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ARTICLE *in* INORGANICA CHIMICA ACTA · FEBRUARY 2007

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Copper(II) complexes with β-cyclodextrin–homocarnosine conjugates and their antioxidant activity

Francesco Bellia [a], Diego La Mendola [b], Giuseppe Maccarrone [a], Placido Mineo [a], Daniele Vitalini [c], Emilio Scamporrino [a], Salvatore Sortino [a], Graziella Vecchio [a], Enrico Rizzarelli [a,b,*]

[a] *Dipartimento di Scienze Chimiche, Università di Catania Viale A. Doria, 6, Catania CT 95125, Italy*
[b] *Istituto di Biostrutture e Bioimmagini-CNR-Unità Organizzativa di Supporto di Catania Viale A. Doria, 6, Catania 95125, Italy*
[c] *Istituto di Chimica e Tecnologia dei Polimeri, CNR-Sezione di Catania, Catania, Viale A. Doria, 6, Catania 95125, Italy*

Received 20 June 2006; accepted 6 July 2006
Available online 22 July 2006

Dedicated to Professor Vincenzo Balzani.

Abstract

Copper(II) complexes of the β-cyclodextrin (β-CD) functionalized with homocarnosine (HC) in the primary (CDHC6) and secondary rim (CDHC3) were characterized by means of different spectroscopic techniques such as UV–Vis absorption, circular dichroism, electron paramagnetic resonance and electron-spray mass spectrometry. Taken together, all the spectroscopic parameters indicate the formation of different copper(II) complex species at various pH values. In the CDHC3 copper(II) complex species, a direct involvement of the secondary hydroxyl group 2 of functionalized β-CD's ring has been pointed out.

The antioxidant activity of the copper(II) complexes of the two derivatives was determined through pulse radiolysis measurements. The results obtained provide direct evidence for a high catalytic activity of both complexes towards the dismutation of the superoxide anion radical. It is also demonstrated that the complex formation is not detrimental to the excellent scavenger activity exhibited by the ligands alone towards hydroxyl radicals. These copper complexes then represent very intriguing antioxidant agents against well known toxic reactive oxygen species.

Keywords: Copper(II) complexes; Antioxidant activity; Cyclodextrin derivatives

1. Introduction

Homocarnosine (γ-aminobutyryl-L-histidine) is a dipeptide related to a group of natural histidine-containing molecules of which carnosine (β-alanyl-L-histidine) is the archetype [1,2]. These dipeptides are present in considerable amounts in several vertebrate tissues including skeletal muscle, the eye, the olfactory system and the brain [3–6]. Their specific biological function is not yet known, however, different hypotheses have been postulated: as being neurotransmitters, metal ion-chelating, having buffering effect, or as being antioxidants and antiglycating agents [7–13]. Homocarnosine is present in the human brain in greater amounts than in other mammals such as rats or mice (0.3–1.6 mM, with higher levels in subcortical gray matter), and its concentration is three to six times higher in adults than in infants [14]. It has been suggested as being a precursor for the neurotransmitter GABA hence serving as an important inhibitory neuromodulator in the human neocortex [15].

Homocarnosine can bind zinc in vivo and so modulate synaptic transmission directly by altering the availability of endogenous zinc as well as carnosine [7]. These peptides

* Corresponding author. Address: Dipartimento di Scienze Chimiche, Università di Catania Viale A. Doria, 6, Catania CT 95125, Italy. Tel.: +39 0957385070; fax: +39 095337678.
E-mail address: erizzarelli@unict.it (E. Rizzarelli).

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doi:10.1016/j.ica.2006.07.028

have been shown to be efficient copper chelating agents therefore having a neuroprotective role in copper-mediated toxicity [16]. Moreover, their copper complexes exhibit a strong SOD-like activity so as to suggest a possible role as antioxidants in vivo [17] and in this respect the copper–homocarnosine system has been reported to show higher activity than carnosine and anserine copper complexes [18].

Little is known about the regulation of homocarnosine synthesis but it has been demonstrated that it is rapidly hydrolyzed by carnosinase, a specific enzyme present in plasma, so its use as a potential exogenous antioxidant agent is limited [19].

Recently, we have synthesized β-cyclodextrin (β-CD) containing homocarnosine covalently attached to both β-CD rims, in order to stabilize the dipeptide and maintain its potential biological activity as antioxidant agent in vivo [20]. β-Cyclodextrin is a cyclic oligosaccharide currently used and investigated as a pharmaceutical excipient, mainly as a solubilizing and stabilizing agent so as to improve the bioavailability in vivo of lipophilic drugs [21–23].

We think that a key strategy in developing a new potential SOD-mimic (SOD = superoxidismutase) system has to be directed towards copper(II) complex systems which are not only able to catalyze the superoxide reduction but are also able to eliminate the hydroxyl radical that can be generated by H_2O_2 produced in the SOD-like enzymatic cycle.

As a consequence, we have synthesized and characterized copper(II) complexes of new β-cyclodextrin derivatives functionalized with carnosine and have evaluated their antioxidant activity [24–27].

In the present paper, we report the characterization of copper complex species of two derivatives 6^A-[(4-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-4-oxobutyl)amino]-6^A-deoxy-cyclomaltoheptaose (CDHC6) and (2^A*S*,3^A*R*)-3^A-[(4-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-4-oxobutyl)amino]-3^A-deoxy-cyclomaltoheptaose (CDHC3) by means of spectroscopic techniques UV–Vis, CD, EPR, and ESI-MS spectrometry, comparing the data with those pertinent to copper(II) complex of free dipeptide homocarnosine. We have found that the coordination features of copper(II) complexes with cyclodextrin–homocarnosine conjugates are quite different from those of analogous carnosine derivatives.

Then SOD-like activity of cyclodextrin–homocarnosine derivative copper(II) complexes was investigated as well as their ability to act as hydroxyl radical scavengers by means of pulse radiolysis measurements, trying to correlate the activity with the structure of copper(II) complexes.

2. Experimental

2.1. Materials

All reagents were of analytical grade, unless otherwise stated. β-Cyclodextrin was purchased from Fluka and homocarnosine from Sigma; anhydrous *N*,*N*-dimethylformamide was purchased from Aldrich. They were used without further purification.

The synthesis of the ligands (purity higher than 99%) was carried out as reported elsewhere [20]. Copper(II) nitrate was prepared from copper(II) basic carbonate by adding a slight excess of HNO_3. The concentration of stock solutions was determined by EDTA titrations using murexide as an indicator. Stock solutions of HNO_3 and KOH were standardized by titration with primary standard tris(hydroxymethyl)aminomethane (THAM) and potassium hydrogen phthalate, respectively. Potassium nitrate (Suprapur Merck) was used without further purification. All solutions were prepared with doubly distilled water.

2.2. Spectroscopic measurements

A Bruker Elexsys E500 CW-EPR spectrometer driven by a PC running an XEPR program under Linux and equipped with a Super X-band microwave bridge operating at 9.3–9.5 GHz and a SHQE cavity was used throughout this work. All spectra were recorded at 150 K using quartz tubes with 3 mm inner diameters. Solutions of $^{63}Cu(NO_3)_2$ (2×10^{-3} mol dm^{-3}) and ligand at different molar ratios were prepared in water solution, containing a small quantity of methanol (not exceeding 10%) and varying the pH by addition of potassium hydroxide. The $g_\parallel$ and $A_\parallel$ values were taken directly from the experimental spectra.

UV–Vis spectra of the copper(II) complexes were recorded on a Cary 500 spectrophotometer in 1 cm path length quartz cells.

CD spectra of the ligands and their copper(II) complexes were recorded on a JASCO 810 spectropolarimeter at a scan rate of 50 nm/min and 0.1 nm resolution. The pathlengths were 1 or 0.1 cm, in the 190–800 nm range. The spectra were recorded as an average of 10 or 20 scans. Calibration of the CD instrument was performed with a 0.06% solution of ammonium camphorsulfonate in water ($\Delta\varepsilon = 2.40$ (mol dm^{-3})$^{-1}$ cm^{-1} at 290.5 nm). The spectral range between 200 and 700 nm was covered by using quartz cells of various path lengths. Dilution of the solution was therefore not required. Results are reported in terms of ε (molar adsorption coefficient) and $\Delta\varepsilon$ (molar CD coefficient) in mol^{-1} dm^3 cm^{-1}.

2.3. EMF measurements

Potentiometric titrations were performed with a computer-controlled Metrohm digital pH meter (Model 654) and Hamilton digital dispenser (mod microlabm). The titration cell (2.5 ml) was thermostated at 25.0 ± 0.2 °C and all solutions were kept under an atmosphere of argon, which was bubbled through another solution under the same conditions of ionic strength and temperature. KOH solution was added through a Hamilton burette equipped with 0.25 or 0.50 cm^3 syringes. The combined microelectrode (ORION 9103SC) was calibrated on the

$pH = -\log[H^+]$ scale by titrating HNO_3 with CO_2 free base. The ionic strength of all solutions was adjusted to 0.10 mol dm^{-3} (KNO_3). The analytical concentrations of ligands were varied from 2.5×10^{-3} to 5.0×10^{-3} mol dm^{-3}. Stability constants for proton complexes were calculated from three or four titrations carried out over the pH range 2.5–10.6. Calculations of the electrode system, E°, E_j and K_W values as well as ligand purity were determined by the least square ACBA computer program [28]. Potentiometric data were handled using the HYPERQUAD program [29].

2.4. ESI mass spectrometric analysis

Positive ESI mass spectra were acquired on a Mariner ESI-TOF mass spectrometer (PerSeptive Biosystems) equipped with an API ion source. The ionspray voltage was fixed at 5 kV and the orifice potential (declustering potential) was varied from 50 to 250 V. The aqueous solutions of the samples investigated, diluted by a CH_3OH/H_2O (60/40) solution up to a solute concentration of approximately 10^{-4} mol dm^{-3}, were introduced into the source at a flow rate of 7 μL/min. The mass spectra were elaborated with the "BioSpec Data Explorer ver. 3.0.0.1" software (from PerSeptive Biosystems) and, to make the peak assignments in the spectra easier, a nitrate salt of pure ^{63}Cu(II) isotope was used.

Each species (identified as singly, doubly or triply charged ion on the basis of the ΔT_h values among the peaks) is indicated in the following with the m/z value of the first peak of its isotopic cluster. For a more accurate structural assignment, the relative intensity of the peaks in each cluster was compared with that of the peaks in the corresponding simulated modelling.

2.5. Pulse radiolysis assay

Pulse radiolysis was performed by using electron pulses (≈20 ns duration) from the 12 MeV electron linear accelerator at the ISOF-CNR Institute in Bologna. The irradiations were carried out at room temperature, 22 ± 2 °C, on samples contained in Spectrosil cells of 2 cm optical path length. Solutions were protected from the analyzing light by means of a shutter and appropriate cutoff filters. The monitoring light source was a 450 W Xe arc lamp. The radiation dose per pulse was monitored by means of a charge collector placed behind the irradiation cell and calibrated with a N_2O-saturated solution containing 0.1 mol dm^{-3} HCO_2^- and 0.5×10^{-3} mol dm^{-3} methyl viologen (1,1′-dimethyl-4,4′-bipyridinium dication; MV^{2+}) using $G\varepsilon = 9.66 \times 10^{-4}$ m^2 J^{-1} at 602 nm [30]. G(X) represents the number of moles of species X formed or consumed per joule of energy absorbed by the system.

The radiolysis of water predominantly produces the species shown in Eq. (1) where the values in parentheses represent the yields expressed in terms of G-values (μmol J^{-1}) [31].

$$H_2O \rightarrow e_{aq}^-(0.27), {}^{\cdot}OH(0.28) \text{ and } H^{\cdot}(0.062),\ H_2(0.047),\ H_2O_2(0.073),\ H^+(0.27) \quad (1)$$

2.5.1. Reaction of $O_2^{\cdot-}$ radical with Cu–CDHC3 and Cu–CDHC6

If the solutions are saturated with oxygen in the presence of 10^{-2} mol dm^{-3} formate ion, the following reactions take place in the irradiated solution:

$$e_{aq}^- + O_2 \rightarrow O_2^{\cdot-} \quad (2)$$

$$H^{\cdot} + O_2 \rightarrow HO_2^{\cdot} \quad (3)$$

$$HO_2^{\cdot} \leftrightarrows H^+ + O_2^{\cdot-} \quad pK_a = 4.69 \quad (4)$$

$$^{\cdot}OH + HCOO^- \rightarrow CO_2^{\cdot-} + H_2O \quad (5)$$

$$CO_2^{\cdot-} + O_2 \rightarrow CO_2 + O_2^{\cdot-} \quad (6)$$

Under these experimental conditions, the superoxide radical anion is generated within less than 1 μs after the pulse, with a yield of 0.61. Since the dose used was ≈16 Gy, the concentration of $O_2^{\cdot-}$ was ≈10^{-5} mol dm^{-3}. The characteristic absorption band of $O_2^{\cdot-}$ in the UV region [32] enables the direct measurements of its decay at 250 nm.

2.5.2. Reaction of $^{\cdot}OH$ radicals with Cu–CDHC3 and Cu–CDHC6

Samples containing either Cu–CDHC3 or Cu–CDHC6 were irradiated after saturating the aqueous solutions with N_2O. Under these experimental conditions, the e_{aq}^- are converted into OH according to

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^{\cdot}OH + OH^- + N_2 \quad (7)$$

and the yield of this species increases to ca. 0.55. Since the dose used in our experiments was ≈16 Gy, the concentration of $^{\cdot}OH$ generated is ≈10^{-5} mol dm^{-3}.

The error in catalytic constants is ±15%.

3. Results and discussion

3.1. Copper(II) complexes with HC

Homocarnosine is a dipeptide similar to carnosine but with an atom of carbon more in the aliphatic chain. Differently from carnosine, dimeric complex species were not observed in all pH range investigated.

Based upon potentiometric data reported in a previous paper [33], the monomeric species CuLH is the prevailing one in the acidic pH range 5–6. The copper ion has been supposed to be bound by imidazole nitrogen.

Our EPR parameters indicate a type 2 copper(II) coordination environment at all pH values investigated [34,35]. At pH 5–6 the EPR data (see Table 1) clearly indicate the binding of one nitrogen, without the involvement of other donor atoms except the water oxygens. Raising the pH to 6–7, the magnetic parameters ($g_\parallel = 2.291$, $A_\parallel = 0.00166$ cm^{-1}) are consistent with the replacement of

Table 1
Spectroscopic data of copper(II) complexes with HC, CDHC(3) and CDHC(6)

Ligands	CD	Vis	EPR		pH
	λ_{max}/nm ($\Delta\varepsilon$/M^{-1} cm^{-1})	λ_{max}/nm (ε/M^{-1} cm^{-1})	$g_{\parallel}$	$A_{\parallel} \times 10^{-4}$/$cm^{-1}$	
HC	192 (9.8); 219 (2.5)	797 (17)			4
	192 (9.1); 218 (2.7); 752 (0.015)	771 (22)	2.369	130	5
	195 (6.1); 217 (3.3); 296 (−0.080); 719 (0.062)	736 (37)	2.291	166	6
	214 (4.9); 320 (0.070); 659 (0.075)	690 (44)	2.286	169	7
	208 (5.8); 236 (0.23); 318 (0.90); 501 (−0.053); 614 (0.52); 746 (−0.12)	624 (57)	2.277	175	8
	205 (7.0); 234 (−0.62); 318 (1.2); 509 (−0.071); 614 (0.16); 745 (−0.17)	606 (63)			9
	205 (7.4); 232 (−1.2); 312 (1.3); 511 (−0.07); 619 (0.11); 745 (−0.14)	596 (66)	2.236	185	10
	204 (8.7); 227 (−1.9); 252 (−0.92); 303 (1.6); 571 (−0.075); 654 (0.071); 759 (−0.082)	582 (68)	2.235	186	11
CDHC(3)	195 (7.4); 217 (3.6)	798 (14)			4
	194 (7.4); 217 (3.5)	782 (18)	2.373	126	5
	201 (10.6); 218 (4.0); 300 (1.7); 680 (0.26)	679 (53)	2.268	172	6
	202 (11.5); 247 (−1.9); 290 (2.4); 553 (0.47); 644 (0.22); 751 (−0.096)	615 (63)	2.238	180	7
	202 (10.7); 220 (−4.3); 245 (−3.7); 285 (3.5); 551 (0.87); 726 (−0.37)	586 (72)	2.237	180	8
	202 (10.8); 221 (−5.2); 242 (−4.4); 284 (3.6); 553 (0.96); 723 (−0.44)	579 (74)	2.237	180	9
	202 (11.1); 221 (−5.4); 244 (−4.4); 285 (3.7); 512 (0.98); 723 (−0.46)	570 (74)	2.236	175	10
	202 (11.0); 221 (−5.6); 244 (−4.5); 285 (3.7); 554 (0.97); 724 (−0.47)	574 (74)	2.236	175	11
CDHC(6)	194 (5.5); 217 (3.5)	786 (28)			4
	135 (5.8); 215 (3.7)	751 (27)			5
	197 (3.6); 215 (4.4); 702 (0.028)	734 (40)	2.284	168	6
	214 (6.9); 780 (0.016)	690 (60)	2.269	176	7

one oxygen by one nitrogen donor atom with the formation of bis-complex species where each ligand acts as a unidentate and copper(II) is coordinated by two imidazole nitrogens [36,37]. The blue shift of λ_{max} values in the UV–Vis and in the CD spectra are consistent with a 2 N complex species. Furthermore, the small positive Cotton effect between 640 and 700 nm and the absence of nitrogen amide charge transfer in the CD spectra give further evidence that only imidazole nitrogen was involved in the copper(II) binding.

When the pH was increased to 8, a band at 300 nm, diagnostic of amide backbone coordination, became evident in the CD spectra. The shoulder around 340 nm is due to imidazole nitrogen $\pi \rightarrow Cu^{2+}$ charge transfer. At this pH the CuL species is the predominant one and the enhancement of ligand strength can be explained by assuming that amide nitrogen and N_{Im} take part in coordination so as to form a six-chelate ring, with the contemporary protonation of the more basic amino group. EPR parameters ($g_{\parallel} = 2.277$, $A_{\parallel} = 0.00175\ cm^{-1}$) are nearly identical to those determined for analogous systems [38], bringing additional support to the coordination mode.

The amide CT band intensity increases with the pH increase and it is shifted towards higher energy, showing the involvement of another nitrogen donor atom. The same effect is observed in the d–d band. UV–Vis spectroscopic parameters at pH 10 are indicative of a stronger ligand field due to the involvement of another nitrogen atom in the coordination environment, the amino group with the formation of seven- to six-membered fused chelate rings. The low $g_{\parallel}$ and the high $A_{\parallel}$ constant are characteristic of copper(II) complexes with at least three nitrogen atoms in the equatorial plane.

Due to the excess of ligand, the formation of bis complexes $Cu(LH_{-1})(L)$ could not be ruled out. In this case the copper coordination involves the imidazole, the deprotonated amide, the amino group and a fourth nitrogen atom from an imidazole or amino of a second homocarnosine. The previously reported potentiometric data indicate that the complex species $Cu(LH_{-1})(L)$ is negligible [33], in agreement with ESI-MS data; thus this coordination environment around metal ion can be excluded. At strongly basic pH 11–12 another species forms. The small blue shift of UV–Vis spectra and the non-variation in molar coefficient extinction indicate the involvement of an OH group, excluding the contribution of a stronger deprotonated amide group binding. EPR parameters at pH 11 are pertinent to the same complex species observed at pH 10.

3.1.1. β-Cyclodextrin–homocarnosine conjugate proton complexes

When homocarnosine is covalently attached to β-cyclodextrin, the protonation constants of the amino nitrogen decrease significantly (Table 2). The pK values are nearly 2 logarithmic units lower than amino group of free homocarnosine, while only slight differences are observed for imidazole nitrogen and carboxylate group protonation constants. In fact the amino group bound to cyclodextrin in position 6 is adjacent to the ring of six primary OH groups delineating the narrow rim of the cavity, so the possible hydrogen bonding between them and the amino nitrogen, the steric hindrance and hydrophobicity of the cavity,

Table 2
Stepwise stability constant values of the proton complex of HC, CDHC6 and CDHC3 at 25 °C and $I = 0.10$ mol dm^{-1} (KNO_3) (3σ in parentheses)

Equilibrium	log K
$HC^- + H^+ \leftrightarrows HC$	10.04(3)
$HC + H^+ \leftrightarrows [(HC)H]^+$	6.81(2)
$[(HC)H]^+ + H^+ \leftrightarrows [(HC)H_2]^{2+}$	2.68(4)
$CDHC6^- + H^+ \leftrightarrows CDHC6$	8.22(3)
$CDHC6 + H^+ \leftrightarrows [(CDHC6)H]^+$	6.65(4)
$[(CDHC6)H]^+ + H^+ \leftrightarrows [(CDHC6)H_2]^{2+}$	2.73(2)
$CDHC3^- + H^+ \leftrightarrows CDHC3$	7.98(5)
$CDHC3 + H^+ \leftrightarrows [(CDHC3)H]^+$	6.70(2)
$[(CDHC3)H]^+ + H^+ \leftrightarrows [(CDHC3)H_2]^{2+}$	2.78(3)

may explain the amino group basicity decrease in comparison to free homocarnosine; this is in agreement with previously reported data for analogous systems [39].

These differences are more evident for the CDHC3 derivative as observed for the corresponding carnosine derivative system [26]. In this case, the amino group replaces an hydroxyl group in position 3 involved in hydrogen bonding with the 2-OH group and this explains the further basicity decrease.

3.1.2. Copper(II) complexes with CDHC6

Spectroscopic data collected at pH 6 for the Cu–CDHC6 system indicate the presence of a complex species where copper ion is coordinated by two nitrogens in a similar way to the Cu–HC system. The involvement of two imidazole nitrogens can be justified with at least three reasons: (1) the decrease of amino group basicity; (2) the steric hindrance of the cyclodextrin's cavity; and (3) the low intensity CD signals indicate that the metal ion chromophore is distant from the chiral cavity.

The EPR parameters, obtained at pH 7, suggest the formation of a complex with a stronger field and CD data show a weak signal in the amide CT region around 300 nm. EPR data are very similar to those observed for the Cu–HC system at pH 8. They are indicative of a 2 N copper(II) coordination environment characterized by the involvement of the imidazole and the deprotonated amide nitrogens involved in the formation of a six-membered chelate ring (see Fig. 1a).

When raising the pH above 7 a precipitate is observed. The decrease of basicity and steric hindrance of the cyclodextrin cavity do not allow the formation of two fused seven and six membered chelate rings as observed in the Cu–HC system.

3.1.3. Copper(II) complexes with CDHC3

The Cu–CDHC3 system shows a behaviour similar to those found in Cu–CDHC6 and Cu–HC systems only at low pH values. At pH 5, spectroscopic and EPR parameters are indicative of a copper complex species in which the metal ion is coordinated by one nitrogen. But, differently from the other two systems, at pH 6 a band at 300 nm diagnostic of amide nitrogen deprotonation is

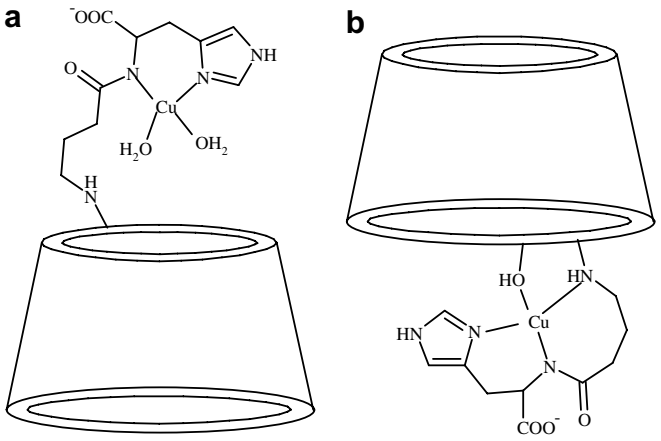

Fig. 1. Tentative structures of (a) the [Cu(CDHC6)(H_{-1})] and (b) [Cu(CDHC3)(H_{-1})] complexes.

clearly present in the CD spectrum. The CuL species, with one of the basic groups protonated, is formed at a pH value lower than that evidenced in the Cu–HC and Cu–CDHC6 systems. In addition, the dichroic bands show a higher intensity than those found for the Cu–CDHC6 system, suggesting a major proximity to the cyclodextrin chiral cavity of metal ion chromophore. Similar to the data reported for the analogous carnosine derivative CDAH(3) [26], we can consider the amino group as the anchoring site for the copper ion, assisted by the hydroxyl in position 2 of the cyclodextrin, giving rise to a chelate ring which counterbalances the decrease of amino group basicity.

The EPR parameters are slightly different from that of Cu–CDHC6 species. On increasing the pH, $CuLH_{-1}$ is formed with the imidazole ring that is ready to form a bis-chelate complex and complete the tetragonal coordination environment (see Fig. 1b). After pH 8 we observe a stronger equatorial coordination field around the copper ion, determined by the deprotonation of cyclodextrin OH. This generates also a slight distortion of the coordination plane indicated by the imidazole $\pi_1 \rightarrow$ CT sign change from positive to negative in the CD spectrum, diagnostic of a slight shift out of the chelate plane by the imidazole ring [24].

EPR parameters are indicative of this distortion considering that the $A_{\parallel}$ value decreases and it is lower than the corresponding $Cu(HC)H_{-1}$ complex species of homocarnosine.

The higher intensity of all dichroic signals compared to those found for Cu–HC and Cu–CDHC6 systems strongly suggests that metal ion chromophore is near to cavity and in all Cu–CDHC3 copper complex species at pH higher than 5.

Even in this system, the formation of mixed bis complexes Cu[(CDHC3)H_{-1}](CDHC3) has to be taken into account, but ESI-MS data indicate that actually there are no bis-complex species.

4. Near UV CD spectra

Near UV CD spectra show marked differences between the two β-CD-derivatives after copper addition (Fig. 2).

The spectra of HC, CDHC3 and CDHC6 essentially show a band that can be assigned to the $n - \pi^*$ transition of the carbonyl group [24]. For the sake of simplicity, in Fig. 1a only the spectra carried out at pH 7 are reported, the band with λ_{max} at 215 nm being almost unchanged in a wide pH range (4–10). Probably all protonation forms of the dipeptide show a similar conformation, and are indicative that the homocarnosine chain is not included in the cavity of β-cyclodextrin in the β-CD-derivatives [40].

After copper(II) addition, the CD spectra show marked differences. The band at 215 nm of Cu–HC spectrum is blue shifted and its intensity enhances as the pH increases. Starting from pH 7, the π–π^* transition of the amide group and the π–π^* transition of the imidazole ring could contribute to a negative band at 230 nm and a shoulder at 250 nm, respectively.

Cu–CDHC6 shows a pH dependent band shift similar to that of Cu–HC, but the intensity is higher because of the contribution of the chiral cavity.

In the Cu–CDHC3 spectra, the band of carbonyl transition is modified as occurs for Cu–CDHC6, but two negative bands, also observed in Cu–HC spectra, appear at pH 7 and become much intense and are substantially unchanged in pH values of 8 and higher.

On the whole, the CD spectra carried out in the near UV show that the greatest change in band shape and intensity is observed for the Cu–CDHC3 system. This clearly indicates that the metal ion chromophore is closer to cyclodextrin moiety in the last β-CD-derivative.

5. ESI-MS measurements

ESI-MS data are particularly useful in the study of copper(II) complexes with ligands synthesized in low yield. The high sensitivity and the great accuracy in the mass assignments give direct information on the formation of copper(II) complexes and their stoichiometry [24].

5.1. HC, CDHC6 and CDHC3 ESI-MS features

The ESI mass spectrum of HC in aqueous solution at pH 6.0 (Fig. S1) shows a base peak at m/z 263.1 corresponding to singly charged HC molecule detected as sodiated species $[(HC)Na]^+$ (Table 3); the peak due to protonated ion $[(HC)H]^+$ appears with lower intensity at m/z 241.2. Furthermore, the peak corresponding to the

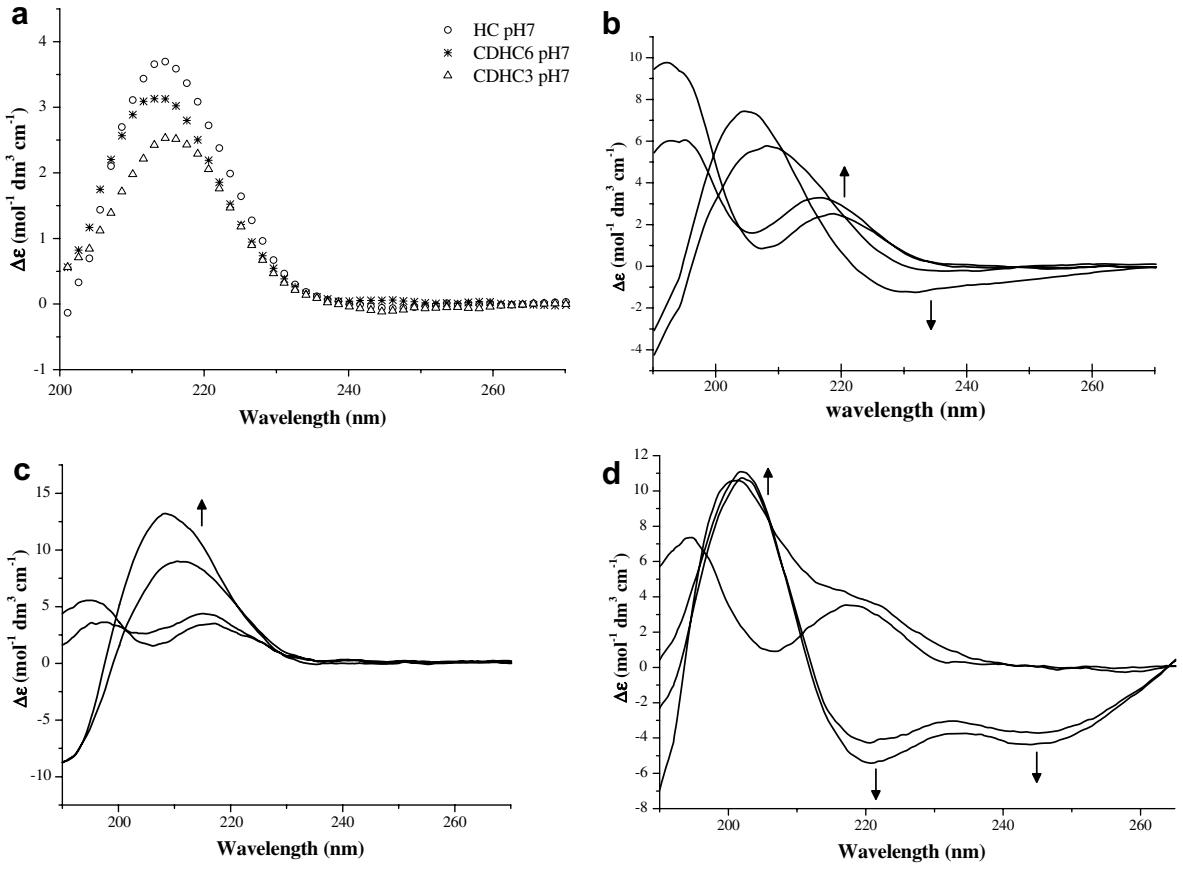

Fig. 2. Near UV CD spectra of: (a) HC, CDHC6 and CDHC3 at pH 7, (b) Cu–HC, (c) Cu–CDHC6, and (d) Cu–CDHC3 at different pH values and at a 1:3 metal-to-ligand ratio. Arrows indicate the curve changes from pH 4 to 10.

Table 3
Assignment of ions detected in the ESI mass spectra of HC and Cu–HC

Species	*m/z*
$[(HC)H]^+$	241.1
$[(HC)Na]^+$	263.1
$[(HC)H_{-1}Na]Na^+$	285.1
$[(HC)_2H]^+$	481.3
$[(HC)_2]Na^+$	503.3
$[(HC)_2]K^+$	519.3
$[Cu(HC)]^+$	303.0
$[(HC)_2H_{-1}Na]Na^+$	525.3
$[(HC)_2H_{-2}Na_2]Na^+$	547.3
$[Cu(HC)H_{-1}]Na^+$	325.1
$[Cu(HC)_2H_{-1}]^+$	542.2
$[Cu(HC)_2H_{-2}]Na^+$	564.1

Table 4
Assignment of ions detected in the ESI mass spectra of CDHC3 and Cu–CDHC3

Species	*m/z*
$[(CDHC3)H]^+$	1357.5
$[(CDHC3)Na]^+$	1379.5
$[(CDHC3)K]^+$	1395.3
$[(CDHC3)_2H]^+Na^+$	1368.5
$[(CDHC3)H_2]^{2+}$	679.3
$[(CDHC3)H]^+Na^+$	690.3
$[(CDHC3)H]^+K^+$	698.3
$[(CuCDHC3)H_{-1}]^+$	1418.4
$[Cu(CDHC3)H_{-2}]Na^+$	1440.3
$[Cu(CDHC3)H_{-2}]K^+$	1456.3
$[Cu(CDHC3)H_{-3}Na]Na^+$	1462.3
$[Cu(CDHC3)H_{-3}Na]K^+$	1478.3
$[Cu(CDHC3)H_{-3}K]K^+$	1494.1
$[Cu(CDHC3)]^{2+}$	709.7
$[Cu(CDHC3)H_{-1}]^+Na^+$	720.7
$[Cu(CDHC3)H_{-1}]^+K^+$	728.7
$[Cu(CDHC3)H_{-2}]Na_2^{2+}$	731.7
$[Cu(CDHC3)H_{-2}]Na^+K^+$	739.7
$[Cu(CDHC3)H_{-2}]K_2^{2+}$	747.6
$[Cu(CDHC3)H_{-3}Na]Na_2^{2+}$	742.7
$[Cu(CDHC3)H_{-3}Na]Na^+K^+$	750.7
$[Cu(CDHC3)H_{-3}Na]K_2^{2+}$	758.7
$[Cu(CDHC3)H_{-3}K]K_2^{2+}$	766.5

sodiated HC salt, in which a sodium ion substitutes the carboxyl hydrogen, is also present at *m/z* 285.1 $[(HC)H_{-1}Na]Na^+$. The peak at *m/z* 223.1 is due to an HC fragment formed by loss of NH_3 probably during the desorption process, as also observed in the carnosine case [41]. The peaks at *m/z* 279.1 and 301.2, present in almost all the ESI spectra examined in this work as background signals, are due to a contamination of phthalates contained in the tubes as plasticizers and will be neglected in the following discussion.

Signals due to species formed by two HC molecules are present at *m/z* 481.3 and 503.3 as $[(HC)_2H]^+$ or $[(HC)_2Na]^+$ and, with a low intensity, at *m/z* 525.3 and 547.3 as sodiated salt species, $[(HC)_2H_{-1}Na]Na^+$ or $[(HC)_2H_{-2}Na_2]Na^+$ (very small traces of heavier HC structures also appear at higher *m/z* values).

The ESI mass spectrum of CDHC3 in aqueous solution at pH 5.8 is constituted essentially by peaks due to singly charged (at *m/z* 1357.5 and 1379.5, as $[(CDHC3)H]^+$ and $[(CDHC3)Na]^+$ ions) and doubly charged CDHC3 species (at *m/z* values between 679.3 and 698.3 as $[(CDHC3)M]^{2+}$ with M = different combinations of H, Na and/or K ions, for the structural assignments see Table 4). No signals due to both unreacted pure HC and β-cyclodextrin species are present in the spectrum. The presence of the peak at *m/z* 1368.5 indicates the formation of species constituted by two CDHC3 molecules detected as $[(CDHC3)_2]H^+Na^+$ ion; the peaks corresponding to the doubly charged protonated or sodiated species $[(CDHC3)_2H_2]^{2+}$ and $[(CDHC3)_2Na_2]^{2+}$ are superimposed to those of more intense singly charged ions at *m/z* 1357.5 and 1379.5.

The ligand CDHC6 has the same molecular mass as CDHC3 and the ESI mass spectra are exactly coincident with that of the CDHC3 (data not shown).

5.2. Copper(II) complexes ESI-MS features

5.2.1. Cu–HC system

Ions detected in the ESI mass spectrum of an aqueous solution of HC mixed with $^{63}Cu(II)$ nitrate (molar ratio 3:1) at pH 5.5 are reported in Table 3. Peaks due to pure HC species are still present at *m/z* values between 241.2 and 285.1. However, the spectrum is essentially constituted by a very intense peak at *m/z* 303.0 corresponding to the singly charged Cu(I) HC salt protonated species $[Cu(HC)]^+$, in which a monovalent copper ion substitutes the carboxyl hydrogen in the HC unit (the corresponding sodiated species appears at *m/z* 325.1, with low intensity). As demonstrated in a previous case, the partial reduction of the copper(II) ions is induced by the hydrazine present in the commercial HC sample [41]. The peaks due to the dimeric HC species are almost absent, whereas new peaks at *m/z* 542.2 and 564.1 due to $[Cu(HC)_2H_{-1}]^+$ or $[Cu(HC)_2H_{-2}]Na^+$ ion in which the copper(II) ion bonds two HC units by means of their carboxylate groups appear in the spectrum.

5.2.2. Cu–CDHC6 and Cu–CDHC3 systems

ESI mass spectra of CDHC6 aqueous solutions treated with different amounts of $^{63}Cu(II)$ nitrate (molar ratio from 3:1 to 1:3) at pH 7 result almost coincident with the spectrum of pure CDHC6 (omitted for brevity). The only difference observed was a reduced intensity of the peak at *m/z* 1357.5 and 1379.5, as a consequence of the increase of the relative amount of copper nitrate.

This is a direct consequence of the low solubility of copper complex species with this ligand even at a molar concentration of 10^{-4} M.

Different data were obtained for the Cu–CDHC3 system. In this case, besides the signals due to pure ligand, signals of copper complex species are detected as singly (from *m/z* 1418.4 to 1494.1) or doubly charged ions (from *m/z* 709.7 to 766.5) (see structural assignments in Table 4). In

the ESI-MS, all the copper(II) complex species were found obtained by means of spectroscopic data.

6. Pulse radiolysis assay

As regards indirect assays, the measurements of SOD activity by means of pulse radiolysis avoid the possibility of artefacts because they directly follow the disappearance of the superoxide radical and the eventual effect of a SOD-mimic system on this. The rate of $O_2^{\cdot -}$ decay in the blank solution (data not shown), in the absence of copper(II) complexes, was slow and followed second order kinetics with a spontaneous dismutation as $k_1 = 10^5\ M^{-1}\ s^{-1}$, a value in good agreement with the literature data [32,42]. Differently, in the solution containing the copper(II) complexes with ligands: either CDHC6 or CDHC3, the reaction was clearly accelerated and the time profile for the $O_2^{\cdot -}$ decay was described well by first order kinetics (Fig. 3a and b) with a pseudo-first order rate constant k_{obs} of $2.10 \times 10^3\ s^{-1}$ for the Cu–CDHC6 system and $k_{obs} = 1.25 \times 10^4\ s^{-1}$ for the Cu–CDHC3 system. The measurements were carried out with high $O_2^{\cdot -}$ concentration ($\approx 10^{-5}$ mol dm^{-3}) because of its low molar absorbivity, so the concentration of the complexes was lower than the initial $O_2^{\cdot -}$ concentration. The fact that the kinetics observed did not change, during repeated electron pulses on the same solution, was a clear evidence that both complexes were functioning in a catalytic fashion. The second order rate constants for Cu–CDHC3 and Cu–CDHC6 catalyzed disproportionation of superoxide anion were obtained by using Eq. (8), in agreement with that reported for other copper(II) complexes showing catalytic activity [43,44].

$$k_{cat} = k_{obs}/[\text{complex}] \quad (8)$$

The k_{cat} values obtained for the copper complexes were $2.3 \times 10^9\ M^{-1}\ s^{-1}$ and $3.0 \times 10^9\ M^{-1}\ s^{-1}$ for Cu–CDHC6 and Cu–CDHC3, respectively, and did not change significantly with the complex concentration. These constant values are of the same order of magnitude of the wild-type SOD enzyme [45], indicating a high superoxide dismutase activity in the two systems. These results are also similar to those obtained for the analogous carnosine derivatives [26]. However, taking into account the experimental error, it is possible to assert that homocarnosine systems show a slightly higher SOD activity. We suppose that this tendency could be due to the longer aliphatic chain and the greater flexibility in the copper(II) coordination environment. Control experiments carried out with the ligands alone up to 10 μM ruled out any effects of these on the rate of $O_2^{\cdot -}$ disappearance.

By taking into account the fact that the ligands are able to react with the hydroxyl radical [20], we have carried out experiments in order to verify if copper(II) complexes maintain the same scavenger activity.

Pulse radiolysis measurements of N_2O saturated solution in the presence of copper complex species with CDHC6 showed the formation of transient absorption spectra characterized by relevant absorption below 280 nm, a shoulder around 300 nm and a better-defined band in the 350–450 nm range (Fig. 4). This spectrum,

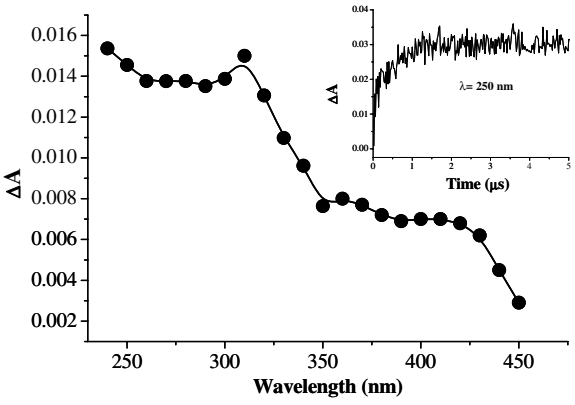

Fig. 4. Absorption spectrum obtained by means of the pulse radiolysis experiment of N_2O-saturated 10^{-4} mol dm^{-3} solution of Cu–CDHC3 and taken 6 μs after the pulse. The inset shows the build-up monitored at 240 nm.

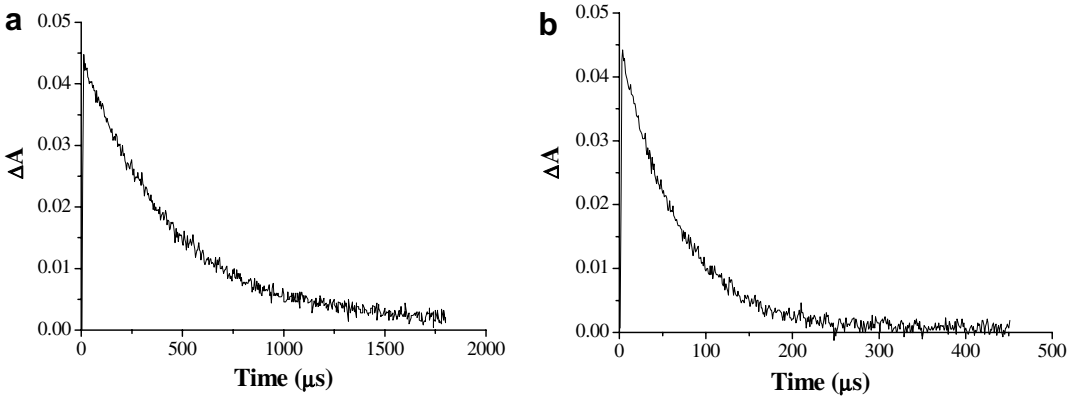

Fig. 3. Absorption decay at 250 nm obtained by means of the pulse radiolysis experiment of formate 10^{-2} mol dm^{-3} solution of (a) Cu–CDHC6 and (b) Cu–CDHC3.

due to the reaction of OH radicals with the systems, is very similar to that observed in the case of ligand alone [20], where two different sites of radical reaction have been characterized: the imidazole ring and the β-cyclodextrin moiety. This result clearly indicates that copper(II) binding did not affect the scavenger activity towards hydroxyl radicals.

The time profile shown in the inset of Fig. 4 is described fairly well by a first order fit with a pseudo-first order rate constant, $k_{obs} \sim 1 \times 10^6\ s^{-1}$. A bimolecular quenching constant $k_{Cu-CDHC6} \sim 8 \times 10^9\ M^{-1}\ s^{-1}$, related to the reaction of OH with Cu–CDHC6, is obtained by using the following equation:

$$k_{(\cdot OH+complex)} = k_{obs}/[complex] \quad (9)$$

Such a value did not change significantly with the concentration of the complex, confirming that under these experimental conditions ·OH radicals react almost exclusively with the quencher. Furthermore, it is interesting to note that this bimolecular quenching constant is identical to that reported for the ligand alone [20].

Cu–CDHC3 exhibited similar spectral and kinetic features to Cu–CDHC6 and a bimolecular quenching constant $k_{Cu-CDHC3} \sim 7 \times 10^9\ M^{-1}\ s^{-1}$ (data not shown). This value is exactly the same as that of the ligand CDHC3 alone.

On the whole, the results underlined that there are no differences in the scavenger activity of Cu–CDHC6 and Cu–CDHC3. In addition, differently from what is reported for the analogous carnosine derivatives [26], the copper coordination does not modulate the relative distribution of the ·OH reaction between the two different sites of attack (β-CD or imidazole). This is instead in agreement with the absence of dimeric copper(II) complex species for homocarnosine derivatives and with the absence of significative interactions between the imidazole ring and β-CD cavity in both the compounds [26].

7. Concluding remarks

The reactive oxygen species are involved in the pathogenesis of many diseases and aging, so many ligands able to bind copper(II) have been synthesized and studied as SOD-mimic models. We report the characterization of copper(II) complexes with the β-cyclodextrin mono-functionalized with homocarnosine at the narrow or wide rim.

The spectroscopic data show peculiar differences between the two investigated compounds. In the CDHC6 derivative the anchoring site for the metal ion is the imidazole nitrogen, analogously to what was found for the underivatised homocarnosine ligand. In contrast, in the copper(II) complexes with CDHC3 derivative, the anchoring group is the amino nitrogen as indicated by the direct involvement of the neighbouring secondary hydroxyl group of β-CD moiety. The high flexibility of CD cavity in the CDHC3 and the presence of another binding site for the copper(II) account well for the different behaviour. The Cu–CDHC6 is not soluble up to pH 7 in the millimolar range. The CDHC3 ligand is able to bind the copper(II) ion tightly, forming fused seven and six-membered chelate rings with the involvement of both amino and imidazole nitrogen atoms.

ESI-MS data strongly support the spectroscopic results; in fact the low solubility of Cu–CDHC6 system does not allow obtaining suitable spectra while we determined the coordination environment of the various deprotonated species of Cu–CDHC3 system formed within the investigated pH range. Both complexes are excellent agents against superoxide radical anion and hydroxyl radical. While the metal centre plays the active role in the catalytic dismutation of the former, it is demonstrated that both the imidazole and the cyclodextrin units of the ligands are involved in the scavenging of the latter by diffusion-controlled formation of adducts and hydrogen abstraction reactions. On these bases, these copper complexes represent very promising agents against the two harmful oxygen species.

Acknowledgements

The authors thank MIUR (PRIN 2004 032851-003 and FIRB RBNE03PX83) for financial support. We express our sincere thanks to Dr. Q.G. Mulazzani (ISOF-CNR Bologna, Italy) for his inestimable help in the pulse radiolysis measurements and to Dr. A. Martelli and A. Monti (ISOF-CNR Bologna, Italy) for their technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ica.2006.07.028.

References

[1] K.G. Crush, Comp. Biochem. Physiol. 34 (1970) 3.
[2] A.A. Boldyrev, Biochemistry (Moscow) 65 (2000) 751.
[3] H. Abe, G.P. Dobson, U. Hoeger, W.S. Parkhouse, Am. J. Physiol. 249 (1985) R449.
[4] P.J. Quinn, A.A. Boldyrev, V.E. Formazuyk, Mol. Aspects Med. 13 (1992) 379.
[5] M.C. Jackson, J.F. Lenney, Inflamm. Res. 45 (1996) 132.
[6] L. Bonfanti, P. Peretto, S. De Marchis, A. Fasolo, Prog. Neurobiol. 59 (1999) 333.
[7] P.Q. Trombley, M.S. Horning, L.J. Blakemore, Neuroreport 9 (1998) 3503.
[8] A.A. Boldyrev, Trends Pharmacol. Sci. 22 (2001) 112.
[9] M.S. Horning, L.J. Blakemore, P.Q. Trombley, Brain Res. 852 (2000) 56.
[10] R. Kohen, Y. Yamamoto, K.C. Cundy, B. Ames, Proc. Natl. Acad. Sci. USA 85 (1988) 3175.
[11] R. Tabakaman, P. Lazarovici, R. Cohen, J. Neurosci. Res. 68 (2002) 463.
[12] M. Salim-Hanna, E. Lissi, L. Videla, Free Radical Res. Commun. 14 (1991) 263.
[13] L.J. Hobart, I. Seibel, G.S. Yeargans, N.W. Seidler, Life Sci. 75 (2004) 1379.
[14] O.A.C. Petroff, Neuroscientist 8 (2002) 562.
[15] O.A.C. Petroff, F. Hyder, D.L. Rothman, R.H. Mattson, Neurology 56 (2001) 709.

[16] C.E. Brown, J. Theor. Biol. 88 (1981) 245.
[17] S.Y. Choi, H.Y. Kwov, O.B. Kwon, J.H. Kang, Biochim. Biophys. Acta 1472 (1999) 651.
[18] R. Kohen, R. Misgav, I. Ginsburg, Free Radic. Res. Commun. 12–13 (1991) 179.
[19] M. Teufel, V. Saudek, J.P. Ledig, A. Bernhard, S. Boulerand, A. Carreau, N.J. Cairns, C. Carter, D.J. Cowley, D. Duverger, A.J. Ganzhorn, C. Guenet, B. Heintzelmann, V. Laucher, C. Sauvage, T. Smirnova, J. Biol. Chem. 278 (2003) 6521.
[20] A.M. Amorini, F. Bellia, V. Di Pietro, B. Giardina, D. La Mendola, G. Lazzarino, S. Sortino, B. Tavazzi, G. Vecchio, E. Rizzarelli, submitted for publication.
[21] J. Szejtli, Chem. Rev. 98 (1998) 1743.
[22] D. Duchene, D. Wouessidjewe, G. Ponchel, J. Control Release 62 (1999) 263.
[23] T. Loftsson, M. Masson, Int. J. Pharm. 225 (2001) 15.
[24] D. La Mendola, G. Vecchio, D. Vitalini, P. Mineo, E. Scamporrino, E. Rizzarelli, J. Supramol. Chem. 1 (2001) 147.
[25] D. La Mendola, S. Sortino, E. Rizzarelli, G. Vecchio, Helv. Chim. Acta 85 (2002) 1633.
[26] R.P. Bonomo, E. Conte, D. La Mendola, G. Maccarrone, F. Nicoletti, E. Rizzarelli, S. Sortino, G. Vecchio, Dalton Trans. (2003) 4406.
[27] P. Mineo, D. Vitalini, D. La Mendola, E. Rizzarelli, E. Scamporrino, G. Vecchio, J. Inorg. Biochem. 98 (2004) 254.
[28] G. Arena, E. Rizzarelli, S. Sammartano, C. Riganò, Talanta 26 (1979) 1.
[29] P. Gans, A. Sabatini, A. Vacca, Talanta 43 (1996) 1739.
[30] Q.G. Mulazzani, M. D'Angelantonio, M. Venturi, M.Z. Hoffman, M.A. Rodgers, J. Phys. Chem. 90 (1986) 5347.
[31] G.V.C. Buxton, L. Greenstock, W.P. Helman, A.B. Ross, J. Phys. Chem. 17 (1988) 513.
[32] J. Rabani, S.O. Nielsen, J. Phys. Chem. 73 (1969) 3736.
[33] I. Sovago, E. Farkas, C. Bertalan, A. Lebkiri, T. Kowalik-Jankowska, H. Kozlowski, J. Inorg. Chem. 51 (1993) 715.
[34] B.G. Malmostrom, B. Reinhammer, T. Vanngard, Biochim. Biophys. Acta 156 (1968) 67.
[35] J. Peisach, W.E. Blumberg, Arch. Biochem. Biophys. 165 (1974) 691.
[36] B. Boka, A. Myari, I. Sovago, N. Hadjiliadis, J. Inorg. Biochem. 98 (2004) 113.
[37] K. Varnagy, J. Szabo, I. Sovago, G. Malandrinos, N. Hadjiliadis, D. Sanna, G. Micera, Dalton Trans. (2000) 467.
[38] D. Sanna, C.G. Agoston, I. Sovago, G. Micera, Polyhedron 20 (2001) 937.
[39] E. Rizzarelli, G. Vecchio, Coord. Chem. Rev. 188 (1999) 343.
[40] G. Vecchio, D. La Mendola, E. Rizzarelli, J. Supramol. Chem. 1 (2001) 87.
[41] P. Mineo, D. Vitalini, D. La Mendola, E. Rizzarelli, E. Scamporrino, G. Vecchio, Rapid Commun. Mass Spectrom. 16 (2003) 722.
[42] D. Behar, C. Czapski, J. Rabani, L.M. Dorfman, H.A. Schwarz, J. Phys. Chem. 74 (1970) 3209.
[43] J. Weinstein, B.H.J. Bielski, J. Am. Chem. Soc. 102 (1980) 4916.
[44] C. Amar, E. Vilkas, J. Foss, J. Inorg. Biochem. 17 (1982) 313.
[45] D. Klug-Roth, I. Fridovich, J. Rabani, J. Am. Chem. Soc. 95 (1973) 2786.