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PAPER

Paramagnetic antibody-modified microparticles coupled with voltammetry as a tool for isolation and detection of metallothionen as a bioindicator of metal pollution

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Low-molecular mass proteins rich in cysteines called metallothioneins (MT) can be considered as markers for the pollution of the environment by metals. Here, we report on suggestion for an automated procedure for the isolation of MT followed by voltammetric analysis. Primarily, we optimized the automated detection of MT using an electrochemical analyser. It was found that the most sensitive and repeatable analyses are obtained at a temperature of 4 °C for the supporting electrolyte. Further, we optimized experimental conditions for the isolation of MT by using antibody-linked paramagnetic microparticles. Under the optimal conditions (4 h long interaction between the microparticles and MT), the microparticles were tested on isolation of various amounts of MT. The lowest isolated amount of MT by antibody-linked paramagnetic microparticles was 5 μg ml^{-1} of MT (50 ng). The automated procedure of MT isolation was further tested on isolation of MT from guppy fish (*Poecilia reticulata*) treated with silver(I) ions (50 μM $AgNO_3$). The whole process lasted less than five hours and was fully automated. We attempted to correlate these results with the standard method for MT isolation. The correlation coefficient is 0.9901, which confirms that results are in good agreement. Moreover, the concentration of silver ions in tissues of fish treated with Ag(I) ions was determined by high performance liquid chromatography with electrochemical detection.

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1. Introduction

Pollution of the environment with toxic metals due to anthropogenic activities poses a threat to plants as well as animals. Mining, fertilizing, using fossil fuels and others belong to the main sources of metal-pollution.[1] Prior to the development and use of silver, the majority of silver occurring in surface waters originated from natural leaching, mining and the photographic industry,[2] with measured concentrations in natural and contaminated waters in the ng l^{-1} and higher range (Sanudo-Willhelmy and Flegal, 1992). Since the 1970s, concentrations of silver have decreased in surface waters due to the restrictions of using Ag in the photographic industry and more stringent

Environmental impact

An easy-to-use and automated procedure for the isolation of metallothioneins (MT) as markers of metal pollution in the environment is missing. In this study, we optimize experimental conditions for the isolation of MT by using antibody-linked paramagnetic microparticles and also for the automated electrochemical determination of the isolated proteins. The automated procedure of MT isolation and determination was tested on isolation of MT from guppy fish (*Poecilia reticulata*) treated with silver (I) ions (50 μM $AgNO_3$). Moreover, we attempted to correlate the MT levels determined using standard procedures with the optimized one based on antibody-linked paramagnetic microparticles. We attempted to correlate these results with standard method for MT isolation. The correlation coefficient is 0.9901, which confirms that results are in good agreement.

environmental regulations.[2] However, silver "is coming back" because of extensive use of this metal in the form of nanoparticles.[3]

Due to exposition of the biosphere with metals, organisms have developed various strategies to protect themselves against adverse effects of these ions and their compounds. Homeostasis of metals in both plant and animal cells is maintained by low-molecular mass compounds rich in –SH moieties. In animals, metallothioneins (MT) play a key role in these processes. MT are the group of low molecular mass (2 to 16 kDa) single-chain proteins. The metal binding domain of MT consists of 20 cysteine residues juxtaposed with basic amino acids (lysine and arginine) arranged in two thiol-rich sites.[4] Based on their metal affinity these proteins are able to transport essential metals to places of need or detoxify toxic metals to protect cells.[5] There have been numerous papers published showing levels of MT in animals as bioindicators for pollution of the environment by metals.[6–8] Various analytical techniques including mass spectrometry, liquid chromatography, capillary electrophoresis, saturation methods and electrochemistry can be employed for detection of MT.[9,10] Electrochemical methods belong to the most sensitive ones, mainly differential pulse voltammetry Brdicka reaction[11–14] and chronopotentiometric stripping analysis.[15,16] Besides electrochemical methods with high sensitivity to MT, there are also immunochemical methods utilized including enzyme-linked immunosorbent assay,[13,17,18] radioimmunoassay[19] or blotting techniques[17] for detection of MT with convenient detection limits. In this study, we test a new protocol for isolation of MT from tissues of guppy fish (*Poecilia reticulata*) treated with silver(I) ions based on antibody-linked paramagnetic particles and compare the results with a standard procedure using only heat treatment of a sample.

2. Results and discussion

2.1 Automated electrochemical detection of MT – Brdicka reaction

The Brdicka reaction belongs to the most sensitive methods for detection of MT.[11] However, it suffers from demands of manual introduction of each sample. Here, we used a unique electrochemical stationary analyser consisting of a) a three electrode system connected to a potentiostat/galvanostat; b) an autosampler and c) a control unit. For analysis of larger set of samples, there is also need for accurate analysis of low volumes of biological samples. Therefore, we tested relative standard deviation (RSD) of measurements of various volumes of MT standards. Volumes between 1 and 50 μl were measured. We determined that the introduction of the lowest tested volume (1 μl) resulted in a RSD of 1.5% ($n = 10$). If we introduced volumes higher than 5 μl, the RSD of MT measurements was below 1% ($n = 10$, concentration range from 1 to 50 μM). Based on these results, we introduced 5 μl in the following experiments. A catalytic signal Cat2 proportional to MT concentration was detected on all voltammograms. The calibration curve measured within the concentration range 1 to 50 μM was strictly linear with $R^2 = 0.9991$. This concentration range fully covers the concentration commonly determined in tissues.[8,20–25] The influence of temperature on the catalytic signal Cat2 was investigated in several papers.[11,14] It was found that the most sensitive and repeatable measurements are carried out at a temperature of between 4 and 10 °C for the supporting electrolyte. We used a 4 °C temperature for the supporting electrolyte.

2.2 Paramagnetic microparticles for MT isolation

Fig. 1 shows the scheme of the whole process that is based on the proteins binding onto paramagnetic microparticles. Prior to MT isolation, microparticles were modified by chicken antibodies against MT. These polyclonal antibodies have been shown to react with MT from human blood serum and human cell lines,[17] and from pigs' and carps' tissue extracts.[13] Moreover, to evaluate the applicability of these antibodies, bioinformatics comparison of structures of MTs isolated from various organisms have been carried out (Table 1). The type of isoform, number of all the amino acids (AA) in sequence and part of cysteins are described for each chosen sequence. Moreover, the number of coupled metal ions are recorded separately for structural domains α and β, if they exist. The allocation of two named domains is caused through specificity in the secondary structure. Sequences from simpler organisms do not create a separate domain. Selected MT sequences were aligned because the study group was not homological (length of sequences vary). The BLOSUM30 scoring matrix was used for multiple alignments.[26,27] After global multiple alignment, biological distances between each pair of sequences were determined by the Jukes–Cantor model normalized for amino acids.[28] Finally, the phylogenetic tree was constructed using the relationship among the distance data by the BIONJ method.[29] The resulting tree structure is shown in Fig. 2A. Moreover, mutual amino-acid motifs were estimated for mammals and for fish. These motifs are shown in Fig. 2B and 2C, respectively. Based on the above-mentioned results, it can be concluded that the sequences are similar with common structural motifs, which can bind with polyclonal MT antibodies, as it was shown experimentally.[13,17]

Modification of the commercially available microparticles was carried out according to the following protocol. The microparticles (100 μl) were washed with PBS buffer (pH 7.4) and then were held by a magnetic field whilst the solution was removed. The washed microparticles were kept in the presence of 900 μl of chicken antibodies (diluted 1 : 500) at room temperature for 24 h with shaking at 10 rpm on a Vortex–2 Genie (Scientific Industries, New York, USA). The antibody-linked paramagnetic microparticles (10 μl) were washed with PBS buffer (pH 7.4) and then held by a magnetic field whilst the solution was removed. The washed antibody-linked paramagnetic microparticles were equilibrated three times with borate buffer (pH 9.5). The equilibrated antibody-linked paramagnetic microparticles were incubated in the presence of borate buffer (pH 9.6) with addition of MT at 24 °C, 400 rpm shaking for two hours. The antibody-linked paramagnetic microparticles were then rinsed four times with borate buffer (pH 9.6). The stability of linking of antibodies and particles is high due to strong interactions between parts of the antibodies and particles and commonly used for such modifying as reviewed by Gupta and Gupta.[30] Captured MT was released from the antibodies linked to paramagnetic microparticles surface by decreasing the pH (Britton-Robinson buffer,

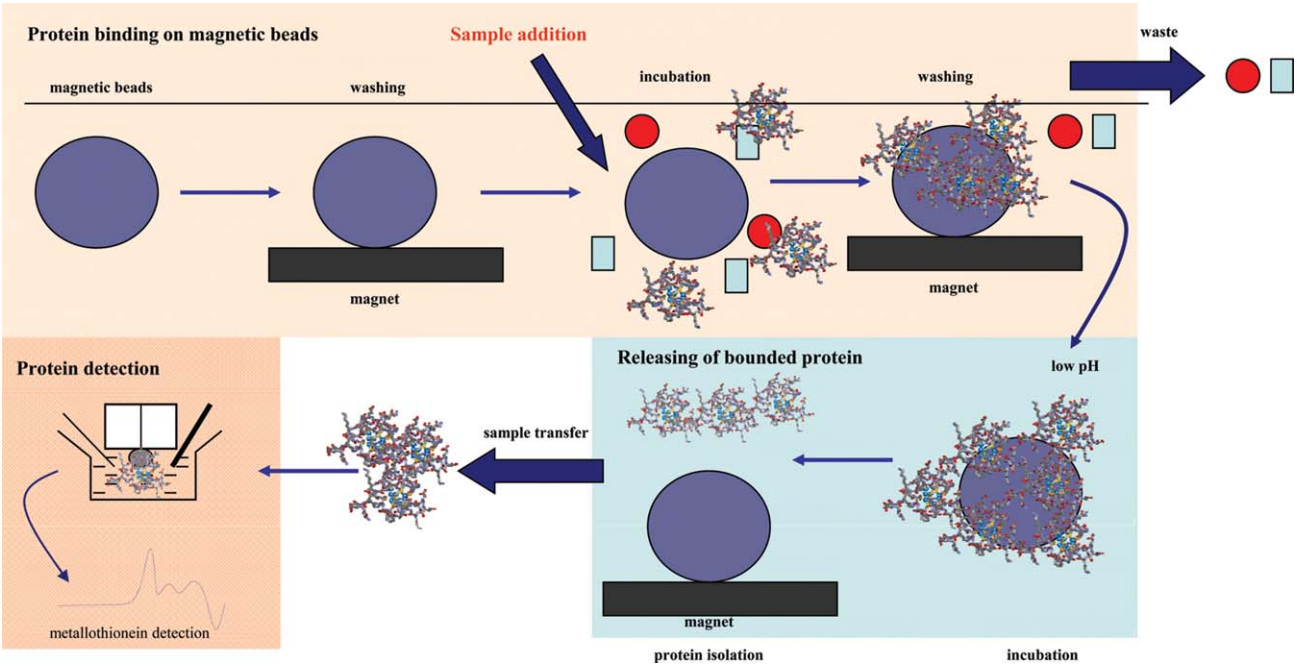

Fig. 1 The scheme of using antibody-linked paramagnetic microparticles for the isolation of metallothioneins. The microparticles are primarily held by a magnet and rinsed several times. The rinsed microparticles are incubated in the presence of metallothionein. The protein binds with the surface of the microparticles *via* antibodies. The anchored proteins are released from the surface of the paramagnetic microparticles by lowering of pH of solution. The microparticles are held by a magnet. The free-of-the-microparticles solution is then electrochemically analysed.

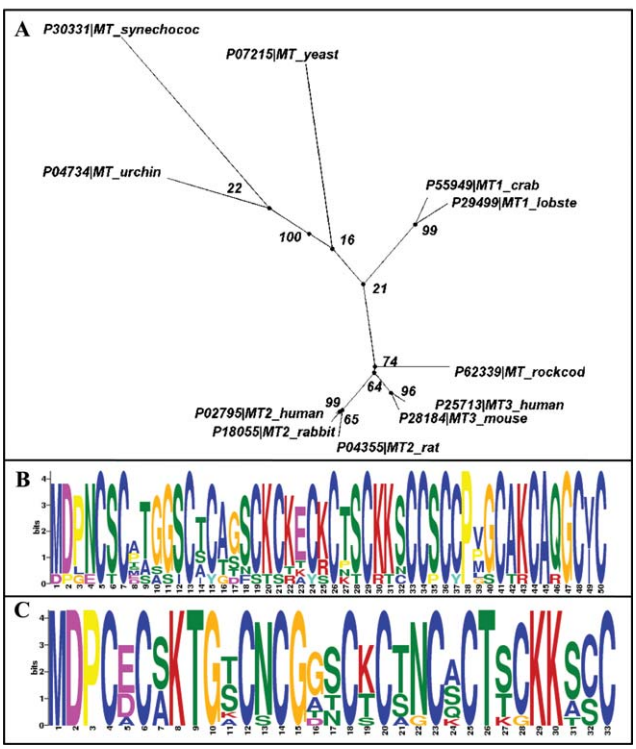

Fig. 2 (**A**) BIONJ tree of a metallothionein. The tree is obtained by a Jukes–Cantor distance model. The number for each interior branch is the bootstrap value from 1000 replications. Mutual amino-acid motifs for (**B**) mammals and for (**C**) fish.

pH 2). Isolated protein was subsequently electrochemically detected by Brdicka reaction.

The dependence of the time of interaction (from 1 to 10 h) on the amount of MT (100 μg ml^{-1}) isolated by microparticles was measured. The dependence of Cat2 peak height on the interaction time is shown in Fig. 3A. The amount of isolated MT increased with the time of interaction. The yield of MT was about 20% after a 1 h long interaction and increased up to 70% after a 5 h long interaction (calculated as the ratio: quantified amount/given amount). To find the optimum between the length of isolation and its yield, we calculated the length-yield-ratio (LYR) as follows: (peak height of Cat2, %)/(time of interaction, h). Based on the results obtained the best LYR ratio was obtained after a 1 h long interaction followed by 2, 6 and 4 h. 1 and 2 h long interactions resulted in a yield under 50%. 10 h was the longest tested interaction time. Therefore, we used a 6 h long interaction in our following experiments, because at this time we obtained the highest recovery. Further, the microparticles were tested on isolation of various amounts of MT. To detect MT isolated by microparticles, we employed an automated electrochemical instrument. The obtained dependence is shown in Fig. 3B. The lowest amount of MT, which can be isolated by the antibody-linked paramagnetic microparticles, was 5 μg ml^{-1} of MT (50 ng). Moreover, we tested the isolation of MT using unmodified microparticles. Due to some un-specific interactions between the surface of the particles and the MT standard, we were able to reach a 3% yield after a 6 h long isolation. This amount is negligible compared to those obtained by antibody-modified microparticles.

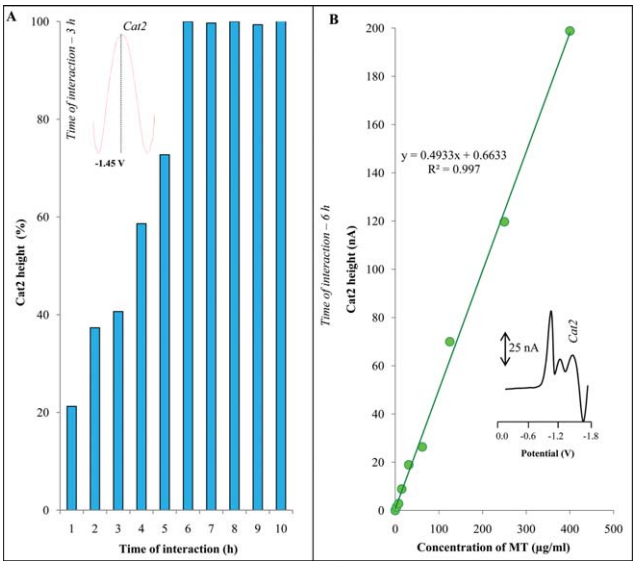

Fig. 3 It has been known more than 70 years that it is suitable to use a catalytic signal of hydrogen evolution in the presence of ammonium buffer containing cobalt solution $Co(NH_3)_6Cl_3$ called Brdicka reaction (signals are measured by differential pulse voltammetry) for the determination of the proteins containing cysteine amino acids, *e.g.* MT. (**A**) Dependence of catalytic peak Cat2 height (amount of isolated MT) on the time of interaction between antibody-linked paramagnetic microparticles and MT standard (100 μg ml^{-1}); in inset: baseline corrected and smoothed (level 2) Cat2 peak of MT after a 3 h long interaction between the microparticles and MT. (**B**) Dependence of Cat2 height on the amount of MT released from the microparticles; in inset: differential pulse voltammogram of MT (125 μg ml^{-1}).

2.3 Metallothionein as an indicator of environment contamination by metal ions

MT levels can be used as a bioindicator of metal pollution, however, age, sex, and other factors have to be considered.[31–33] Guppy fish (*Poecilia reticulate*) were used in our experiment when 50 μM $AgNO_3$ was added into water where the fish were kept. The impact of silver ions in the environment have been intensively studying. The existence of International research organisations such as Silver Coalition (1991–1995) and The Silver Council (from 1996 to date) proves this fact. These organisations also established the International Conference on Transport, Fate and Effects of Silver in the Environment.[34] In water silver(I) exists as various inorganic and organic compounds. Most of these are nonsoluble, but hydrated silver ions, Ag^+, may be also present in surface waters due to competing equilibria and kinetics, which, in turn, are dependent on the conditions of the water such as pH, hardness and many others. This concern relates to the fact that Ag^+ has been shown to be highly toxic to aquatic life, while other species of silver(I) are much less toxic.[35–42]

Silver(I) ion toxicity was manifested as a progressive fish mortality increase as early as 24 h after exposition. At the end of the exposition, the mortality was about 35% (Fig. 4A). The fish were prepared as described in the material and methods section. MT was quantified according to the procedure published by Fabrik *et al.*[8] The results obtained are shown in Fig. 4B. MT concentration in the fish significantly increased after a 72 h long exposure. After that, MT levels declined, which can be associated with the high toxicity of silver(I) ions.

2.4 Microparticles based biosensors for detection of MT

Further, we proposed an automated procedure for the detection of MT in guppy fish treated with silver(I) ions. The tissues were prepared as described in Material and methods and then analysed by using an automated pipetting instrument (EP Motion, Eppendorf, Germany). We programmed the automated analyser as follows: a) introduction of 10 μl of antibody-linked paramagnetic microparticles onto certain positions on PCR96 plate and transfer of the plate onto the magnetic platform; b) rinsing step (repeated four times); c) introduction of the prepared extracts from fish tissues; d) paramagnetic microparticle interaction with MT at 24 °C under shaking (400 rpm), this step lasted four hours; e) rinsing step (repeated four times); f) eluting of the isolated MT molecules and measurement of them using an automated electrochemical analyser. The whole process lasted less than five hours and was fully automated except homogenizing and heat treatment steps. The levels of MT determined in the tissues of fish treated with silver(I) ions are shown in Fig. 4C. The trend in the MT concentration is similar for both preparation protocols. However, by using antibody-linked paramagnetic particles the concentration of MT is slightly lower compared to the standard protocol (app. 10%). This phenomenon can be associated with the description of Krizkova *et al.*, who showed that the differential pulse voltammetry Brdicka reaction slightly overestimates the content of MT in blood serum and tissue extracts samples.[13] This was found by enzyme-linked immunosorbent assay employing chicken yolk antibodies, which were sensitive to the presence of MT only in a heat-treated sample compared to the electrochemical analysis, which was most probably also sensitive to the presence of some heat shock proteins containing –SH moieties. Moreover, we attempted to correlate MT concentrations determined by antibody-modified microparticles coupled with DPV Brdicka reaction with standard Brdicka reaction. We plotted the results measured and found that the dependence was linear with a correlation coefficient of 0.989 (Fig. 4D). It can be concluded that the results presented in Fig. 4B and 4C are in good agreement. Antibody-modified microparticles coupled with the DPV Brdicka reaction, thus, represents a new method for the determination of MT in real samples. Its advantages can be seen in that it is easy-to-use and its possibility to be miniaturized and used *in situ*.

2.5 Determination of silver(I) ion content

Silver(I) ions in aquatic environments are stable in a wide range of pH. Only in alkaline solutions are hydroxides and hydroxide anions like AgOH and $Ag(OH)_2^-$ are formed. There are many other compounds which silver(I) ions can interact with, forming many hardly soluble compounds, which do not pose a threat to organisms. Moreover, silver(I) ions have a high affinity to –SH moieties (Fig. 4E), which clearly relates with the fact that these ions are detoxified by compounds rich in –SH moieties including MT. Therefore, we determined the amount of silver(I) ions in fish tissue extracts by high performance liquid chromatography coupled with electrochemical detection (HPLC-ED).[43] The

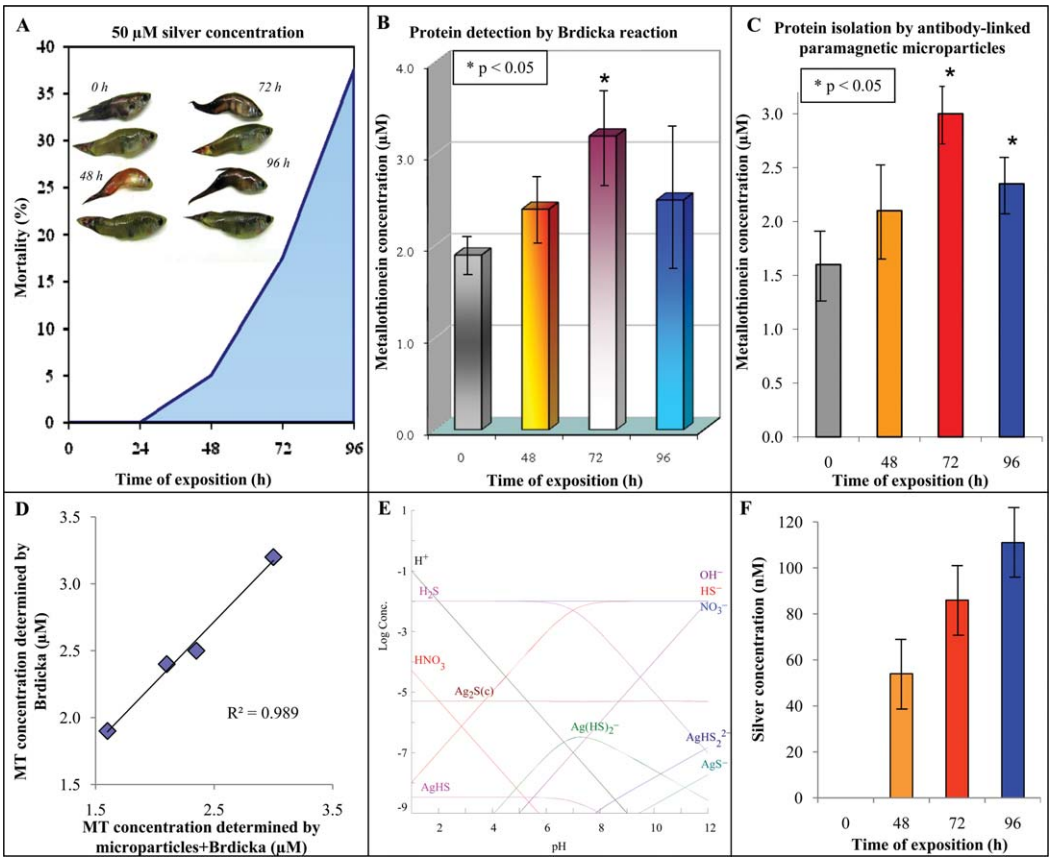

Fig. 4 Guppy fish (*Poecilia reticulata*) were exposed to silver nitrate (50 μM) for four days. (**A**) Mortality of the treated guppy fish. Content of MT in fish tissues: (**B**) standard sample processing and measuring by Brdicka reaction or (**C**) isolating by antibody-linked paramagnetic microparticles and measuring by Brdicka reaction. (**D**) Correlation of MT concentrations determined by antibody-modified microparticles coupled with differential pulse voltammetry Brdicka reaction (Fig. 4C) with the standard differential pulse voltammetry Brdicka reaction (Fig. 4B). (**E**) Distribution diagram of silver(I) ions made by MEDUSA, concentration of Ag(I) = 10 μM, SH^- = 10 mM and NO_3^- = 10 mM. (**F**) Concentration of silver(I) ions determined in tissues of fish treated with 50 μM Ag(I) for 96 h.

applied concentration of silver(I) was hundred times higher compared to those found in natural and contaminated environment in nineties,[2] but there is a threat with using of silver-based nanoparticles.[3] Therefore, some enhancement of silver(I) concentration in the environment can be expected. We found that the concentration of silver(I) ions in tissues increased with the increasing applied concentration of silver(I) ions (Fig. 4F). This fact correlated well with the increasing concentration of MT as the main detoxifier of such ions in an organism. However, a decrease in the amount of MT and an increase in the concentration of silver(I) ions in the last day can be associated with the high mortality of the fish (Fig. 4A).

3. Material and methods

3.1 Chemicals and material

Rabbit liver MT (MW 7143 g mol^{-1}), containing 5.9% Cd and 0.5% Zn, silver nitrate and all other reagents used were purchased from Sigma Aldrich (Sigma-Aldrich, USA) in ACS purity unless noted otherwise. Stock standard solutions were prepared with ACS water and stored in the dark at −20 °C. MT standards were reduced by 1 mM tris(2-carboxyethyl)phosphine, because reduced MT offers better repeatability and higher sensitivity for a determination in comparison with non-reduced ones.[44] Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through a 0.45 μm Nylon filter discs (MetaChem, Torrance, CA, USA). Paramagnetic microparticles were obtained from Dynabead (Invitrogene Corp., Carlsbad, USA).

Chicken antibodies were prepared by HENA, s. r. o., Prague, Czech Republic. Two hens were immunized by the commercially available metallothionein (1 mg of the mixture of horse MT 1 and MT 2, Sigma Aldrich, USA), which was diluted in the water and incubated for seven days at room temperature in order to polymerize. From the egg yolk the IgY fraction with reactivity to MT was obtained. The antibodies in phosphate buffered saline (PBS) were stabilized with 0.1% sodium azide. The protein concentration was 54.7 mg ml^{-1} in immunoglobulin fraction.

3.2 Electrochemical measurement

Electrochemical measurements were performed with a 747 VA Stand instrument connected to a 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was

the reference and glassy carbon electrode was auxiliary electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%), saturated with water for 120 s.

The Brdicka supporting electrolyte containing 1 mM Co $(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used and changed per one analysis. The DPV parameters were as follows: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude −250 mV, E_{ads} = open circuit. The temperature of the supporting electrolyte (4 °C) was maintained by the flow electrochemical cell coupled with a thermostat JULABO F12/ED (Labortechnik GmbH, Germany).

3.3 High performance liquid chromatography with electrochemical detection (HPLC-ED)

The system consisted of a solvent delivery pump operating in the range of 0.001–9.999 ml min^{-1} (Model 582 ESA Inc., Chelmsford, MA, USA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m) and/or a chromatographic column (Polaris C18-A, 4.6 mm, 5 μm particle size), and an electrochemical detector. The electrochemical detector (ED) includes one low volume flow-through analytical cell (Model 5040, ESA, USA), which consisted of a glassy carbon working electrode, a palladium electrode as the reference electrode and an auxiliary carbon electrode, and a Coulochem III as a control module. The sample (5 μl) was injected manually. The obtained data were treated by CSW 32 software. The experiments were carried out at room temperature. The guard cell potential was 0 V. 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.[25,42,43]

3.4 Automated pipetting systems

Fully automated isolation was carried out on an automated pipetting system epMotion 5075 (Eppendorf, Germany). There are 12 positions called by letters and figures such as A1, in which tips, waste or specials such as thermostated or magnetic devices can be placed. The position of B4 is a magnetic separator (Promega). The positions of C1 and C4 can be thermostated (Epthermoadapter PCR96). The pipetting provides a robotic arm with adapters (TS50, TS300, TS1000) and Gripper (TG-T). The samples are placed in the position B3 in an adapter Ep 0.5/1.5/2 ml. A module reservoir is located in the position B1, where washing solutions and waste are available. The device is controlled by the ep*Motion* control panel. The tips are located in the A4 (ePtips 50), A3 (ePtips 300) and A2 (ePtips 1000) positions. PCR 96 plates are used. The resulting volumes of collected samples ranged from 10 to 30 μl depending on the procedure.

3.5 Acute toxicity test on guppy fish

Twenty four specimens of guppy fish (*Poecilia reticulata*), 6 months old, were exposed to 50 μM silver nitrate. The experiment lasted 4 days (96 h, from 3rd to 7th May 2008). Two specimens were sampled per day. The experiment was done in triplicates. The experimental conditions such as pH value of the solution where the fish were kept constant, oxygen concentration and temperature were monitored during the experiment. The oxygen concentration varied within the range 1.7 to 4.0 mg l^{-1}, the pH level from 6.34 to 7.00, and the temperature from 20.2 to 21.5 °C during the 7 day long experiment. The sampled fish were killed by CO_2, washed one time with distilled water and one time

Table 1 Summary of chosen sequences[a]

					Domain	
Notation	Isoform	Organism	Length & cysteine	Metal ions	β	α
P55949	MT1	Callinectes sapidus Blue crab	59 [AA] 18 [Cys]	β 3 α 3	1-29	30–59
P29499	MT1	Homarus americanus American lobster	58 [AA] 18 [Cys]	β 3 α 3	1-28	29–58
P18055	MT2A	Oryctolagus cuniculus Rabbit	62 [AA] 20 [Cys]	β 3 α 4	1-30	31–62
P02795	MT2A	Homo sapiens Human	61 [AA] 20 [Cys]	β 3 α 4	1-29	30–61
P04355	MT2	Rattus norvegicus Rat	61 [AA] 20 [Cys]	β 3 α 4	1–29	30–61
P25713	MT3	Homo sapiens Human	68 [AA] 20 [Cys]	β n α 4	1-30	31–68
P28184	MT3	Mus musculus Mouse	68 [AA] 20 [Cys]	β n α 4	1-30	31–68
P62339	MT	Notothenia coriiceps neglecta Black rockcod	60 [AA] 19 [Cys]	β 3 α 4	1-28	29–60
P04734	MT	Strongylocentrotus purpuratus Purple sea urchin	64 [AA] 18 [Cys]	β 4 α 3	2-37	37–64
P07215	MT	Saccharomyces cerevisiae Baker's yeast	61 [AA] 11 [Cys]	7	9–61	
P30331	MT	Synechococcus elongatus	56 [AA] 9 [Cys]	4	2–56	

[a] AA = amino acids; MT = metallothionein;α, β = domains; Cys = cysteine.

with 0.5 M EDTA. All experiments were authorised by ethic commission of Veterinary and Pharmaceutical University in Brno, Czech Republic.

3.6 Sample preparations

The sampled fish (app. 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 a test-tube and vortexed for 15 min at 4 °C (Vortex Genie, USA). The supernatant was subsequently heat-treated. Briefly, the sample was kept at 99 °C in a thermomixer (Eppendorf Thermomixer Comfort, USA) for 15 min. with occasional stirring, and then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C, 15 000 g for 30 min. (Eppendorf 5402, USA). Heat treatment effectively denatures and removes high molecular weight proteins out from samples.[11,45]

3.7 Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significance of the differences between MT MT level quantified in control and silver(I) ions treated fish was determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using one way ANOVA test (particularly Scheffe test), which was applied for means comparison.

Equilibrium diagrams made using sophisticated algorithms (MEDUSA program) were used for the construction of a distribution diagram of different cadmium chemical forms present in the basic electrolyte. The basic parameters, including equilibrium constants that are necessary for the calculation of distribution diagrams are in the program database.[46,47] The program author is Ignasi Puigdomenech from the Inorganic Chemistry of Royal Institute of Technology, Stockholm, Sweden. The MEDUSA program is freeware and is available on http:\\www.kemi.kth.se/medusa.

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References

1 J. Zehnalek, V. Adam and R. Kizek, *Listy Cukrov. Reparske*, 2004, **120**, 222–224.
2 T. W. Purcell and J. J. Peters, *Environ. Toxicol. Chem.*, 1998, **17**, 539–546.
3 J. Fabrega, S. N. Luoma, C. R. Tyler, T. S. Galloway and J. R. Lead, *Environ. Int.*, 2011, **37**, 517–531.
4 T. Eckschlager, V. Adam, J. Hrabeta, K. Figova and R. Kizek, *Curr. Protein Pept. Sci.*, 2009, **10**, 360–375.
5 F. Hispard, D. Schuler, A. de Vaufleury, R. Scheifler, P. M. Badot and R. Dallinger, *Environ. Toxicol. Chem.*, 2008, **27**, 1533–1542.
6 A. Cravo, B. Lopes, A. Serafim, R. Company, L. Barreira, T. Gomes and M. J. Bebianno, *J. Environ. Monit.*, 2009, **11**, 1673–1686.
7 S. Masson, Y. Couillard, P. G. C. Campbell, C. Olsen, B. Pinel-Alloul and O. Perceval, *J. Environ. Monit.*, 2010, **12**, 143–158.
8 I. Fabrik, Z. Ruferova, K. Hilscherova, V. Adam, L. Trnkova and R. Kizek, *Sensors*, 2008, **8**, 4081–4094.
9 V. Adam, I. Fabrik, T. Eckschlager, M. Stiborova, L. Trnkova and R. Kizek, *TrAC, Trends Anal. Chem.*, 2010, **29**, 409–418.
10 M. Ryvolova, S. Krizkova, V. Adam, M. Beklova, L. Trnkova, J. Hubalek and R. Kizek, *Curr. Anal. Chem.*, 2011, **7**, 243–261.
11 J. Petrlova, D. Potesil, R. Mikelova, O. Blastik, V. Adam, L. Trnkova, F. Jelen, R. Prusa, J. Kukacka and R. Kizek, *Electrochim. Acta*, 2006, **51**, 5112–5119.
12 I. Fabrik, S. Krizkova, D. Huska, V. Adam, J. Hubalek, L. Trnkova, T. Eckschlager, J. Kukacka, R. Prusa and R. Kizek, *Electroanalysis*, 2008, **20**, 1521–1532.
13 S. Krizkova, P. Blahova, J. Nakielna, I. Fabrik, V. Adam, T. Eckschlager, M. Beklova, Z. Svobodova, V. Horak and R. Kizek, *Electroanalysis*, 2009, **21**, 2575–2583.
14 B. Raspor, M. Paic and M. Erk, *Talanta*, 2001, **55**, 109–115.
15 R. Kizek, L. Trnkova and E. Palecek, *Anal. Chem.*, 2001, **73**, 4801–4807.
16 V. Adam, J. Petrlova, J. Wang, T. Eckschlager, L. Trnkova and R. Kizek, *PLoS One*, 2010, **5**, e11441.
17 S. Krizkova, V. Adam, T. Eckschlager and R. Kizek, *Electrophoresis*, 2009, **30**, 3726–3735.
18 D. F. Akintola, B. Sampson and A. Fleck, *J. Lab. Clin. Med.*, 1995, **126**, 119–127.
19 Z. A. Shaikh, S. A. Jordan, T. Kido, W. F. Tang, W. A. Gross and E. Sabbioni, *Toxicol. Mech. Methods*, 1999, **9**, 47–56.
20 V. Adam, J. Baloun, I. Fabrik, L. Trnkova and R. Kizek, *Sensors*, 2008, **8**, 2293–2305.
21 V. Adam, M. Beklova, J. Pikula, J. Hubalek, L. Trnkova and R. Kizek, *Sensors*, 2007, **7**, 2419–2429.
22 V. Adam, O. Blastik, S. Krizkova, P. Lubal, J. Kukacka, R. Prusa and R. Kizek, *Chem. Listy*, 2008, **102**, 51–58.
23 D. Huska, S. Krizkova, M. Beklova, L. Havel, J. Zehnalek, V. Diopan, V. Adam, L. Zeman, P. Babula and R. Kizek, *Sensors*, 2008, **8**, 1039–1047.
24 A. Vasatkova, S. Krizova, O. Krystofova, V. Adam, L. Zeman, M. Beklova and R. Kizek, *Neuroendocrinol. Lett.*, 2009, **30**, 163–168.
25 K. Stejskal, S. Krizkova, V. Adam, B. Sures, L. Trnkova, J. Zehnalek, J. Hubalek, M. Beklova, P. Hanustiak, Z. Svobodova, A. Horna and R. Kizek, *IEEE Sens. J.*, 2008, **8**, 1578–1585.
26 S. Henikoff and J. G. Henikoff, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 10915–10919.
27 R. C. Edgar, *BMC Bioinformatics*, 2009, **10**, 11.
28 K. Takahashi and M. Nei, *Mol. Biol. Evol.*, 2000, **17**, 1251–1258.
29 O. Gascuel, *Mol. Biol. Evol.*, 1997, **14**, 685–695.
30 A. K. Gupta and M. Gupta, *Biomaterials*, 2005, **26**, 3995–4021.
31 P. Irato, G. Santovito, A. Cassini, E. Piccinni and V. Albergoni, *Arch. Environ. Contam. Toxicol.*, 2003, **44**, 476–484.
32 R. van der Oost, J. Beyer and N. P. E. Vermeulen, *Environ. Toxicol. Pharmacol.*, 2003, **13**, 57–149.
33 J. C. Amiard, C. Amiard-Triquet, S. Barka, J. Pellerin and P. S. Rainbow, *Aquat. Toxicol.*, 2006, **76**, 160–202.
34 J. W. Gorsuch and S. J. Klaine, *Environ. Toxicol. Chem.*, 1998, **17**, 537–538.
35 R. A. Bell and J. R. Kramer, *Environ. Toxicol. Chem.*, 1999, **18**, 9–22.
36 W. J. Berry, M. G. Cantwell, P. A. Edwards, J. R. Serbst and D. J. Hansen, *Environ. Toxicol. Chem.*, 1999, **18**, 40–48.
37 A. Bianchini and C. M. Wood, *Environ. Toxicol. Chem.*, 2003, **22**, 1361–1367.
38 D. J. Call, C. N. Polkinghorne, T. P. Markee, L. T. Brooke, D. L. Geiger, J. W. Gorsuch and K. A. Robillard, *Environ. Toxicol. Chem.*, 1999, **18**, 30–39.
39 C. Hogstrand, F. Galvez and C. M. Wood, *Environ. Toxicol. Chem.*, 1996, **15**, 1102–1108.
40 C. Hogstrand and C. M. Wood, *Environ. Toxicol. Chem.*, 1998, **17**, 547–561.
41 T. P. Morgan and C. M. Wood, *Environ. Toxicol. Chem.*, 2004, **23**, 1261–1267.
42 R. Mikelova, J. Baloun, J. Petrlova, V. Adam, L. Havel, J. Petrek, A. Horna and R. Kizek, *Bioelectrochemistry*, 2007, **70**, 508–518.
43 S. Krizkova, O. Krystofova, L. Trnkova, J. Hubalek, V. Adam, M. Beklova, A. Horna, L. Havel and R. Kizek, *Sensors*, 2009, **9**, 6934–6950.
44 V. Adam, J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, L. Trnkova, F. Jelen and R. Kizek, *Electroanalysis*, 2005, **17**, 1649–1657.
45 M. Erk, D. Ivankovic, B. Raspor and J. Pavicic, *Talanta*, 2002, **57**, 1211–1218.
46 I. Puigdomenech and U. Bergstrom, *Nucl. Saf.*, 1995, **36**, 142–154.
47 D. Jagner, E. Sahlin and L. Renman, *Talanta*, 1995, **42**, 1447–1455.