In: Voltammetry: Theory, Types and Applications
Editors: Yuki Saito and Takumi Kikuchi
ISBN: 978-1-62948-057-2

Chapter 5

AUTOMATIZED DETERMINATION OF METALLOTHIONEIN BY ADSORPTIVE TRANSFER STRIPPING TECHNIQUE COUPLED WITH BRDICKA REACTION

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ABSTRACT

Application of electrochemical methods for protein determination was discovered in the thirties of the last century. Rudolf Brdicka was one who focused his attention to the protein electrochemical determination and used discovered analytical method for clinical purposes. Brdicka´s way of protein detection was based on the specific reaction between thiol group of proteins with Brdicka's solution. Metallothionein (MT) as a low-molecular mass protein with high content of sulfhydryl (thiol) groups seems to be ideal target for such detection. This protein is involved in heavy metals homeostasis due to their chelation centers in MT structure. Moreover, it is known that MT enters the intracellular redox homeostasis, heavy metals transport and regulation of gene expression. Principle explanation of electrode reactions during protein Brdicka reaction is presented like the influence of various physico-chemical conditions on this procedure. Improvement and development of this technique due to new technical and technological tools is discussed.

Keywords: Metallothionein, Brdicka reaction, thiol compound, mercury electrode, tumor diseases, automation

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1. INTRODUCTION

Metallothionein (MT) is a low molecular mass protein (6-10 kDa) that was firstly isolated from horse kidneys by Margoshes and Vallee in 1957. This protein contains cysteine moieties and lacks aromatic amino acids in the structure (Figure 1). Four MT isoforms (MT-1 to MT-4) have been found in mammals [1]. Expression and localization of individual MT isoforms are variable at intracellular level (cytosol, nucleus, lysosomes and mitochondria) and in individual tissues. MT-1 and 2 are present in tissues of kidneys, liver and intestine. MT-3 is located in brain and MT-4 in skin [2]. MT-1 and MT-2 primarily provide protection against the effects of heavy metals, but also participate in the maintenance of intracellular homeostasis of zinc [2-7].

The main function of MTs in organism is to transport metal ions, to maintain the oxidative-reducing conditions, and to regulate the gene expression (Figure 2). Thiols including MT and glutathione are known as effective scavengers of free radicals, which create optimal oxidative-reducing conditions. As a result, cell compartments and biologically important compounds including enzymes of the cell cycle or DNA are protected. MT regulates level of free radicals also indirectly by binding metal ions, which represent potential radical producers, e.g. Cu [8]. It has been found that the loss of the protective effects of MT contributes and finally leads to an escalation of pathological conditions. MT has antioxidant effect too. During intoxication of organism by heavy metals (Cd, Hg, Pb), MT is able to bind these metals and detoxifies them by this way.

Due to the involvement of MT in the wide range of the cell processes and variety of physiological functions, MT is considered as an essential protein in organisms. Its main function in organism consists in maintaining the oxidative-reducing conditions, transport and homeostasis of metals ions and regulation of gene expression. Recently, the role of MT in the anticancer therapy has been discussed [9-11].

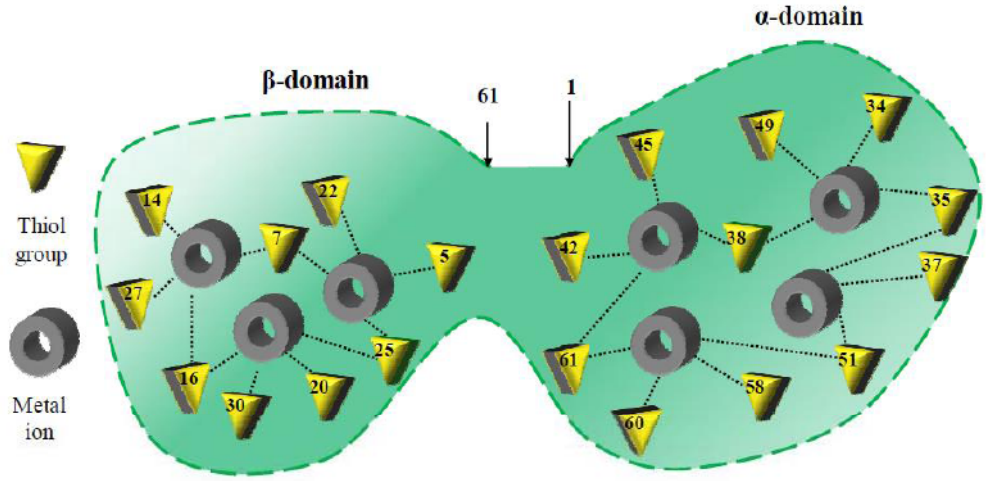

Figure 1. Schematic structure of MT that contains two main domains α and β.

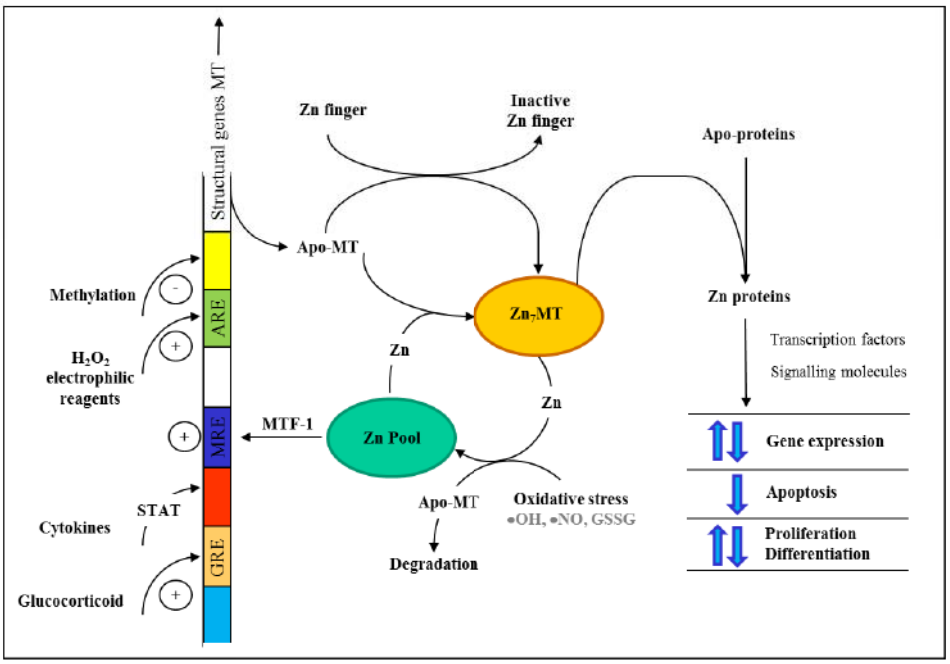

Figure 2. Effects inducing the expression of liver MT.

1.1. Structure of Metallothionein

Metallothioneins of mammals are single-stranded low molecular proteins with 61 – 68 amino acids in the structure. Cysteine accounts for one third of amino acid moieties; cysteine moieties are located in the conservative sequences such as **cys-x-cys, cys-x-y-cys** and **cys-cys** (x and y are amino acids different from cysteine). Metal ions are bound to sulfhydryl groups of cysteine moieties that create tetraedric conformation of thiolated clusters in the case of divalent ions [12]. MT has the highest affinity to Cu^{+} (stability constant 10^{19} - 10^{17}), then to Cd^{2+} (10^{17} – 10^{15}) and Zn^{2+} (10^{14} – 10^{11}) and it is not able to bind Cu^{2+}. Eighteen metal ions that can be bound by MT have been shown; however, only Cu^{+}, Cd^{2+}, Pb^{2+} , Hg^{2+}, Ag^{+} and Bi^{3+} are able to displace bounded Zn^{2+} from the structure of MT. Overall, it can coordinate up to 12 mono- or 7 divalent ions. Its tertiary structure is based on the presence of two domains, α and β (Fig. 1). α-domain (C-terminal) is more stable and contains four binding sites for divalent ions of heavy metals, β-domain (N-terminal) can bind three divalent metal ions [13].

1.2. MT Isoforms

Four isoforms of mammalian MT (from MT-1 to MT-4) have been found; in addition more than 13 metallothionein-like proteins have been discovered in humans [14]. There are 11 genes (MT-1A, B, E, F, G, H, I, J, K, L, and X) for isoform MT-1 and one gene for each another isoform [15]. Differences between individual isoforms arise mainly from posttranslational modifications, small changes in the primary structure. Affinity for various

heavy metals and increased turnover are although related to their localization in different cellular compartments and tissues. MT-1 and MT-2 are prevalent in almost all tissues [16]; MT-3 is expressed in the brain tissue, in cardiac muscle, in kidney and in the tissues of reproductive organs [17]. Isoform MT-4 is known only poorly; it was firstly detected in epithelial cells [16].

1.3. Metallothionein and Tumor Diseases

Metallothionein was being associated with cell proliferation [19], in which this protein serves as a carrier and reservoir Zn^{2+}. Moreover, increased concentration of MT was also observed in many types of tumor cells. Although the use of MT as a tumor marker is still not commonly applied due to difficulties with interpretation, it is known that the expression of MT depends on the degree of differentiation of tumor, stage of disease and other characteristics of the tumor cells [20, 21]. MT can either directly or indirectly (de)activate a number of proteins associated with cancer just via binding Zn^{2+}. Tumor-suppressor protein p53, which active conformation depends on the binding of Zn^{2+}, serves as a very good example. Apo-MT is able to bind Zn^{2+} from the structure of p53 and thereby regulates its function (activation/inactivation). Inactivation leads to a decrease in the affinity of p53 to DNA and thus prevents initiation of apoptosis via pro-apoptotic signals [22]. MT provides metal homeostasis in the cells and maintains the level of free radicals, protects the cells against xenobiotics, potentially harmful substances. This ability can be significantly responsible for the effectiveness of cancer treatment, especially by metal-based cytostatics [23]. Relationship between MT and proliferation of tumor cells has not been fully understood and in this time it is in the center of interest of many scientific groups [24-30].

2. Pre-Determination Steps for Metallothionein Detection

2.1. Isolation of Metallothionein

Applied separation techniques are represented mostly by chromatographic and electromigration methods. These methods enable elimination of potentially interfering compounds and improve significantly the determination of the target analyte.

The method of gel electrophoresis is applicable due to combination of some specific properties of MT – low molecular mass, content of heavy metal(s) and the presence of thiol groups; however, the protocols commonly used for electrophoresis of proteins may bring only poor results. Generally, isolation of metalloproteins with non-covalently bound metal(s) requires mild, non-denaturing conditions [18]. In 2002, the comprehensive review focused on the determination of MT by capillary electrophoresis (CE) was published by Minami *et al.* [31]. This work pays special attention to the elimination of the adsorption by the coating of inner walls of capillary by polyacrylamide, which is one of the most frequently used methods for separation of MT [32, 33]. Other ways of detection of MT by the use of CE is presented in [18].

Chromatographic methods represent the group of suitable techniques for an analysis of complex biological matrices including tissues, blood serum, liver and/or kidney samples. Various modes, such as size exclusion chromatography (SEC) [34, 35], ion exchange (IE) [36] and even two dimensional HPLC [37] have been successfully employed for the analysis of MT.

2.2. Preparation of Sample

Due to the fact that MT is a native protein, its separation from real samples is closely connected with an isolation process. This procedure is being understood as a complex of individual steps, which start with pretreatment methods involving extraction and purification and continue with separation techniques prior the MT detection. At the beginning of the quantification of MT, it is necessary to prepare samples (from cells or tissues) according the following protocol (Figure 3). In the case of the cells that were treated with metal ions, samples should be washed with buffer solution to remove residual culture medium and metals adsorbed on the surface of the cells.

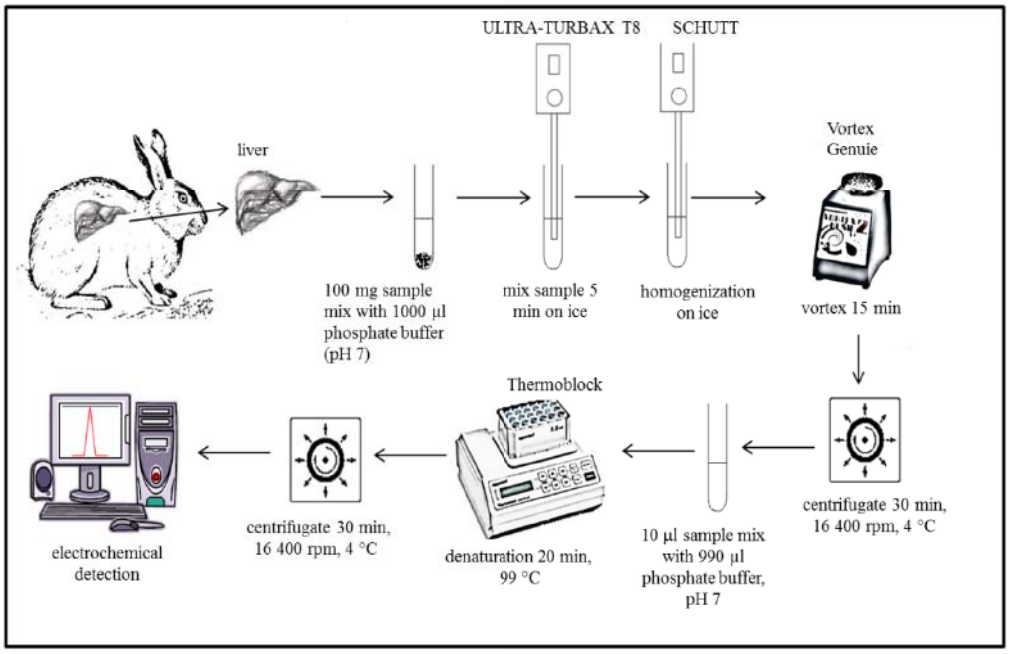

Figure 3. Scheme of the isolation process of metallothionein from the real sample (rabbit liver). Specific conditions of individual steps are accentuated.

The most commonly used buffer is phosphate buffer (Na_2HPO_4 + NaH_2PO_4, 100 mM, pH 7) or Tris-HCl buffer (10 mM, pH 7.4). Samples are further diluted with buffer solution in a quantity equivalent to ten times the tissue weight. An ultrasound sonication apparatus should be used to homogenize the sample. In the case of a solid tissue it is better to use mechanical disruption by a mixer.

Separation of metallothionein from other proteins is done by using its thermostability and this operation is sufficient for denaturation of the sample in a heating block (99 °C, 20 minutes). Subsequent centrifugation (4 °C, 16 400 rpm, 30 min) is necessary to separate solid cellular compartments from disrupted cytoplasm and organelles. The obtained supernatant may be then analyzed.

3. Electrochemical Determination of Metallothionein

Detection of metallothionein is connected with specific structure of MT, i.e. the absence of aromatic amino acids in the native state and disulfide bonds. However, its structure is adapted to incorporate heavy metal ions by sulfhydryl groups, thus, these specific features may be used for the electrochemical determination. The protection by reduction agents is required, because sulfhydryl groups have a tendency to be oxidized. On the other hand, this property allows an application of electrochemical determination. The other opportunity how to detect metallothionein is the catalysis of hydrogen evolution from a supporting electrolyte [38-41].

3.1. Redox Reactions of Proteins on Mercury Electrode

The first group of methods includes the electrochemical methods based on an oxidation or reduction processes of thiol groups included in the structure of MT. Many authors studied electrochemical behavior of metallothionein containing cadmium and/or zinc ions on the surface of a hanging mercury drop electrode (HMDE) by cyclic voltammetry (CV) [38, 39, 42].

Three significant signals of MT, which have been called peaks A, B and C (Figure 4A), were observed and mechanism of their formation has also been described [42]. Determination of MT by cyclic voltammetry (CV) brings worse results compared to other electrochemical methods; nevertheless CV is suitable for the characterization of samples of MT [43]. We optimized detection procedure based on the application of the reducing agent – tris(2-carboxyethyl)phosphine (TCEP) (Figure 4B). This reducing agent was used to detect MT by differential pulse voltammetry too [43].

In general, the method of differential pulse voltammetry has been established as an advantageous method for a sensitive determination of heavy metals. On the other hand, this electrochemical method has been also intensively used to study an electrochemical behavior of MT [44-46].

3.2. Catalytic Reactions of Proteins on Mercury Electrode

Catalytic reactions of proteins are based on the specific reactions between protein(s) and an electrolyte. There are two types of catalytic signals on mercury electrode, H-peak and the Brdicka reaction.

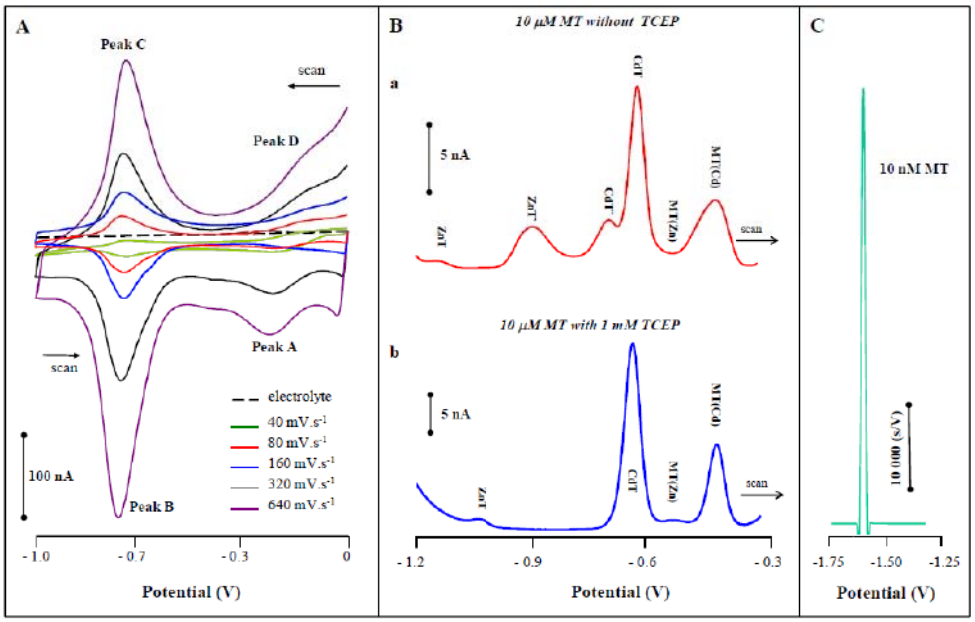

Figure 4. (A) CV voltammograms of basic electrolyte (0.5 M NaCl, pH 6.4) and 10 μM MT at different scan rates of 40, 80, 160, 320, and 640 $mV.s^{-1}$. (B) Typical DPV voltammograms of 10 μM MT without (a) or with (b) 1 mM TCEP measured in 0.5 M NaCl, pH 6.4. (C) CPSA scan of MT in concentration of 100 nM. The supporting electrolyte was composed of 0.1 M H_3BO_3+0.05 M $Na_2B_4O_7$. AdTS CPSA parameters were as follows: starting potential 0 V, ending potential -1.85 V, stripping current 1 μA, time of accumulation 120 s; no reducing agent was added.

3.2.1. H-Peak

The first works focused on an application of H-peak in the analysis of protein were published twenty years ago [47, 48]. This method is based on the catalytic evolution of hydrogen in the presence of a protein. Signal is measured by chronopotentiometry, using an inverse proportion of the time derivation of potential s (dt/dE) (Figure 4C). Chronopotentiometric stripping analysis (CPSA) represents one of the most sensitive methods for detection of peptides and proteins, even in subnanomolar amounts [40]. The character and origin of the catalytic peak H has not been fully elucidated yet. The current accepted opinion lies in the fact that free –SH moieties together with $–NH_2$ ones are involved in the catalysis of hydrogen evolution at very negative potentials. No heavy metal complex present in a supporting electrolyte (as in the Brdicka reaction) is needed. Results described in the work of Strouhal *et al.* [49] showed that optimal buffer composition is borate 0.1 M H_3BO_3 + 0.05 M $Na_2B_4O_7$ (pH 8.0). The value of pH is crucial for the height and position of the signal at a potential about E = -1.7 V [51]. The suitability of application of the borate buffer can be explained by its ability to act as a donor of proton. Catalytic process during analysis of MT also depends on the content of oxygen in the electrolyte; samples with higher concentrations of oxygen show better results [50]. Sensitivity of this method has been showed in the work of Kizek et al., who detected femtomolar concentrations of MT in low volume (5 μl) of analytes [52]. However, the result of analysis strongly depends on many parameters including pH, ionic strength, and pI of the analyzed protein. Lower effect of temperature was observed in the works of Trnkova et al. and Kizek et al. [50-52]. Recently, the ability of

chronopotentiometry to distinguish between native and denatured protein form has been proved [53, 54]. Low cost, low variance coefficient, low detection limits, easy miniaturization, and no interferences are the most important advantages of this technique.

3.2.2. Brdicka Reaction

The Brdicka reaction is a catalytic reaction of protein in so-called Brdicka's solution, which was firstly published by Rudolf Brdicka in the 1930s [55, 56], and onward this method has been developing by other authors [38, 39, 41]. It is necessary to do a short historical overview to perform Professor Rudolf Brdicka (1906-1970). The inventor of the polarographic method, father of the electroanalytical chemistry, and recipient of the Nobel Prize in 1959 in chemistry was Professor Jaroslav Heyrovsky [57-64]. Polarography of Professor Heyrovsky motivated a large scientific community and led to a dramatic expansion and application of electrochemistry, which was accompanied by the establishment the entire research institutions. Besides polarographic determination of metal ions, which have distinct redox properties, the research also focuses on biomolecules. One of the scientists who developed applications of polarography to determine proteins was Heyrovsky´s student Rudolf Brdicka. Works about polarographic determination of proteins and their relation to serious diseases, including cancer, have been published later [65-70]. Brdicka was afterwards appointed to a professor of physical chemistry at Charles University in Prague. He was also the founding member of the Czechoslovak Academy of Sciences and founder and first director of the Institute of Physical Chemistry of the Czechoslovak Academy of Sciences. The details of the reaction are described in the following chapter.

4. BRDICKA REACTION AND ITS APPLICATION TO METALLOTHIONEIN DETERMINATION

4.1. Mechanism of Brdicka Reaction on Mercury Electrode

4.1.1. Hexaamminecobalt(III) Chloride

The Brdicka reaction (procedure) is the frequently employed electrochemical method for determination of metallothionein in a variety of biological samples [71-77]. The method uses Brdicka´s solution. Brdicka´s solution consists of an ammonium buffer (ammonium chloride and ammonium) and hexaamminecobalt(III) chloride complex ($[Co(NH_3)_6]Cl_3$).

Chemical phenomena of this described below is based on the interaction of $[Co(NH_3)_6]Cl_3$ with -SH groups of the protein. The complete scheme is shown in Figure 5. As it was above-mentioned, ammonium buffer with a high pH serves as a buffer. The first step of the process is the irreversible reduction of Co^{3+} to Co^{2+} to create $[Co(NH_3)_6]^{2+}$. Because the amino complex $[Co(NH_3)_6]^{2+}$ is extremely unstable, it immediately undergoes hydrolysis to create aqua complex according to the following reaction:

$$[Co(NH_3)_6]^{2+} + 6\ H_2O \rightarrow [Co(H_2O)_6]^{2+} + 6\ NH_3 \quad (1)$$

Because the cobalt ion is classified as a hard cation and the amino group as a hard anion, the Co(III) complex is stable. After reduction, cobalt ion creates complex, which is larger and

has a smaller surface charge density, therefore, it is soft. Stability of the complex of hard and soft acids and bases is low and the product of reduction (hexaamminecobalt(II) ion) is subsequently hydrolyzed.

Ammonium created during hydrolysis increases pH and by this way, it creates conditions suitable for catalytic reaction, which can continue using NH_4^+ cations. In the presence of ammonia buffer, the reaction is as it follows:

$$NH_{3(aq.)} + H_2O \leftrightarrow NH_4^+ + OH^- \quad (2)$$

and is defined by a dissociation constant $K_b=1.74.10^{-5}$ M. NH_4^+ acts as an acid, the donor of proton, while NH_3 is a conjugated base.

The first reaction of cobalt from oxidative number III to II creates a polarographic wave in the potential approximately Ep = -0.3 V. Subsequent reaction of unstable $[Co(H_2O)_6]^{2+}$ complex to Co^0 proceeds at the potential -1.2 V according to:

$$[Co(H_2O)_6]^{2+} + 2\ e^- \rightarrow Co^0 + 6H_2O \quad (3)$$

The resulting voltammogram of the reduction of $[Co(NH_3)_6]Cl_3$ in the ammonium buffer represents two polarographic waves approximately at the potentials Ep = - 0.3 V ($Co^{3+} \rightarrow Co^{2+}$) and Ep = -1.28 V ($Co^{2+} \rightarrow Co^0$), see in Figure 5b black line.

In general, the inert type of complexes $[Co(NH_3)_6]^{3+}$ prevails in the solution due to a complexity of the interactions. On the other hand, high pH value created by the ammonium buffer is the cause of dissociation of protons from carboxyl and ammino groups of proteins, but not of cysteine groups. Only the formed $[Co(NH_3)_6]^{2+}$ complexes react with the thiol groups of the proteins:

$$[Co(H_2O)_6]^{2+} + R(SH)_2 \rightarrow RS_2Co + 2H^+ \quad (4)$$

The second signal is not based on a reduction of $[Co(H_2O)_6]^{2+}$, it corresponds to binding Co^{2+} ions in a complex with thiol groups. Reduction stages can be described with E_p = -1.35 V as it follows:

$$RS_2Co + 2e^- \rightarrow Co^0 + R(S^-)_2 \quad (5)$$

At higher concentrations of thiols, which chelate metal ions, peak Co1 that is connected with a reduction of $[Co(H_2O)_6]^{2+}$ and is located at the more positive potential than reduction peak RS_2Co can be also visible. Hydrogen ions are due to an exchange of water ligands for sulfhydryl groups absorbed by molecules of ammonia to create ammonium ions. After reduction of Co^{2+} to Co^0, the $R(S^-)_2$ groups immediately protonate NH_4^+ groups and the compound $R(SH)_2$ is recovered and able to bind more hexaaquacobalt(II) ions $[Co(H_2O)_6]^{2+}$.

The last two signals (Cat1 – Ep = -1.35 V and Cat2 – Ep = -1.48 V) represent a catalytic reaction, an addition of the compound with the -SH groups. Cat2 peak is probably the result of reduction of H^+ ions resulting from the reaction between $R(SH)_2$ and $[Co(H_2O)_6]^{2+}$. After the deposition of Co^0 from the complex, the $R(S-)_2$ is rapidly protonated by the proton donor, NH^{4+} ion:

$$\begin{array}{l} 2NH_4^+ \\ \downarrow \\ R(S^-)_2 + 2H^+ \leftrightarrow R(SH)_2 \\ \downarrow \\ NH_{3(aq.)} \end{array} \quad (6)$$

The response of $R(SH)_2$ with $[Co(H_2O)_6]^{2+}$ takes place on the surface of the electrode and $R(SH)_2$ is the catalyst of the hydrogen evolution from the electrolyte, which regenerates on the surface of electrode [73].

It has been found that the concentration of MT determined in a real sample by the use of the Brdicka reaction corresponds to the height of the last signal (-1.48 V) in the obtained voltammogram [78].

4.1.2. Cis-Diamminedichloroplatinum(II) Complex

Cis-diamminedichloroplatinum(II) is the possible substitution for hexaamminecobalt(III) chloride complex in the Brdicka procedure where the buffer system is the same. This system was described using the method of cyclic voltammetry with the following description of the mechanism [79]:

Present double wave can be explained by a reaction of cisplatin with the –SH group(s) of the protein:

$$[Pt(NH_3)_2]^{2+} + R(SH)_2 \rightarrow RS_2Pt(NH_3)_2 + 2H^+ \quad (7)$$

The first wave at E_P = -1.35 V corresponds to a reduction of the complex created on the electrode according to

$$RS_2Pt(NH_3)_2 + 2H_2O + 2e^- \rightarrow Pt^0 + R(S^-)_2 + 2\ (NH_3.H_2O) \quad (8)$$

Electroactive species $RS_2Pt(NH_3)_2$ are created by a direct reaction presented in Eq. (7), but also by an exchange reaction of metals (Cd, Zn, Cu) bound to the -SH groups of MT.

The second wave at Ep = -1.55 V is assigned to a reduction of the protons liberated in Eq. (7), named the catalytic hydrogen evolution (cat):

$$2H^+ + 2e^- \rightarrow H_2 \quad (9)$$

An advantage of using platinum complex is that reduction of platinum(II) occurs at positive potentials and does not influence the resolution of the double wave unlike cobalt in the Brdicka reaction.

Ammonia buffer was used to buffer the solution to the pH required as well as the donor of proton regenerate the catalyst of $R(SH)_2$, but also other buffers with suitable pH can be used including borate buffer [80].

This regeneration consists of an addition of proton (H^+) to the product of the electrochemical reduction of the catalyst by acids (here NH_4^+), which are the donors of protons in the solution [79].

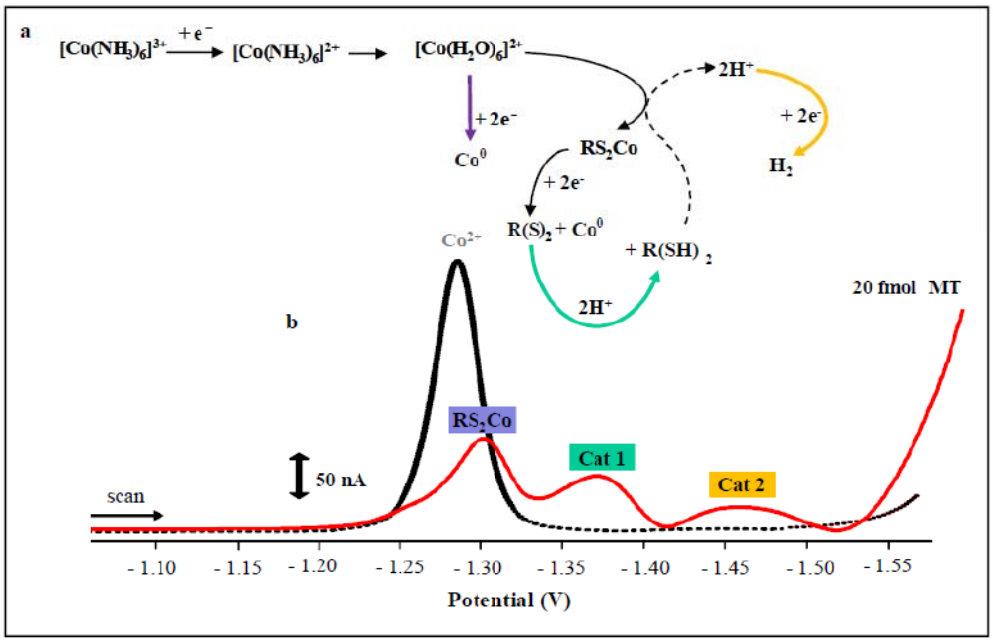

Figure 5. (a) Probable scheme of the catalytic hydrogen evolution in the Brdicka reaction. (b) Real voltammograms of 20 fmol metallothionein (red line) and the Brdicka´s solution as a supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M $NH_3(aq)$ + NH_4Cl, pH 9.6; (black line). Parameters of DPV method were as follows: initial potential -0.35 V, end potential –1.8 V, modulation time 0.057 s, time interval 0.2 s, step potential 1.05 mV/s, modulation amplitude 250 mV, time of accumulation of MT 120 s, E_{ads} = 0 V. All measurements were carried at temperature of 5 °C.

4.1.3. Brdicka Reaction in the Presence of Nickel(II) Ions

Analogically to Co^{2+}-containing system for determination of metallothionein, the evolution of hydrogen catalyzed by a particular form of nickel(II) ions (sulfide) is possible. The possible mechanism suggested for low-molecular thiols has been shown [81-86]. Application of nickel ions instead of the cobalt ions leads to the formation of a similar catalytic peak at the potential app. -1.5 V. The mechanism of the reaction is practically the same as the mechanism presented for cobalt ions [87]. Several differences arise from the particular behavior of each thio-amino acid as a ligand in chelate complexes with the metal ions. Thus, thiols give rise to two specific catalytic processes: (i) the catalytic hydrogen prewave - type reaction that occurs at near-neutral pH and in the presence of Ni^{2+}, and (ii) the Brdicka reaction that takes place in a slightly alkaline system and in the presence of Co^{2+}. Metal complexes that are present in the solution phase cause both these processes. Consequently, both reactions are not affected by a preliminary accumulation of the mercury thiolate in particular. Chelation of metal ions in these complexes involves the amino and thiol groups.

Two different forms of nickel sulfide were detected by characteristic cathodic reactions, i) adsorbed free molecules that are reduced at -1.0 V and are able to take a part in a catalytic reduction of Ni^{2+}; and ii) an aggregated form that is irreversibly reduced at negative potentials beyond -1.2 V and acts as a catalyst for hydrogen evolution.

4.2. Physico-Chemical Conditions of Brdicka Reaction

Due to the fact that the Brdicka reaction belongs to the catalytic processes, its development depends on various physico-chemical parameters that influence the height of signal of the hydrogen catalytic wave (Cat), which is most frequently used for the quantitation of MT. Brdicka used a direct current (DC) mode on a dropping mercury electrode (DME) [1,16] for systematic investigations of the effect of temperature and the concentration of depolarizer $Co(NH_3)_6Cl_3$ on the height of catalytic signal. Since then more selective and sensitive electrochemical techniques like differential pulse (DP) mode on the hanging mercury drop electrode (HMDE) have been developed and used.

Modification of the original Brdicka´s method with the differential pulse method led to the necessity to optimize the experimental conditions, mainly temperature and concentration of depolarizer. The first parameter, temperature, is closely associated with the evolution of individual peaks and helps to identify the nature of individual processes in the Brdicka reaction. The peak corresponds to a reduction process of Co^{3+} to Co^{2+} at the potential Ep = -1.28 V and is caused by the diffusion-controlled reaction [78]. This fact was deduced from the changes in the detected signal (an increase for 30 %) with changing temperature within the range from 7 to 25 °C. The other signal created in the presence of protein and that corresponds to the reduction of RS_2Co complex at the potential Ep = -1.35 V is created by diffusion-controlled reaction [78]. The third peak detected at potential Ep = -1.48 V corresponds to the reduction of protons by forming RS_2Co complex. This signal is related to the catalytic evolution of hydrogen. The decrease of the peak height with the increasing temperature indicates that the signal is controlled by a surface reaction [78]. It has been published that the analysis of MT by the Brdicka reaction is effective to perform at temperatures within the range from 5 to 10 °C [78, 80]. In the view of the fact that the height of Cat2 peak closely corresponds to the concentration of protein, influence of the temperature on this specific signal is very important. It is obvious from several published works that the optimal temperature is the lowest possible temperature, in our case 5°C [43, 78]. The concentration of depolarizer is the second parameter that influences the evaluated peaks. The concentration range of the depolarizer from 0.12 to 1 mM $Co(NH_3)_6Cl_3$ was tested [43, 78]. The obtained results show that the highest concentration of $Co(NH_3)_6Cl_3$ is the best for the evaluation of the Cat peak height, i.e. that the concentration of 1 mM $Co(NH_3)_6Cl_3$ indicates the highest sensitivity of the response.

4.3. Adsorptive Transfer Stripping (AdTS) Technique Connected with Brdicka Reaction

Adsorptive Transfer Stripping (AdTS) technique was firstly used for detection of MT by Palecek [88]. Principle of the AdTS is based on the strong adsorption of the studied analyte on the surface of electrode at an open electrode circuit. The excess of analyte is rinsed from the surface of the working electrode in the buffer. The adsorbed analyte is finally detected in the presence of indifferent electrolyte. It is possible to describe the individual steps of this technique as follows (Figure 6A): (1) renewed surface of HMDE is placed into a drop containing MT, (2) MT binds on the surface of HMDE only, (3) low molecular compounds,

such as heavy metals, are washed out in this step, (4) the modified HMDE electrode is placed into a supporting electrolyte, (5) and analyzed by DPV.

The great advantage of the adsorptive technique is the possibility to study the behavior of MT adsorbed on a surface of working electrode without interferences from both sample and supporting electrolyte and the analysis itself takes only few minutes [89]. Usually a volume of 5 µl of sample is applied. This fact makes this procedure very suitable for determination of very small volumes and concentrations of analytes. Thanks to this technique, we are able to detect changes in electrochemical signals caused, first of all by experimental conditions.

Comparison of three electrochemical methods that are connected with application of adsorptive technique is shown in Table 1 [43]. This table presents limits of detection for individual techniques in the detection of MT. As it is obvious from presented results, the Brdicka reaction provides the best limit of detection.

Table 1. Limits of detection of metallothionein estimated by three different electroanalytical methods [43]

Method	Limit of detection[a]		R.S.D.[c]
CV	120 nM	0.5 pmol[b]	9.5
DPV	0.8 nM	4 fmol[b]	7.9
Brdicka reaction	2 pM	10 amol[b]	5.7

[a]Limit of detection (3S/N). [b]Limit of detection per 5 µl. [c]Relative standard deviations.

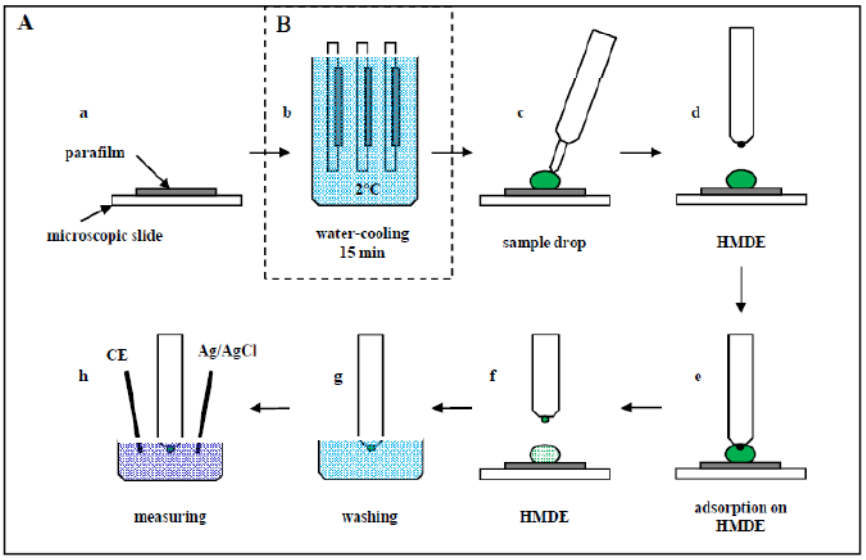

Figure 6. (A) Scheme of the adsorptive transfer technique (AdTS). (B) Scheme of improvement of the transfer to detect MT in very low volume of a sample. Procedure steps were done as follows: (a) microscopic slide, degreasing and seaming of a small square from parafilm (10 × 10 cm, Sigma-Aldrich); (b) transferring it to a beaker filled with distilled water and placed in the tempered water bath (temperature of 2°C, at least 15 min., Julabo, Germany); (c) drying it using cellulose and pipetting of a sample on it; (d) creating new drop of mercury; (e) adsorbing MT on the surface of HMDE; (f) transferring the electrode and (g) washing it; (h) transferring the electrode and measuring in the supporting electrolyte.

The adsorptive transfer stripping technique has been unable to generate reproducible results when analyzing very low volume (nanolitres) of sample. This obstacle can be overcome technically by modifying the current transfer technique including cooling step of the adsorbed analyte. The detailed description of the modification of the method is presented in Figure 6B.

The detection limit (3 S/N) of MT was evaluated as 500 zeptomoles per 500 nl (1 pM) and the quantification limit (10 S/N) as 1.500 zeptomoles per 500 nl (3 pM) [71]. Based on the obtained results it can be concluded that the improved technique can be used to detect MT in very low volumes of sample and can prevent interferences during the washing and transferring steps.

4.4. Automation of the Brdicka Reaction

Electrochemical methods are generally used in clinical laboratories. Value of pH and detection of metal ions by the use of ion selective electrodes belong to the most measured parameters. However, voltammetric analysis is practically not applied in the clinical laboratories. There are several reasons for this phenomenon, but the requirement of the qualified operation of the analyzer and its servicing belong to the most important. Despite of these disadvantages, electrochemical analyzers have a lot of advantages such as the excellent repeatability of the analysis, low demands on the adjustment and manipulation with sample and above all low costs of operation of apparatus (one analysis costs less than 1 Euro). Possibilities of the electrochemical analysis are especially in detection of heavy metals ions, but now we can observe renewed interest in the area of analysis of nucleic acids and detection of proteins.

For the purposes to analyze thiol compounds in biological samples, an electrochemical analyzer from Metrohm Company (the unique arrangement in the whole Central Europe) was tested (Figure 7) [90]. The arrangement is conceived as an automat consisting of an electrochemical module (potentiostat/galvanostat, VA Stand 747) and an electrochemical cell placed on it. Three electrodes (working, reference, and auxiliary electrode) are positioned in this electrochemical cell. Another part of the arrangement is a special handler of samples (Autosampler 695), which fulfills the request on the minimization of the dosing the samples. The sample is taken into a Teflon® capillary filled by the hydraulic liquid. The capillary is attached to an air pump, which supplies the accurate dosing the samples. The applied volume of sample may vary between 1 and 100 µl due to very precise dosing. The capillary is placed on a moving arm with the possibility of the movement in three directions x, y and z. The samples and eventually other reagents are placed in three different carousels. Individual carousels are identified by the arrangement according to the placing of magnets on their bottom. It was necessary to place the samples into the cooled space in arrangement tested by us. For this purpose, a simple water-cooled holder of sample, which can carry more than 80 different samples, has been suggested and subsequently constructed in our laboratory. Remaining carousels serve as the holders for the chemicals (reagents), which can be used during the analysis. In the light of above-mentioned facts, there is a scope for the inexhaustible possibilities of the modifications of electrochemical analysis; these modifications can provide higher selectivity of the determination, eventually lower limits of detection. Due to the necessity to provide a supporting electrolyte and to carry out rinsing

steps, three pumps (700 Dosino) are available. Two of these pumps supply the perfect rinsing of the working cell (maximum capacity 50 ml) and the third pumps the supporting electrolyte (maximum capacity 20 ml). The arrangement is controlled by a microprocessor (746 VA Trace Analyser). The automatic analyzer facilitates an application of the small volume of the supporting electrolyte (2 ml), which is not so common in the case of the electrochemical analysis. In addition, it is possible to set and customize almost all parameters from the volumes of electrolyte, the sample, or the washing buffer to the automated measurement of calibration curve and real samples (Figure 5).

After the choosing the acceptable method, which is submitted to the control module, filling the electrochemical cell by the supporting electrolyte starts firstly. In our arrangement, the amount of the used solutions was minimized significantly. The maximal volume necessary for the analysis does not exceed 2 ml [90]. In the next step, the dosing arm is initialized, rinsing of the dosage needle, and loading of the volume of the sample for dosing follow. Then the dosage arm locates the determined position with sample and takes it up. Washing of the dosage needle and movement of the arm to the electrochemical cell follow. After the dosing of the sample into the electrochemical cell, electrochemical analysis is initiated. In our case, the time from the washing the cell until application of the sample is about 5 minutes, but the duration of the analysis may be surely reduced. It depends on the character of the analyte and the procedure of the detection.

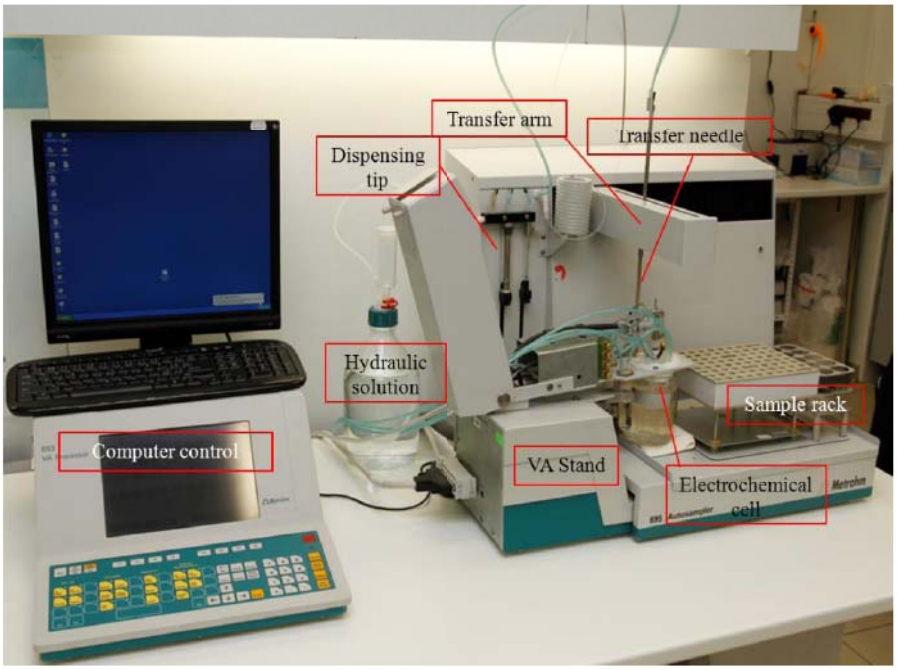

Figure 7. The automated electrochemical analysis of the low-molecular thiol compounds. The electrochemical measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and a cooled sample 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 a glassy carbon electrode was the auxiliary electrode.

4.5. Importance of Determination of Metallothionein

The importance of the determination of metallothionein is closely associated with its biological functions. There is an issue in the exact characterization of biological functions of metallothionein. MT has been revealed to be involved in tens of physiological processes, usually varying in different organisms and also in different isoforms in the same organism [91]. Therefore, it seems to be useful to assume that MT has not only one specific function in organisms, but plays many specific roles in various processes. The biological function of MT is closely connected with its functions at molecular level and is based on its unique structure given by the primary structure based on amino acid composition: (i) binding of metals and (ii) redox activity [92]. The problem is that these two functions are necessary in a large number of different biological processes. In addition, involvement of MT depends on the individual physiological needs of various organisms. Therefore, the following part is divided into several parts that describe a range of application of metallothionein.

4.5.1. Basic Electrochemical Behavior of MT

The Brdicka reaction can be carried out in the case of all peptides and proteins that contain cysteine moieties in the structure. These peptides and proteins include metallothionein [38, 39, 43, 50, 71, 73, 76, 89, 93-104], glutathione [72, 78, 80, 105-108], phytochelatins [72, 95, 107, 109, 110], and others [111, 112]. The approach to determine metallothionein electrochemically varied over time with the development and modifications of known electroanalytical methods. The need to reduce detection limits of metallothionein and generally thiols led to the modification of the Brdicka reaction with DPV method [78, 105, 106]. Electrochemical determination of MT is not limited to DPV method only, but also other methods are applicable and have been intensely studied in connection with Brdicka reaction [51, 71, 79, 113-115].

4.5.2. Structure of MT and Binding of Metals

Investigation of the structure of metallothionein has been done many times and published in various types of journals [3-5, 116-121]. There are 88 review entries in ISI Web of Knowledge related to the key word "metallothionein structure". These materials cover the time line from the half of eighties to the present. Most works focus on the description of the structure of metallothionein in connection with metal ions, especially on the binding metal ions into the structure of MT. Binding metal ions into the structure of MT is closely connected with its biological functions [92].

Exchange of metal ions in MT starts with a metal-loaded MT (with either Zn^{2+} or Cu^{+}), which exchanges, totally or partially, initially bound metal ions by others. This possibility relies on the well-known series of the affinity of heavy metal ions to the thiolate ligands: $Fe^{2+} \approx Zn^{2+} \approx Co^{2+} < Pb^{2+} < Cd^{2+} < Cu^{+} < Au^{+} \approx Ag^{+} < Hg^{2+} < Bi^{3+}$ [92]. The highest affinity has been shown for Cu^{I}, but in the most cases metallothionein binds Zn^{2I}, thus, these ions are very intensively studied in the connection with the homeostasis of zinc in organisms [10, 27, 122-124].

While molecular zinc is inert in redox properties, sulfur ligands in cysteine moieties in MT can be oxidized and reduced concomitantly with the releasing and binding zinc, and creating and cleaving disulfide bonds. This redox mechanism confers redox activity of MT

[125]. In the zinc transfer reactions, MT does not release all of the seven zinc atoms. The stability constants (K) of most enzymes that contain zinc are by three orders of magnitude lower than the total stability constant measured for MT ($K \sim 10^{12}$-10^{13} M^{-1}). This makes transfer of zinc from MT thermodynamically unfavorable. However, the redox mechanism in the MT zinc-thiolate cluster can overcome this thermodynamic barrier and can lead to release additional zinc in accordance with demands [126]. All seven atoms of zinc in MT seem to have similar coordination environments, but three distinct classes of zinc sites, whose stability constants covering four orders of magnitude, have been observed. The first class of the zinc sites (Zn 1–4) binds zinc more strongly than the second (Zn 5 and Zn 6). The stability constant of one zinc atom, Zn 7, is significantly lower ($K_d = 2.1.10^8$ M^{-1}) than that of the remaining six atoms (Zn1 – 6). This enables thermodynamically the transfer of one zinc atom from MT to other zinc binding proteins [127].

The most often studied complexes of MT with metals are complexes of zinc, copper and cadmium ions. Nuclear magnetic resonance (NMR) [3, 5], circular dichroism, and ultraviolet absorption spectroscopy [4, 121] are enabling to study the structure of these complexes.

4.5.3. MT as an Environment Biomarker

Anthropogenic activities lead to the accumulation of different types of pollutants in the environment and to its changes. Anthropogenic activities include especially industry, source of both inorganic and organic pollutants in the environment. Acidification in the geo-environment changes availability and mobility of the elements including highly toxic elements, such as arsenic, mercury, and cadmium. In the geo-environment, chemical reactions such as methylation and demethylation occur. They can contribute to the modification of relatively non-toxic compounds to toxic or highly toxic ones.

It is also known that the toxic effects of heavy metals may be reduced in many organisms by binding to specific ligands. Metallothionein plays a crucial role among these specific ligands. In relation to trace elements, MT might serve as an indicator of an environmental pollution and exposure to this pollution. Increased level of MT-I and -II in tissue(s) indicates an exposure to trace elements, respectively heavy metals. MT-III is non-inducible and probably plays an important role in the metabolism of zinc and elements that are involved in neurotoxicity [128].

While the most of metallothionein research has been carried out on mammals or vertebrates, there are only few studies focused on invertebrates. Application of invertebrates as a suitable model for detection and monitoring the metal pollution of the environment has been shown in several works [129-134]. MT was usually determined as a biomarker of contamination of aquatic environment by heavy metals. Connection between increased levels of metallothionein as a biomarker in different fish tissues and environmental pollution has been published in many papers [73, 76, 93, 135-142]. On the other hand, application of MT as a biomarker of metal pollution has been shown for the other animal species too [25, 26, 102, 103, 143-152].

4.5.4. MT as a Toxicological Biomarker

Because of the MT is, in the first place, the transporter of metal ions [9, 91, 153-156], its importance as an environmental marker is obvious. During the intoxication by heavy metals, such as Cd^{2+}, Pb^{2+}, and Hg^{2+}, metallothionein is capable to bond these metal ions (under releasing Zn^{2+}) and these ions in the complex with MT are harmless for the cell [156-159].

The subsequent detoxification probably proceeds in kidneys. MT has also significant antioxidant role. In connection with reduced glutathione (GSH), MT constitutes the oxidation-reduction couple, which regulates the occurrence of the free oxygen radicals [9, 127]. They together generate reduction background, which contributes to the protection of biomolecules - nucleic acids, phospholipids, an integral part of biomembranes, and proteins - against the ionizing effects of the high-energetic radiation and the chemooxidative activity of the toxic reagents [9].

Recently, there is more and more pointed at the capability of metallothionein to regulate the genome expression. As a reservoir of the zinc, metallothionein is able to transport the essential metals to the transcription factors and activate them. The activated transcription factors subsequently bind to the specific DNA sequences (regulatory sequences) and initialize the transcription [153, 160, 161].

Besides the intoxication by heavy metal, the stress caused by free radicals represents next threat for organism. The role of MT in this field was therefore investigated too [162]. Damage of complexes of plant thiols (metallothionein-like proteins) and Zn^{2+} and Cd^{2+} ions by reductive radical stress was investigated by Raman spectroscopy [7, 162]. Cysteine moieties have been shown to be among the most sensitive toward radical attack. The authors concluded that MT residues are more sensitive to the reductive radical attack when the protein binds Cd^{2+} ions.

4.5.5. MT as a Marker of Tumor Diseases

Usage of metallothionein as a marker of tumor diseases is widely discussed [27, 73, 76, 93, 100, 163-167]. Diagnosis of tumor in the early stage increases significantly the chances for successful treatment and curing. The applicability of the expression of MT as a diagnostic marker is intensively investigated in the last decade. Several studies that correlate the progression of tumor and resistance to cytostatics in a variety of malignancies (e.g. leukemia, melanoma, breast, ovarian, renal, lung, pancreatic, etc.) to the MTs level have been published [166]. On the other hand, some studies focused to colorectal, bladder, and some other types of cancer have showed no significant correlation between the expression of MT and prognosis of disease [165, 168, 169].

The relationship between MT and cancer has been recently discussed in the review by Eckschlager *et al.* [9]. This comprehensive review summarizes the studies focused on the correlation between expression of MT and prognosis of different types of cancer. Our research group published series of works to explain the correlation between metallothionein and tumor disease [27, 74, 76, 93, 170-173]. The application of Brdicka reaction has been shown in the study that focuses determination of MT in patients with childhood solid tumors by differential pulse voltammetry in Brdicka electrolyte. As a control, 58 healthy volunteers (average age 27.3 years) were gained from Institute of Sports Medicine, Brno, Czech Republic. Samples of blood were obtained from 38 children hospitalized at Department of Pediatric Hematology and Oncology of Faculty Hospital Motol with newly diagnosed different oncology illness; ependymoma (n = 4), Ewing sarcoma (n = 4), solid tumors medulloblastoma (n = 10), neuroblastoma (n = 12), and osteosarcoma (n = 8); (average age 7.3 years). The blood samples were collected before chemo- and radiotherapy. Serum was separated and prepared for electrochemical measurement according the method described in Krizkova et al. [174]. It has been found out that voltammograms vary in dependence on the type of cancer (Figure 8). As a control, serum samples were used. Particularly, in patients

with Ewing sarcoma, medulloblastoma and ependymoma the voltammetric curves were deformed and all signals had the same height comparing with the other diagnoses.

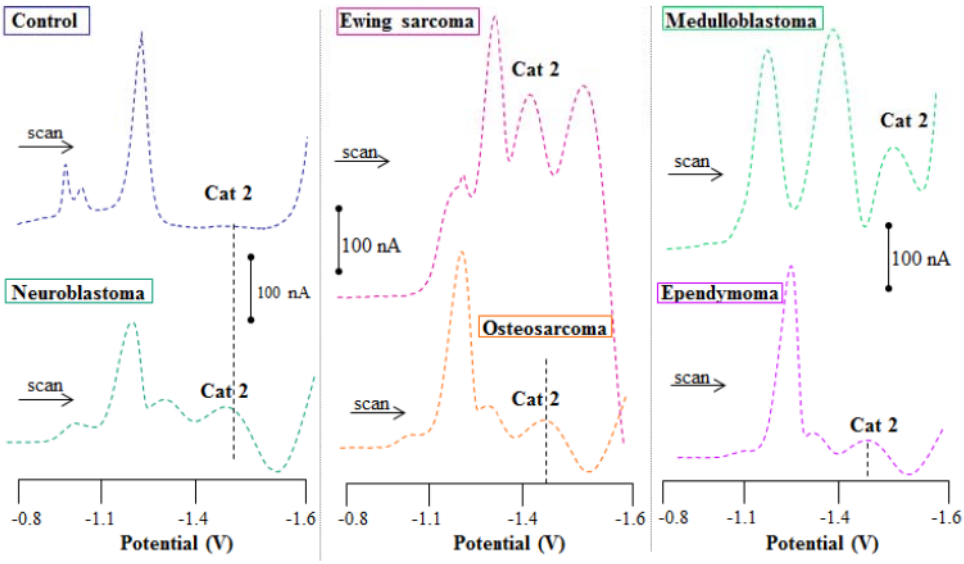

Figure 8. The voltammograms for different type of cancer: control, Ewing sarcoma, medulloblastoma, neuroblastoma, osteosarcoma and ependymoma.

CONCLUSION

This work provides a summary of the current stage in the determination of metallothionein by the Brdicka reaction. This method based on catalytic process is more sensitive to this analyte than other electrochemical methods. Due to this fact, the Brdicka reaction plays the key role in the determination of metallothionein in biological samples. Importance of determination of metallothionein could be obvious in connection with the fact that this protein is probably involved in some diseases, especially tumor diseases.

ACKNOWLEDGMENT

Financial support from the following project CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged.

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