



Short communication

Determination of metallothionein in *Daphnia magna* by modified square wave cathodic stripping voltammetry



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ABSTRACT

Metallothioneins (MTs) are widely used as biomarkers in environmental studies. However, eliminating interfering factors for MT determination in vivo by electrochemical detection is difficult. In this study, NO_3^- and Cl^- were found to negatively affect MT determination in *Daphnia magna* by square wave cathodic stripping voltammetry (SWCSV). The relevant mechanism was analyzed, and results were used to modify the original electrochemical testing parameters and environmental conditions. This modified SWCSV achieved accurate MT concentrations in *D. magna*, leading to detection limits as low as picomolar levels.

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1. Introduction

Metallothioneins (MTs) are a specific class of low-molecular weight proteins (6–7 kDa for the mammalian protein) that are ubiquitous in vivo. The expression of these proteins can be induced by metals, such as Cu, Pb, Zn, and Hg, and MTs have high affinity for heavy metals. MTs perform important functions in toxic metal detoxification; that is, the proteins are used as common biomarkers for evaluating environmental contamination. Several studies have positively correlated MT concentration with environmental contamination levels [1–3].

Several techniques have been applied to determine MT in vivo [4]. Voltammetric methods are advantageous because they are highly reproducible, sensitive, and fast. In 1933, Brdicka [5] proposed the concept of the electrochemical determination of proteins that contain cysteine residues; this technique depended on the complex reaction between –SH groups and hexamine cobalt chloride from a supporting electrolyte. Olafson and Sim [6] applied the Brdicka reaction using differential pulse polarography (DPP) for MT determination, and the resulting method was independent of the concentration or type of the metal combined with MT. Thus, this method is widely used for determinations in organisms [7,8]. Voltammetric methods in other modes, including chronopotentiometric stripping analysis (CPSA) [9,10], differential pulse anodic stripping voltammetry [11], and square wave cathodic stripping voltammetry (SWCSV) [12], have been applied to detect MT.

In a study of novel voltammetric methods for MT determination, Chen et al. developed a modified electrode, a glassy carbon electrode covered with a nanocomposite architecture layer to carry antibodies, which combined the advantages of immunoassays and electrochemical methods [13].

Despite encouraging advances in this field, eliminating the interfering factors in commonly used electrochemical methods is difficult, and accurate MT determination in vivo cannot be guaranteed. Pedersen et al. showed that DPP may be unsuitable for crustaceans and that detection by DPP may result in 5- to 20-fold overestimation of MT levels [14]. In addition, given the low MT concentrations in zooplankton and phytoplankton, DPP based on the Brdicka reaction may not meet the requirements in such cases. CPSA can determine MT concentrations as low as a few femtomoles in 5 μL aliquots of an analyte [9]. However, Petrlova et al. found that the CPSA signal of MTs depended on the metal content of the sample, and lower CPSA signals could be produced by MT–metal complexes compared with those obtained from metal-free MTs [15]. Ureña [16] and Chen [13] provided viable procedures to obtain accurate MT values in biological samples. Unfortunately, both procedures are not convenient for monitoring MT concentrations in the natural environment.

The SWCSV technique used for MT determination, a method developed by El Hourch et al., achieved low detection limits comparable with those obtained from CPSA and yielded an assay that was unaffected by combined metals [12,17]. In the current study, this method was modified for accurate determination in *Daphnia magna*, which is specified by OECD for chemical testing. Furthermore, the study of MT determination in *D. magna* provided a promising protocol for other crustaceans.

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In this study, the factors that interfere with measurement accuracy were determined. A convenient and accurate SWCSV that features detection limits as low as picomolar levels was finally developed.

2. Experimental

2.1. Reagents and solutions

MT from rabbit liver (MT-1, containing 9% Cd and Zn) was purchased from ENZO Life Sciences (Switzerland) and used as a standard. Hexamine cobalt chloride and *cis*-diamine dichloroplatinum (II) were purchased from Sigma Chemical Co. (USA). All solutions were prepared with ultrapure water from a Millipore system (Dura 12FV).

2.2. Apparatus

Two voltammetric methods were performed using a 797 VA computrace system (Metrohm, Switzerland) connected to a personal computer. A multimode electrode operated in hanging Hg drop electrode mode was used as the working electrode, and Ag/AgCl (3 M KCl) and glassy carbon electrodes were used as the reference and auxiliary electrodes, respectively. All potentials were obtained versus the Ag/AgCl (3 M KCl) electrode.

2.3. Procedure

2.3.1. Exposure of *D. magna*

D. magna was cultured in natural water for 14 days until the commencement of the experiment. In the experiment, a simplified Elendt M7 medium was used for exposure instead of ultrapure water to control medium conditions. During the experiment, the *D. magna* samples were not fed. The animals were assigned to five groups with 50 individuals each and were exposed to 0, 10, 30, 50, or 80 $\mu\text{g/L}$ Cu(II). Two parallel samples were set up for each group. After 48 h of exposure, live *D. magna* samples were collected for MT determination.

2.3.2. MT determination

After exposure, *D. magna* samples were weighed after the removal of residual water from the surface of their bodies. Tissues were homogenized by ultrasonication in 1.0 mL of sucrose buffer (0.25 mol L⁻¹ sucrose, 0.1 mol L⁻¹ Tris-HCl, pH 8.6) and centrifuged at 16,000 g in a refrigerated centrifuge for 20 min. The supernatant was then diluted and prepared for MT determination by DPP [18], SWCSV [12], and the modified silver saturation method [1]. In addition, the supernatant was heated at 99 °C for 5 min and centrifuged before electrochemical testing. This procedure has been proven to be an effective way to separate MT, which is a thermostable protein, from the interfered high-molecular weight (HMW) proteins through precipitation of HMW proteins by heating.

In the present study, the modified silver saturation method was set as the standard method to evaluate the accuracy of the two other voltammetric methods. The modified silver method is based on the high affinity between Ag⁺ and MT, and the thermostabilization of MT. Many works have proven its reliability on MT determination in *D. magna* [1,19,20].

2.3.3. MT determination after gel filtration

To determine the factors that interfere with the accurate determination of MT in *D. magna*, the unheated supernatant was divided into 20 tubes through gel filtration (Superdex 75 column); each tube represented an interval of molecular weight. Then 50 μL of the solution in each tube was added to a vessel that contains 20 mL of 10 μM Pt(NH₃)Cl₂ + 1 M NH₄Cl + NH₄OH (pH 10) in descending order according to molecular weight. The peak current determined by SWCSV was recorded each time a new solution was added. Voltammograms were recorded using negative potential scanning from

–1.2 V to –1.75 V under the following parameters: –0.06 V deposition potential, 60 s deposition time, 3 mV voltage step, 50 mV amplitude, 50 Hz frequency, 2000 rpm stirring rate during deposition, and 20 °C temperature. The SWCSV parameters were not changed unless specifically mentioned in the sections below.

3. Results and discussion

3.1. Testing conditions of SWCSV

In a previous experiment, the testing environment and electrochemical parameters of SWCSV by El Hourch [12] were verified. Five deposition potentials (i.e., –0.06, –0.08, –0.1, –0.12, and –0.14 V) were applied to select the most effective potential (Fig. 1A). Among the potentials surveyed, the deposition potential of –0.06 V proved to be the most suitable; at –0.06 V, a high peak current and unaffected peak were observed. A standard curve was subsequently obtained at the –0.06 V deposition potential (Fig. 1B). The detection limit was estimated to be 5×10^{-8} g L⁻¹ (S/N = 3); this value decreased with increased deposition time. The detection limit obtained in the present work is comparable with those observed in immunoassays (10^{-8} g L⁻¹) [21] and constant current stripping chronopotentiometry (10^{-7} g L⁻¹) [9].

3.2. MT determination in *D. magna* by different techniques

Fig. 2 shows that neither the unmodified SWCSV method nor the DPP method reflected positive correlations between MT concentration in *D. magna* and Cu²⁺ concentration in the environment [20]. In addition, no correlation was found between the MT levels determined by SWCSV and DPP. In theory, SWCSV and DPP are based on the reactions of –SH groups [12,22]. However, the values obtained by these methods and their trends differed. These phenomena indicate that the factors that interfere with accurate MT determination by the two methods vary.

Furthermore, in Fig. 2, the values obtained by SWCSV and DPP differed from those obtained through the silver saturation method. The

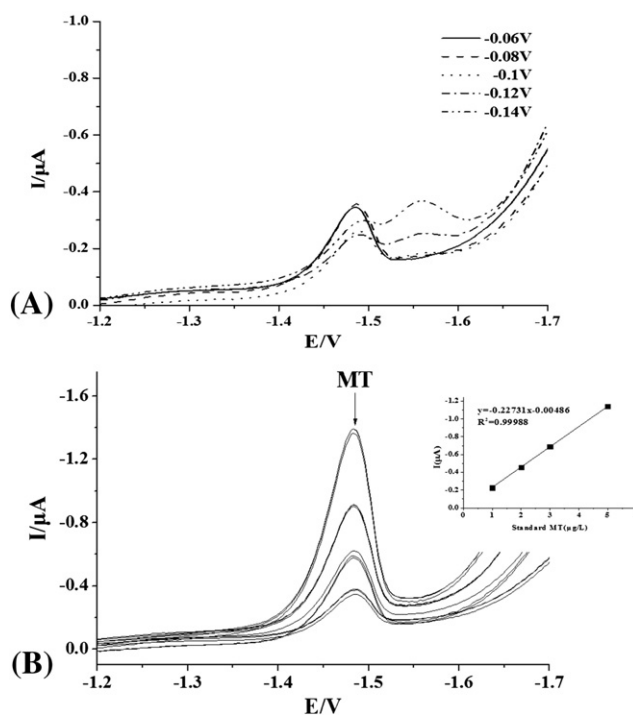


Fig. 1. (A) Square wave cathodic stripping voltammogram of standard MT (1 $\mu\text{g/L}$). (B) Square wave cathodic stripping voltammograms of calibrated MT at –0.06 V deposition potential and the corresponding standard curve.

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