# Use of mass spectrometry technique (MALDI-TOF/TOF) for the characterization of metallothionein in biological systems

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Metallothioneins (MTs) are intracellular, low molecular mass and cysteine-rich proteins having several interesting biological roles associated with the protection against DNA damage, oxidative stress and apoptosis. Recent developments in the mass spectrometry have brought clinical proteomics to the forefront of diagnosis and treatment of diseases, offering reliable, robust, and efficient analytical methods for discovery and monitoring of biomarkers. MALDI-TOF/TOF mass spectrometry has been proven an effective tool not only for analysis of MTs in biological samples, but also for the identification of its isoforms in various types of samples. Importantly, it has been reported that MTs play a role in oncogenesis and prognosis of cancer, and there is an evidence of possible involvement of these proteins in the development of the resistance of cancer cells to anticancer metal-based drugs including cisplatin as the most used cytostatics. We review MALDI-TOF profiling techniques as tools for the MTs detection in cancers.

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## Biochemistry of Metallothionein

Metallothioneins (MTs) were discovered in 1957 and identified as low-molecular weight sulfhydryl-rich proteins. MTs belong to a superfamily of intracellular metal-binding proteins, present in virtually all living organisms, with features common to the archetypal. MT was first isolated from horse kidney and characterized by Margoshes and Vallee[1]. In this work, we wish to briefly summarize the current knowledge regarding the MT forms. All vertebrates examined contain two or more distinct MT isoforms designated MT-1 through MT-4. The three-dimensional structures of MTs from mammalian that have been determined so far show a monomeric protein composed of two globular domains, each encompassing a metal–thiolate cluster. The metallothionein isoform A (MTA) is a 64-residue metalloprotein, which contains essentially the same number of metal-chelating Cys–Cys and Cys–Xxx–Cys motifs (where Xxx stands for any amino acid, other than Cys) and metal ions[2,3]. These cysteine-rich proteins are localized in cytoplasm and some organelles, predominantly in mitochondria, where their presence is sensitively and strictly regulated by the oxidative state induced by mitochondrial respiration. Depending on the cell state, but especially presence of oxidative stress, MTs are rapidly translocated to the nucleus through nuclear pore complexes. MT localized in the nuclei is oxidized there and it is transported to cytosol; this system is balanced[3].

## Interaction of metallothionein and metals

MTs are currently classified into 15 families. Mammalian MTs are single-chain polypeptides of 61 to 68 amino acid residues. There are no free thiol groups, and divalent metals are bound by sulfur atoms in thiolate clusters with a tetrahedral geometry (or trigonal for $Cu^{+}$).

The binding affinity varies between metals, with Cu having the greatest stability constant ($10^{19}$–$10^{17}$) followed by Cd ($10^{17}$–$10^{15}$) and then Zn ($10^{14}$–$10^{11}$). As many as 18 different metals may associate with MT, but only Cu(I), Cd(II), Pb(II), Ag(I), Hg(II) and Bi(II) can displace Zn[4]. MT can incorporate up to 7 divalent metal or 12 monovalent Cu atoms per molecule. The Figure 1 shows the photos of 3-dimensional structure of MT isolated from liver rabbit liver without and with heavy metal. $Cu^{+}$ binds in multiple stoichiometry with a minimum of 7 Cu(I)/mol[5]. MT has two subunits: the more stable a domain (C-terminal), which incorporates four divalent metal atoms, and the more reactive b-domain (N-terminal), which contains only three [6]. The three-dimensional protein structure of this was reported by both X-ray crystallography and NMR spectroscopy in the 1990s. Structural studies have shown that this unusual protein with 61 amino acids (mammalian MT) can bind with both essential metals (Zn and Cu) and toxic metals (Cd and Hg) in two distinct cluster structures within the molecule. One cluster is closer to the N-terminal and three metal atoms are bound to nine cysteines with three bridging sulfur atoms, while in the second cluster closer to the C-terminal and four metal atoms are bound to 11 cysteines with five bridging sulfur atoms[7].

## Metallothionein physiological functions

MTs have many important and crucial functions (Figure 2). Expression of MTs is induced by many factors including physical stress, chemical stress and endogenous factors[8,9]. The most important of them includes detoxification of essential as well as non-essential heavy metal ions, such as Cd(II) or Hg(I, II), homeostasis and control of Zn(II) and Cu(II) ions and metal transfer reactions [10]. This role is still claimed by most authors working in the MT field and often supported by data from species ranging from fungi to mammals, which could explain the wide variety of MT isoforms[11]. A copper-specific MT isoform was shown to preferentially bind 12 copper ions in the snail's taxonomic Helix pomatia[12]. Thus, one of the most important MT functions consists in cell protection against free radicals[13]. It is clear that MT is induced by oxidative stress. Free oxygen radicals are associated with ubiquitous cell functions, especially by the mitochondrial electron transport system and by NADPH oxidase in cells. The danger of free radicals consists of the ability of damage to biomolecules, including proteins and polyunsaturated fatty acids, major components of cell biomembranes[10], and protection against DNA damage[14,15].

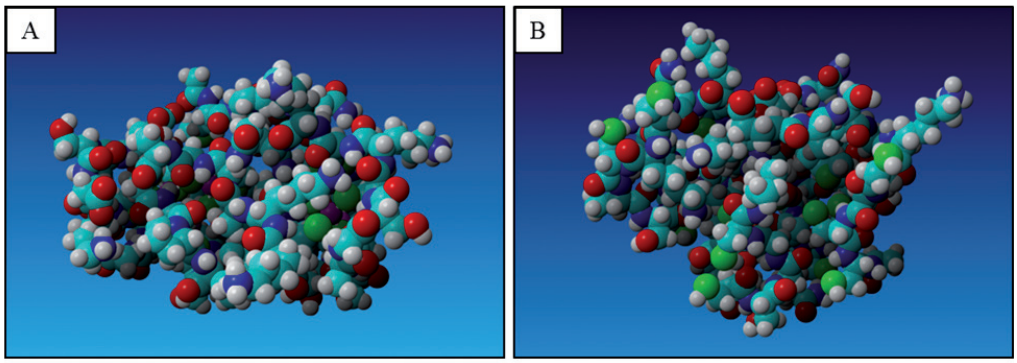


**Figure 1**: Photos of 3-dimensional structure of MT isolated from liver rabbit liver without (A) and with heavy metal (B). These photos were created by an advanced molecule editor (Avogadro 1.1.1) in our laboratory

## Regulation of expression of metallothionein in biological systems

Although the metals, Zn, Cu, Cd, Hg, Au and Bi all induce MTs, Zn is the primary physiological inducer since, Cu excepted, the other metals can be regarded as environmental toxicants. Interestingly, nontoxic Cu levels do not induce MT, although it is often bound to MT in vivo. The binding of Zn to metal transcription factor (MTF-1) allows the protein to bind to metal response elements (MREs) in the promoter region which, in turn, initiates MT--gene transcription. It has been proposed that MTF-1 regulates the free zinc concentration by controlling the expression of MT as well as that of a Zn-transporter protein, ZnT-1[16]. The binding of Zn to MTs has proven to be a physiologically relevant. Several studies have produced strong evidence to support the idea that MTs function as zinc chaperones for the regulation of gene expression and activity of proteins, such as metalloproteins and metal--dependent transcription factors[17]. A hallmark of the mouse MT-1 and MT-2 genes is their transcriptional induction by Zn and Cd. Essential for this induction are DNA motifs, termed metal response elements (MRE), present in multiple copies in the proximal promoters of MT genes. MREs were shown to confer response to Zn and Cd and to oxidative stress[18,19].

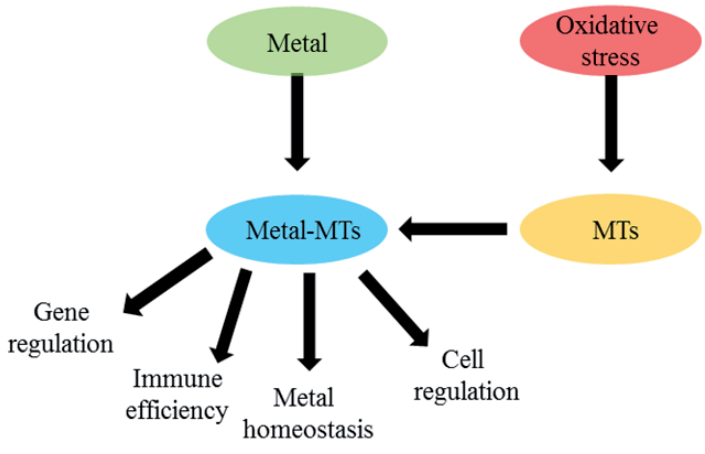


**Figure 2:** Overview of MT function

## Metallothionein and tumor pathology

A number of studies have demonstrated the presence or enhanced synthesis of MTs in rapidly proliferating normal cells, regenerating cells and cancer cells[20]. MTs have been shown to protect cells against the cytotoxic effects of electrophilic anticancer drugs. The enhanced expression of MT in cells induces the antiapoptotic effects and a lack of MT in MT-null cells increases the susceptibility to apoptic cell death after exposure to certain anticancer drugs[21]. MTs have also been shown to be involved in the development of resistance to anticancer drug cisplatin, one of the most widely used chemotherapeutic metal-based drugs. The increase in cellular content of MTs was considered to be a possible biomarker of resistance to treatment with cisplatin[21,22]. MT overexpression has been revealed in variety of human tumors. Positive correlation between MT overexpression and aggressive biological behaviour as well as poorer prognosis have been found in many of them (e.g. for carcinomas of urinary and digestive tract, breast cancers, lung carcinomas, squamous cell carcinomas of oral cavity and larynx as well as malignant melanoma) [23-26]. Knocking down MT1X by siRNA could sensitize cells to cisplatin through increased apoptosis of cancer cells and inhibition of cell proliferation. The study suggests that inhibitors of MT1X may have potential therapeutic application in inducing apoptosis in oral squamous cell carcinoma. These findings may help in the developing better cancer chemotherapy strategies[27]. Moreover, MTs might be involved in the protection against Helicobacter pylori induced-gastric chronic inflammation associated with gastric carcinogenesis [28]. p53, p21, BAX, c-kit, and MTs may have different roles in the pathogenesis of ovarian tumors. p53 and MTs may be helpful in the typing the borderline and malignant ovarian tumors[29].

Different studies have shown that MT has important functions in hematopoietic cells; these studies consider also possible role of MTs in these cells. MT has been reported to be involved in the differentiation and proliferation of hematopoietic cells[30,31], and prevention of apoptosis[32]. The MT1A, E, X and MT2A isoforms have been revealed to play an important function in prostate cancer. It has been shown that MT1 and MT2 isoforms may be related to the proliferative activity of breast, colon and prostate human cancers[33, 34]. Five isoforms of MT were overexpressed in non-small cell lung cancer; overexpression of the MT1F and MT2A isoforms predicted patient's poor prognosis. Both these isoforms might be involved in progression of this type of cancer; this fact has been confirmed by the correlation analysis of up-regulated MT1F expression, size of primary tumor and rate of grade of malignancy[35]. Connection between zinc and MTs in central nervous system is still studied. In the central nervous system, zinc is released along with glutamate during neurotransmission and, in excess, can promote neuronal death. Experimental studies have shown that MT1 and MT2, which chelate free zinc, can affect seizures and reduce neuronal death after status epilepticus[36]. MTs have role in the pathogenesis of autoimmune diseases. The expression of MT1 and MT2 and the concentrations of Zn and Cu in tissues of the brain, spinal cord and in the liver during the periods of attacks and remissions in chronic relapsing experimental autoimmune encephalomyelitis have been estimated to have a role in the disorders of central nervous system. This data, obtained by clinical assessment, immunohistochemistry and inductively coupled plasma spectrometry, showed that MT1 and MT2 were markedly up-regulated in the subarachnoid regions and perivascular space in astrocytes, microglia and spinal neurons; copper in the liver was significantly increased [37].

## MALDI-TOF-MS as an analytical technique for the detection of Metallothionein

Recent developments in mass spectrometry have introduced clinical proteomics to the forefront of diseases diagnosis, offering reliable, robust and efficient analytical method for biomarker discovery and monitoring.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers high sample throughput and the flexibility to couple with different off-line sample fractionation techniques. MALDI-TOF MS is an extremely sensitive technique that permits the detection of chemical and biological compounds at abundances below sub-femtomole (< 10– 15 mol). The technique offers soft ionization potential, a relatively low degree of fragmentation, and uncomplicated spectra comprised of mostly singly charged ions[38]. MALDI-TOF is a powerful tool for surveying proteins and peptides comprising the realm for clinical analysis. MALDI-TOF MS has the potential to revolutionize cancer diagnostics by facilitating biomarker discovery, enabling tissue imaging and quantifying biomarker levels[39].

## Detection of metallothionein

Andon et al. established a method for the separation and characterization of rabbit liver MTs subisoforms by capillary electrophoresis coupled to electrospray ionization time-of-flight mass spectrometry (CE-ESI-TOF MS). The analysis described here revealed the presence of the apothioneins MT1a, MT1d, and MT1e, belonging to MT1 sample, and MT2a, MT2b, and MT2c belonging to MT2. Similar results were found when MALDI-TOF experiments were performed; they enable to identify all the sequenced rabbit liver MTs as apo-MT--forms, as in the CE-ESI-TOF MS coupling [40]. Other study verified that the subisoforms of MT in rabbit liver have a different apparent molecular mass under different conditions. This experiment predicted that there probably exist a stable peptide structure of MT2 using MALDI-TOF MS to study the subisoforms of MTs and get their exact primary structure[41]. Moreover, two-dimensional gel electrophoresis (2DGE), MALDI-TOF MS, the peptide mass fingerprinting (PMF) map, and bioinformatic analysis used for studying differentially expressed proteins between multidrug resistant cells HL-60/DOX and drug sensitive cells HL-60 of

acute myeloblastic leukemia were potential methods for identification of proteins in these cells. The results revealed presence of MTs only in HL-60 cells [42].

## Cancer

MALDI-TOF MS acts as one of the most comprehensive and versatile tools for research in proteomics [43]. Wang et al. have shown a simple and rapid method for identification of MTs isoforms in cultured human prostate cells (RWPE-1 cell line) by MALDI-TOF/TOF mass spectrometry[44] and they demonstrated that MS method allows correlation between expression of isoform-specific proteins and expression of isoform-specific mRNA by providing information about expression of MTs isoforms in a rapid fashion. The lack of publications is well evident in the area of MALDI analysis of MTs in cancer. For example, MALDI imaging of cancer tissue could be very beneficial to confirm hypotheses about possible connection of MTs with matrix metalloproteinases, which due to presence of zinc ions in peripheral tumor tissue [45].

## Anticancer drugs

The MALDI-TOF MS was used for comparative study focused on interactions of cisplatin and ruthenium arene anticancer complexes with MTs. The results showed that the novel ruthenium arene anticancer complexes are much less reactive with thiol-rich MTs, which overexpression in the cancer tissues is closely connected with increased resistance to cisplatin. This finding may be helpful to understand better the distinct pharmacological profile of ruthenium arene anticancer complexes, such as reduced toxicity and no cross-resistance to cisplatin[22]. Platinum(II) complexes have been demonstrated to form covalent bonds with sulfur-donating ligands (in MTs, GSH and other sulfur-containing biomolecules) or coordination bonds with nitrogen-donating ligands (such as histidine and guanine). Terpyridine platinum(II) (TP-Pt(II)) complexes was used as model system. Moreover, it has been demonstrated that the TP-Pt(II) complex formed a covalent bond with the active-site cysteine residue in two other types of cysteine protease by using MALDI-TOF MS. This results showed unequivocally that TP-Pt(II) complexes can selectively bind into the active site of most of cysteine proteases and can be useful in the design of new platinum(II) compounds with promising anti-cancer, anti-parasitic or anti-viral activities[46].

## MALDI-TOF optimization for metallothionein determination in cancer cells

In this review, we summarize the different parameters and materials used in the detection and identification of MTs in biological samples (matrixes) by MALDI-TOF MS. Researchers have discussed the importance of choosing the matrix, conditions of crystallization of the matrix and analyte, concentration of matrix, and the use of matrix additives, for different proteins and peptides elsewhere. The matrix consists of small organic compounds, which show strong resonance absorption at the applied laser wavelength. Pulsed laser systems are used to enable an explosive disintegration of a laser-light-excited matrix-analyte volume, and thus subsequent desorption with ionisation. In the most of the studies reviewed, 2,5-dihydroxybenzoic acid (2,5-DHB) and $\alpha$-cyano-4-hydroxycinnamic acid (CHCA) were the constituents of matrix used for appropriate determination of MTs[44, 47]. In few studies published focused on the determination of MTs in cancer tissues by MALDI-TOF MS, both 2,5-DHB and CHCA were used for detection and the samples of MTs were prepared in TFA and ACN in different concentrations (50% ACN containing 0.1% TFA or 30% ACN with 0.1% TFA) for obtain better signa[44]. An increase in the intensity and the signal-to-noise ratio of peaks (signals) of peptide in MALDI-TOF mass spectra was observed benefit of the addition of ammonium monobasic phosphate to samples. Combining both of the approaches, addition of ammonium salts into the CHCA matrix followed by one post-crystallization washing step with ammonium buffer provided a substantial improvement of the sensitivity of MALDI-MS detection compared to unwashed sample spots. This method of

preparation of sample is necessary to improve quality of spectra obtained and is essential for successful searching in databases for subnanomolar concentrations of protein digests[48].

## Conclusion

Despite impressive scientific, medical and technological achievements over the past few decades, cancer is still a leading cause of death, largely because most cancer patients are diagnosed when disease is advanced. The early detection is associated with improved survival rates. The MALDI-TOF/TOF mass spectrometry has the potential to revolutionize cancer diagnostics by facilitating biomarker discovery and quantifying biomarker levels. 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. As summarized, MALDI-TOF MS is a rapid and simple method for identification and characterization of MT isoforms in cancer cells.

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