# Analytical Methods for Metallothionein Detection

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**Abstract:** Metallothioneins (MTs) are a group of low molecular mass, cysteine-rich proteins with a variety of functions including involvement in metal homeostasis, free radical scavenging, protection against heavy metal damage, and metabolic regulation *via* Zn donation. The overexpression of MTs in proliferating cells is turning the attention to the study of MTs as novel promising marker of tumor diseases. Besides, levels of MTs in invertebrates and aquatic vertebrates well correlate with heavy metal pollution of an environment and, thus, serve as bio-environmental marker. It is not surprising that these proteins are of great interest not only for biochemists, molecular biologists clinical chemists, but also for environmental chemists and ecologists.

Detection and quantification of MTs, which is needed in all above mentioned areas, is challenging due to the high cysteine content and relatively low molecular mass. This review is giving an overview of the analytical methods used for determination of MTs comprising a broad range of immunochemical, electrochemical as well as spectrometric methods, which have been optimized and even hyphenated with different separation methods to detect MTs. More attention is paid to the structural analysis of these proteins as well as the employment of analytical instruments for environmental, biochemical and clinical purposes.

**Keywords:** Metallothionein, Electrochemistry, Immunochemistry, Gel electrophoresis, Capillary electrophoresis, Chromatography, Spectroscopy, Mass spectrometry, Saturation assays, Cancer, Environmental analysis.

## 1. INTRODUCTION

Metallothioneins (MTs) were discovered by Margoshes and Valee in 1957 as newly identified proteins isolated from a horse renal cortex tissue [1]. These proteins occur in whole animal kingdom with high degree of homology. Similar proteins are expressed by bacteria, fungi and even plants. MTs are low molecular mass (from 2 to 16 kDa) proteins with unique abundance of cysteine residues (more than 30 % from all other aminoacids). Other interesting structural property is the presence of no aromatic amino acids. However, as discovered recently the exception is represented by a group of certain yeast and bacterial species rarely containing histidine [2]. The main function of MTs in organism is a metal ion transport, maintenance of the oxidative-reducing conditions and expression regulation (Fig. **1**). Thiols including MT and glutathione are known as effective radical scavengers creating optimal oxidative-reducing conditions to protect cell compartments and biologically important compounds including cell-cycle-enzymes or DNA. MT regulates free radical level also indirectly by binding of metal ions which are potential radical producers, e.g. Cu [3]. As confirmed by several studies [4, 5], MT expression in cells is induced also by superoxide and hydroxyl radicals generated by γ-radiation. It is supposed that MT acts either as a scavenger of radicals or zinc donor for enzymes participating in repairing processes (Fig. **1**).

MTs expression is induced by various factors including presence of metal ions, cytokinins and/or stress hormones [6-8]. Expression of MTs is started by binding of metal regulatory-transcription factor – 1 (MTF-1) to the regulative region of MTs gene called metal responsive element (MRE). Transcription of MTs through the MRE can be initiated by several metal ions (Zn, Cd, Cu, Hg, Pb, Au and Bi) however only Zn can activate MTF-1 [9]. Moreover MRE is capable to interact with many proteins, which can regulate MT expression [10, 11]. Induction of MT expression by chemicals producing free radicals as well as various organic solvents, e.g. ethanol [12] chloroform [13] or tetrachlormethan [14];

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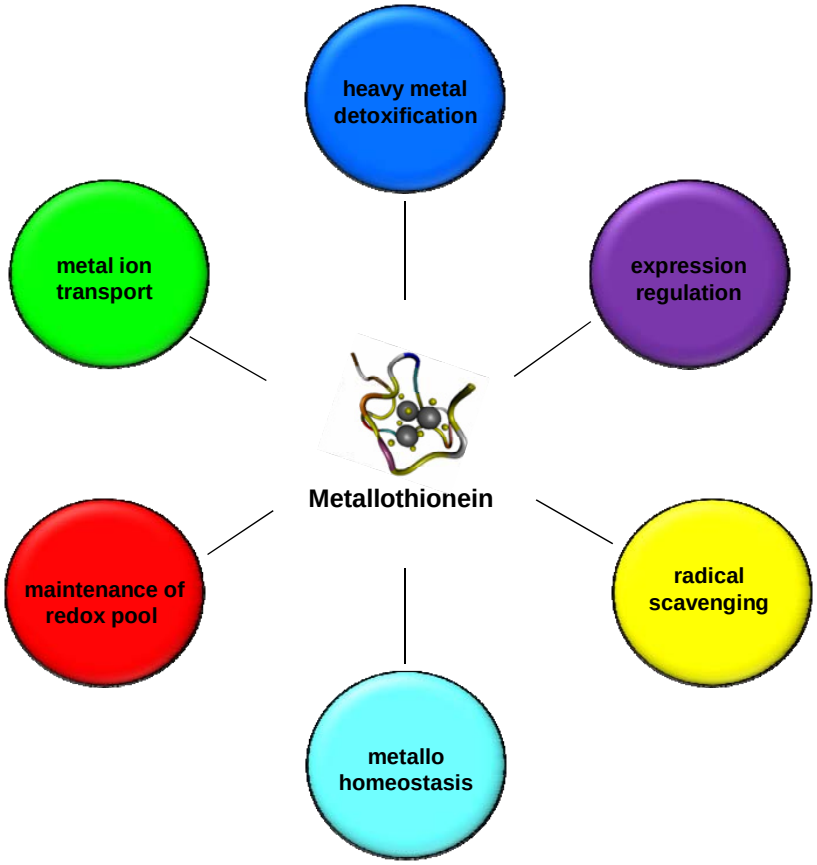


**Fig. (1).** Main functions of metallothionein in an organism. MT participate mainly in maintaining of metal ions homeostasis, toxic metal ions detoxification and metal ions transport. Further, their roles in maintaining redox pool, scavenging of radicals and regulation of expression are discussed.

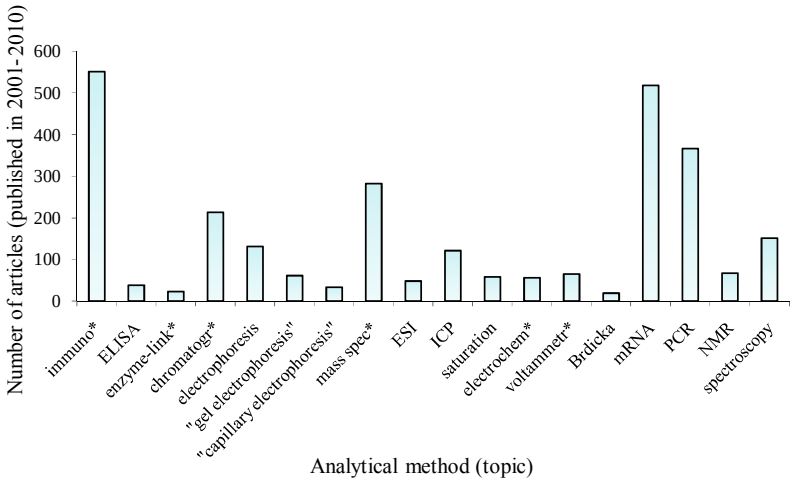


**Fig. (2).** Articles published in 2001-2010 divided according to the specific analytical method (Metallothionein* + method in the chart).

and alkylating agens (bromobenzen [15] and others) has been shown. It has been found that the expression reaches the highest levels in the late G1 phase and during entering the S phase [16]. Nowadays the attention is focused on MT's role in cancerogenesis and on its relation to cancer cell cycle [17-20]. Cytostatics used in chemotherapeutic treatment of tumor disease (bleomycin, adriamycin) are capable to initiate the MT synthesis as well [21].

Due to the involvement in the wide range of cell processes and variety of vital functions, MT is seen as an essential protein in living organisms. Also the potential as an environmental pollution as well as cancer marker has been extensively studied. Moreover the progress in the instrumental analytical techniques enables to study MT in details and in context of the organism. The interest attracted in scientific society can be expressed as a number of publications devoted to this compound. In Fig. (**2**) the number of publication dedi-

**Table 1. Summary of Reviews Related to the Analytical Techniques Used for MT**

| Title | Year Published | First Author | Number of Citations | Ref. |
|---|---|---|---|---|
| Vertebrate metallothioneins as target molecules for analytical techniques | 2010 | Adam V. | 1 | [148] |
| Raman spectroscopy a promising technique for investigations of metallothioneins | 2010 | Torreggiani A. | 0 | [222] |
| The emerging role of ICP-MS in proteomic analysis | 2009 | Bettmer J. | 8 | [223] |
| Fluorescent probes for the structure and function of metalothionein | 2009 | Maret W. | 2 | [34] |
| Elemental mass spectrometry for quantitative proteomics | 2008 | Sanz-Medel A. | 38 | [224] |
| Capillary electrophoresis-high resolution sector field inductively coupled plasma mass spectrometry | 2007 | Sonke J.E. | 18 | [225] |
| The application of inductively coupled plasma mass spectrometry in pharmaceutical and biomedical analysis | 2006 | Huang J.Q. | 19 | [226] |
| Application of CE-ICP-MS and CE-ESI-MS in metalloproteomics: challenges, developments, and limitations | 2005 | Prange A. | 41 | [227] |
| Capillary electrophoresis-inductively coupled plasma-mass spectrometry: A report on technical principles and problem solutions, potential, and limitations of this technology as well as on examples of application | 2005 | Michalke B. | 39 | [228] |
| Recent advances in the elemental speciation by hyphenated techniques | 2003 | Yan X.P. | 7 | [229] |
| Recent developments in quantification methods for metallothionein | 2002 | Dabrio M. | 85 | [230] |
| Multielement trace-element speciation in metal- biomolecules by chromatography coupled with ICP-MS | 2002 | Ferrarello C.N. | 30 | [231] |
| Study of metallothionein using capillary electrophoresis | 2002 | Minami T. | 17 | [123] |
| Hyphenated techniques for the characterization and quantification of metallothionein isoforms | 2002 | Prange A. | 67 | [132] |
| NMR spectroscopic studies of I = 1/2 metal ions in biological systems | 1998 | Oz G.L. | 44 | [232] |
| CD-113 nuclear-magnetic-resonance applied to metalloproteins | 1993 | Coleman J.E. | 73 | [233] |

cated to study of MT by individual analytical method in last decade is shown. It can be concluded that despite the selection of applicable methods, immunoanalytical methods remain the key techniques employed in MT analysis. Also investigation of MT-mRNA is in the centre of interest and rapid expansion of mass spectrometric detection is evident.

The aim of this article is to summarize the analytical methods applied to the structural characterization, localization as well as quantification of various MT forms. Selected review articles related to the analytical techniques employed for determination of MT are summarized in Table **1**.

### 1.1. MT Classification

Historically, MTs were ordered in three classes. Class I comprising all proteinaceous MT with locations of cysteine closely related to those in mammals. Some molluscs and crustacean MT belonged to this class, such as those characterized in mussels, oysters, crabs and lobsters. Class II including proteinaceous MT lacks this close similarity to mammalian MTs, while Class III consisting of non-proteinaceous MTs, in to which some authors included plant heavy-metal-binding peptides called phytochelatins.

However due to the enormous diversity of MT group, complex classification system has been introduced by Binz and Kägi. This system involves families, subfamilies, subgroups and isoforms. Fifteen families include vertebrate, mollusc, crustacean, echinodermata, diptera, nematode, ciliate, fungi-I, fungi-II, fungi-III, fungi-IV, fungi-V, fungi-VI, prokaryota and planta. The biggest family – vertebrate- is subdivided into eleven subfamilies including five mammalian, three avian, and one of batracian, anura and teleost subfamily. Complete MT classification is summarized in Table (**2**). Probably the most widely studied group of MTs is the mammalian subfamily. There are 4 mammalian MT isoforms (MT-1 – MT-4) known and 13 MT-like human proteins were identified [22]. Eleven genes have been discovered for MT-1 isoform (MT-1A, B, E, F, G, H, I, J, K, L a X) and one gene for each other isoform [23]. Differences of constituent forms come mainly from post-translational modifications, small changes in primary structure, type of incorporated metal ion and speed of degradation. Despite the physical-chemical similarity of the forms, their roles and occurrence in tissues vary significantly [7]. MT-1 a MT-2 are present almost in all type soft tissues [24], MT-3 is expressed mostly in brain tissue but also in heart, kidneys and

**Table 2. MT Classification According to Binz and Kägi**

| Family | Subfamily |
|---|---|
| vertebrate MTs | m1: mammalian MT-1 |
| | m2: mammalian MT-2 |
| | m3: mammalian MT-3 |
| | m4: mammalian MT-4 |
| | m: n.d. mammalian MT |
| | a: n.d. avian MT |
| | a1: avian MT-1 |
| | a2: avian MT-2 |
| | b: batracian MT |
| | ba: anura MT |
| | t: teleost MT |
| mollusc MTs | mo1: mussel MT-1 |
| | mo2: mussel MT-2 |
| | mog: gastropod MTs |
| | mo: other mollusc MTs |
| crustacean MTs | c1: crustacean MT-1 |
| | c2: crustacean MT-2 |
| | c: other crustacean MT |
| echinodermata MTs | e1: echinoidea MT-1 |
| | e2: echinoidea MT-2 |
| diptera MTs | d1: diptera MT-1 |
| | d2: diptera MT-2 |
| nematoda MTs | n1: nematoda MT-1 |
| | n2: nematoda MT-2 |
| ciliata MTs | ci |
| fungi-I MTs | f1 |
| fungi-II MTs | f2 |
| fungi-III MTs | f3 |
| fungi-IV MTs | f4 |
| fungi-V MTs | f5 |
| fungi-VI MTs | f6 |
| prokaryotic MTs | pr |
| plants MTs | p1: plant MT type 1 |
| | p2: plant MT type 2 |
| | p2v: clan of variants to p2 |
| | p3: plant MT type 3 |
| | pec: plant EC protein |
| | p21: plant MT type 2x1 |

n.d. – refer to the sets of sequences of insufficient phyligenetic or structural information an assignment to an already specified subfamily is not feasible

reproductive organs [25] and MT-4 gene was detected in epithelial cells [26]. MT without metal ion, apo-MT, is present in zinc deficient cells. Recently, this form was discovered also in tumor cells. It was observed that apo-MT can remove Zn from zinc fingers (Sp-1) and transcription factors (TFIIIA) [27, 28]. Among others this mechanism is probably used by tumors to avoid apoptosis and accelerate its proliferation [29].

As mentioned previously, mussels belonged to the class I, however in classification according to Binz and Kägi there is a family devoted to mollusc, which is further divided into four subfamilies. Two of them are specifically dedicated to mussels. In contrary to the mammals, two groups of isoforms; MT-10 and MT-20 were identified in this species. The most significant difference between MT-10 and MT-20 is the presence of two additional cysteine residues in the MT-20 isoforms. The exact role of each specific MT isoform or isoform group has not been clarified yet, but there are some indications that MT-20 could play a role in detoxification of cadmium in exposed mussels [30].

Analytical methods used for study of particular MT isoforms are summarized in Table (**3**). It clearly follows from these data that polymerase chain reaction is the most commonly used technique for detection of MT. However separation techniques combined with selective and sensitive detection method are also often used.

## 2. STRUCTURAL ANALYSIS OF METALLOTHIONEIN

### 2.1. Metallothionein Structure

MTs are single-chain proteins with amino acid number oscillating between app. 20 and more than 100 residues according to organisms. Almost one third of this number is cysteine occurring in conserved sequences *cys-**x**-cys*, *cys-**x**-**y**-cys* a *cys-cys* where **x** and **y** represent other amino acid. Divalent metal ions bonded to sulfhydryl groups of cysteines are creating tetraedric configuration of thiolate clusters [7]. MT exhibit the highest affinity for $Cu^{+}$ (stability constant $10^{19}$ - $10^{17}$), followed by $Cd^{2+}$ ($10^{17}$ – $10^{15}$) and $Zn^{2+}$ ($10^{14}$ – $10^{11}$); however it is not capable of binding $Cu^{2+}$. Generally, 18 metal ions suitable to be bonded by MT are known but only $Cu^{+}$, $Cd^{2+}$, $Pb^{2+}$, $Hg^{2+}$, $Ag^{+}$ a $Bi^{2+}$ can replace $Zn^{2+}$ in MT structure. Binding capacity of MT is 7 and 12 atoms for divalent and monovalent ions, respectively. MT`s tertiary structure consists of two domains: more stable α (C-terminal), containing 4 ion binding sites, and β (N-terminal) capable to incorporate 3 ions [31].

### 2.2. Analytical Methods for MT Structure Determination

$^{113}Cd$ and $^{1}H$ NMR nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), Raman spectroscopy, circular dichroism (CD), Mossbauer spectroscopy and techniques based on absorption and diffraction of X-rays have been utilized for structural analysis of MTs [32]. X-ray diffraction results together with NMR data have established that the metals are tetrahedrally coordinated in two isolated domains in mammalian Cd-7-MT and Zn-7-MT, and recently, combination of $^{113}Cd$ NMR, CD and mass spectrometry showed that α domain of MT was capable of binding more than 5 $Cd^{2+}$ ions [33].

**Table 3. Summary of Analytical Methods Used for Detection and Analysis of Particular Isoform**

| Isoform | Analytical Method | Sample Type | Ref. |
|---|---|---|---|
| MT 1 | mRNA RT-PCR | human peripheral blood lymphocytes | [234] |
| | semi-quantitative RT-PCR | rat testes and liver (Cd injection) | [235] |
| | RT-PCR | mouse cerebral cortex | [236] |
| | HPLC-ICP-TOF-MS | cytosols of gibel carp | [141] |
| | RT-PCR ,Western blotting | AML-2 cell line | [237] |
| | CE | horse kidney | [238] |
| | CE-ICP | rabbit liver | [239] |
| MT 1 and 2 | mRNA QRT-PCR | in situ prostate tissue | [240] |
| | real-time PCR | HeLa cells | [241] |
| | 2D micro HPLC- ICP-MS<br>RT-PCR | isoform-specific knockdown cells | [142] |
| | saturation with cadmium chloride,<br>ion-exchange HPLC, Western blot<br>AAS-ICP | human placenta | [242] |
| | Immunohistochemical staining<br>Real time RT-PCR, immunoblot | human breast and the MCF-10A cell line | [243] |
| | ELISA | in plasma of humans and experimental animals (rabbit, mouse rat) | [244] |
| | SDS-PAGE, Western blot immunofluorescence staining | prostate cells (BPH, PC-3 and HPR-1 cells) | [48] |
| | RT-PCR<br>agarose gel electrophoresis | $CD61^+$ megakaryocytes | [245] |
| | real-time PCR | liver tissue of lake trout | [246] |
| | cadmium–hemoglobin assay<br>real-time RT-PCR | liver and kidney tissue, blood of human and rats | [247] |
| | tryptic digestion, MALDI-TOF/TOF MS | RWPE-1 cells derived from normal human prostate epithelium | [197] |
| | Western blotting | superoxide dismutase 1 transgenic (SOD1 Tg) mouse | [248] |
| | RT–PCR and in situ hybridisation | hippocampus of old rats | [249] |
| | quantitative real-time RT-PCR | breast cancer cell lines | [250] |
| | real-time RT-PCR | keloid keratinocytes | [251] |
| MT 2 | semi-quantitative RT-PCR - densitometry scanning<br>ELISA<br>AAS | testicular interstitial cells (Sprague-Dawley rats) | [36] |
| | CZE | cytosol of the pancreas and liver in mice | [124] |
| | anion-exchange HPLC, RP-HPLC<br>MALDI-TOF-MS | rabbit liver | [252] |
| | RT-PCR | bone tissue of rats | [253] |
| | RT-PCR | dental pulp of rat incisors | [254] |
| | immunohistochemistry | hyperplastic, dysplastic and neoplastic prostatic lesions | [255] |
| | TaqMan(R) RT-qPCR | earthworms (*Lumbricus rubellus*) | [256] |

**Table 3. contd……**

| | | | |
|---|---|---|---|
| MT 3 | Immunohistochemical staining<br>RT- PCR | transformed human urothelial cells | [257] |
| | RT-qPCR<br>microarray | pituitary adenomas | [258] |
| | Immunoaffinity chromatography<br>SDS-PAGE<br>trypsin digestion, ESI-MS | Swiss-Webster mice brain extract | [259] |
| | RT-PCR | Human kidney (human proximal tubule cells) | [260] |
| | RT-PCR | prostate cells (BPH, PC-3 and HPR-1 cells) | [48] |
| | RT-PCR | mouse cerebral cortex | [236] |
| | immunoaffinity chromatography, SDS-PAGE<br>trypsin digestion<br>MS<br>Immunohistochemical Localization | mouse brain | [261] |
| | RT-PCR | human salivary gland | [262] |
| | RT–PCR and in situ hybridisation | hippocampus of old rats | [249] |
| | RT-qPCR | nasopharyngeal cancer (NPC) cell lines with laryngeal carcinoma and embryonic lung cell lines | [263] |
| | ESI-MS | zinc-enriched neurons | [264] |
| | RT-PCR<br>immuno-blot | estrogen receptor positive (MCF-7 and T-47D)<br>estrogen receptor negative cell lines (Hs578T and MDA-MB-231) | [265] |
| MT 5 | RT-PCR<br>immuno-blot | human kidney (proximal tubule cells) | [266] |
| | RT-PCR | dental pulp of rat incisors | [254] |
| | Rt-qPCR | *Tetrahymena thermophila* | [267] |
| MT 10 and 20 | Circular dichroism<br>UV spectra at 254nm<br>Emission intensity at 569 nm (different Cu:MT ratios)<br>SDS–PAGE | sea mussel (*Mytilus galloprovincialis*) | [268] |
| | Semi-quantitative RT–PCR<br>Non-radioactive in situ hybridization | mussels (*Mytilus galloprovincialis*) | [71] |
| | Northern blot | mussel (*Mytilus edulis*) | [269] |

Optical spectroscopy, and in particular CD and luminescence have provided details of a complicated metal binding chemistry whether metals are added directly to the metal free, apo-metallothionein, or to the zinc containing protein. Due to the absence of aromatic amino acids in MTs primary structure the UV-VIS spectrometric methods are applicable for apo-MT analysis only in deep UV range (below 220 nm), however metal-MT can be determined at wavelengths between 250-800 nm due to the MT-metal bond. Similarly, CD can be employed for analysis of more or less metal saturated MT and for evaluation of effects on kinetic and thermodynamic of MT-metal bond formation [32]. Recently, Wolfgang Maret published a review paper highlighting the importance of fluorescence based method particularly fluorescence resonance energy transfer (FRET) for structural analysis of MT [34].

## 3. QUANTITATIVE AND QUALITATIVE METALLOTHIONEIN DETERMINATION

### 3.1. Molecular Biology Methods

#### *3.1.1. Immunochemical Methods*

Immunochemical methods (immunoassays) are based on the interaction of antigen and antibody and include a) enzyme-linked immunosorbent assay (ELISA) b) radioimmunoassay (RIA), c) fluorescence immunoassay (FIA), and d) western blotting. Polyclonal as well as monoclonal antibodies can be employed for all mentioned immunoassays of MTs. Rat and rabbit, sheep, goats and chickens can be used as a source of polyclonal antibodies.

ELISA is a routine method for the determination of target protein based on the reaction between protein of interest (an-

tigen) and specific antibody immobilized on solid support. Subsequently, the conjugate of antibody with certain enzyme is added to visualize the antigen-antibody interaction by addition of the enzyme substrate and measuring of the product signal. Direct, indirect, competitive, sandwich and multiplex types of the assays have been used. ELISA has been applied for MT determination is number of samples including hepatic cell lines [35], Sertoli cells of rats exposed to cadmium [36-38] and urine of children living in polluted environment [39]. The investigation of relationships between MT level and tumor diseases has shown down regulation of MTs in thyroid carcinoma [40] and MT overexpression in oral squamous cell carcinoma [41], in hepatocellular carcinoma [42] and also in laryngeal carcinoma [43]. MTs was also detected in actinic skin non-melanoma carcinoma [44], breast cancer [45] and adjacent tissue [46], in human ovarian and abdominal wall endometriomas [47]. In another study, the influence of zinc intake on MTs expression was investigated in malignant and healthy prostate cells [48].

RIA is an immunochemical method requiring isotopically labelled antigens, which competitively bind to antibodies. Amount of antigen present in the sample is calculated from remaining radioactivity after separation of unbound molecules. Despite the limitation caused by special precautions since using the radioactive materials it has been utilized in range of studies such as RIA monitoring of MT levels in hepatocytes of patients with viral hepatits C [49], in tumor tissues of gastrointestinal carcinomas [50, 51] and in ovarian cancer tissues [52]. It was shown that expression of MT was lower in mice with knock-out gene for Interleukin-6 compared to wild-type mice after exposition to ultraviolet B irradiation [53]. It was also used to detect MTs in transgenic mice with neurological disorder, resembling many of the features of human diseases (multiple sclerosis, Aicardi-Goutieres syndrome and others) [54, 55].

FIA is based on the use of fluorescently labelled antibodies. Several modes such as time-resolved FIA (TR-FIA) and/or fluorescence quenching FIA can be identified. TR-FIA method allows time-resolved detection of antibody binding using specific fluorescence of rare earth ions measured after short-life background fluorescence in the sample. Thus the interference of background fluorescence is eliminated and the sensitivity is enhanced. Moreover the advantage over the RIA is the fact that the radioactive labeling process is omitted. TR-FIA protocol for MT analysis has been developed by Butcher *et al.* allowing measurement of low MT levels that are undetectable by RIA and ELISA methods [56].

Fluorescence quenching immunoassay can be based on two principles. At the first type of the fluorescence immunoassay quenching occurs due to the change in the microenvironment of the fluorophore. The second type of fluorescence quenching immunoassay utilizes analyte–fluorophore conjugates which fluorescence is quenched due to antibody binding [57]. Fluorescence quenching immunoassay has been successfully applied for MT determination by Yao *et al.* [58] The MTs recovery in human serum within the range from 95 % to 100 % was reported with the relative standard deviation of immunoassay less than 10 %.

Western blot is another commonly used immunological method. Proteins are separated by gel electrophoresis and then transferred to a membrane (typically nitrocellulose or PVDF), where they are detected using antibodies specific to the target protein. To obtain good results both electrophoretic separation and immunodetection has to be optimized. For more information about gel-electrophoretic techniques see section 3.2.1.1. Already in 1996 Mizzen *et al.* described a method able to detected isoforms MT-1, -2 and -3 by immunoblotting on both membrane types (nitrocellulose and PVDF) [59]. Later on, MT and endothelial nitric oxide synthase were analyzed by Western blot for evaluation of quercetin effect on cadmium induced hepatotoxicity in rats [60]. The same method was applied also for determination of MT in cells of prostate cancer in hypoxia [61] or mesothelial and mesothelioma cells treated by zinc [62]. *In vitro* study monitoring MT by Western blot and dot blot during incubation with GSNO, GSH with $H_2O_2$ and GSH with diamide considering possible nitrosilation of MT cysteines was conducted by Casadei *et al.* in 2008 [63]. Recently, the use of PVDF membranes was compared to other types of membranes 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 detection limit estimated as 3 pg of MT [64].

It can be concluded that immunochemical methods are essential techniques used for MT detection. The main obstacles in using ELISA and other immune methods are the need to avoid degradation of the target molecule, cross reactivity of polyclonal antibodies and possible interferences of higher metal content, thus optimization for sample and antibody type is necessary.

### *3.1.2. MT-mRNA Analysis*

Gene family of MT consists of 17 sub-genes on human $16^{th}$ chromosome (thirteen for MT-1, two for MT-2, one for MT-3 and one for MT-4). Monitoring of MT-mRNA is commonly carried with real time polymerase chain reaction (RT-PCR) or microarrays. Advantage of this approach is distinguishing of MT isoforms, however the fact that mRNA amount could not match with protein concentration, is a concern [65]. Protocols were suggested for semiquantitative analysis and were utilized for MT-mRNA detection in human kidneys [66] and on lymphocytes of workers exposed to cadmium [67]. The quantitative protocols using cRNA as inner standard of known concentration were used for detection of prostatic MT-mRNA of rats [68]. Based on these and many other results it can be concluded that expression of MT is detected in several cases. Significant attention is paid to MT-mRNA level of various animals due to environment pollution monitoring and for determination of homology level among MT isoforms. Cockle [69], catfish (QRT-PCR) (quantitative reverse transcription – polymerase chain reaction) [70], mussel (RT-PCR) (reverse transcription – polymerase chain reaction) [71, 72], seal pups (RT-PCR) [73], fish *Hemiborbus mylodon* [74], puffer fish *Takifugu obscurus* [75], calm *Mytilus galloprovincialis* [76], oyster *Crassostrea gigas* [77] and *Crassostrea virginica* (QRT-PCR) [78] or medaka (QRT-PCR) [79] were analysed due to pollution of environement. Mutants of *Drosophila* [80] and localization of isoforms specifically binding certain metals at snail [81]

were investigated due to studying of homology level among MT isoforms. Processes connected with the effects of zinc(II) ions on an organism were examined by studies aimed at MT expression changes in men supplemented with this essential metal [82], analyzing hepatic and intestinal transcriptome of rats under various dietetic conditions [83, 84], mice with mutant gene for zinc transport protein ZnT4 by QRT-PCR [85], cells of human colon adenocarcinoma which were treated with $Zn^{2+}$ [86], intestinal absorption of zinc in broilers (MT, ZnT1 and ZnT5) [87] or in chicks, at which appropriate zinc(II) dose in food was investigated (RT-PCR) [88]. In the paper focused on procyanidins it was discovered using RT-PCR that polyphenols down regulated hepatic MT gene expression in healthy rats to 30 % of control groups. Similar effect was observed in human hepatic cell lines HepG2 [89]. In rats treated with cadmium(II) ions MT expression increased due to application of *Chlorella vulgaris* which helped lower heavy metal toxicity [90]. MT-mRNA in different parts of rhesus monkey brain was monitored in association with airborne-manganese exposure [91]. Lithium cation effects on gene expression in mice brain were investigated by microarrays and RT-PCR and results showed MT-3 gene overexpression [92]. In mice with high cadmium(II) absorption from food (thanks to low-Ca diet) MT-mRNA overexpression was detected in liver and kidneys [93]. Effects of copper(II) ions and insecticide esfenvalerate on MT transcription were quantified using QRT-PCR in stripped bass (*Morone saxatilis*) [94]. Last but not least MT-mRNA studies were aimed also at antioxidative activity of MT. Expression of MT, SOD2 and spermidine/spermidine N-1-acetyltransferase were monitored in cows by QRT-PCR with respect to involution of the bovine mammary gland [95]. Further, olive flounder (*Paralichtys olivaceus*) were treated with benzo[a]pyrene and quantitative PCR showed the increase of transcription of MT and SOD genes [96].

### 3.2. Bioanalytical Methods

#### *3.2.1. Separation Methods*

The complexity of real biological samples requires employment of not only sample pre-treatment methods involving extraction and purification but also separation techniques prior to the selected detection. Chromatographic and electromigration methods are well established commonly used methods enabling elimination of potentially interfering compounds and improving significantly the determination of the target analyte. Even though the separation step is vital, it can not exist on its own. Therefore, sufficient detection technique is necessary, which is discussed separately.

##### *3.2.1.1. Gel Electrophoresis*

Gel electrophoresis (GE) is routine method used in biochemical laboratories for identification of bioanalytes such as proteins and/or nucleic acids.

Due to its structural and chemical properties – low molecular weight, heavy metal content and high-abundant thiol groups, the protocols commonly used for proteins electrophoresis may bring poor results. Generally, isolation of metalloproteins with non-covalently bound metal requires a mild, non-denaturing condition. Treatment of metalloprotein with denaturing sodium dodecyl sulfate (SDS) gel electrophoresis will result in a loss of metal [97]. Due to low molecular mass of MTs and their easy reoxidation during electrophoretic run, GE analysis may be challenging and gels with acrylamide concentration app. 15 – 17.5 % or gradient gel electrophoresis has to be used. Traditionally radioactive isotope labeling is used for detection of heteroatom-containing proteins in GE. Another alternative represents gel electrophoresis with laser ablation and mass spectrometry.

SDS polyacrylamide gel electrophoresis, (SDS-PAGE) and electrochemical methods were used for monitoring arsenic influence on MT levels of clams (*Corbicula fluminea*) [98]. Metallothionein-like proteins and metalloproteins in sunflower were also separated by SDS-PAGE followed by synchrotron radiation X-ray fluorescence (SRXRF) detection and data were evaluated by neural network [99]. In the paper focusing on protein with affinity for Zn and Cd in HeLa cells (cell line with unlimited proliferative potential derived from cervical cancer [100]) separation was achieved by metal-chelating column chromatography for protein separation according to their affinity for these metals and bond strength [101]. Gel electrophoresis was following separation step for mass fractionation. Transcription factor for MTs of fungus *Neurospora crassa* responsible for MT expression in the presence of copper was analyzed by two-dimensional electrophoresis. Protein binding on calcineurin-dependent response element was digested by trypsin after the isolation and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [102]. Moreover, SDS-PAGE combined with specific fluorescence detection has been showed as a very sensitive method for determination of MTs in biological samples of various types [103-105].

Tricine - SDS-PAGE is commonly used to separate proteins in the mass range 1 - 100 kDa. Tricine (N-(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine), used as the trailing ion, allows a resolution of small proteins at lower acrylamide concentrations than in glycine-SDS-PAGE systems. It is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa [106-108]. A method using the thiol-selective fluorescent tag, monobromobimane, coupled with Tricine-sodium dodecyl sulphate-urea polyacrylamide gel electrophoresis for a sensitive determination of both PCs and MTs was reported [109].

Isoelectric focusing (IEF), also known as electrofocusing, is a technique for separating different molecules by their electric charge differences. It is a type of zone electrophoresis, usually performed in a gel, but also in a capillary [110, 111], that takes advantage of the fact that a molecule's charge changes with the pH of its surroundings. Proteins are introduced into an immobilized pH gradient gel composed of polyacrylamide, starch, or agarose where a pH gradient has been established. This method was for the first time applied for separation of two MT isoforms in 1972 [112]. Metallothionein-II (MT-II) and Cu, Zn-superoxide dismutase (Cu,Zn-SOD) interacted with mercury were detected by a method utilizing isoelectric focusing-agarose or -polyacrylamide gel electrophoresis (IEF-AGE or IEF-PAGE) and nondestructive one-dimensional synchrotron radiation X-ray fluorescence (SR-XRF) analysis [113]. IEF was also used to separate and detect metallothioneins and metalloproteins from human liver cytosol [114, 115], mammalian tissues

[116], urine [117] and to study metallothionein-like substance from cyanobacterium *Synechocystis* [118]. Isoelectric focusing is the first step in two-dimensional gel electrophoresis, in which proteins are first separated by their pI and then further separated by molecular weight through SDS-PAGE. This method is used for whole proteomes analysis or for analysis of complex protein mixtures extracted from cells, tissues, or other biological samples [119-121], the proteins are then identified immunochemically or by mass spectroscopy or the images of stained gels are compared by the software.

#### *3.2.1.2. Capillary Electrophoresis*

Capillary electrophoresis (CE) is nowadays well established and very powerful separation tool for analysis of complex biological samples. The extreme separation power combined with short time of analysis and low sample requirements are main advantages of this effective analytical technique. In comparison with classical electrophoresis, the use of thin capillaries (25-100 μm I.D.), removes temperature gradient created by current flow and, therefore, eliminates the molecule diffusion and band broadening [122]. Another important factor in CE is an electroosmotic flow enabling simultaneous detection of positively and negatively charged analytes as well as neutral molecules. On the other hand, conventional gel electrophoresis is not limited by protein sorption on the capillary wall, which has a great impact on the CE analysis and has to be addressed. In 2002 a comprehensive review on MT determination by CE has been published by Minami *et al.* [123]. This paper is paying special attention to the elimination of the wall adsorption by polyacrylamide coating of inner capillary walls which is often used method for MT separation [124, 125]. Another attempt of MT isoforms separation improvement by addition of cyclodextrines was done by Wilhelmsen *et al.* [126]. The results showed that the presence of cyclodextrines improves significantly the separation efficiency of the various MT forms from horse kidney both below and above isoelectric point of MTs.

CE is suitable for the separation of low $M_r$ molecules and therefore it is optimal for MT analysis. One of the advantages of CE is the possibility of coupling with a range of detectors providing a variety of information about the sample. Standard UV/Vis absorbance detectors were used in experiments in 1990's [127-129]. The advantage of this type of detection is the relative versatility in the deep UV range; on the other hand this is the main reason of interferences. Therefore other detection types such as CE with inductively coupled plasma mass spectrometry (ICP-MS) are tested [130, 131]. The main limitation of this method has been the coupling of the separation and ionization. The efficient design of the nebuliser and interface of CE-ICP-MS is nowadays commercially available. This method is, thus, highly sensitive and applicable in metalloproteomics. Most CE-ICP-MS applications are focused on MT isoform characterization [132, 133]. ICP-MS and electrospray ionization mass spectrometry (ESI-MS) have been successfully applied in parallel to the characterization of metallothionein. In 2000, Mounicou *et al*. used the combination of CE-ICP-MS and CE-ESI-MS to identify MT isoforms. Moreover metal stochiometries of the different isoforms in commercially available MT samples were determined [134]. Later similar approach to characterize the MT isoforms from liver extracts based on the Cu:Zn:Cd stoichiometric ratio was used [135, 136]. It should be noted that CE is very suitable for miniaturization a chip-based CE methods are of a great interest in last decade. Obviously also in MT analysis, chip-based CE with laser-induced fluorescence detection has been applied in work of Krizkova *et al*. focused on the MT oxidation by $H_2O_2$ [137].

#### *3.2.1.3. Chromatography*

One of the advantages of chromatographic methods is the diversity of this group offering the choice of the appropriate method optimal for particular analyte. In principle, two ways of liquid chromatographic (LC) methods classification can be distinguished. The first way is based on the retention mechanism including adsorption, partition, size exclusion, affinity, and ion exchange. The other is based on the separation principle, where the methods of removing the analytes from the column are elution, displacement, and frontal analysis [138].

Chromatographic methods are indispensable techniques used for analysis complex biological matrices including blood serum, liver and/or kidney samples. Various modes such as size exclusion chromatography (SEC) [139, 140], ion exchange (IE) [141] and even two dimensional HPLC [142] have been successfully employed in MT analysis. Due to the relatively poor sensitivity and no metal-specific capabilities of UV detection hyphenation with more sensitive element- or molecule-specific detectors is essential. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) and/or ICP-MS are fulfilling the requirements, however coupling with conventional chromatographic techniques is significantly easier due to the higher flow volumes in comparison with CE. During research of MLP (metallothionein-like proteins) of mussels (*Mytilus galloprovincialis*) MT isoforms were separated by IE-HPLC with ICP-OES detection and for isoform isolation from muscle tissue and gills pressurized-liquid extraction was employed [143, 144]. For study of zinc transport between MT and carbonic anhydrase SEC and IE-HPLC with ICP-MS detection were used [145]. Isoforms of metallothionein were identified and quantified from liver extracts based on their Cu, Zn and Cd stoichiometry using online and offline HPLC or CE-ESI-MS methods [46, 47]. All of the mentioned applications show that LC is convenient technique for separation of MTs, however, without broader usage due to high costs and demands on operator and sample preparation.

### *3.2.2. Detection Techniques*

#### *3.2.2.1. Electrochemical Methods*

Latest developments of electrochemical methods yielded the most significant advances in the field. Hence voltammetry, chronopotentiometry and polarography applications have lowered the detection range to as low as $10^{-7}$ to $10^{-10}$ M, and recently even zeptomolar sensitivity has been reported. Despite these attractive perspectives, electrochemical approaches entail high preparation purity, due to the interference caused by different compounds in the sample [146]. Electrochemical methods are used mainly detection of MTs

as environmental pollution biomarker. Determination of MT by electrochemical methods is based on electroactivity of -SH moieties, which tend to be oxidized or catalyze evolution of hydrogen from a supporting electrolyte. An adsorptive transfer stripping technique is often used to increase the sensitivity. MT is accumulated on surface of hanging mercury drop electrode (HMDE), then ballast substances are washed out and MT-modified electrode is inserted into measuring vessel with appropriate supporting electrolyte [147]. Voltammetric methods in various modes including linear sweep, cyclic, differential pulse and square wave have been used to detect MT [148]. Nevertheless, the most sensitive ones are based on measuring of catalytic evolution of hydrogen from a supporting electrolyte.

- ***Brdicka Reaction***

Brdicka reaction is often used electrochemical method for MT determination in biological samples [149-156]. The mechanism is discussed elsewhere [157]. Briefly, the reaction is based on the interaction of hexaamminecobalt chloride complex ($[Co(NH_3)_6]Cl_3$) with protein containing –SH group. The height of the last signal of Brdicka reaction voltammogram of real sample depends on the MT concentration [149]. It is obvious that sample pre-treatment has a strong impact on the analysis. In case of MTs, its thermostability is successfully utilized and interfering components can be easily removed from the mixture by heat treatment [158]. MT is not the only thermostable protein remaining in the solution after heat treatment, but it is usually the only species having significant electrochemical activity [159].

Electrochemical methods have been effectively used for determination of MT in clams [98, 160-162] showing optimal sensitivity. Various methods for determination of MT-like proteins in plants [163, 164], yeasts [165], fishes [166, 167], earthworms [168] and other species [169, 170] have been optimized. Moreover, electrochemical methods were used for determination of MT in blood serum of patients with brain injury [171] and with tumor disease [148-150, 154, 172, 173]. Good agreement of results provided by Brdicka reaction with results obtained by immunoanalysis was demonstrated [154, 174]. Moreover, an automated electrochemical analyzer was recently utilized for detection of metallothionein [152].

- ***Peak H***

The first papers focused on application of H-peak in protein analysis were published twenty years ago [175, 176]. 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 time and potential derivations (dt/dE). Chronopotentiometric stripping analysis (CPSA) represents one of the most sensitive methods for detection of peptides and proteins even in subnanomolar amounts [177]. The character and origin of the catalytic peak H is not clear yet. 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 supporting electrolyte (as in Brdicka reaction) is needed and results described in a work of Strouhal *et al.* [165] showed that optimal buffer composition was borate 0.1 M $H_3BO_3$ + 0.05 M $Na_2B_4O_7$ (pH = 8.0). pH is crucial for the height and position of signal and suitability of borate buffer can be explained by the ability to act as proton donor [178]. Catalytic process during MT analysis also depends on oxygen content in electrolyte; higher concentrations show better results [178]. MT analysis result is signal at a potential about E = -1.7 V [173]. The sensitivity of the method is demonstrated by femtomolar concentrations of MT detected in low volumes (5 µl) [179]. However it is strongly dependent on many parameters including pH, ionic strength and protein pI. Lower influence was observed in relation to the temperature [173, 178, 179]. Recently, the ability of chronopotentiometry to distinguish between native and denatured protein form was proved [180, 181]. The advantage of this technique is low cost, a low variance coefficient, low detection limits, easy miniaturization and no interference.

#### *3.2.2.2. Metallothionein as a Part of Metal-Detecting Electrochemical Biosensors*

MTs can be used as a protein component of biosensors for heavy metals [147, 182-190]. Gonzalez-Bellavista *et al.* proposed a metallothionein-based silver biosensor where MTs was a part of ion-selective electrode with detection limit in orders of $10^{-5}$ M in model solutions [191]. Very good adsorption of MTs to the surface of gold and hanging mercury drop (HMDE) electrodes has been reported in numerous papers [166, 178, 184, 187, 192]. Therefore adsorption of MTs was used to introduce a metallothionein-based silver biosensor based on HMDE with 500 nM detection limit of Ag(I) ions [186]. In comparison to the bare carbon paste electrode the detection limit of the sensor was ten times lowered making it comparable to modified carbon paste electrodes. A precise, accurate and, significantly, selective electrodes for MT1 quantification, were presented by Capdevila *et al.* [146] The membrane was created by embedding mammalian Zn-7-MT1 complexes as ionophores in a polysulfone matrix. It was proved that the presence of other mammalian MT isoforms do not interfere in MT1 measurement.

#### *3.2.2.3. ESI-, MALDI- and ICP-Mass Spectrometry*

Mass spectrometry represents in coupling with electrospray ionization (ESI), matrix assisted laser desorption/ionization (MALDI) and inductively coupled plasma (ICP) ionization techniques the most important techniques in metallomics deserving special attention. These techniques are providing essential information about protein identity and structure (ESI, MALDI) and elemental specific analysis (ICP). One of the biggest advantages is the compatibility with various separation techniques especially liquid chromatography and capillary electrophoresis. Coupling can be arranged in on-line (ESI, ICP) as well as off-line mode (MALDI). ESI and MALDI are specific for CE due to its low flow rate. Although ICP can be coupled either to CE or LC, the coupling CE-ICP can be challenging.

Electrospray ionization provides multiply charged molecular ions without fragmentation and very accurate molecular weight determination of polypeptides [193]. ICP-MS is highly sensitive type of detection capable of the determination of a range of metals and several non-metals at concentrations of ppt. It is suitable for metalloprotein analysis due

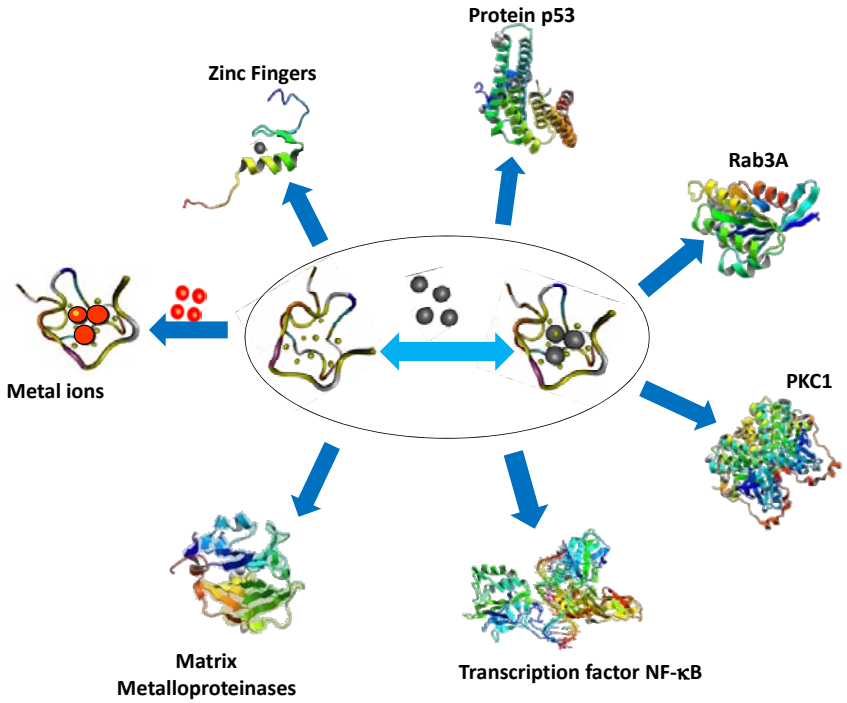


**Fig. (3).** The scheme of MT interactions with other biologically important proteins such as Zinc Fingers Family, Protein p53, Rab3A, PKC1, Transcription factor NF-κB and Matrix Metalloproteinases. The interactions between proteins is carried out through the exchange of essential metal ions (e.g. zinc(II) and copper(II) ions) between MT and other proteins.

to the elemental specific character determining the metal content of the biomolecule.

As mentioned previously, ESI, MALDI and ICP key methods for detection of metalloproteins including MT and large number of studies utilizing these methods has been published. Most recently, bismuth interaction with human metallothionein was monitored by ESI-MS in the work of Ngu *et al.* [194]. However also Cd(II) and Cu (I) binding studies have been performed by ESI-MS [195, 196]. ICP-MS in combination with separation technique proved to be efficient tool analyses of MT isoforms [131, 139, 141, 142]. Also MALDI can be used [197].

### *3.2.2.4. Saturation Assays*

These techniques are based on saturation of MT binding sites by metal ions having high affinity to sulphur. Hg(II) [34, 35], Ag(I) [32, 33] as well as Cd(II) [30, 31] are used in this type of measurements. (affinity decreases in order Hg(II) > Ag(I) ~ Cu(I) > Cd(II) > Zn(II) [198]. These methods have been used for analysis of MT content in a wide range of species including water organisms [199-207], mice [208], rats [209] and in chicken chondrocytes [210]. The work aimed on MT determination in crustaceans using Ag-saturation method coupled with AAS detection of silver was described by Delramo *et al.* demonstrating detection limit of 0.03 ppm [211]. Cadmium thiomolybdate assay is based on replacing of Cu ions bonded to the MT by Cd ions using the affinity of tetrathiomalybdate to the Cu ions [212, 213].

## 4. METALLOTHIONEIN AND TUMOR DISEASE

Worldwide, over 12 million people are cancer diagnosed each year and 7.6 million die of the disease according to International Union Against Cancer. In Europe, cancer is the second most important cause of death responsible for 20 % of all deaths. Two and half of million new cases are diagnosed each year according to statistics coming from World Health Organization. This alarming number is motivating researchers to improve the diagnostic and treatment methods as well as develop new anti-cancer drugs. Early stage diagnosis increases significantly the chances for successful treatment and therefore reliable biomarkers are desperately looked for. All above mentioned analytical methods, separately or in combination, serve as more or less efficient and powerful tools for MT determination, trying to meet the main goal – verify the hypothesis that MT is suitable marker of tumor diseases.

The applicability of MTs expression as diagnostic marker is intensively investigated in the last decade. Several studies correlating the tumor progression and drug resistance in a variety of malignancies (e.g. leukaemia, melanoma, breast, ovarian, renal, lung, pancreatic, etc.) to the MTs level have been published [214]. On the other hand in some other studies devoted to colorectal and bladder cancer and others, no significant correlation between MTs expression and prognosis was observed [215-217]. The relationship between MTs and cancer has been discussed in details recently in review by Eckschlager *et al.* [218]. This comprehensive summary is over viewing studies focused on correlation between MT expression and variety of cancer types.

The hypothesis that MTs is protecting tumor cells against apoptosis, supporting the metastatic behaviour and cancer cell proliferation is being verified. It is known that overexpressed apo-MT can remove Zn from tumor-suppressor protein p53 decreasing significantly its capability of forming complex with DNA triggering the apoptosis [219]. It also should be noted that the metal transporting and scavenging

function of MT can noticeably negatively influence the effectiveness of anti-tumor treatment [220, 221], however, this issue remain still without larger support coming from clinical studies.

It can be concluded that many more comprehensive studies have to be carried out before the detection of MTs as a prognostic marker become a routine clinical practice.

## 5. CONCLUDING REMARKS

Proteomics and metalloproteomics is rapidly developing interdisciplinary field combining the areas of biochemistry, clinical chemistry, molecular biology as well as environmental chemistry and food analysis. About one-third of all proteins are associated with a metal and therefore metallomic techniques, such as *in vivo* analytical methods, *in vitro* functional analysis and *in silico* bioinformatic analysis are of extreme interest. The impact of mass spectrometric detection in combination with various ionization techniques such as ICP, ESI and/or MALDI and separation techniques is incomparable.

Due to the increased metal binding capacity, MT has been proposed as indicator of both environmental and biological monitoring reflecting exposure to metal. Futhermore, MT might play an important role as a zinc donor for many other essential metalloproteins including matrix metalloproteinases, zinc fingers (Fig. **3**). Also the role in cancerogenesis and the potential applicability of MT as a biological marker of disease progress is in the centre of interest. Large number of studies have been published demonstrating benefits of MT in cancer diagnostics. On the other hand, there are studies showing no direct correlation between MT level and the stage of the disease. These are the main reasons for further extensive exploration of this field in near future.

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## ABBREVIATIONS

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

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