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Paramagnetic particles for immobilization of metallothionein – promising biomarker of head and neck cancer

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Currently, metallothioneins (MTs) are investigated as the biomarkers of various pathological states. High positive association of MT amount was determined particularly in head and neck cancer (comparison with healthy tissue). Thus we suggested paramagnetic particles, based on nanomaghemite core, modified with polyvinylpyrrolidone and gold nanoparticles. Utilizing the natural affinity of thiol, contained in MT cysteines and gold, the MT was immobilized with approximately 25% recovery, when using the ideal conditions 100 mM borate buffer, pH 6 and incubation temperature 37 °C. Our particles may be helpful in isolation of MT as biomarker for subsequent analyses as well as for MT immobilization via external magnetic field on electrode surface for development of heavy metal biosensors.

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1. Introduction

Metallothioneins (MTs) are a group of low-molecular weight (~6 -10kDa), cysteine-rich, evolutionarily conserved proteins, found in generally all life forms. The MTs are multi-functional and play crucial roles in the transport of essential trace elements as well as in the detoxification of harmful metallic elements[1]. Four major MT isoforms, MT-1, MT-2, MT-3 and MT-4, have been identified in mammals[2]. Specifically, MT-3 has been reported to be secreted mainly in neural cells. Recent reports established that this isoform play an important protective role in brain injury and metal-linked neurodegenerative diseases[3]. In general, MT is known to modulate three basic processes: the release of hydroxyl radical or nitric oxide[4], apoptosis[5] and the binding and exchange of heavy metals such as zinc[6], cadmium[7] or copper[8]. Previous studies have shown a positive correlation between the expression of MT with invasion, metastasis and poor prognosis in various cancers, including head and neck cancers[9], where increasing levels of MT concentration in tumor (vs. healthy tissues) were observed[10]. Moreover; due to high affinity towards heavy metals, MT can be employed for modification of electrodes in development of electrochemical biosensors[11]. In view of these facts, immobilization of MT on paramagnetic particles (PMPs) may provide many possibilites, such as simplification of biosensing of low levels of MT, through its pre-concentration. The present study demonstrates immobilization of MT on a surface of paramagnetic particles (gold modified). For confirmation of MT presence two detection techniques were employed: matrix-assisted laser desorption/ionization - time of flight mass spectrometry and differential pulse voltammetry with adsorptive transfer technique utilizing Brdicka solution as supporting electrolyte. For binding capacity evaluation MT isolated from rabbit liver was employed and ideal particles for its immobilization were chosen, simultaneously with optimization of immobilization conditions (pH and temperature).

2. Materials and Methods

2.1 Chemicals

$HAuCl_4$, $NaBH_4$, 2,5-dihydroxybenzoic acid (DHB) and/or α-cyano-4-hydroxycinnamic acid (HCCA) were obtained from (Sigma-Aldrich, St. Louis, MO, USA) in ACS purity. Furthermore, we used sodium citrate, trifluoroacetic acid, Brdicka supporting electrolyte containing 1 mM $Co(NH_3).6Cl_3$ and 1 M ammonia buffer (NH_3(aq) NH_4Cl, pH = 9.6). All buffer solutions were prepared with ACS H_2O from (Sigma-Aldrich, USA).

2.2 Preparation of rabbit liver for MT isolation by using FPLC and confirmation of purity

MT was isolated from rabbit liver and purified by using fast-protein liquid chromatography (FPLC) according to our previous study[12].

The purity was evaluated by using MALDI-TOF/TOF (Bruker ultrafleXtreme, Bruker Daltonik GmbH, Germany). The matrix used in the MALDI method was 2,5-dihydroxybenzoic acid (DHB) and/or α-cyan-4-hydroxycinnamic acid (HCCA) (Sigma-Aldrich). The saturated matrix solution was prepared in 30% acetonitrile and 0.1% trifluoroacetic acid (TFA). Mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic GmbH, Germany) for 2 minutes at 50% of intensity at laboratory temperature. Sample preparation crystallization method for MALDI-TOF was *dried-droplet* method (DD) – the sample solutions for analysis were mixed with matrix solution in volume ratio of 1:1. After obtaining a homogeneous solution, 1 µL was applied on the MTP 384 polished steel target plate (Bruker) and dried under atmospheric pressure at laboratory temperature. The preparation yielded relatively large crystals on the target surface as well as regions without matrix or analyte. All measurements were performed in linear positive mode in the *m/z* range 4-17 kDa. The mass spectra were typically acquired by averaging 500 sub spectra from a total of 500 shots of the laser with laser power set 5-10% above the threshold.

2.3 Preparation of paramagnetic core-shell particles

Nanometric maghemite core was prepared by $NaBH_4$ reduction of $FeCl_3.6H_2O$, according to protocol, as follows: 1 g of $FeCl_3.6H_2O$ was dissolved in 80 mL of MilliQ water and a solution of 0.2 g of $NaBH_4$ in ammonia (3.5%, 10 mL) was poured into the first solution under vigorous stirring. The obtained solution was boiled for 2 h. After cooling down the magnetic nanoparticles were separated by external magnetic field and washed with water for several times. Resulting mixture was stirred overnight, separated using an external magnetic force field and dried at 40 °C. To form a shell structure on a nanoscaled maghemite several various modification processes were employed: in case of MAN18 100 mL of nanomaghemite solution was mixed with 20 mL of $Ti(isopropox)_4$. MAN32 were formed by bare nanomaghemite without shell structure. MAN38 formed addition (3.33 mL) of 3-amiopropyl triethoxysilan (APTES). MAN53 constituted 1.5 g of polyvinylpyrrolidone (PVP-40k) with reduced $HAuCl_4$ (1 mM). MAN57 were modified with 10 mL of 1% hyaluronic acid (HA), MAN59 with 2 mL of 18% poly(4-styrenesulfonic acid, 75k), MAN63 with 1 g of glucose and 100 mg of grafen oxide, MAN65 with 3 g of PVP (10k) with addition of 7.48 g $Fe(NO_3)_3.9H_2O$ and finally MAN70 with 0.5 g of PVP (10k), 1.5 g of glucose and 30 mL of 1 mM $HAuCl_4$. All complexes were washed with water three times and dried at 40 °C prior to use.

2.4 Preparation of Sample PMPs for MT Isolation

For isolation was employed 50 µL of dispersion, comprising 40 $mg.mL^{-1}$ of each type of PMPs in 1 mL PBS (pH 7). After incubation in thermomixer (37°C, 60 min, 800 rpm) (Eppendorf, Hamburg, Germany), the sample was dissolved in 3 M hydrochloric acid (250 µL) and evaporated using nitrogen evaporator Ultravap RC (Porvair Sciences, Leatherhead, UK). Finally, the evaporated sample was resuspended with ACS H_2O (250 µL) and analysed using Differential pulse voltammetry with adsorptive transfer technique (AdT DPV) and MALDI-TOF/TOF.

2.5 Differential pulse voltammetry with adsorptive transfer technique

Quantification of MT bound on the surface of paramagnetic nanoparticles was carried out using differential pulse voltammetry (DPV), using parameters according to[13]. The recovery of MT binding was calculated as difference between applied and analysed concentration of MT.

2.6 Descriptive statistics

Mathematical analysis of the data and their graphical interpretation were realized by Microsoft Excel®, Microsoft Word® and Microsoft PowerPoint®.

3. Results and Discussion

For all experiments MT was isolated from rabbit liver (MT was induced by intraperitoneal injection of 10 mg of $CdCl_2$.kg^{-1} of weight) by using fast-protein liquid chromatography from liver homogenates. After isolation, MT was lyophilized and after resuspendation in water subsequently analyzed by using MALDI-TOF/TOF for its purity. In **Fig. 1** are shown MALDI-TOF/TOF mass spectra obtained in two types of matrixes (**Fig. 1A**) HCCA and (**Fig. 1B**) DHB. In both conditions, monomers of MT were identified (major peaks ~6 kDa), however in DHB matrix ionization was carried out more readily (intensity about 4400 a. u.), and thus it was applied in subsequent measurements. Moreover, it was shown that purity of our isolated protein is very high, hence its applicability for further isolation experiments was confirmed. The iron oxide nanoparticles (γ-Fe_2O_3 and Fe_3O_4) are versatile materials, exploitable for magnetic separation of various types of analytes[14,15], in particular due to possibility of surface modifications, which leads to introduction of broad spectrum of binding sites. In our study we employed broad spectrum of various surface modifications, including metals, polymers, sugars, or carbon nanoparticles (for details see chapter **2.3 Preparation of paramagnetic core-shell particles**).

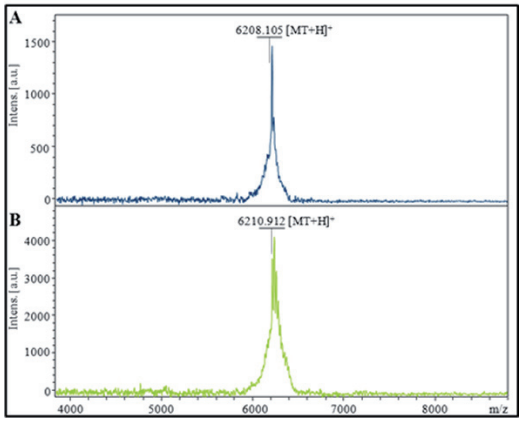

Figure 1. MALDI-TOF/TOF mass spectra of MT fractions after the fast protein liquid chromatography purification (concentration of MT - 2.0 µM). In analyses the linear positive mode, (**A**) HCCA and (**B**) DHB matrixes were employed.

The resulting paramagnetic particles were employed for screening of their binding capacity towards MT (1 µM) and by using MALDI-TOF/TOF it was shown that only paramagnetic particles with acronym MAN53, composed of γ-Fe_2O_3 covered with PVP layer with gold nanoparticles resulted from reduction of $HAuCl_4$ with citric acid[16], are able to bind MT (**Fig. 2**). The bond in other types of particles was likely to weak to withstand the washing steps or was not present at all. In this case MALDI-TOF/TOF was shown as a perfect tool for rapid screening tests of protein binding with very low requirements for sample volume (1 µL).

Since MT is constituted of more than 30% of cysteine residues[17], containing sulfhydryl moieties with high affinity towards metals, surface modification with gold nanoparticles seems to be the most suitable for MT binding (it was described that affinity of MT towards Au is much higher than towards Zn or Cd[18]). Moreover, the thiol/gold bond is strong enough to withstand washing steps, involved to remove the undesired impurities.

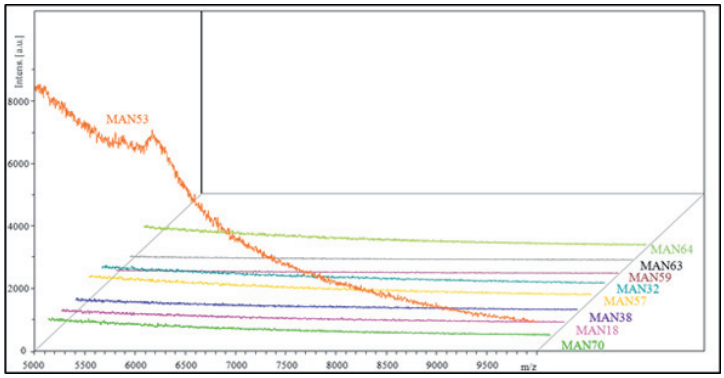

Figure 2. MALDI-TOF/TOF mass spectra showing the results of screening of various types of paramagnetic particles (MANX) for their ability to bind MT (1 µM) on their surface. MS measurements were carried out in linear positive ion mode, with DHB matrix and laser power established to 80%.

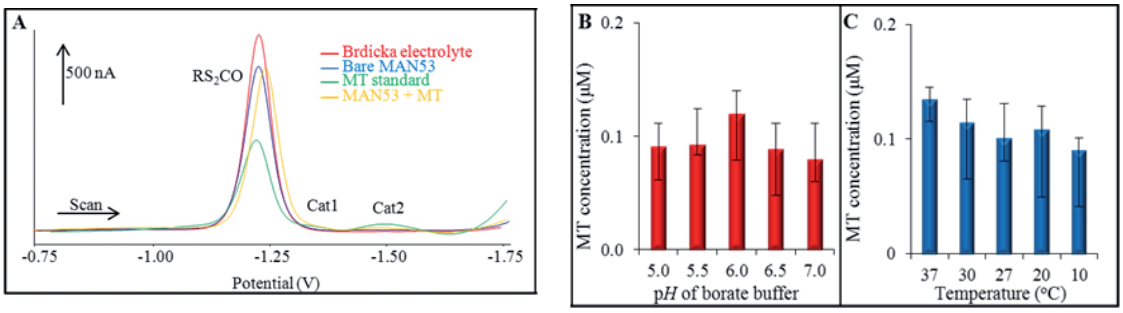

Figure 3. (**A**) Typical voltammogram, obtained by using AdT DPV with Brdicka electrolyte, where (**a**) stays for Brdicka electrolyte (1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) and NH_4Cl, pH = 9.6), (**b**) for bare MAN53, (**c**) for MT standard (500 nM) and (**d**) for MAN53 + MT conjugate. (**B**) Optimization of ideal binding conditions – pH of binding borate buffer (5.0 - 7.0). (**C**) Influence of temperature (10 – 37 °C) on binding of MT on MAN53. All optimization conditions were evaluated by using AdT DPV. Values are means of three independent replicates (n = 3). Vertical bars indicate standard error.

Since electrochemistry offers many advantages in determination of thiol compounds[13], we employed AdT DPV for quantification of MT immobilized on MAN53 surface. In **Fig. 3A** are depicted typical electrochemical voltammograms for PMPs, MT and their interleaving records (labelled PMPs + MT). Voltammograms obtained in Brdicka solution show three characteristic peaks (RS_2Co, Cat1 and Cat2). RS_2Co is around -1.25 ± 0.05 V and is associated with the reduction of protein thiol moieties[19]. Cat1 (around -1.30 ± 0.05 V) and Cat2 (around -1.50 ± 0.05 V) are related to the reduction of hydrogen developed from electrolyte catalyzed by thiol groups on the mercury electrode[19] and the height of Cat2 peak is related to the amount of MT in sample[20]. Further in **Fig. 3A** is obvious the absence

Although it was shown that MAN53 particles immobilize MT, we further optimized the conditions of incubation. In particular, pH of buffer in which binding is carried out and incubation temperature can significantly influence the immobilization efficiency[15].

As it is represented in **Fig. 3B** the largest immobilization yields were achieved in 100 mM borate buffer with pH 6.0 (0.12 µM MT). With both - decreasing and increasing pH the amount of electrochemically determi-

ned MT on particles was reduced (0.09 μM in pH 5.0 or 0.08 μM in pH 7.0). Thus, further optimization of incubation temperature was carried out with 100 mM borate buffer, pH 6. In case of temperature of incubation (10 - 37°C), the highest yields were observed when using 37°C, while lowering of temperature lead to decrease of MT amount. After application of ideal optimized conditions the recovery of MT immobilization was established to be about approximately 25%. Paramagnetic particles modified with gold nanostructures can thus serve for immobilization of MT through strong bonds. This phenomenon can be employed for isolation of MT from biological matrixes and moreover for immobilization of MT on electrodes surface to enhance the sensitivity and specifity towards heavy metal detection.

4. Conclusion

In particular, because of low fabrication cost and easy manipulation paramagnetic particles have currently attracted much attention. In this present study nanomaghemite core was synthesized and subsequently functionalized with shell, composed of PVP and gold nanoparticles evolving from reduction of $HAuCl_4$ with sodium citrate. Resulting structure binds MT with approximately 25% recovery, when using optimized conditions of incubation - 100 mM borate buffer, pH 6 and incubation temperature 37°C. Such paramagnetic particles can be applicable in isolation of MT from various biological matrixes for its subsequent determination as a biomarker of various pathological states, including head and neck cancer. Moreover, particles can be used for immobilization of MT in separation part of heavy metal biosensor.

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The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements“ for biomedical papers.

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