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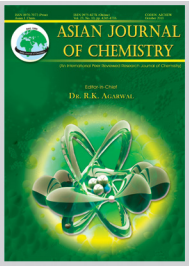

Fluorescence Study on the Interaction of Human-Like Collagen and Copper(II)

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Fluorescence spectroscopy and UV-VIS absorption spectroscopy were employed to analyze the binding of Cu^{2+} to human-like collagen (HLC) with Cu^{2+} concentrations from 0 to 1.0×10^{-4} mol L^{-1} at 273 and 298 K. The fluorescence titration results indicated that Cu^{2+} quenched the fluorescence intensity of human-like collagen and the quenching mechanism should belong to both dynamic and static quenching according to the Stern-Volmer equation. The binding constants and the corresponding thermodynamic parameters at different temperatures were calculated, which showed that hydrophobic interactions were the main binding force. The conclusion that Cu^{2+} could bind to human-like collagen was clarified by all these experimental results and theoretical data, and it could be a useful guideline for further study of human-like collagen-Cu complex.

Key Words: Human-like collagen, Fluorescence, Copper.

INTRODUCTION

Copper is an essential trace metal found in all living organisms in the oxidized Cu(II) and reduced Cu(I) states. It is required for survival and serves as an important catalytic cofactor in redox chemistry for proteins that carry out fundamental biological functions required for growth and development[1]. The average intakes of copper by human adults vary from 0.6 to 1.6 mg/d[1-4]. Although copper deficiency is rare, it may occur when there is a genetic defect in the functioning of a copper transporter (ATP7A), resulting in Menkes disease or the milder Occipital Horn Syndrome. Menkes and Wilson's diseases (WDs), human genetic diseases in Cu transport, have revealed the importance of maintaining appropriate Cu homeostasis[5-9]. Moreover, Cu is essential for efficient iron uptake and mobilization in mammals[10].

Traditionally, supplementation of copper has been achieved through inorganic salts, such as sulfate. However, the mineral availability from this source was inhibited by some antinutritional substances, such as phytate and tricalcium phosphate. Hence, there has been a great deal of interest in alternative mineral sources for copper supplementation, particularly the copper(II) complexes of amino acids, small peptides and other low molecular weight proteins, due to their higher bioavailability compared to conventional (inorganic) sources[11-13].

Besides nutritive supplies for humans and animals mentioned above, copper(II) complexes present a variety of important pharmacological effects, including antiinflammatory, antibacterial, antiulcer, anticonvulsant and even antitumoral activity[14-19]. Moreover, it has been found that the pharmacological activity of certain copper complexes is usually more active than that of the free ligands[14-16].

Studies on copper complexes of amino acids and peptides have been performed[20-24], and as a conti-nuation of them, human-like collagen (HLC) has now been chosen as a ligand in the present study, which is recombinant protein and its gene coding is produced by cloning a partial cDNA that was reversed by mRNA from human collagen and transferred to *E. coli*. The previous study demonstrated that it has several special characteristics which are significantly different from animal collagen, such as virus-free, chemically defined structure, biocompatibility, processability, water solubility, little immunogenic reaction and so on[25-27]. To the best of our knowledge, the use of human-like collagen in coordination study on copper complex has not hitherto been reported.

Fluorescence quenching is a powerful technique used to obtain adequate information on the interaction between small molecule and bio-macromolecule owing to its high sensitivity, selectivity, reproducibility, convenience and abundant theoretical foundation[28,29]. In this experiment, the binding characteristics and the binding constants were obtained by fluorescence and absorption spectroscopic methods. Besides, the nature of the binding forces was analyzed on the base of the calculated thermodynamic parameters. These results are of crucial importance to understand the mechanism of photodynamic

actions of Cu(II) on human-like collagen and offer theoretical foundation for the preparation of HLC-Cu chelate.

EXPERIMENTAL

Human-like collagen (HLC, China Patent No. ZL01106757.8, M_r = 97,000) was purchased from Xi'an Giant Biogene Technology (XABTC) Co. All other chemicals used were of analytical reagents grade and double distilled water was used throughout.

Steady-state fluorescence measurements were carried out on a spectrophotometer (HITACHI F-4500, Japan) equipped with 1.0 cm quartz cell. The absorption spectra were recorded with an UV-VIS spectrophotometer (Unico 2802pcs, USA). All pH measurements were made with a pH meter (Mettler-Toledo EF20, Switzerland).

Methods: The absorption spectroscopy measurement was performed at room temperature (298 ± 1 K). The spectra were recorded between 200 and 350 nm. The fluorescence spectra were performed at different temperatures (273 and 298 K). The concentration of human-like collagen was stabilized at 1.0×10^{-5} mol L^{-1} and the concentration of Cu(II) varied from 0-1.0 to 10^{-4} mol L^{-1}. The excitation wavelength of 280 nm was chosen and the emission spectra in the range of 300-540 nm were recorded.

RESULTS AND DISCUSSION

Absorption spectra: The electronic absorption spectra for human-like collagen in the absence and presence of copper ions are given in Fig. 1. Free ligand and complex exhibit similar spectra in UV region. A common feature of these spectra is the presence of absorption peak near 220 nm, which is contributed by $n \rightarrow \pi^*$ transition of C=O in the peptide bond[30]. The detected increase in the absorption and a bathochromic shift of the absorption peak at about 220 nm with increasing Cu(II) concentration compared to the free human-like collagen indicate that there exists interaction in human-like collagen with Cu(II) and ground state complex forms.

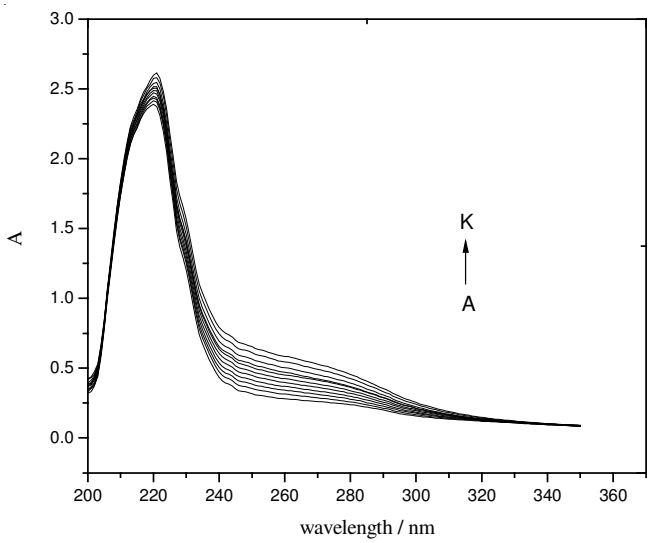

Fig. 1. Absorption spectra of HLC and copper ions. [HLC] = 1.0×10^{-5} mol L^{-1}, [Cu^{2+}] = 0-1.0×10^{-4} mol L^{-1}

Fluorescence quenching spectra: There are three types of fluorophores in protein, namely, tryptophan residue, tyrosine residue and phenylalanine residue. When 280 nm excitation wavelength is used, the intrinsic fluorescence of protein comes from both tryptophan and tyrosine residues, whereas 293 nm wavelength only excites tryptophan residues[31]. There is no tryptophan residue in the molecule of human-like collagen, therefore 280 nm was chosen as the excitation wavelength throughout the experiment.

The fluorescence quenching spectra of human-like collagen at various concentration of Cu(II) are shown in Fig. 2. It illustrates that HLC had a strong fluorescence emission band at 348 nm, while Cu^{2+} had no intrinsic fluorescence by fixing the excitation wavelength at 280 nm. And the fluorescence emission intensity of HLC decreased regularly as the concentration of Cu^{2+} increased. These results indicated that there were strong interactions and energy transfer between HLC and copper ions and a HLC-Cu^{2+} complex formed.

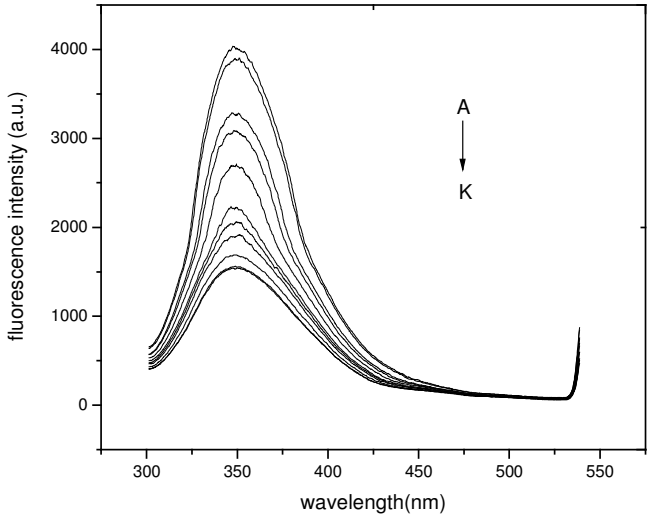

Fig. 2. Fluorescence quenching spectra of HLC with various concentrations of copper ions. $C_{HLC} = 1.0 \times 10^{-5}$ mol L^{-1}; curves A → K: C_{Cu} = 0-1.0×10^{-4} mol L^{-1}. (λ_{ex} = 280 nm, T = 298 K)

Study of fluorescence quenching mechanism: Fluorescence quenching refers to a process that fluorescence intensity of a fluorophore decreases by variety of molecular interactions with quencher molecule. Under the fixed pH, temperature and ionic strength, fluorescence quenching are normally due to the molecular interactions such as ground-state complex formation, exciplex formation and energy transfer in biochemical systems[32].

Fluorescence quenching is usually classified into static quenching and dynamic quenching. The formation of non-fluorescent ground-state complex between the fluorophore and the quencher results in static quenching and if the fluorophore and the quencher come into contact during the lifetime of the excited state, then dynamic quenching takes place. In general, static and dynamic quenching can be distinguished by their different dependence on temperature and viscosity or by lifetime measurements[33]. Higher temperature typically results in the dissociation of weakly bound complexes, whereas it leads to faster diffusion. Therefore, the quenching rate constants decrease with increasing temperature for the static quenching,

but the reversed effect was observed for the dynamic quenching[34].

The fluorescence quenching data are usually analyzed by the stern-Volmer equation[33].

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. K_q is the quenching rate constant of the biomolecule. τ_0 is the average lifetime of the fluorescent molecule without quencher. [Q] is the concentration of quencher. In this paper, [Q] is the concentration of copper ions (II). $K_{SV} = K_q\tau_0$ is the Stern-Volmer quenching constant. Because the fluorescence lifetime of the biopolymer is about 10^{-8} s[35], the quenching constant can be obtained by the slope.

The F_0/F-[Q] (Stern-Volmer) plots of human-like collagen with Cu(II) at different temperatures (273 and 298 K) are displayed in Fig. 3. In the range of Cu(II) concentration from 1×10^{-5} to1×10^{-4} mol L^{-1} (concentration of HLC was 1×10^{-5} mol L^{-1}), the curves exhibit good linear relationships and the slops increase with the temperature increasing. Therefore, dynamic quenching interaction between HLC and Cu(II) should occur and may be the quenching mechanism. However, the static quenching effect can not be completely precluded in the HLC and Cu(II) system. According to eqn. 1, the quenching rate constant K_q (L mol^{-1} s^{-1}) was obtained at different temperatures and listed in Table-1. Generally, the maximum dynamic quenching constant K_q of various kinds of quenchers with biopolymer is 2.0×10^{10} L mol^{-1} s^{-1} [36]. However, K_q of HLC quenching procedure initiated by Cu(II) are greater than 2.0×10^{10} L mol^{-1} s^{-1}, which means that the process is static. In conclusion, it is considered that there are two modes of interaction, dynamic and static quenching, between HLC and Cu(II).

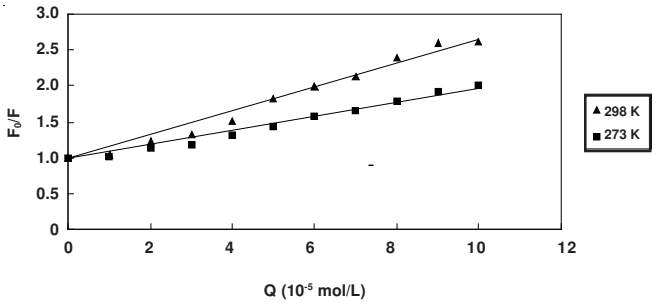

Fig. 3. Stern-Volmer plots for the quenching of HLC by copper(II) ions at different temperatures

TABLE-1
QUENCHING CONSTANTS OF HLC BY COPPER IONS

T (K)	Stern-Volmer equation	Correlation coefficient	K_{SV} ($\times 10^4$ L mol^{-1} s^{-1})	K_q($\times 10^{12}$ L mol^{-1} s^{-1})
273	Y = 0.0954X + 1	0.9724	0.954	0.954
298	Y = 0.1638X + 1	0.9722	1.638	1.638

Determination of binding constants and binding points: When small molecules bind independently to a set of equivalent sites on a macro-molecule, the equilibrium between free and bound molecules is given by the following quation[37,38].

$$\log\frac{F_0 - F}{F} = n\lg K - n\log\frac{1}{[Q_t] - \frac{F_0 - F}{F_0}[P_t]} \quad (2)$$

where in the present case, K is the binding constant to a site, n is the number of binding sites per HLC molecule and $[Q_t]$ and $[P_t]$ is the concentration of Cu^{2+} and human-like collagen, respectively. According to eqn. 2, the binding parameters can be obtained by the plot of lg $[(F_0\text{-}F)/F]$ against lg $[1/([Q_t]\text{-}(F_0\text{-}F)[P_t]/F_0)]$.

The fitted plots of $\log\frac{F_0 - F}{F}$ *versus* log $[1/([Cu^{2+}]\text{-}(F_0\text{-}F)[HLC]/F_0)]$ were got by the measured fluorescence data at different temperatures. The values of K and n were calculated from the values of intercept and slope of the plots (Fig. 4), respectively, and the corresponding results are listed in Table-2. The value of K increases with the temperature rising and n is about 1.5, which exhibits that HLC and Cu(II) formed the complex of mol ratio 2:3 and the complex would not be decomposed when temperature increased from 273 to 298 K.

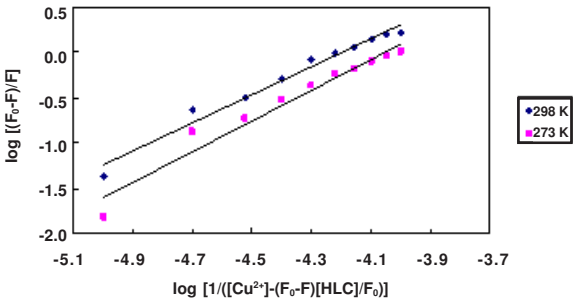

Fig. 4. Plot of quenching of HLC by copper ions at different temperatures. The relationship of log $[1/([Cu^{2+}]\text{-}(F_0\text{-}F)[HLC]/F_0$ *versus* log [Q]

Thermodynamic parameters and type of the binding forces: The binding forces between small molecule and biomacromolecule may include hydrophobic forces, electrostatic interactions, van der Waals interactions, hydrogen bonds, *etc*.[39]. The thermodynamic parameters of binding reaction are regarded as the main criterion for judging the nature of binding forces. In order to clarify the interaction between HLC and Cu(II), the thermodynamic parameters were calculated based on the binding constant at different temperatures. Because the temperature effect is very small, the enthalpy change (ΔH) can be regarded as constant if the temperature range studied is not too wide. Therefore, the values of ΔH, ΔS (entropy change) and ΔG (Gibbs free energy change) can be determined from the following equations:

$$\log(K_2 - K_1) = \frac{\Delta H\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}{R} \quad (3)$$

$$\Delta G = -RT\ln K \quad (4)$$

where K is the binding constant at the corresponding temperature[40], R is the gas constant and T is the absolute temperature.

The values of ΔH, ΔS and ΔG are listed in Table-2. For the system of HLC and Cu^{2+}, ΔH and ΔS are positive and ΔG is negative. The negative values of ΔG and positive ΔH mean that the binding process was spontaneous and the formation of the HLC-Cu complex was an endothermic reaction. According to the rules on relationship between the sign and magnitude of the thermodynamic parameters and various kinds of interaction in the binding process[41], the positive ΔH and ΔS indicate that the reaction is entropy-driven and the hydrophobic forces play a major role in the interaction of human-like collagen with copper ions.

TABLE-2
BINDING PARAMETERS FOR THE SYSTEM OF HLC AND COPPER IONS (II)

T (K)	K ($\times 10^4$ L mol^{-1} s^{-1})	n	ΔH (KJ mol^{-1})	ΔS (J K^{-1} mol^{-1})	ΔG (KJ mol^{-1})
273	1.138	1.70	8.66	109.38	-21.20
298	1.567	1.54	8.66	109.38	-23.94

Conclusion

The interaction between human-like collagen and copper ions (II) has been studied by spectroscopic methods including fluorescence spectroscopy and UV-VIS absorption spectroscopy in this paper. The experimental results demonstrate that Cu(II) can quench the intrinsic fluorescence of human-like collagen. Both dynamic and static quenching mechanisms are simultaneously involved in the binding procedure. And the number of binding points indicates that human-like collagen (HLC) and Cu formed the complex of mol ratio 2:3. In addition, the binding process is spontaneous and is predominantly owing to hydrophobic interaction. These results may contribute to some useful evidence for further understanding the photodynamic process and mechanism and provide great importance for preparation of HLC-Cu complex.

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