Full Paper

Determination of apo-Metallothionein Using Adsorptive Transfer Stripping Technique in Connection with Differential Pulse Voltammetry

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Abstract
Links between metallothionein (MT), its structure and many biologically important pathways demonstrate the necessity of taking into account of studying the behavior of MT in different well defined mediums, which could help to model conditions in an organism easily. The main aim of this work was to prepare and determine apoMT by adsorptive transfer stripping technique (AdTS) in connection with differential pulse voltammetry (DPV). Particularly, we investigated the electrochemical behavior of MT measured on the surface of hanging mercury drop electrode in the presence of sodium chloride as supporting electrolyte using AdTS DPV with respect to study the effects of MT signals by different concentrations and pH's of the electrolyte. Then, we aimed at utilizing this technique to observe changes in MT which are dependent on using strong chelating compounds ethylendiamine-*N*, *N*, *N*′, *N*′-tetraacetic acid (EDTA). Thanks to ability of EDTA to bind heavy metals from active center of enzymes and regulation proteins, we were able to prepare apoMT; that means, MT without any metal ion bound in its structure. Detection limit of apoMT at very short time of accumulation ($t_A = 120$ s) was 3 nM (20 ng/mL and/or 15 fmol in 5 μL drop; $RSD = 2-5\%$) estimated by dilution of the analyzed solution until the signal disappeared. In addition, it was possible to decrease detection limit by extending of time of accumulation of apoMT on the surface of HMDE. We were able to detect 30 pM of apoMT (200 pg/mL and/or 150 amol in 5 μL drop) at $t_A = 500$ s.

Keywords: Differential pulse voltammetry (DPV), Adsorptive transfer stripping (AdTS) technique, Hanging mercury drop electrode (HMDE), Metallothionein, Reaction of thiol group, Heavy metals biosensor, EDTA

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1. Introduction

Metallothioneins (MT) belongs to group of intracellular, high molecular and cysteine-rich proteins with molecular weight from 6 to 10 kDa [1–6]. The MT was discovered in 1957, when Margoshes and Valee isolated it from horse kidney [7]. MTs consist of two binding domains (α, β) that are assembled from cysteine clusters. Cysteine sulfhydryl groups participate in covalent bindings with heavy metals [8] (see Fig 1A). The *N*-terminal part of the protein is marked as α-domain, which has four binding places for divalent ions. β-Domain (C-terminal part) has the ability to bind three divalent ions of heavy metals. In the case of univalent ions of heavy metals, MT is able to bind twelve metal ions. A crystal structure of metallothionien binding cadmium and zinc was described in 1986 [9].

The assumed structure of MT has been well described by number of different analytical techniques. Nevertheless arrangement and placement of metals binding in MT has been still discussing intensively [10, 11]. Moreover, it seems that MT could have more crucial biological properties than regulation of content of toxic metals in an organism [11–15]. Particularly, very interesting links between MT concentration and cell proliferation or fetus development have been described. For that reasons a question was raised: "How can be level of MT regulate in an organism, and thus, how can be MT degrade?" A few papers describing regulation of MT gene expression have been published, but not of all mechanisms are clear yet [16].

On the other hand about "fate" of metals bound to MT molecule is not clear yet. Several models and hypotheses showing their active transport to excretory system (kidney) have been suggested, but, unfortunately, what happen with

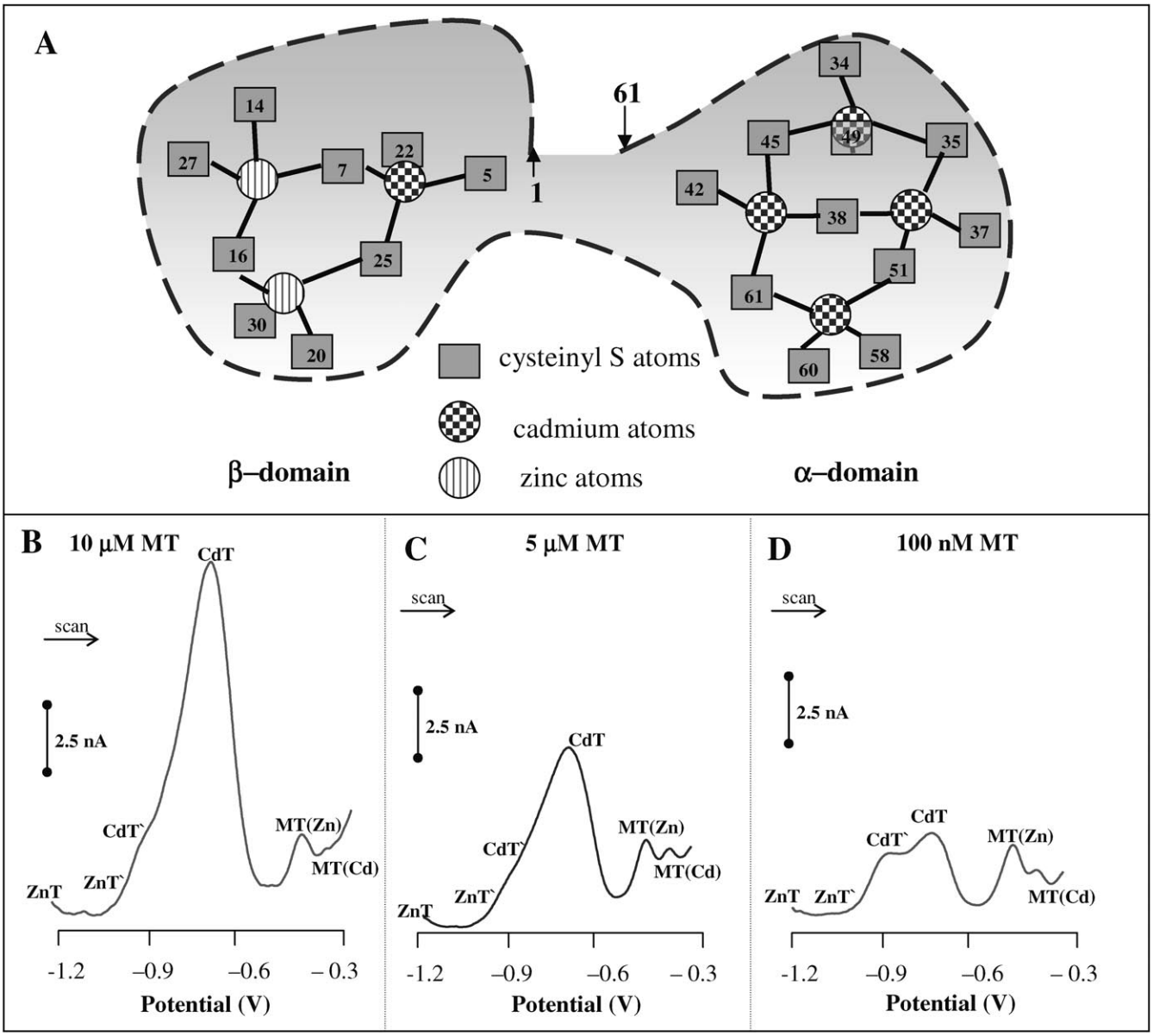

Fig. 1. Model of structure of rabbit liver metallothionein (A). The metallothionein with five bound atoms of cadmium and two of zinc is shown. Adsorptive transfer stripping technique in connection with differential pulse voltammetry. Typical DP voltammograms of 10 μM MT (B), 5 μM MT (C), 0.1 μM MT (D) measured in the presence of 0.5 M NaCl, pH 6.4. AdTS DPV parameters were as follows: initial potential – 1.2 V, end potential – 0.3 V, modulation time 0.057 s, time interval 0.2 s, step potential of 1.05 mV/s, modulation amplitude of 250 mV, time of accumulation of MT 120 s, $E_{ads} = 0$ V. For other details see Section 2.

MT in kidney has not been described sufficiently. There could proceed a number of interactions between heavy metals, reactive oxygen species and kidney cells [17, 18] including releasing of heavy metal ions to urine, which take place in glomerulus cells. The ions release thanks to decrease of pH. This mechanism is well known and used for preparation of MT without any metal (apoMT).

Facts mentioned above demonstrate the necessity of taking into account studying of behavior of MT in different well defined medium, which could help to model conditions in an organism easily [19]. Electrochemical technique seems to be very promising tool for these purposes thanks to high sensitivity and selectivity to thiols. A number of published papers dealing with study of thiols (e.g., rabbit MT-I, MT-II , horse MT, human MT and peptide Lys-Cys-Thr-Cys-Cys-Ala thionein fragment MT I) by electrochemical techniques in the presence of various supporting electrolytes and pHs using different working electrodes has been published [20 – 39]. Moreover, catalytic signals (peak H and Brdicka reaction) are of great importance for sensitive analysis of MT in biological samples [40]. It is supposable that a SH-groups-rich protein and ions such as Co, Ni and/or cisplatin serve as catalysts of these signals [40 – 52]. It follows from these experimentally results that a supporting electrolyte (its chemical composition and concentration of single compounds consisted from, ionic strength and pH) probably

influence electrochemical signal of MT [23]. The changes observed relates with different MT structure including releasing of bound metal ions in the presence of the supporting electrolyte with low pH [23]. Published papers and results obtained describing electrochemical determination of MT, from 1980 to June of 2006, according to Web of Science, are summarized in Table 1.

The main aim of this work was to prepare and determine apoMT by adsorptive transfer stripping technique (AdTS) in connection with differential pulse voltammetry (DPV). Particularly, we investigated the electrochemical behavior of MT measured on the surface of hanging mercury drop electrode in the presence of sodium chloride as supporting electrolyte using AdTS DPV. Then, we aimed on utilizing of this technique to observe changes of MT in dependence on different pH and concentration of supporting electrolyte, and on using of strong chelating compounds ethylendiamine-*N*, *N*, *N*′, *N*′-tetraacetic acid (EDTA) with respect to prepare apoMT.

2. Materials and Methods

2.1. Chemicals

Rabbit liver MT (MW 7143), containing 5.9% Cd and 0.5% Zn, was purchased from Sigma Aldrich (St. Louis, USA). Sodium chloride, ethylendiamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) and other chemicals used were purchased from Sigma Aldrich. Stock standard solutions of MT (10 μg mL^{-1}) were prepared by ACS water (Sigma-Aldrich, USA) and stored in the dark at the temperature of $-20\,^{\circ}C$. Working standard solutions were prepared daily by dilution of the stock solutions. The pH of prepared solution was measured using WTW inoLab Level 3 (Weilheim, Germany). The pH-electrode (SenTix-H, pH 0 – 14/3 M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2. Spectrophotometric Measurements

Spectra were recorded by means of UV-viz diode array detector (HP-Packard) in the range of 190 – 400 nm.

Table 1. Summary of electroanalytical determination of metallothionein from 1980 to June 2006.

Metallothionein	Electrochemical method	Supporting electrolyte	References
Rabbit liver (MT-I, MT-II, MT-I + MT-II, horse kidney peptidic fragment Lys-Cys-Thr-Cys-Cys-Ala thionein human MT (MT-I, MT-II)	DPP	0.01 M phosphate buffer pH 7.5 pH (2 – 12) adjusted by NaOH, HCl	[21, 22, 58, 66, 67]
Human MT rabbit liver MT peptide Lys-Cys-Thr-Cys-Cys-Ala thionein fragment MT I	DPP	0.02 and 0.01 M phosphate HEPES buffer pH (6 – 9) adjusted by NaOH, HCl	[23, 60, 61, 68]
Rabbit liver MT	DPP-Brdicka reaction	0.6 and/or 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq) + NH_4Cl$, pH 9.6)	[40 – 43, 69 – 71]
Rabbit liver MT	DPV	borate buffer pH 8.5	[25]
		borate buffer (pH 9.1) + 0.1 M $NaClO_4$ + 10% vol CH_3OH	[72]
Rabbit liver MT	AdTS DPV	0.5 M NaCl, pH 6.4	[34]
Peptide Lys-Cys-Thr-Cys-Cys-Ala thionein fragment Rabbit liver MT	SWV	0.02 M phosphate HEPES buffer pH (6 – 9) adjusted by NaOH, HCl	[60, 61, 68, 73]
Rabbit liver MT	SVW-modified Brdicka reaction	0.5 M borate buffer pH 9.2 + 10 μM cisplatin	[52, 74]
Rabbit liver MT	CV	borate buffer pH 8.5	[25]
Peptidic fragment Lys-Cys-Thr-Cys-Cys-Ala MT I, rabbit liver MT	CV	0.1 and/or 0.01 M phosphate HEPES buffer pH 8	[75, 76]
apoMT	CV	Tris-buffer pH 8.5	[72]
Rabbit liver MT	CPSA	borate buffer pH 8.5	[25]
Rabbit liver MT	CPSA	0.4 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq) + NH_4Cl$, pH 9.6)	[77]
Rabbit liver MT	AdTS CPSA	0.1 M H_3BO_3 + 0.05 M $Na_2B_4O_7$, pH 8.0	[29, 78, 79]
Rabbit liver MT	EVLS	borate buffer pH 8.5	[25]

According to ISI Web of Knowledge

Table 2. Recovery of preparation of apoMT by means the interacting of EDTA with MT.

Concentration of MT (nM)	Concentration of EDTA (nM)	Height of CdT peak (nA)	Maximal CdT peak height [a] (nA)	Recovery (%)
100	0	10.0	17.0	58.7
100	100	10.8	17.0	61.0
100	200	10.5	17.0	61.6
100	300	10.9	17.0	63.9
100	400	11.2	17.0	65.7
100	500	12.0	17.0	70.4
100	550	13.7	17.0	80.3
100	600	16.9	17.0	99.1
100	650	17.0	17.0	100
100	700	17.0	17.0	100
100	740	17.0	17.0	100
100	770	17.0	17.0	100
100	800	17.0	17.0	100

[a] Peak height of CdT measured at the highest dose of EDTA (800 nM).

2.3. Electrochemical Measurements

Electrochemical measurements were performed with the AUTOLAB Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode (HMDE) with the drop area of 0.4 mm^2. The reference electrode was the Ag/AgCl/3 M KCl electrode and the auxiliary electrode was the graphite electrode (GE). The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%), saturated with water for 120 s. All experiments were carried out at room temperature. For smoothing and baseline correction [53], the software GPES 4.4 supplied by EcoChemie was employed.

2.4. Adsorptive Transfer Stripping (AdTS) Differential Pulse Voltammetry (DPV)

MT has been analyzed using AdTS DPV (principles of the transfer technique was described by Palecek [54]). The supporting electrolyte (sodium chloride: 0.5 M NaCl, pH 6.4) from Sigma Aldrich in ACS purity was purchased. DPV parameters were as follows: the initial potential of -1.2 V, the end potential -0.3 V, the modulation time 0.057 s, the interval 0.2 s, the step potential of 1.05 mV/s, the modulation amplitude of 25 mV.

3. Results and Discussion

3.1. Electrochemical Behavior of Metallothionein

Primarily, we attempted to characterize metallothionein used electrochemically (rabbit liver, M_r 7065, $MTCd_5Zn_2$). We picked up the threads of our previous works and analyzed MT in the presence of 0.5 M NaCl (pH 6.4) [34, 55–57]. Our experiments are based on original methods using adsorptive transfer technique (MT is adsorbed on the surface of HMDE from low volume of samples – 5 μL drop). Typical well reproducible AdTS DP voltammograms of rabbit liver MT (100 nM, 5 and 10 μM) measured in the presence of 0.5 M NaCl (pH 6.4) at t_A of 120 s is shown in Figs. 1B, C, D, respectively. We observed all assumed electrochemical signals of MT complexes with Cd(II) and Zn(II) – MT(Cd): -0.42 V; MT(Zn): -0.49 V, ZnT′: -0.87 V, CdT: -0.65 V, CdT′: -0.71 V and ZnT: -0.99 V (more detailed description of the signals are shown in following papers [23, 34, 56, 58]). In addition, we can see strong influence of MT concentration on the signals (Figs. 1B, C, D), more will be published elsewhere. The great advantage of the adsorptive technique is the possibility to study behavior of MT adsorbed on a surface of working electrode without interferences both from samples and supporting electrolyte, where the analysis last only few minutes. Thanks to this technique we are able to observe the changes of electrochemical signals caused, first of all, by experimental conditions.

3.2. Influence of a Supporting Electrolyte and its pH on Electrochemical Signal of MT

As it is shown in Table 1, a supporting electrolyte and its pH influence markedly the electrochemical records of MT. A few authors shown in Table 1 have used sodium chloride (0.5 M, pH 6.4) as a supporting electrolyte that has similar pH and ionic strength as physiological environment [34, 55, 56]. We again picked up the threads of a previous work and observed influence of changes of supporting electrolyte pH (0.5 M NaCl) within the range from 6.0 to 7.6 (adjusted by additions of HCl and/or NaOH) on electrochemical signal of MT. We found out that signals called as MT(Cd) and MT(Zn) decreased with increasing pH, whereas at the highest tested pH values they were poorly detectable (Fig. 2A). When we work on an assumption that MT(Cd) and MT(Zn) signals are associated with reducing of metal

ions bound in MT, afterwards it clearly follows from the results obtained that MT molecule is going to be "packed" with increasing pH. This cause that metal ions can not be electrochemically changed on the surface of working electrode. On the other hand, if we use a lower pH, MT molecule is slightly "unpacking" its structure, which means that sulfhydryl moieties and metal ions bound in MT are more accessible to the surface of working electrode. Moreover, marked decrease of CdT and ZnT signals with increasing pH affirm this hypothesis (Fig. 2A). Thus, the decrease probably relates with worse accessibility of SH groups to HMDE surface. Besides that behaviour of ZnT′ and CdT′ signals is very interesting, because they increase with increasing pH, which shows that they could correspond to aminoacids or metal ions, which are not contained directly in the MT clusters (Fig. 1A). That means, if the structure of MT is really going to be "packed", other metal ions have to be bound in the outer part of the clusters. Thus, they are more accessible to the surface of HMDE. The increase in heights of ZnT' and ZnT signals have been shown during polarographic analysis of rabbit liver MT-2 [59], but the authors done their experiments directly in electrochemical cell, and then MT analysis could be influenced by metals contained in the supporting electrolyte. In spite of the differences between transfer technique and analysis in the cell, similar dependences of MT signals have been obtained [60, 61]. The exact explanations about mechanisms influencing of MT are rather difficult. Conclusions would be suitable to support by physiological experiments, when lab animals would be feed by cadmium(II) and zinc(II) ions with consequently kidney sampling, where content of MT would be analyzed. It can be expected that the whole process including binding and releasing of metal ions from MT molecule will be based not only on the changing and reorganizing of cluster structure but also on other non-described mechanisms.

3.3. Influence of NaCl Concentration on MT Signal

As we studied the influence of pH of NaCl on MT signals, we attempted to investigate the influence of different concentrations of NaCl within the range from 0.05 to 0.5 M under constant pH 6.4 adjusting by additions of HCl and/or NaOH. We found out that MT signals were influenced by different concentrations of NaCl but not so much as by different pHs. MT(Cd) and MT(Zn) signals changed according to different ionic strength markedly, whereas MT(Zn) signal measured in the presence 0.5 M NaCl was very low in comparison with 0.4 M NaCl. Contrariwise, CdT and ZnT signals did not change with increasing ionic strength of the supporting electrolyte. In addition the ZnT' and CdT' signals changed evidently than CdT and ZnT signals (Fig. 2B). As we discussed above, the results could be associated with structural changes of MT structure and with interaction of the structures formed with the supporting electrolyte [62]. It is likely that MT structure strongly depends on the electrolyte used, thus, if it will be used other electrolyte, the results will differ.

3.4. Influence of EDTA on AdTS DPV Signals of Rabbit Liver Metallothionein

Here, we aimed on investigation of structural changes of MT causing by affecting of strong chelating compounds. These investigations are strongly needed for following experiments, which enable to study of important regulation processes such interaction between transcription factors and a metal [63]. One of the most commonly used chelating compounds for biological purposes is ethylenediamine-*N*, *N*, *N*′, *N*′-tetraacetic acid (EDTA). Surprisingly, this compound is able to bind heavy metals from active center of enzymes and regulation proteins. The experiment has been suggested

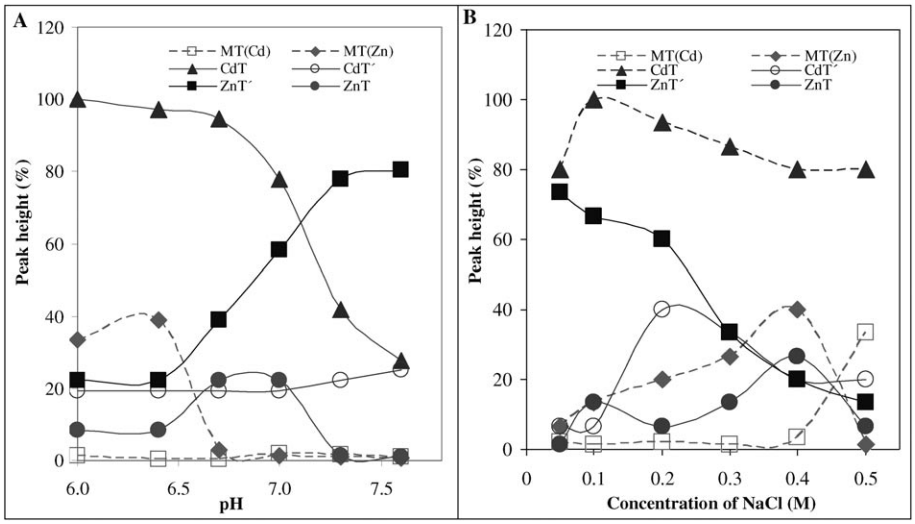

Fig. 2. Dependences of heights of ZnT, ZnT′, CdT′, CdT, MT(Zn), and MT(Cd) signals on pH (6.0 – 7.8) (A) and on concentration of the supporting electrolyte (0.05 – 0.5 M) (B). pH was adjusted by additions of NaOH and/or HCl. Other experimental conditions are the same as in Figure 1.

as follows: low concentration of MT (100 nM) was added to microtest-tube (20 μL) followed by addition of EDTA. This solution has been shaken slightly for 2 min. Aliquot (5 μL) has been analyzed by AdTS DPV. The experiment was repeated with different concentrations of EDTA. MT was adsorbed ($t_A = 120$ s) on the surface of HMDE, whereas EDTA and heavy metals were washed from the surface (Fig. 3A). All of the typical DPV signals of rabbit liver MT were observed: MT(Zn), MT(Cd), CdT, ZnT, ZnT' and CdT′. We found out that the signals observed except CdT decreased with increasing EDTA concentration up to 600 nM, then did not change much (Fig. 3B). On the other hand CdT signal markedly increased with increasing concentration of EDTA up to 600 nM. It could be suggested that interaction between EDTA and MT proceeds as follows: i) $MTCd_5Zn_2 + EDTA =$ cluster collapse M–S-protein (M-metal; T-thionein) ii) M–S-protein $= M^+ S^-$ protein $= M^+ +$ S-protein and iii) $M^+ + EDTA^- = MEDTA$ [64]. Structure of MT clusters has been probably damaging markedly from 400 nM EDTA, which was confirmed by the following experiments. If we studied the change of CdT signal in the presence of 400 nM according to different times of interactions (from 30 s to 25 min.), we observed that CdT signal did not change much within the range of experimental deviation (about 10%). Thanks to ability of EDTA unbound all metal ions from the MT structure; we assumed that apoMT can be obtained by addition of 600 nM EDTA (Fig. 3B). Comparison between AdTS DP voltammograms of 100 nM rabbit liver MT without and with addition of 600 nM EDTA are shown in Figure 3C. It clearly follows from the results obtained that MT without addition of EDTA gave all typical expected signals (see in Fig 1B),

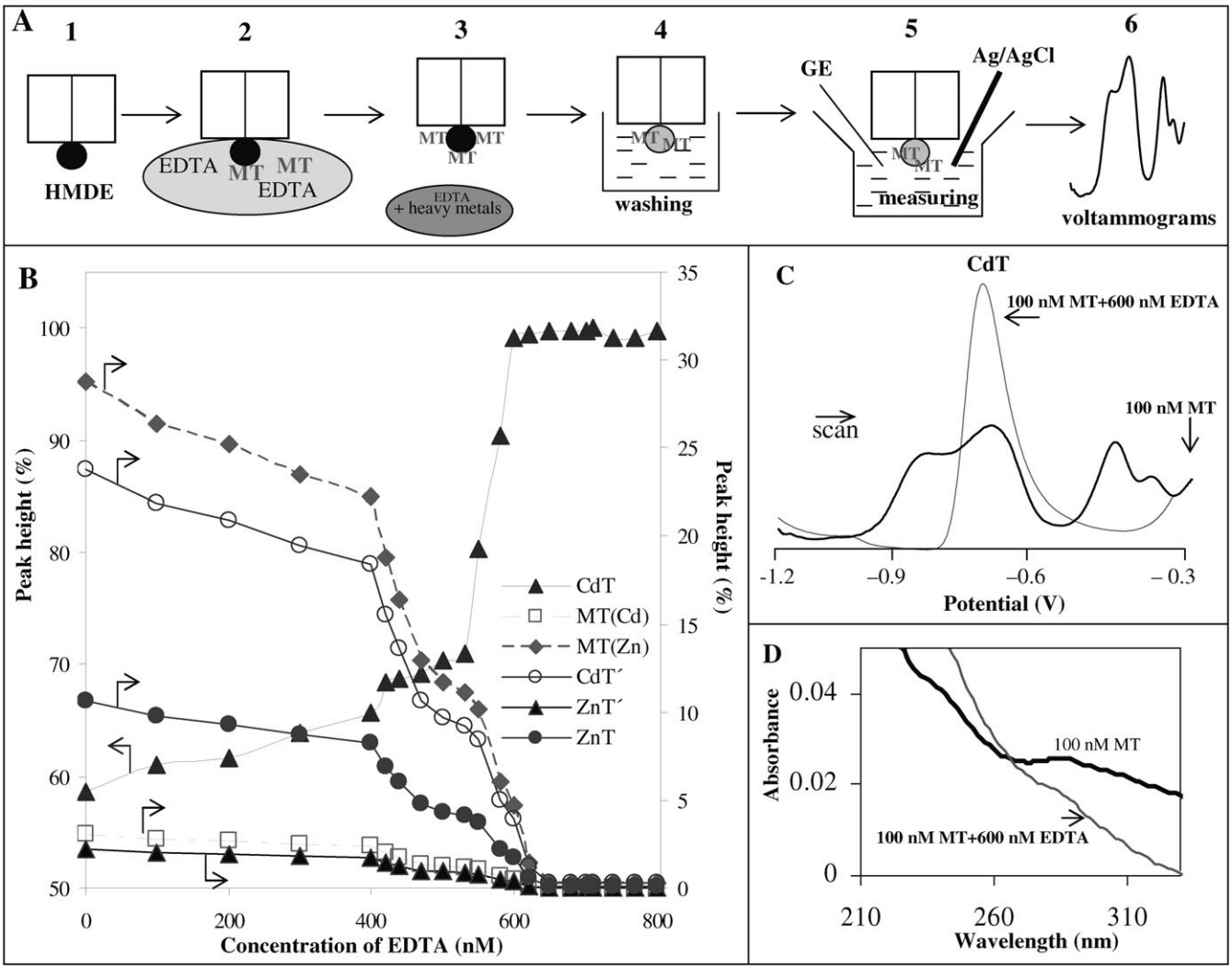

Fig. 3. Scheme of using of adsorptive transfer stripping technique for study of changes of MT in the presence of EDTA (A). Renewed surface of HMDE (1) is placed to drop containing rabbit liver MT and EDTA (2); MT binds on the surface of HMDE only (3); low molecular compounds such as heavy metals and EDTA are washed out in the following step (4); the modified HMDE electrode is placed to supporting electrolyte (5) and analyzed by DPV (6). Dependence of heights of ZnT, ZnT′, CdT′, CdT, MT(Zn), and MT(Cd) signals on different concentrations of EDTA (B); the heights are expressed as ration to highest signal, whereas heights of CdT signals are shown in the left axis, other ones in the right axis. DP voltammograms (C) and UV-vis spectra (D) of 100 nM rabbit liver MT (bold line) and 100 nM MT + 600 nM EDTA (thin line). Other experimental conditions are the same as in Figure 1 and Section 2.

whereas MT with EDTA gave only two signals: CdT that corresponds to free SH groups in MT clusters) and peak at potential of −1 V that corresponds to Zn from the supporting electrolyte (Fig. 3C). Moreover, the same solutions were analyzed using UV-VIS spectrophotometer. The changes observed are shown in Figure 3D. It follows from the results obtained that EDTA can really unbound metal ions from MT, which means that apoMT is presented in this solution. This procedure could be an useful alternative tool for simple, rapid and undemanding preparation of apoMT to different purposes such as a suggestion of heavy metal biosensor [65].

3.5. Electroanalytical Determination of apoMT

As we mentioned above, we were able to prepare apoMT which has been characterized consequently by AdTS DPV. We found out that apoMT gave CdT signal at potential of -0.70 ± 0.01 V ($n = 5$). Therefore, we utilized this signal to study of recovery of the preparation of apoMT by means of EDTA interacting with rabbit liver MT. We found out that this signal increased with increasing concentration of EDTA up to 600 nM and then did not change (Table 1). We assumed that we prepared apoMT, which was confirmed by spectrophotometric measurements (Fig. 3D) and by results published in [64]. Based on the obtained results we used ratio of (rabbit liver MT)/EDTA as 1/6 to prepare apoMT in the following experiments.

After the optimizing of apoMT preparation step, the changes in its signal with its changing concentration have been investigated. This signals slightly shifted to more positive potential with decreasing concentration of apoMT, whereas the signal appeared at potential of −0.585 V at 2 nM apoMT. In addition, we observed dependence of CdT signal on concentration of apoMT. Due to sustaining of constant experimental conditions, apoMT was diluted by 0.5 M NaCl (pH 6.4). We obtained the strictly linear dependence with following equation ($y = 0.1935x + 0.1926$; $R^2 = 0.9986$) within the whole range of concentrations used (Fig. 4A). If we analyzed concentration of apoMT from 0.15 to 12 nM, we again obtained the strictly linear dependence ($y = 0.2053x + 0.1052$; $R^2 = 0.9931$; Fig. 4B). The CdT signals were well developed (Fig 4B). Detection limit of apoMT at very short time of accumulation ($t_A = 120$ s) was 3 nM (20 ng/mL and/or 15 fmol in 5 μL drop; $RSD = 2–5\%$) estimated by dilution of the analyzed solution until the signal disappeared. In addition, it was possible to decrease detection limit by extending of time of accumulation of apoMT on the surface of HMDE. We were able to detect 30 pM of apoMT (200 pg/mL and/or 150 amol in 5 μL drop) at t_A 500 s (Fig. 4C). In addition the limit quantification of apoMT was 100 pM at t_A 500 s. Moreover, apoMT has been successfully used for suggestion of EDTA MT biosensor for determination of heavy metals [65]. We found out that the suggested biosensor had similar behavior as MT and/or PC biosensor, whereas the biological part of EDTA MT biosensor enabled to reach lower detection limits about 10–30% [34, 55].

4. Conclusions

Utilizing of electrochemical biosensors using heavy metal binding peptides and proteins as a biological part belongs to new tools for sensitive analysis of heavy metals. We can use EDTA for unbinding of metals naturally occurs in the structure of these peptides and proteins, whereas consequently formed compounds without any metal embody better properties than the ones with heavy metals.

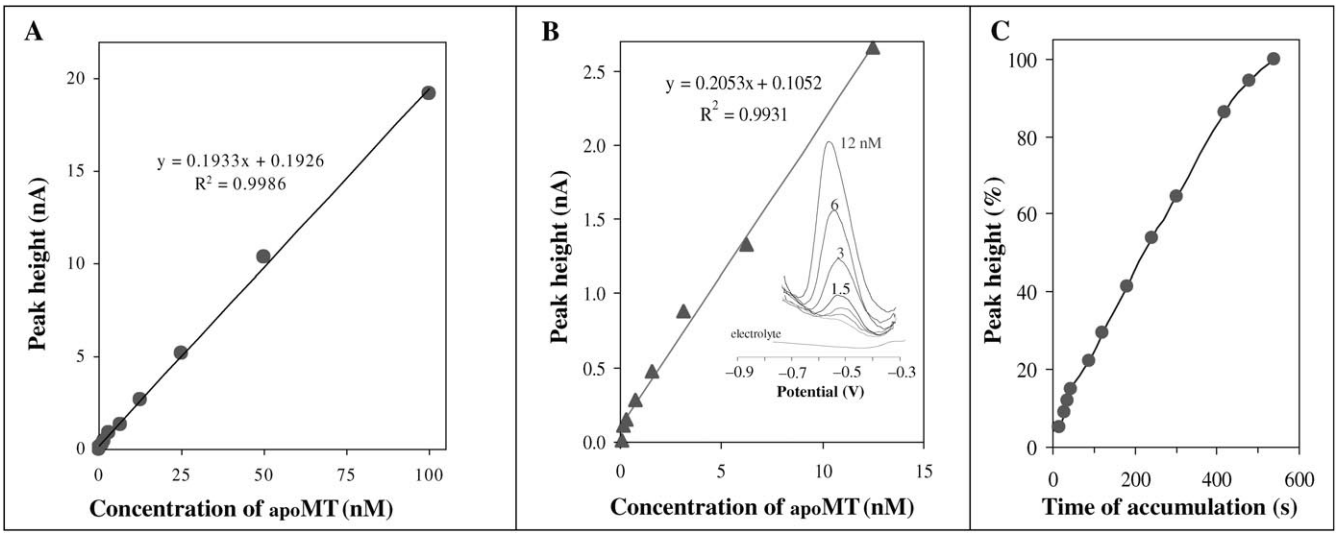

Fig. 4. Electroanalytical determination of prepared apoMT. Dependence of heights of AdTS DPV signals of rabbit liver MT on its concentration within the range of 0.075–100 nM (A) and 0.015–12 nM (B); inset: typical CdT signals measured at (0.188, 0.375, 0.75, 1.5, 3, 6, and 12 nM). Dependence of CdT peak height (5 nM of MT) on time of accumulation (C). Other experimental conditions are the same as in Figure 1 and Section 2.

5. Acknowledgements

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6. References

[1] J. H. R. Kagi, A. Schaffer, *Biochemistry* **1988**, *27*, 8509.
[2] M. Vasak, *J. Trace Elem. Med. Biol.* **2005**, *19*, 13.
[3] N. Romero-Isart, M. Vasak, *J. Inorg. Biochem.* **2002**, *88*, 388.
[4] G. Nordberg, T. Jin, P. Leffler, M. Svensson, T. Zhou, M. Nordberg, *Analusis* **2000**, *28*, 396.
[5] M. Nordberg, G. F. Nordberg, *Cell. Mol. Biol.* **2000**, *46*, 451.
[6] M. Nordberg, *Talanta* **1998**, *46*, 243.
[7] M. Margoshes, B. L. A. Vallee, *J. Am. Chem. Soc.* **1957**, *79*, 4813.
[8] M. Studnickova, J. Turanek, H. Zabrsova, M. Krejci, M. Kysel, *J. Electroanal. Chem.* **1997**, *421*, 25.
[9] W. F. Furey, A. H. Robbins, L. L. Clancy, D. R. Winge, B. C. Wang, C. D. Stout, *Science* **1986**, *231*, 704.
[10] W. Troger, B. Ctortecka, P. Faller, M. Vasak, *J. Inorg. Biochem.* **2001**, *86*, 460.
[11] G. I. Giles, N. M. Giles, C. A. Collins, K. Holt, F. H. Fry, P. A. S. Lowden, N. J. Gutowski, C. Jacob, *Chem. Commun.* **2003**, 2030.
[12] M. Roch, J. A. McCarter, A. T. Matheson, M. J. R. Clark, R. W. Olafson, *Canad. J. Fisher. Aquatic Sci.* **1982**, *39*, 1596.
[13] J. D. Otvos, R. W. Olafson, I. M. Armitage, *J. Biol. Chem.* **1982**, *257*, 2427.
[14] R. W. Olafson, *Int. J. Peptide Protein Res.* **1984**, *24*, 303.
[15] F. Brady, *Trends Biochem. Sci.* **1982**, *7*, 143.
[16] R. D. Palmiter, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 1219.
[17] M. C. M. Mateo, C. Herreros, R. Melero, J. Bustamante, *Renal Failure* **2003**, *25*, 719.
[18] G. Hellemans, A. Soumillion, P. Proost, J. Van Damme, H. Van Poppel, L. Baert, M. De Ley, *Nephron*. **1999**, *83*, 331.
[19] J. Kukacka, J. Petrlova, R. Prusa, V. Adam, B. Sures, M. Beklova, L. Havel, R. Kizek, *FASEB J.* **2006**, *20*, A75.
[20] A. R. Rodriguez, M. Esteban, *Cell. Mol.Biol.* **2000**, *46*, 237.
[21] A. Munoz, A. R. Rodriguez, *Analyst* **1995**, *120*, 529.
[22] C. Ruiz, J. Mendieta, A. R. Rodriguez, *Anal. Chim. Acta* **1995**, *305*, 285.
[23] C. Ruiz, A. R. Rodriguez, *Anal. Chim. Acta* **1997**, *350*, 305.
[24] M. Dabrio, A. R. Rodriguez, G. Bordin, M. J. Bebianno, M. De Ley, I. Sestakova, M. Vasak, M. Nordberg, *J. Inorg. Biochem.* **2002**, *88*, 123.
[25] N. Serrano, I. Sestakova, J. M. Diaz-Cruz, *Electroanalysis* **2006**, *18*, 169.
[26] I. Sestakova, T. Navratil, *Bioinorg. Chem. Appl.* **2005**, *3*, 43.
[27] H. Vodickova, V. Pacakova, I. Sestakova, P. Mader, *Chem. Listy* **2001**, *95*, 477.
[28] R. Kizek, J. Vacek, L. Trnkova, B. Klejdus, L. Havel, *Chem. Listy* **2004**, *98*, 166.
[29] R. Kizek, L. Trnkova, E. Palecek, *Anal. Chem.* **2001**, *73*, 4801.
[30] M. J. Lopez, C. Arino, S. Diaz-Cruz, J. M. Diaz-Cruz, R. Tauler, M. Esteban, *Environm. Sci. Technol.* **2003**, *37*, 5609.
[31] M. Esteban, C. Arino, J. M. Diaz-Cruz, *Trac-Trends Anal. Chem.* **2006**, *25*, 86.
[32] M. S. Diaz-Cruz, J. M. Diaz-Cruz, M. Esteban, *Electroanalysis* **2002**, *14*, 899.
[33] M. S. Diaz-Cruz, M. J. Lopez, J. M. Diaz-Cruz, M. Esteban, *J. Electroanal. Chem.* **2002**, *523*, 114.
[34] V. Adam, J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, L. Trnkova, F. Jelen, R. Kizek, *Electroanalysis* **2005**, *17*, 1649.
[35] I. Sestakova, P. Mader, *Cell. Mol. Biol.* **2000**, *46*, 257.
[36] I. Sestakova, D. Miholova, H. Vodickova, P. Mader, *Electroanalysis* **1995**, *7*, 237.
[37] R. W. Olafson, *Bioelectrochem. Bioenerg.* **1988**, *19*, 111.
[38] J. Szpunar, *Analyst* **2005**, *130*, 442.
[39] J. G. Lan, D. B. Luo, Y. L. Feng, *Microchim. Acta* **2004**, *148*, 299.
[40] J. Petrlova, D. Potesil, R. Mikelova, O. Blastik, V. Adam, L. Trnkova, F. Jelen, R. Prusa, J. Kukacka, R. Kizek, *Electrochim. Acta* **2006**, *51*, 5112.
[41] R. W. Olafson, P. E. Olsson, *Meth. Enzymol.* **1991**, *205*, 205.
[42] R. W. Olafson, R. G. Sim, *Anal. Biochem.* **1979**, *100*, 343.
[43] B. Raspor, M. Paic, M. Erk, *Talanta* **2001**, *55*, 109.
[44] M. Erk, B. Raspor, *Cell. Mol. Biol.* **2000**, *46*, 269.
[45] B. Raspor, S. Kozar, J. Pavicic, D. Juric, *Fresenius J. Anal. Chem.* **1998**, *361*, 197.
[46] B. Raspor, J. Pavicic, *Croatica Chem. Acta* **1997**, *70*, 247.
[47] B. Raspor, J. Pavicic, *Fresenius J. Anal. Chem.* **1996**, *354*, 529.
[48] J. Pavicic, B. Raspor, D. Martincic, *Marine Biol.* **1993**, *115*, 435.
[49] F. G. Banica, A. Ion, *Coll. Czech. Chem. Comm.* **1998**, *63*, 995.
[50] A. Ion, F. G. Banica, C. Luca, *Coll. Czech. Chem. Commun.* **1998**, *63*, 187.
[51] F. G. Banica, A. G. Fogg, A. Ion, J. C. Moreira, *Anal. Lett.* **1996**, *29*, 1415.
[52] M. El Hourch, A. Dudoit, J. C. Amiard, *Anal. Bioanal. Chem.* **2004**, *378*, 776.
[53] J. Vacek, Z. Andrysik, L. Trnkova, R. Kizek, *Electroanalysis* **2004**, *16*, 224.
[54] E. Palecek, I. Postbieglova, *J. Electroanal. Chem.* **1986**, *214*, 359.
[55] V. Adam, J. Zehnalek, J. Petrlova, D. Potesil, B. Sures, L. Trnkova, F. Jelen, J. Vitecek, R. Kizek, *Sensors* **2005**, *5*, 70.
[56] V. Adam, J. Petrlova, D. Potesil, P. Lubal, J. Zehnalek, B. Sures, R. Kizek, *Chem. Listy* **2005**, *99*, 353.
[57] J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, V. Adam, L. Trnkova, R. Kizek, *Electrochim. Acta* **2006**, *51*, 5169.
[58] O. Nieto, G. Hellemans, G. Bordin, M. De Ley, A. R. Rodriguez, *Talanta*. **1998**, *46*, 315.
[59] M. Dabrio, A. R. Rodriguez, *Electroanalysis* **2000**, *12*, 1026.
[60] M. Dabrio, A. R. Rodriguez, *Anal. Chim. Acta* **1999**, *385*, 295.
[61] M. Dabrio, A. R. Rodriguez, *Anal. Chim. Acta* **2000**, *406*, 171.
[62] M. Erk, B. Raspor, *Anal. Chim. Acta* **2001**, *442*, 165.
[63] M. Huang, C. F. Shaw, D. H. Petering, *J. Inorg. Biochem.* **2004**, *98*, 639.
[64] S. Yue, W. Q. Zhong, B. L. Zhang, L. Y. Zhu, W. X. Tang, *J. Inorg. Biochemistry* **1996**, *62*, 243.
[65] S. Krizkova, V. Adam, J. Petrlova, O. Zitka, K. Stejskal, J. Zehnalek, B. Sures, L. Trnkova, M. Beklova, R. Kizek, *Electroanalysis* **2007**, *19*, 331.
[66] A. Munoz, A. R. Rodriguez, *Electroanalysis* **1995**, *7*, 674.
[67] C. Ruiz, A. R. Rodriguez, *Anal. Chim. Acta* **1996**, *325*, 43.
[68] O. Nieto, A. R. Rodriguez, *Electroanalysis* **1999**, *11*, 175.
[69] M. Erk, B. Raspor, *Anal. Chim. Acta* **1998**, *360*, 189.
[70] D. B. Luo, J. G. Lan, C. Zhou, C. X. Luo, *Anal. Chem.* **2003**, *75*, 6346.
[71] J. A. J. Thompson, R. P. Cosson, *Marine Environ. Res.* **1984**, *11*, 137.
[72] M. Fedurco, I. Sestakova, *Bioelectrochem. Bioenerg.* **1996**, *40*, 223.

[73] O. Nieto, A. R. Rodriguez, *Bioelectrochem. Bioenerg.* **1996**, *40*, 215.
[74] M. El Hourch, A. Dudoit, J. C. Amiard, *Electrochim. Acta* **2003**, *48*, 4083.
[75] C. Harlyk, G. Bordin, O. Nieto, A. R. Rodriguez, *J. Electroanal. Chem.* **1998**, *446*, 139.
[76] C. Harlyk, O. Nieto, G. Bordin, A. R. Rodriguez, *J. Electroanal. Chem.* **1998**, *458*, 199.
[77] M. Tomschik, L. Havran, E. Palecek, M. Heyrovsky, *Electroanalysis* **2000**, *12*, 274.
[78] M. Strouhal, R. Kizek, J. Vacek, L. Trnkova, M. Nemec, *Bioelectrochemistry* **2003**, *60*, 29.
[79] L. Trnkova, R. Kizek, J. Vacek, *Bioelectrochemistry.* **2002**, *56*, 57.

