5^{2-}$	(11)
$SO_5^{2-} + H^+ \rightleftharpoons HSO_5^-$ pK = 9.4	(12)
$Cu^{II}L + HSO_5^- \rightarrow Cu^{III}L + SO_4^{\bullet-} + OH^-$	(13)
$Cu^{II}L + HSO_5^- \rightarrow Cu^{III}L + SO_4^{2-} + HO^{\bullet}$	(14)
$Cu^{II}L + SO_4^{\bullet-} \rightarrow Cu^{III}L + SO_4^{2-}$	(15)
$2Cu^{III}L \rightarrow Cu^{II}L + Cu^{II}L'$	(16)
L' is the oxidized form of L	

The synergistic effect of traces of Ni(II) can be explained by the faster oxidation of $Ni^{II}L$ by O_2 (equation 8), producing Ni(III), which rapidly reacts with sulfite to form the $SO_3^{\bullet-}$ radical (equation 9). $Ni^{II}L$ can also be oxidized to $Ni^{III}L$ by $SO_5^{\bullet-}$, SO_5^{2-}, HSO_5^- and $SO_4^{\bullet-}$, and participate in the redox cycling similarly to the Cu(II) reactions (see Scheme 1). The chain propagation, product formation and termination reactions involving sulfite, HSO_5^-, $SO_3^{\bullet-}$ and $SO_5^{\bullet-}$ are already described in the literature.[7-11,13]

Margerum and co-workers have investigated and characterized several Cu(II) and Cu(III) complexes with some peptides with respect to their reactivity, structure and products.[43-47] These studies showed that Cu(III) complexes with tripeptides or tetrapeptides decompose with rapid oxidative degradation of the peptides. In addition, some of the authors[29] suggested Cu(III) as an intermediate in the oxidative degradation and cleavage of DNA in reactions involving Cu^{II}GGHG/H_2O_2/ascorbic acid (GGHG = glycylglycylhistidylglycine).

Our spectrophotometric and EPR studies also suggest the involvement of Cu(III) intermediates, which decompose rapidly during the reaction between $Cu^{II}G_3$ (or Cu^{II}GGA) and S(IV), in the presence of oxygen. The decomposition should regenerate some of the initial Cu(II) complex and partially oxidize the ligand.

After the addition of S(IV) to an air saturated solution of $Cu^{II}G_3$, a characteristic absorption band of $Cu^{III}G_3$ (λ_{max} = 375 nm[34] which rapidly shifts to 385 nm, Figure 1b) was observed, followed by an absorption decrease, indicating that an instable complex is formed. In the case of Cu^{II}GGA, only the absorption band at 385 nm is observed (Figure 1a), which also decays with time. As these

intermediate complexes are instable, they could not be detected by EPR (item "Direct EPR of copper complexes"), which showed no formation of Cu(III). If some Cu(III) is formed during the first second, the life time is so short that it could not be detected by EPR (Figure 4D, E and F).

The increase in the EPR signal after addition of S(IV), (Figure 4F, I-IV) seems to indicate that initially the most of the total Cu(II) is present as Cu^{II}GGA and a small portion forms other species. After addition of S(IV), the species Cu^{III}GGA and/or Cu^{I}GGA could likely form Cu^{II}GGA' (GGA' is the oxidized form of GGA), but always keeping the same coordination geometry. The oxidation of the ligand could occur in some region of the molecule such as the initial coordination to the Cu(II) by the GGA peptide was not modified.

In the case of $Cu^{II}G_4$, EPR studies[21] showed the oxidation of DMPO by some species resulting from the decomposition of $Cu^{III}G_4$, probably a peroxyl radical. According to Kurtz *et al.*,[29] after decomposition of $Cu^{III}G_4$, generated by oxidation of $Cu^{II}G_4$ by oxygen, some reactive intermediate (a carbon-centered free radical, a Cu(I) complex, a peroxyl radical or a copper(III) peroxide) may be formed.

Conclusions

The results represented in Figure 2 demonstrate that the loss of native SC DNA is controlled by the ligand (L = G_3, G_4 and GGA) coordinated to Cu(II) and the presence of S(IV) and dissolved oxygen. However, DNA damage occurs in a similar way in the presence of Cu(II) and S(IV) when Cu(II) is added as $Cu(NO_3)_2$. This result may be attributed to the proximity of Cu^{II}L or Cu(II) to DNA deoxyl ribose rings. Cu(II), a divalent cation, binds DNA strongly. In some cases the DNA can partially displace the ligand since the charge of the Cu(II) complex can be controlled by the pH, due to the different degree of protonation of the coordinated ligand.[19,21] In fact, if the complex is anionic, direct interaction of the Cu(II) ion with DNA would be more difficult. Figures 2 and 3 also show, in some cases, that at higher S(IV) concentrations, the percentage of OC decreases, showing a less effective damage. It can be explained by the lack of dissolved oxygen when S(IV) is added in a large excess over oxygen (0.25 mmol L^{-1}, air saturated solution), the formed Cu(III) can be reduced by S(IV) still remaining in the solution (equation 4), and the redox cycling (Scheme 1) is no longer observed.

In the cases of Cu(II) complexes with G_3, G_4, GGA, GHG, GHK and GGYR, the efficiency of DNA damage must be related to the redox process represented in Scheme 1. In the spectrophotometric studies, Cu(III) complexes formation (λ_{max} = 360-390 nm) was possible to be observed, in the presence of S(IV) and dissolved oxygen (at pH 7) only for $Cu^{II}G_3$ ($E_{Cu(III)/Cu(II)}$ = 0.92 V *vs* NHE),[34] $Cu^{II}G_4$ ($E_{Cu(III)/Cu(II)}$ = 0.64 V *vs* NHE)[25] and Cu^{II}GGA ($E_{Cu(III)/Cu(II)}$ = 0.88 V *vs* NHE).[25] As $Cu^{III}G_3$, $Cu^{III}G_4$ and Cu^{III}GGA decompose fast, the intermediate formed may also damage DNA. For the Cu(II) complex with GGH, GHG, GHK and GGYR, Cu(III) formation and DNA damage were not observed even in the presence of traces of Ni(II).

The strong oxidant species (Scheme 1) formed in the redox cycling of $Cu^{II}L/Cu^{III}L$ (L= G_3, G_4 and GGA) may oxidize anyone of the four nucleosides in DNA. However, guanine is the most susceptible to undergo oxidative damage ($E^{o}Guo^{\bullet}/Guo$ = 1.29 V *vs* NHE for guanosine).[48] In addition, the guanine redox potential can be lower in the DNA molecule. $SO_3^{\bullet-}$ radical ($E^{o}SO_3^{\bullet-}/SO_3^{2-}$ = 0.76 *vs* NHE)[49] initially formed (equation 3), and $SO_5^{\bullet-}$ ($E^{o}SO_5^{\bullet-}/HSO_5^{-}$ = 1.10 *vs* NHE)[49] are unlikely to oxidize guanine because of their low redox potential, therefore other species must be involved in DNA damage. HSO_5^{-} anion ($E^{o}HSO_5^{-}/SO_4^{2-}$ = 1.75 *vs* NHE)[49] and $SO_4^{\bullet-}$ radical ($E^{o}SO_4^{\bullet-}/SO_4^{2-}$ = 2.43-3.08 *vs* NHE)[49] could easily oxidize not only guanine but also the other nucleosides.

In the case of DNA damage in the presence of Cu(II), BCA and S(IV), the mechanism must be completely different. The reduction of Cu(II) by S(IV) with Cu^{I}BCA complex formation (similar to equation 7) in the presence of DMPO, did not generate sulfite radicals, but the radical adducts DMPO/$HO^{\bullet}$ (*via* equations 1 and 2) and DMPOX (5,5-dimethylpyrrolidone-(2)-oxy-(1) were detected. The strong oxidant possibly formed, may be a carbon centered radical of BCA or peroxyl radical ($ROO^{\bullet}$), which is capable of oxidizing DMPO (1.87 V *vs* NHE) and the DNA bases. As already discussed, the order of the addition of reagents is extremely important to observe DNA damage. The present study is the first observation of DNA strand breaks in the presence of S(IV) and Cu^{II}BCA.

On the contrary, in literature it is reported that the presence of bathocuproine, a chelator for Cu(I), inhibited the DNA damage. Most of these studies are on the Cu(II)-mediated DNA damage via generation of hydrogen peroxide and suggested the involvement of hydrogen peroxide, superoxide and Cu(I).[50-56]

Acknowledgments

We gratefully acknowledge the financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico (CNPq) (Brazilian Agencies) and Prof. Dr. Ohara Augusto (IQ-USP) for allowing us the use of the EPR equipment.

References

1. Karlin, K. D.; Tyeklár, Z.; *Bioinorganic Chemistry of Copper*, Chapman & Hall: New York, 1993, p. 213.
2. Mack, D. P.; Iverson, B. L.; Dervan, P. B.; *J. Am. Chem. Soc.* **1988**, *110*, 7572.
3. Ueda, J. I.; Shimatu, Y.; Ozawa, T.; *Free Radical Biol. Med.* **1995**, *18*, 929.
4. Liu, C.; Zhou, J.; Li Q.; Wang, L.; Liao, Z.; Xu, H.; *J. Inorg. Biochem.* **1999**, *75*, 233.
5. Liu, J.; Zhang, T.; Lu, T.; Qu, L.; Zhou, H.; Zhang, Q.; Ji, L.; *J. Inorg. Biochem.* **2002**, *91*, 269.
6. Burke, S. K; Xu, Y.; Margerum, D. W.; *Inorg. Chem.* **2003**, *42*, 5807.
7. Coichev, N.; van Eldik, R.; *Inorg. Chem.* **1991**, *30*, 2375.
8. Coichev, N.; van Eldik, R.; *Inorg. Chim. Acta* **1991**, *185*, 69.
9. Coichev, N.; Reddy, K. B.; van Eldik, R.; *Atmos. Environ.* **1992**, *26 A*, 2295.
10. Pezza, H. R.; Bonifácio, R. L.; Coichev, N.; *J. Chem. Res. (M)* **1999**, 1520.
11. Lima, S.; Bonifacio, R. L.; Azzellini, G. C.; Coichev, N.; *Talanta* **2002**, *56*, 547.
12. Alipázaga, M. V.; Bonifácio, R. L.; Kosminsky, L.; Bertotti, M.; Coichev, N.; *J. Braz. Chem. Soc.* **2003**, *14*, 713.
13. Alipázaga, M. V.; Coichev, N.; *Anal. Lett.* **2003**, *36*, 2255.
14. Alipázaga, M. V.; Moreno, R. G. M.; Coichev, N.*; Dalton Trans.* **2004**, *13*, 2036.
15. Pezza, H. R.; Coichev, N.; *J. Coord. Chem.* **1999**, *47*, 107.
16. Alipázaga, M. V.; Kosminsky, L.; Coichev, N.; Bertotti, M.; *Talanta* **2002**, *57*, 375.
17. Alipázaga, M. V.; Lowinsohn, D.; Bertotti, M.; Coichev, N.; *Dalton Trans.* **2004**, *2*, 267.
18. Carvalho, L. B.; Alipázaga, M. V.; Moreno, R. G. M.; Coichev, N.; *J. Braz. Chem. Soc.* **2007**, *18*, 1247.
19. Moreno, R. G. M.; Alipázaga, M. V.; Medeiros, M. H. G.; Coichev, N.; *Dalton Trans.* **2005**, *6*, 1101.
20. Alipazaga, M. V.; Moreno, R. G. M.; Linares, E.; Medeiros, M. H. G.; Coichev, N.; *Dalton Trans.* **2005**, *23*, 3738.
21. Moreno, R. G. M.; Alipázaga, M. V.; Gomes, O. F.; Linares, E.; Medeiros, M. H. G.; Coichev, N.; *J. Inorg. Biochem.* **2007**, *101*, 866.
22. Alipázaga, M. V.; Moreno, R. G. M.; Linares, E.; Medeiros, M. H. G.; Coichev, N.; *Dalton Trans.* **2008**, *41*, 5636.
23. Muller, J. G.; Burrows, C. J.; *Inorg. Chim. Acta* **1998**, *275*, 314.
24. Guo, D. S.; Yuan, X. Y.; Liang, J. Q.; *Spectrochim. Acta, Part A* **2006**, *65*, 459.
25. Bossu, F. P.; Chellapa, K. L.; Margerum, D. W.; *J. Am. Chem. Soc.* **1977**, *99*, 2195.
26. Kirksey, S. T.; Neubecker, T. A.; Margerum, D. W.; *J. Am. Chem. Soc.* **1979**, *101*, 1631.
27. Anast, J. M.; Margerum, D. W.; *Inorg.Chem.* **1981**, *20*, 2319.
28. Sòvàgò, I.; Sanna, D.; Dessi, A.; Vàrnagy, K.; Micera, G.; *J. Inorg. Biochem.* **1996**, *63*, 99.
29. Kurtz, J. L.; Burce, G. L.; Margerum, D. W.; *Inorg. Chem.* **1978**, *17*, 2454.
30. Gershuns, A. L.; Koval, V. L.; *Visnik Kharkivs'kogo Universitetu* **1970**, *46*, 69.
31. Flaschka, H. A.; *EDTA Titrations*, Pergamon Press: London, **1959**, p.78.
32. Buettner, G. R.; Oberley, L. W.; *Biochem. Biophys. Res. Commun.* **1978**, *83*, 69.
33. Rybka, J. S.; Kurtz, J. L.; Neubecker, T. A.; Margerum, D. W.*; Inorg. Chem.* **1980**, *19*, 2791.
34. Lowinshon, D.; Alipazaga, M. V.; Coichev, N.; Bertotti, M.; *Electrochim. Acta* **2004**, 1761.
35. Pulyayeva, I. V.; Yegorova, N. L.; Experiandova, L. P.; Blank, A. B.; *Anal. Chim. Acta* **1997**, *357*, 239.
36. Cerchiaro, G.; Aquilano, K.; Filomeni, G.; Rotilio, G.; Ciriolo, M. R.; Ferreira, A. M. D. C.; *J. Inorg. Biochem.* **2005**, *99*, 1433.
37. Mottley, C.; Mason, R. P.; *Arch. Biochem. Biophys.* **1988**, *267*, 681.
38. Augusto, O.; Gatti, R. M.; Radi, R.; *Arch. Biochem. Biophys.* **1994**, *310*, 118.
39. Burkitt, M. J.; Tsang, S. Y.; Tam, S. C.; Bremner I.; *Arch. Biochem. Biophys.* **1995**, *323*, 63.
40. Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A.; *J. Biol. Chem.* **1991**, *266*, 4244.
41. Rosen, G. M.; Rauckman, E.; *J. Mol. Pharmacol.* **1980**, *17*, 233.
42. Berglund, J.; Fronaeus, S.; Elding, L. I.; *Inorg. Chem.* **1993**, *32*, 4527.
43. McDonald, M. R.; Scheper, W. M.; Lee, H. D.; Margerum, D. W.; *Inorg. Chem.* **1995**, *34*, 229.
44. McDonald, M. R.; Fredericks, F. C.;.Margerum, D. W.; *Inorg. Chem.* **1997**, *36*, 3119.
45. Hamburg, A. W.; Nemeth, M. T.; Margerum, D. W.; *Inorg. Chem.* **1983**, *22*, 3535.
46. Neubecker, T. A.; Kirksey Jr., S. T.; Chellappa, K. L.; Margerum, D. W.*; Inorg. Chem.* **1979**, *18*, 444.
47. Diaddario, L. L.; Robinson, W. R.; Margerum, D. W.; *Inorg. Chem.* **1983**, *22*, 1021.
48. Steenken, S.; Jovanovic, S.; *J. Am. Chem. Soc.* **1997**, *119*, 617.
49. Brandt, C.; van Eldik, R.; *Chem. Rev.* **1995**, *95*, 119.
50. Midorikawa, K.; Murata, M.; Oikawa, S; Tada-Oikawa, S.; Kawanishi, S.; *Chem. Res. Toxicol.* **2000**, *13*, 309.
51. Hirakawa, K.; Mori, M.; Yoshida, M.; Oikawa, S.; Kawanishi, S.; *Free Radical Res.* **2004**, *38*, 439.

52. Murata, M.; Ohnishi, S.; Seike, K.; Fukuhara, K.; Miyata, N.; Kawanishi, S;. *Chem. Res. Toxicol.* **2004**, *17*, 1750.
53. Oikawa, S.; Yamada, K.; Yamashita, N.; Tada-Oikawa, S.; Kawanishi, S.; *Carcinogenesis* **1999**, *20*, 1485.
54. Oikawa, S.; Nishino, K.; Oikawa, S.; Inoue, S.; Mizutani, T.; Kawanishi, S.; *Biochem. Pharmacol.* **1998**, *56*, 361.
55. Yunbo, L.; Trush, M. A.; *Carcinogenesis* **1993**, *14*, 1303.
56. Lloyd, D. R.; Phillips, D. H.; *Mutat. Res.* **1999**, *424*, 23.

Received: November 7, 2008
Web Release Date: June 12, 2009

FAPESP helped in meeting the publication costs of this article.