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Vertebrate metallothioneins as target molecules for analytical techniques

Vojtech Adam, Ivo Fabrik, Tomas Eckschlager, Marie Stiborova, Libuse Trnkova, Rene Kizek

Metallothioneins (MTs) are a family of ubiquitous, biologically interesting proteins that have been isolated and studied in a wide variety of organisms, including prokaryotes, plants, invertebrates and vertebrates. Due to the property of MTs being metal-inducible and their high affinity to metal ions, homeostasis of heavy-metal levels is probably their most important biological function.

MTs are also involved in other important biochemical pathways, including scavenging of reactive oxygen species, activation of transcription factors and participation in carcinogenesis. Detection and quantification of MTs are not simple due to the unique primary structure and their relatively low molecular mass. Analytical methods are based on: a) detection of bound metal ion; b) detection of free –SH groups; c) protein mobility in electrical field; and, d) interaction with different types of sorbent.

This review highlights techniques used for detection and determination of MTs with discussion of the advantages and the disadvantages of particular approaches.
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Keywords: Capillary electrophoresis; Electrochemistry; ELISA; Gel electrophoresis; High-performance liquid chromatography; Immunochemistry; Metallothionein; Saturation assay; Spectrometry; Voltammetry

Abbreviations: AAS, Atomic absorption spectrometry; AE-HPLC, Anion-exchange HPLC; CE, Capillary electrophoresis; CPSA, Chronopotentiometric stripping analysis; CV, Cyclic voltammetry; CZE, Capillary-zone electrophoresis; DPASV, Differential pulse anodic stripping voltammetry; DPV, Differential pulse voltammetry; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immunosorbent assay; ESI, Electrospray ionization; FD, Flame detector; GF-AAS, Graphite-furnace AAS; HPLC, High-performance liquid chromatography; ICP, Inductively coupled plasma; ICP-OES, ICP optical emission spectroscopy; ID, Isotope-dilution method; MALDI, Matrix-assisted laser desorption-ionization; MS, Mass spectrometry; MT, Metallothionein; PAGE, Polyacrylamide gel electrophoresis; PVDF, Polyvinylidene fluoride; QMS, Quadrupole mass spectrometry; RIA, Radioimmunoassay; RP-HPLC, Reversed-phase HPLC; SBD-F, 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole; SDS, Sodium dodecyl sulfate; SEC, Size-exclusion chromatography; SFMS, Sector-field mass spectrometry; SPE, Solid-phase extraction; SPE-TDI-AAS, SPE terylenedi-imide AAS; SWCSV, Square-wave cathodic stripping voltammetry; TOF, Time of flight; UV, UV spectrometer

Vojtech Adam, Ivo Fabrik, Rene Kizek*
Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic

Vojtech Adam
Department of Animal Nutrition and Forage Production, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic

Tomas Eckschlager
Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine, Charles University, V Uvalu 84, CZ-150 06 Prague 5, Czech Republic

Marie Stiborova
Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic

Libuse Trnkova
Department of Chemistry, Faculty of Science, Masaryk University, Kotlarska 2, CZ-611 37 Brno, Czech Republic

*Corresponding author.
Tel.: +420 5 4513 3350;
Fax: +420 5 4521 2044
E-mail: kizek@sci.muni.cz.

1. Introduction

Metallothioneins (MTs) belonging to the group of intracellular and low-molecular-mass proteins (2–16 kDa) were discovered in 1957, when Margoshes and Vallee isolated them from a horse renal cortex tissue [1]. These proteins have been isolated and studied in a wide variety of organisms, including prokaryotes, plants, invertebrates and vertebrates [2]. Vertebrates MTs are rich in cysteine and have no aromatic amino acids. The metal-binding domain of MT comprises 20 cysteine residues juxtaposed with basic amino acids (lysine and arginine) arranged in two thiol-rich sites called α and β (Fig. 1A). The cysteine-sulfhydryl groups can bind 7 mol of divalent metal ions per mol of MT, while the molar ratio for monovalent metal ions (Cu and Ag) is 12. Although the naturally-occurring protein

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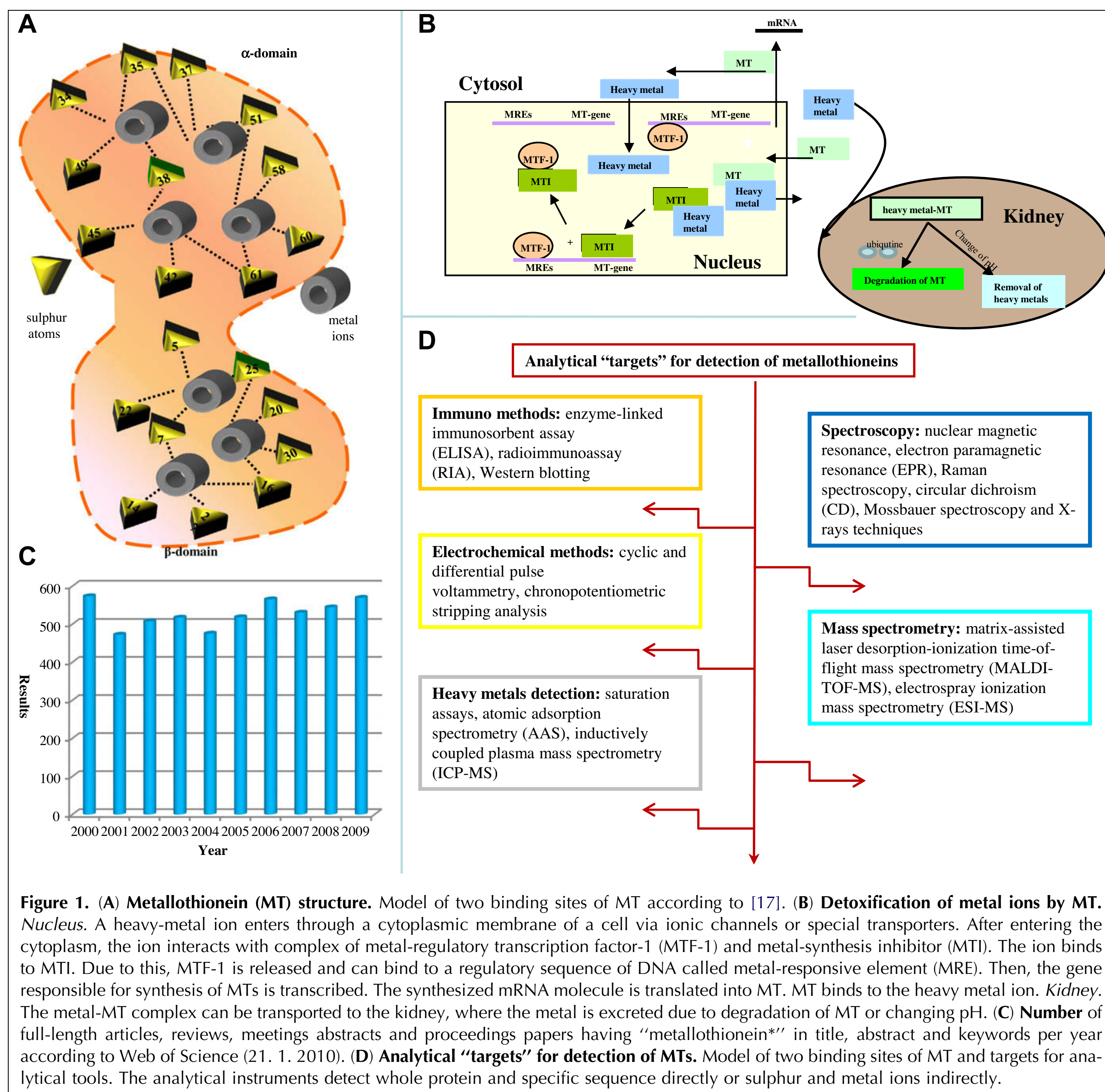

Figure 1. (**A**) **Metallothionein (MT) structure.** Model of two binding sites of MT according to [17]. (**B**) **Detoxification of metal ions by MT.** *Nucleus.* A heavy-metal ion enters through a cytoplasmic membrane of a cell via ionic channels or special transporters. After entering the cytoplasm, the ion interacts with complex of metal-regulatory transcription factor-1 (MTF-1) and metal-synthesis inhibitor (MTI). The ion binds to MTI. Due to this, MTF-1 is released and can bind to a regulatory sequence of DNA called metal-responsive element (MRE). Then, the gene responsible for synthesis of MTs is transcribed. The synthesized mRNA molecule is translated into MT. MT binds to the heavy metal ion. *Kidney.* The metal-MT complex can be transported to the kidney, where the metal is excreted due to degradation of MT or changing pH. (**C**) **Number** of full-length articles, reviews, meetings abstracts and proceedings papers having "metallothionein*" in title, abstract and keywords per year according to Web of Science (21. 1. 2010). (**D**) **Analytical "targets" for detection of MTs.** Model of two binding sites of MT and targets for analytical tools. The analytical instruments detect whole protein and specific sequence directly or sulphur and metal ions indirectly.

has Zn^{2+} in both binding sites, this ion may be substituted for another metal ion that has a higher affinity for thiolate (e.g., Pb, Cu, Cd, Hg, Ag, Fe, Pt and/or Pd) [3,4].

Besides the metal-thiolate clusters and the absence of aromatic amino acids, MTs do not have other characteristic structural features. The primary structure is extremely variable, whereas it is conserved only within closely related species, which makes classification of MTs problematic [5]. The classification system containing three groups of MTs has been proposed and revised several times [6]. Class I comprises all proteinaceous MTs with locations of cysteine closely related to those in mammals. Some molluscs and crustacean MTs belong to this class (e.g., those characterized in mussels, oysters, crabs and lobsters) [6]. Often several paralogues (mostly called isoforms) exist in the genome of a species; four major isoforms (MT-1–MT-4) have been identified in mammals [7]. Class II includes proteinaceous MTs that lack this close similarity to mammalian MTs, while Class III comprises non-proteinaceous MTs, in which some authors include plant heavy-metal-binding peptides called phytochelatins [8].

Based on their metal-inducible properties and their high affinity for metal ions, homeostasis of heavy-metal levels is probably the most important biological function of MTs (Fig. 1B). MTs can also serve as "maintainers" of the redox

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pool of a cell [9]. In mammals, these proteins may serve as a reservoir of metals (mainly zinc and copper) for synthesis of apoenzymes and zinc-finger-transcription regulators. Moreover, new roles of these proteins have been discovered, including those needed in carcinogenesis [10].

According to Web of Science, since 2000, ~500 papers including the metallothionein* term in the title, the abstract and/or the keywords have been published per year (Fig. 1C).

Considerable attention is paid mainly to the involvement of these proteins in various biochemical pathways and their probable using as markers of stress or diseases. To fulfill the requirements of multifarious studies, a wide range of bio-analytical instruments is needed. It is not surprising that techniques using for detection and determination of MTs have been reviewed several times [6,7,11–15]. Isolation, separation, detection and/or quantification of MTs are not easy tasks in modern bioanalytical chemistry. Because of the low molecular mass and unique primary structure of MTs, commonly used methods for detecting proteins suffer from many deficiencies including insufficient specificity and sensitivity. The methods most frequently used for detection of MTs are indirect and based on quantifying heavy-metal ions occurring in their structure or the high content of sulfhydryl groups (Fig. 1D).

2. Isolation procedures

2.1. Blood, blood serum and cells

Isolation and consequent detection of MTs in blood and/or blood-serum samples are not so frequently carried out when compared with their analysis in tissues. Heat treatment of a sample (~100°C for more than 5 min) to denature and to remove high-molecular-mass proteins from samples proposed by Erk et al. [16] is successfully applied to blood and blood-serum samples [17,18]. Moreover, Petrlova et al. showed that using tris(2-carboxyethyl)phosphine as a reducing agent could be beneficial for quantification of MT. The modified method was utilized to prepare blood and blood-serum samples from patients with various tumor diseases [19,20] or fish sperm [21], in which these proteins have not been quantified before.

Caulfield et al. used heat treatment for preparing human red-blood cells [22], which were disrupted by repeated freeze-thawing cycles. The lysates obtained were heat treated and analyzed. The authors had drawn blood from patients by venipuncture into tubes containing heparin. The presence of heparin or other compounds [e.g., ethylenediaminetetraacetic acid (EDTA)] can seriously influence quantification of MT in blood, blood serum or blood fractions, when electrochemical methods are used. Adam et al. showed that the presence of EDTA influenced voltammetric signals markedly [23].

2.2. Animal tissues

To isolate MTs from animal tissues, preparation and purification of crude extract from a tissue by using gel filtration is one of the most commonly used protocols [24]. Tissue extract is prepared in the presence of Tris HCl with added sucrose [25], glucose and antioxidant species (e.g., mercaptoethanol, dithiothreitol and/or TCEP) [26]. This extract is centrifuged or heat treated with subsequent centrifugation.

Erk et al. reported on comparison of different procedures to purify MT from the digestive glands of mussels (*Mytilus galloprovincialis*) exposed to cadmium: heat treatment (at 70°C and 85°C), solvent precipitation and gel filtration [16]. They found that it was most convenient to use heat treatment for preparing both heavy-metal stressed and non-stressed tissues with consequent voltammetric detection. Moreover, Beattie et al. successfully utilized solid-phase extractors for MT isolation [27].

2.3. Plant tissues

Preparation of plant tissues, cells and parts to isolate phytochelatins (included in MT Class III) has been shown in many papers and also reviewed [8]. MT Classes I and II cannot be found in plant tissues without genetic modification of a plant genome.

Macek et al. inserted MT genes from yeast and human into tobacco to enhance their ability to accumulate metal ions [28]. To detect MTs, Diopan et al. prepared crude extracts from these plants and heat treated the extracts. Results on content of MT were similar to those detecting expression of mRNA [29].

3. Direct detection of metallothioneins

Although MTs are low-molecular-mass proteins with very unusual primary structure, several methods have been proposed to detect them directly without using robust electrophoretic and chromatographic separation techniques. Apart from the commonly used immunochemistry and mass spectrometry (MS), electrochemistry can be also used for this purpose (Fig. 1).

3.1. Electrochemical methods

Determination of MTs by electrochemical methods is based on the electroactivity of –SH moieties, which tend to be oxidized or to catalyze evolution of hydrogen from a supporting electrolyte (Fig. 1). To prevent interferences and lower limits of detection (LODs), an adsorptive transfer stripping technique (AdTS) is often coupled with electrochemical methods. The main improvement of AdTS is based on removing the electrode from a solution after accumulating a target molecule on its surface, rinsing the electrode and transferring it to a pure supporting electrolyte, where no interferences are present [23].

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To detect MTs, linear sweep, cyclic, differential pulse and square-wave voltammetry have been used, and reviewed by Sestakova and Navratil [30]. Besides the previously mentioned voltammetric methods, differential pulse voltammetry with a modification called after its founder ''Brdicka reaction'' is the most commonly used electrochemical method for detection of MTs in various types of samples since Olafson optimized it on fish tissues [31]. Over several decades, the method has been optimized with LODs under fM [17]. Temperature of the supporting electrolyte (~5°C) and concentration of cobalt(III) ions (~1 mM) play key roles in reaching the lowest LOD.

Raspor attempted to elucidate the exact mechanisms of this reaction [32]. Based on these results, Raspor and her colleagues have done a lot of work to propose physical and chemical conditions to achieve comparable results in various laboratories [33]. Moreover, sample-preparation steps, including heat treatment (mentioned in Section 2) must precede measurement. Measurements can also be automated and thus used for larger set of samples, as shown by Fabrik et al. [34].

In spite of the fact that the Brdicka reaction is commonly used for detecting MTs, Pedersen et al. showed that differential pulse polarography was found to be unsuitable for crustacean tissues due to unidentified interfering compounds that led to 5–20-fold overestimation of MT levels [35]. The interfering compounds such as other low molecular mass thiols, ionic strength or surfactants contained in a sample can be considered [36].

Besides voltammetric methods, chronopotentiometric stripping analysis (CPSA) can also be utilized for detecting MTs. This method is the most sensitive analytical tool for detection and determination of MT with LODs estimated as units of aM [18]. Reaction and therefore the sensitivity of determination depend on many parameters (e.g., pH and ionic strength of a supporting electrolyte, and isoelectric point of measured protein). Temperature is not a concern compared to Brdicka reaction.

Another study discovered that addition of $[Co(NH_3)_6]Cl_3$ to a supporting electrolyte can increase sensitivity up to 30% [37]. Signal amplification is probably

Table 1. Limits of detection of methods used for metallothioneins (MTs)

Determination	Sample	Method	Limit of detection (μg/ml)	Ref.
MTs	Human-blood serum	DPV-Brdicka reaction ($[Co(NH_3)_6]Cl_3$)	0.0000068*	[93]
MTs	Standard	CV DPV DPV-Brdicka reaction ($[Co(NH_3)_6]Cl_3$)	0.816* 0.00544* 0.0000136*	[17]
MTs	(*Cyprinus carpio L.*) spleen, liver and testes	CPSA	0.0016	[18]
MTs	Cultured human Chang liver cells	HPLC-UV	0.031	[94]
MT-1, MTLP	Earthworm *Eisenia andrei*	HPLC-UV (SBD-F derivatization)	0.06 (5 μg/g of tissue)	[95]
MT-1,2	Mussel hepatopancreas cytosol	CZE-UV	4 (MT-1) 3 (MT-2)	[96]
MTs	Sheep fetal liver	SPE-CE-UV	0.028 (sheep standard MT-1) 0.272 (sheep liver samples)	[97]
MTs	*Caretta caretta, Chelonia mydas*	GFAAS AAS	Cd - 0.2 ng/g of tissue Zn - 0.08 μg/g of tissue Cu - 0.12 μg/g of tissue	[98]
MT-1,2	Rat livers	AE-HPLC-AAS	0.46 (MT-1) 0.31 (MT-2)	[99]
Cd-MTs	Bakers' yeast	ICP-OES	0.0012 (Cd-MT)	[100]
MT-II	Standard	HPLC-ICP-MS	0.000102 (MT-II via sulphur)*	[86]
^{113}Cd-MT	Mouse Hepa 1–6 cells	2D μHPLC-ICP-MS	22.2 (^{113}Cd-MT-I,II)	[101]
MTs	Porcine liver	SEC-RP with ICP-MS and ESI-MS	<0.6 (Cd_7-MT)	[102]
Cd-MTs	*Synechococus sp.*	CE-ICP-MS	0.001 (Cd-MT-I,II, CPN nebulizer) 0.0001 (Cd-MT-I,II MCN nebulizer)	[103]
MTs	Brain tissue	CE-ICP-SFMS	0.011 (MT-1 via ^{114}Cd) 0.023 (MT-2 via ^{114}Cd)	[104]
MTs	*Artemia, Procambarus clarkii*	Saturation method (Ag)	Ag 0.03 ppm	[105]
MTs	Urine, hepatic cytosol	ELISA	0.008	[45]
MT-1	Rabbit-liver and kidney cytosols	ELISA	0.004	[106]
MT-3	Cerebrum (rats)	Western blot	10 μg of protein	[107]

*MT (Mr) = 6800 Da.

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caused by formation of complex inorganic salt-protein. AdTS coupled with the CPSA method was used for detecting MTs expressed in yeast *Yarrowia lipolytica* exposed to Zn, Ni, Co and Cd [38]. However, Petrlova et al. found that the CPSA signal of MT depends on metal content of the sample, because an MT-metal complex gives a lower CPSA signal than metal-free MTs [39]. However, the results can be re-calculated on the content of metals. LODs of electrochemical methods are summarized in Table 1. It is obvious that electrochemical methods are the most sensitive, but they can suffer from misinterpreting the measured data or inadequate sample preparation.

3.2. Immunochemical methods

The second group of methods for MT detection includes use of antibodies (Fig. 1D). It comprises techniques based on immunological detection of MTs in whole tissues [40], particularly, enzyme-linked immunosorbent assay (ELISA) with enzymatically-labeled antibodies, radioimmunoassay (RIA) using isotopically-labeled antibodies and Western blotting. In pathology, immunohistochemistry is widely used to visualize MT distribution in tissues. Frequent targets of analysis are tumor tissues, in which MT expression is changed (e.g., between perineural and non-perineural prostate cancer) [41]. Techniques using fluorescence detection and lanthanide-labeled antibodies have been proposed for detection of MT as more sensitive than RIA [42]. ELISA can be widely modified and optimized for direct MT detection in many types of samples without difficult sample preparation procedures. Potential oxidation of MTs, which can cause 10-fold overestimation of results can be avoided by addition of reducing or chaotropic agents (e.g., mercaptoethanol [43] or Tween-20 [44], respectively). To enhance sensitivity, gel separation can be used before immunoanalysis [45].

A competitive ELISA method using IgG rabbit antibodies was also proposed [46]. This method can be applied for MT detection in both tissues and serum. ELISA was also compared with Brdicka reaction and the results were in good agreement [47]. LODs of the ELISA method are in the region of ng/mL (Table 1).

Another group of immunological methods for MT detection – blotting techniques – use immobilization of protein on membrane [nitrocellulose or polyvinylidene fluoride (PVDF)]. Recently, PVDF membranes were compared to other types of membrane and it was found that combination of PVDF membrane, chicken-yolk antibodies and 3-aminoethyl-9-carbazole as chromogenic substrate was the most sensitive with the LOD estimated as 3 pg MT/μL [48].

In an *in vitro* study, researchers detected MTs by Western blot and dot blot during incubation with S-nitroso-glutathione, reduced glutathione and hydrogen peroxide, and reduced glutathione, and diamide considering possible nitrosylation of MT cysteines, which can occur in a real sample [49].

The main obstacles in using ELISA and other immunological methods are the need to avoid degradation of the target molecule, cross reactivity of polyclonal antibodies and possible interferences of higher metal content. These methods are more suitable for qualitative detection of MTs than their quantification.

3.3. Mass spectrometry

For analysis of proteins by MS, two main ionization techniques are used – electrospray ionization (ESI) and matrix-assisted laser desorption-ionization (MALDI). These techniques are characterized by a soft type of ionization that lowers the probability of protein destruction.

ESI is used in detection systems for on-line protein measurements often within masses in the range 500–3000 *m/z*, in which the separation step is preceded by capillary electrophoresis (CE) or liquid chromatography (LC). The presence of multiple charged ions is not unique to this ionization technique. Thanks to this phenomenon, ESI can be used for masses even above 3000 Da [50]. Soft ionization using ESI can preserve formation of metal-protein complexes, so coupling ESI with MS can help identification of protein isoforms and stoichiometry of metal complexes [51].

Recently, CE coupled with ESI-MS was used for validation of results obtained from electrophoretic mobility equations of MT isoforms depending on pH [52]. An ESI-time-of-flight analyzer (TOF) was used for MT detection in *Mytilus edulis*, whose gene was inserted into *Escherichia coli* culture [53].

MALDI-TOF is soft-ionization technique suitable for protein analysis and is a more tolerant than ESI to higher sample mass and to content of salts, buffers and other substances. MALDI coupled with a TOF/TOF analyzer was used for rapid identification and characterization of MT isoforms from a prostate cell line [54]. MALDI-MS was used for studying of cadmium bonds in MT of the fungus *Heliscus lugdunensis* after cadmium exposure [55]. Binding characteristics of human MT-2 for As^{3+} were investigated among other methods by MALDI-TOF [56] and results were shown to be strongly pH dependent. Moreover, MALDI-TOF seems to be suitable technique for *in vitro* interaction studies of MT with other molecules, mainly metals and metal-based drugs [57].

We can conclude that ESI-MS and MALDI-TOF and their analogues are very convenient for detecting MTs and analysis of *in vivo* prepared mixtures. However, using these techniques for quantification or for analysis of crude or impure samples without good purification steps should cause concern, which needs to be taken into account.

4. Separation methods

The detection methods mentioned above can be used separately, but coupling with appropriate separation

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techniques can bring many advantages (e.g., sensitivity, selectivity, and reduced interferences). Among separation techniques used for MT analysis, various types of electrophoresis and chromatographic instruments can be used.

4.1. Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) belongs to the so-called gold standard in proteomics. Native or denaturating protocols can be utilized for MT detection. Due to low MT mass and their easy reoxidation during an electrophoretic run, it is necessary to use gels with acrylamide concentration ∼15–17.5% or gradient gel electrophoresis.

Native electrophoresis in Tris-glycine buffer with Coomassie Blue and silver-staining visualization was used for densitometric quantification of MT-1 and MT-2 isoforms in animal tissues. Due to this staining protocol, the authors could specifically amplify MT-band intensity, but the LOD (2 μg of MT) was relatively high compared with other proteins [58]. If a real sample is analyzed with good resolution, the proteins can be denaturated by sodium dodecyl sulfate (SDS) [14]. Despite all the advantages of SDS-PAGE, it is not unusual for bands to contain different proteins with the same mobility. Occasionally, the formation of artifacts can be seen due to re-oxidation, which can be observed as a smear or can cause smudgy bands and/or migration of protein of interest at molecular masses higher than expected. This can be prevented by carboxymethylation of a protein by iodoacetic acid prior to the electrophoresis [59]. Nevertheless, LODs of unspecific Coomassie Blue staining, which is difficult due to the absence of aromatic amino acids in MT structure, or silver staining, which is time consuming and may be problematic as some types of metal ions in MTs, mainly Cu [58], vary by μg amounts.

Lower LODs can be reached by autoradiography, storage phosphorimaging or fluorescent staining [60]. Among these types of detection, fluorescent staining is very convenient for detection and quantification of MTs in biological samples, mostly of invertebrates [61,62].

Orthogonal separation techniques are needed to separate MT in the presence of large amounts of other low-molecular-mass proteins or to analyze a sample containing numerous MT isoforms (e.g., coupling isoelectric focusing with PAGE [63], or immunoelectrophoresis based on protein interactions with antibodies incorporated in a carrier [64]).

Another interesting coupling was done by Otsuka et al., who used metal-chelating column chromatography for protein separation according to affinity for Zn and Cd metals and then employed SDS-PAGE [65].

Krizkova et al. used chip-based CE for quantitative study of MT oxidation. After MT oxidation by H_2O_2, they observed marked decrease in peak heights and shift of peak positions to higher molecular mass, which corresponded with the time of oxidation [66].

In general, MT detection and quantification by gel electrophoresis is a relatively difficult task thanks to their small protein mass, but the method is popular for its indisputable advantages, including low cost and ease of use.

4.2. Capillary electrophoresis

CE is a hot topic in proteomics, mainly for its excellent resolution, rapidity, low sample volume demands and ability to separate differently charged and neutral molecules in a run [67]. However, this technique has limitations, most of all, protein adsorption, which can however be prevented by use of lower pH of background electrolytes. Nevertheless, many proteins then become unstable.

To suppress protein adsorption, capillaries are coated by derivatives of cellulose, polysterene nanoparticles, polyamides, polyacrylamides and other polymers also utilized for electro-osmotic flow modulation [68].

A recently published study aimed at separation and detection of MT isoforms by CE coupled with ESI-TOF reported on the ability of these techniques to distinguish even sub-types of MT-1 and MT-2 isoforms [69].

Other authors compared the separation properties of capillary LC and CE using MTs and superoxide dismutase as models. Both separation instruments were coupled with inductively coupled plasma (ICP)-MS detectors [70]. Results, although very similar, support using capillary LC instead of CE, although opinions differ [67].

After several optimization steps, Chamoun and Hagege enhanced the sensitivity of CE-ICP-MS for detection of metalloproteins by more than 6-fold [71].

The great advantages of CE in detection and quantification of MTs are its ability to distinguish MT isoforms in crude samples and its ultralow LODs (Table 1). Commercially-available standards of MT isoforms are not adequate to be used for CE due to impurities and poorly defined mixtures of various MT isoforms. In addition, heat treatment, molecular filtration or solid-phase extraction must be performed before the analysis, so it is necessary to add internal standards (e.g., carbonic anhydrase) to a crude sample.

4.3. Chromatographic methods

Electrophoretic methods mentioned above are convenient for detection of MTs, but they have several limitations, also discussed above. Due to the relatively small molecular mass of MTs, we can also use chromatographic methods.

Gel chromatography fractionating molecules according to their size, is obviously the first separation step. For MT separation, silicates and organic polymers with pores (10–100 nm) are employed as column packing. Water or cytosol-like buffers are used as the mobile phase for preservation of MT nativity, MT-metal complexes and

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minimization of ligand competition. However, under these conditions, MTs bind tightly to the stationary phase. Higher ionic strength required to disrupt the interaction between stationary phase and MT can damage higher structures of MT and their complexes [72].

Another solution to the problem of the interaction between MTs and stationary phases is to use different column-packing materials (e.g., co-polymeric styrenedivinylbenzene). For samples with a higher content of heavy metals, it is appropriate to use orthogonal separation techniques [e.g., CE or high-performance LC (HPLC), particularly ionex chromatography or reversed-phase-HPLC (RP-HPLC)]. Anion-exchange molecules (e.g., weakly basic diethylaminoethyl cellulose) represent suitable ionex chromatography column packings for MT separation [13]. MT isoforms from the bivalve *Laternula elliptica* after cadmium exposure were separated using anion-exchange chromatography [73].

As an alternative, anion-exchange chromatography columns for RP-HPLC can be packed with aliphatic hydrocarbons (C_8 and C_{18}). Decreasing mobile-phase polarity by adding methanol or acetonitrile is often used to elute MTs from hydrophobic chains. Based on the results published, we can conclude that RP-HPLC is more convenient for metallo-complexes, including MT separation, than anion-exchange chromatography.

However, due to the absence of potent interacting moieties for MTs in the stationary phase, RP-HPLC analysis is time consuming [13,74].

Size-exclusion chromatography (SEC) followed by RP-HPLC was used for separating MT isoforms of snails (*Helix aspersa*), which were exposed to Cd^{2+} ions [75].

MS is most often used to identify proteins from fractions obtained after chromatographic separation. If there are no library spectra available, enzymatic digestion of analyte or tandem MS (MS^2) can be used to determine the amino-acid sequence.

Of concern is the connection of hyphenated separation techniques with mass detectors. Unfortunately, each separation technique has its own characteristic flow rate, which does not match the optimum for ionization sources for mass detectors, and, moreover, salt and organic solvents in the mobile phase needed for separation are the main cause of interference. A flow rate suitable for ICP ionization may be very similar to that for HPLC, but the high content of organic substances results in carbon sedimentation on the ICP torch and the plasma thus becomes unstable. This can be prevented by adding oxygen to the plasma gas and removing the solvents. Another solution to this adverse phenomenon is to use capillary HPLC or nanoHPLC, due to the lower content of interfering substances, but flow rates (capillary HPLC 4 μL/min and nanoHPLC 200 nL/min) are insufficient for ICP [13].

Trace-element analysis of cerebrospinal fluid was performed by high-resolution-ICP-MS, whereas MT isoforms in this liquid were separated by SEC-HPLC. Due to non-denaturating conditions in the mobile phase, MT-metal complex formation was preserved [76].

SEC-HPLC coupled with ICP-MS was also used to discover Cu-MT complexes in whey from bovine milk [77].

Chromatographic methods under properly chosen conditions are very suitable tools for detecting MTs. Nevertheless, LODs are higher than for CE and others techniques (Table 1). The advantage of chromatography is the possibility of using an extract from a crude sample without numerous laborious and time-consuming purification steps.

5. Indirect detection of metallothioneins

Besides direct separation and detection of MTs, we can detect MTs indirectly via the content of heavy-metal ions occurring in their molecules. This way of determination has several advantages, especially sensitivity and precision. Saturation protocols and spectroscopic methods are most commonly used for this purpose.

5.1. Saturation assays

Saturation assays, whose principle depends on the different affinity of MTs to heavy-metal ions (affinity decreases in order Hg(II) > Ag(I) ~ Cu(I) > Cd(II) > Zn(II) [78]), can be used for indirect MT detection and determination (Fig. 1). Unsurprisingly, the displacement of metals by Hg(II) (mostly unstable isotope ^{203}Hg), for which MTs have strong affinity, is the most commonly used approach [79].

Recently a new method with stable Hg isotope in low concentrations was proposed [80]. Removal of non-specifically bound metals can be achieved by adding egg-white solution [81].

In addition to mercury, titration by Cd(II) or Ag(I) can be also employed for detecting MTs [78,82]. When using Ag(I), it is necessary to take into account that higher chloride levels cause precipitation with Ag(I) ions.

Van Campenhout et al. quantified MT level by cadmium thiomolybdate titration in carp tissues (*Cyprinus carpio*), but the total content of individual metal ions was determined by ICP-TOF [83]. Saturation assays are the first methods used for quantification with sufficient LODs (Table 1), but there are several limitations in using this method, mainly when copper content is high.

5.2. Spectroscopic methods

The main advantage of metal detection in proteomics is that we can easily quantify target protein, because direct protein detection can be difficult [13,51]. ICP-MS, which can even distinguish heteroatoms other than metals, is the most commonly used tool in indirect detection of proteins. It is possible to detect and to distinguish single elements and their isotopes in real samples [84]. In spite of the fact that ICP-MS is highly sensitive, specificity in

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detection of individual proteins is doubtful, because it is very difficult to distinguish the origin of the elements detected when a mixture of different analytes is measured. ICP-MS is therefore often connected with appropriate separation techniques, mainly CE and/or LC [67]. As detectors, quadrupole, sector-field and/or TOF mass spectrometers are employed. Sector-field MS (SFMS) is overtaking the most frequently used quadrupole MS (QMS), particularly in resolution. ICP-TOF-MS has higher LODs than QMS, but new technological devices to overcome this lack are still being proposed. Moreover, there is no trouble with time between analysis of different masses (spectral skew error) [85]. This distortion can negatively influence the signal height of different masses from fractions with the same retention time.

Because MTs are rich in sulphur and heavy-metal ions, the isotope-dilution (ID) method using ^{34}S isotope can be used for their detection [86]. Comparing non-isotope and isotope signal intensities, it is possible to detect sulphur content and, thus, protein content. Wider application of these techniques remains limited due to un-proportional sulphur distribution in proteins (particularly amino acids cysteine and methionine) or due to the method of detection itself, because sulphur with high first ionization potential gives a low yield of ionized molecules (10%). Moreover, a high-resolution analyzer is required (e.g., SFMS) due to the possible overlap of $^{32}S^+$ with ion $^{16}O_2^+$, which common quadrupole mass spectrometers lack [87]. The overlap problem can also be solved by using a collision cell. The ID method can also be used for detection of metals, as shown by Van Campenhout et al., who utilized SEC-HPLC coupled with ICP-TOF-MS with post-column addition of ^{65}Cu, ^{67}Zn and ^{106}Cd isotopes for detection of MT levels in eels [88].

SEC-HPLC coupled with ICP-MS was utilized to determine MT content in gills, kidneys, livers and muscles of carps (*Cyprinus carpio L.*), which were exposed to Hg, Cd and Pb [89]. Results show disproportional distribution of MTs in such tissues, whereas quantification of MTs in gills is an appropriate biomarker of Cd and Hg pollution in an environment.

The same technique enabled Esteban-Fernandez et al. to investigate the formation of aqua-complexes of cisplatin with MTs *in vitro* and also *in vivo* in kidney, liver and inner-ear cytosol extracts obtained from rats treated with this drug [90].

A narrow-bore HPLC-ICP-MS technique was used in the study of copper metabolism in mutant mice with a functionless gene for ATP-ase transporters of copper Atp7a [91]. Measurements were taken of superoxide dismutase and MT as proteins with copper-binding ability. Results, which were correlated with mRNA detection, showed increased occurrence of Cu-MT in intestine and kidney. However, higher content of Cu-MT did not correlate with mRNA expression, which can be explained by a higher transport rate of Cu-MT into that organ.

In addition to the methods described above, roentgen methods (X-ray fluorescence) and ICP-MS with laser ablation coupled with gel electrophoresis are also used for detection of MTs in gels or on membranes [92]. Although the metal content determined by these techniques can be proportional to MTs, an obstacle to their use is the noise signal generated from the other elements (S or P) and, moreover, the techniques make higher demands on instrumentation and operators. For these reasons, the techniques are not commonly used for MT detection and quantification [15]. Besides these two specific types of detector, spectrometric techniques are the most versatile instruments for MT quantification. They can be coupled with almost all separation techniques, so they can be utilized for quantification of content of all and/or specific MT isoforms and large-scale analyses of impure samples without difficulties.

6. Conclusions and outlook

MTs are of interest in various fields, including environmental chemistry, biochemistry, clinical chemistry, and analytical chemistry. Due to their unique primary structure (no aromatic amino acids, rich in cysteine moieties), MTs are involved in many biochemical pathways (e.g., scavenging of reactive oxygen species, detoxifying various xenobiotics and metal ions, transporting essential metal ions, and cell proliferation). To cast the light on these issues, a battery of precise, sensitive and selective analytical instruments is needed. Due to trends to miniaturize whole detection systems, newly designed instruments based on paramagnetic particles and small, readily portable devices have become the focus of much attention. Moreover, it is reasonable to assume that hyphenated spectroscopic instruments will reveal new transport mechanisms and interactions of MTs with other biologically-active compounds, because it seems that these proteins could also serve as reservoirs of essential heavy metals for other heavy-metal binding proteins, including transcription factors.

Acknowledgements

Financial support from GA AV IAA401990701 and INCHEMBIOL MSM0021622412 is highly acknowledged. The authors wish to express their great thanks to Dr. Harry Martin and Dr. Jitka Petrlova for language correction.

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