

Bio-Assessing of Environmental Pollution via Monitoring of Metallothionein Level Using Electrochemical Detection

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Abstract—In this paper, we report on an investigation of affecting guppy fishes (*Poecilia reticulata*) by silver ions (0, 0.3, 0.6, 1.2, 2.5, and 5 μM) for seven days under well-controlled experimental conditions. To observe the physiological changes, we attempted to determine metallothionein (MT) as a biomarker of heavy metal stress. For this purpose, we proposed a sensor utilizing a carbon electrode coupled with flow injection analysis. The experimental conditions, which have been optimized, were as follows—applied potential: 750 mV, mobile phase: Britton–Robinson buffer (pH 1.9) with flow rate of 0.6 ml/min, time filter: 2.5 s, “current R”: 1 μA . Under these conditions, the detection limit of MT was estimated as 100 pM. After the optimizing step, the fish tissues were measured. Based on the results obtained, MT content increased with increasing dose of silver ions and time of the treatment. The results obtained were in good correlation with those obtained by adsorptive transfer stripping technique coupled with differential pulse voltammetry Brdicka reaction, which was used as the reference technique.

Index Terms—Amperometric detection, biomarker, electrochemical sensors, fish, flow injection analysis, heavy metal, metallothionein, poecilia reticulata, silver.

Manuscript received March 31, 2007; revised June 30, 2007 and September 28, 2007; accepted January 6, 2008. This work was supported under Grants GACR 526/07/0808, MSMT 6215712402, MSMT 2636/F4b, and MSMT INCHEMBIOL 0021622412. This is an expanded paper from the Sensors 2006 Conference. The associate editor coordinating the review of this paper and approving it for publication was Prof. Okyay Kaynak.

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I. INTRODUCTION

A. Sensors in Environmental Chemistry

ROBUST analytical techniques, which cannot be miniaturized easily [1], are not useful for furnishing online monitoring of environmental pollution. Therefore, new analytical approaches allowing easy and rapid assessment of environmental pollution are suggested [2]–[7]. These chemical sensors and biosensors have superior properties over existing analytical instruments fitting perfectly for the aforementioned purposes [8], [9]. The sensor system can be composed from three parts: 1) sensor; 2) converter of the signals; and 3) data acquisition instrument [Fig. 1(A)]. Nevertheless, a sensor can be used for analyzing of environmental samples after the optimizing according to its type and properties of target molecule (e.g., gas, liquid) [8], [10]. From the wide spectrum of sensors the electrochemical sensors (i.e., potentiometric, amperometric) are intensively used due to their very low costs, low detection limits, and spectrum of applications [11]–[13]. Electrochemical sensors also allow us to obtain both qualitative and quantitative character of the target molecule and can give information about its physicochemical properties (oxidation state, type of ligand, etc.) and concentration in one measurement [14]–[16].

B. Bio-Indication of Environmental Pollution

To assess environmental pollution we can use three strategies: 1) to detect a pollutant directly; 2) to determine a substance called biomarker, which can be used to indicate exposure of an organism to a pollutant; or 3) to observe physiological changes of an organism living in a polluted environment. Detection of pollutant directly in such a complex matrix as an environmental sample can be a difficult task for an analytical chemist. Moreover, concentration of a certain pollutant can reveal only the immediate level of polluting but reveal nothing about the effect of the pollutant on the environment in a long-term scale. This lack of information can be partly supplemented via analysis of biomarkers or observation of physiological changes of organisms exposed to the pollutant.

Numerous plant and animal species can be used as bio-indicators of heavy metals pollution of the environment [17]–[22]. Aquatic animals, most of all various species of fishes, are very suitable for these purposes [23], [24]. To assess the quality of the Digital Object Identifier 10.1109/JSEN.2008.928500

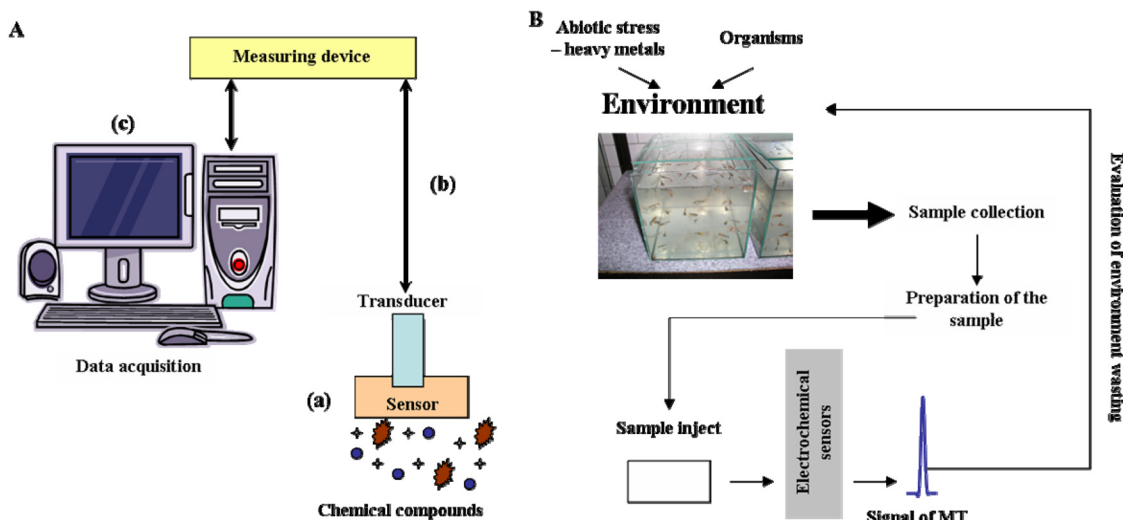


Fig. 1. (A) Typical arrangement of electrochemical sensor: (a) sensor, (b) measuring device, and (c) data acquisition. (B) Scheme of environmental monitoring by heavy metals. Environmental samples are collected and processed for electroanalytical determination according to current protocol. The analysis of one individual is sufficient, which is advantageous for screening programs assessing environment quality [56]–[58].

environment investigations, i.e., the changes in behavior, morphology, habitation, or changes of basic morphometric properties (body weight, color, length, etc.), are often used. All these data are only of qualitative character and are hard to obtain due to requirements on large population of the target specie and the time period of the experiment. Therefore, one may suggest that the most reliable investigations about the effect of heavy metals on an aquatic environment can be proposed using fish specie supplemented with analyzing a certain biomarker.

C. Biomarkers

It has been shown that low-molecular peptides and/or proteins rich in cysteine can be considered as biomarkers, referring to various types of pollutants including heavy metals [25]–[28]. Metallothioneins (MT) as low molecular cysteine rich proteins belong in the group of molecules [29]. Their molecular weight is within the range from 6 to 10 kDa. Due to their affinity to heavy metals, they are involved in their detoxifying and in maintaining of heavy metals homeostasis. MT contains two binding domains α and β , which are composed from cysteine clusters. In this paper, we report on investigations affecting the guppy (*Poecilia reticulata*) by silver ions for seven days under well-controlled experimental conditions, because silver ions pose a threat to aquatic organisms due to their very high toxicity [30]. To observe the physiological changes we determined MT. For this purpose, we proposed a simple and rapid electrochemical detection utilizing carbon electrode coupled with flow injection analysis.

II. MATERIAL AND METHODS

A. Chemicals

Rabbit liver MT (MW 7143), containing 5.9 % Cd and 0.5 % Zn, was purchased from Sigma Aldrich (St. Louis, MO). Tris(2-carboxyethyl)phosphine (TCEP) was produced by Molecular Probes (Evgen, OR). $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ and other chemicals used were purchased from Sigma Aldrich (Sigma-Aldrich,

USA) in ACS purity unless noted otherwise. Stock standard solutions were prepared with ACS water (Sigma-Aldrich, USA) and stored in the dark at -20°C . Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through $0.45\text{-}\mu\text{m}$ Nylon filter discs (Millipore, Billerica, MA) prior to electrochemical analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by a personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3 mol/dm³ KCl) was calibrated by set of WTW buffers (Weilheim, Germany).

B. Experimental Model

Guppy fishes (*Poecilia reticulata*), 2 or 3 months old, were exposed to silver nitrate, always seven individuals per dose 0, 0.3, 0.6, 1.2, 2.5, or $5\ \mu\text{M}$. The experiment lasted seven days (168 h); a fish from each experimental variant was sampled per day. The experimental conditions such as pH value of the solution where the fishes were kept constant, oxygen concentration, and temperature were monitored during the experiment. The oxygen concentration varied within the range from 1.7 to $4.0\ \text{mg/l}$, the pH level from 6.34 to 7.00, and the temperature from 20.2 to 21.5°C during the seven-day-long experiment. The sampled fish was killed by CO_2 and washed one time with distilled water and one time with $0.5\ \text{M}$ EDTA.

C. Preparation of Biological Samples for Electrochemical Analysis

The sampled fishes (approximately $0.2\ \text{g}$) were frozen with liquid nitrogen and spread in mortar, and then exactly $1000\ \mu\text{l}$ of $0.2\ \text{M}$ phosphate buffer (pH 7.2) was added to the homogenized sample. The obtained homogenate was transferred into test-tube and vortexed for 15 min at 4°C (Vortex Genie). The supernatant was subsequently heat treated. Briefly, the sample was kept at 99°C in a thermomixer (Eppendorf 5430, Germany) for 15 min with occasional stirring and then cooled to 4°C . The denatured homogenates were centrifuged at 4°C , $15000\ \text{g}$ for 30 min

(Eppendorf 5402, Germany). Other experimental details related to this methodology are described in [31]–[33].

D. Adsorptive Transfer Stripping Technique (AdTS) Coupled With Differential Pulse Voltammetry (DPV) Brdicka Reaction

Electrochemical measurements were performed using an AUTOLAB analyser (EcoChemie, The Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The three-electrode system consisted of a hanging mercury drop electrode as working electrode, an Ag/AgCl/3 M KCl reference electrode, and a carbon counter electrode. For smoothing and baseline correction the software GPES 4.4 supplied by EcoChemie was employed. The Brdicka supporting electrolyte containing 1 mmol/dm³ Co(NH₃)₆Cl₃ and 1 mol/dm³ ammonia buffer (NH₃ (aq) + NH₄Cl, pH = 9.6) was used; surface-active agent was not added. AdTS DPV Brdicka reaction parameters were as follows: an initial potential of −0.6 V, an end potential −1.6 V, a modulation time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV, a modulation amplitude of 250 mV, $E_{\text{ads}} = 0$ V. Temperature of supporting electrolyte was 4 °C. For other experimental conditions see [33].

E. Flow Electrochemical Measurement

A flow injection analysis with electrochemical detection (FIA-ED) system consisted of a solvent delivery pump operating in the range of 0.001–9.999 ml/min (Model 582 ESA Inc., Chelmsford, MA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m), and an electrochemical detector. The electrochemical detector (ED) includes one low volume flow-through analytical cell (Model 5040, ESA, USA), which consists of a glassy carbon working electrode, a palladium electrode as a reference electrode, an auxiliary carbon electrode, and a Coulochem III as a control module. The sample (5 μl) was injected via autosampler (Model 540 Microtiter HPLC, ESA, USA). The obtained data were treated with CSW 32 software. The experiments were carried out at room temperature (22 °C). A glassy carbon electrode was polished mechanically by 0.1 μm of alumina (ESA Inc., USA) and sonicated at room temperature for 5 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W.

III. RESULTS AND DISCUSSION

The investigation of affecting various organisms by abiotic factors directly in their environment is rather difficult [32], [34]–[37]. Therefore, scientists have been suggesting experimental models to simplify this monitoring with use of biomarkers as whole organisms, enzymes, or proteins [38]. As we mentioned above, the level of MT in an animal organism increases with increasing concentration of the toxic abiotic factor, most of all, of heavy metal ions [39]–[43]. Here, we focused on suggesting sensor methodology allowing monitoring of this biomarker, as experimental model guppy fishes treated with silver ions were used. The experimental scheme is shown in Fig. 1(B).

A. Electrochemical Measurement of Metallothionein Content by Brdicka Reaction

Previously, we analyzed metallothionein content in wide range of biological samples using electrochemical methods measured catalytic signal, especially methods called Brdicka reaction and peak H [32], [33], [38], [42]. In spite of the fact that peak H is more sensitive than a Brdicka reaction signal, a Brdicka reaction is suitable for routine analysis [26], [27], [33]. Therefore, we utilized a Brdicka reaction to detect MT in the biological samples of interest. MT measured using a Brdicka reaction gave the signals as follows: RS2Co, Cat1 and Cat2 [Fig. 2(A)]. For quantification of MT in the real samples Cat2 signals were used [33]. MT content increased more than twofold at all experimental variants exposed to silver ions compared to control in first day of the exposition [Fig. 2(B)]. The highest MT levels were determined in the second and third day of the exposition, and then the levels changed gradually. In the last day of the exposition the marked decrease of MT content at all experimental variants occurred [Fig. 2(C)], whereas experimental animals at the highest doses of silver ions (2.5 and 5 μM) died before the end of the experiment. The changes in behavior caused by metabolic disruption as a consequence of silver ions action in vital organs were observable at the treated fishes, which was in good agreement with the published papers [44]–[46]. The other details devoted to this phenomenon will be published elsewhere.

As it is shown, the MT level corresponds very well with silver ions contamination in water. Although the Brdicka reaction is a very sensitive method, it is not suitable for further miniaturization and application directly in the environment. This lack of its application is associated with working mercury electrode, which is absolutely unacceptable to be utilized for environmental online monitoring. Therefore, the method of metallothionein detection in the flow system on glassy carbon electrode was designed.

B. Flow Injection Analysis With Electrochemical Detection (FIA-ED) Using Glassy Carbon Electrode to Analyze Thiols

It has been shown that thiols can be analyzed using a carbon electrode as the working one [4], [21], [47]–[50]. The signals of the thiols related to oxidation of their cysteine residues were observed. Moreover, the shifting of the potential of the thiol signals are assigned to bond heavy metal ions in their molecule [47]. Nevertheless, to our knowledge, MT has not been measured by FIA-ED yet.

Influence of Applied Potential, Flow Rate, and Mobile Phase Composition on Electrochemical Response of MT: MT was measured using FIA with a one-channel electrochemical detector and the sample was injected using an autosampler (ESA, USA); see Fig. 3. A Britton–Robinson buffer under room temperature was used as mobile phase. At working potential of 400 mV, the mobile phase flow rate of 0.4 ml/min metallothionein (100 μM) gave a very well-developed oxidation signal [Fig. 4(A)]. To optimize the amperometric detection of MT in a flow system the influence of applied potential on oxidation signal of MT was studied. The potential within the range from +400 to +900 mV was applied on the working and the amperometric response was recorded. The obtained

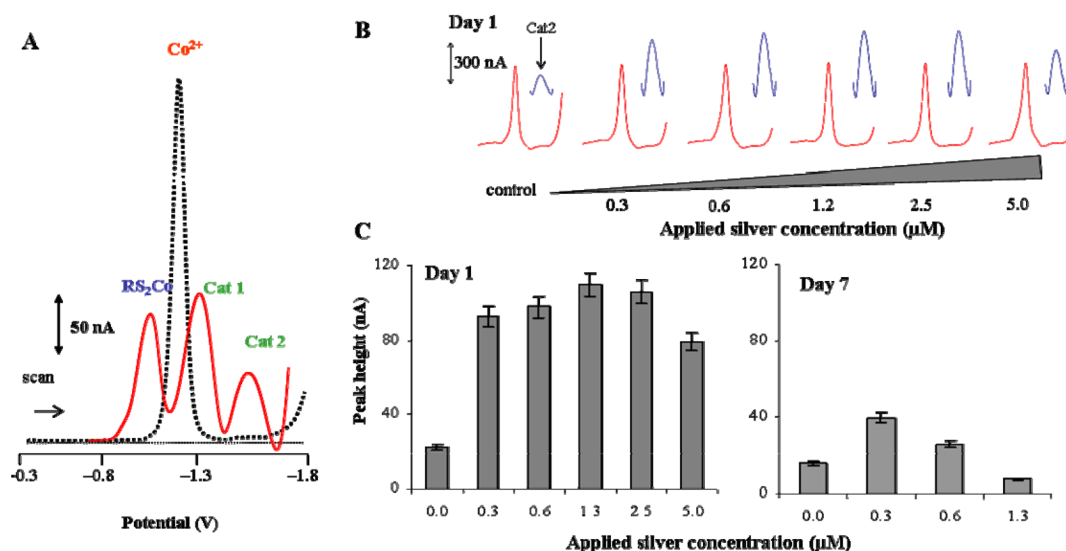


Fig. 2. AdTS DP voltammograms of supporting electrolyte (dotted line) and of metallothionein (500 nM, red solid line). (B) AdTS DP voltammograms of samples obtained from fishes exposed to silver ions in the first day of the treatment. Inset: Cat2 peaks treated with baseline correction. (C) Changes of metallothionein level (as current response) at fish samples in the first and seventh day of the treatment (at highest concentrations the experimental animals died). The Brdicka supporting electrolyte containing $1 \text{ mmol/dm}^3 \text{ Co(NH}_3)_6 \text{ Cl}_3$, and 1 mol/dm^3 ammonia buffer ($\text{NH}_3 \text{ (aq)} + \text{NH}_4 \text{ Cl}$, pH = 9.6) was used; surface-active agent was not added. AdTS DPV Brdicka reaction parameters were as follows: an initial potential of -0.6 V , an end potential -1.6 V , a modulation time 0.057 s , a time interval 0.2 s , a step potential of 1.05 mV/s , a modulation amplitude of 250 mV , and $E_{\text{ads}} = 0 \text{ V}$. Temperature of supporting electrolyte was $4 \text{ }^\circ\text{C}$.

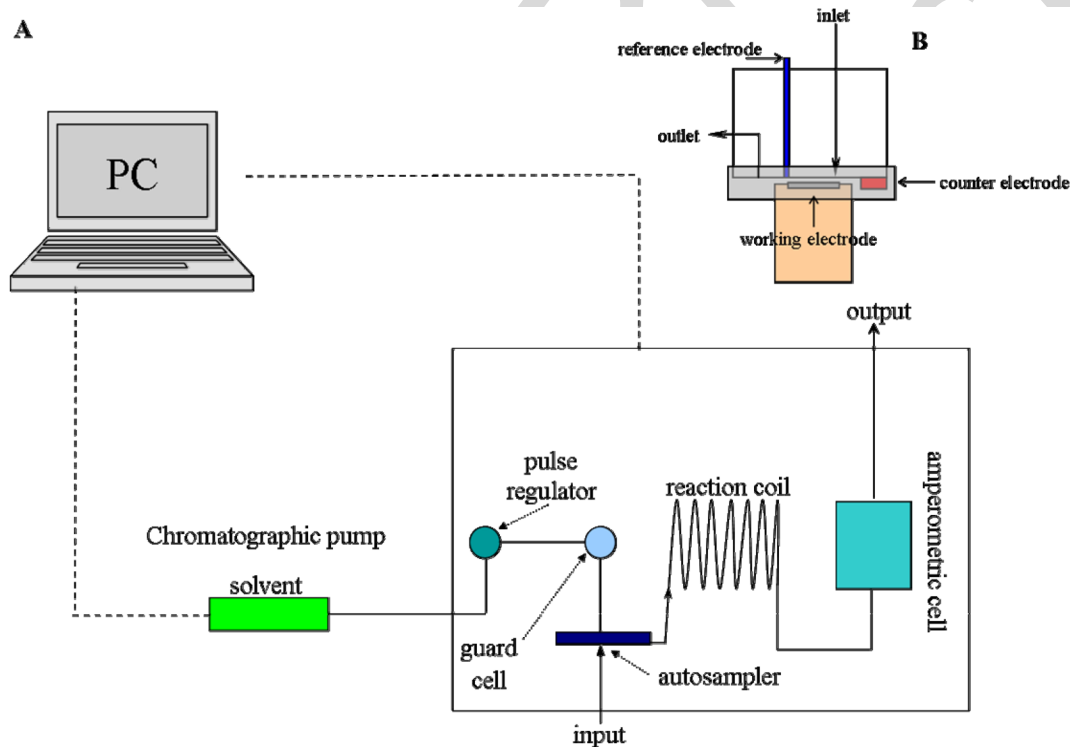


Fig. 3. Scheme of flow injection (FIA) system with amperometric detection. Inset: scheme of electrochemical cell.

oxidation signals of MT at mobile phase flow rate of 0.4 ml/min are shown in Fig. 4(A). The hydrodynamic voltammogram with inflection point at about 600 mV and with maximum at 750 mV was obtained ($n = 5$, relative standard deviation 1.8%). Under the applied potential higher than 750 mV metallothionein oxidation signal changed only gradually [Fig. 4(B)]. The observed oxidation is likely related to cysteine residues present in the MT molecule. In the following FIA-ED experiments, the

potential of 750 mV was used because under this potential MT gave the highest response.

The influence of pH in mobile phase within the range from 1.9 to 4.5 on the MT signal was also investigated. The signal of MT decreased markedly up to pH 3 and then changed gradually. The maximum was reached at pH 1.9 . The observed decrease of the signal was more than 70% . This phenomenon is probably associated with pI of MT (about 8.0). In acidic pH the posi-

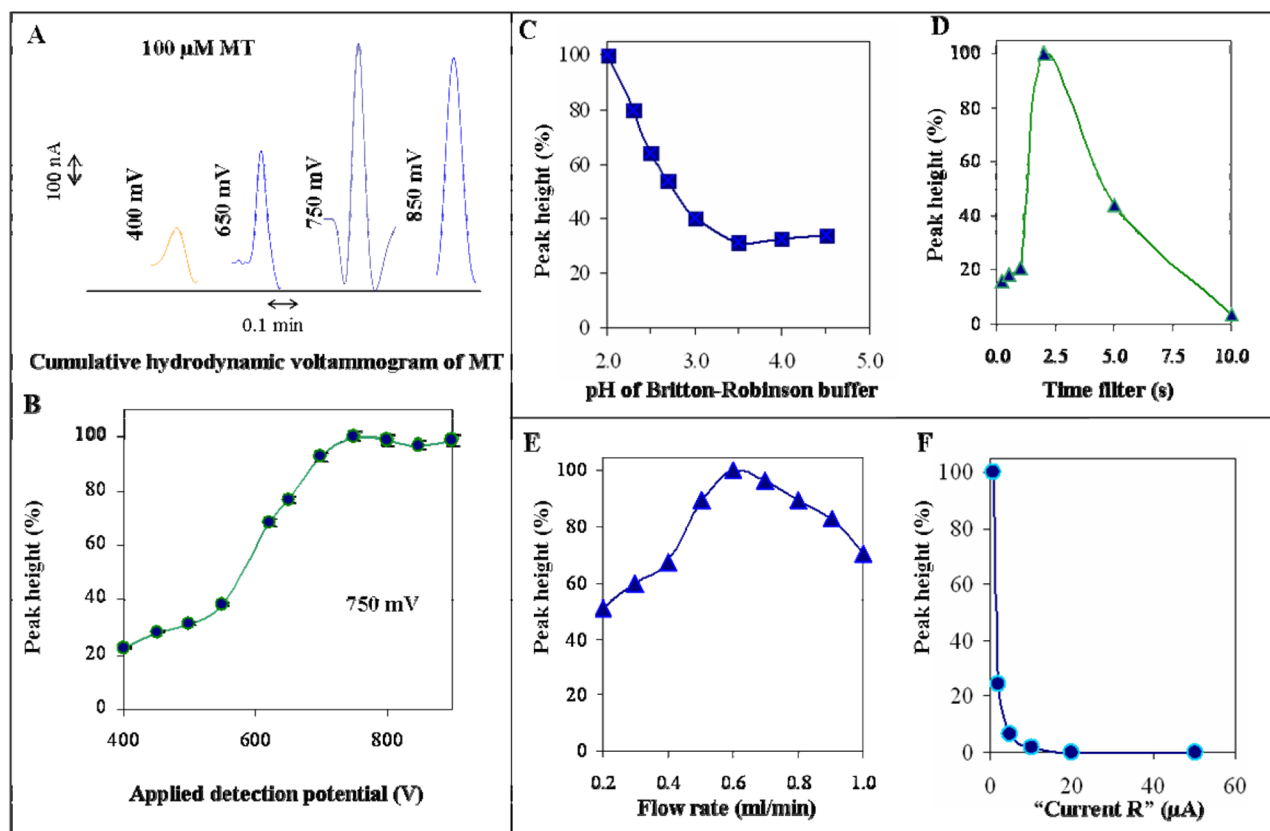


Fig. 4. (A) Typical amperometric response of MT (1 μM) at applied potential 400, 650, 750, and 850 mV. Mobile phase was Britton–Robinson buffer (pH 1.89), flow rate 0.4 ml/min, temperature 25 $^{\circ}\text{C}$, current range 1 μA , time filter 2 s, the sample (10 μl) at 4 $^{\circ}\text{C}$ was injected by autosampler. (B) The hydrodynamic voltammogram. Dependence of MT signals height on (C) pH of Britton–Robinson buffer, (D) time filter, (E) mobile phase flow rate, and (F) applied “current R.”

TABLE I
ANALYTICAL PARAMETERS OF FIA-ED ANALYSIS OF METALLOTHIONEIN

Target molecule	Equation ^a	R ²	LOD (pM)	LOQ (pM)	R.S.D. ^b (%)
Metallothionein	$y = 828.3x + 263.85$	0.9903	100	325	1.98

^a ... The concentration range was within from 5 to 100 nM.

^b ... R.S.D. – relative standard deviation.

tive charge of the molecule can contribute to better interaction with carbon electrode surface and thus to higher electrochemical response. The other phenomenon, which can contribute to higher electrochemical response, relates with releasing of metal ions occurring naturally in MT molecule with decreasing pH, because the metal ions are bounded via $-\text{SH}$ group. Thus, after releasing of the metal ions the free $-\text{SH}$ moieties can be oxidized more easily [Fig. 4(C)].

In addition, the instrument used enables us to utilize a simple mathematical filter to treat the data obtained. The interval of values tested changed the signal height markedly [Fig. 4(D)]. The highest current response was measured at a time filter set as 2.5 s. After that, the influence of flow rate the mobile phase on MT signal was investigated. As we have shown previously, the mobile phase flow rate can influence the electrochemical response of target molecule markedly [35], [51]. The height of the MT signal increased within the range from 0.2 to 0.6

ml/min, and then the signal height decreased. At a flow rate of 1.0 ml/min, the signal height decreased up to 60% of the highest value [Fig. 4(E)]. The last instrument parameter we tested is called “current R.” The tested range was set from 1 to 50 μA . It clearly follows from the results obtained that the maximal response was observed at lowest “current R” applied [Fig. 4(F)].

C. Influence of MT Concentration

Under the optimized experimental conditions the basic analytical parameters were evaluated. Primarily, the calibration curve was measured within the range from 5 to 100 nM with the relative standard deviation (R.S.D.) about 2 % (Table I). The detection limit for MT expressed as 3 S/N was estimated as 100 pM. The detection limit was calculated according to Long [52], whereas \bar{N} was expressed as standard deviation of noise determined in the signal domain. The other approaches for estimation of detection limits were reported by Lavagnini *et al.* [53].

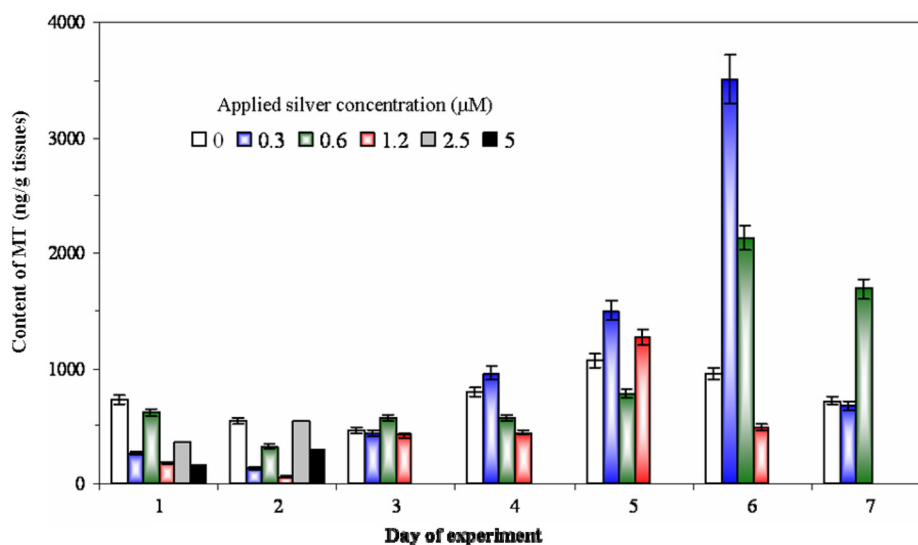


Fig. 5. MT content in guppy fishes (*Poecilia reticulata*) exposed to silver ions (0, 0.3, 0.6, 1.2, 2.5, and 5 μM) for seven days.

D. Utilizing of MT Sensor for Monitoring of Environment Pollution

The proposed sensor was applied for monitoring of the environmental pollution by heavy metal. As an experimental model we used guppy fishes (*Poecilia reticulata*) treated with silver ions, analyzed by Brdicka reaction (Section III-A). As we mentioned, the animals treated with the highest doses of silver ions (2.5 and 5 μM) died up to 48 h. In all survival experimental animals the MT content was determined by the sensor (Fig. 3). Based on the results obtained, MT content increased with the increasing dose of silver ions and time of the treatment (Fig. 5). The highest content of MT was determined at fishes treated with 0.3 μM of silver ions in the sixth day of treatment. The results obtained were in good agreement with those obtained by Brdicka reaction (Figs. 2 and 5).

IV. CONCLUSION

The limit of detection evaluated as 100 pM for metallothionein measured by FIA-ED is several orders higher than those limits obtained using Brdicka reaction published by Petrlova *et al.* [33] and others [54], [55]. Such lower detection limits (Petrlova *et al.* [33]) have been reached via analysis of catalytic signals, but we measured redox signals. These more sensitive techniques suffer from nonautomated or high cost analysis. The proposed technique is automated and sensitive enough to determine MT in real samples. Therefore, we can conclude that the proposed sensor is suitable for simple, rapid, and routine analysis of metallothionein. Moreover, the sensor could be used for evaluation of influence of heavy metals on environment.

ACKNOWLEDGMENT

The coauthors of the paper are holders of the Certificate issued by the Central Commission for Animal Welfare (CCAW) and completed the preparation and successfully passed the qualifying examination according to Section 17 of the Act No. 246/1992 Coll. of Laws on Prevention of Cruelty to Animals, as amended.

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Bio-Assessing of Environmental Pollution via Monitoring of Metallothionein Level Using Electrochemical Detection

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Abstract—In this paper, we report on an investigation of affecting guppy fishes (*Poecilia reticulata*) by silver ions (0, 0.3, 0.6, 1.2, 2.5, and 5 μM) for seven days under well-controlled experimental conditions. To observe the physiological changes, we attempted to determine metallothionein (MT) as a biomarker of heavy metal stress. For this purpose, we proposed a sensor utilizing a carbon electrode coupled with flow injection analysis. The experimental conditions, which have been optimized, were as follows—applied potential: 750 mV, mobile phase: Britton–Robinson buffer (pH 1.9) with flow rate of 0.6 ml/min, time filter: 2.5 s, “current R”: 1 μA . Under these conditions, the detection limit of MT was estimated as 100 pM. After the optimizing step, the fish tissues were measured. Based on the results obtained, MT content increased with increasing dose of silver ions and time of the treatment. The results obtained were in good correlation with those obtained by adsorptive transfer stripping technique coupled with differential pulse voltammetry Brdicka reaction, which was used as the reference technique.

Index Terms—Amperometric detection, biomarker, electrochemical sensors, fish, flow injection analysis, heavy metal, metallothionein, *poecilia reticulata*, silver.

Manuscript received March 31, 2007; revised June 30, 2007 and September 28, 2007; accepted January 6, 2008. This work was supported under Grants GACR 526/07/0808, MSMT 6215712402, MSMT 2636/F4b, and MSMT INCHEMBIOL 0021622412. This is an expanded paper from the Sensors 2006 Conference. The associate editor coordinating the review of this paper and approving it for publication was Prof. Okyay Kaynak.

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I. INTRODUCTION

A. Sensors in Environmental Chemistry

ROBUST analytical techniques, which cannot be miniaturized easily [1], are not useful for furnishing online monitoring of environmental pollution. Therefore, new analytical approaches allowing easy and rapid assessment of environmental pollution are suggested [2]–[7]. These chemical sensors and biosensors have superior properties over existing analytical instruments fitting perfectly for the aforementioned purposes [8], [9]. The sensor system can be composed from three parts: 1) sensor; 2) converter of the signals; and 3) data acquisition instrument [Fig. 1(A)]. Nevertheless, a sensor can be used for analyzing of environmental samples after the optimizing according to its type and properties of target molecule (e.g., gas, liquid) [8], [10]. From the wide spectrum of sensors the electrochemical sensors (i.e., potentiometric, amperometric) are intensively used due to their very low costs, low detection limits, and spectrum of applications [11]–[13]. Electrochemical sensors also allow us to obtain both qualitative and quantitative character of the target molecule and can give information about its physicochemical properties (oxidation state, type of ligand, etc.) and concentration in one measurement [14]–[16].

B. Bio-Indication of Environmental Pollution

To assess environmental pollution we can use three strategies: 1) to detect a pollutant directly; 2) to determine a substance called biomarker, which can be used to indicate exposure of an organism to a pollutant; or 3) to observe physiological changes of an organism living in a polluted environment. Detection of pollutant directly in such a complex matrix as an environmental sample can be a difficult task for an analytical chemist. Moreover, concentration of a certain pollutant can reveal only the immediate level of polluting but reveal nothing about the effect of the pollutant on the environment in a long-term scale. This lack of information can be partly supplemented via analysis of biomarkers or observation of physiological changes of organisms exposed to the pollutant.

Numerous plant and animal species can be used as bio-indicators of heavy metals pollution of the environment [17]–[22]. Aquatic animals, most of all various species of fishes, are very suitable for these purposes [23], [24]. To assess the quality of the Digital Object Identifier 10.1109/JSEN.2008.928500

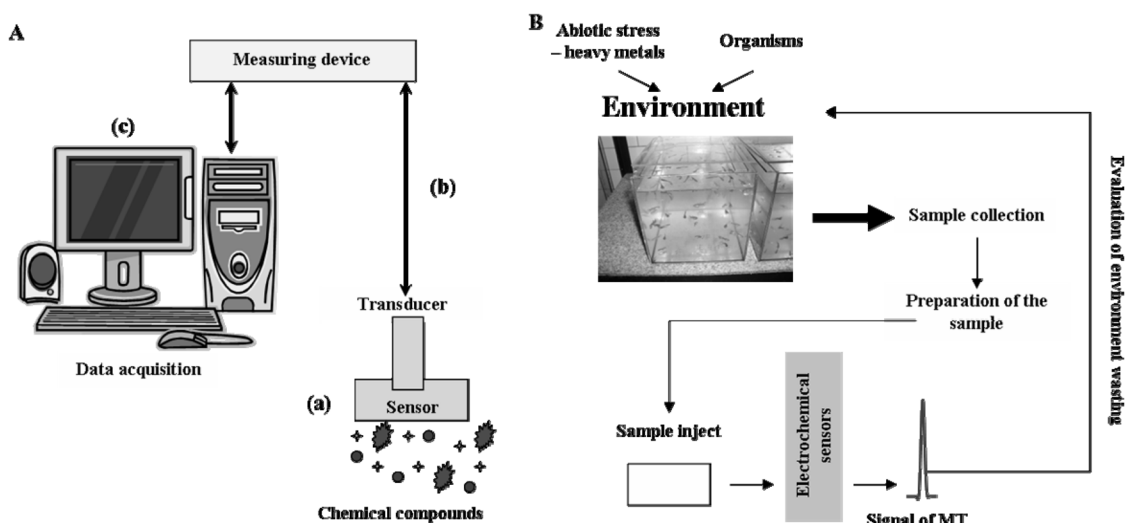


Fig. 1. (A) Typical arrangement of electrochemical sensor: (a) sensor, (b) measuring device, and (c) data acquisition. (B) Scheme of environmental monitoring by heavy metals. Environmental samples are collected and processed for electroanalytical determination according to current protocol. The analysis of one individual is sufficient, which is advantageous for screening programs assessing environment quality [56]–[58].

environment investigations, i.e., the changes in behavior, morphology, habitation, or changes of basic morphometric properties (body weight, color, length, etc.), are often used. All these data are only of qualitative character and are hard to obtain due to requirements on large population of the target specie and the time period of the experiment. Therefore, one may suggest that the most reliable investigations about the effect of heavy metals on an aquatic environment can be proposed using fish specie supplemented with analyzing a certain biomarker.

C. Biomarkers

It has been shown that low-molecular peptides and/or proteins rich in cysteine can be considered as biomarkers, referring to various types of pollutants including heavy metals [25]–[28]. Metallothioneins (MT) as low molecular cysteine rich proteins belong in the group of molecules [29]. Their molecular weight is within the range from 6 to 10 kDa. Due to their affinity to heavy metals, they are involved in their detoxifying and in maintaining of heavy metals homeostasis. MT contains two binding domains α and β , which are composed from cysteine clusters. In this paper, we report on investigations affecting the guppy (*Poecilia reticulata*) by silver ions for seven days under well-controlled experimental conditions, because silver ions pose a threat to aquatic organisms due to their very high toxicity [30]. To observe the physiological changes we determined MT. For this purpose, we proposed a simple and rapid electrochemical detection utilizing carbon electrode coupled with flow injection analysis.

II. MATERIAL AND METHODS

A. Chemicals

Rabbit liver MT (MW 7143), containing 5.9 % Cd and 0.5 % Zn, was purchased from Sigma Aldrich (St. Louis, MO). Tris(2-carboxyethyl)phosphine (TCEP) was produced by Molecular Probes (Evgen, OR). $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ and other chemicals used were purchased from Sigma Aldrich (Sigma-Aldrich,

USA) in ACS purity unless noted otherwise. Stock standard solutions were prepared with ACS water (Sigma-Aldrich, USA) and stored in the dark at -20°C . Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through $0.45\text{-}\mu\text{m}$ Nylon filter discs (Millipore, Billerica, MA) prior to electrochemical analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by a personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3 mol/dm³ KCl) was calibrated by set of WTW buffers (Weilheim, Germany).

B. Experimental Model

Guppy fishes (*Poecilia reticulata*), 2 or 3 months old, were exposed to silver nitrate, always seven individuals per dose 0, 0.3, 0.6, 1.2, 2.5, or 5 μM . The experiment lasted seven days (168 h); a fish from each experimental variant was sampled per day. The experimental conditions such as pH value of the solution where the fishes were kept constant, oxygen concentration, and temperature were monitored during the experiment. The oxygen concentration varied within the range from 1.7 to 4.0 mg/l, the pH level from 6.34 to 7.00, and the temperature from 20.2 to 21.5 $^\circ\text{C}$ during the seven-day-long experiment. The sampled fish was killed by CO_2 and washed one time with distilled water and one time with 0.5 M EDTA.

C. Preparation of Biological Samples for Electrochemical Analysis

The sampled fishes (approximately 0.2 g) were frozen with liquid nitrogen and spread in mortar, and then exactly 1000 μl of 0.2 M phosphate buffer (pH 7.2) was added to the homogenized sample. The obtained homogenate was transferred into test-tube and vortexed for 15 min at 4°C (Vortex Genie). The supernatant was subsequently heat treated. Briefly, the sample was kept at 99°C in a thermomixer (Eppendorf 5430, Germany) for 15 min with occasional stirring and then cooled to 4°C . The denatured homogenates were centrifuged at 4°C , 15000 g for 30 min

(Eppendorf 5402, Germany). Other experimental details related to this methodology are described in [31]–[33].

D. Adsorptive Transfer Stripping Technique (AdTS) Coupled With Differential Pulse Voltammetry (DPV) Brdicka Reaction

Electrochemical measurements were performed using an AUTOLAB analyser (EcoChemie, The Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The three-electrode system consisted of a hanging mercury drop electrode as working electrode, an Ag/AgCl/3 M KCl reference electrode, and a carbon counter electrode. For smoothing and baseline correction the software GPES 4.4 supplied by EcoChemie was employed. The Brdicka supporting electrolyte containing 1 mmol/dm³ Co(NH₃)₆Cl₃ and 1 mol/dm³ ammonia buffer (NH₃ (aq) + NH₄Cl, pH = 9.6) was used; surface-active agent was not added. AdTS DPV Brdicka reaction parameters were as follows: an initial potential of −0.6 V, an end potential −1.6 V, a modulation time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV, a modulation amplitude of 250 mV, $E_{\text{ads}} = 0$ V. Temperature of supporting electrolyte was 4 °C. For other experimental conditions see [33].

E. Flow Electrochemical Measurement

A flow injection analysis with electrochemical detection (FIA-ED) system consisted of a solvent delivery pump operating in the range of 0.001–9.999 ml/min (Model 582 ESA Inc., Chelmsford, MA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m), and an electrochemical detector. The electrochemical detector (ED) includes one low volume flow-through analytical cell (Model 5040, ESA, USA), which consists of a glassy carbon working electrode, a palladium electrode as a reference electrode, an auxiliary carbon electrode, and a Coulochem III as a control module. The sample (5 μl) was injected via autosampler (Model 540 Microtiter HPLC, ESA, USA). The obtained data were treated with CSW 32 software. The experiments were carried out at room temperature (22 °C). A glassy carbon electrode was polished mechanically by 0.1 μm of alumina (ESA Inc., USA) and sonicated at room temperature for 5 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W.

III. RESULTS AND DISCUSSION

The investigation of affecting various organisms by abiotic factors directly in their environment is rather difficult [32], [34]–[37]. Therefore, scientists have been suggesting experimental models to simplify this monitoring with use of biomarkers as whole organisms, enzymes, or proteins [38]. As we mentioned above, the level of MT in an animal organism increases with increasing concentration of the toxic abiotic factor, most of all, of heavy metal ions [39]–[43]. Here, we focused on suggesting sensor methodology allowing monitoring of this biomarker, as experimental model guppy fishes treated with silver ions were used. The experimental scheme is shown in Fig. 1(B).

A. Electrochemical Measurement of Metallothionein Content by Brdicka Reaction

Previously, we analyzed metallothionein content in wide range of biological samples using electrochemical methods measured catalytic signal, especially methods called Brdicka reaction and peak H [32], [33], [38], [42]. In spite of the fact that peak H is more sensitive than a Brdicka reaction signal, a Brdicka reaction is suitable for routine analysis [26], [27], [33]. Therefore, we utilized a Brdicka reaction to detect MT in the biological samples of interest. MT measured using a Brdicka reaction gave the signals as follows: RS2Co, Cat1 and Cat2 [Fig. 2(A)]. For quantification of MT in the real samples Cat2 signals were used [33]. MT content increased more than twofold at all experimental variants exposed to silver ions compared to control in first day of the exposition [Fig. 2(B)]. The highest MT levels were determined in the second and third day of the exposition, and then the levels changed gradually. In the last day of the exposition the marked decrease of MT content at all experimental variants occurred [Fig. 2(C)], whereas experimental animals at the highest doses of silver ions (2.5 and 5 μM) died before the end of the experiment. The changes in behavior caused by metabolic disruption as a consequence of silver ions action in vital organs were observable at the treated fishes, which was in good agreement with the published papers [44]–[46]. The other details devoted to this phenomenon will be published elsewhere.

As it is shown, the MT level corresponds very well with silver ions contamination in water. Although the Brdicka reaction is a very sensitive method, it is not suitable for further miniaturization and application directly in the environment. This lack of its application is associated with working mercury electrode, which is absolutely unacceptable to be utilized for environmental on-line monitoring. Therefore, the method of metallothionein detection in the flow system on glassy carbon electrode was designed.

B. Flow Injection Analysis With Electrochemical Detection (FIA-ED) Using Glassy Carbon Electrode to Analyze Thiols

It has been shown that thiols can be analyzed using a carbon electrode as the working one [4], [21], [47]–[50]. The signals of the thiols related to oxidation of their cysteine residues were observed. Moreover, the shifting of the potential of the thiol signals are assigned to bond heavy metal ions in their molecule [47]. Nevertheless, to our knowledge, MT has not been measured by FIA-ED yet.

Influence of Applied Potential, Flow Rate, and Mobile Phase Composition on Electrochemical Response of MT: MT was measured using FIA with a one-channel electrochemical detector and the sample was injected using an autosampler (ESA, USA); see Fig. 3. A Britton–Robinson buffer under room temperature was used as mobile phase. At working potential of 400 mV, the mobile phase flow rate of 0.4 ml/min metallothionein (100 μM) gave a very well-developed oxidation signal [Fig. 4(A)]. To optimize the amperometric detection of MT in a flow system the influence of applied potential on oxidation signal of MT was studied. The potential within the range from +400 to +900 mV was applied on the working and the amperometric response was recorded. The obtained

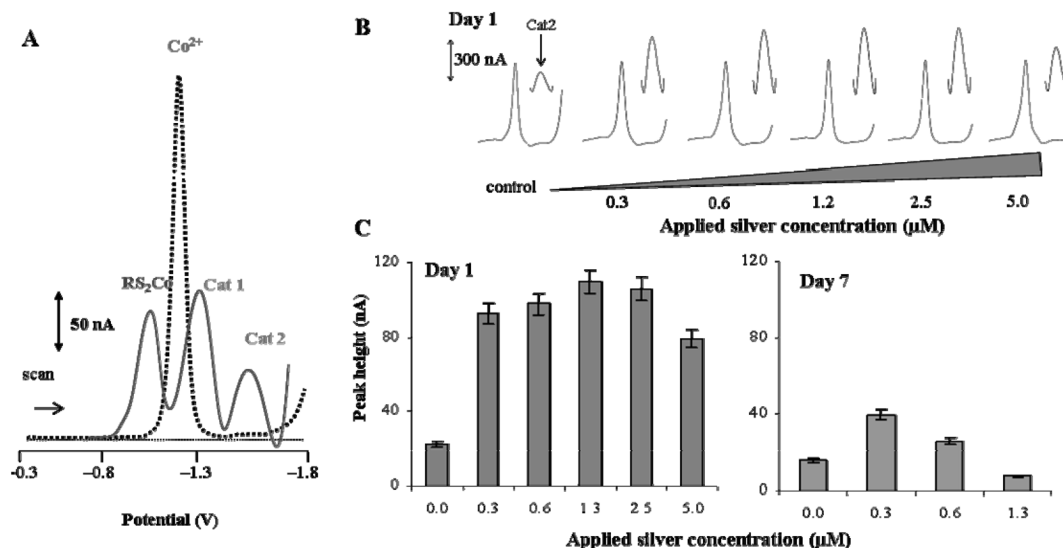


Fig. 2. AdTS DP voltammograms of supporting electrolyte (dotted line) and of metallothionein (500 nM, red solid line). (B) AdTS DP voltammograms of samples obtained from fishes exposed to silver ions in the first day of the treatment. Inset: Cat2 peaks treated with baseline correction. (C) Changes of metallothionein level (as current response) at fish samples in the first and seventh day of the treatment (at highest concentrations the experimental animals died). The Brdicka supporting electrolyte containing $1 \text{ mmol/dm}^3 \text{ Co(NH}_3)_6 \text{ Cl}_3$, and 1 mol/dm^3 ammonia buffer ($\text{NH}_3 \text{ (aq)} + \text{NH}_4 \text{ Cl}$, pH = 9.6) was used; surface-active agent was not added. AdTS DPV Brdicka reaction parameters were as follows: an initial potential of -0.6 V , an end potential -1.6 V , a modulation time 0.057 s , a time interval 0.2 s , a step potential of 1.05 mV/s , a modulation amplitude of 250 mV , and $E_{\text{ads}} = 0 \text{ V}$. Temperature of supporting electrolyte was $4 \text{ }^\circ\text{C}$.

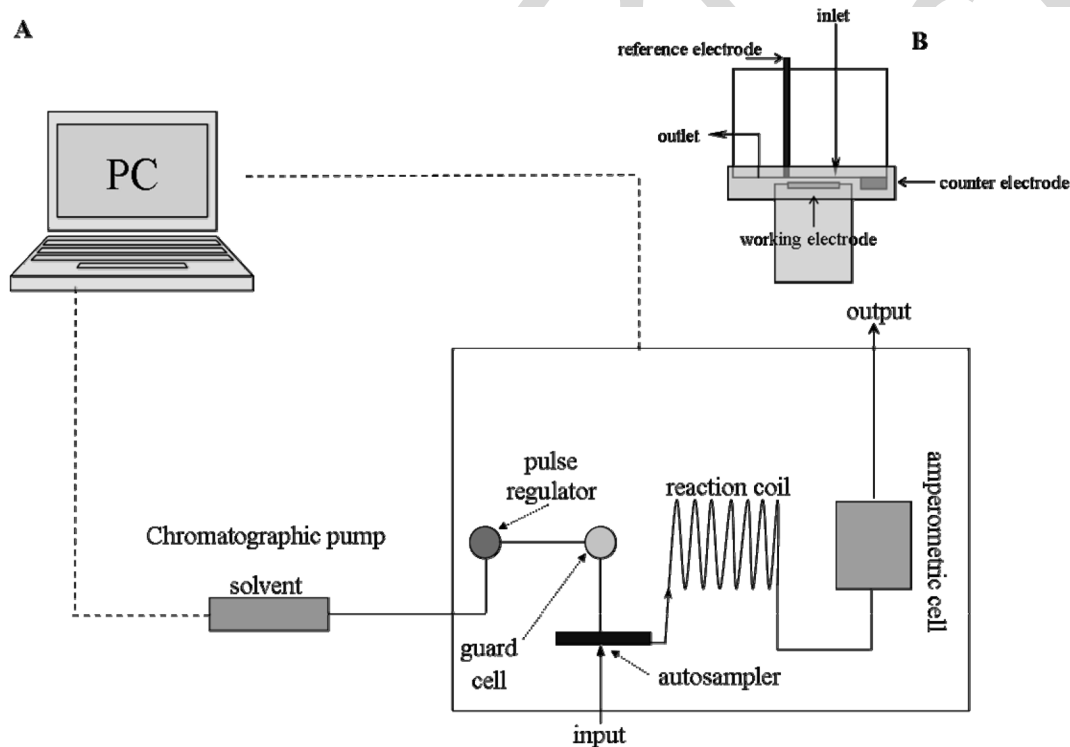


Fig. 3. Scheme of flow injection (FIA) system with amperometric detection. Inset: scheme of electrochemical cell.

oxidation signals of MT at mobile phase flow rate of 0.4 ml/min are shown in Fig. 4(A). The hydrodynamic voltammogram with inflection point at about 600 mV and with maximum at 750 mV was obtained ($n = 5$, relative standard deviation 1.8%). Under the applied potential higher than 750 mV metallothionein oxidation signal changed only gradually [Fig. 4(B)]. The observed oxidation is likely related to cysteine residues present in the MT molecule. In the following FIA-ED experiments, the

potential of 750 mV was used because under this potential MT gave the highest response.

The influence of pH in mobile phase within the range from 1.9 to 4.5 on the MT signal was also investigated. The signal of MT decreased markedly up to pH 3 and then changed gradually. The maximum was reached at pH 1.9 . The observed decrease of the signal was more than 70% . This phenomenon is probably associated with pI of MT (about 8.0). In acidic pH the posi-

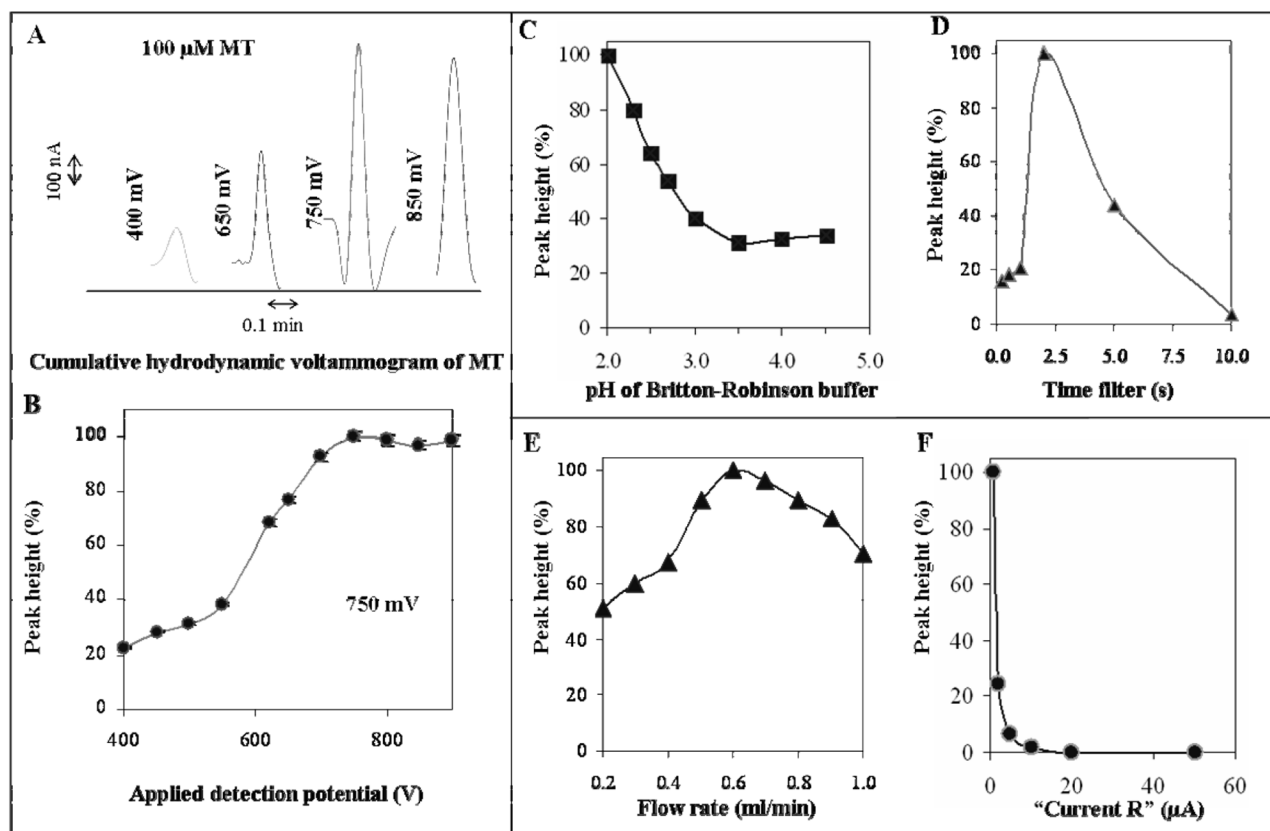


Fig. 4. (A) Typical amperometric response of MT (1 μM) at applied potential 400, 650, 750, and 850 mV. Mobile phase was Britton–Robinson buffer (pH 1.89), flow rate 0.4 ml/min, temperature 25 $^{\circ}\text{C}$, current range 1 μA , time filter 2 s, the sample (10 μl) at 4 $^{\circ}\text{C}$ was injected by autosampler. (B) The hydrodynamic voltammogram. Dependence of MT signals height on (C) pH of Britton–Robinson buffer, (D) time filter, (E) mobile phase flow rate, and (F) applied “current R.”

TABLE I
ANALYTICAL PARAMETERS OF FIA-ED ANALYSIS OF METALLOTHIONEIN

Target molecule	Equation ^a	R ²	LOD (pM)	LOQ (pM)	R.S.D. ^b (%)
Metallothionein	$y = 828.3x + 263.85$	0.9903	100	325	1.98

^a ... The concentration range was within from 5 to 100 nM.

^b ... R.S.D. – relative standard deviation.

tive charge of the molecule can contribute to better interaction with carbon electrode surface and thus to higher electrochemical response. The other phenomenon, which can contribute to higher electrochemical response, relates with releasing of metal ions occurring naturally in MT molecule with decreasing pH, because the metal ions are bounded via –SH group. Thus, after releasing of the metal ions the free –SH moieties can be oxidized more easily [Fig. 4(C)].

In addition, the instrument used enables us to utilize a simple mathematical filter to treat the data obtained. The interval of values tested changed the signal height markedly [Fig. 4(D)]. The highest current response was measured at a time filter set as 2.5 s. After that, the influence of flow rate the mobile phase on MT signal was investigated. As we have shown previously, the mobile phase flow rate can influence the electrochemical response of target molecule markedly [35], [51]. The height of the MT signal increased within the range from 0.2 to 0.6

ml/min, and then the signal height decreased. At a flow rate of 1.0 ml/min, the signal height decreased up to 60% of the highest value [Fig. 4(E)]. The last instrument parameter we tested is called “current R.” The tested range was set from 1 to 50 μA . It clearly follows from the results obtained that the maximal response was observed at lowest “current R” applied [Fig. 4(F)].

C. Influence of MT Concentration

Under the optimized experimental conditions the basic analytical parameters were evaluated. Primarily, the calibration curve was measured within the range from 5 to 100 nM with the relative standard deviation (R.S.D.) about 2 % (Table I). The detection limit for MT expressed as 3 S/N was estimated as 100 pM. The detection limit was calculated according to Long [52], whereas \bar{N} was expressed as standard deviation of noise determined in the signal domain. The other approaches for estimation of detection limits were reported by Lavagnini *et al.* [53].

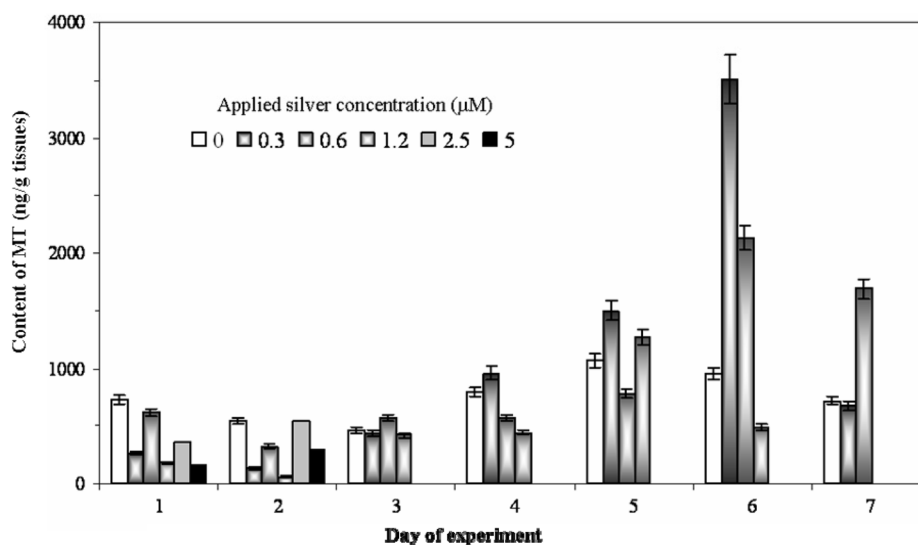


Fig. 5. MT content in guppy fishes (*Poecilia reticulata*) exposed to silver ions (0, 0.3, 0.6, 1.2, 2.5, and 5 μM) for seven days.

D. Utilizing of MT Sensor for Monitoring of Environment Pollution

The proposed sensor was applied for monitoring of the environmental pollution by heavy metal. As an experimental model we used guppy fishes (*Poecilia reticulata*) treated with silver ions, analyzed by Brdicka reaction (Section III-A). As we mentioned, the animals treated with the highest doses of silver ions (2.5 and 5 μM) died up to 48 h. In all survival experimental animals the MT content was determined by the sensor (Fig. 3). Based on the results obtained, MT content increased with the increasing dose of silver ions and time of the treatment (Fig. 5). The highest content of MT was determined at fishes treated with 0.3 μM of silver ions in the sixth day of treatment. The results obtained were in good agreement with those obtained by Brdicka reaction (Figs. 2 and 5).

IV. CONCLUSION

The limit of detection evaluated as 100 pM for metallothionein measured by FIA-ED is several orders higher than those limits obtained using Brdicka reaction published by Petrlova *et al.* [33] and others [54], [55]. Such lower detection limits (Petrlova *et al.* [33]) have been reached via analysis of catalytic signals, but we measured redox signals. These more sensitive techniques suffer from nonautomated or high cost analysis. The proposed technique is automated and sensitive enough to determine MT in real samples. Therefore, we can conclude that the proposed sensor is suitable for simple, rapid, and routine analysis of metallothionein. Moreover, the sensor could be used for evaluation of influence of heavy metals on environment.

ACKNOWLEDGMENT

The coauthors of the paper are holders of the Certificate issued by the Central Commission for Animal Welfare (CCAW) and completed the preparation and successfully passed the qualifying examination according to Section 17 of the Act No. 246/1992 Coll. of Laws on Prevention of Cruelty to Animals, as amended.

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