Determination of Trace Metallothioneins at Nanomolar Levels Using Phenanthroline–Copper Coordination by Fluorescence Spectra

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A direct fluorescence spectra method was applied for the determination of metallothioneins at nanomolar levels. In Britton-Robison (B-R) buffer (pH 7.0), the interaction of bis(1,10-phenanthroline)copper(II) complex cation [Cu(phen)₂]⁺ and metallothioneins enhanced the fluorescence intensity of system. The fluorescence enhancement at 365 nm was proportional to the concentration of metallothioneins. The mechanism was studied and discussed in terms of the fluorescence and UV-absorption spectra. Under the optimal experimental conditions, at 365 nm, there was a linear relationship between the fluorescence intensity and the concentration of the metallothioneins in the range of 8.30 × 10⁻⁹ - 7.70 × 10⁻⁷ mol L⁻¹. The linear regression equation was ΔF = 8.96 + 38.01c (mol L⁻¹), with a correlation coefficient of r = 0.998 and detection limit 2.50 × 10⁻⁹ mol L⁻¹. The relative standard deviation was 0.47% (n = 11), and the average recovery 97.2%. The proposed method was successfully reliable, selective and sensitive in determining trace metallothioneins in fish visceral organ samples with the results in good agreement with those obtained by HPLC.

Keywords Metallothioneins, copper, 1,10-phenanthroline, fluorescence

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Introduction

Metallothioneins (MTs) are a class of inducible metal-binding proteins characterized by low molecular weight (1 - 10 kDa), a high cysteine content (about 30%), lack of aromatic acid residues and heat stability. Under normal physiochemical conditions, MTs are composed of two metal clusters (α and β). MTs are found to occur in a widespread variety of organisms, including vertebrates, invertebrates, microorganisms and various kinds of plants.¹ It is believed that these proteins play important roles in protecting cells from oxidative stress through metal binding/release dynamic mechanisms.²–³ Moreover, MTs are also involved in other important biology functions, such as detoxification, transport and storage of heavy metals, such as cadmium and mercury, by sequestering and preventing them from binding to sensitive biochemical sites in cells,⁴ as well as in the homeostasis and storage of essential metals, like zinc and copper, which act as scavengers of free radicals and reactive oxygen metabolites.

The induction of MTs in a variety of aquatic animals in response to increased exposure to heavy metals has been extensively reported. Owing to their significant responses to heavy metal exposure, and a statistically significant correlation between the content of MTs in aquatic animals and the levels of environmental pollution from metals, MTs have also been proposed as potential biomarkers for metal pollution in the environment.⁵ In the 1950s and 1960s, Minamata disease and bone ache disease were attributed to mercury and cadmium contamination, respectively, in Japan. Today international organizations are seriously concerned about aquatic environmental disruption and dangers to humans resulting from heavy metal contamination, especially from cadmium, mercury, and lead from industrial sources.¹ Other studies have shown a statistically significant correlation between the content of MTs in human urine and the levels of environmental pollution due to metals. Accordingly, the study of MTs as biomarkers for metal contamination has already become a major focus in many fields, such as environmental science, biological science, medical science, toxicology, etc.,⁶–⁷ especially in regard to the widespread application perspectives of MTs.

The detection and quantification of MTs are not easy tasks in modern bioanalytical chemistry due to the low molecular mass and unique primary structure of MTs. Over the past 10 years, several analytical methods for the determination of MTs have been reported,³ such as metal saturation assays,⁹ atomic absorption spectroscopy (AAS),¹¹ and so on.¹² These methods are primarily indirect and based on the content of coordinated metal ions in MT molecules or the high content of sulphydryl groups, and suffer from many deficiencies, including insufficient specificity and sensitivity. Other quantification methodologies, such as enzyme immunoassay (ELISA), have also been proposed to detect MTs directly based on the use of specific antibodies.¹³–¹⁴ The main obstacles in using ELISA methods are the need to avoid any cross reactivity of polyclonal antibodies. The quantification of MTs by electrochemical methods, based on the
reactivity of the –SH group, allows for quantification at very low concentration levels.\textsuperscript{15–16} Unfortunately, only a couple of groups are applying this method for quantification purposes. In recent years, capillary zone electrophoresis (CZE),\textsuperscript{17} real-time PCR, high-performance liquid chromatography (HPLC),\textsuperscript{18} and ICP-mass spectrometry\textsuperscript{19} methods on have been reported. However, these methods require specialized instrumentation, and the limits of detection of some assays are still not sufficient to measure MTs in biological samples. Therefore, the development of a convenient and reliable method for the direct determination of MTs is urgently needed for monitoring environmental quality and for assessing occupational and environmental health risks. In addition, direct monitoring of MT fluctuations under physiological conditions could play an important role in investigations related to the pathophysiological and toxicological effects of cellular exposure to additional metal ions or reactive species.

Recently, fluorescence spectroscopy (FS) has been increasingly applied to the study and determination of some biological macromolecules, organic substances, inorganic ions, etc.\textsuperscript{20–22} Compared with other analytical methods, FS has the distinct advantages of being less time-consuming, requiring only simple instrumentation, and having high sensitivity and stabilization.\textsuperscript{23–24} Furthermore, it can be accomplished with a common fluorescence spectrometer using inexpensive and safe reagents. Although the fluorescence method allows for the sensitive analysis of bio-macromolecules with fluorophore, it is difficult to provide a promising potential for the detection of MTs.\textsuperscript{25} To the best of our knowledge, there is no report on the determination of MTs using the Phenanthroline (Phen)-Cu chelate as a fluorescence probe.

In the present work, a simple, sensitive and inexpensive method has been applied for the determination of trace MTs in fish visceral organ samples based on an enhancement of the FS intensity resulting from the interaction of the Phen-Cu chelate with MTs in a Britton-Robinson (B-R) buffer solution (pH 7.0). Our investigation shows that the high sensitivity of FS can provide a promising potential for the detection of protein in biological samples, and can extend the applications of FS. Furthermore, the proposed method has some advantages over others, such as greater sensitivity, wider linear ranges, and lower limits of detection. In addition, the interaction between MTs and Phen-Cu chelate, as well as the mechanism of the FS enhancement were investigated.

**Experimental**

**Reagents and chemicals**

All experiments were performed with analytical reagent-grade chemicals and doubly distilled water. MTs was purchased from Sigma. The stock solution (1.54 × 10^{-4} mol L^{-1}) was prepared by dissolving an appropriate amount of MTs in water. The standard working solution was prepared by diluting the stock solution with water in a 100 mL volumetric flask to give a final concentration of 7.70 × 10^{-6} mol L^{-1}. Then, 0.25 mL of Phen (3.0 × 10^{-4} mol L^{-1}), 0.5 mL of B-R buffer (pH 7.0), 0.10 mL of copper (II) (2.0 × 10^{-4} mol L^{-1}), were added, followed by an appropriate amount of MTs (7.7 × 10^{-6} mol L^{-1}). The mixture was diluted to the 5 mL mark with doubly distilled water and mixed thoroughly at room temperature. After 25 min, the fluorescence emission spectra were recorded with λ_{ex} = 270 nm over a range of 220 - 550 nm. The fluorescence intensity (F) was determined for the product and F_{0} for the reagent blank at the emission maximum (365 nm). The enhanced fluorescence intensity was calculated as ΔF = F - F_{0}, where F and F_{0} were the FS intensities of the reaction system with and without MTs, respectively.

**High-performance liquid chromatography method**

Samples containing 0.00, 1.54, 3.08, 4.42, 6.16 × 10^{-3} mol L^{-1} MTs were prepared in 1 mL centrifuge tubes. Before the determination, the calibration standards and samples were filtered using a 0.22 μm membrane (Whatman Company). After the instrument stabilized, a syringe was used for 100 μL injections.

**Results and Discussion**

**Spectral properties and study of the system reaction mechanism**

As shown in Fig. 1, a Cu^{2+}, MTs, Cu^{2+}-MTs in the B-R buffer solution (pH 7.0) had no absorption in the range of 240 - 300 nm (curves 1 - 3). Phen absorbed in the range of 250 - 280 nm (curve 6) with a λ_{max} of 262 nm. The absorbance...
increased slightly after the addition of MTs (curve 7). After scanning Phen–Cu²⁺, the absorption peak was found at 270 nm (curve 4). After MTs were added, the absorbances increased with a red shift of the absorption peak (curve 5). This experimental result indicated that MTs perhaps react with Phen–Cu²⁺ to form the triple complex.  

There was no fluorescence of Cu²⁺, MTs alone in pH 7.0 B-R buffer. There was a strong fluorescent signal of Phen in pH 7.0 B-R buffer as shown in Fig. 2, and the addition of MTs enhanced the fluorescent signal in a pH 7.0 B-R buffer. The addition of Cu²⁺ quenched the fluorescence of Phen solution, but the fluorescence intensity increased after adding MTs. After repeated experiments, it was found that an excitation wavelength of 270 nm and emission wavelength of 365 nm gave a stable signal intensity with high sensitivity. Thus, a wavelength of 365 nm was selected for all further measurements.

Optimization of experimental reaction conditions

Because the pH greatly influenced the fluorescence intensities of the system, four buffer solutions, including B-R, HAc-NaAc, and triethanolamine, were investigated for adjusting the pHs. The results showed that the fluorescence intensity of the system in B-R buffer was the highest. A possible reason was that the phosphoric acid group in the B-R buffer could react with the system and help to form the Phen–Cu²⁺–MT triple complex. Thus, the B-R buffer (pH 7.0) was selected to control the pH value of the solution. The effect of the pH value on the fluorescence intensity of system was also tested, the results are given in Fig. 3. It was found that the fluorescence intensity of the reagent blank ($F_0$) gradually increased in the range of pH 3.0 – 10.0. The fluorescence intensity of the system ($F$) and the fluorescence intensity change value ($ΔF$) both increased as the pH increased between pH 3.0 – 6.0. There was a plateau between pH 5.5 – 8.5, which is above the MT isoelectric point (pH 4 or so).  

Because of the large number of negative charges on MTs, they bind strongly with Cu²⁺ in this pH range, leading to the maximum fluorescence intensity. When the pH was greater than 8.5, $F$ and $ΔF$ both declined rapidly possibly due to a decrease in the binding capacity of Cu²⁺ at a high-pH solution, resulting in fluorescence quenching. The $ΔF$ was maximum at pH 7.0, which was chosen as the optimum value, with 0.50 mL B-R buffer.

In optimization experiments, the effect of the Phen...
The effect of the Cu²⁺ concentration was studied by varying the volume of 3.0 × 10⁻⁴ mol L⁻¹ Phen added to the system. As shown in Fig. 4, as the Phen concentration increased, the values of F and F₀ both increased, resulting in a maximum ΔF at 0.25 mL 3.0 × 10⁻⁴ mol L⁻¹ Phen (1.5 × 10⁻⁴ mol L⁻¹).

The effect of the Cu²⁺ concentration was studied by varying the volume of 2.0 × 10⁻⁴ mol L⁻¹ Cu²⁺ added, the experimental results are shown in Fig. 5. It was found that the fluorescence intensity of reagent blank (F₀) was gradually quenched with increasing concentration of Cu²⁺, and was quenched completely when 0.40 mL was added. After adding MTs, the fluorescence intensity (F) gradually increased in the range of 0.0 – 0.06 mL (0.00 – 2.40 × 10⁻⁶ mol L⁻¹) Cu²⁺, but above 0.1 mL (4.0 × 10⁻⁶ mol L⁻¹) Cu²⁺, it gradually decreased. The fluorescence enhancement (ΔF) gradually increased in the range of 0.02 – 0.12 mL (8.0 – 48.0 × 10⁻⁷ mol L⁻¹) Cu²⁺ and significantly decreased above 0.15 mL (6.0 × 10⁻⁶ mol L⁻¹) Cu²⁺, with a maximum region between 0.05 – 0.15 mL (2.0 – 6.0 × 10⁻⁶ mol L⁻¹) Cu²⁺. Thus, 0.10 mL of 2.0 × 10⁻⁴ mol L⁻¹ Cu²⁺ (4.0 × 10⁻⁶ mol L⁻¹) was chosen as the optimum.

Tests of the influence of temperature on the reaction system showed that the fluorescence intensity changed little between 5 and 50°C. Therefore, room temperature was chosen for further experiments. For five minutes after mixing the reagents, the fluorescence remained stable for 3 h. Thus, a 20 min reaction time was used. Studies of the sequence of reagent addition showed that Phen-Cu²⁺–BR–MTs gave the largest ΔF (Table 1). This experimental result indicated that Phen may react with Cu²⁺ to form a coordinated complex, with the addition of MTs to form the Phen-Cu²⁺–MTs triple complex, which is helpful to enhance the fluorescence signal.

**Interferences**

Under the experimental conditions, 3.08 × 10⁻⁷ mol L⁻¹ MTs was determined with a relative error of less than 25%, and the following selectivity factors (allowable times) were observed: histidine (25000), glycine (260), HSA (15), BSA (50), glucose (500), urea (8000), Pb²⁺ (630), Cd²⁺ (1150), Ag⁺ (1500), Cr³⁺ (13000), Al³⁺ (1050), NH₄⁺ (52), SO₄²⁻ (200), Fe³⁺ (3). HSA, BSA, and Fe³⁺ were the most serious interferences. The reason may be that the sulfhydryl in albumins also interacts with Cu²⁺ from Phen-Cu²⁺ to release Phen, and to form a triple complex, resulting in increasing fluorescence intensity. The interference of Fe³⁺ may be owing to interact with Phen to form Phen-Fe³⁺ complexes, and to reduce the formation of the Phen-Cu²⁺–MTs triple complex. Their interference could be excluded through sample pretreatment (water bath, ethanol precipitation).

**Calibration curve, the detection limits and precision**

A 0.25 mL 3.0 × 10⁻⁴ mol L⁻¹ Phen, 0.10 mL 2.0 × 10⁻⁴ mol L⁻¹ copper sulfate solution, 0.5 mL aliquot of B-R buffer solution (pH 7.0) and different volumes of 7.7 × 10⁻⁶ mol L⁻¹ MTs were added to a 5.0 mL colorimetric cylinder. Then, distilled water was added to the mark and the solution was mixed. The fluorescence intensity was measured according to the experimental procedure, and the resulting standard curve is shown in Fig. 6. At the selected optimal experimental conditions, the linear range of the detection system was 8.30 × 10⁻⁹ mol L⁻¹ – 7.70 × 10⁻⁷ mol L⁻¹, and the linear regression equation was ΔF = 8.96 + 38.01F₁ (mol L⁻¹), with r = 0.998.

After parallel determinations of nine blanks, in accordance with Cᵢᵢ = Sᵢᵢ/k (Sᵢᵢ represents the standard deviation of the blank; k represents the slope of the working curve), the detection limit for MTs was calculated to be 2.50 × 10⁻⁹ mol L⁻¹.

After nine parallel determinations of the concentrations of 1.54 × 10⁻³, 3.08 × 10⁻³, and 4.62 × 10⁻³ mol L⁻¹ standard MT solutions, the relative standard deviations were 0.82, 0.27, 0.33%, respectively, indicating good precision.

**Handling fish tissue samples and the recovery experiment**

The kidney (sample 1) and liver (sample 2) of locally purchased fish (crucian) were cut into pieces and ground to a fluid-like consistency. Fish tissue samples were pretreated according to the literature with appropriate modifications. A specified volume of tissue was extracted with an equal volume of 2.0 × 10⁻² mol L⁻¹ pH 8.6 Tris-HCl buffer, followed by...
centrifugation (4°C, 4000 rpm, 20 min). The supernatant was heated at 80°C for 10 min, cooled to 4°C and centrifuged (4°C, 4000 rpm, 20 min). The supernatant was added to absolute ethanol at –20°C (supernatant: ethanol = 1:3), stored overnight at –20°C and centrifuged (4°C, 4000 rpm, 30 min). The supernatant was redissolved in pH 8.6 Tris–HCl buffer and centrifuged (4°C, 4000 rpm, 20 min). The fluorescence intensity of the sample was measured as described in the experimental methods. After calculating the MT concentration in the sample, a known volume of MT solution was added to determine the % Recovery.

The HPLC method (standard curve $S = 1554.4c – 11344, r = 0.9838$) was used to analyze the same samples, the results of the two methods are given in Table 2.

Samples were measured by both methods at the same time, and the averages were compared using the $t$-test. For sample 1, $t = 1.986, 0.1 < p < 0.2$, so the two methods are not significantly statistically different. For sample 2, $t = 1.139, 0.2 < p < 0.4$, also showing no significant statistical difference. Thus, the detection results of the new fluorescence method and the HPLC method were basically the same.

### Conclusions

The experiments showed that the fluorescence intensity of the Phen-Cu$^{2+}$-MTs system ($λ_{ex} = 365$ nm) increases as the concentration of MTs increases in pH 7.0 B-R buffer solution. Thus, a new fluorescence method for the determination of MTs was established, with a correlation coefficient of $r = 0.998$. The linear range is $8.30 \times 10^{-9} - 7.70 \times 10^{-7}$ mol L$^{-1}$, the detection limit is $2.50 \times 10^{-7}$ mol L$^{-1}$, and the average recovery rate is 97.2%. The method is simple, sensitive and selective. Detection results showed no significant statistical difference between the fluorescence and HPLC methods by the $t$-test.

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