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Evaluation of the binding of four anti-tumor Casiopeínas® to human serum albumin

Isabel Correia[a], Sladjana Borovic[a,b], Isabel Cavaco[a,c], Cristina P. Matos[a], Somnath Roy[a,d], Hugo M. Santos[e,f], Luz Fernandes[e,f], José L. Capelo[e,f], Lena Ruiz-Azuara[g], João Costa Pessoa[a,*]

[a] Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa, Av Rovisco Pais, 1049-001 Lisboa, Portugal
[b] University Business Academy in Novi Sad, Faculty of Pharmacy Novi Sad, Trg Mladenaca 5, 21000 Novi Sad, Serbia
[c] Departamento de Química e Farmácia, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[d] Department of Chemistry, Ananda Chandra College, Jalpaiguri, West Bengal, India
[e] UCIBIO, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
[f] PROTEOMASS Scientific Society, Madan Park, Rua dos Inventores, 2825-152 Caparica, Portugal
[g] Facultad de Química, Departamento de Química Inorgánica y Nuclear, Universidad Nacional Autónoma de México, México DF 04510, Mexico

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ABSTRACT

The metal complexes designated by Casiopeínas® are mixed-ligand Cu^{II}-compounds some of them having promising antineoplastic properties. We report studies of binding of Cu(glycinato)(4,7-dimethyl-1,10-phenanthroline) (Cas-II-Gly (**1**)), Cu(acetylacetonato)(4,7-dimethyl-1,10-phenanthroline) (Cas-III-Ea (**2**)), Cu(glycinato)(4,4′-dimethyl-2,2′-bipyridine) (Cas-IV-Gly (**3**)) and Cu(acetylacetonato)(4,4′-dimethyl-2,2′-bipyridine) (Cas-III-ia (**4**)) to human serum albumin (HSA) by circular dichroism (CD), Electron paramagnetic resonance (EPR) and fluorescence spectroscopy. The results indicate that HSA may bind up to three molecules of the tested Casiopeínas. This is confirmed by inductively coupled plasma – atomic absorption spectroscopy measurements of samples of HSA-Casiopeínas after passing by adequate size-exclusion columns. The binding of Cas-II-Gly to HSA was also confirmed by MALDI-TOF mass spectrometric experiments. In the physiological range of concentrations the Casiopeínas form 1:1 adducts with HSA, with conditional binding constants of ca. 1×10^9 (**1**), 4×10^7 (**2**), 1×10^6 (**3**) and 2×10^5 (**4**), values determined from the CD spectra measured, and the fluorescence emission spectra indicates that the binding takes place close to the Trp214 residue. Overall, the data confirm that these Casiopeínas may bind to HSA and may be transported in blood serum by this protein; this might allow some selective tumor targeting, particularly in the case of Cas-II-Gly. In this work we also discuss aspects associated to the reliability of the frequently used methodologies to determine binding constants based on the measurement of fluorescence emission spectra of solutions containing low concentrations of proteins such as HSA and BSA, by titrations with solutions of metal complexes.

1. Introduction

Metal-based therapeutic molecules offer many possibilities for the design of therapeutic agents not accessible to organic compounds. The several metals available, distinct redox states, the diverse range of coordination numbers and geometries, thermodynamic and kinetic characteristics, as well as the huge variety of available ligands offer the medicinal chemist a broad range of properties to be explored.

Copper is an essential element for humans and most aerobic organisms [1,2], and most Cu-based complexes have been investigated for therapeutic applications on the assumption that endogenous metal ions such as Cu^{2+} may be less toxic for normal cells when compared to non-endogenous metal ions. However, copper can also be toxic, namely because of its redox activity and affinity for binding sites that should be

Abbreviations: EPR, electron paramagnetic resonance; HSA, human serum albumin; BSA, bovine serum albumin; ATCUN site, amino terminal Cu(II) and Ni(II) binding site; MBS site, multi metal binding site of HSA; Cas-II-Gly (**1**), Cu(glycinato)(4,7-dimethyl-1,10-phenanthroline); Cas-III-Ea (**2**), Cu(acetylacetonato)(4,7-dimethyl-1,10-phenanthroline); Cas-IV-Gly (**3**), Cu(glycinato)(4,4′-dimethyl-2,2′-bipyridine); Cas-III-ia (**4**), Cu(acetylacetonato)(4,4′-dimethyl-2,2′-bipyridine); ROS, reactive oxygen species; bipy, 2,2′-bipyridine; Me $_2$bipy, 4,4′-dimethyl-2,2′-bipyridine; phen, 1,10-phenanthroline; Me $_2$phen, 4,7-dimethyl-1,10-phenanthroline; NN, aromatic aromatic diamine such as bipy or phen and their derivatives; PBS buffer, phosphate buffered saline buffer; TRIS, tris(hydroxymethyl)aminomethane; K_{SV}, Stern-Volmer dynamic quenching constant; EPR, electron paramagnetic resonance spectroscopy; CD, circular dichroism spectroscopy; UV/Vis, absorption spectroscopy; MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight; AFM, atomic force microscopy; XRD, X-ray

circular dichroism spectroscopy, UV/Vis, absorption spectroscopy, MALDI-TOF, Matrix-assisted laser desorption/ionization time-of-flight, AFM, atomic force microscopy, XRD, X-ray diffraction; K_{BC}, binding constant (defined by eq.); Δε, differential absorptivity per mole of HSA

* Corresponding author.
E-mail address: joao.pessoa@ist.utl.pt (J.C. Pessoa).

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occupied by other metals. It is the altered metabolism of cancer cells and differential response between normal and tumor cells to copper that is the basis for development of Cu-complexes with prospective anti-neoplastic characteristics [1,3–10].

The rich coordination chemistry of copper and the flexible Cu(I/II) redox behavior enable wide perspectives for the design of more potent and less toxic Cu-based antitumor drugs. The majority of compounds developed so far are mononuclear Cu(II)-species [4,9]; their mode of action is miscellaneous but differs from that of cisplatin; it includes DNA interaction, mitochondrial toxicity, reactive oxygen species (ROS) generation and proteasome inhibition [1,3–9,13,15], and a great variety of Cu-compounds has been tested as cytotoxic agents and showed antitumor activity in many in vitro tests and a few in vivo experiments [1,2,10], namely complexes containing 2,2′-bipyridine (bipy) or 1,10-phenanthroline (phen) [4–6,8,9,11,12].

Among the most representative copper complexes with antitumor potential are the Casiopeínas®. These are mixed-chelate Cu^{II}-compounds of the general formula $[Cu(OO)(NN)]NO_3$ or $[Cu(ON)(NN)]NO_3$, where NN is a substituted aromatic diimine (phen, bipy or derivatives), ON is an α-amino acidate or peptide and OO is acetylacetonate or salicylaldehydate. These complexes have been patented and registered under the name of Casiopeínas® [13,14] and have been tested in several models in vitro and in vivo, showing antiproliferative [8,15–18], cytotoxic [19], genotoxic [3,20] and antitumor activities [4], and low toxicity towards healthy cells Lymphocytes and macrophages [16].

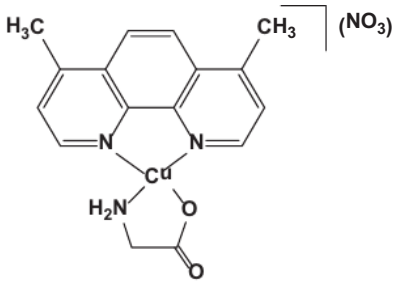
Preclinical studies have revealed that the substitution on the NN ligand as well as changes in the co-ligand, modifies the magnitude of the biological activity [10,17]. A similar behavior was observed for the antiproliferative activity on human tumor cell lines tested in vitro [9,15,17–19] and for the genotoxic activity [8,19]. In a recent work [20] on the physiological responses of cervical cancer cell cultures to Cas-II-gly (**1**) (abbreviations of the Casiopeínas **1**–**4** are defined in Fig. 1), it was reported that, besides producing pro-apoptotic processes in the malignant cells, Cas-II-Gly also inhibits estrogen-mediated G1/S cell cycle progression, down-regulates transformation processes in fibroblasts, inhibits uncontrolled cell migration of cancer cells and increased molecular mechanisms of liver protection. Currently, Cas-II-Gly is in phase I clinical trials [21,22] and this Casiopeína, which corresponds to the $[Cu(Gly)(4,7\text{-}Me_2phen)]NO_3$ complex, will be the one that we will address more extensively.

Regarding DNA interaction, the bidentate NN ligands included in the coordination sphere are nitrogen-donor ligands with a high affinity for copper, and they have been reported to bind DNA by intercalative and non-intercalative interactions, either as free ligands or included in the structure of the metal complexes [5,6,15]. On the other hand, copper-phen complexes have been characterized as agents for binding, cleavage and oxidative modification of DNA [6,9,11,19,23,24]. Oxidative damage of DNA and/or other targets may constitute important mechanisms for Casiopeínas' antitumor activity [2,10,18,25].

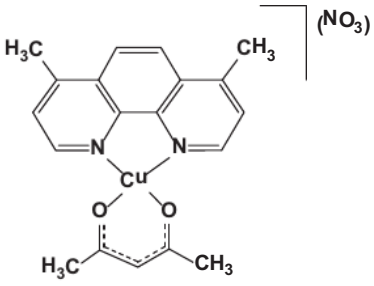
Proteosome inhibition or interactions with proteins involved in the cell cycle, such as tubulin, or in the cytoskeleton formation, as integrin, or those present in the extracellular medium, like fibronectin, can offer many binding sites to metal ion complexes. The interaction of the Cas-III-Ea (**2**) with the biologically relevant proteins tubulin, integrin and fibronectin was demonstrated by atomic force microscopy (AFM) imaging [26]. In all the cases strong modifications were induced in the structure of the proteins which would have consequences on the life cycle of tumor cells, showing that these proteins may also be targets for Casiopeínas.

Cas-II-Gly (**1**) and Cas-III-ia (**4**) probably have been the two most investigated of a large number of similar complexes. Their molecular structure was determined by single-crystal X-ray diffraction (XRD), the coordination geometry corresponding to four-coordinate, slightly distorted square planar molecules [21,27]. The molecular structure of $[Cu(Gly)(phen)(H_2O)]NO_3{\cdot}1.5H_2O$ was also determined by single-crystal XRD [28]; the Cu(II) coordination geometry is distorted square pyramidal as in Cas-II-Gly, with a water molecule in the apical position. Similar binding sets were also determined by single-crystal XRD for $[Cu(DL\text{-}Ala)(phen)(H_2O)]NO_3{\cdot}2.5H_2O$ and $[Cu(Me_2Gly)(phen)(H_2O)]NO_3{\cdot}$(Me$_2$Gly = 2,2-dimethylglycine) [7].

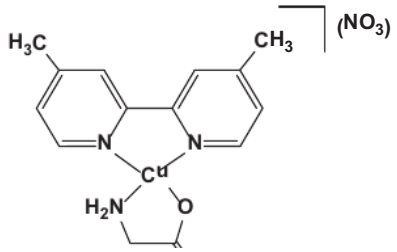
Whatever the treatment envisaged for a compound or its therapeutic target, how it is transported in blood is a relevant issue, as this may also determine their biological action. When (and if) Cu(II) is administered as a complex it may undergo transformations in the gastrointestinal tract, through the circulation and finally in the tissues and cells. Thus, it is important to know the transformation of the complexes in the blood serum at the physiological conditions, since the original metal-binding



(a) Cas-II-Gly (**1**)



(b) Cas-III-Ea (**2**)



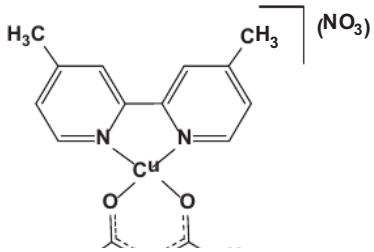


Fig. 1. Structural formulae of the four Casiopeínas studied in this work: (a) Cas-II-Gly, $[Cu(glycinate)(4,7\text{-dimethyl-}1,10\text{-phenanthroline})]NO_3$; (b) Cas-IV-Gly, $[Cu(glycinate)(4,4'\text{-dimethyl-}2,2'\text{-bipyridine})]NO_3$; (c) Cas-III-Ea, $[Cu(acetylacetonate)(4,7\text{-dimethyl-}1,10\text{-phenanthroline})]NO_3$; (d) Cas-III-ia, $[Cu(acetylacetonate)(4,4'\text{-dimethyl-}2,2'\text{-bipyridine})]NO_3$. Throughout the text 4,7-dimethyl-1,10-phenanthroline will be designated by Me$_2$phen and 4,4′-dimethyl-2,2′-bipyridine by Me$_2$bipy.

ligands may be displaced during the absorption processes or during their transport in the blood.

HSA can carry different hydrophobic and hydrophilic drugs through circulation. Cu(II) is known to bind to the N-terminal binding site of HSA, often designated as the "amino terminal Cu(II) and Ni(II) binding site" (ATCUN site); it may also bind to its secondary site, the multi metal binding site (MBS) [29]. Cu(II)-complexes, namely Cu(II)-phen compounds bind to human serum albumin (HSA) [19,30]. Binding to HSA can alter pharmacokinetic and pharmacodynamic properties of drugs and the determination of the binding sites in HSA and their relative affinities for different compounds is an important aspect in drug development [31]. Therefore, in this work we report studies of binding of four Casiopeínas: Cas-II-Gly (**1**), Cas-III-Ea (**2**), Cas-IV-Gly (**3**) and Cas-III-ia (**4**) (Fig. 1) to HSA. The systems were studied through the combined application of electron paramagnetic resonance (EPR), circular dichroism (CD), absorption spectroscopy (UV/Vis) and fluorescence spectroscopy, as well as mass spectrometry.

2. Experimental part

2.1. Reagents and solutions

The $CuCl_2{\cdot}2H_2O$ and 1,10-phenanthroline hydrate ($C_{12}H_8N_2{\cdot}H_2O$) were from Merck. The chemical reagents and albumin were used as received from the supplier without further purification. Millipore® water was used for the preparation of solutions; it was produced using a Millipore Milli-Q Academic water purification system. Phosphate buffered saline (PBS) from Sigma-Aldrich was used in most experiments.

The composition of one PBS tablet dissolved in 200 mL of Millipore water yields 0.010 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M NaCl, pH 7.4, at 25 °C. The PBS buffer was prepared by dissolving the PBS buffer tablets in Millipore® water. Freshly prepared buffer (200 mL) was used for each experiment. The Cu(II) solutions used in the studies were freshly prepared by dissolving $CuCl_2{\cdot}2H_2O$ in Millipore® water. When used, the required amounts of 1,10-phenanthroline were dissolved in absolute ethanol (Panreac Química, S. A), adequate volumes of this solution being then measured.

The group of four Cu(II)-complexes named Casiopeínas®, used in this work are: Cas-II-Gly, Cas-IV-Gly, Cas-III-ia and Cas-III-Ea (Fig. 1). They were synthesized using procedures reported in the literature [13,14,32].

Human serum albumin (HSA, Sigma Aldrich, A3782) was a lyophilized powder, fatty acid free, with a molecular mass (MM) of 66,440 Da. Albumin solutions were prepared by first dissolving the solid in PBS buffer; the solutions were allowed to stand for at least 60 min to allow equilibration. During this period, they were gently swirled. The concentration was estimated spectrophotometrically taking the absorbance measured at 280 nm and the molar extinction coefficient $\varepsilon_{280} = 36{,}850\ M^{-1}\,cm^{-1}$ [33,34,35].

Circular dichoism spectra were run in the 200–250 nm range with HSA solutions in PBS buffer and in mixtures of aqueous PBS buffer and % of EtOH up to 20%. These only showed very small changes, suggesting that the HSA does not significantly change its secondary conformation with addition of EtOH (up to 20%). For measurements involving the HSA-Cu-phen system the solutions were prepared in 95% PBS and 5% EtOH. For all other measurements, namely those involving spectroscopic studies with the Casiopeínas, no ethanol was added.

All pH readings were obtained with Denver Model 15 pH meter, previously calibrated with pH 7.00 and pH 4.00 standard buffer solutions (Panreac Química, S. A).

2.2. Desalting procedure with size exclusion columns

A solution of albumin in tris(hydroxymethyl)aminomethane (TRIS) buffer (0.10 M, pH = 7.4) was prepared and volumes of this solution concentrations were ~100 μM in each. Solutions of the Casiopeínas (ca. 1 mM) were prepared with exactly known concentrations, and aliquots of these solutions were added to the 5 mL volumetric flasks, so that the molar ratios Casiopeínas:HSA were: 0, 1, 2 and 4. The volume of solution in each of the 5 flasks were set to 5.00 mL and these solutions were allowed to equilibrate at 37 °C for ~3 h. A fixed volume (2.50 mL) of each sample was then measured and applied to the top, and passed through a PD-10 MiniTrap G-25 column (GE Healthcare), by elution with the TRIS buffer, as indicated in the SI section. The first component to elute from these size exclusion columns is the protein, which may contain (or not) bound Cu-complexes. A volume of 3.5 mL was collected for each sample. The sample only containing HSA was used to estimate the albumin concentration after passing the column: by measuring its absorbance at 280 nm a concentration of 67.9 μM was determined for HSA upon elution, and we assume this concentration is the same for all columns. The samples of protein-metal solutions collected from these exclusion columns were used to evaluate the metal content by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) using a Horiba Jobin-Yvon apparatus, model Ultima. In ICP-AES metal concentration was determined in mg/L from calibration curves established on the same day.

2.3. Mass spectrometry

2.3.1. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)

Mass spectra were obtained using a Bruker Daltonics Ultraflex MALDI TOF/TOF Mass Spectrometer operating in linear mode with positive ion extracting at 25,000 V and a pulsed ion extraction of 480 ns. Each final spectrum was the accumulated result of at least 1000 laser shots that were obtained from 10 different manually selected regions of the same sample, over a range of 14,000–160,000 Da. Prior to calibration, the spectra were processed with Compass 1.3 using smoothing and baseline subtraction for reproducible peak annotation. The spectra were externally calibrated using 50 pmol of albumin from bovine serum ($[M + H]^+$ 66,430).

2.3.2. Solution preparation

Cas-II-Gly stock solutions were prepared in water with ca. 3 mM immediately before mixing. Solutions of HSA were prepared with ca. 300 μM by dissolving the protein in NH_4HCO_3 buffer (pH 7.4, 25 mM). These solutions were allowed to stand overnight to allow equilibration. Samples for MALDI-TOF MS were prepared with Cas-II-Gly:HSA molar ratios of 0:1, 3:1 and 5:1 by mixing different volumes of the stock solutions with buffer. The final concentration of HSA was 50 μM.

2.3.3. Sample preparation, dried droplet procedure

2 μL of each sample was mixed with 2 μL of matrix solution (saturated solution of sinapinic acid in 1 mL of 30% (v/v) acetonitrile and 0.1% (v/v) aqueous trifluoroacetic acid). Then 1 μL of the sample-matrix solution was deposited by duplicate onto a MTP 384 ground steel BC target and allowed to dry at room temperature. The HSA concentration of the final samples was thus 25 μM.

Several spots were applied, as described above, for each solution prepared. The masses obtained for the several spots of the same solution somewhat varied and average values were obtained for the masses of HSA and Cas-II-Gly + HSA (samples with molar ratios of 3:1 and 5:1).

2.4. Spectroscopic measurements

2.4.1. UV–Visible spectra

The UV–Visible absorption spectra (UV–Vis spectra) of the solutions were recorded on Perkin Elmer Lambda 35 UV–Vis spectrophotometer in the range 800–250 nm. The measurements were done with quartz

were added to 5.00 mL volumetric flasks so that the total final HSA

Suprasil® cells of 0.1 cm, 0.2 cm or 1 cm optical path.

2.4.2. Circular dichroism (CD) spectra

The CD spectra were recorded on a JASCO J-720 spectropolarimeter (JASCO, Hiroshima, Japan) either with a UV photomultiplier as detector (300–800 nm), or with a red-sensitive photomultiplier (400–1000 nm range). Most of the measurements were done at ~37 °C (using a TRADE Raypa thermostat) with quartz Suprasil® cells of 0.2, 0.5, 1 or 2 cm optical path. One or three accumulations were made for each spectrum.

In solutions containing Cu(II)-compounds and chiral ligands, in this case the HSA protein, several complex species may form. The ΔA values recorded (taken as $\Delta A = \Delta\varepsilon \times b \times C$, where b is the optical path and C is the total concentration of HSA present) may be due to one or more types of species. Therefore, the Δε values (differential absorptivity per mole of HSA) correspond to [36–39]:

Δ (1)

where $\Delta\varepsilon_i$ is the differential molar absorptivity of species i of molar concentration C_i. The Σ includes all chiral species present. For λ > ~330 nm the contribution of HSA is ~zero, thus the Δε values measured are only due to species that interact with HSA. For λ > ~400 nm the Δε values are due to HSA-Cu-containing species (i = 1 to n), corresponding to either charge transfer, imine based or d-d transitions; thus, C_i represents the concentration of each of these species in each particular solution prepared.

Each CD spectrum obtained was processed by first subtracting the baseline; then, the spectra, recorded in ellipticity units, θ, expressed in millidegrees, were converted into Delta Epsilon (Δε) values expressed in $M^{-1}\,cm^{-1}$ taking the total concentration of albumin in the solution under consideration and the Jasco 32 software available with the CD instrument. When required, the processing and smoothening of the CD spectra was done also using the JASCO 32 software.

For several of the systems studied the spectra obtained from CD experiments were used in the input files for the determination of binding constants using the computer program PSEQUAD [40]. These normally required smoothing; the version of the program used has a limit of 99 absorbance values per spectrum, therefore the input files contained data with a step of 4 nm. The computer program HySS (Hyperquad Simulation and Speciation version 4.0.31) [41] was used to provide the speciation diagrams, by introducing the values of binding constants calculated with the PSEQUAD computer program, as well as those relevant for each system and previously published.

2.4.3. Fluorescence spectra

Fluorescence spectra were measured on a Horiba Jobin Yvon fluorescence spectrometer model FL 1065 at room temperature using a quartz cuvette of 1 cm optical path. Fluorescence titrations were done for each complex by addition of increasing amounts of solutions of complexes to an HSA solution (1.5 μM in PBS). The samples were exited at 295 nm (slits = 5 nm) and the emission spectra were recorded between 305 and 500 nm. UV–Vis absorption spectra were collected to correct the data for reabsorption and inner filter effects [42–44]. Stock solutions of complexes (ca. 160 μM) were prepared in PBS (Cas-IV-Gly) or deionized water (Cas-II-Gly and Cas-III-ia). The complex concentrations were varied from 0 to ~8.6 μM, by adding different aliquots of the stock solutions of complexes (from 2 to 8 μL). The Stern-Volmer dynamic quenching constant, K_{SV}, was calculated according to the classical Stern–Volmer equation:

/ (2)

where IF_0 and IF are the fluorescence intensities of HSA in the absence and in the presence of each complex, respectively, and [Q] is the complex concentration (the quencher). For the Stern–Volmer plot the emission at 337, 341 and 339 nm was used for complexes Cas-II-Gly,

2.4.4. Electron paramagnetic resonance (EPR) spectroscopy

All EPR measurements were done using 3 mm quartz tubes (Wilmad 707-SQ-250M) filled with ~250 μL of solution. After introducing the sample in the tube, this was then immersed in a Dewar containing liquid nitrogen (at 77 K) to keep the solution frozen. The 1st derivative X-band EPR spectra of the frozen solutions were recorded on a Bruker ESP 300E spectrometer at 77 K using a Bruker WG-816-B-Q Finger Liquid Nitrogen Dewar Flask. The ESP 300E spectrometer was operated at ~9.51 GHz with a frequency modulation of 100 kHz. While keeping the resolution at 2048 points, the microwave power was adjusted to 20 dB attenuations and the receiver gain was set to either 3.2×10^5 or 4.0×10^5. To improve the signal to noise ratio 10 scans were accumulated for each sample. The spectral acquisition parameters were constant for each set of experiments.

The EPR spectra were processed with Bruker's WIN-EPR SimFonia program and simulated with a program developed by Rockenbauer and Korecz [45]. Through the simulations, the A_x, A_y and A_z components of the hyperfine coupling constant (A) and the g_x, g_y and g_z components of the g-factor (g) were obtained.

2.4.5. Binding constant determination of Copper-Serum Components

For the system HSA-Cu^{II}-phen, the binding constants are defined as $\beta_{pqr} = [P_pM_qN_r]/[P]^p[M]^q[N]^r$ where P is the protein, M is the metal ion (Cu^{2+}) and N is a co-ligand (Me_2phen or Me_2bipy) that can yield adducts $\{P_pM_qN_r\}$. p, q and r identify the stoichiometric indexes of the species involved; in the present systems p is always equal to one. For the systems HSA-(Cu^{II}-complex) it is assumed that the complex binds to the protein as the whole parent complex (even if one of the Cu^{II}-donor atoms bond is broken), thus the binding constants are defined as $\beta_{pn} = [P_pQ_n] / [P]^p[Q]^n$ where P is the protein and Q is the Cu^{II}-compound that can yield adducts $\{P_pQ_q\}$. p and n identify the stoichiometry of the species involved. In the present systems p is always equal to one, thus the binding constants are defined as:

H (3)

The measurements were carried out at pH 7.4, therefore these binding constants are 'conditional constants' only valid for the experimental conditions used.

3. Results and discussion

3.1. Circular dichroism spectra

3.1.1. Studies with Cas-II-Gly

Cas-II-Gly (**1**) is a mixed-ligand Cu(II) complex containing Gly^- and Me_2phen and to our knowledge the characterization of the aqueous equilibria of the ternary system Cu(II)-Me_2phen-Gly was never reported. However, the system Cu(II)-phen-Gly was previously studied, and the corresponding formation constants determined [46,47], therefore we use these values in speciation calculations. We predict that the formed species, formation constants and calculated speciations for aqueous solutions of Cas-II-Gly and of Cu(II)-phen-Gly are similar. As Me_2phen is more basic than phen the Cu-species formed with Me_2phen probably are slightly more stable.

From the speciation of the 1:1:1 Cu(II):phen:Gly ternary system [47] (1.0 mM of each reagent) at pH 7.4 the Cu(Gly)(phen)$^+$ complexes are the predominant species (ca. 77% of total Cu), some Cu(phen)$_2^{2+}$, Cu(Gly)$_2$, Cu(Gly) and Cu(OH)$^+$ also existing (see Fig. SI-32). Therefore, once dissolving Cas-II-Gly and mixing it with HSA, besides HSA-(Cas-II-Gly)$_n$ species, several others may possibly form by partial decomposition of the [Cu(Me_2-phen)(Gly)] complex. Namely, it is known that HSA-$(Cu)_n$ and HSA-$(Cu(phen)_m)_n$ species may form [19,29,30], thus, their relative importance must be checked.

With a solution containing 150 μM HSA and 2.0 mol equivalents of

Cas-IV-Gly and Cas-III-Ia, respectively.

Cas-II-Gly the CD spectrum shown in Fig. 2 was obtained. Some features

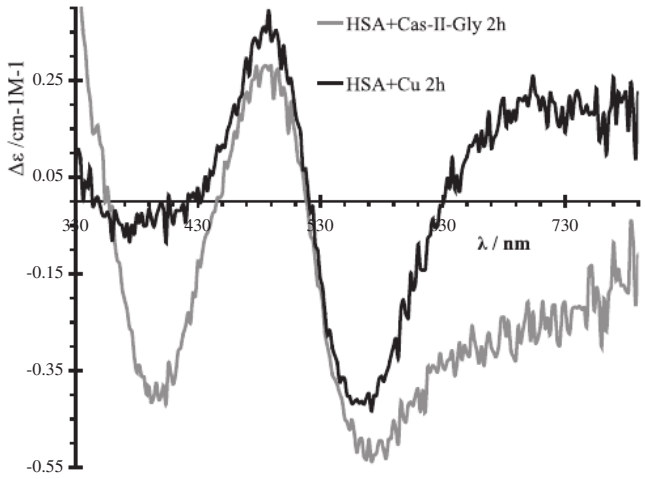


Fig. 2. CD spectra of 150 μM HSA in PBS buffer (pH = 7.4) loaded with either 2.0 mol equivalents of $CuCl_2$ or of Cas-II-Gly (**1**) after 2 h of addition of the Cu-compound to the solution containing HSA. Measurements were carried out with a cell of 2 cm optical path.

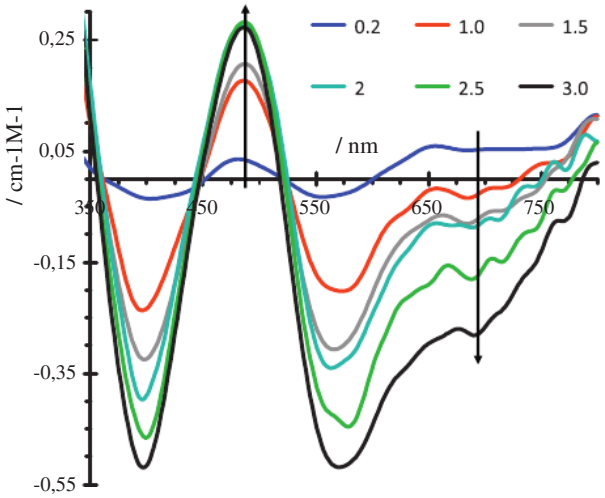


Fig. 3. CD spectra of a 200 μM HSA solution in PBS buffer (pH = 7.4) upon additions of Cas-II-Gly (**1**). The arrows indicate the changes observed as the Cas-II-Gly:HSA molar ratios are increased (0.2, 1.0, 1.5, 2.0, 2.5, 3.0). Only a selection of molar ratios of Cas-II-Gly:HSA are included and several other spectra for this system are included in the SI section. Measurements were carried out with a 2 cm optical path quartz cell.

of the spectra of HSA-(Cas-II-Gly) in Fig. 2 resemble features of the HSA-$(Cu)_2$ CD spectra [29,30], namely a positive band at ~490 nm and a negative band at ~560 nm, but clearly novel distinct negative bands are recorded with λ_{max} ~ 390 and ~700 nm. Some of the CD spectra in the visible range measured with solutions of HSA (200 μM) and increasing mol equivalents (0.2 to 3.0) of Cas-II-Gly are shown in Fig. 3; several other related CD spectra are depicted in the SI section. As albumin alone does not absorb in the range 350–800 nm all CD bands shown in these figures are due to bands of Cu-species bound to HSA. If Cas-II-Gly {more specifically some Cu^{II}-species} was not bound to HSA, no CD bands would be measured in this λ range.

As Cas-II-Gly is progressively added to HSA, the intensity (and pattern) of the CD bands change and initially three bands consistently increase in intensity: at ~390 nm ($\Delta\varepsilon < 0$), at ~490 nm ($\Delta\varepsilon > 0$) and at ~570 nm ($\Delta\varepsilon < 0$). The broad band at 650–750 nm, initially has positive Δε values (Fig. 3 and Fig. SI-2), corresponding to very low $|\Delta\varepsilon|$ values up to a 1:1 (complex:HSA) molar ratio; only after addition of 1.2–1.5 mol equiv. of complex its intensity consistently increases (with negative Δε values). Thus, two Cu-containing species co-exist, one with

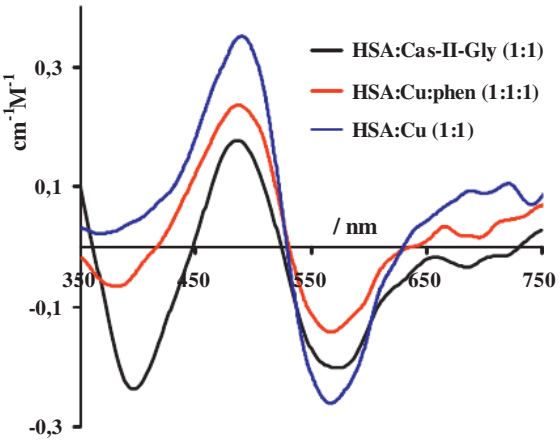


Fig. 4. CD spectrum of a solution of ~150 μM HSA loaded with 1 mol equivalent of Cas-II-Gly (PBS buffer, black line), CD spectrum of a solution of 200 μM HSA + $CuCl_2$ (200 μM) + phen (200 μM) (in 5% EtOH + 95% PBS, red line) and CD spectrum of a solution of HSA (200 μM) + $CuCl_2$ (200 μM) (PBS buffer, blue line). All spectra were measured with a 2 cm optical path cell after 4 h of addition of the Cu-complex to the HSA solution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

positive (but small) Δε values in the range 650–750 nm, and the 2nd one (2:1 stoichiometry) with negative Δε values in the same range. After a Cas-II-Gly:HSA molar ratio of ca. 1.5, another negative band clearly develops with λ_{max} ~700 nm. Overall these observations suggest the formation of three distinct HSA-Cas-II-Gly complexes (see also SI section); however, partial formation of HSA-Cu and HSA-Cu-Me_2phen complexes, resulting from partial decomposition of Cas-II-Gly, cannot be ruled out.

Fig. 4 depicts a comparison between the CD spectra of solutions containing HSA and either Cu^{2+}, Cas-II-Gly or Cu-phen complexes. The spectra of HSA-Cas-II-Gly species differ from those of HSA-$Cu(phen)_n$ complexes [19,29,30], particularly in the range 330–450 nm, this indicating the presence of distinct species in both systems: while HSA-$Cu(phen)_n$ complexes form in solutions containing HSA, Cu(II) and phen, HSA-$(Cas\text{-}II\text{-}Gly)_n$ complexes form in the system containing HSA and Cas-II-Gly. No clear indication for the hydrolysis of Cas-II-Gly exists, but it cannot be ruled out.

3.1.2. Studies with Casiopeina-III-Ea ( )

Cas-III-Ea (**2**) is a mixed-ligand Cu(II) complex with $acac^-$ and Me_2phen as ligands and to our knowledge the aqueous equilibria of the ternary system Cu(II)-Me_2phen was not studied, but the formation constants of $Cu(Gly)(acac)^+$ was determined (log β = 17.4) [46]. As observed with the Cas-II-Gly system, the CD spectra of the solutions containing HSA and $CuCl_2$ differ from those of HSA and Cas-III-Ea, this indicating that the HSA-Cu-complex species responsible for the CD spectra differ in the two systems.

Fig. 5 depicts CD spectra of solutions containing Cas-III-Ea and HSA (~100 μM) after 4 h of addition of the complex, as well as similar experiments with solutions containing $CuCl_2$ and phen or only $CuCl_2$. The spectra for the three systems have some similarities but also depict some quite distinct features. Namely, significant differences are observed for $\lambda > 550$ nm; while for the HSA:Cu:phen (1:1:1) solutions the Δε values are positive for $\lambda > 600$–630 nm, for the Cas-III-Ea (1:1) solutions the Δε values are negative up to ca. 700 nm. This indicates that a significant fraction of the HSA-Cu species formed in the systems differs.

Fig. 6 depicts CD spectra of solutions of HSA upon additions of Cas-III-Ea. In Fig. 6A it may be seen that while the band at ca. 490 nm progressively increases its intensity, the band at ca. 650–750 nm initially increases its intensity (up to a molar ratio of ~0.6), and then the

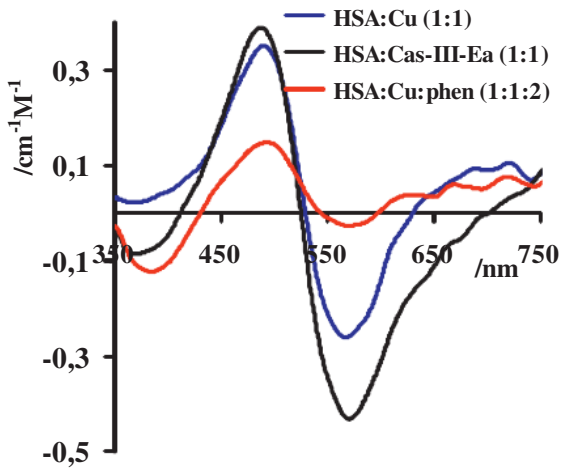


Fig. 5. CD spectra of solutions of HSA with 1.0 mol equivalent of $CuCl_2$ (blue line), with 1.0 mol equivalents of $CuCl_2$ and 2.0 mol equivalents of 1,10-phenanthroline (red line) and with 1.0 mol equivalent of Cas-III-Ea. Measurements were carried out in PBS buffer (pH = 7.4) after ~4 h of addition of the Cu-complex with a 2 cm optical path cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

species. This means that at least two distinct HSA-Cas-III-Ea species form up to the molar ratio of 1. In Fig. 6B the same global trend continues upon further additions of Cas-III-Ea (the Δε values of the band at ~490 nm increase, and those for λ > 550 nm become more negative), but for molar ratios greater than ~2.25, a distinct new negative band at ~670 nm builds up. This suggests the formation of another distinct HSA-Cas-III-Ea species, thus the formation of a HSA(Cas-III-Ea)$_3$ complex. Therefore, if it is assumed that no HSA-Cu or HSA-Cu-Me$_2$phen species are formed, the pattern of the spectra and the changes observed upon additions of Cas-III-Ea to solutions of HSA, suggest the formation of at least 3 distinct HSA-Cas-III-Ea species.

3.1.3. Studies with Casiopeina-IV-Gly ( )

Cas-IV-Gly (**3**) is a mixed-ligand Cu(II) complex with Gly^- and Me_2bipy and to our knowledge the aqueous equilibria of the ternary systems Cu(II)-bipy-Gly or Cu(II)-Me$_2$bipy-Gly have not been studied. Fig. SI-14A depicts CD spectra for the systems HSA-Cu and HSA-(Cas-IV-Gly), measured with time up to 24 h of addition of the Cu-complexes to the solutions containing HSA. The spectra show only minor changes, indicating that the HSA-(Cas-IV-Gly) species formed remains the same up to 24 h. Fig. SI-14B includes a comparison of the CD spectra after 24 h. Comparing these CD spectra for the Cu-HSA and HSA-(Cas-IV-Gly) systems, it can be seen that some of the bands of the two spectra partly overlap (positive band at ~490 nm and negative band at ~560 nm), but quite significant differences are observed mainly in the region below ~430 nm and above ~600 nm. This means that distinct HSA-

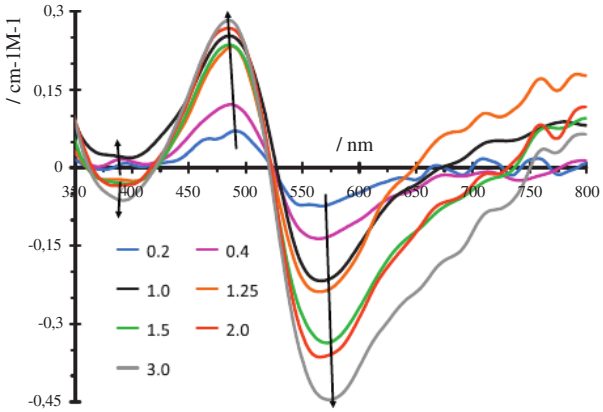


Fig. 7. CD spectra of a 200 μM HSA solution in PBS buffer (pH = 7.4) upon additions of Cas-IV-Gly. The arrows indicate the trends observed as the Cu-complex is progressively added. A selection of molar ratios of Cas-IV-Gly:HSA are included and several other spectra for this system are included in the SI section. Measurements were carried out with a 2 cm optical path cell.

copper complexes form in the two systems. Therefore, as there are no indications of decomposition of the HSA-(Cas-IV-Gly)$_n$ complexes formed, we may interpret the CD results as corresponding to the binding of [Cu(Me$_2$bipy)(Gly)] to HSA, not of [Cu(Me$_2$bipy)$_n$] or of [Cu(Gly)$_n$] (but formation of low amounts of the latter complexes cannot be ruled out).

The spectra shown in Fig. 7 and Figs. SI-15 and -16 indicate that when a ~200 μM HSA solution is titrated with Cas-IV-Gly, at least two types of species form. Upon incremental addition of 0.2–3.0 mol equivalents of Cas-IV-Gly, the bands at ~490 nm and ~570 nm increase in intensity. There is one novel negative band appearing at ~390 nm after addition of > 1 mol equivalent of Cas-IV-Gly (Fig. 7, and Figs. SI-15 and -16); a positive band also builds up at ~800 nm and after addition of ~1 mol equivalent of Cas-IV-Gly.

3.1.4. Studies with Casiopeina-III-ia ( )

Cas-III-ia (**4**) is a mixed-ligand Cu(II) complex with $acac^-$ and Me_2bipy and to our knowledge the aqueous equilibria of the ternary systems Cu(II)-Me$_2$bipy-acac or Cu(II)-bipy-acac were not previously studied. After addition of 2 mol equivalents of Cas-III-ia to a solution containing HSA (~150 μM), at least 4 bands can be observed in the CD spectrum (Fig. 8 and SI-19): a negative band with spectral maxima at ~390 nm, a positive band at ~490 nm, a negative band at ~560 nm and another positive one at λ > 780 nm. At least during the first 4 h the CD signals in the visible range do not change much with time (Fig. SI-19). Assuming the Cas-III-ia bound to HSA does not decompose (as the CD spectra recorded with time indicate), we may interpret the CD data as resulting from the binding of [Cu(4,4′-Me$_2$bipy)(acac)] to HSA, not of [Cu(4,4′-Me$_2$bipy)$_n$] or of [Cu(acac)$_n$] complexes.

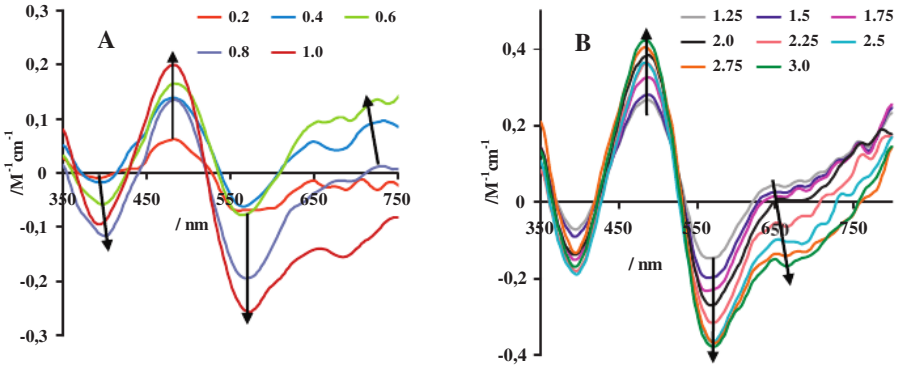


Fig. 6. CD spectra of solutions of HSA (100 μM)) upon additions of Cas-III-Ea to a solution containing HSA. The molar ratios Cas-III-Ea:HSA are indicated, as well as trends observed in the spectra as the Cu-complex is added. Measurements were carried out in PBS buffer (pH = 7.4) with a 2 cm optical path cell. Several other measurements were carried out with solutions containing HSA and Cas-III-Ea and some of these spectra are included in the SI section (Figs. SI-6 to SI-12).

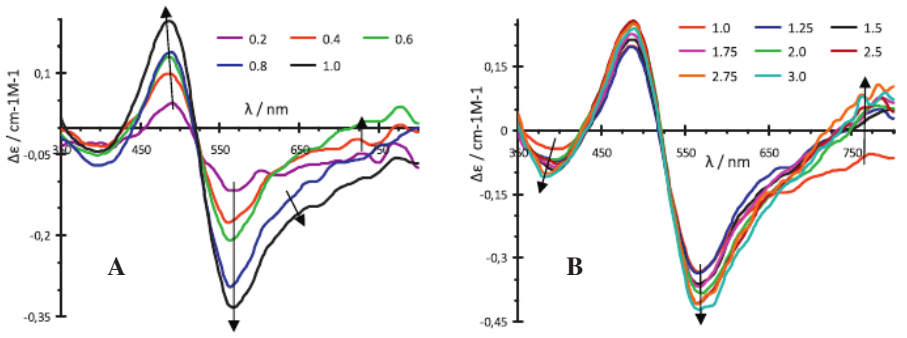


Fig. 8. CD spectra of a solution of HSA (~200 μM) upon stepwise additions of Cas-III-ia; the molar ratios of (Cas-III-ia):HSA are indicated. The approximate trends observed as the Cu-complex is added are indicated by the arrows. Several other CD spectra are depicted in the SI section. The measurements were carried out in PBS buffer (pH = 7.4) with a 2 cm optical path cell.

Fig. 8 (A and B) depicts the changes in CD spectra in the visible range upon additions of Cas-III-ia to a HSA solution (~200 μM). In (A) it is clear that for the bands at λ ~640–800 nm, upon addition of Cas-III-ia, initially the Δε values are negative and increase up to a Cas-III-ia:HSA molar ratio of ~0.6. Then the Δε values start decreasing and become negative again. This means that there are at least two distinct chiral (Cas-III-ia)-HSA species being formed up to the molar ratio of 1.0. In Fig. 11B, it may be seen that for molar ratios higher than ~1.0, the Δε values of the bands at 640–800 nm increase again upon additions of Cas-III-ia, a positive band forming for λ > ~680 nm, this suggesting that a 3rd distinct (Cas-III-ia)-HSA species forms.

3.1.5. Estimation of binding constants of HSA from the CD spectra measured

3.1.5.1. Cas-II-Gly. To estimate binding constants of HSA-Cas-II-Gly complexes, it is assumed that HSA-Cu and HSA-Cu-Me $_2$phen species do not form. As mentioned above, the binding constants are "conditional binding constants", valid at pH 7.4, in the medium used in experiments, defined by Eq. (3).

As mentioned, the CD spectroscopic data obtained for the HSA-Cas-II-Gly system suggest the formation of 3 distinct species (here assumed as HSA-(Cas-II-Gly)$_n$ species) as the Cu-compound is added: HSA-(Cas-II-Gly), HSA-(Cas-II-Gly)$_2$ and HSA-(Cas-II-Gly)$_3$; these correspond to binding constants β_{11} (n = 1), β_{12} (n = 2) and β_{13} (n = 3) and to the molar extinction coefficients of HSA-(Cas-II-Gly)$_n$: $\Delta\varepsilon_{11}$, $\Delta\varepsilon_{12}$ and $\Delta\varepsilon_{13}$, respectively, each of the $\Delta\varepsilon_{11}$, $\Delta\varepsilon_{12}$ and $\Delta\varepsilon_{13}$, being dependent of the wavelength.

To obtain the log values of binding constants (log β_{1n}) and molar absorptivities ($\Delta\varepsilon_{1n}$) for HSA-(Cas-II-Gly)$_n$ species the computer program PSEQUAD [40] was used. The experimental CD spectra were included in the input file and the values obtained were: log β_{11} = 9.0 ± 1.5, log β_{12} = 14.6 ± 1 and log β_{13} = 19.8 ± 1. The calculated $\Delta\varepsilon_{11}$, $\Delta\varepsilon_{12}$ and $\Delta\varepsilon_{13}$ values obtained with PSEQUAD are compared with experimental data in Fig. SI-4. In physiological conditions only the 1:1 (HSA:Cas-II-Gly) will be relevant, as the concentration of Cas-II-Gly in blood serum will never be high enough so that the 1:2 species (HSA:Cas-II-Gly) might form.

3.1.5.2. Cas-III-Ea. The experimental CD spectra measured with the solutions containing HSA and Cas-III-Ea were used in the input file for calculations with the PSEQUAD computer program [40] assuming the formation of HSA-(Cas-III-Ea)$_n$ species (n = 1–3). The binding constants obtained were: log β_{11} = 7.6 ± 0.9, logβ_{12} = 13.0 ± 0.8 and log β_{13} = 18.4 ± 0.8, for HSA-(Cas-III-Ea), HSA-(Cas-III-Ea)$_2$ and HSA-(Cas-III-Ea)$_3$ complexes, respectively. The calculated $\Delta\varepsilon_{11}$, $\Delta\varepsilon_{12}$ and $\Delta\varepsilon_{13}$ values obtained with PSEQUAD [40] are compared with the experimental data in Figs. SI-9 to -11, indicating that the calculated CD spectra for species HSA-(Cas-III-Ea)$_n$ are compatible with those measured and with their trend as the relative amount of Cas-III-Ea increases. As for the HSA-Cas-II-Gly system, in physiological conditions

3.1.5.3. Cas-IV-Gly. To obtain the log values of binding constants (log β_{11} and log β_{12}) and molar absorptivity ($\Delta\varepsilon_{11}$ and $\Delta\varepsilon_{12}$ values) for the HSA-(Cas-IV-Gly)$_n$ (n = 1–2), the experimental spectra from CD experiments done with Cas-IV-Gly additions were used to create the input file for PSEQUAD [40]. The binding constants obtained were log β_{11} = 6.0 ± 0.5 and log β_{12} = 9.4 ± 0.6, for the HSA-(Cas-IV-Gly)$_1$ and HSA-(Cas-IV-Gly)$_2$, complexes, respectively. The results presented below using size-exclusion columns and determinations of the Cu-content in the protein fractions indicate that up to Cu:HSA ratios of 3:1 are achieved in these solutions. Probably the 2nd and 3rd complexes formed correspond to very similar binding to HSA and chiral environment, explaining why the 3rd Cas-IV-Gly complex formed is not distinguished by CD spectral measurements. It was not possible to refine any binding constant for HSA-(Cas-IV-Gly)$_3$ complexes when including log β_{13} values in the input file of PSEQUAD. The calculated $\Delta\varepsilon_{11}$ and $\Delta\varepsilon_{12}$ values are compared with the experimental data in Fig. SI-18.

3.1.5.4. Cas-III-ia. As mentioned above, the pattern of the CD spectra obtained and their changes as Cas-III-ia is added may be interpreted as due to the formation of three distinct complex species: HSA-(Cas-III-ia)$_n$, with n = 1–3. To obtain the corresponding binding constants (log β_{1n}, n = 1–3) and molar absorptivities ($\Delta\varepsilon_{1n}$, n = 1–3) for the HSA-(Cas-III-ia)$_n$ species formed, the CD spectra measured were used in the input file of the PSEQUAD program [40]. The binding constants obtained were: log β_{11} = 5.3 ± 0.5, log β_{12} = 9.1 ± 0.3 and log β_{13} = 13.2 ± 0.3, for the HSA-(Cas-III-ia), HSA-(Cas-III-ia)$_2$ and HSA-(Cas-III-ia)$_3$ complexes; the calculated $\Delta\varepsilon_{11}$, $\Delta\varepsilon_{12}$ and $\Delta\varepsilon_{13}$ values are compared with the experimental data in Figs. SI-23.

The log values of binding constants log $_{pq}$ for binding of Casiopeinas to HSA obtained from the CD spectra measured in each system, by using the PSEQUAD program [40] are summarized in

Table 1
The log values of binding constants $_{pq}$ calculated by the PSEQUAD [40] computer program from the CD spectra measured for each of the systems HSA–(Cas-II-Gly), HSA–(Cas-IV-Gly), HSA-(Cas-III-ia) and HSA-(Cas-III-Ea).

Species forming for each system considered	Log β_{pq} (± error)[a]	
{HSA-(Cas-II-Gly)}	Log β_{11}	9.0 (± 1.5)
{HSA-(Cas-II-Gly)$_2$}	Log β_{12}	14.6 (± 1)
{HSA-(Cas-II-Gly)$_3$}	Log β_{13}	19.8 (± 1)
{HSA-(Cas-IV-Gly)}	Log β_{11}	6.0 (± 0.5)
{HSA-(Cas-IV-Gly)$_2$}	Log β_{12}	9.4 (± 0.6)
{HSA-(Cas-III-ia)}	Log β_{11}	5.3 (± 0.5)
{HSA-(Cas-III-ia)$_2$}	Log β_{12}	9.1 (± 0.4)
{HSA-(Cas-III-ia)$_3$}	Log β_{13}	13.2 (± 0.4)
{HSA-(Cas-III-Ea)}	Log β_{11}	7.6 (± 0.9)
{HSA-(Cas-III-Ea)$_2$}	Log β_{12}	13.0 (± 0.8)
{HSA-(Cas-III-Ea)$_3$}	Log β_{13}	18.4 (± 0.8)

[a] The SDs presented are not those directly obtained in the output files of PSEQUAD. They correspond to errors estimated from results of different runs using distinct sets of CD

only the 1:1 (HSA:Cas-III-Ea) will be relevant.

Table 2
Determination of the number of copper atoms per mole of HSA, by analysis of concentrations of HSA (by UV absorption at 280 nm) and Cu (by ICP-AES analysis), in samples of HSA (≈ 100 μM), incubated with the indicated ratios of complex, collected after elution through a size exclusion column (see text and SI section). [a]

Molar Ratios {total Cu(II)}/{HSA}		Molar Ratios {total Cu(II)}/{HSA}	
Initial	After elution from PD-10 column	Initial	After elution from PD-10 column
Cas-II-Gly		Cas-III-ia	
1:1	0.89:1 [b]	1:1	1.1:1
1:1	0.9:1 [b]		
2:1	1.69:1	2:1	1.6:1
3:1	2.35:1	4:1	2.2:1
4:1	2.87:1 [b]		
4:1	2.9:1 [b]		
Cas-IV-Gly		Cas-III-Ea	
1:1	1.1:1	1:1	0.9:1
2:1	1.7:1	2:1	1.5:1
4:1	2.7:1	4:1	2.2:1

[a] Upon passing the size exclusion columns the concentration of HSA, as measured by absorbance at 280 nm for the eluted solution not containing any Copper atoms was 68 μM.
[b] From two distinct experiments.

Table 1. Due to the approximations done to make the calculations, the formation constants should be considered as an estimate of the log $_{pq}$ values.

3.2. Use of size exclusion columns

The concentration of the albumin solution in TRIS buffer (0.10 M, pH = 7.4) prepared for these experiments was ~100 μM in each of the several 5 mL volumetric flasks used for each system. In each flask the required volumes of solutions of each Casiopeína (ca. 1 mM) were added, so that the molar ratios Casiopeínas:HSA were: 0, 1, 2 or 4 (for Cas-II-Gly the ratio of 3 was also analysed). After equilibration each sample was applied to the top of the columns, eluted and collected (see experimental and SI section). The first component to elute from this size exclusion column is the protein, which may contain (or not) bound Cu-complexes. In the collected samples the amount of Cu was analysed by ICP-AES; this correspond to the total Cu(II) bound to HSA in each sample.

Table 2 includes the initial ratios of Casiopeínas:HSA before elution, as well as the final molar ratios determined from the concentration of copper analysed after elution of each sample. For all systems the amount of Cu analysed confirms that (at least) up to 3 molecules of Casiopeínas may bind to HSA, this confirming the CD data. In physiological conditions the amount of Casiopeínas in blood serum will be low, therefore only the first (1:1) HSA-Casiopeínas' complex will be relevant.

3.3. Electron paramagnetic Resonance spectroscopy experiments

To further characterize the HSA-Casiopeína systems EPR spectroscopy was also used. Fig. 9 depicts EPR spectra of solutions containing HSA and Cas-II-Gly. For the spectra with 1:1 and 2:1 molar ratios (Cas-II-Gly:HSA) the signal-to-noise ratio is low because the total Cu ^{II} concentration in the solutions is low. The similar pattern for the three EPR spectra suggests that similar type of donors groups are participating in binding of all HSA-(Cas-II-Gly) species present.

In each of the EPR spectra of Fig. 9 at least two distinct species, which we designate by **1-I** and **1-II**, are observed. The corresponding spin Hamiltonian parameters are: **1-I** - $A_z = 155 \times 10^{-4}\,cm^{-1}$ and $g_z = 2.24(5)$; **1-II** - $A_z = 177 \times 10^{-4}\,cm^{-1}$ and $g_z = 2.19(5)$ (estimated). The EPR spectra of Cu-HSA and Cu-Cas-II-Gly species differ

spectra in the input file.

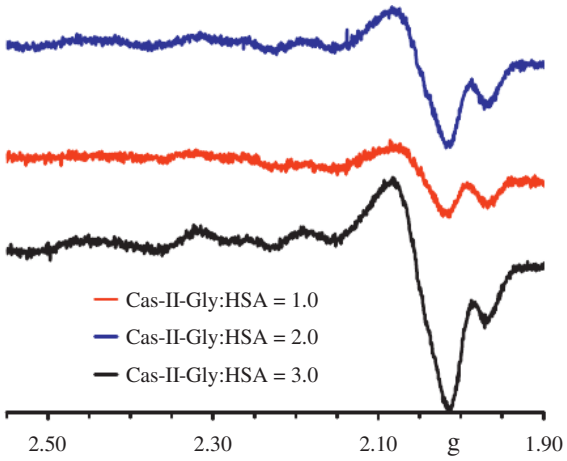


Fig. 9. First derivative of the X-band EPR spectra recorded with frozen solutions (77 K) containing HSA (200 μM) and Cas-II-Gly with the indicated molar ratios. The measurements were carried out in PBS buffer at pH 7.4.

I and **1-II** differ from those reported by Bal et al. [29] for the HSA-Cu system: {ATCUN: $g_z = 2.18$, $A_z = 211 \times 10^{4}\,cm^{-1}$ and MBS: $g_z = 2.29$, $A_z = 177 \times 10^{4}\,cm^{-1}$.

The system HSA-Cu-phen was also studied by CD and EPR [30], and four complex species were considered to be formed: HSA-Cu(phen), HSA-Cu(phen)$_2$, HSA-Cu$_2$(phen) and HSA-Cu$_2$(phen)$_2$ and the corresponding binding constants were determined. The spin Hamiltonian parameters determined were ca. $g_z = 2.26(7)$, $A_z = (150–155) \times 10^{4}\,cm^{-1}$, not equal to those determined for **1-I**, but not much different.

Fig. 10 depicts EPR spectra of solutions containing HSA and either Cas-III-Ea or Cas-II-Gly, and Fig. SI-25 and Fig. SI-26 compare the frozen solution EPR spectra of solutions containing HSA and Cas-III-ia or $CuCl_2$, and of Cas-IV-Gly and $CuCl_2$, respectively, all solutions with Cu(total):HSA molar ratio of 2. Globally the EPR spectra of solutions containing HSA (150 μM) and Casiopeínas differ significantly from those containing HSA and $CuCl_2$.

Fig. 11 depicts EPR spectra of solutions containing HSA and Cas-III-ia. The measured EPR spectra of Cas-III-ia:HSA molar ratio of 1 produced EPR spectra with low intensity. As expected there is an increase in intensity of the spectra with the concentration of complexes, the relative intensity of species with higher g_z value change and the superhyperfine structure becomes more clearly visible for the spectra with 2:1 and 3:1 molar ratios of Cas-III-ia:HSA. While the pattern of the two later EPR spectra are quite similar, thus the Cu(II)-binding set should

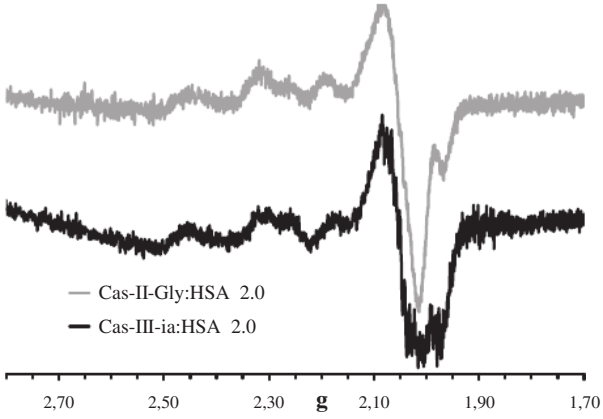


Fig. 10. Comparison of the frozen solution 1st derivative EPR spectra of solutions containing HSA (150 μM, in PBS buffer, pH = 7.4) and either Cas-III-ia or Cas-II-Gly, with Casiopeína:HSA molar ratios of 2.

significantly (see Fig. SI-24), and the spin-Hamiltonian parameters of **1**-

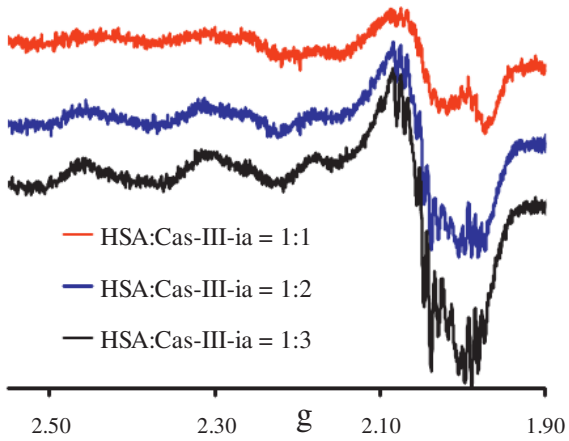


Fig. 11. First derivative of the X-band EPR spectra recorded with frozen solutions (77 K) containing HSA (150 μM) and Cas-III-ia with the indicated molar ratios. The measurements were carried out in PBS buffer at pH 7.4.

also be similar in both cases, the EPR spectrum for the solution with 1:1 molar ratio slightly differs.

Additional splitting due to coupling with ^{14}N nuclei is visible in these spectra. The corresponding EPR parameters are: **4-I** - $A_z = 166 \times 10^{-4}\,cm^{-1}$ and $g_z = 2.24$ (different from those of the Cu(bipy)$^{2+}$ and Cu(bipy)$_2^{2+}$); **4-II** – $A_z = 182 \times 10^{-4}\,cm^{-1}$ $g_z = 2.19$ (estimation). The spectra of Cu(bipy)$^{2+}$ and Cu(bipy)$_2^{2+}$ do not depict such superhyperfine pattern [30] and this is not much visible also in the spectra of the HSA-Cu-phen system [30]; the EPR parameters again differ from those reported by Bal et al. [29] for Cu^{2+} binding to HSA. Therefore, the X-band EPR spectra shown in Fig. 11 are compatible with the observations made from CD measurements, namely they cannot be explained by decomposition of Cas-III-ia and binding to HSA of either Cu^{2+} or Cu-phen species. It is plausible that the EPR spectrum of the third species overlaps with the other ones (namely of **4-I**, as the intensity of its components increase upon addition of the complex). Thus, globally the CD and EPR data may be considered compatible with the existence of HSA-(Cas-III-ia), HSA-(Cas-III-ia)$_2$ and HSA-(Cas-III-ia)$_3$.

In Fig. 12 it is clear that all EPR spectra of solutions containing HSA and Cas-IV-Gly (**3**) differ from the EPR spectrum of the solution containing $CuCl_2$ and HSA, although the spectrum with Cas-IV-Gly:HSA = 1 shows similarities in the low field range up to ca. g = 2.0. As observed in the Cas-III-ia + HSA system, the EPR spectra of solutions containing Cas-IV-Gly with molar ratios Cas-IV-Gly:HSA of 2 and 3

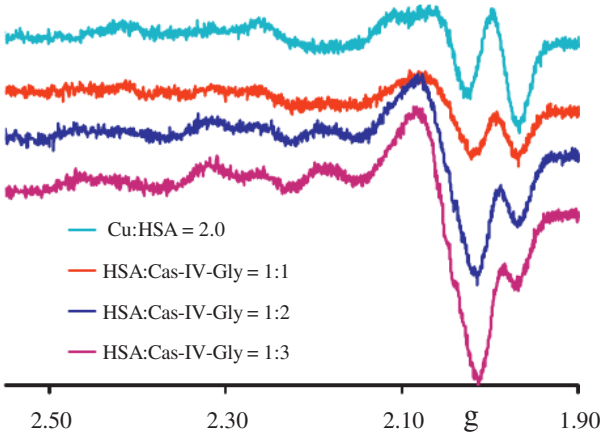


Fig. 12. First derivative of the X-band EPR spectra recorded with frozen solutions (77 K) containing HSA and either $CuCl_2$ (~400 μM of HSA) or Cas-IV-Gly (~300 μM of HSA) with the indicated molar ratios. The measurements were carried out in PBS buffer at pH 7.4.

are quite similar (but that for the molar ratio of 1, differs). This indicates the presence of species with similar donor atoms coordinated to the metal centre in the case of the solutions with molar ratios Cas-IV-Gly:HSA of 2 and 3. The corresponding EPR parameters are similar to those obtained for Cas-II-Gly: **3-I** - $A_z = 155 \times 10^{4}\,cm^{-1}$ and $g_z = 2.25$; **3-II** – $A_z = 177 \times 10^{4}\,cm^{-1}$ and $g_z = 2.19$ (estimation). Also in this system **3-I** and **3-II** are not simple HSA-Cu^{II}-bipy complexes.

Fig. SI-26 depicts EPR spectra of solutions containing HSA and either Cas-IV-Gly or $CuCl_2$, after a few minutes and after 24 h of addition of the Cu-complex. No significant differences are observed in the EPR spectra of either system up to 24 h.

Table 3 collects the spin Hamiltonian parameters estimated for HSA-Casiopeinas' systems.

The similarity of the EPR spectra and of the spin Hamiltonian parameters (Table 3) for species **I** and **II** in the HSA-(Casiopeina) systems suggest similar types of Cu(II) environment in all HSA-(Casiopeina) systems. It is clear that both the CD and EPR data recorded with HSA concentrations in the range 150–400 μM do not indicate extensive hydrolysis of the Casiopeínas and subsequent binding of free Cu^{2+} ions at the ATCUN or MBS sites. However, the EPR data for species **I** do not differ much from those obtained for the HSA-Cu^{II}-phen system [30]. This means that, from the EPR spectra obtained it would not be possible to sort out if one of the species formed might not be Cu-bipy or Cu-phen complexes bound to HSA, i.e., if one of the several species detected might consist of Cu binding upon release of acac (**2** and **4**) or Gly (**1** and **3**). However, taking also into account the CD data (above), we can conclude that the binding of Casiopeinas **1**–**4** to HSA does not correspond to simple binding of either Cu-Me$_2$bipy or Cu- Me$_2$phen complexes bound to HSA.

Peisach and Blumberg compiled experimental g_z and A_z values obtained from a wide range of model Cu^{II}-compounds and constructed a g_z/A_z plot [48]. This representation allows to establish correlations between geometry, donor group and experimental g_z and A_z data obtained from EPR spectra. The Peisach-Blumberg plot allows the prediction of the donor group set and geometry of a Cu^{II} complex. Addison and co-workers [49] published an updated version of this plot and introduced another empirical relation known as the tetrahedral distortion index, which is obtained by dividing g_z for A_z (in cm^{-1}). The larger this index is, the greater the extent of the tetrahedral distortion. Values ranging from 100 to 135 cm indicate a square-planar geometry with minimal distortion. For the HSA-Casiopeinas most of the g_z/A_z values are within this range (Table 3).

In the present case the binding of the Casiopeinas to HSA involves coordination numbers for Cu^{II} higher than 4. The representation of (g_z, A_z) values obtained for the spin Hamiltonian parameters (Fig. SI-27) all fall close to the line corresponding to the N_4 binding set. It is plausible that the complexes bind to HSA at N-imidazole donors from His residues, or N-amino from Lys residues, the O-Gly atom probably binding axially. It is possible that a second donor atom from the protein may bind (and other possibilities of binding exist), however, the data available does not allow indicating definite assignments for the binding mode.

3.4. MALDI-TOF mass spectrometry

As described in the experimental section the samples for MALDI-TOF MS were prepared with Cas-II-Gly:HSA molar ratios of 0:1, 3:1 and 5:1 by mixing different volumes of the stock solutions with a NH_4HCO_3 buffer (pH 7.4, 25 mM). The results hereby reported were obtained with samples prepared as described in Section 2.3. The concentrations of HSA in the final samples were 25 μM, thus lower than those used in the CD and EPR spectroscopic measurements.

In a set of experiments the average mass obtained for HSA was 66,477 (± 30), while the average mass obtained with samples containing 3:1 Cas-II-Gly:HSA molar ratios was 66,892 (± 15). Thus, the

Table 3
Spin Hamiltonian parameters obtained for the HSA-(Cas-II-Gly), HSA-(Cas-IV-Gly) and HSA-(Cas-III-ia) systems at 77 K. Solutions were prepared in PBS buffer (pH 7.4). The values obtained for solutions containing HSA and Cu(II), HSA, Cu(II) and phen, and for the Cu^{II}-bipy system are also included [30].

	g_z[a]	A_z ($\times 10^4$ cm^{-1})	g_z/A_z	g_z	A_z ($\times 10^4$ cm^{-1})	g_z/A_z
HSA-(Cas-IV-Gly)	2.25 (**3-I**)	155 (**3-I**)	145	2.19 (**3-II**)	177 (**3-II**)	124 [b]
HSA-(Cas-III-ia)	2.24 (**4-I**)	166 (**4-I**)	135	2.19 (**4-II**)	182 (**4-II**)	120 [b]
HSA-(Cas-II-Gly)	2.24 (**1-I**)	157,155 (**1-I**)	143	2.19 (**1-II**)	179,177 (**1-II**)	122 [b]
Cu(II) + HSA	2.29[a]	177[a]	129[a]	2.18[b]	211[b]	103[b]
HSA:Cu:phen = 1:2:0	~2.29	177	129	~2.18	211	103
HSA:Cu:phen = 1:2:1	2.274	150	152	2.187	204	107
HSA:Cu:phen = 1:2:2	2.267	154	147	2.190	205	107
HSA:Cu:phen = 1:2:3	2.267	154	147			
HSA:Cu:phen = 1:2:4	2.267	155	146			
Cu:phen = 1:3 or 1:4	2.271	154	147			
$Cu(bipy)^{2+}$	2.307	170	136			
$Cu(bipy)_2^{2+}$	2.263	162	140			
$Cu(bipy)(OH)(H_2O)_3$	2.253	177	127			
$Cu(bipy)(OH)_2(H_2O)_2$	2.232	196	114			

[a] ATCUN site of HSA.
[b] MBS site of HSA.

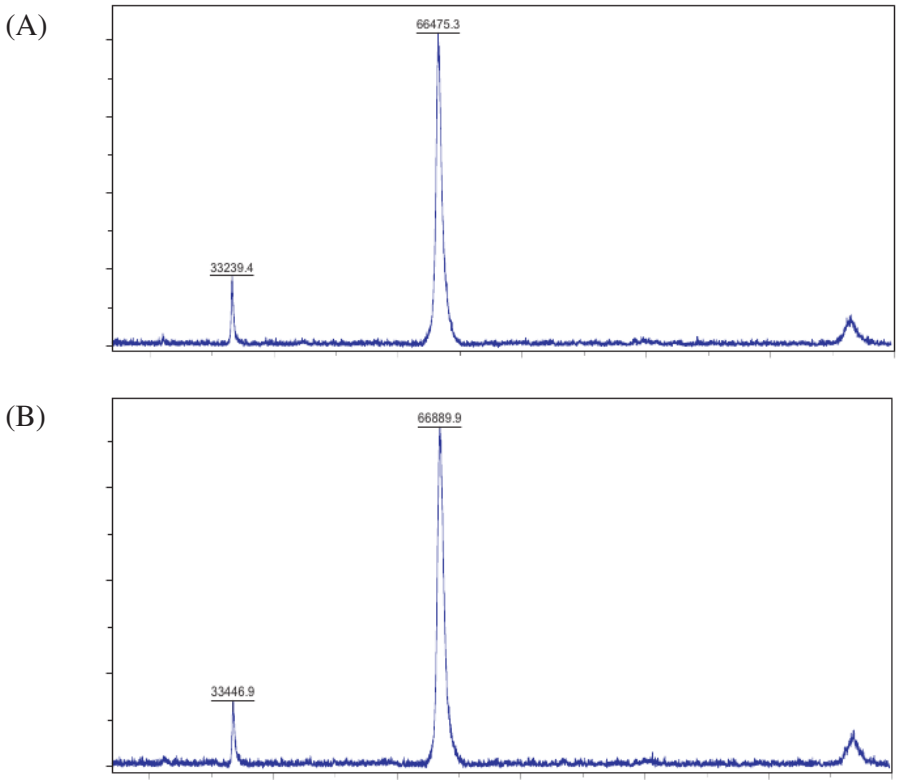


Fig. 13. MALDI-TOF mass spectra obtained for samples of (A) HSA and (B) Cas-II-Gly:HSA (with a 3:1 molar ratio).

difference to the binding of one Cu(II) and one $Cu(Gly)(Me_2phen)^+$ molecule to HSA (total increase in mass of 408 Da). The possibility of the binding of one $Cu(Me_2phen)_2^{2+}$ corresponds to an increase in mass of 479 Da, and that of one $Cu(Me_2phen)^{2+}$ and one Cu(II) moieties to 542 Da, which may be ruled out to explain our MALDI-TOF mass spectrometry data. Fig. 13 depicts two representative Maldi-TOF spectra.

Interestingly, in the set of MALDI-TOF MS experiments carried out with 5:1 Cas-II-Gly:HSA molar ratios the obtained average difference in mass between the samples of HSA and of Cas-II-Gly + HSA was 665 (± 20, considering the several spectra obtained for different samples of these solutions). This is consistent with the binding of HSA to e.g. one Cu(II), one $Cu(Gly)(Me_2phen)^+$ and one $Cu(Me_2phen)^{2+}$ moiety, further discussed below but we highlight here that as the concentrations used in the Maldi MS experiments were ca. 25 μM, lower than those used in CD and EPR studies, it is expected that significant proportion of the Casiopeina, $Cu(Gly)(Me_2phen)^+$, hydrolyzes to form Cu-HSA and $Cu\text{-}Me_2phen$ species, in agreement with the known global trend in aqueous solution that release of the ligands is favored by decreasing the total metal concentration.

3.5. Use of fluorescence spectroscopy

The use of the quenching of fluorescence to study the binding of complexes to BSA or HSA relies mainly on the quenching of tryptophan residues (Trp214 in the case of HSA, located in subdomain II-A, being

which correspond to a total increase in mass of 680 Da. This subject is part of the so-called drug binding Site I), and to some extent of the Tyr

residues. If the binding of the drug to HSA takes place close to the Trp214 residue, the quenching effect may be considered to result from a quite direct effect on this residue, but if the binding takes place very far from Trp214, either there is an effective mechanism for the quenching to occur, or the quenching will be hardly detected.

Fluorescence spectroscopy (FS) has been frequently used to study the interaction of metal complexes with proteins, namely BSA and HSA, but to our knowledge FS has not been used to study the interaction of Casiopeina-type complexes with HSA, but there are reports of studies with BSA [37,44,50–52].

Fluorescence spectroscopy is useful to study the interaction of compounds to BSA or HSA, but should applied critically and used for the determination of binding constants only when this determination is valid and if the experimental conditions allow it. For the binding of organic compounds to proteins the use fluorescence quenching methods was discussed by Weert [53,54], but for metal complexes further aspects must be checked if binding constants may be determined by FS applying these methodologies (see below). In most cases Stern-Volmer or modified Stern-Volmer equations have been used, the quenching considered to be static and the binding constant, K_{BC} and the number of binding sites per HSA molecule (n) calculated with the equation:

l / (4)

For the Casiopeína-type complexes with BSA the values determined for K_{BC} are in the range 10^3–10^6[50–52].

3.5.1. Determination of binding constants of Casiopeínas' to HSA by fluorescence quenching measurements

Fluorescence spectroscopy was used in this work to monitor changes induced by the interaction with the Casiopeina complexes **1**, **3** and **4**, following the procedure typically used in this type of studies. Titrations of HSA solutions in PBS buffer at pH 7.4 were carried out adding aliquots of the Casiopeinas' stock solutions and the corresponding fluorescence emission spectra were measured. Fluorescence spectra of HSA in the absence and in the presence of different amounts of Cas-II-Gly are shown in Fig. 14 (for the other Casiopeinas, see Fig. SI-29). The emission maximum was observed at 337 nm, and addition of the complex to HSA in the molar ratio 0.1 to 6 strongly quenches (by 57%) the tryptophan fluorescence at pH 7.4.

The Stern–Volmer plots obtained for all complexes **1**, **2** and **4** are shown in Fig. SI-30. Linear fittings of the experimental data were obtained for all complexes, affording the K_{SV} values included in Table 4. The complexes have similar K_{SV} constants, in the 10^4 range, with Cas-II-Gly showing the strongest quenching and highest constant. Since $K_{SV} = k_q\tau_0$ - where k_q is the bimolecular quenching rate constant and τ_0 is the HSA fluorescence lifetime - and in average the molecular lifetime of HSA in the absence of quencher is ca. 5 ns [55], the bimolecular rate constant can be calculated and is also included in Table 3. For dynamic quenching, the maximum scattering collision quenching constant of various quenchers is 2.0×10^{10} L·mol^{-1} s^{-1} [56]. Continuing with what has been typically done in this type of studies, it is normally argued that since the k_q values obtained here are much greater, this would indicate that the quenching is not caused by dynamic collision but through formation of complexes between HSA and the Casiopeinas' - static quenching.

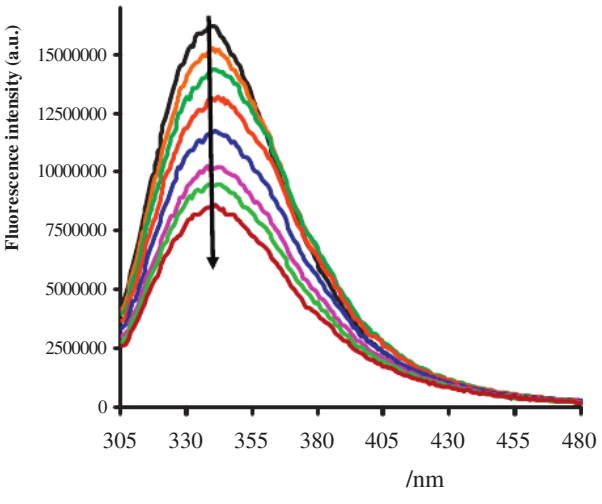


Fig. 14. Emission fluorescence spectra (λ_{exc} = 295 nm) of HSA (1.5 μM) in the absence and presence of increasing amounts of Cas-II-Gly (from 0 to 8.5 μM) after subtraction of the corresponding spectra of solutions containing the complex in the absence of HSA.

Fig. SI-31 shows the plots corresponding to Eq. (4) and the values obtained for K_{BC} and n are listed in Table 4. The values obtained for n are equal to 1 or very close to it, suggesting that there is one single binding site in HSA for the Casiopeinas' complexes. The binding constants follow the order Cas-II-Gly < Cas-IV-Gly ≤ Cas-III-ia. While the fitting for Cas–II-Gly and Cas-III-ia was consistent, the one for Cas-IV-Gly depended on the concentration range used. The order of magnitude of the binding constants of the Casiopeinas (**1**, **2** and **4**) to HSA, determined by fluorescence spectroscopy, is of the same order of magnitude or significantly higher than those determined for Casiopeína-type compounds to BSA using similar methodologies [57–59].

3.5.2. Speciation considerations and consequences to the reliability of binding constants measurements

Having described the methodology frequently applied in literature and the results obtained from it, if the drug is a metal complex, as is the case of the Casiopeínas, the data based on quenching of fluorescence should be critically analysed. In fact, while the visible absorption, CD and EPR spectroscopic experiments are usually done with concentrations in the mM range, the fluorometric measurements are normally carried out with protein solutions of 1–3 μM concentrations, and up to 20 μM of complex. With labile complexes of metal ions such as Cu(II), which may hydrolyze, it may be questionable if such complexes maintain their integrity at such low concentrations. Taking the example of Cas-II-Gly, the relevant formation constants are not known, but have been reported for the related system: Cu(II) – Gly – phen [46,47].

Figs. SI-32 to Fig. SI-35 depict speciation diagrams for the system Cu(II)–Gly–phen for several concentrations of each component, with $[Cu(II)]_{total} = [Gly]_{total} = [phen]_{total}$. It is clear that the speciations may differ significantly; namely, at pH 7.4, while at total 1 mM concentrations the amount of $Cu(Gly)(phen)^+$ is ca. 77% of total Cu(II), at 200 μM it is ~70% and at 2 μM concentration (approximately the conditions chosen for the fluorescence quenching determinations), $[Cu(Gly)(phen)]^+$ is ca. 16% of total Cu(II). At 2 μM total concentration the main complex now present is $Cu(phen)^{2+}$ (ca. 52%), relevant amounts of Cu^{2+} and $Cu(OH)^+$ being also present. Fig. 15 depicts concentration distribution diagrams calculated for the Cu(II)-containing species of the Cu(II) + Gly + phen + HSA system at pH = 7.4 in the range 1 to 20 μM of total complex concentration. As $Cu(phen)^{2+}$ and Cu^{2+} may also bind to HSA (or BSA) [30] it is plausible that the quenching of fluorescence may be due to at least two/three species bound to HSA: $Cu(Gly)(phen)^+$ and $Cu(phen)^{2+}$ and Cu^{2+}, thus, not only one species, so the methodology described above, and frequently used in the literature, cannot be applied to determine the binding constant of $Cu(Gly)(phen)^+$ (or of Cas-II-Gly) to HSA or BSA.

Notwithstanding, at higher concentrations, as those used in the CD and EPR measurements, and considering the log K values determined for the binding of Cas-II-Gly to HSA (see above), the main species present in the system Cu^{II}-Gly-phen-HSA at pH ~7.4 are $[Cu(Gly)(phen)]_n$-HSA species (see e.g. Figs. SI-38 and -39). This also indicates that in this system, the binding constant for $[Cu(Gly)(phen)]_1$-HSA determined from CD measurements (ca. 10^9) should be closer to its correct value than those determined by the fluorescence quenching

the corresponding spectra of solutions containing the complex in the absence of HSA.

methodology (ca. 10^{-3}–10^{-4}), which are wrong.

Table 4
Quenching %, Stern-Volmer constant (K_{SV}), maximum emission wavelength (λ_{em}) and bimolecular rate constant (k_q), binding constant (K_{BC}) and n binding sites for the interaction of HSA (1.5 μM in PBS 0.1 M, pH 7.4) with the studied Casiopeinas'.

Complex	% quenching	K_{SV}/M^{-1} [a]	λ_{em}/nm	$k_q/M^{-1}s^{-1}$	K_{BC}/M^{-1} [a]	n
Cas-II-Gly	57	$8.6(4) \times 10^4$	337	$1.7(2) \times 10^{13}$	$2.3(9) \times 10^4$	0.90
Cas-IV-Gly	62	$7.1(0) \times 10^4$	341	$1.4(2) \times 10^{13}$	$9.8(9) \times 10^4$	1.0
Cas-III-ia	61	$7.5(6) \times 10^4$	339	$1.5(1) \times 10^{13}$	$1.3(5) \times 10^5$	1.0

[a] While the fitting for Cas–II-Gly and Cas-III-ia was consistent, the one for Cas-IV-Gly depended on the concentration range used.

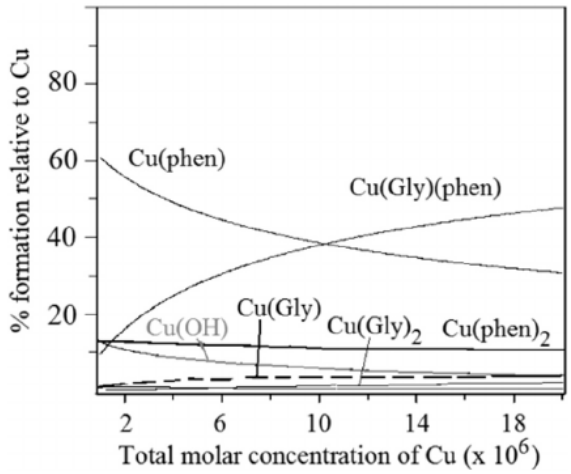


Fig. 15. Distribution diagram for the system Cu(II) + Gly + phen, with concentrations ($[Cu(II)]_{total} = [Gly]_{total} = [phen]_{total}$) varying in the range 1 to 20 μM at pH ~7.4. The formation constants were taken from Turkel et al. [47] and $Cu(OH)^+$ with log β = − 6.4. The charges of species were omitted for clarity.

In the SI section we present several speciations for the Cu^{II}-Gly-phen-HSA system in distinct conditions, starting by assuming that only $Cu(Gly)(phen)^+$ species bind to HSA (case A), then assuming that also $(Cu^{2+})_1$(HSA) and $(Cu^{2+})_2$(HSA) may form [29,30,60], (case B), and finally assuming that also $Cu(phen)_n$ may bind to HSA [30] (case C).

As a general comment regarding the use of fluorescence quenching experiments and the methodology described in Section 3.5.1 for the determination of binding constants (complex + HSA complex-HSA) should be only be applied after critically examining the system and its speciation behavior at low concentrations (ca. 10^{-6} M), namely taking into consideration the hydrolysis of the metal ion and the capacity of all relevant species at these low concentration, namely the ligands, to bind the protein. In metal complex systems, particularly involving labile hydrolysable metals that may bind to HSA (e.g. Cu, Zn, V) and where the values of binding constants are lower than ca. 10^6, the fluorescence quenching methodology described above probably will not yield reliable results. Moreover, if it is experimentally observed, as often reported, that the quenching continues to increase when several moles of drug per mole of HSA (or BSA) have been added, e.g. 8 or more, it is not probable that all molecules will bind to the protein and/or will do it at identical/very similar binding sites.

Once understanding that the use of emission fluorescence experiments may have limitations for the determination of binding constants of drugs to proteins, particularly of metal complexes, but having clear indications that the Casiopeínas bind to HSA, the observation of significant quenching of fluorescence when using λ_{exc} = 295 nm (Fig. 14 and Fig. SI-29), is an indication that the binding of the first molecule of Casiopeína to HSA takes place not far from the Trp214 residue, thus at sub-domain II-A. Possible residues that may be involved in the binding to the Cu centre might be e.g. His242 and Lys199. Other residues belonging to drug binding site I such as His288 and Lys195 are also plausible N-donors for coordination to Cu(II). The plausibility of co-

single crystal X-ray diffraction of the complex formed with a copper(II)-benzoyl hydrazone Schiff base [61]. The binding to HSA might allow selective tumor targeting. Noteworthy, in the study of Gou et al. [61] it was found that, compared with the Cu(II)-salts, the HSA-complexes enhanced cytotoxicity in MCF-7 cells (around 3 − 5-fold), but did not increase cytotoxicity levels in normal cells in vitro, possibly by, to some extent, selectively accumulating in cancer cells.

3.5.3. Other comments on speciation

In this work we are not discussing mechanisms of cytotoxic action, but it may be relevant to make some further comments concerning in vitro and in vivo studies involving metal complexes.

It is known that the release of the ligands is favored by increasing pH and decreasing the total metal concentration, this being particularly relevant for metal ions susceptible to hydrolysis such as V(IV) or V(V). This has been discussed in several recent publications [62–69]. Reactions with cell culture media may lead to significant change in metal speciation, thus give rise to modification of biological activities or between different in vitro assays. Additionally, and due to the possibility of many other types of interactions and media involved, results from in vivo assays may also differ significantly from in vitro observations. The effects observed may also not be directly related to the metal complex under study. Besides the expected relevant role of serum proteins, particularly HSA and transferrin both in the speciation and biological activity, namely in the case of vanadium and copper it is feasible that the interactions at the level of cell membranes will be relevant, as well as inside cell compartments such as e.g. lysosomes [70]. Further development in the tools to study speciation and the fate of both metal and ligands upon addition of metal complexes to biological media in contact with cells are required to clarify the mechanism of biological action of metal complexes.

4. Conclusions

The group of Cu(II)-complexes known as Casiopeínas have been reported to demonstrate benefits in the treatment of cancer, particularly by increasing specificity and decreasing side effects in comparison to currently used drugs, and Cas-II-Gly (**1**) is in phase I of clinical trials [21].

Binding to HSA can alter pharmacokinetic and pharmacodynamic properties of drugs and the determination of their binding sites in HSA is an important aspect in the drug development process. In this work we report studies of binding of Cas-II-Gly (**1**), Cas-III-Ea (**2**), Cas-IV-Gly (**3**) and Cas-III-ia (**4**) to HSA, which confirm that these Casiopeínas may bind to HSA and may be transported in blood serum by this protein. MALDI-TOF mass spectrometry experiments carried out for **1** confirm the binding of Cas-II-Gly to HSA.

In the physiological range of concentrations that may be found in blood, Casiopeínas may form 1:1 adducts with HSA and the conditional binding constants determined were ca. 1×10^9 (**1**), 4×10^7 (**2**), 1×10^6 (**3**) and 2×10^5 (**4**), values calculated from the CD spectra measured. This might allow some selective tumor targeting, particularly for Cas-II-Gly, without corresponding to an irreversible binding of the complexes to HSA. Fluorescence emission spectra suggest that the

ordination of N-atoms of His242 and Lys199 to Cu(II) was confirmed by

binding probably takes place close to the Trp214 residue and we

suggest that the residues involved in the binding to the Cu centre might be e.g. His242 and Lys199.

In this work we also discuss aspects associated to the reliability of the frequently used methodologies based on the measurement of fluorescence emission spectra of solutions containing low concentrations of proteins such as HSA and BSA, by titrations with solutions of metal complexes. It is demonstrated by making speciation calculations that for complexes such as Cas-II-Gly these methodologies that are based on very low concentration of metal complexes may confirm the binding of metal complexes to the protein, but are not adequate for the determination of the corresponding binding constants.

Speciation calculations are also important to fully predict/understand how Cas-II-Gly and other Casiopeínas may be transported by HSA. As the concentration of Cas-II-Gly decreases, the extent of decomposition of this mixed-ligand compound into Cu^{2+}, $Cu(Me_2phen)$ and Gly increases; at the low concentrations of Cas-II-Gly expected to be found in blood it is predictable that the transport involves more than one type of Cu-species, namely Cu-HSA, $[Cu(Me_2phen)]$-HSA and $[Cu(Me_2phen)(Gly)]$-HSA will all be involved.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2017.07.025.

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