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Analytical detection of biological thiols in a microchip capillary channel

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ABSTRACT

Sulfur-containing amino acids, such as cysteine and homocysteine play crucial roles in biological systems for the diagnosis of medical states. In this regard, this paper deals with separation, aliquot and detection of amino thiols on a microchip capillary electrophoresis with electrochemical detection in an inverted double Y-shaped microchannel. Unlike the conventional capillary electrophoresis, the modified microchannel design helps in storing the separated thiols in different reservoirs for further analysis, if required; and also eliminates the need of electrodes regeneration. The device was fabricated using conventional photolithographic technique which consisted of gold microelectrodes on a soda lime glass wafer and microchannels in PDMS mold. Multiple detections were performed using in-house fabricated dual potentiostat. Based on amperometric detection, cysteine and homocysteine were analyzed in 105 s and 120 s, respectively after diverting in branched channels. Repeated experiments proved the good reproducibility of the device. The device produced a linear response for both cysteine and homocysteine in electrochemical analysis. To prove the practicality of device, we also analyzed cysteine and homocysteine in real blood samples without any pre-treatment. Upon calculation, the device showed a very low limit of detection of 0.05 μM. The modified microchip design shall find a broad range of analytical applications involving assays of thiols and other biological compounds.

1. Introduction

Biological thiols are compounds of main interest due to their importance in biological processes. Cysteine (Cys) and homocysteine (Hcys) are naturally occurring thiol containing amino acids, and are structurally similar and metabolically linked. Abnormal levels of these amino thiols in human plasma and urine are linked with a number of diseases (Refsum et al., 1998; Seshadri et al., 2002; Ueland and Vollset, 2004). Increased levels of both Cys and Hcys have been often associated with neurotoxicity (Janaky et al., 2000) and Cys induced hypoglycemic brain damage has been studied as an alternative mechanism to excitotoxicity (Gazit et al., 2004). Additionally, low level of Cys is associated with slow growth, hair depigmentation, edema, lethargy, liver damage, muscle and fat loss, skin lesions, and weakness (Shahrokhian, 2001), as Cys is an active site in the catalytic function of certain enzymes called cysteine proteases. It is also widely used in the food industry as an antioxidant and in the pharmaceutical industry in drug formulations. High level of Hcys is associated with increased risk of myocardial infarction, stroke, and venous thromboembolism (Refsum et al., 1998). Hyperhomocysteinemia has also been linked to increased risk of Alzheimer's disease (Seshadri et al., 2002), neural tube defects (Steegers-Theunissen et al., 1991), complications during pregnancy (Ueland and Vollset, 2004), inflammatory bowel disease and osteoporosis. Furthermore, alterations in Hcys metabolism have also been observed clinically in diabetic patients (Hofmann et al., 1998). Hcys is of interest as an analyte for the screening of inborn errors of methionine metabolism. Therefore, the rapid, sensitive and selective detection of Cys and Hcys is of much importance for investigating their functions in cells and medical diagnosis. Currently available methods for determination of thiols in body fluid samples focus on chromatographic (Chwatko and Bald, 2000) or electrophoretic separation methods (Arlt et al., 2001) coupled with spectrometric (Tanaka et al., 2004), colorimetric and fluorimetric detection (Wang et al., 2005b) or immunoassay (Frantzen et al., 1998; Kusmierek et al., 2006; Wang et al., 2005b). Therefore the development of a simpler and rapid assay method is highly desirable.

Recent advances in microfabrication technique have facilitated the creation of on-chip capillary electrophoresis (CE) devices coupled with optical or electrochemical detection methods. Particularly, the CE analysis can achieve unparalleled sensitivity of up to attomoles levels when combined with amperometric

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detection (AD) technique. Electrochemical measurements of amino thiols have attracted considerable interest because of their high sensitivity, simplicity, low cost and feasibility to the development of in vivo sensors and detectors (Chen et al., 1990; Perez et al., 1998; Shi et al., 1999). Electrochemical detection of thiols was done in conjunction with HPLC. Hiraku et al. and Cataldi et al. used gold electrode in HPLC for pulsed amperometric detection of Cys and other thiols which helped in regeneration of gold surface (Cataldi and Nardiello, 2005; Hiraku et al., 2002). A variety of chemically modified and specially prepared electrodes have also been used for the voltammetric detection of thiols. Electrodes like, coenzyme pyrroloquinoline quinone, ferrocenedicarboxylic acid and ordered mesoporous carbon modified carbon electrodes were employed for the detection of thiols (Inoue and Kirchhoff, 2002; Raoof et al., 2007; Zhou et al., 2007). The electrochemical method of detection offers better chances of miniaturizing CE system compared with optical or spectrometric techniques, owing to the simplicity and ease in microfabrication of on-chip microelectrodes. Some of the reported electrodes suffer from certain drawbacks, such as instability (Chen et al., 1990), large overpotential (Tang et al., 2010) and low sensitivity (Perez et al., 1998) which reduces detection selectivity, especially in the case of biological and real samples. A majority of these methods is based on pretreatment of electrodes for specific modification which makes it cumbersome and increases the overall cost. Fabrication of special electrodes and its modification loses the practicality of the device. Another major drawback of conventional microchip CE–AD system is that after separation, the analytes migrates over the detection electrode and then mixes up again in the waste reservoir which limits the use of separated analytes for further analysis.

Therefore, in the present work, we devised a simple on-chip CE–AD process to separate, aliquot and detect Cys and Hcys. The electrodes were fabricated on glass wafers while microchannels were laid in PDMS. Gold was used as the material for electrode to separate and detect thiols. Gold is a very common material for electrode and has been used in several forms to detect thiols. Pure gold has been used for pulsed amperometric detection while Agui et al. used gold nanoparticle modified electrode and Wirde et al. used self assembled monolayer of thiols on gold surface, to detect thiols (Agui et al., 2007; Wirde et al., 1999). The microchannel had an inverted double Y-shaped structure to collect the separated analytes in different reservoirs. A handy potentiostat array was also fabricated to simultaneously detect analytes in different channels. We also detected Cys and Hcys in real blood samples which were achieved without any electrode or sample pretreatment, which is an added advantage of this report. Use of in house built potentiostat and other experimental setup led to simultaneous determination of Cys and Hcys with an extremely low limit of detection (LOD).

2. Experimental

2.1. Materials and equipment

The testing analytes included cysteine and homocysteine purchased from Sigma Aldrich (USA). All other solvents and chemicals were of analytical grade. Heparinized blood samples were obtained from university clinic and were used as received. Deionized water was used throughout this study. Electrochemical measurement including cyclic voltammetry (CV) and chronoamperometry (i–t curve) were carried out using an electrochemical analyzer CHI 800B (CH Instruments, USA) or in-house built dual potentionstat.

2.2. CE–AD microchip configuration and fabrication procedure

The configuration of the CE–AD microchip is shown in Fig. 1A. Our CE–AD microchip was fabricated by standard photolithographic procedures (Jang et al., 2011). The chip was built on single soda lime glass substrate with microchannel engraved in PDMS. For negative molding, a silicon wafer cleaned with piranha solution was spin coated with SU8-2075 negative photoresist (Micro-Chem, USA) and patterned using a photomask and UV exposure (SUSS Microtec, Germany). The microchannels were subsequently cast by pouring of a degassed mixture of Sylgard 184 silicone elastomer and curing agent (10:1) (Dow Corning, USA) on this master wafer, followed by curing for at least 1 h at 75 °C. The positive patterns formed the inverted relief of the desired microstructures and were equal to the channel depth created on the PDMS layer. The cured PDMS was peeled off from the mold, and reservoirs were made at the end of each channel using a 3-mm circular punch. The microchannel had an inverted double Y-shaped structure. The straight microchannel had the dimensions of 6 cm in length, 250 μm in width and 120 μm in height. The straight channel had branches in the opposite direction with funnel shaped nodes, 2 cm in length at a distance of 1 cm and 1.5 cm respectively measured from the outlet reservoir. All the electrodes used in this study were made from Au/Ti layers. The three sets of electrode each containing three electrodes for the electrochemical detection, viz. working, reference and counter; in addition electrodes for applying separation electric field were fabricated on the sodalime glass wafer using the vacuum thermal evaporation method. The Au layer was fabricated by first spin coating AZ-1512 positive photoresist on the bare glass and then patterning with a photomask. Subsequently, 50 nm thick Ti and then 300 nm thick Au layers were deposited on the patterned surface. The remaining photoresist was subsequently removed by using an ultrasonic cleaner. The width of reference, working and counter electrodes created in this process was 250 μm. The electrodes were separated by a distance of 200 μm. In the end, the PDMS mold carrying microchannel was bonded to the glass substrate containing Au microelectrodes by UV-ozone treatment.

2.3. Fabrication of dual potentiostat

The potentiostat array device having two potentiostat channels was assembled in our laboratory using simple op-amp circuits. The electronic circuit of the potentiostat consisted of several op-amps for each potentiostat unit. One of the op-amp (LM 348 N, Texas Instruments, USA) was used as voltage followers and comparator. The precision op-amp OP 177AZ (Analog Devices, USA) was used as current to voltage converter. This analog circuit was interfaced with analog to digital converter card (NI USB 6212) from National Instruments, USA. The device was controlled using a program developed with LabVIEW (National Instruments, USA) code. It was possible with this device–software interface to apply fixed or variable bias on counter/reference electrode combinations and read the output current as a consequence of redox activity on working electrodes of each channel, simultaneously and sensitively. The measurement data could be plotted online as well as stored for offline use. The instrument was first validated through a series of conventional electrochemical studies, including CV of potassium ferricyanide ($K_3[Fe(CN)_6]$).

2.4. Electrochemical detection and electrophoresis procedure

At first, conventional CV studies were carried out on Cys and Hcys with a three electrode system using an electrochemical analyzer. The voltammetric analysis of Cys (100 μM) and Hcys (100 μM) in 100 mM NaOH solution were performed using planar gold working (2 mm dia.), Pt wire counter and Ag/AgCl reference (3 M NaCl) electrodes. Through these CV experiments, we could find the detection voltage(s) to be applied in CE–AD device and peak current range that these chemicals would generate. Subsequently, the disposable microchip was subjected to CE–AD for the analysis of