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Copper complexes for biomedical applications: Structural insights, antioxidant activity and neuron compatibility

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ABSTRACT

Copper coordinated with amino acid residues is essential for the function of many proteins. In addition, copper complexed to free L-Histidine, as $[Cu(His)_2]$, is used in the treatment of the neurodegenerative Menkes disease and of cardioencephalomyopathy. This study was aimed to coordinate copper(II) with four small ligands (L-Serine, L-Histidine, Urea and Biuret) and to evaluate structural features, stability, antioxidant activity and neuronal compatibility of the resulting complexes. All complexes were synthesized with $CuCl_2$ and purified by precipitation in alcohol. Elemental composition, X-rays diffraction and FTIR indicated that the complexes were in form of $[Cu(ligand)_2]$ and exhibited tridentate (L-Histidine), bidentate (L-Serine and Biuret) or monodentate (Urea) coordination with copper. UV–Vis absorbance profiles in physiologically relevant solutions and cyclic voltammetry revealed that, contrarily to $[Cu(Urea)_2Cl_2]$ and $[Cu(Biuret)_2Cl_2]$, the $[Cu(Ser)_2]$ and $[Cu(His)_2Cl_2]$ complexes were stable in different media including water, physiological saline and intestinal-like solutions. All complexes and their ligands had antioxidant capacity as evaluated by DPPH (1,1-diphenyl-2,2-picrylhydrazyl) and DPD (*N*,*N*-diethyl-*p*-phenylenediamine) methods, and the $[Cu(His)_2Cl_2]$ complex was the most potent. Neuronal compatibility was assessed through cell viability measurements using cultured neurons derived from mouse P19 stem cells. Although only $[Cu(His)_2Cl_2]$ showed a good neurocompatibility (about 90% at concentrations up to 200 μM), the cytotoxicity of the other copper complexes was lower compared to equivalent concentrations of $CuCl_2$. These findings open new perspectives for the use of these copper complexes as antioxidants and possibly as therapeutic agents for neurodegenerative diseases. Furthermore, study of these complexes may help to improve chelation therapy for copper dysfunctions.

1. Introduction

Metal ions are involved in a wide range of biological functions in the intra and extracellular contexts of life systems [1]. Among metal cations, copper(II) plays an essential role in all living organisms, for instance as a structural component of several enzymes (ex: laccase, ascorbate oxidase, ceruloplasmin, amine oxidases, superoxide dismutase, cytochrome *c* oxidase) [2,3]. In contrast to copper bound to proteins or peptides, unbound copper can be highly toxic due to its ability to generate free radicals *via* Fenton reaction [4]. Copper ions are transported in biological systems by proteins such as ceruloplasmin (carrying about 90–95% of circulating of copper(II)) and serum albumin in mammals, and hemocyanine in crustaceans. Blue-copper proteins, such as ascorbate oxidase and laccase enzymes, are also frequent in vegetables. Also, small biologically active molecules, such as complexes with amino acids, capable to bind and transport copper have various physiological functions and may possess therapeutic properties [5]. The copper complexes Dicuprene, Alcuprin and copper salicylate can act as

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anti-inflammatory agents that help to reduce symptoms (pain and stiffness of rheumatoid arthritis) [6]. Cu(II)ATSM: copper(II)-diacetyl-bis(N4-methylthiosemi-carbazone) and kynurenine precursor of anthralinic acid copper ligand exert antioxidant and cardio-protective effects [7,8], and could be beneficial in the treatment of neurodegenerative disorders such as Parkinson's and Alzheimer's diseases [9]. Ceruloplasmin, the circulatory blue copper protein, possesses antioxidant, cardioprotective and neuromodulatory properties [10]. Its active site consists of six copper atoms, and three of them are coordinated by histidine residues [2]. Despite its therapeutic potential, ceruloplasmin cannot be used as a medication due to its protein nature with immunogenic and hazardous risks of animal origin. It was then hypothesized that small copper complexes may be of interest in certain aspects, being comparable to copper proteins, but with an easier administration and a better regulatory acceptability.

Copper amino acid complexes synthesized decades ago have received increasing attention in the past years, particularly for their potential biomedical application [11–13]. One of the important copper chelators is the amino acid L-histidine (His). Copper bound with His represents an exchangeable pool of copper(II) with albumin in blood [5]. His possesses an imidazole group that can coordinate metal ions in metalloproteins and is also part of the catalytic site of various enzymes [14]. Copper(II)-histidine in blood has a key role in copper transportation and availability for cells and tissues [11]. Dysregulation of copper metabolism leads to various diseases. The transport properties of copper(II)-histidine complex ([$Cu(His)_2$]) led to its application with success in the treatment of Menkes disease that was considered lethal in the first decade of life [11]. This neurodegenerative disorder consists in a deficient absorption of copper(II) due to an inherited genetic dysfunction of the *ATP7A* gene which codes for a transmembrane protein acting as a copper transporter [15]. There was no cure for Menkes disease until mid-seventies when Sarkar et al. [11] showed that early treatment by intravenous injection of copper(II)-histidine complex can greatly increase life expectancy and delay neurodegeneration [14,16]. Furthermore, it has been reported that sub-cutaneous administration of copper(II)-histidine is probably effective in the treatment of infantile hypertrophic cardioencephalomyopathy [17], a fatal disease related to a deficit of the copper-enzyme cytochrome *c* oxidase. However, injectable daily administration of this complex complicates its use and alternative routes of administration are desirable. For instance, in case of cardioencephalomyopathy, the intestinal ATP7A-mediated absorption of copper is not affected; consequently an oral copper administration may be beneficial. Therefore, the isolation of copper(II)-histidine in solid state may be useful for a better oral formulation and for transdermal administration in various copper-related diseases.

L-Serine (Ser) is a non-essential amino acid that can be synthesized by many cells except some cell types, such as neuronal and glial cell subpopulations that rely on the uptake of Ser to support their survival and development [18]. Copper complexed with Ser could be uptaken by cells through Ser transporters in situations of downregulated copper transporters. Urea is an endogenous product of the catabolism of proteins and amino acids, and is present at different concentrations in blood and various organs [19]. Under physiological conditions, urea was shown to act as an antioxidant and as a cardioprotective agent [20]. Biuret is often used together with urea in animal feeding as an excellent non-protein nitrogen additive having a better palatability compared to urea [21]. It contains two acylamino groups and one imino group, a structure compatible with its use as a neutral ligand in various complexes mostly for structural studies or bioanalytical applications (*i.e.* biuret dosage of proteins). Complexes of rare earth metals [22], actinide metals [23,24], and alkaline earth metals [22,24] based on urea or biuret ligands have been described. However, the complexes of transition metals such as copper with these ligands have been rarely reported [25].

This study describes the preparation and characterization of four low molecular weight copper complexes: [$Cu(His)_2Cl_2$], [$Cu(Ser)_2$], [$Cu(Urea)_2Cl_2$] and [$Cu(Biuret)_2Cl_2$] with special emphasis given to aspects related to the stability of these complexes in physiologically relevant media, to their potential antioxidant activity and to their biocompatibility with neuronal cells in view of their eventual use in the treatment of neurological diseases. Furthermore, study of these complexes may help to improve chelation therapy for copper dysfunctions and to better understand copper metabolism in human.

2. Experimental procedures

2.1. Synthesis of copper complexes

All chemicals were reagent grade and were used without further purification. Copper complexes were prepared in isopropanol following Pop et al.'s procedure [25]. The copper(II)-histidine complex was prepared using an 1:2 M ratio of $CuCl_2$:His and a 50:1 v/v isopropanol:H_2O solution. A cold solution (400 mL) of $CuCl_2$ (1.93×10^{-3} mol/L) in isopropanol was added dropwise to an equal volume of aqueous His solution (3.84×10^{-3} mol/L) at pH 6.8, and the mixture was continuously stirred on ice during 45 min. The complexation was done in isopropanol to facilitate the recovery of copper-histidine which precipitates in these conditions. The resulting solid was collected by filtration, washed with cold isopropanol and dried at ambient temperature. The copper-urea and copper-biuret complexes were prepared similarly but using an 1:4 M ratio of $CuCl_2$:ligand. The copper-serine complex was prepared in ethanol using an 1:2:1 M ratio of $CuCl_2$:L-Ser:NaOH. A cold solution (15 mL) of $CuCl_2$ (2.64×10^{-2} mol/L) in ethanol was added dropwise to a solution of 30 mL Ser (5.19×10^{-2} mol/L) in ethanol containing sodium hydroxide (0.01 mol/L). The mixture was stirred on ice during 30 min, and the resulting solid was filtered, washed with ethanol and dried at room temperature.

2.2. Structural characterization

The content of carbon (C), hydrogen (H) and nitrogen (N) was determined by the Dynamic flash combustion method which is based on complete oxidation of samples, using an EAS1108 (Fisons instruments SpA) and an ECS4010 (Costech Co. California. USA). Melting points were measured using open capillary tubes on a Gallenkamp melting point apparatus (London, England). Analysis by X-ray diffraction was done with a Bruker X8 Venture Metal jet diffractometer (Geesthacht, Germany). The experimental parameters for X-ray analysis are presented in Table 1S (supplementary data). Single crystals were obtained by slow evaporation of alcoholic solution of copper complexes during 3 days. The crystals were kept at 100 K during data collection. The samples were exposed to GaKα X-rays with a wavelength of 1.34 Å and analyzed at an angular range of 2θ from 10 to 121°. The structure was solved using the Olex2 software [26], with the SHELXT structure solution program [27] using Direct Methods and the SHEXLXL [28] refinement package with the Least Squares minimization method. Data collection and refinement parameters are presented in Section 3.2. The infrared (IR) spectra of the complexes and of their ligands were recorded on a Thermo-Nicolet 6700 (Madison WI USA) FTIR spectrophotometer (4000–400 cm^{-1}) by using potassium bromide pellets (2%).

2.3. Stability in different media

2.3.1. Spectrophotometric measurements

Solutions of 20 or 40 mM of His, Ser, Urea, Biuret and their copper (II) complexes were prepared in water, in physiological saline (0.9% NaCl), Simulated Gastric Fluid (SGF: 0.26% HCl, 0.2% NaCl, pH 1.2) and in Simulated Intestinal Fluid (SIF: 0.68% KH_2PO_4, 0.0615% NaOH, pH 7.2). The absorbance spectra (360–960 nm) were taken after 2 h of incubation at room temperature. Time stabilities of the complexes were

investigated by mesuring the absorbance values at 37 °C and at constant wavelenghts, from 0 to 48 h. Measurements were done using a Visible Ultrospec 100 Pro (USA) Spectrophotometer.

2.3.2. Electrochemical measurements

A sealed three necked flask was used for electrochemical measurements. The working electrode was a 3 mm diameter glassy carbon electrode (BASi), the reference electrode was a NaCl saturated Ag/AgCl electrode and the counter electrode was either copper wire or platinum mesh (AlfaAesar). The electrochemical solutions contained copper complexes freshly prepared in physiological saline (0.9% w/v NaCl in deionized water) and were purged by argon for 5 min. All measurements were carried out under argon atmosphere.

2.4. Antioxidant capacity

2.4.1. DPD (N,N-diethyl-p-phenylenediamine) colorimetric method

The DPD reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA). Reactive oxygen species (ROS) were generated by electrolysis (10 mA, 400 V, 3 min [29]) of 3 mL of modified Krebs-Henseleit (KH) buffer solution (in mM: NaCl 118, KCl 4.8, $CaCl_2$ 1.8, $MgSO_4$ 0.86, KH_2PO_4 1.2, $NaHCO_3$ 2.54, glucose 11 and EDTA 0.027). The electrolysis cell contained 2.7 mL of KH buffer in the presence of 0.3 mL of different concentrations of the copper complex or ligand. A volume of 0.2 mL of the electrolyzed (ELS) solution (ELS KH buffer + complex or ligand) was then added to 0.8 mL of 25 mg/mL DPD solution. The electrolysis-generated ROS react rapidly with DPD forming an oxidation product with a specific absorbance at 515 nm [20,30]. In the presence of antioxidant molecules, ROS may be neutralized and thus unavailable to react with DPD. The antioxidant capacities of ligands and complexes were expressed as a percentage of ROS still remaining in the ELS solution and was calculated according to the following equation:

$$\%ROS = A_{sample}/A_{100\%ROS} \times 100$$

where $A_{100\%ROS}$ is the absorbance of ELS KH buffer containing DPD (100% amount of ROS in solution) and A_{sample} is the absorbance of ELS KH buffer containing DPD and in the presence of the copper complex or ligand.

2.4.2. 1,1-Diphenyl-2,2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH is a stable free radical (DPPH·) characterized by an absorption band at about 517 nm. In the presence of a substance that can donate a hydrogen atom, DPPH is reduced by losing its violet color. The DPPH assay was performed in conditions of Marinova et al. [31]. The DPPH reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA). A solution of 0.5 mM DPPH was prepared and its absorbance was approximately 0.7 at 517 nm. A volume of 0.2 mL of sample (copper(II) complexes or ligands) was added to 1.8 mL of DPPH solution. After 30 min, the absorbance was measured at 517 nm. The percentage of scavenged DPPH radical was calculated as follows:

$$\%DPPH\cdot = (A_{br} - A_{ar})/A_{br} \times 100$$

where A_{br} is the absorbance before reaction and A_{ar} is the absorbance after reaction with the antioxidant (complex or ligand).

2.5. Neuronal cell culture and treatment

Mouse P19 embryonic stem cells were differentiated to neurons by exposure to 0.5 μM *all-trans*-retinoic acid for 4 days [32,33]. On day 4, an amount of 3.8×10^5 neurons were seeded in gelatinized 12 well-plates containing supplemented Neurobasal (SNB) medium [Neurobasal containing 2% v/v B27 supplement (Fisher Scientific, St-Laurent, QC, Canada), 0.5 mM L-glutamine, 50 U/mL penicillin and 50 U/mL streptomycin].

Neuron treatment with His, Ser, urea or biuret ligands as well as with their copper(II) complexes and $CuCl_2$ began on day 4, concomitantly with cell seeding into the SNB medium. The eight studied agents (ligands and corresponding Cu(II) complexes) were freshly solubilized in phosphate buffer saline (PBS) as 10-fold concentrated stock solutions and added individually to the culture medium at the indicated final concentrations. After 48 h of incubation (37 °C, 5% CO_2), cultures were analyzed for cell viability. Control cultures contained only the cells in SNB medium.

2.6. Cell viability

Cell viability was determined by the Neutral Red (NR) uptake assay as recently described [34]. One mL of freshly prepared NR solution (138 μM in 20 mM HEPES, 140 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM D-glucose, pH 7.2) was added to each culture well prewashed delicately with PBS. After 2 h incubation (37 °C, 5% CO_2), NR solution was removed and cultures were washed rapidly with 1% formaldehyde-l% calcium chloride. Then, 1 mL of NR eluent (EtOH:-H_2O:acetic acid 50:49:1) was added to extract the NR from cells. After 10 min agitation at room temperature, the absorbance was read at 540 nm using an ELX800 UV universal microplate reader. The 100% viability was established with control (untreated) cultures. Assays were done in duplicate using three independent cell cultures.

3. Results and discussion

3.1. Chemical analysis of copper complexes

The elemental analysis of copper complexes prepared in isopropanol as well as their physical properties (color, molecular weight and melting point) are presented in Table 1. The results of elemental

Table 1
Analytical and physical data of the Cu (II) complexes of Ser, His, Urea and Biuret.

Compound	Formula	Elemental analysis[a] % found (% calc.)			Color	MW[b] g/mol	MP °C
		C	H	N			
$[Cu(His)_2Cl_2]$	$C_{12}H_{18}Cl_2CuN_6O_4$	30.74 (32.34)	3.96 (4.05)	17.96 (18.29)	Light blue	444.00	185 ± 1.6
$[Cu(Ser)_2]$	$C_6H_{12}CuN_2O_6$	26.28 (26.49)	4.35 (4.46)	10.08 (10.33)	Navy blue	271.72	186 ± 0.6
$[Cu(Urea)_2Cl_2]$	$C_2H_8Cl_2CuN_4O_2$	9.33 (9.42)	3.23 (3.14)	23.61 (21.99)	Blue-green	254.56	161.6 ± 2.3
$[Cu(Biuret)_2Cl_2]$	$C_4H_{10}Cl_2CuN_6O_4$	14.59 (14.11)	2.98 (2.94)	25.02 (24.67)	Blue-green	340.61	171.6 ± 1.1

MP, melting point.
[a] The calculated values of elemental analysis are given in parentheses.
[b] The MW were determined from the formula calculated from elemental analysis.

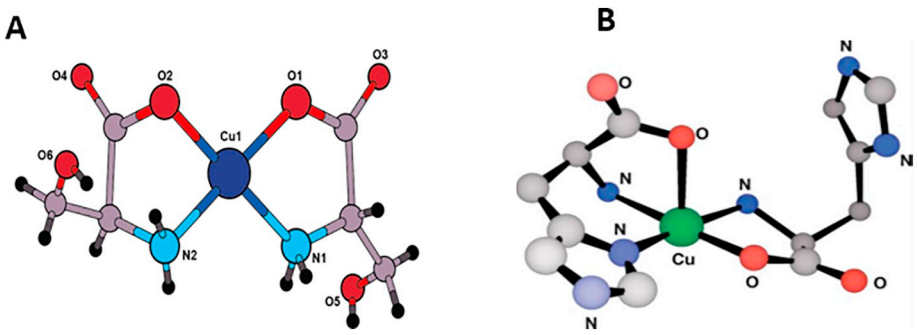

Fig. 1. Structure of $[Cu(Ser)_2]$ bidentate obtained from crystal X-ray diffraction (A) and of tridentate $[Cu(His)_2]$ complex as elucidated in [5].

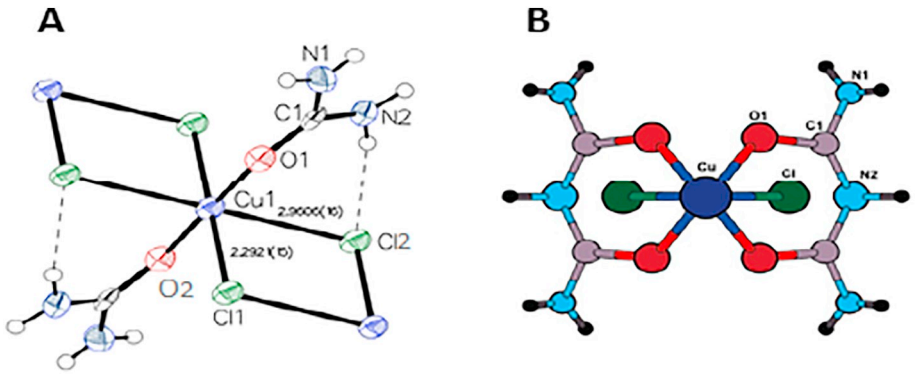

Fig. 2. Structure of $[Cu(Urea)_2Cl_2]$ obtained from crystal X-ray diffraction (A) and molecular structure proposed for $[Cu(Biuret)_2Cl_2]$ complex (B).

analysis as well as the proposed chemical formula were in good agreement with the expected stoichiometry (Table 1). The melting points of copper complexes were similar to those of previous investigations [14,35].

The chemical formula of $[Cu(Ser)_2]$ complex was corroborated by mass spectroscopy (data not shown). In dry phase (as powders), the complexes are stable in air, easily soluble in water, and insoluble in alcoholic media.

3.2. X-ray crystal structure of copper complexes

Only $[Cu(Ser)_2]$ and $[Cu(Urea)_2Cl_2]$ generated adequate crystal for X-ray analysis. The $[Cu(His)_2]$ and $[Cu(Biuret)_2Cl_2]$ data were from references Deschamp et al. [36] and Freeman et al. [37], respectively.

The X-ray structural analysis of single crystals of $[Cu(Ser)_2]$ complex revealed two serine molecules each bound to the copper ion in a bidentate manner (Fig. 1A). In amino acids with non-coordinating side chains, the more common mode of coordination is bidentate chelation [38]. The structure of $[ML_2]$ species showed that two amino nitrogen (N_{am}) and two carboxyl oxygen ($O_{carboxyl}$) are coordinated to the copper (II) ion (Fig. 1A). The mode of coordination around the copper(II) center is a square pyramid that gives rise to the more thermodynamically stable five-membered chelate rings (Fig. 1A). The length values of the Cu—O (1.97 Å) and Cu—N (2.00 Å) bonds in this study are in good agreement with the previously published theoretical values for this complex [39,40]. According to the elemental analysis (Table 1), chloride atoms are not present in structure of $[Cu(Ser)_2]$ complex, differing from the other copper complexes of this study. This could be due to several factors influencing the structural arrangement, including metal-ligand bond strength, stereochemistry of the complex and proton displacement reactions [41].

In the case of $[Cu(His)_2Cl_2]$ prepared in conditions similar to those described for $[Cu(Urea)_2Cl_2]$ by Pop et al. [25], we did not obtain single crystals. However, the X-ray patterns of our preparation showed structures similar to those of other related complexes previously described [36,42].

A neutral five-coordinated complex ($[Cu(His)_2]$) was described by Deschamps et al. [36]. This study proposed a distorted square pyramidal geometry with bidentate and tridentate L-histidine ligands (Fig. 1B). On the other hand, the crystal X-ray diffraction structure of another $[Cu(His)Cl_2]$ complex obtained by Bujacz et al. [42] showed copper coordinated (semi-coordination) by one histidine only, in a bidentate manner involving the carboxyl ($O_{carboxyl}$) and the amine (N_{am}) from the main chain group of His. Two additional chloride anions completed the square coordination of the central Cu^{+2}. The structures reported by Deschamps et al. [36] and Bujacz et al. [42] are not in agreement with the chemical formula ($C_{12}H_{18}Cl_2CuN_6O_4$) found from elemental analysis in the present study. We have obtained a different copper histidine complex consistent with two moleculse of His and two atoms of chloride bound to the central Cu^{2+} cation as $[Cu(His)_2Cl_2]$.

The crystallographic investigation of the urea complex, $[Cu(Urea)_2Cl_2]$, revealed a chain structure, with the chloride ions acting as bridges as shown in Fig. 2A. The Cu(II) ions are hexacoordinated by two chloride bridges and by the oxygen atoms from two urea molecules, showing an elongated octahedral stereochemistry. The apical positions seem occupied by two semi-coordinated chloride bridging ligands (Cu1-Cl1 = 2.96 Å); the equatorial plane consists of two urea ligands and two chloride bridges (Cu1-l1 = 2.29 Å) (Table 2).

The Cu1-Cl1 distance (2.96 Å) seems too large for a covalent binding (r_{Cl} = 0.99 Å) and is more consistent with an ionic bonding ($r_{Cl}-$ = 1.84 Å, $r_{Cu}{}^{2+}$ = 0.81 Å) [42]. Although urea has two potential electron donor atoms in its structure ($O_{carbonyl}$ and N_{am}), copper is frequently coordinated only through the oxygen atom in a monodentate pattern and the C=O—M angle is often smaller than 180° [43,44]. To the best of our knowledge, the crystal structure ascribed to $[Cu(Urea)_2Cl_2]$ is novel.

As for $[Cu(His)_2Cl_2]$, the crystallisation of $[Cu(Biuret)_2Cl_2]$ has not been successful in isopropanol. Therefore, a possible structure for this complex is proposed (Fig. 2B) on basis of the crystal X-ray diffraction previously described by Freeman et al. [37], which is in accordance

Table 2
Bond lengths for $[Cu(Urea)_2Cl_2]$.

Atom	Atom	Length (Å)
Cu1	$Cl1^1$	2.9605(16)[a]
Cu1	Cl1	2.2921(15)
Cu1	$Cl1^2$	2.9605(16)
Cu1	$Cl1^3$	2.2921(15)
Cu1	O1	1.955(4)
Cu1	$O1^3$	1.955(4)
O1	C1	1.262(8)
N1	C1	1.324(8)
N2	C1	1.345(9)

[a] The numbers in parentheses in this column refer to the unit cell defined by Bernal and Fowler [65].

Table 3
Structural features of the first coordination shell of copper for the four copper (II) complexes.

Complexes	Coordination binding	Involved atom	Distance Cu–X (Å)	Reference
$[Cu(His)_2Cl_2]$	Tridentate	N_{am}	2.003	[11][a]
		N_{im}	1.984	
		$O_{carboxyl}$	2.277	
$[Cu(Ser)_2]$	Bidentate	N_{am}	2.001	This work
		$O_{carboxyl}$	1.972	
$[Cu(Urea)_2Cl_2]$	Monodentate	$O_{carbonyl}$	1.955	This work
$[Cu(Biuret)_2Cl_2]$	Bidentate	$O_{carbonyl}$	1.982	[44]

[a] Reference describes $[Cu(His)_2]$.

with our elemental and FTIR analyses (Table 1 and *cf.* Section 3.3 below). In the $[Cu(Biuret)_2Cl_2]$ complex, the copper atom has a coordination number 6 and a tetragonally distorted octahedral environment [37]. Its binding to the four oxygen atoms of ligands is covalent, as indicated by the Cu−O bond lengths while the Cu−Cl distances revealed ionic bonding between Cl^- and Cu^{2+} ions, similar to those found for urea copper complexes [37]. The biuret molecules act as bidentate chelates *via* their oxygen atoms [37]. The coordination data and structural features of the four copper(II) complexes are summarized in Table 3.

3.3. FTIR characterization of copper complexes and ligands

FTIR pattern of $[Cu(His)_2Cl_2]$ showed changes in the positions and profiles of some bands compared to that of free His (Fig. 3A, B). Generally, in amino acids, NH stretching vibration is observed in 3130–3030 cm^{-1} region [45]. Theoretically, in metal complexes, their NH stretching vibration (νNH_3^+) should disappear due to coordination [46]. In the metal complexes, however, a band was observed at 3300 cm^{-1}, which could arise from other vibrations appearing in this region [47]. Free His shows the antisymmetric COOH stretching frequency at 1634 cm^{-1}(Fig. 3B), and $[Cu(His)_2Cl_2]$ exhibits this band at 1616 cm^{-1} (Fig. 3A). This shift in the stretching frequency is consistent with the findings of Sarkar and Wigfield [48] who observed a similar shift for $[Cu(His)_2]$ and other Cu(II)-amino acid complexes. These results indicate the participation of the amino acid groups in copper coordination and the tridentate chelation pattern of $[Cu(His)_2Cl_2]$ complex.

Fig. 3 (C, D) shows the FTIR spectra for $[Cu(Ser)_2]$ and serine. The bands between 3444 and 3259 cm^{-1} assigned to the NH stretching vibration in the spectrum of the amino acid are observed almost at the same respective frequencies in the $[Cu(Ser)_2]$ spectrum. The NH stretching vibration bands of Ser (at 3346 and 3258 cm^{-1} respectively) are shifted to higher wavenumbers (3481–3398 cm^{-1}) in metal complex [49]. These results clearly suggest deprotonation of the NH_3^+ group of the Ser molecule and binding to the metal ion through its nitrogen atom [50]. On the other hand, the Ser absorption bands at 1640 and 1601 cm^{-1} (corresponding to the C=O stretching bond) are shifted to the lower wavenumbers (1631 and 1587 cm^{-1}) in the $[Cu(Ser)_2]$ spectrum. Moreover, the symmetric (M-N) stretching band appearing at 523 cm^{-1} in this study is in agreement with previous studies [49]. Altogether, these observations indicate that Ser binds the metal ion *via* its carboxyl and amino groups.

Fig. 3 (E, F) presents the FTIR spectra obtained for urea and $[Cu(Urea)_2Cl_2]$. The bands between 3444 and 3259 cm^{-1} assigned to the NH stretching vibration in the spectrum of urea are observed almost at the same respective frequencies in the $[Cu(Urea)_2Cl_2]$ spectrum. However, the intense absorption bands for urea at 1680 and 1602 cm^{-1}, corresponding to the C=O stretching and to the NH_2 bending vibrations, are shifted to lower frequencies (1616 and 1576 cm^{-1}) in the spectrum of the complex. This may indicate a possible implication of oxygen from the carbonyl group of urea in the coordination with copper (II) ions [43]. This is also supported by the 419 cm^{-1} band corresponding to M-O vibration [20,44] in $[Cu(Urea)_2Cl_2]$ spectra.

The FTIR spectra of biuret and $[Cu(Biuret)_2Cl_2]$ complexes are shown in Fig. 3 (G, H). The bands ascribed to the NH stretching vibration observed in the $[Cu(Biuret)_2Cl_2]$ spectrum are similar but not identical to the bands in the range of 3458–3016 cm^{-1} observed in the spectrum of free biuret. This might indicate that probably not all nitrogens are involved in the formation of the complex. A symmetric vibration frequency of C=O stretching appears near 1718 cm^{-1} in the biuret spectrum (Fig. 3H). When coordination occurs, a shift of the C=O stretching mode to lower wavelength was reported [22] indicating that coordination could be produced with participation of the oxygen [43]. In fact, a vibration band in the $[Cu(Biuret)_2Cl_2]$ complex was detected at 1679 cm^{-1} (Fig. 3H). As in the case of $[Cu(Urea)_2Cl_2]$ complex, the presence of a 490 cm^{-1} band ascribed to M−O vibration suggests the participation of oxygen in a coordinative complexation between biuret and copper(II) ion fitting well previous data of Wang et al. [22]. Overall, the FTIR results are in accordance with the structures depicted (Figs. 1, 2).

3.4. Stability of copper complexes in physiologically-relevant media

Evaluation of the stability of copper complexes in physiologically relevant conditions will help in the design of their pharmaceutical formulations. As oral administration of copper complexes will be eventually sought in treatment of cardioencephalomyopathy and in Menkes disease, it was of interest to explore their stability in conditions simulating the gastic and intestinal system. Absorbance spectra and cyclic voltammetry were used as indications of structural conformation of studied complexes in various media. Fig. 4 shows the UV–Vis spectra of the copper(II) complexes incubated for 2 h, at room temperature, in H_2O, SGF (pH 1.2), SIF (pH 7.2), and in physiological saline solution 0.9% NaCl (pH 7.2).

3.4.1. Stability at room temperature

The spectra of $[Cu(His)_2Cl_2]$ in H_2O, in physiological saline (0.9% NaCl) and in SIF showed maximal absorption at 620–640 nm (Fig. 4A). The absorption in SIF was about twice than that observed in H_2O and in 0.9% NaCl, which could be attributed to the changes of pH. The addition of 20 mM copper(II)-histidine in H_2O or in 0.9% NaCl media decreased the pH to about 5.32 whereas the final pH of the complex in SIF was 6.8, probably due to better buffering capacity. According to Deschamps et al. [11], distribution of the copper(II) histidine species differs as a function of pH. For instance, $[MHL_2]$ was found at pH 5 and below whereas $[ML_2]$ is found at pH 7. When the pH value is low (pH ≤5), the nitrogen of imidazole group (similarly to carboxyl) is protonated and thus it is no more involved in binding to copper(II); therefore 50% or more of copper may be unbonded. The presence or absence of the hydrogen atom would have influence on the absorbance properties. In SGF, absorption was maximal at 750 nm probably due to unbonded

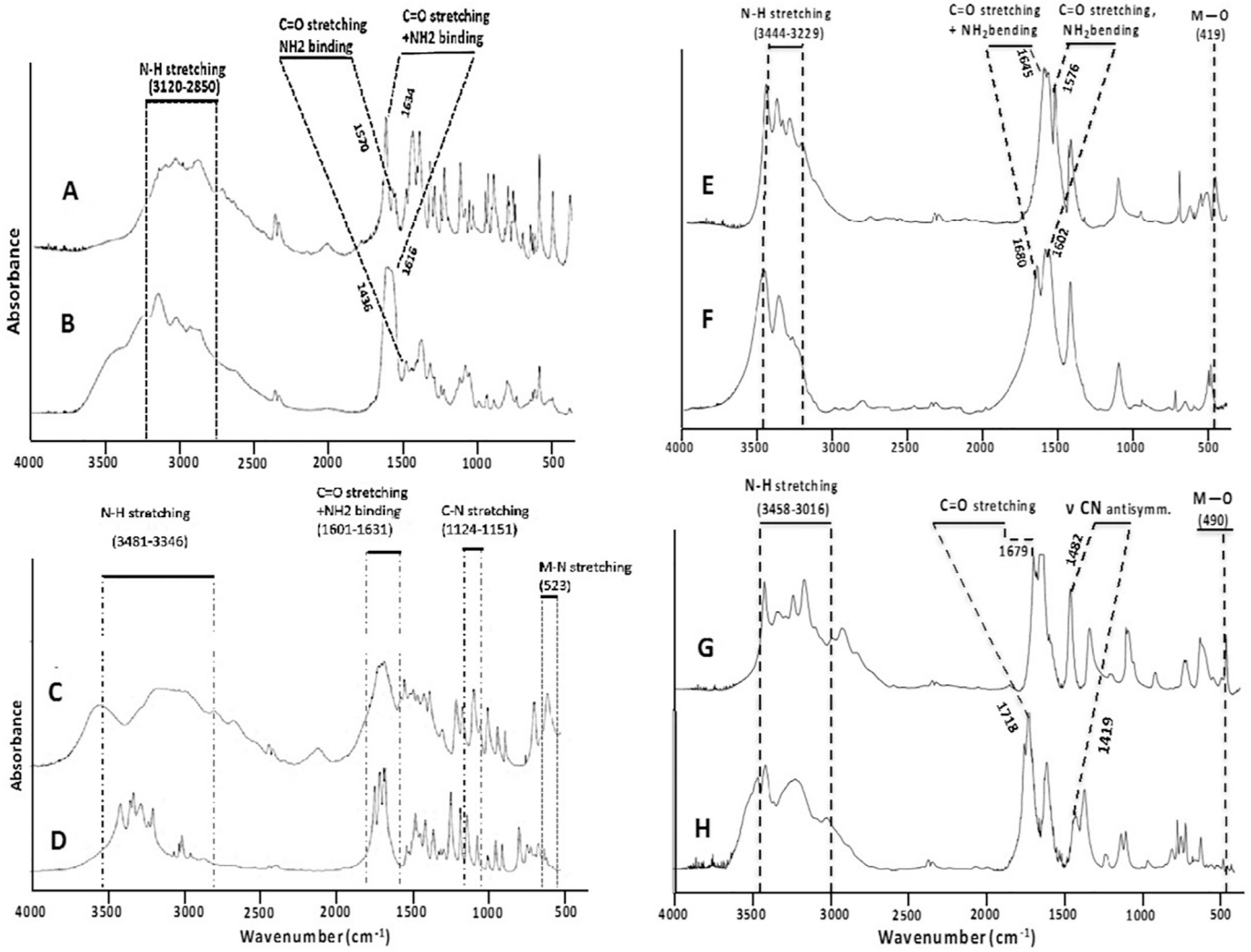

Fig. 3. FTIR spectra of $[Cu(His)_2Cl_2]$ (A), His (B), $[Cu(Ser)_2]$ (C), Ser (D), $[Cu(Urea)_2Cl_2]$ (E), urea (F), $[Cu(Biuret)_2Cl_2]$ (G) and biuret (H) in the wavenumber range of 4000–400 cm^{-1}.

copper in view of Deschamps et al. [11] suggesting that at pH ≤3.7 most of the remaining ligand is unbound. At physiological pH, the major structure proposed for $[Cu(His)_2]$ in aqueous solution was $[ML_2]$ [11]. Upon increase of pH (pH > 7), the amine group is deprotonated to give a negatively charged ligand, and the addition of HO^- or the ionization of pyrrole-like nitrogen in the imidazole group may induce the complex to adopt, in basic medium, a $[ML_2(OH)]$ form which in our case may be $[Cu(His)_2OH]$.

The addition of $[Cu(Ser)_2]$ did not change the pH of the four media. The spectra of this complex were similar in H_2O, NaCl 0.9%, and SIF with maximal absorption at 630 nm (Fig. 4B). However, in SGF, similarly to copper(II) histidine, the maximal absorbtion was at 750 nm, which could be attributed to protonation of coordinating groups and eventually to unbonded copper.

The $[Cu(Urea)_2Cl_2]$ and $[Cu(Biuret)_2Cl_2]$ complexes showed a strong band in the visible light, similarly to amino acid copper complexes in acidic conditions. These data fit well those of Corradi [43]. The $[Cu(Urea)_2Cl_2]$ spectra (Fig. 4 C) were similar in H_2O, 0.9% NaCl (neutral) and in acidic SGF, with maximal absorption at 810 nm. In SIF, the maximal absorbency was slightly shifted to 830 nm and with a markedly lower signal intensity (about 50% from that in 0.9% NaCl) probably due to the presence of phosphate in SIF which can form a precipitate by the displacement of the two chloride anions generating copper(II) urea phosphate complex [51]. Similarly, the spectra of $[Cu(Biuret)_2Cl_2]$ (Fig. 4D) in H_2O, 0.9% NaCl and SGF exhibited maximal absorption at 810 nm and with various intensities of the signal in each medium. In SIF, the formation of a green precipitate was observed with $[Cu(Biuret)_2Cl_2]$, which may explain the disparition of the signal at 810 nm. An absorption still observed in the 800–830 nm region (copper region) suggests a degradation of the complex and the presence of free copper ions.

3.4.2. Cyclic voltammetry

Cyclic voltammetry measurements of each complex were carried out in physiological saline. Each complex (2 mM) showed a quasi-reversible response (Fig. 5). A background voltammogram in physiological NaCl saline (Fig. 5A) without the presence of a complex is shown for reference. The onset of the reduction, and mid-potential value of $CuCl_2$, $[Cu(Biuret_2)Cl_2]$ and $[Cu(Urea_2)Cl_2]$ are quite similar, suggesting a small difference in their reduction potential (Fig. 5B). When compared with $CuCl_2$, urea and biuret copper complexes, the onset of reduction of both $[Cu(Ser_2)]$ and $[Cu(His_2)Cl_2]$ is cathodically shifted, and displays weakly defined peak current values making assignment of a mid-potential value difficult (Fig. 5C). If the onset of reduction of each of the complexes is taken as an indication of stability, then based on the results from voltammetric measurements $[Cu(Ser_2)]$ and $[Cu(His_2)Cl_2]$ have higher stabilities when compared with $CuCl_2$, $[Cu(Biuret_2)Cl_2]$ and $[Cu(Urea2)Cl_2]$ [52], in accordance whit absorbance profiles.

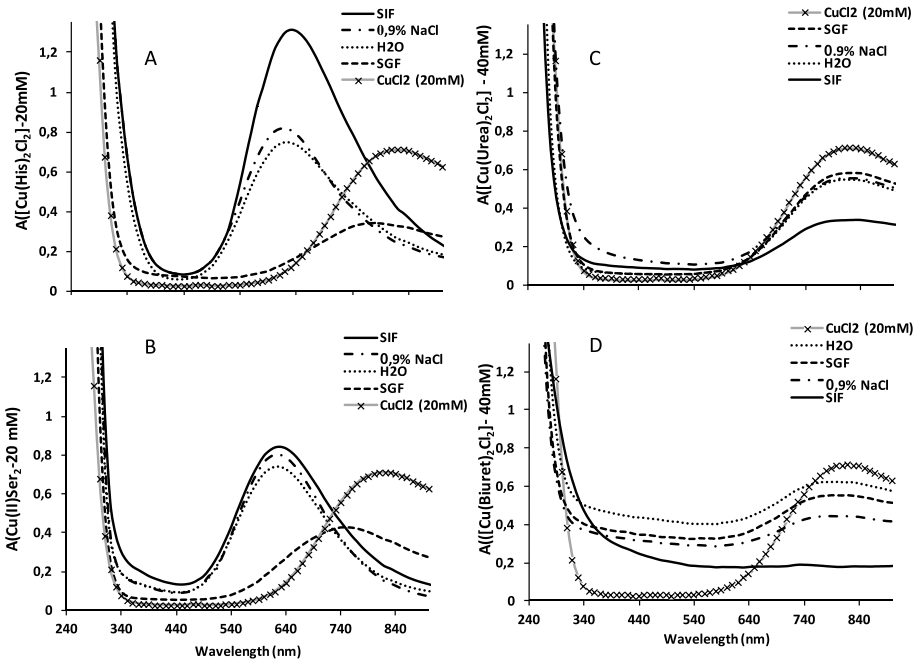

Fig. 4. UV–Vis absorbance spectra of $[Cu(His)_2Cl_2]$ [A], $[Cu(Ser)_2]$ [B], $[Cu(Urea)_2Cl_2]$ [C], and $[Cu(Biuret)_2Cl_2]$ [D]in different physiologically-relevant media: H_2O, 0.9% NaCl (pH 7.4), SGF (pH 1.2), and SIF (pH 7.2), after 2 h at room temperature. The spectrum of aqueous solution of $CuCl_2$ is included for comparison.

According to Deschamps et al. [11], the tridentate ligation confers greater stability compared to bidentate and monodentate ligations. Although the mode of coordination of Ser around copper (II) center is bidentate, the relative stability of the serine complex compared to that of the urea and biuret complexes could result from the thermodynamically stable five-membered chelate ring architecture. The differences in $[Cu(His)_2Cl_2]$ spectra are due to the distribution of the copper(II) histidine species at different pH values [11].

3.4.3. Stability at 37 °C

The stability of the copper complexes in the different media was also evaluated by absorbance over 48 h at the physiological temperature of 37 °C (Fig. 6). The absorption values at 630 nm of the histidine copper complex (Fig. 6A) in SIF, in water and in 0.9% NaCl were similar: the values gradually decreased with time up to 24 h and then remained constant. The absorption value in SGF was constant and low throughout the whole period. The absorption values of the serine copper complex (Fig. 6B) at 620 nm were similar in H_2O and 0.9% NaCl and constant up to 48 h. In contrast, the absorbance in SIF decreased rapidly from 1 to 0.3 after 2 h (probably due to the phosphate ions). The absorbance value was low initially and remained low during the whole interval up to 48 h, indicating a lower stability of $[Cu(Ser)_2]$ in SIF at 37 °C compared to room temperature.

In the case of the urea and biuret complexes, the spectral profiles at 37 °C (Fig. S1 in Supplementary data), like those at room temperature (Fig. 4), showed maximal absorbencies at 810–830 nm due to free copper(II). It was not surprising that the absorbances at this wavelength range remained almost constant in view of the instability of these complexes (Supplementary data, Fig. S2).

In conclusion, compared to urea and biuret copper(II) complexes, the amino acid, His and Ser, copper(II) complexes presented relatively higher stability at room temperature as well as over 5 h at 37 °C. The highest stability was seen for copper(II)-serine in H_2O and 0.9% NaCl media.

3.5. In vitro antioxidant capacity

Given the complexity of oxidative processes and the diversified hydrophilic and hydrophobic nature of the antioxidants, there is no a unique well-defined method for quantitative measurement of antioxidant activity. In most cases it is necessary to combine the responses of different and complementary tests in order to have an indication of the antioxidant capacity of a sample [53].

Practically, according to the reactions involved in the scavenging processes, the antioxidants can neutralize free radicals by two different mechanisms: Hydrogen Atom Transfer (HAT) and Single Electron

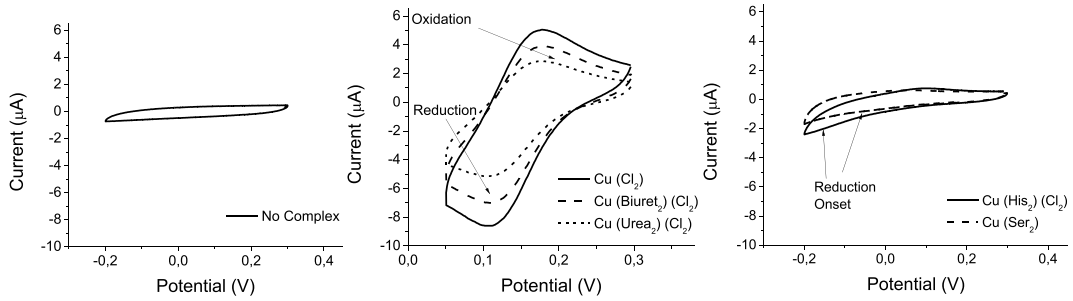

Fig. 5. Cyclic voltammetry measurements of (A) background response of glassy carbon electrode (blank), (B) $CuCl_2$ (control), $[Cu(Biuret_2)Cl_2]$ and $[Cu(Urea_2)Cl_2]$ and (C) $[Cu(Ser_2)]$ and $[Cu(His_2)Cl_2]$, in physiological NaCl saline. A saturated Ag/AgCl reference electrode and a scan rate of 20 mV/s was used for all measurements.

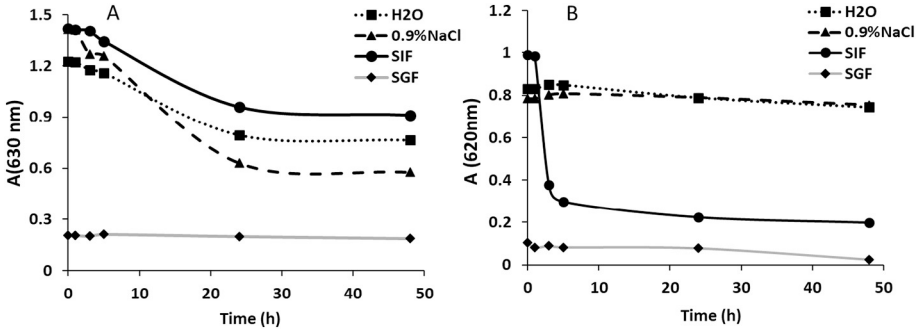

Fig. 6. Time course of absorbance values of [Cu $(His)_2Cl_2$] (A) and $[Cu(Ser)_2]$ (B) complexes at their λ_{max} and 37 °C, and in different physiologically-relevant media (20 mM final concentration). Readings were taken at 0, 1, 2, 3, 5, 10, 24 and 48 h, and values are presented as means of three separate determinations. The 360–960 nm individual absorbance spectra are given in Fig. S1, in Supplemental data.

Transfer (SET) [54]. HAT–based methods measure the ability of an antioxidant to scavenge free radicals by H-atom donation. SET − based methods measure the capacity of an antioxidant to transfer one electron to reduce an oxidant, often associated to a change of color [29,55]. In the present study, the antioxidant capacities of the copper complexes and their ligands were evaluated by using two types of assays, the DPPH assay for the HAT mechanism and the DPD assay for the SET mechanism. The electrolysis of KH buffer (10 mA, 400 mV, 1 min) can generate ROS such as superoxide anion ($\cdot O_2^-$), singlet oxygen ($^1O_2$), hydroxyl radical (·OH) and their by-products: hydrogen peroxide (H_2O_2) and hypochlorite ion (ClO^-) [53]. The generation of ROS is determined by the percentage of the oxidation of DPD. In the presence of the copper complexes or ligands, part of ROS may be scavenged leaving less of these species available to oxidize DPD.

Fig. 7 shows that all compounds have a certain capacity to scavenge ROS compared to the control condition (100% ROS). Kohen et al. reported that histidine and its derivatives having an imidazole group have an antioxidant activity due to hydrogen donation [56]. $[Cu(His)_2Cl_2]$ complex, at concentrations equal to or > 0.02 M, presented a higher scavenging activity than that of the ligand alone and of the other copper complexes (Fig. 7A). Differently, serine and urea copper complexes presented ROS scavenging capacities close to that of their free ligand (Fig. 7B, C). Interestingly, the antioxidant activity of $[Cu(Biuret)_2Cl_2]$ was about twice that of its free ligand (Fig. 7D) and about the same activity as that of urea and of $[Cu(Urea)_2Cl_2]$. The amide groups in the biuret and nitrogens of imidazole groups of His may act as efficient electron donating groups to neutralize ROS.

The antioxidant capacity of copper(II) complexes and of their ligands were also evidenced by the DPPH assay which is recommended for compounds containing SH, NH and OH groups [57,58]. Fig. 8 shows that the eight compounds can scavenge DPPH·. Among the amino acids and their copper complexes, $[Cu(His)_2Cl_2]$ exhibited the highest antioxidant capacity in comparison to histidine itself (Fig. 8A) and to the other copper complexes and ligands (Fig. 8B, C, D). The Biuret and [Cu $(Biuret)_2Cl_2$] (Fig. 8D) also exhibited antioxidant capacity but at concentration 10 to 20-fold higher than those of $[Cu(His)_2Cl_2]$. The ROS scavenging activities of $[Cu(His)_2Cl_2]$ and $[Cu(Ser)_2]$ complex appear as concentration-dependent. Differently, in the case of $[Cu(Urea)_2Cl_2]$ and of $[Cu(Biuret)_2Cl_2]$, as for their ligands, there were two steps: an initial strong scavenging capacity exerted at low concentration (0–0.5 M) followed by another step when the scavenging capacity was almost maintained at high concentration (up to 5 M, Fig. 8C, D). It has been reported that the reaction between an antioxidant and DPPH· apparently occurred in two steps [59,60]: (i) a fast step (1–2 min) essentially consisting in the abstraction of the most labile H-atoms from the antioxidant, and (ii) a slow neutralization step (30 min) reflecting a remaining activity on the oxidation/degradation products derived from the first step. It is now reported that Cu(II) complexes have the capacity to scavenge DPPH radical by hydrogen donation from the NH-groups, with the mention that the ascorbic acid, an antioxidant used as a standard (0.002–5 M), showed stronger antioxidant activity than that of synthesized copper complexes (data not shown). Worth to mention is

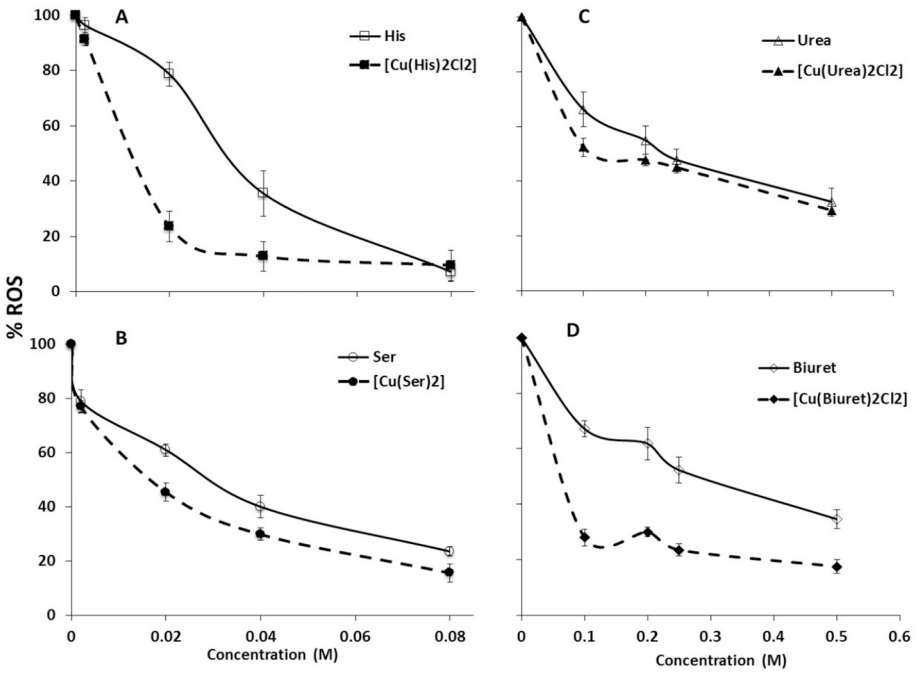

Fig. 7. The ROS scavenging capacity of copper complexes and of free ligands determinated by the DPD assay. The antioxidant capacities are inversely related to the percentages of remaining ROS after electrolysis of KH solutions in the presence of the indicated concentrations of copper(II) complexes or ligand. Notice the different concentration scales between A, B (0.002–0.08 M) and C, D (0.1–0.5 M). Values are presented as mean ± SEM (n = 3).

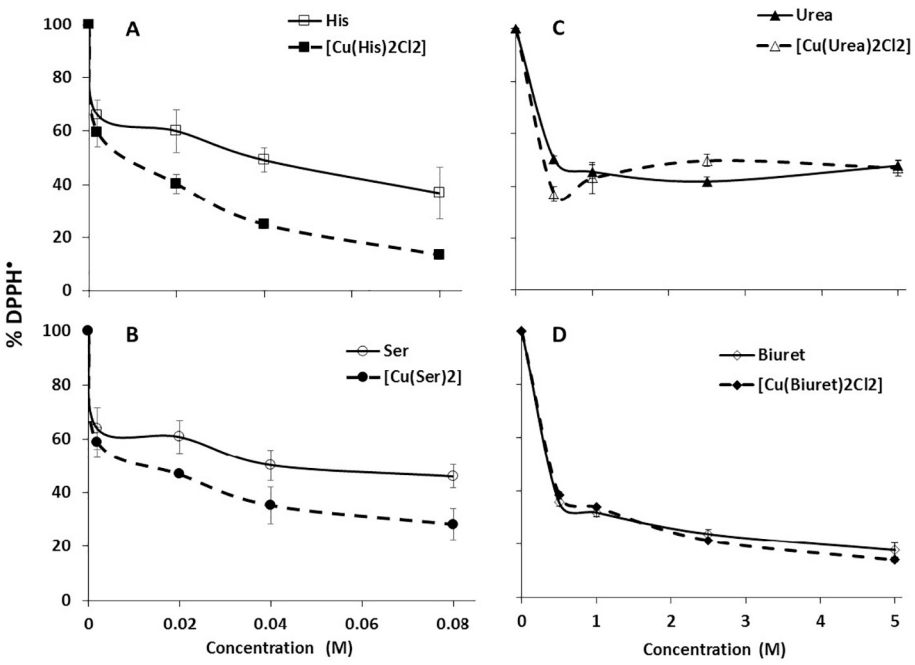

Fig. 8. The free radical scavenging capacity of copper complexes and free ligands evaluated by the DPPH assay. The antioxidant capacities are inversely related to the percentages of remaining DPPH· in the presence of the indicated concentrations of the copper(II) complexes or ligands. Notice the different concentration scales between A, B (0.002–0.08 M) and C, D (0.5–5 M). Values are presented as mean ± SEM (n = 3).

that for the investigated copper complexes, as for their ligand, data obtained with the DPPH method also were in agreement with those of DPD method. Complexation with Cu(II) can modulate the feature of the amino acids and influence the antioxidant capacity. In the case of histidine, copper complexation markedly increased this capacity, whereas for $[Cu(Ser)_2]$ this increase was lower. No differences were found for $[Cu(Urea)_2Cl_2]$ and for $[Cu(Biuret)_2Cl_2]$ in comparison with their ligands. Probably due to their lower stability the $[Cu(Urea)_2Cl_2]$ and $[Cu(Biuret)_2Cl_2]$ complexes may lose the Cu^{2+}, liberating thus the ligands. The urea and biuret alone also exhibit marked radical scavenging capacity. These observations are in agreement with previous data on urea as antioxidant and as cardioprotector [20].

3.6. Biocompatibility of copper complexes and ligands

The biocompatibility was evaluated using P19 neuronal cultures and the NR assay [34] which was recommended for mesuring the viability of cells exposed to concentrations of copper compounds exceeding those of physiological copper.

Histidine and urea presented good biocompatibility in concentrations up to 0.4 mM (Fig. 9A, C). The good biocompatibility of urea is in line with urea levels of 2.3–6.5 mM encountered in normal adult human brain [61]. Serine and biuret caused a concentration dependent moderate loss of viability at 400 μM concentration, with a remaining viability of about 70% in both cases (Fig. 9B, D). The cytotoxicity of serine could be due to the racemisation by a neuronal serine racemase of L-Serine into D-Serine known to be involved in neurodegeneration [62]. In contrast to urea, biuret was found to induce a decrease of viability (Fig. 9D). This cytotoxicity could be due to the transformation of biuret into products different from urea or to an eventual stronger chaothropic action compared to urea. However, the metabolic action of biuret in neuronal cells is still unknown.

The normal physiological concentration of copper in brain parenchyma is 70 μM, and concentrations up to 1300 μM were found in the brain of patients affected by Wilson's disease [63]. Our findings of markedly decreased neuronal viability in the presence of $CuCl_2$ concentrations of 100 up to 400 μM (Fig. 9) are in line with these data. Copper toxicity in cell culture is well known and can be attributed to its capacity to generate ROS. Worth of note, all copper complexes were less toxic to neurons than equivalent $CuCl_2$ concentrations (Fig. 9).

Compared to free histidine, serine, urea and biuret, the corresponding copper complexes induced a loss in cell viability in a concentration dependent manner (Fig. 9). In retrospective, $[Cu(His)_2Cl_2]$, $[Cu(Ser)_2]$ and $[Cu(Urea)_2Cl_2]$ showed similar biocompatibility with P19 neurons (70% cell viability at 200 μM) while the toxicity of $[Cu(Biuret)_2Cl_2]$ was close to that of $CuCl_2$ (about 50% cell viability at 200 μM), in line perhaps with the low stability of this complex. Therefore, $[Cu(His)_2Cl_2]$, $[Cu(Ser)_2]$, $[Cu(Urea)_2Cl_2]$, $[Cu(Biuret)_2\ Cl_2]$ but not His and urea, moderately altered neuronal viability. It is not excluded that the bidentate $[Cu(Ser)_2]$ and $[Cu(Biuret)_2Cl_2]$ and also the monodentate $[Cu(Urea)_2Cl_2]$ copper complexes could release copper ions in the culture medium resulting in alteration of the environmental pH, generation of ROS, and promotion of apoptosis and DNA damages [64]. In contrast, the tridentate $[Cu(His)_2Cl_2]$ complex presented a good antioxidant activity and, in addition, a moderate cell viability loss up to 200 μM. The cytotoxicity of the synthesized copper (II) complexes was lower compared to equivalent concentrations of $CuCl_2$. Although $[Cu(Urea)_2Cl_2]$ appeared less stable than $[Cu(His)_2Cl_2]$, the loss of viability was similar in both cases. It is not excluded that urea, which has an excellent antioxidant capacity [this report and 20] could protect cells against eventual oxidative damage due to the *in situ* liberation of copper (II) proxidant. Among the studied compounds, $[Cu(His)_2Cl_2]$ had the best cell biocompatibility and pronounced *in vitro* ROS scavenging activity.

4. Conclusion

Four copper complexes: $[Cu(His)_2Cl_2]$, $[Cu(Ser)_2]$, $[Cu(Urea)_2Cl_2]$ and $[Cu(Biuret)_2Cl_2]$ have been synthesized in isopropanol and they structurally exhibited tridentate (His), bidentate (Ser and Biuret) or monodentate (Urea) coordination with copper(II). Furthermore, *in vitro* antioxidant activity of all copper complexes presented effective scavenging of radicals when tested with DPD and DPPH assays. The results of biocompatibility studies showed that they may affect, in a concentration-depend manner, the neuronal viability but to a lesser extent than does $CuCl_2$. The copper complexes of amino acid ligands

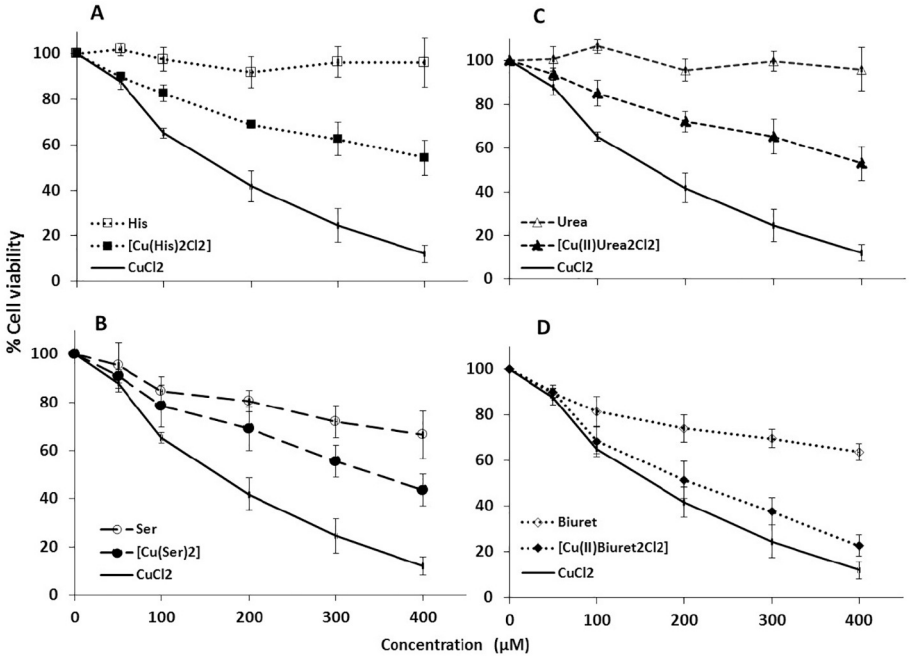

Fig. 9. Cell viability in the presence of different concentrations of copper complexes and ligands. P19 neurons were incubated for 48 h in the presence of each complex or corresponding ligand, and their viability was evaluated by the NR uptake assay and reported as percentages from that of control cultures (no complex and no ligand). The viability of cells treated with $CuCl_2$ was included for comparison. Values (mean ± SEM) are for three independent cell cultures.

(His, Ser) presented higher stability in different simulating physiological media than those of urea and biuret, except Simulated Gastric Fluid in which all complexes were instable. $[Cu(His)_2Cl_2]$, closely followed by $[Cu(Ser)_2]$, was stable, biocompatible and the best in terms of antioxidant capacity. The other copper complexes showed a relatively higher cytotoxicity, but much lower than that of $CuCl_2$ generated by the free copper(II) ions.

Abbreviations

DPD *N,N*-diethyl-*p*-phenylenediamine
DPPH diphenyl-2,2-picrylhydrazyl
ELS electrolysed
HAT Hydrogen Atom Transfer
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His L-histidine
KH modified Krebs-Henseleit
ML and ML_2 M (metal), L (ligand)
MP melting point
NR Neutral Red
PBS phosphate buffer saline
ROS reactive oxygen species
Ser L-serine
SET Single Electron Transfer
SGF Simulated Gastric Fluid
SIF Simulated Intestinal Fluid
SNB Supplemented Neurobasal (medium)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2018.12.010.

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