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Fully automated two-step assay for detection of metallothionein through magnetic isolation using functionalized γ-Fe_2O_3 particles

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ABSTRACT

Metallothioneins (MTs) are involved in heavy metal detoxification in a wide range of living organisms. Currently, it is well known that MTs play substantial role in many pathophysiological processes, including carcinogenesis, and they can serve as diagnostic biomarkers. In order to increase the applicability of MT in cancer diagnostics, an easy-to-use and rapid method for its detection is required. Hence, the aim of this study was to develop a fully automated and high-throughput assay for the estimation of MT levels. Here, we report the optimal conditions for the isolation of MTs from rabbit liver and their characterization using MALDI-TOF MS. In addition, we described a two-step assay, which started with an isolation of the protein using functionalized paramagnetic particles and finished with their electrochemical analysis. The designed easy-to-use, cost-effective, error-free and fully automated procedure for the isolation of MT coupled with a simple analytical detection method can provide a prototype for the construction of a diagnostic instrument, which would be appropriate for the monitoring of carcinogenesis or MT-related chemoresistance of tumors.

1. Introduction

Metallothioneins (MTs) make up a superfamily of low molecular-weight and cysteine-rich proteins that can specifically bind to closed-shell metal ions like zinc (II), cadmium (II), and copper (II) using the sulfur atoms of cysteine rich residues [1–3]. MTs are widely distributed in invertebrates and vertebrates, plants, prokaryotes and even in fungi kingdoms [4–7]. Despite the diversity of the amino acid sequences, the MTs are generally analogous in structure with respect to a high content of cysteine residues (up to 30%), and lack of aromatic amino acids [4]. MTs are involved in the metabolism of heavy metal ions, including their metal detoxification, homeostasis [8,9], storage of zinc [7], radical scavenging [2,10] and stress response [11]. MTs are overexpressed in several tumors and this overexpression is accompanied by an increased proliferation and protection against apoptosis. Therefore, MTs can be considered as a sign of worse prognosis in some malignancies [12–15]. Some previous studies showed that the MT level correlates inversely with tumor grade [16], which led us to consider the MTs as a potential cancer marker [6,7,17]. The biosynthesis of MT was shown to be elevated to protect the cells against heavy metal toxicity, cytotoxicity [18], and radiation and/or DNA damage [19]. Hence, the quantification of MTs should point out some pathological states in living organisms. The determination of MTs is usually coupled with several analytical methods as follows: capillary electrophoresis, mass spectrometry, immunoassays, inductively coupled plasma mass spectrometry, liquid chromatography, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrochemistry [20–23]. In the present study, we have particularly aimed at the utilization of the three last mentioned methods (liquid chromatography, MALDI-TOF MS and electrochemistry). Many studies demonstrated the method of liquid chromatography is highly specific, sensitive, and reliable to

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improve the resolution of MTs separations [24–31]. MALDI-TOF MS has the potential to revolutionize the cancer diagnostics by facilitating biomarker discovery [32], enabling tissue imaging [32], and quantifying biomarker levels at abundances below sub-femtomole ($<10^{-15}$ mol) [33]. Herein, we present the use of MALDI-TOF MS to characterize the MTs isolated from rabbit liver under the optimized conditions. MTs can be used to modify electrodes to develop electrochemical biosensors on the basis of their ability to bind heavy metals [20,21]. Such biosensors can be applied in the field of point-of-care testing for the patients. In a view of these facts, the immobilization of MTs on paramagnetic particles (PMPs) may provide many possibilities, such as simplification of biosensor systems for the low levels of MTs due to its pre-concentration. This study focuses on the design and optimization of the method based on the isolation of MTs by using PMPs (functionalized nano-maghemite γ-Fe_2O_3 core) with consequent electrochemical detection. The recovery of MTs was determined by differential pulse voltammetry (DPV). We tested six types of particles, which differ from each other in their composition and functionalization procedure. A comparative study was carried out between automated and manual procedure for the accuracy, efficiency and the consumption of the time of the assay. The optimization and automation of the assay increased its sensitivity and made it simple and fast. For the detection of the isolated products an electrochemical analysis by Brdicka reaction was carried out.

The trends in electroanalytical detection of MTs by Brdicka reaction were described previously by Adam et al. [20].

2. Experimental

2.1. Chemicals

$Fe(NO_3)_3{\cdot}9H_2O$, $NaBH_4$, $HAuCl_4$ and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise. For the isolation procedure, phosphate buffered saline (PBS, pH = 7.4) and borate buffer (pH = 6.0) were used. The buffers and solutions were prepared with ACS H_2O (Sigma-Aldrich, St. Louis, MO, USA). High purity deionized water (Milli-Q Millipore 18.2 $M\Omega\,cm^{-1}$, Bedford, MA, USA) was used for washing and rinsing.

2.2. Preparation of samples for MT isolation

MT was isolated from rabbit liver and purified by using fast-protein liquid chromatography (FPLC) according to our previous study [31]. The males of New Zealand rabbits were kept in separate cages on regular pelleted laboratory chow (MaK-Bergman, Kocanda, Prague, Czech Republic) and allowed free access to drinking water. Intraperitoneal injection of 10 mg $CdCl_2{\cdot}kg^{-1}$ were given to the rabbits in three equal doses (day 1, day 3 and day 5). In the aforementioned day intervals, animals were anaesthetised with Ketamine (30 mg kg^{-1}) and Xylazine (3 mg kg^{-1}) (Vétoquinol Biovet, France) and subsequently the livers were collected. 2 g of liver was homogenised on ice using Ultra-turrax T8 (Scholler instruments, Germany) in 8 mL of 10 mM Tris–HCl buffer (pH 8.6). The obtained sample was subsequently vortexed (Vortex Genuie, Germany) and centrifuged (Universal 320, Hettich Zentrifugen, Germany) at 5000 rpm for 30 min at 4 °C. The supernatant was again centrifuged (Eppendorf centrifuge 5417R) at 25,000 rpm, 4 °C for 30 min and new supernatant than was subsequently heated in a thermomixer (Eppendorf thermomixer comfort, Germany) at 99 °C for 10 min and centrifuged again with same condition.

2.3. Fast protein liquid chromatography (FPLC)

The FPLC (Biologic DuoFlow system, Biorad, USA) was composed of two chromatographic pumps for the transportation of the elution buffers, a gel filtration column (HiLoad26/60, GE Healthcare, Uppsala, Sweden), a UV–VIS detector and an automatic fraction collector. The separation was carried out using the isocratic elution of a mobile phase (150 mM NaCl in 10 mM Tris-HCl buffer (pH = 8.6)). The flow of mobile phase was 4 mL min^{-1}. Before the separation, the column was washed with the mobile phase for 60 min. For validation, a standard of bovine serum albumin (BSA)(1.5 mg mL^{-1}) was used. 254 nm UV was used to detect MT. The fractions (15 mL per fraction) were collected from min 46 of the separation. After the isolation, the fractions of MT samples were pipetted into the microtiter plate (Sigma-Aldrich, St. Louis, MO, USA), and concentrated using a nitrogen evaporator Ultravap Mistral (Porvair Sciences, Norfolk, UK). Thereafter, each well of a microtiter plate has been washed with ACS to the final sample volume of 1.5 mL. Then, the samples were lyophilized and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and MALDI-TOF MS was carried out to confirm the purity.

2.4. MALDI-TOF MS for verification of MT

The isolated MT was validated for its molecular weight and purity. The mass spectrometry experiments were performed using a MALDI-TOF MS Bruker Ultraflextreme (Bruker Daltonik GmbH, Germany) equipped with a laser operating system at a wavelength of 355 nm with an accelerating voltage of 25 kV (cooled with nitrogen) and a maximum energy of 43.2 μJ with repetition rate 2000 Hz in a linear and positive mode. The data acquisition and processing were performed using the softwares flexControl version 3.4 and flexAnalysis version 2.2 respectively. The matrices used in the MALDI method were 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (HCCA) and sinapinic acid (SA) (Bruker). All the matrices were prepared in TA30 (30% acetonitrile, 0.1% trifluoroacetic acid solution). The mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin Electronic GmbH, Germany) for 2 min, 50% of intensity at ambient temperature. The working matrix standard solutions were prepared freshly by diluting the stock solutions.

The used concentrations of MTs samples were 1.5, 3, 6 and 12 μg mL^{-1}. The sample solutions were prepared with TA30. The solutions for the analysis were mixed in a ratio of 1:1 (matrix/substance). After obtaining a homogeneous solution, 1 μL was applied on the MTP ground target plate (Bruker Daltonik GmbH) and dried under atmospheric pressure and ambient temperature. A mixture of protein calibration standards I (Bruker) was used to externally calibrate the instrument. The protein mixture allowed the calibrations and testing of MALDI-TOF MS in a mass range between ~4,000 and 20,000 Da.

The samples preparation method for MALDI-TOF was carried out in two different ways: Dried Droplet (DD) and Thin Layer (TL) for HCCA matrix. For DD, a saturated matrix solution was prepared by mixing the matrix solution with the sample solution in a ratio of 1:1. The sample solution was prepared with TA30. The mixture was pipetted on the target (1 μL) and dried at ambient temperature. The preparation should yield relatively large crystals on the target surface without the matrix or analyte. For TL, the matrix was prepared on the target to form a thin layer of very small and homogenous crystals. Then, the solution was deposited on the target and evaporated. The thin matrix layer remained on the surface of the target. The MTs sample (in TA30 solution) was applied on the top of this thin layer. After drying of the sample, the analyte molecules remained on top of the matrix. Before measuring the samples, a mixture of protein calibrations standard I