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ARTICLE

Quantification of Metallothioneins in the Earthworm by Lomefloxacin-Europium(□) Fluorescent Probe

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

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A new fluorimetric method was established for the determination of trace amounts of metallothioneins (MTs) in earthworm, using lomefloxacin-europium(□) (LMLX-Eu3+) complex as a fluorescent probe. In the pH=6.5 Tris-HCl buffer solution, MTs can remarkably decrease the fluorescence intensity of the LMLX-Eu^{3+} at λ = 613 nm and the decreased fluorescence intensity of LMLX-Eu^{3+} was in direct proportion to the concentration of MTs. The linear range was 0.08-20 mg•L^{-1} with the detection limit of 0.022 mg•L^{-1}, and the recovery was in the range of 91.9-104.4%. The results show that the fluorimetric method was more accurate, sensitive and wide linear range. This method has been successfully applied to the determination of MTs in *Eisenia andrei* induced by heavy metal ions (Cd^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}). The amounts of MTs increased significantly in a dose-dependent to heavy metals exposure, they can be used as biomarkers to assess the impact of heavy metals contamination in soils. The method offered higher sensitivity as well as accuracy with simple instrumentation and suitable for direct quantification of total *Eisenia andrei* MTs.

Introduction

Metallothioneins (MTs) are metal-binding proteins characterized by low molecular weight, high content of cysteine residues, lack of aromatic amino acids and high resistance to heat. Cysteine contains a sulfhydryl group (-SH) that has the ability to bind a number of metals including zinc, mercury, copper and cadmium.[1] What's more, MTs have been implicated in the homeostasis of essential metals,[2] and in the detoxification of excess levels of essential and non-essential metals (Cd, Hg, Ag, Pb) in invertebrates.[3] While MTs can be induced by toxic metal, there is an increasing interest in MTs as molecular markers for heavy metal exposure. Earthworms have long been recognized as excellent biomonitors of trace metal contamination in soil.[4] Studies by Scott Fordsmand[5] have shown a significantly increased expression of MTs in native earthworms collected from a heavily Cd(Ⅱ) polluted mine.

The quantification of MTs in biological samples remains challenging. Metal saturation assays are commonly used for environmental monitoring of MTs in fish[6] and terrestrial organisms.[7] The indirect methods quantify MTs assuming a total saturation of -SH by metal ions.[8] However, these methods have been shown to present a risk of over-evaluating the quantities of metals bound to MTs due to the presence of other biological metal-binding ligands.[9,10] The main problem of using immunochemical technique[11] is difficult to obtain corresponding metallothionein antibodies, high sensitivity and specificity though it has. A relatively improved sensitivity has been achieved using fluorescence detection following fluorescent reagents.[12,13] This derivation reaction was shown to proceed by nucleophilic substitution of the MTs thiolate for the fluorescent reagents, such as ammonium-7-fluorobenz-2-oxa-1,3-diazole-4-sulfonate(SBD-F),[14] monobromobimane(mBBr).[15] However, these indirect detections required either MT purification before the derivatization reaction or a tandem column system to achieve a good separation of the derivatized compounds to eliminate the background interference of biological samples.[16] Recent advances in speciation analysis have made a variety of promising techniques including high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC–ICP–MS), high-performance liquid phase chromatography - electrospray tandem mass spectrometry (HPLC–ESI–MS).[17-19] In spite of the sensitivity and accuracy of the new methods, the analysis of biological samples with these hyphenated techniques requires the presence of expensive equipment and well-trained persons, which quite often is lacking in most laboratories working in this field. Therefore, a fast, simple and sensitive quantification method is still required for MTs.

In this work, we demonstrate that highly sensitive, direct quantification of MTs from crude earthworm extract can be achieved by measuring the fluorescence of LMLX-Eu^{3+}-MTs. After MTs labelled by fluorescent probe of lomefloxacin-europium(Ⅲ) (LMLX-Eu^{3+}), the characteristics of the fluorescence peak at λ = 613 nm, which can eliminate the background interference of biological samples better, the sensitivity improved greatly. The idea of this method was based on earlier results obtained from fluorescence probes for proteins and our proposed fluorescence system.[20,21] We also show that earthworm MTs are metals-responsive in a concentration- and time-dependent manner. Cadmium ion, lead ion, zinc ion, copper ion were used in this study because they have been shown to induce the production of MTs in earthworms.[22,23]

Experimental

Chemicals

Rabbit liver metallothioneins (MTs) standard 95% was purchased from United Botai Biotechnology Co. (Dalian, China). Lomefloxacin (LMLX) was supplied by the institute of pharmaceutical and biological products (Beijing, China). Eu_2O_3 99.99% was obtained from the non-ferrous metal Co. of Yuelong (Shanghai, China). Tris-hydroxymethyl-aminomethane (Tris), Hydrochloric acid (HCl) Lead nitrateand ($PbNO_3$), Cupric sulfate ($CuSO_4$), Zinc sulfate ($ZnSO_4$), Chromium chloride ($CdCl_2$) were from Guangfu chemical industry research institute (Tianjin, China). All aqueous solutions were prepared using ultra-pure water.

Eisenia andrei was supplied by Jia Liming earthworm breeding Co. (Tianjin, China).

Apparatus

All fluorescence measurements were carried out on a F-7000 fluorescence spectrophotometer (Hitachi, Japan). A TU-1900 type double beam UV-visible spectrophotometer (Puxi, Beijing, China) was used for the UV detection. A tissue grinder (Ruiqi glass products factory, Hebei, China) was used to blend and homogenize earthworm samples. A refrigerated centrifuge TGL-16M (Xiangyi centrifuge instrument co., LTD, Changsha, China) were used to separate the solid biological material from the cytosolic extract. All pH measurements were made by a PHB-10 pH-meter with a glass–calomel electrode (Hongyi, Shanghai, China).

Design of Exposure experimental and extraction of MTs in earthworm

Earthworms (*Eisenia andrei*) weighing 0.4 to 0.6 g were randomly assigned into 48 groups (5 worms per group), then exposed respectively to sublethal nominal concentrations of Cd^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+} (0, 2, 20, and 200 mg•kg^{-1} of artificial wet soil) in triplicate. Earthworms were maintained at 25±3℃ and fed weekly with corn flour. After 28 d of exposure, five earthworms were removed from the soil, dischared 24 h. The depurated earthworms were weighed prior to MTs extraction.

About 2.5 g of earthworms were homogenized in 10 mL extracting solution (0.01 mol·L^{-1} pH 8.6 Tris-HCl, 1.0×10^{-5} mol·L^{-1}, phenylmethylsulfonyl fluoride (PMSF), 5.0×10^{-3} mol·L^{-1} 2-mercaptoethanol (2-MEC) and 0.025 mol·L^{-1} NaCl), using a glass homogenizer. The mixture was transferred to centrifuge tubes and centrifuged at 10000 rpm for 30 min at 4℃. The pellets were separated by decantation and the supernatant was warmed in a water bath at 80℃ for 10min. A second centrifugation step at 4℃ and 10000 rpm for 20 min was carried out to separate the tertiary structure proteins which denatured at high temperature. The supernatant was transferred to centrifuge tubes containing 30 mL ethanol of -20℃ and placed in the refrigerator at -20℃ for 12 hours or overnight. A third centrifugation step at 4℃ and 10000 rpm for 30 min was carried out to separate the low molecular weight proteins which have a better solubility in organic solvent. The supematant was discarded and the microsomal pellets were resuspended in the original volume of Tris-HCl buffer and the earthworms MTs extraction was obtained. The crude MTs extraction was purified by the Sephadex^{TM}G-75 column (sigma, 1.6×40 cm)，the fractions containing MTs was collected.

MTs determination

Optimum concentrations of Eu^{3+}, LMLX, pH, and the time of reaction were determined using rabbit MTs standard. The final concentrations in the reaction mixture were: 1.0×10^{-4} mol•L^{-1} Eu^{3+}, 2.5×10^{-5} mol•L^{-1} LMLX, pH=6.5 Tris-HCl. Different concentrations of MTs were added to the solution above, incubated for 20 min. The fluorescence of LMLX-Eu^{3+} labelled proteins was measured with excitation at 285 nm and emission at 613 nm, using rabbit liver MTs as a reference standard. The amount of MTs in sample were determined using a standard curve obtained by rabbit MTs labelled LMLX-Eu^{3+}. The MTs concentration was expressed as mg MTs per kg earthworm. Metallothionein concentrations in earthworm were determined by the developed fluorescent probe.

Statistical analysis

All analyses were carried out in five replicates. Results are expressed as the means. The data were analyzed by one-way analysis of variance (ANOVA) using the SPSS software. The means obtained from each set were compared using the Duncan's Multiple Range test at 0.05 confidence level.

Results and discussion

Characteristics of fluorescence and absorption spectra

The fluorescence spectra of Eu^{3+}, LMLX-Eu^{3+} and LMLX-Eu^{3+}-MTs are shown in Fig. 1. The LMLX-Eu^{3+} showed an emission peak at 592 nm and 613 nm (Curve 2 in Fig. 1) while the Eu^{3+} solution had almost no peak (Curve 1 in Fig. 1), which indicated that the LMLX and Eu^{3+} formed a binary complex by efficient energy transfer from LMLX to Eu^{3+}. After the addition of MTs to LMLX-Eu^{3+}, the fluorescence intensity of the emission peak of LMLX-Eu^{3+} at λ = 613 nm was markedly enhanced (Fig. 1, curve 3 to 5) and the enhanced fluorescence intensity was proportional to the concentration of MTs. Therefore, the results indicate that MTs reacts with the LMLX-Eu^{3+} system and forms a stable LMLX-Eu^{3+}-MTs ternary complex.

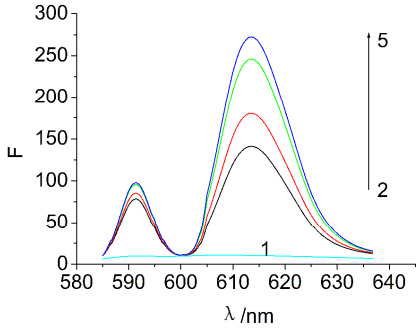

Fig. 1 Fluorescence spectra of Eu^{3+}, LMLX-Eu^{3+} and LMLX-Eu^{3+}-MTs of different concentrations of MTs.
1. Eu^{3+}; 2. LMLX-Eu^{3+};. LMLX-Eu^{3+}-MTs(5 mg•L^{-1}); 4. LMLX-Eu^{3+}-MTs(15 mg•L^{-1}; 5. LMLX-Eu^{3+}-MTs(20 m g•L^{-1})

According to the Förster non-radiation energy transfer theory,[24] the rate of energy transfer depends upon the extent of overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the relative orientation of the donor and acceptor transition dipoles and the distance between these molecules. It can be seen from Fig. 2 that energy transfer easily occurred between MTs and LMLX for the large spectral overlap between the fluorescence spectrum of the donor (MTs(a)) and the absorption spectrum of the

acceptor (LMLX(b)). Comparing curve 2 to 5 in Fig. 1, it could be seen that the characteristic peak of Eu^{3+} at 613 nm can be enhanced remarkably after the addition of MTs, which indicates that LMLX can form a ground state complex with MTs and energy transfers from MTs to LMLX.

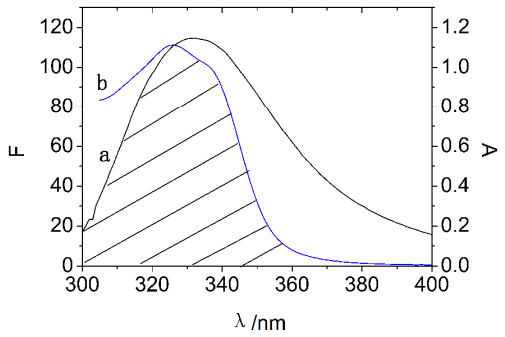

Fig. 2 Fluorescence emission spectra of MTs(a) and absorption spectra of LMLX(b)

Optimization of the experimental condition

Effect of pH

The effect of pH from 5.3 to 7.4 on the fluorescence intensity was examined. The results are listed in Fig. 3.

As shown in Fig. 3, the pH had a great influence on the FL intensity of the LMLX- Eu^{3+}-MTs system. LMLX is an amphoteric specie with one piperazinyl and one quinolone ring. Degree of protonation of nitrogen atoms and oxygen atoms in different acid and alkali solution will directly affect the distribution of electron. In the strong acid condition, it is hard ionization for –COOH, so the formation of the LMLX-Eu^{3+}-MTs ternary complex is not complete. In alkaline environment, Eu^{3+} is easy to hydrolysis, and generates europium hydroxide precipitation. In the near neutral environment, iomefloxacin mainly exists in the form of amphoteric ion. Therefore, the fluorescence intensity of the LMLX-Eu^{3+} system with MTs is strongly dependent upon the pH. The fluorescence intensity reached a maximum at pH 6.5. Therefore, in order to obtain maximum fluorescence signal of the system, a pH of 6.5 was selected for all the experiments.

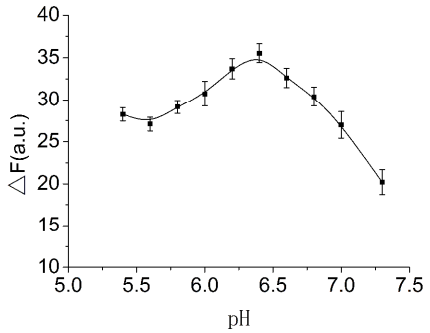

Fig. 3 Effect of pH on the enhanced fluorescence intensity(ΔF)

As the volume of buffer solution added was varied from 0.5 to 2.5 mL, as shown in Fig.4, ΔF reached a maximum at 1.0 mL, then remained constant. Thus, 1.0 mL was chosen for the subsequent experiments.

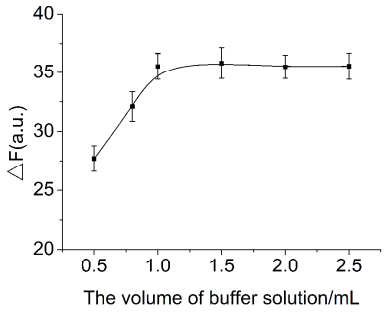

Fig. 4 Effect of the volume of Tris-HCl on the enhanced fluorescence intensity(ΔF)

Effect of the concentration of Eu^{3+} and LMLX

A series of solutions with different amounts of Eu^{3+} and the same concentration of the other reagents were measured at $\lambda_{ex}/\lambda_{em}$ = 285/613 nm. The influence of Eu^{3+} concentration of on the fluorescence intensities of the solutions is shown in Fig. 5.

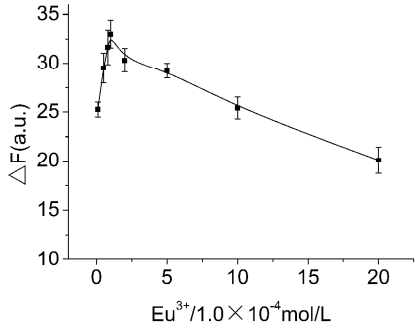

Fig. 5 Effect of the concentration of Eu^{3+} on the enhanced fluorescence intensity

The enhanced fluorescent intensity ΔF increased at first and then decreased with the increasing amounts of Eu^{3+}. The experimental results showed that the ΔF reached its maximum when Eu^{3+} solution was added 1.0 mL, that is, when the concentration of Eu^{3+} was 1.0×10^{-4} mol•L^{-1}. When the Eu^{3+} concentration is less than 1.0×10^{-4} mol•L^{-1}, with the increase of the Eu^{3+}, the luminous complexes is increased, so the system of the fluorescence intensity enhanced. However, when the concentration of Eu^{3+} is too high, the interaction between Eu^{3+} increased, which resulted in no radiation transition, so that the luminous efficiency drops, fluorescence intensity is reduced, namely "concentration quenching". Thus, 1.0 mL 1.0×10^{-4} mol•L^{-1} Eu^{3+} was selected for further study.

The amount of LMLX was also optimized, as shown in Fig. 6. The enhanced fluorescent intensity ΔF increased with the increasing ratio of [Eu^{3+}]/[LMX] up to 4.0 and then decreased. When the concentration of Eu^{3+} was 1.0×10^{-4} mol•L^{-1}, the composition ratio for the Eu^{3+} to LMLX $^{+}$ in the LMLX-Eu^{3+}-MTs system was 4.0, and the concentration of LMLX was 2.5×10^{-5} mol•L^{-1}. Thus, 2.5×10^{-5} mol•L^{-1} LMLX was selected for further study.

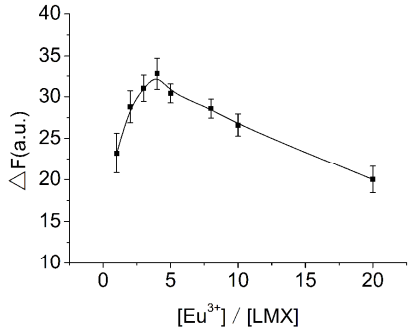

Fig. 6 Effect of the concentration of $[Eu^{3+}]$/[LMLX] on the enhanced fluorescence intensity (ΔF)

Stability of the LMLX-Eu^{3+}-MTs fluorescence system

The effect of the reaction time on fluorescence intensity was investigated. A 10.0 mL solution containing LMLX (1.0×10^{-5} mol•L^{-1}), Eu^{3+} (1.0×10^{-4} mol•L^{-1}), and MTs (5.0 mg•L^{-1}) and pH 6.5 Tris-HCl buffer solution was added. The fluorescence intensity was determined. The result was shown in Fig. 7.

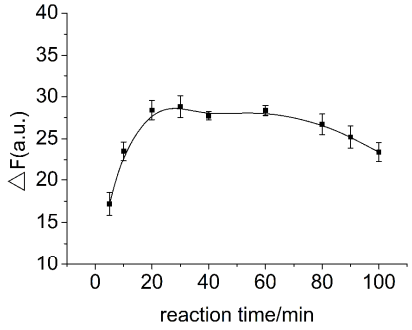

Fig. 7 Effect of reaction time on the enhanced fluorescence intensity

The fluorescence intensity was obviously increased with the increasing of reaction time from 5 to 20 min. This result suggested the chelation reaction time of the LMLX-Eu^{3+}-MTs system at room temperature needed at least 20 min. The fluorescence intensity remained constant from 20 to 80 min. Therefore, the chelation reaction was carried out for 20 min and all measurements were made within 80 min.

Effect of coexisting substances

Under the optimal conditions, a systematic study of various Metal ions and amino acids in the determination of MTs 5 mg•L^{-1} was carried out. The criterion for interference was fixed at a ±5% variation of the average fluorescence intensity calculated for the established level of MTs, and the experimental results are shown in Table 1. From Table 1, it can be seen that most coexisting substances were found to show no influence.

Table 1 Effect of coexisting substances

Interfering substance	Concentration (mol•L^{-1})	△F (%)
$Zn^{2+}(Cl^-)$	1.0×10^{-5}	3.15
$Na^+(Cl^-)$	2.0×10^{-4}	3.23
$Ca^{2+}(Cl^-)$	2.0×10^{-4}	3.58
$Mg^{2+}(SO_4^{2-})$	4.0×10^{-4}	4.70
$K^+(Cl^-)$	1.2×10^{-3}	0.87
$NH_4^+(Cl^-)$	2.0×10^{-3}	-2.94
Glucose	2.0×10^{-3}	4.90
Adenine	3.0×10^{-4}	2.45
L-lysine	2.5×10^{-4}	-2.50
Guanine	3.0×10^{-4}	-2.79
L-glutamic acid	3.0×10^{-4}	1.92
Glycine	4.0×10^{-4}	-0.25
L-serine	2.0×10^{-4}	4.65

Analytical performance

Linear range and limit of detection

Linear range and detection limit (LOD) of the proposed method had been established taking into account the specific literature for analytical performances of analytical methods. The comparative results were listed in Table 2.

Under the optimum experimental conditions, there was a linear relationship between the enhanced fluorescence intensity and MTs concentration in the range of 0.08 to 20 mg•L^{-1} with a correlation coefficient of 0.998. The regression equation was $\Delta F = 14.29+6.81C$ (C-concentration, mg•L^{-1}). The result was shown in Fig. 8.

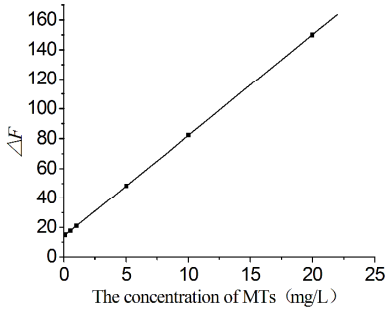

Fig. 8 The calibration curve of MTs detection

The limit of detection of the proposed method was determined to be 0.022 mg•L^{-1} when the standard deviation was 0.05 obtained from a series of 11 reagent blanks. In comparison with some existing methods, as shown in Table 2, the current method has advantages of high sensitivity and wide linear range.

Table 2 Comparison of methods for the determination of MTs

Method	Linear range (μg•mL^{-1})	Detection limit (ng•mL^{-1})	Reference
HPLC, UV detection	2.79-1.24	0.31	[14]
HPLC, fluorescence detection	0.70-50	0.025	[15]
CZE, UV detection	5.0-100	/	[25]
This method	0.08-20	0.022	

Recovery and Precision

In order to obtain recovery percentage, MTs in *Eisenia Andrei* sample were analyzed. The results were shown in Table 3. For the assay of MTs in *Eisenia andrei*, the fresh samples must be diluted appropriately to be within the linear range of determination of MTs. A portion (1.0 mL) of this sample solution was analyzed by the method developed above, using the standard calibration method. From Table 3, recovery percentage was ranged between 85-93 % and the relative standard deviation (RSD) was 4.60 to 5.25% for five measurements. The developed method can be easily performed and affords good precision and accuracy when applied to real samples.

Table 3 Results for the determination of MTs in *Eisenia andrei*

Concentration of MTs ($mg•L^{-1}$)	Added ($mg•L^{-1}$)	Detected ($mg•L^{-1}$)	Recovery (%)	RSD (%, n=25)
2.00	1.60	3.31	91.9	5.25
	2.00	3.90	97.5	4.60
	2.50	4.70	104.4	4.85

The evaluation of this method

In blank control group, MTs determined by the enzyme linked immuno sorbent assay(ELISA)[26,27] method resulted in basal levels of MTs of 22 mg MTs per kilogram earthworm. The same samples subjected to the fluorescence method gave basal levels of 30 mg MTs per kilogram earthworm. The overestimation by the fluorescence method was due to high levels of nonmetallothioneins thiol groups relative to the metallothioneins concentrations expressed under unexposed conditions. Both methods showed a significant increase in the MTs concentrations in the earthworm exposed to different concentrations of heavy metals. As the sample was pured by gel filtration, the purity of extraction MTs was satisfied the demands of fluorescence measurement. However, the differential pulse polargraphy-analysis of gel filtration fractions was not accurate, approximately higher 10-20 fold than the amount found by the ELISA method[28]. In some of the investigations in which MTs has been determined by means of differential pulse polarography, a low metal to MTs ratio strongly suggests that the MTs concentrations have indeed been overestimated. Legras et al.[29] present very detailed information about soluble concentrations of Cu^{2+}, Zn^{2+} and Cd^{2+} in gills and midgut gland of crabs with the concentrations of MTs determined by differential pulse polarographic. The results show that each molecule of metallothioneins would only contain approximately 1.2 and 1 metal atom in midgut gland and gills, respectively.Given the normal metal binding capacity of crustacean metallothioneins (six Zn/Cd atoms or nine Cu atoms[30]), this indicates either an unusually low degree of metal saturation on the metallothioneins, a very large proportion of apo-metallothioneins or more likely in view of the results of the present investigations that the differential pulse polarography determination of MTs has overestimated the real MTs concentrations. In a word, the accuracy of fluorescence method was superior to the differential pulse polarography.

Effect of heavy metal stress on earthworm MTs

The average concentrations (n=9) of MTs detected in earthworm were shown in Fig. 9.

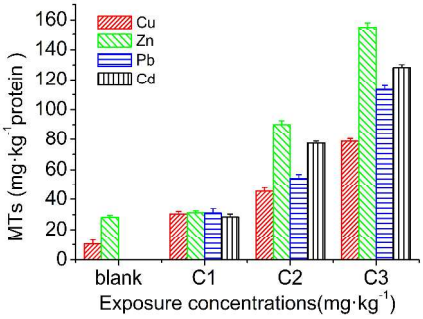

Fig. 9 MTs concentration in earthworms exposed to heavy metals (blank; C1=2mg•kg^{-1}; C2=20mg•kg^{-1}; C3=200mg•kg^{-1})

No mortality was observed in earthworms exposed for 4 weeks to different concentrations of Cd^{2+}, Pb^{2+}, Cu^{2+}, and Zn^{2+}. Decreases in body weight and food consumption were observed in worms exposed to the highest concentrations of Cd^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+} (20 and 200 mg•kg^{-1} of artificial wet soil) as compared to the blank control group. The results in Fig. 9 revealed significant increases in MTs with the increasing heavy metal concentrations. However, little changes in MTs concentrations were observed in all groups exposed to the lowest concentration (2.0 mg•kg^{-1} of artificial wet soil) as compared to the control group. Significant increases in MTs concentrations were noted when earthworms were exposed to the higher concentrations (20 and 200 mg•kg^{-1} of artificial wet soil) for 4 weeks. This would suggest that MTs can be induced by Cd^{2+}, Pb^{2+} Cu^{2+} and Zn^{2+}. However, there is a lag time between metals exposure and MTs induction.

The data from 200 mg•kg^{-1} Cd^{2+} of artificial wet soil group were analyzed by one-way analysis of variance (ANOVA) using the SPSS software. The regression equation was $Y = 0.115 + 0.213X$ ($F = 180.15$, $P < 0.001$). It was significantly associated at 0.01 level, the correlation coefficient was greater than 0.958. It was demonstrated that the MTs content in earthworms has good correlation with exposed concentration of Cd^{2+}. The regression equation of $Pb^{2+}$was $Y = 0.055 + 0.123X$ ($F = 159.20$, $P < 0.001$). The response sensitivity of MTs to Cd^{2+} was better than Pb^{2+}. However, the linear relationship between MTs to Cu^{2+}, Zn^{2+} was not well, as the earthworms itself have Cu-MTs and Zn-MTs. The content of MTs increased with the concentration of the four metal increasing. The critical concentration of Cd^{2+}, Pb^{2+}, Cu^{2+}, and Zn^{2+} causing earthworm death and the maximum allowable metallothioneins content in earthworms remains to be determined. The results show that the MTs can be used as the biomarkers of heavy metals contaminated soils to a certain level.

Conclusions

This experiment provides a new luminescent probe method to determine MTs. The proposed method appeared to be rapid, sensitive, and suitable for direct measuring total MTs in earthworms. The fluorescence peak of biological macromolecules generally between 400-500 nm, however, the fluorescence peak of LMLX-Eu^{3+} labled MTs at $\lambda = 613$ nm, which can eliminate the background interference of biological samples perfectly. The sensitivity of this method is greatly increased with fluorescence probe of rare earth ions using a simple fluorescence spectrometer.

Our results suggest that MTs assay in the earthworm could provide information on heavy metals bioavailability in soil. Thus, this method could be a valuable tool for ecotoxicological bio-

monitoring of Cu^{2+}, Zn^{2+}, Pb^{2+} and Cd^{2+} contamination. As earthworms are central to soil quality and fertility, any toxic insult to earthworm populations represents a potential threat to the soil ecosystem and human health.

Acknowledgements

This study was supported by the Hebei Province Special Fund for Environmental Protection Research in the Public Interest (2013).

Notes

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Environmental impact statement

Cadmium is to be of toxicological importance. The high chronic toxicity of cadmium is explainable on the basis of its long retention time and accumulation. Based on experience of the toxicity of cadmium for animals and humans, the determination of Cd is of importance.

This work established a new sensitive method for the determination of metallothioneins (MTs) using lomefloxacin-europium(Ⅲ) (LMLX-Eu^{3+}) complex as a fluorescent probe. It was successfully applied to the direct quantification of MTs in *Eisenia andrei* induced by cadmium. The results suggested MTs can be used as biomarkers to assess the impact of Cd contamination in soils. Thus, this method could be a valuable tool for assessment of Cd contamination level.