AUTOMATED ISOLATION AND DETECTION OF METALLOTHIONEIN USING FUNCTIONALIZED MAGHEMITE AND DIFFERENTIAL PULSE VOLTAMMETRY

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Abstract

Currently, it is well known that metallothioneins (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 diagnostics, an easy-to-use and rapid detection method is required. The aim study was to develop a fully automated and high-throughput assay for the estimation of MT levels. We focused on the design and fabrication of a method based on isolation of MTs using paramagnetic particles (functionalized nanomaghemite γ-Fe_2O_3 core) with consequent detection by differential pulse voltammetry (DPV) utilizing Brdicka electrolyte. We designed and tested six types of particles, which differed in the surface functionalization [polyvinylpyrrolidone and $HAuCl_4$; polyethyleneimine; polytheleneglycol (MW 1500 and 4000) and $HAuCl_4$; poly(4-styrenesulfonic acid and $HAuCl_4$; and polyacrylamide and $HAuCl_4$]. The best conditions were employed in the automation of isolation and detection procedure, which made it simple and fast. As a proof-of-concept we successfully applied our protocol for isolation and detection of MT in serum collected from Wistar rats. The designed easy-to-use, cost-effective and fully automated procedure for the isolation of MT coupled with a simple electrochemical detection can serve for the construction of a diagnostic instrument, which would be appropriate for the monitoring of carcinogenesis or MT-related chemoresistance of tumors.

Keywords: Automation, electrochemistry, magnetic isolation, MALDI-TOF MS, metallothionein

1. INTRODUCTION

Metallothioneins (MTs) are involved in the metabolism of heavy metal ions, including their metal detoxification, homeostasis [1, 2], storage of zinc, radical scavenging [3, 4] and stress response. MTs are overexpressed in several tumors and their 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 [5-8]. 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 [9-12]. Herein, we present the use of MALDI-TOF MS to characterize the MTs isolated from rabbit liver under the optimized conditions. This study focuses on the design and optimization of the method based on the isolation of MTs by using paramagnetic nanoparticles (PMPs) with consequent electrochemical detection. 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.

2. MATERIALS AND METHODS

2.1. MT isolation and its characterization by MALDI-TOF MS

MT was isolated from rabbit liver and purified by using fast-protein liquid chromatography (FPLC) according to our previous study [13]. 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 matrices used 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).

2.2. Synthesis and functionalization of PMPs

1.5g of $Fe(NO_3)_3 \cdot 9H_2O$ was dissolved in water (80 mL). Under stirring 0.2 g of $NaBH_4$ was added, which was dissolved in 10 mL of 3.5% NH_3 and heated (2 h, 100 °C). After cooling, the mixture was left overnight and the superparamagnetic nanoparticles were separated by an external magnetic field, and subsequently washed several times with water and used as a core for the surface modifications. MAN-53: The procedure of this material fabrication was published previously [14]; MAN-131: The nanoparticles were mixed with a water solution of polyethyleneimine (0.5 g) and stirred for 1 h. 25 mL of $HAuCl_4$ (1 mM) was added, stirred 1 h and sodium citrate (0.75 mL, 26 $mg \cdot mL^{-1}$) was poured into the solution. The mixture was stirred overnight, separated by magnet, washed 3 times with water and dried at 40 ºC (this last step was the same for all PMPs); MAN-132: The maghemite was suspended in a water solution (20 mL) of polyethylene glycol (PEG 4000), mixed with 25 mL of 1 mM $HAuCl_4$ and finally 0.75 mL of trisodium citrate (26 $mg \cdot mL^{-1}$) was added; MAN-133: The preparation way was similar to the preparation of MAN-132, only PEG of molecular weight 1500 (0.5 g) was used instead of 4000; MAN-134: A maghemite suspension was added into a solution of poly(4-styrenesulfonic acid) (0.5 g) and mixed for 1 h. Further 25 mL of 1 mM $HAuCl_4$ was mixed with the solution for 1 h and finally 0.75 mL of trisodium citrate (26 $mg \cdot mL^{-1}$) was added; and MAN-135: maghemite suspension was mixed with a solution of polyacrylamide (0.5 g) and stirred for 1 h. Solution was mixed with 25 mL of 1 mM $HAuCl_4$ for 1 h and finally 0.75 mL of trisodium citrate (26 $mg \cdot mL^{-1}$) was added.

2.3. Manual and automated isolation of MTs using PMPs

For the manual isolation, the MT from rabbit liver (50 $\mu g \cdot mL^{-1}$) was incubated with the PMPs (0.5 $mg \cdot mL^{-1}$) at 37 ºC, 1190 rpm in a thermomixer for 30 min. The PMPs with the bound MT were separated using an external magnetic field and washed six times with PBS or combination of PBS (3 × 250 µL, pH = 7.0) and 200 mM borate buffer (3 × 250 µL, pH = 6.0). In order to detect MTs, the PMPs with the bound MT were dissolved in hydrochloric acid (250 µL, 3 M). The obtained solution was evaporated using a nitrogen evaporator Ultravap RC (Porvair Sciences, Leatherhead, United Kingdom). Finally, the evaporated sample was resuspended in H_2O (250 µL) and the final product was detected by DPV. The fully automated MTs isolation procedure used the same specific parameters of the manual procedure. Here, the whole procedure was carried out using an automated pipetting system epMotion 5075 (Eppendorf, Hamburg, Germany). As real samples, we used the sera of four Wistar rats. We followed the European Community Guidelines as accepted principles for the use of experimental animals.

2.4. Electrochemical detection of isolated MT

The products of the isolation procedure were detected by differential pulse voltammetry (DPV) coupled with a hanging mercury drop working electrode (HMDE). For the detection of the isolated products an electrochemical analysis by Brdicka reaction was performed according to our previous study [15]. For the data processing, VA Database 2.2 (Metrohm, Switzerland) was employed.

3. RESULTS

3.1. Study of the optimal conditions for the determination of MTs by MALDI-TOF MS

To study the optimal conditions for the determination of MT, three different matrices were used: DHB, HCCA and SA. The MALDI-TOF MS spectra showed the presence of the MT monomer (major peak ~ 6 kDa) in case of all the matrices. The main observed signal for MT was assigned as follows: $[M+H]^+$ (m/z 6126 Da).

3.2. MT Isolation procedure

The scheme and the results of the manual isolation and detection of MT using functionalized PMPs is shown in **Figure 1**. Only MAN-131 was found to be unsuccessful in the suggested assay, whereas MAN-53 appeared to be the most successful (recovery more than 40%). This phenomenon was likely caused by the functionalization of the surface of nanomaghemite core using reduced gold, which attracted MT thiol moieties [16]. The MT recovery was found to be higher when PBS alone was employed. The scheme of the automated isolation and detection of MT using the functionalized PMPs is shown in **Figure 2**, and displays that the automated isolation and detection procedure provided higher recoveries of MT than the manual method. In comparison with model MAN-132 and -133, the model MAN-135 showed a lower MT recovery. According to the obtained results, the man-131 PMPs were not suitable for the isolation of MT by the suggested method because the final product was not electrochemically detectable.

While the manual detection exhibited a high accuracy, the automatic mode (higher LOD and LOQ) showed a faster and less time consuming procedure with a sufficiently accuracy, summarized in **Table 1**.

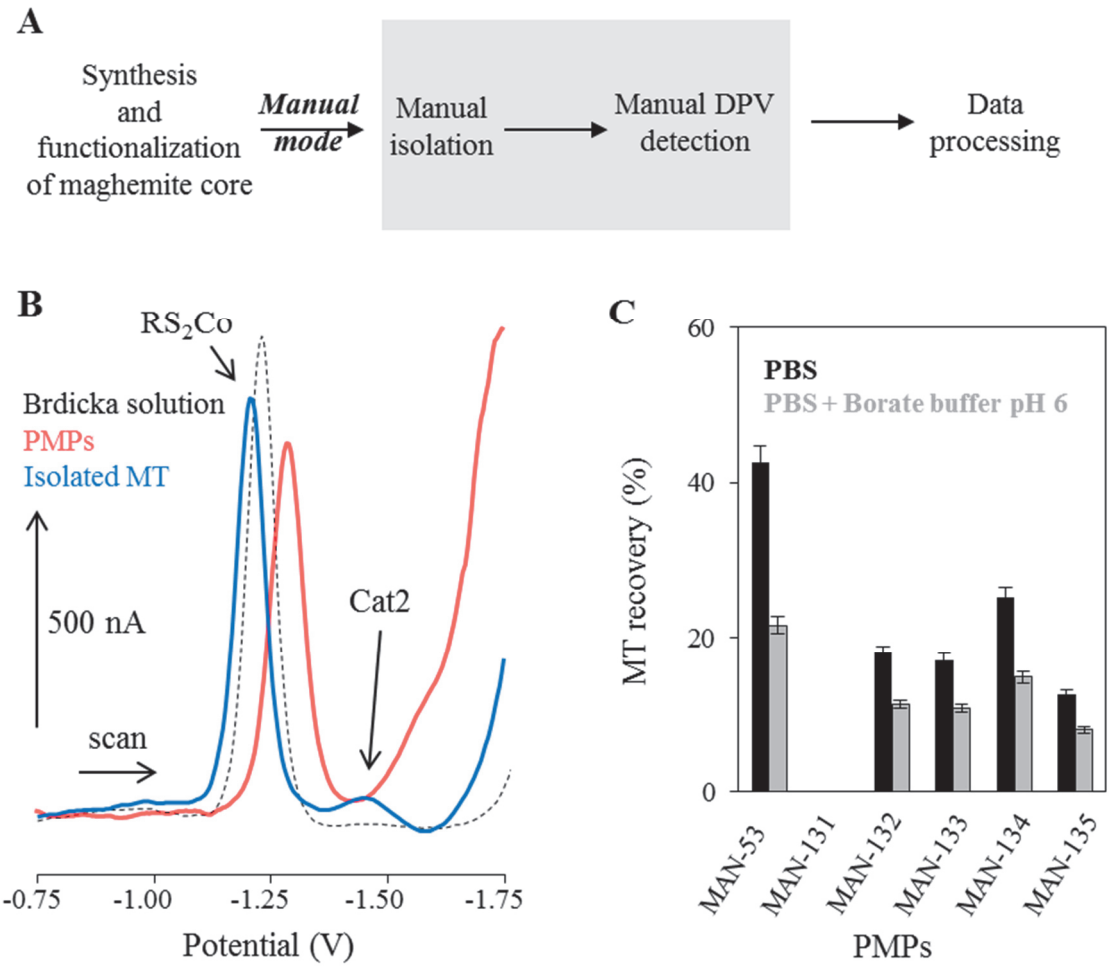

Figure 1 (**A**) Scheme of manual isolation and detection of MT using functionalized PMPs. (**B**) Typical voltammograms of isolated MT in Brdicka solution (blue line), Brdicka solution itself (black dashed line) and PMPs itself (red line) with highlighted peaks (RS_2Co and Cat2). (**C**). Influence of washing buffer composition on the obtained relative recovery of MT using six various PMPs

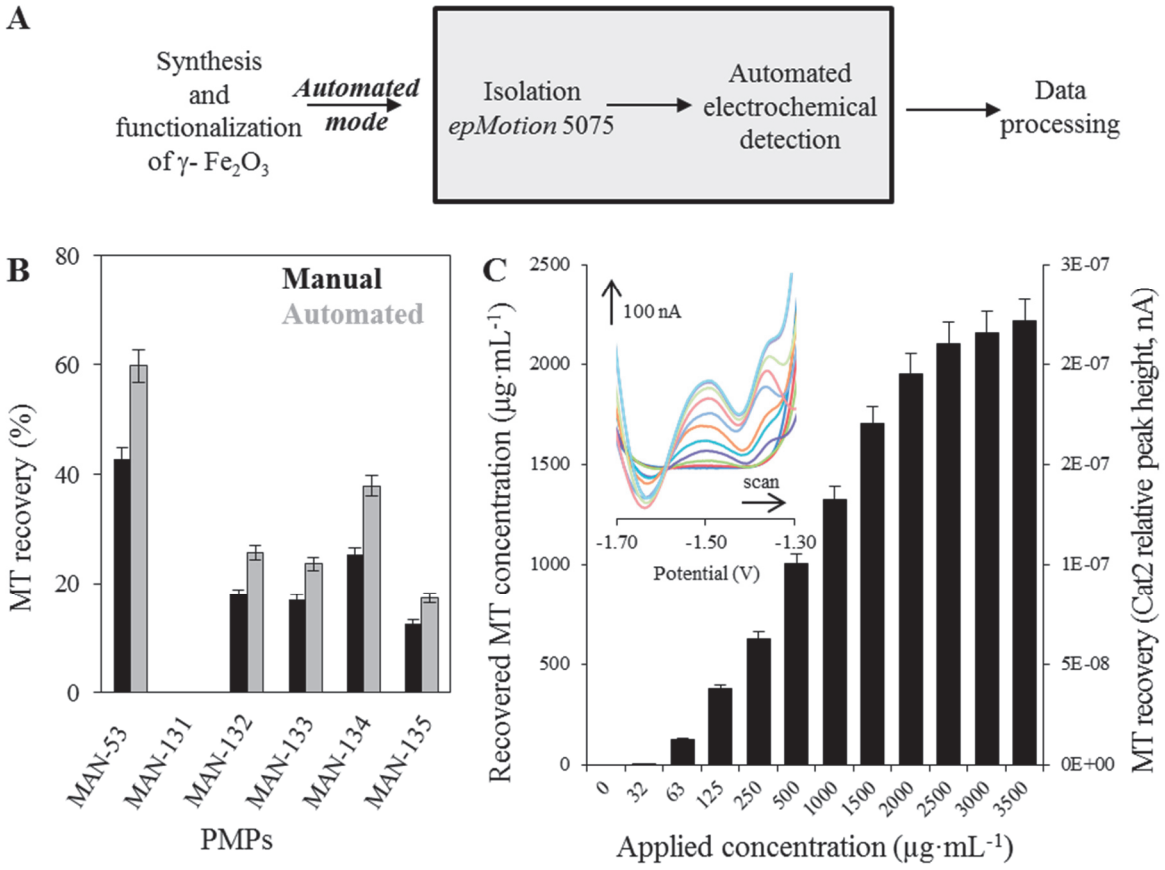

Figure 2 (**A**) Scheme of automated isolation and detection of MT using functionalized PMPs. (**B**) Influence of mode of isolation and detection on obtained relative recovery of MT using six various PMPs (MAN-53, MAN-131-135). (**C**) Recovery values for MT automatically isolated using the MAN-53. Saturation curve of the most successfully PMPs (MAN-53), the detail of the Cat2 peaks from interleaved real voltammograms are shown in the inset

Table 1 Analytical data about regression equation, correlation (R^2), limit of detection (LoD), limit of quantification (LoQ) and relative standard deviation (RSD)

Method	Regression equation	R^2	LoD (nM)	LoQ (nM)	RSD (%)
Manual	y = 10987x + 3.0743	0.997	12	39	7.7
Automated	y = 11623x + 3.8254	0.997	9	29	10.0

3.3. Validation of manual and automated procedure on real samples

Finally, both methods were validated for the real serum samples. The isolation of MT from Wistar rat serum was carried out using the MAN-53. As shown in **Table 2**, the automated procedure provided higher recoveries of MT than the manual procedure from all of the rat serum samples. If we compare the total time, consumed during the sample processing, the automated procedure (50 min) was found to be four times faster than the manual procedure (210 min) (**Table 2**). These results suggested that the modified PMPs coupled with automated isolation and detection procedure can be applied efficiently to isolate MT from various biological samples for the characterization of various pathological states including cancer.

Table 2 The comparison of the isolation and detection efficiency between automated and manual procedure. The isolation of MT was carried out from Wistar rat serum using the MAN-53. The recoveries (%) are recalculated to total MT amount prior own isolation

	Automated isolation protocol		Manual isolation protocol	
Sample	Concentration (μg.mL^{-1})	Recovery (%)	Concentration (μg.mL^{-1})	Recovery
1	70.9	28.9	51.9	21.2
2	69.3	20.0	47.6	13.8
3	47.5	15.6	36.3	11.9
4	52.5	14.0	45.2	12.1
Total time	50 min		210 min	

CONCLUSION

A large number of samples for isolation MT can be handled conveniently by the automated procedure, which was found to be four times faster than the manual procedure. The suggested procedure can also be used for the detection of other important biomolecules. We anticipate that this simple and cost-effective procedure could be helpful to estimate the MT serum levels in cancer patients before and during their treatment, which should allow specifying their clinical outcomes linked with possible development of MT-related chemoresistance.

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