ISOLATION OF REDUCED AND OXIDIZED GLUTATHIONE USING MAGHEMITE PARTICLES

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Abstract

Here we report the optimal conditions for the isolation of reduced glutathione (GSH) and oxidized glutathione (GSSG) from various types of matrix, such as blood, plasma or cells and their characterization using ion-exchange liquid chromatography with VIS detector. In our study, we aimed to synthesize paramagnetic microparticles able to isolate and immobilize GSH, GSSG from blood or plasma and thus preconcentrate it for subsequent analysis on ion-exchange liquid chromatography with VIS detector. We modified amberlite and Dowex by nanomaghemite (γ-Fe_2O_3), as the functional carriers providing perfect paramagnetic properties. Both the paramagnetic microparticles were able to bind GSH and GSSG. Our paramagnetic microparticles have potential to improve GSH and GSSG isolation and in future can serve for application as a platform of biosensors or delivery system.

Keywords: Ion exchange chromatography, maghemite microparticles, glutathione, reactive oxygen species

1. INTRODUCTION

Oxidative stress causes profound alterations of various biological structures, including cellular membranes, lipids, proteins and nucleic acids, and it is involved in various malignancies [1]. Reduced glutathione (GSH) is a water-soluble tripeptide composed of glutamine, cysteine and glycine. GSH is considered to be one of the most important scavengers of reactive oxygen species (ROS), and its ratio with oxidized glutathione (GSSG) may be used as a possible marker of oxidative stress [2]. GSH is very important antioxidant in cells [2,3], animals [4,5], plants, bacteria [6] and human [7]. In the process, glutathione is converted to its oxidized form, glutathione disulfide, also called L-glutathione. Glutathione is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals [8]. It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side chain and the amine group of cysteine and the carboxyl group of cysteine is attached by normal peptide linkage to a glycine. Nowadays, numerous methods are developed for GSH and GSSG determination. Most of them are based on chromatography (gas chromatography, liquid chromatography, and/or high performance liquid chromatography [9] with tandem of mass spectrometry [10], capillary electrophoresis or ion exchange chromatography (IEC) [6]. In our study, we decided to use IEC with post-column ninhydrin derivatization and visible light photometric detector for GSH and GSSG determination [11].

2. METHODOLOGICAL BASES AND EXPERIMENTAL PART

2.1. Chemicals

GSH, GSSG, citric acid, NaCl, N_3Na, TDG, HCl (35%) and ninhydrin of purity 99% were obtained from Sigma Aldrich (St. Louis, Missouri, USA). Solutions of GSH and GSSG for preparing of calibration curve were

prepared in the dilution buffer Na: TDG (N_3Na-0.10 g, NaCl - 11.5 g, $C_6H_8O_7$ - 14 g per 1L H_2O). Methylocellosolve and $SnCl_2$ were supplied from Ingos, (Prague, Czech Republic).

2.2. Ion exchange chromatography

An AAA 400 (Ingos, Czech Republic) liquid chromatography apparatus was used for determination of amino acids. The system consists of a glassy filling chromatographic column and steel precolumn, two chromatographic pumps for transport of elution buffers and derivatization reagent, cooled carousel for 25 test tubes of 1.5-2.0 mL volume, dosing valve, heat reactor, visible light photometric detector and cooled chamber for derivatization reagent. Chromatographic columns for transfer of elution buffers and derivatization reagent are able to work at flow 0.01-10 $mL \cdot min^{-1}$ under a maximum pressure of 40 MPa. Volume of injected sample was 100 µL with an accuracy of application relative standard deviation of about 1%. A two-channel visible light photometric detector with a 5 µL flow volume cuvette was operated at wavelengths of 440 and 570 nm.

2.3. Scanning electron microscopic (SEM) characterization of DOWEX and amberlite modified microparticles

Structure and elemental composition of paramagnetic microparticles were characterized by electron microscope Tescan, a.s., Brno, Czech Republic. This model is equipped with a high brightness Schottky field emitter for low noise imaging at fast scanning rates. The SEM was fitted with Everhart-Thronley type of SE detector, high speed YAG scintillator based BSE detector, panchromatic CL Detector and EDX spectrometer. The MIRA 3 XMU system is based on a large specimen chamber with motorized stage movements 130×130 mm. Samples were coated by 10 nm of carbon to prevent sample charging. A carbon coater K950X (Quorum Technologies, Grinstead, United Kingdom) was used. For automated acquisition of selected areas, a TESCAN proprietary software tool called Image Snapper was used. An accelerating voltage of 15 kV and beam currents about 1 nA gives satisfactory results regarding maximum throughput.

2.4. Scanning electrochemical microscopic (SECM) characterization of DOWEX and amberlite modified microparticles

The scanning electrochemical microscope SECM 920C from CH Instruments, Austin, Texas, USA was used for measurements. Gold substrate electrode with a diameter of 3 mm has been cleaned by sonication in ethanol, and then was treated for two minutes with a piranha solution, which was made from 30% hydrogen peroxide and concentrated sulfuric acid in a ratio of 1:3. Then cyclic voltammetry was started in 0.5 M sulfuric acid from -0.2 to +1.5 V. Finally, the electrode was washed with water and dried with nitrogen.

2.5. X-ray fluorescence (XRF) analysis of PMPs

XRF analysis of PMPs was carried out on Xepos (SPECTRO analytical instruments GmbH, Kleve, Germany) fitted with three detectors: Barkla scatter - aluminum oxide, Barkla scatter - HOPG and Compton/secondary molybdenum respectively. Analyses were conducted in Turbo Quant cuvette method of measuring. Analysis parameters were set to - measurement duration: 300 seconds, tube voltage from 24.81 to 47.72 kV, tube current from 0.55 to 1.0 mA, with zero peak at 5000 cps and vacuum switched off.

2.6. Preparation of PMPs

MAN 3

Maghemite was prepared by sodium borohydride reduction of iron chloride. 10 g of $FeCl_3 \cdot 6H_2O$ was dissolved in 800 ml of water followed by addition of 2 g $NaBH_4$ in 100 ml of 3.5% NH_3. Product was separated by a magnet and washed 5 times with water. Final volume was 100 ml. To 20 ml of this solution was added amberlite with stirring at Biosan OS-10 overnight. Then the product was separated by a magnet, washed with water and dried at 40 °C.

MAN 16

It was prepared in a similar way, only 1 g of Dowex was added instead of amberlite.

3. RESULTS AND DISCUSSION

Our major idea was an isolation and 2D separation of the low mass molecules, able to serve as the potential ROS biomarkers, based on the adsorption on the PMPs with various modifications and subsequent determination using IEC. We modified amberlite with nanomaghemite and these PMPs showed the best properties for binding of GSH (recovery 36.83%) (**Figure 1D**). Our PMPs showed also relatively good selectivity, which is probably based on principles of workflow process used for deprivation of undesired impurities from beads. Britton-Robinson buffer with pH 2 causes GSH and GSSG protonation which leads into a positive charging of molecules due to its pI = 5.0. Interaction between positively charged molecule and surface of PMP provides the forming of bond between them. These interactions depend on isoelectric points of amino acids, which are in this manner behaving as the ion-exchangers. Other amino acids showed also relative good recoveries (serine recovery 1.1%, leucine 1.2%, sarcosine 1.3%, GSSG 3.1%, glutamic acid 6.7%, glycine 10.08%, valine 10.35%, arginine 10.48% and GSH 36.83%, respectively); nevertheless, their amounts in urine are not so high to interfere during GSH binding to PMPs **Figure 1D**. Moreover, our second separation step - IEC - eliminates the influence of interferents to minimum.

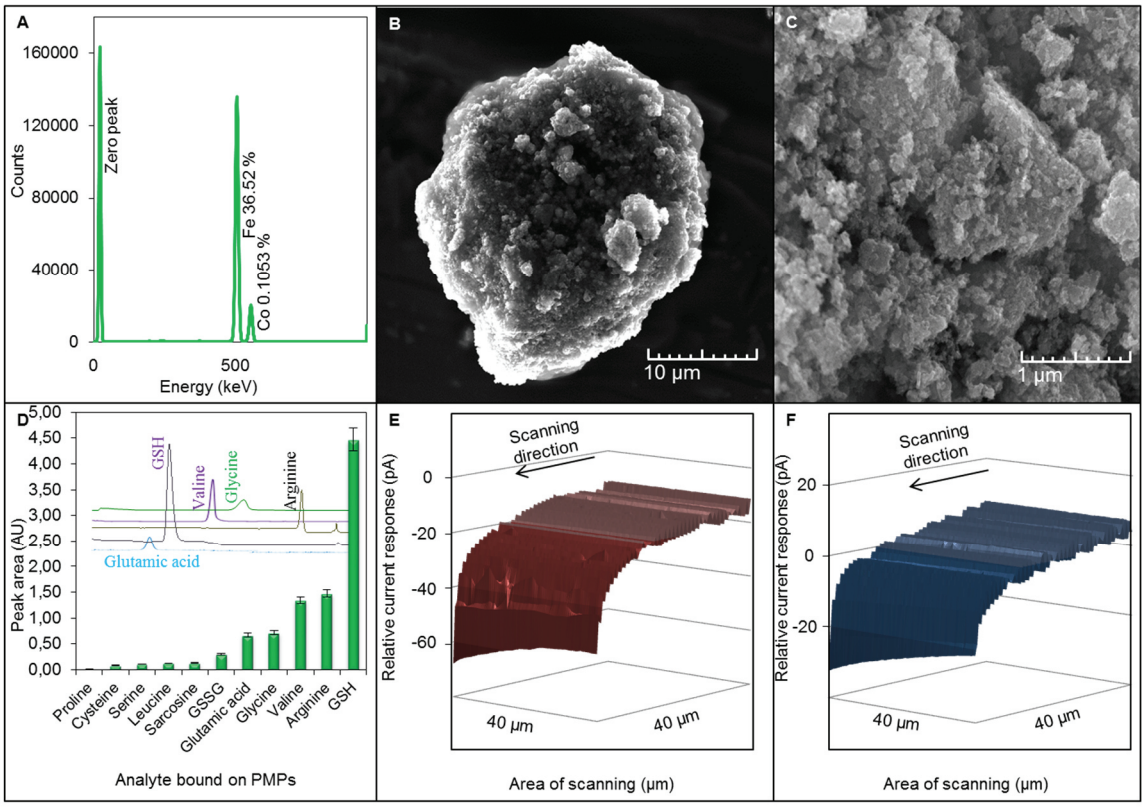

Figure 1 Characterization of PMPs MAN3. (**A**) XRF showing elemental composition of paramagnetic microparticles. (**B**) SEM image in resolution of 10 µm. (**C**) SEM scan in resolution of 1 µm. (**D**) IEC results showing ability of MAN3 bound required substances specifically. (**E**) SECM scan showing relative current response of MAN3 without GSH bound. (**F**) SECM scan of MAN3 with GSH bound showing increased relative current response.

After confirmation of ability to bind GSH, we carried out a characterization of PMPs. First characterization step was XRF analysis providing information about element composition of microparticles. In the **Figure 1A** can be

seen that iron was determined to be the most abundant element (Fe represented in 36.52%). This information was not surprising, because we carried out SECM analysis for recognition of PMPs surface relative current response changes in dependence on GSH binding. In **Figure 1B** can be seen a micrograph expressing microparticles surface and morphology in resolution of 10 µm for MAN16 and in **Figure 1C** is SEM micrograph of PMP surface in resolution of 1 µm. In **Figure 1E**, F can be seen 3D images, expressing a relative current response of PMP surface without GSH and with GSH bound. Comparing the figures, considerable changes of surface current response can be observed. Relative current response after establishing of GSH binding is higher than before binding. This obvious difference (approximately 20 pA) is indicating that the GSH binding leads to a change of PMPs attributes. As mentioned above, GSH gets protonated under influence of acidic pH maintained by Britton-Robinson buffer used during workflow process. Positively charged molecule increases current response of PMP. Hence, we received a confirmation that PMPs bind GSH and GSSG properly and therefore are usable for their isolation and preconcentration from various types of matrices.

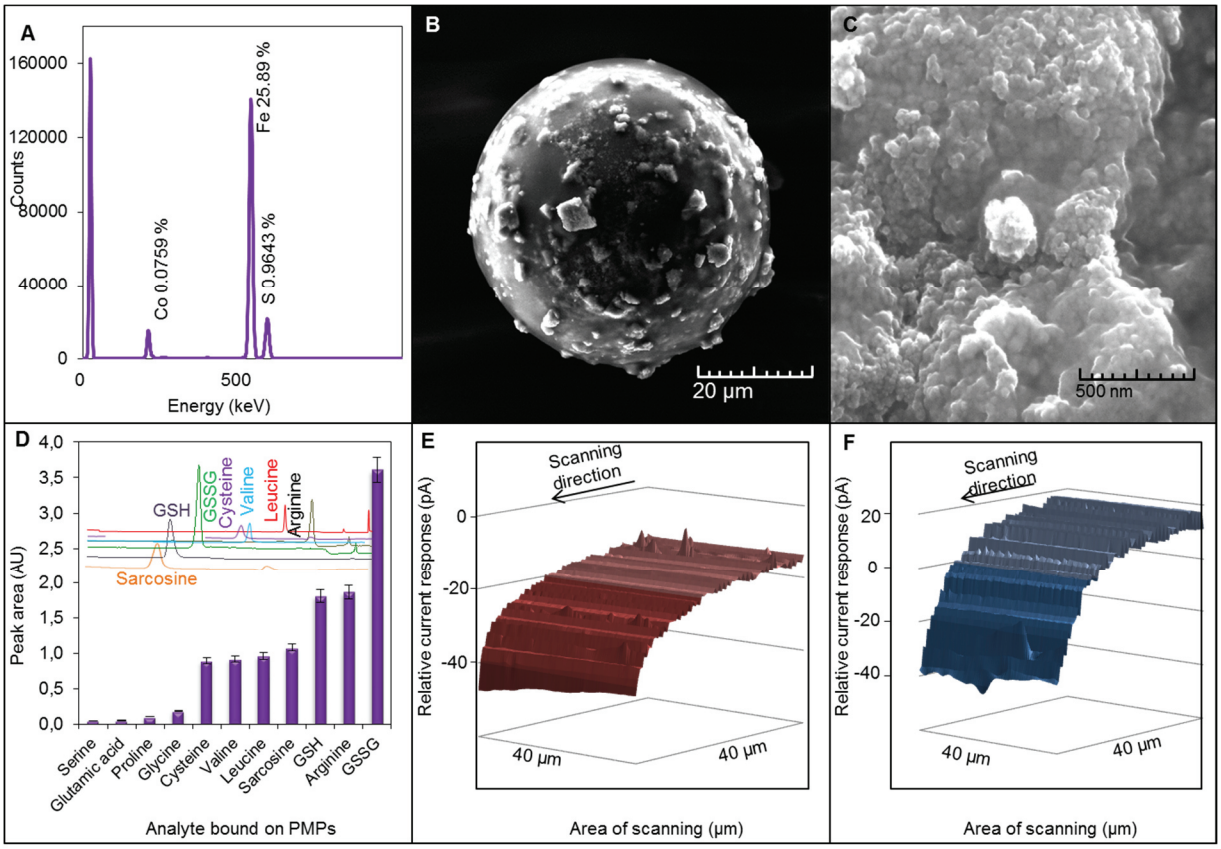

Figure 2 Characterization of PMPs MAN16. (**A**) XRF showing elemental composition of MAN16. (**B**) SEM scan of MAN16 in resolution of 20 µm. (**C**) SEM micrograph of MAN16 in resolution of 500 nm. (**D**) IEC results showing ability of MAN16 bound required substances specifically. (**E**) SECM scan showing relative current response without GSSG bound. (**F**) SECM scan with GSSG.

In the second step of our experiment, we modified Dowex with nanomaghemite, most important parts of PMPs are Dowex, Fe_2O_3 microparticles and SO_3^- functional groups and these PMPs showed the best properties for binding of GSSG (recovery 29.66%) (see **Figure 2D**) In the **Figure 2A** can be seen that the most abundant elements were iron (25.89%) and S (0.96%). Basic characterization of synthesized PMPs can been seen in **Figure 2B**, showing microparticles surface and morphology imaged by SEM in resolution of 20 µm. **Figure 2C** is a SEM micrograph of PMP surface in resolution of 500 nm. In **Figure 2D**, IEC analysis of amino acids with relatively good recoveries (serine recovery 1.1%, leucine 1.2%, sarcosine 1.3%, GSSG 3.1%, glutamic acid 6.7%,glycine 10.08%,valine 14.05%,GSH 14.05%, arginine 14.83% and GSSG 29.66%) are shown. In

Figure 2E, a **3D** image expressing a relative current response of PMPs surface without GSSG interaction can be seen. Comparing with **Figure 2F**, considerable changes of surface current response can be observed. Relative current response after establishing of GSSG binding is higher than before binding. This obvious difference (approximately 20 pA) is indicating that the GSSG binding leads to a change of PMPs attributes.

4. CONCLUSION

In this study, we synthesized new paramagnetic microparticles able to bind GSH and GSSG - as possible markers of oxidative stress and ROS such as free radicals, peroxides, lipid peroxides and heavy metals. The paramagnetic microparticles (MAN 3, MAN 16) have potential to better isolation in the samples of blood or plasma and in future can serve for application in biosensors.

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