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Quantification of carnosine-related peptides by microchip electrophoresis with chemiluminescence detection

Shulin Zhao [a,b], Yong Huang [a], Ming Shi [a], Junming Huang [a], Yi-Ming Liu [b,*]

[a] College of Chemistry and Chemical Engineering, Guangxi Normal University, Guilin 51004, People's Republic of China
[b] Department of Chemistry, Jackson State University, Jackson, MS 39217, USA

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ABSTRACT

A microchip electrophoresis (MCE) method with chemiluminescence (CL) detection was developed for the determination of carnosine-related peptides, including carnosine, homocarnosine, and anserine, in biological samples. A simple integrated MCE–CL system was built to perform the assays. The highly sensitive CL detection was achieved by means of the CL reaction between hydrogen peroxide and *N*-(4-aminobutyl)-*N*-ethylisoluminol-tagged peptides in the presence of adenine as a CL enhancer and Co^{2+} as a catalyst. Experimental conditions for analyte labeling, MCE separation, and CL detection were studied. MCE separation of the above-mentioned three peptides took less than 120 s. Detection limits (signal/noise ratio [*S*/*N*] = 3) of 3.0×10^{-8}, 2.8×10^{-8}, and 3.4×10^{-8} M were obtained for carnosine, anserine, and homocarnosine, respectively. The current MCE–CL method was applied for the determination of carnosine, anserine, and homocarnosine in human cerebrospinal fluid (CSF) and canine plasma. Homocarnosine was detected at the micromolar (μM) level in the CSF samples analyzed, whereas the levels of carnosine and anserine in these samples were below the detection limit of the assay. Interestingly, both carnosine and anserine were detected in the canine plasma samples, whereas homocarnosine was not.

Carnosine-related peptides, including carnosine (Car),[1] homocarnosine (Hcar), and anserine (Ans), are a group of histidine-containing small peptides that occur widely in vertebrates [1,2]. Over the past years, studies have demonstrated that these peptides are involved in important biological roles such as antioxidant action, chelation of heavy metals, and modulation of enzyme activities [3,4]. It was also shown that the abnormality of their metabolism in the nervous system might be related to certain pathological conditions, including Huntingon's disease [5].

Several analytical methods based on high-performance liquid chromatography (HPLC) have been reported for the determination of Car-related peptides present in biological samples [4,6–8]. HPLC methods have good selectivity. However, HPLC has shortcomings, including long analysis time and limited column lifetime. A capillary electrophoresis (CE) method with laser-induced fluorescence (LIF) detection was also developed for quantifying these peptides in meat and human cerebrospinal fluid (CSF) samples [9]. This method had detection limits of 4.7, 4.4, and 3.9 nM for Car, Ans, and Hcar, respectively. However, the CE–LIF system required sophisticated and expensive instrumentation. Microchip electrophoresis (MCE), as a miniaturized version of classical CE, has recently attracted considerable interest for its potential of miniaturization and integration of an entire chemical or biological analysis process on a single device. MCE has many advantages compared with HPLC and CE, including higher separation efficiency, shorter run time, lower sample and reagent consumption, and ease of integration and automatization [10–13].

Chemiluminescence (CL) detection is a highly sensitive detection scheme. In addition, it is simple in instrumental setup and low in costs for operation and maintenance. Because a CL detector can be easily miniaturized, CL detection is particularly well suited for coupling with MCE separations to perform sensitive and selective MCE–CL bioassays. Over the past years, several MCE–CL methods have been reported. Mangru and Harrison performed an interesting study on CL detection following MCE to monitor horseradish peroxidase (HRP) and fluorescein-conjugated HRP using the luminol CL system [14]. Tsukagoshi and coworkers developed an MCE system with CL detection for assaying dansyl amino acids, hu-

* Corresponding author. Fax: +1 601 979 3674.
E-mail address: yiming.liu@jsums.edu (Y.-M. Liu).

[1] *Abbreviations used:* Car, carnosine; Hcar, homocarnosine; Ans, anserine; HPLC, high-performance liquid chromatography; CE, capillary electrophoresis; LIF, laser-induced fluorescence; CSF, cerebrospinal fluid; MCE, microchip electrophoresis; HRP, horseradish peroxidase; CL, chemiluminescence; ABEI, *N*-(4-aminobutyl)-*N*-ethylisoluminol; GSH, glutathione; Tau, taurine; GABA, γ-amino-*n*-butyric acid; DSC, *N*,*N*′-disuccinimidyl carbonate; H_2O_2, hydrogen peroxide; SDS, sodium dodecyl sulfate; PDMS, polydimethylsiloxane; PMT, photomultiplier tube; UV, ultraviolet; S, sample; SW, sample waste; B, buffer; BW, buffer waste; R, oxidizer reagent; MEKC, micellar electrokinetic chromatography; DA, dopamine; NE, noradrenalin; Agm, agmatine; Oct, octopamine; *S*/*N*, signal/noise ratio; RSD, relative standard deviation.

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man serum albumin, and immunosuppressive acidic protein [15,16]. However, because the microfluidic channels were extremely small, the sensitivity of these MCE–CL assays fell mostly in the range of 10^{-5} to 10^{-7} M (detection limit).

In the current work, we report on the development of a rapid and sensitive MCE–CL method for the determination of small peptides present in biomedical samples. Car-related peptides were taken as the model analytes. To make the peptides CL detectable with high sensitivity, labeling of the peptides with *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) was performed prior to the MCE separation. ABEI is a derivative of isoluminol and is highly chemiluminescent on being oxidized. It has been used to tag biogenic amines for HPLC [17], and precolumn CL labeling with ABEI has also been used in CL detection following CE separations of arginine, glycine, and biogenic amines [18,19]. We recently reported the development of an MCE–CL method based on precolumn CL labeling with ABEI to determine biogenic amine [20]. A big advantage of using the precolumn labeling technique is to avoid adding the CL reagent into the MCE running buffer, further reducing the background noise of CL detection. In addition, a CL enhancer (i.e., adenine) and a catalyst (i.e., Co^{2+}) were used to further improve the MCE–CL assay sensitivity. The use of adenine and Co^{2+} as enhancer and catalyst in MCE–CL assays has not been reported before. The MCE–CL method has the advantages of being fast, convenient, sensitive, and reproducible. The use of a microfluidic device coupled with CL detection for peptide analysis with a detection limit at the 10^{-8}-M level was demonstrated for the first time in this work.

Materials and methods

Chemicals and solutions

Car, Ans, Hcar, adenine, glutathione (GSH), taurine (Tau), γ-amino-*n*-butyric acid (GABA), 20 protein amino acids, biogenic amines, and *N*,*N*′-disuccinimidyl carbonate (DSC) were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). ABEI was purchased from Fluka (Buchs, Switzerland). Hydrogen peroxide (H_2O_2) was obtained from Taopu Chemicals (Shanghai, China). Sodium dodecyl sulfate (SDS) was provided by Shanghai Reagents (Shanghai, China). All other chemicals used in this work were of analytical grade. Water purified by a Milli-Q Plus 185 device from Millipore (Bedford, MA, USA) was used throughout the work. The electrophoretic buffer was 15 mM borate buffer (pH 9.8, adjusted with a 1-M NaOH solution) containing 1.0 mM Co^{2+}, 1.0 mM adenine, and 35 mM SDS. The CL oxidizer solution was 40 mM sodium bicarbonate buffer (pH 12.5, adjusted with 1 M NaOH solution) containing 110 mM H_2O_2. Stock solutions of ABEI and DSC were prepared in methanol and acetonitrile, respectively. A stock solution of peptides was prepared in water and diluted with water as needed. All solutions were filtered through 0.22-μm membrane filters before use.

MCE–CL system

The glass/polydimethylsiloxane (PDMS) hybrid microchip assembly was mounted on the *X*–*Y* translational stage of an inverted microscope (Olympus CKX41) that also served as a platform of CL detection. The CL signal was collected by means of a microscope objective. After passing a dichroic mirror and a lens, CL photons were detected by a photomultiplier tube (PMT, Hamamatsu R105). The PMT was mounted in an integrated detection module, including an HV power supply, a voltage divider, and an amplifier. The output signal of the PMT was recorded and processed with a computer using a Chromatography Data System (Zhejiang University Star Information Technology, Hangzhou, China). A multiterminal high-voltage power supply, variable in the range of 0 to 8000 V (Shandong Normal University), was used for sample loading and MCE separation. The inverted microscope was placed in a dark box.

Microchip fabrication

A double T glass/PDMS hybrid microfluidic chip was used in this work. The schematic layout of the chip is illustrated in Fig. 1. Fabrication of the microchip is described as follows. First, the glass substrate with microchannels was fabricated through standard photolithography and wet chemical etching techniques [21,22]. Sylgard 184 PDMS prepolymer (Dow Corning, Midland, MI, USA) was mixed thoroughly with its curing agent at 10:1 (w/w) and then was degassed by a vacuum pump. The mixture was cured against the As–Ga mold at 80 °C for 2 h. After the replica was peeled from the mold, holes were punched as reservoirs. The obtained glass substrate and PDMS cover plate were cleaned ultrasonically with acetone, methanol, and water for 25 min, and then they were dried under an infrared lamp. After being exposed to ultraviolet (UV) light (6 W, mercury lamp) from a distance of 3 cm for 3 h, the PDMS cover was bonded to the glass substrate. Microchannels measured 65 μm wide by 25 μm deep for sample introduction, separation, and waste delivery and measured 250 μm wide by 25 μm deep for oxidizer introduction and CL detection. All reservoirs were 4.0 mm in diameter and 1.5 mm deep. The channel between the sample (S) and sample waste (SW) reservoirs was used for sampling, the channel between the buffer (B) and buffer waste (BW) reservoirs was used for the separation, and the channel between the oxidizer reagent (R) and BW reservoirs was used for the oxidizer introduction. The joining point of the oxidizer introduction channel with the separation channel was used for the collection of CL.

Sample preparation

The human CSF samples were collected from patients with various neurological conditions: (i) cephalitis, (ii) brain tumor, and (iii) surgical brain damage. A portion (400 μl) of a CSF sample was diluted with ice-cold 800 μl of acetonitrile and shaken vigorously for 5 min to precipitate proteins. After centrifuging at 16,000 rpm for 10 min, the supernatant was transferred into a 1.5-ml vial and dried with an N_2 stream. The residue was redissolved in 20 μl of 20 mM borate buffer at pH 9.0. The solution was kept at 4 °C until analysis.

Canine plasma samples were obtained from 3 healthy dogs. The same procedure as stated above for sample preparation was used.

CL labeling

To label the samples for CL detection, 25 μl of a 5-mM ABEI solution was added to an equal volume of 10 mM DSC solution.

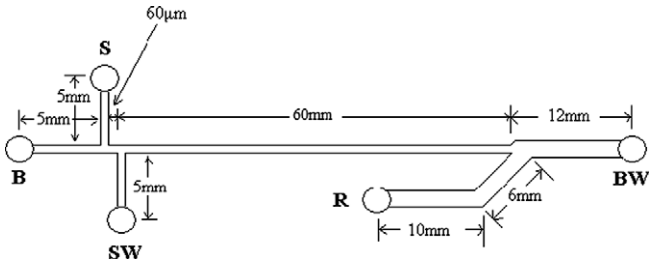

Fig. 1. Schematic diagram of the layout of the glass/PDMS microchip used in this work. Reservoirs: S, sample; B, buffer; SW, sample waste; BW, buffer waste; R, oxidizer reagent.