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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

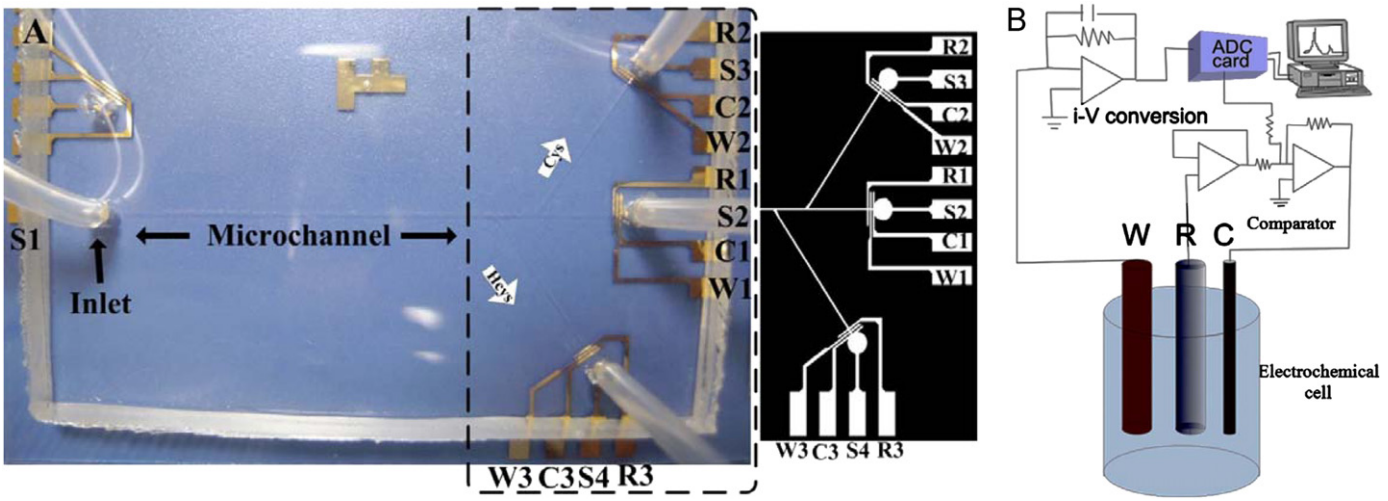

Fig. 1. (A) Image of CE–AD microchip showing microchannel engraved in PDMS mold, sample reservoirs, silicon tubes carrying sample and NaOH solution into the microchannel, gold microelectrodes (W1–3=working; C1–3=counter; R1–3=reference; S1–4=separation electrodes). (B) Schematics for electronic circuit and the operation of the in-house built dual potentiostat.

free thiols while applying the detection voltages as obtained from their voltammograms. At first, the microchannel was cleaned using NaOH solution and then 1.5% (w/v) agarose gel prepared in 100 mM NaOH was filled in the channel. The gel was allowed to settle for 30 min and then electrophoresis was performed. The amperometric detection was carried out in two parts, first the separation was done in the central channel to calculate the electrophoretic mobility of analytes; and then based on calculated detection time, analytes were diverted into branched channels by switching the direction of electric field. First, 2 μL of the sample mixture was injected into the sample reservoir close to the microchannel opening using a micropipette and an electric field of 100 V was applied immediately between the inlet reservoir and the waste reservoir. AD was performed with three-electrode configuration placed in the path of analyte flow near the end of the channel. Redox reaction of Cys and Hcys on the working electrode generated current peaks in the i–t curve, which was recorded and stored on a computer using the commercial electrochemical analyzer or the dual potentiostat. The electropherogram gave the migration pattern of Cys and Hcys within the straight channel. Calculation of electrophoretic mobility and migration time provided the time (x) taken by Cys to travel up to the first branch. In the second stage, a new CE–AD chip was processed, as mentioned before. Later on, 2 μL of the sample mixture was injected into the sample reservoir and an electric field of 100 V was applied immediately between the inlet reservoir and the waste reservoir of the straight channel. After time x, the electric field between inlet reservoir and the waste reservoir of the straight channel was cut off and an electric field of 100 V was applied immediately between inlet reservoir and the waste reservoir of the branched channels. The change in electric field direction diverted the Cys and Hcys in two separate channels which were then detected using an in-house built dual potentiostat. The change in electric field and switching-on of dual potentiostat was controlled using the LabVIEW program.

3. Results and discussion

3.1. Device fabrication

The CE–AD microchip used in this study was fabricated on a glass substrate with PDMS mold containing microchannel pattern. PDMS is a widely used material for microfluidic devices due to their transparent and flexible properties and as they can retain microstructures for a considerably long period without fatigue. The choice for all-gold electrode material was due to their inertness to redox reactions and ease of fabrication over having an Au, Pt and Ag/AgCl microelectrode configuration (Jang et al., 2011; Jha et al., 2009). Agarose gel in the microchannel creates a drag force on analytes due to its sieving action, which is essential for the effective separation of amino acids as the molecular weight difference between Cys and Hcys is too low. The design of our CE–AD separation channel is considerably different from previous reports incorporating double T structure (Kim et al., 2005) or straight channel configurations (Jang et al., 2011). Conventional microchip CE employ separation and detection of analyte within a microchannel but post detection, analyte mixes up again in the waste reservoir and cannot be reused for other analysis. In our system we used an inverted double Y-shaped channel which diverts the Cys and Hcys into two different branched microchannels after separation which is then detected separately using the dual potentiostat. As the detection is performed in two different channels, the two waste reservoirs contain pure Cys and Hcys respectively. The schematic of fabricated dual potentiostat is shown in Fig. 1B.

3.2. CV of cysteine and homocysteine

The cyclic voltammetric analysis of Cys and Hcys was the prerequisite for the knowledge of detection voltage to be applied in CE–AD procedure. Therefore, conventional CV analysis of these amino acids was performed using three electrodes in a beaker. The NaOH was used as the supporting electrolyte and proved to be useful, as amino acids were redox active at alkaline pH with negative charge which facilitates better electrophoretic mobility. Several reports mention the use of alkaline media for the detection of thiol containing amino acids (Casella et al., 2002).

The voltammetric experiments of Cys and Hcys were performed separately in 0.1 M NaOH solution with 100 μM concentrations of each. The electrochemical measurements were performed at least three times for each condition ($n=3$) except otherwise stated. The potential was cycled from −0.2 to 0.8 V with a scan rate 100 mV/s for each of the analyte. Fig. 2 shows the cyclic voltammograms of Cys and Hcys on the gold electrode in 0.1 M NaOH solution. A stable voltammogram was obtained without any sign of thiols fouling the electrode surface. Both

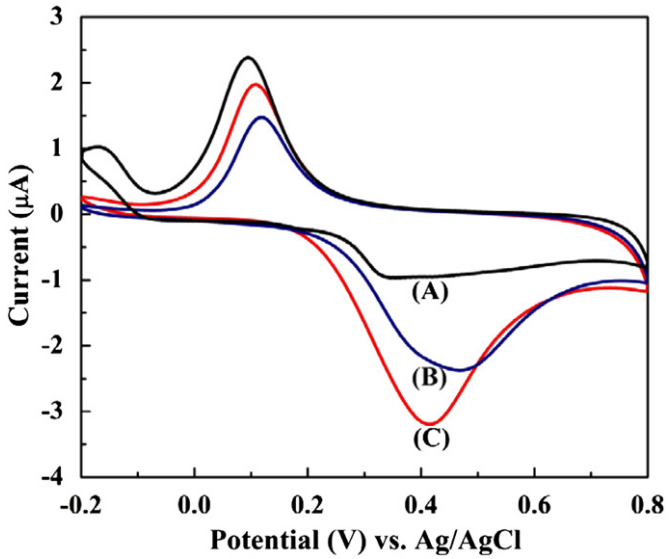

Fig. 2. CV of amino thiols using conventional disk electrodes: (A) 100 mM NaOH, (B) Hcys (100 μM) in 100 mM NaOH and (C) Cys (100 μM) in 100 mM NaOH; scan rate: 100 mV/s.

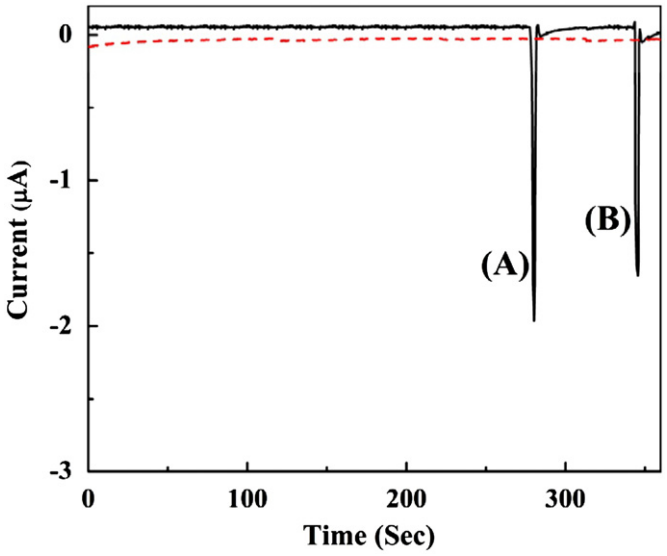

Fig. 3. Electropherograms of (A) Cys, (B) Hcys at 5 μM concentrations each and (—) 100 mM NaOH in straight channel at a separation voltage of 100 V DC and detection voltage of 0.5 V.

Cys and Hcys produced defined oxidation peaks in the anodic scan at 0.42 and 0.48 V, respectively. To detect sample in straight microchannel a detection voltage of 0.5 V was applied, which could simultaneously detect Cys and Hcys. In the branched channel, specific 0.42 V and 0.48 V were applied using the dual potentiostat to detect Cys and Hcys respectively.

3.3. CE–AD of cysteine and homocysteine in straight microchannel

Capillary electrophoretic detection of amino acids has long been a topic of interest and has been summarized by Carlucci and Tabucchi (2009), Poinsot et al. (2012). In the recent past, several groups tried to detect cysteine, homocysteine and other biological thiols electrophoretically in capillary channels. Batz et al. and Antwi et al. based their system on flow injection detection using amalgam electrodes (Antwi et al., 2011; Batz and Martin, 2009). The microchip involved just a detection system and did not include a separation system. Chen et al. suggested a microchip CE–AD device using a thin wire carbon nanotube electrode for the detection of amino thiols but it lacked the idea of miniaturization (Chen et al., 2004). Lately, Pasas et al. and Kim et al. demonstrated a microchip CE–AD with on-chip microelectrodes to detect amino acids and amine amperometrically (Kim et al., 2005; Pasas et al., 2002). The majority of such research involved expensive and toxic modification of electrodes and used flow injection based detection.

As Cys and Hcys are closely related compounds with only a small difference in molecular weight, low sensitivity and overlapping of the signals are the main disadvantage of many sensors. A modified channel design and use of agarose gel inside the microchannel, achieved a distinct separation and also prevented the fouling of electrodes. Agarose gel is a well known polymer to achieve electrophoretic separation with its molecular sieving action. 1.5% (w/v) agarose was dissolved in 0.1 M NaOH and cooled down to around 40 °C before injecting it into the pre-washed microchannel using a syringe. The agarose gel was allowed to settle for 30 min.

Cysteine (5 μM) and homocysteine (5 μM) mixture were subsequently analyzed on the microfluidic chip at 0.5 V by injecting a small volume of 2 μL into the reservoir. The separation voltage between the straight channel reservoirs was kept at 100 V, which is one of the mildest conditions used in any CE–AD analysis. A low separation voltage ensures less corrosion of the electrodes and the less shearing force is experienced by biomolecules, thereby preserving their native state. The resulting electropherogram is shown in Fig. 3. In this experiment, migration time of Cys and Hcys was found to be 280 s ($n=3$, $SD=2.8$) and 345 s ($n=3$, $SD=3.5$) respectively. The average current response for Cys was 2 ± 0.1 μA while for Hcys the average current response was 1.8 ± 0.2 μA. The electropherogram, thus proved the effectiveness of using such device in separation of Cys and Hcys.

3.4. CE–AD of cysteine and homocysteine in branched microchannel

The aim of the present study was to separate, deviate and detect amino acid separately in branched channels by the CE–AD method. The success of this methodology would decide the usefulness of the proposed device in rapid detection and analysis of thiols in body fluids. After the initial success of separation and detection of Cys and Hcys mixture in a straight microchannel, we performed their diversion and detection in branched channels. In order to perform such separation, we first calculated the electrophoretic mobility and the time taken by Cys to reach up to the first branching, using the following equation:

$$\mu_p = (L/T_r)\,(L_t/V) \quad (1)$$

where, μ_P is the electrophoretic mobility, L is the distance from the inlet to the detector, T_r is the detection time, L_t is the total length of the microchannel and V is the voltage of the applied electric field. It was calculated that Cys would take 230 s to reach the node of first branching.

Subsequently, we injected 2 μL of an equiproportionate mixture of Cys and Hcys into a fresh device filled with 1.5% (w/v) agarose gel, and applied an initial separation voltage between the inlet and outlet reservoir of straight channel. After 230 s, the electric field was switched in the direction of outlets of branched channels. The change in the direction of electric field allowed the electrophoresis of Cys and Hcys to continue towards the branched channel rather than towards the outlet of straight channel. Thereafter, simultaneous detection of both the components was performed using the dual potentiostat. The detection voltage was set to 0.42 V and 0.48 V for Cys and Hcys respectively. Fig. 4

shows the resulting electropherograms of Cys and Hcys in the branched microchannels. The detection time for Cys in the branched channel was 105 s ($n=6$, $SD=2.5$) while for Hcys it was 120 s ($n=6$, $SD=2.8$). The average current response for Cys (5 μM) was 2.4 ± 0.1 μA while for Hcys (5 μM) it was 2.2 ± 0.1 μA.

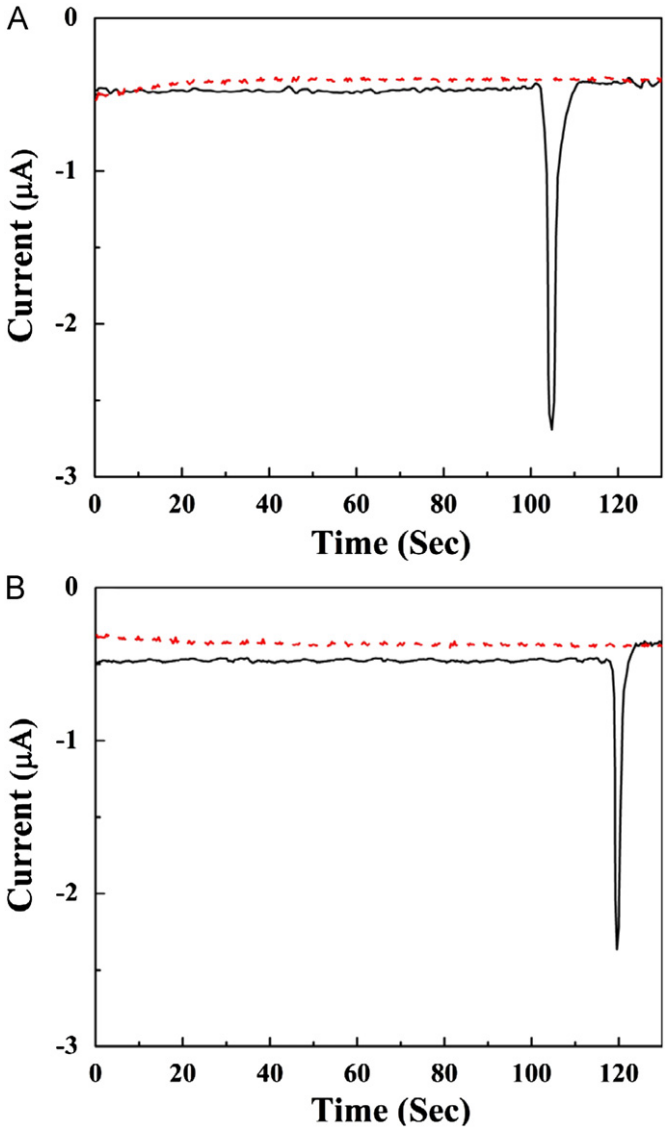

Fig. 4. Electropherograms of (A) Cys, (B) Hcys at 5 μM concentrations each and (—) 100 mM NaOH in branched channel at a separation voltage of 100 V DC and detection voltages of 0.42 V for Cys and 0.48 V for Hcys respectively.

In order to accurately calibrate the system for quantitative analysis, a correlation between electropherogram peak heights and the concentration of analytes was desirable. The relation between the baseline corrected current response and the concentration of Cys and Hcys is shown in Fig. 5. Each concentration was measured three times or more. The calibration plots obtained for each analyte over a concentration range 0.1–5 μM represented a typical sigmoidal correlation between peak current and concentration. A linear response was obtained for a range from 0.5 μM to 3 μM. Moreover, the calculated limit of detection (LOD) for the sensor was 0.05 μM (S/N=3 without the analytes). Though a direct comparison of the obtained result with previous literature is not exactly possible as most of the attempts to separate and detect amino thiols were performed using HPLC, conventional CE or flow injection based detection setup. A few attempts to separate thiols on a microchip electrophoretically along with AD were demonstrated by Chen et al. (2004), Pasas et al. (2002). Chen et al. detected Cys and Hcys in about 75 s while Passas et al. detected Hcys in 50 s which is faster than our findings but they used a very high separation voltage of 2000 V and 1200 V respectively which is detrimental for stability and safety of handheld devices. Similarly, Wang et al. detected selenocysteine in microchip based setup in 200 s with a LOD of 42 μM (much higher than our LOD of 0.05 μM), albeit, with an application of 2000 V for electrophoretic separation (Wang et al., 2005a). Another advantage of our chip was the peak resolution. Though the use of agarose gel and the lower separation voltage increased the detection time, we could efficiently resolve the two analytes having very similar molecular weights. On the other hand, results obtained by Chen et al. and Wang et al. showed overlapping of the signals. In the same experiment, Chen et al. stated a LOD of Cys as 0.8 μM which is much higher than our result. This suggested that our microchip can successfully detect the concentrations of Cys and Hcys with high sensitivity and lower LOD compared with previous studies and the detection range was well closer to the normal amount present in biological fluids.

3.5. Detection of cysteine and homocysteine in blood sample

The CE–AD device was further tested for the detection of amino thiols in biological fluid. The applicability of the proposed biosensor for Cys and Hcys determination in blood samples was investigated and results are presented in Table 1. The standard addition method was adopted for amino acid detection in real

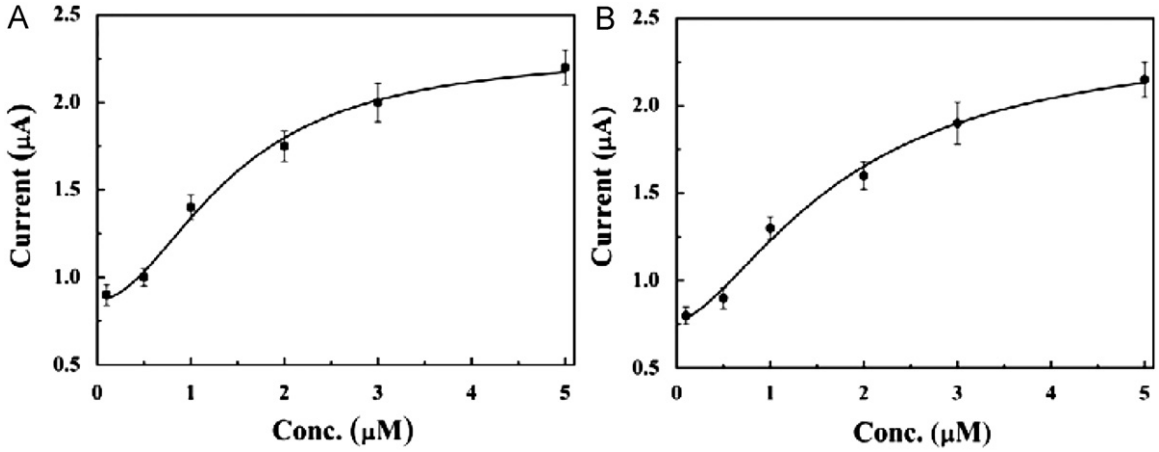

Fig. 5. Calibration curves for (A) Cys and (B) Hcys. Concentration range: 0.1–5 μM.

Table 1
Detection of cysteine and homocysteine in spiked blood sample through CE–AD.

	Cys added (μM)	Cys recovered (μM)	% Recovery
Blood sample 1	5	4.9	98
Blood sample 2	0.5	0.475	95
	Hcys added (μM)	Hcys recovered (μM)	% Recovery
Blood sample 1	5	4.85	97
Blood sample 2	0.5	0.47	94

sample. The blood samples obtained from the university clinic were spiked with a defined amount of Cys and Hcys and were subjected to similar CE–AD procedure in branched channels. The injections of blood samples with varying concentrations of thiols led to reproducible peaks, while blank sample injections did not show any response. The results summarized in Table 1 demonstrate good recoveries, varying between 95% and 98% with respect to the concentrations of spiked analytes. As expected, the device showed high signal to noise ratio because most of the interfering components in blood including cells and other heavier molecules such as proteins either could not enter the agarose gel or were electrophoretically migrated into the waste reservoir of central microchannel, thereby collecting only the pure analytes in the outlets of branched microchannel. In similar experiments, Antwi et al. and Batz et al. detected thiols in lysed red blood cells and showed no interference of other components which supports our findings (Antwi et al., 2011; Batz and Martin, 2009). This proved the successful miniaturization of a CE process on a microchip, with inverted double Y-shaped microchannel for effective separation and detection of amino thiols and the proposed CE–AD chip can be used for detection of thiol in real samples without the need of any sample pretreatments.

4. Conclusion

In conclusion, we have demonstrated in the present work, a method for the analysis of amino thiols using the CE–AD system. The device fabricated using glass substrate and PDMS based microchannel could successfully detect Cys and Hcys after their electrophoretic separation in the microchannel and collecting them in separate reservoirs. Initial separation in straight channel provided time of flight of Cys and Hcys which was later used to separate them into the branched channels. Unlike previous studies, the device did not require any preconditioning of gold electrode or sample. Detection of thiols in blood samples proved the applicability of the device. The in-house built dual potentiostat coupled with the modified channel design allowed simultaneous detection of analytes and such system can be miniaturized on a handheld architecture. The success of this work provides a great opportunity to detect thiols on a portable lab-on-a-chip for disease diagnosis. Further work is being carried out to couple further analysis of Cys and Hcys stored in the separate reservoir.

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