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Microfluidic chip with electrochemiluminescence detection using 2-(2-aminoethyl)-1-methylpyrrolidine labeling

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Abstract

A tertiary amine derivative, 2-(2-aminoethyl)-1-methylpyrrolidine (AEMP) was successfully developed as electrochemiluminescence (ECL) probe within microfluidic chip using ECL detection in this paper. The system was characterized by the interaction between biotin and avidin. In principle, tertiary amine derivatives containing active group can be used as a potential alternative of traditional tris(2, 2′-bipyridine)ruthenium(II) [$Ru(bpy)_3^{2+}$] label. Firstly, The ECL efficiency of AEMP was characterized via comparing with that of two coreactants enhancing $Ru(bpy)_3^{2+}$ ECL, TPA and proline. At same condition, AEMP has a similar ECL efficiency to TPA, and much higher than proline. After AEMP reacted with NHS-LC-biotin (succinimidyl-6-(biotinamido) hexanoate), the products and their ECL were analyzed by directly injecting it in the microfluidic chip. A 4.5 cm microchannel was used to separate the mixture of AEMP and biotinylated AEMP. The present works indicated that AEMP has a good reactivity to the analytes containing carboxyl group with a similar ECL efficiency to TPA. Under optimal condition, the detection limits (based on 3 S/N) of AEMP was 2.7 μM. The system was also validated by the reaction between biotin and avidin. The calculated binding ratio between avidin and biotin based on the present method was 4.4.
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Keywords: Electrochemiluminescence; Microfluidic chip; Tertiary amine label

1. Introduction

Since Tokel and Bard's work [1] about the electrochemiluminescence (ECL) of tris(2,2′-bipyridine)ruthenium(II) [$Ru(bpy)_3^{2+}$] in aqueous solution, ECL based on $Ru(bpy)_3^{2+}$ has an extensive application in analytical science because of its good spatial and temporal resolution [2–4]. As an alternative to tradition detection methods [2–9], such as electrochemical [10,11] and optical [12], ECL technique was used to characterize some bio-specific reactions, such as antigen–antibody, nucleic acid hybridization and protein–ligand reactions [10,11]. Moreover, compared with optical methods, ECL technique needs no external excitation light source and additional instrumentation to cut out the scattered light and thus, makes the optical system for ECL detection much simple, only consisting of a photomultiplier tube (PMT) and a light-tight observation chamber [2,9].

DNA assays and immunoassays based on ECL generally included a $Ru(bpy)_3^{2+}$ labeling procedure [2,7,8,13]. But the procedure possesses some limitations. For example, ruthenium is a heavy metal, which maybe precipitate some target analytes, such as protein; ruthenium-labeling at multiple sites may result in the loss of biological activity of the molecules [8,13]; the synthesis of the label and the labeling procedure are complex because the labels are photo- and thermo-sensitive. Therefore, the searching of the stable, easy to preparation, and highly biocompatible labels is of critical importance for the analysis of biological substance.

If triporpylamine (TPA), the most important coreactant in traditional ECL, can be tagged on the biomaterials, an ECL signal will be generated when they were in contact with $Ru(bpy)_3^{2+}$ solution and a potential was applied.

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Morita and Konishi [14,15] found some tertiary amine derivatives, such as *N*-(3-aminopropyl)pyrrolidine (NAPP), 2-(2-aminoethyl)-1-methylpyrrolidine (AEMP), *N*-methyl-L-proline (NMP), 3-(diethylamino)propionic acid (DEAP) and 4-(dimethylamino)butyric acid (DMBA) displayed strong ECL intensity and applied the compounds to label carboxylic acids and amine. The previous work [16] indicated that the tertiary amines, as labels, have some advantages over $Ru(bpy)_3^{2+}$ and conventional fluorophore (e.g. fluorescein), such as high label efficiency, good biocompatibility and so on. We also used DMBA as label to detect bovine serum albumin (BSA) and immunoglobulin G (IgG) in our previous work [17]. In principle, the tertiary amine derivatives above should be good labels in terms of stability and biocompatibility for biological material analysis.

Microfluidic chip based on capillary electrophoresis (CE) has attracted much attention recently [18]. Its advantages included short analysis time, portability, disposability, and minute sample and reagents consumption. Moreover, the integration of sample-handling, separation, and detection makes the chip CE system much simple and user-friendly [18]. In this work, AEMP, as a tertiary amine derivative was developed as a sensitive ECL probe for investigation of biotin–avidin reaction. To simply the analysis procedure, the mixture was directly injected in a microchip CE system. After separation with chip, AEMP and biotin-AEMP were detected by ECL technique.

2. Experimental

2.1. Reagents

All the reagents employed were used as received and without any further purification. Double distilled water (DDW) was used throughout. Unless otherwise stated, all solutions were freshly prepared. AEMP, tripropylamine (TPA), and tris(2,2′-bipyridine)ruthenium(II) chloride hexahydrate [$Ru(bpy)_3Cl_2{\cdot}6H_2O$] were purchased from Sigma–Aldrich (St. Louis, MO, USA). NHS-LC-biotin [succinimidyl-6-(biotinamido) hexanoate] and avidin from Pierce (Rockford, IL, USA) were used to investigate their interaction. $Ru(bpy)_3^{2+}$ (20 mM) was dissolved in DDW as a stock solution and stored in refrigerator. The working solution of 5 mM $Ru(bpy)_3^{2+}$ was prepared freshly by diluting the stock solutions with 10 mM phosphate salt solution (pH 7.5) and degassed ultrasonically for 10 min just before use. AEMP, L-proline (Pro, Shanghai Biochemical Co., Shanghai, China), and TPA were dissolved in DDW directly at concentration of 10 mM, respectively, as stock solution. The work solution was obtained by stepwise dilution of the stock solutions above. NHS-LC-biotin was dissolved freshly in dry dimethyl formamide (DMF) for labeling with AEMP. All buffer solutions, such as the matrix of reaction between NHS-LC-biotin and AEMP, separation and detection electrolyte, were 10 mM phosphate buffer (pH 7.5) prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate (Beijing Chemicals, Beijing, China).

2.2. Instrumentation

The technique used to fabricate indium tin oxide (ITO) electrodes and poly(dimethysiloxane) (PDMS) layers was described as previously[19]. Briefly, ITO working electrode was fabricated by wet chemical etching ITO-coating glass slide (7 cm × 2 cm, HIVAC Technology, Shenzhen, China) after a photolithographic procedure of developing photoresist (RZJ-390, Suzhou Ruihong Electronic, Suzhou, China). The width of ITO electrode was 100 μm in this work. Master for PDMS micromolding was made from flat soda-lime glass substrates with a thin layer of chromium and positive photoresist (SG2506, Shaoguang Microelectronics, Changsha, China). The PDMS monomer and curing agent were mixed in a 10:1 (w/w) ratio, degassed, poured onto the master, and cured under an infrared lamp for 1 h. The configuration of the chip used in this work was a simple cross and the microchannels were typically ∼15 μm deep, 48 μm wide at the bottom, and 60 μm wide at the top. The efficient length of the separation channels and the distance between sample reservoir and the cross were 45 mm and 4 mm, respectively. At the end of the separation and injection channels were four reservoirs: buffer reservoir, detection reservoir where $Ru(bpy)_3^{2+}$ solution was filled with, sample reservoir, and an unused reservoir. The PDMS layer containing microchannels and reservoirs was rinsed with ethanol and DDW and dried. The clean PDMS layer was reversibly bound to the electrode plate. A distance of 80 μm between the end of separation channel and ITO electrode was set with the help of an optical microscope. The schematic diagrams of PDMS layer, electrode plate, and the entire microfluidic chip system were shown previously [19].

Model GY-4 Programmable Source (Research Center of Analytical Science, Northeast University, Shenyang, China) was used to perform the electrokinetic sample injection and CE separation. The chip channels were treated before experiment by rinsing with 0.1 M NaOH, DDW, and running buffer with the aid of a vacuum pump. External platinum wires were used to provide electrical contact from the high voltage to the solutions in the reservoirs. The detection reservoir was filled with $Ru(bpy)_2^{2+}$ solution and grounded all the time. Sample injection was carried out by applying a high potential between the sample and detection reservoirs for 5 s with the buffer reservoir floating. After sample injection, the high voltage was set between the buffer and detection reservoirs with sample reservoir floating.

The electrochemical measurement for ECL experiments was carried out with Model CH800 Voltammetric Analyzer (Shanghai ChenHua Instruments, Shanghai, China). A three-electrode system was employed with Pt wire as counter electrode, Ag/AgCl/KCl (sat) as reference electrode, and ITO on the chip plate as working electrode. The ECL emission was detected with a Model MCFL-A Chemiluminescence Ana-

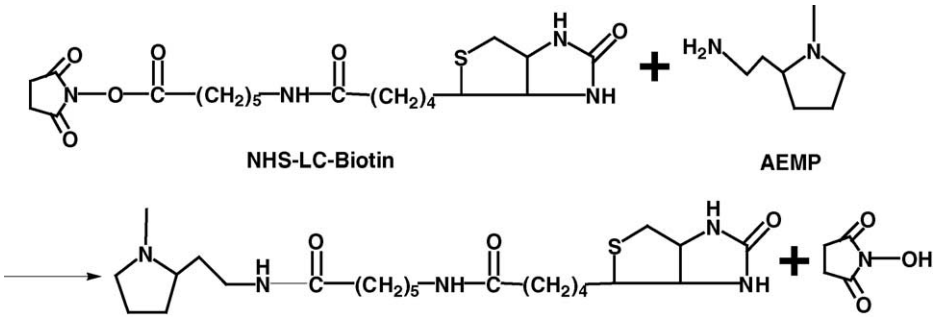

Fig. 1. The molecule structures of AEMP and NHS-LC-biotin and the reaction between AEMP and NHS-LC-biotin.

lyzer Systems (Xi'An Remax Science & Technology, Xi'An, China). The voltage of the PMT used in Model MCFL-A Chemiluminescence Analyzer was set at 750 V.

2.3. Formation of AEMP-LC-biotin and biotin–avidin reaction

NHS-LC-biotin is a reactive biotin analog with an extended spacer arms. The long chain reduces the steric hindrance associated with binding the biotinylated molecules on avidin. For being tagged biotin with AEMP, NHS-LC-biotin was firstly dissolved in 100 μl dry DMF, and then, added in 100 μl 10 mM phosphate buffer (pH 7.5) solution containing AEMP. A 1:1 mole ratio of AEMP to NHS-LC-biotin was used. The reaction was allowed for 40 min at 37 °C. The molecule structures of AEMP and NHS-LC-biotin and their reaction were shown in Fig. 1 and the product was denoted as AEMP-LC-biotin.

For investigation of avidin–biotin reaction, different concentration of 100 μl avidin was added into 400 μl AEMP-LC-biotin solution (300 μM) prepared above. The mixture of avidin and AEMP-LC-biotin was incubated for 40 min at 37 °C. The reacted product was injected directly into the microfluidic chip. The primary results indicated that avidin can be adsorbed on the surface of the separation channel and affect the detection of AEMP and AEMP-LC-biotin. To eliminate the effect of the adsorption of avidin on the detection, A 500 μl solution was dialyzed with 1 ml DDW overnight. The molecule weight cutoff of the dialyser (Pierce) is 7 kDa. The dialyzed solution was injected into microfluidic chip.

3. Result and discussion

3.1. AEMP ECL efficiency

Firstly, AEMP ECL efficiency was investigated compared with that of the two most important ECL coreactants, TPA and proline (Pro). To be consistent with matrix of formation of AEMP-LC-biotin and avoid the denaturation of avidin, 10 mM phosphate buffer (pH 7.5) solution was used as the separation electrolyte of the chip system for the further works. Fig. 2A and B were the electropherograms of 40 μM TPA and the mixture of 40 μM AEMP and 100 μM Pro. The results indicated that AEMP has similar ECL efficiency to that of TPA and much high ECL efficiency over Pro and proved that possibility of AEMP as a highly efficient ECL label. Fig. 2C was the electropherogram of three consecutive injection of 100 μM AEMP, given a good reproducibility with the present system.

3.2. ECL of biotinylated AEMP

The preparation of biotinylated AEMP with 1:1 mole ratio of AEMP to NHS-LC-biotin was shown as in the experiment section. The electropherograms of three consecutive injection of 50 μM AEMP-LC-biotin were illustrated in Fig. 3A. There were three peaks given on the electropherogram. To qualify the peaks, AEMP was added in the product of biotinlyation. Fig. 3B was the electropherogram of biotinylated AEMP spiked with 15 μM AEMP. It can be found that the peak of AEMP in the electropherogram of biotinylated AEMP was the first peak. The low peak intensity of AEMP indicated a high reaction efficiency between AEMP and NHS-LC-biotin. One of the products in Fig. 1 is *N*-hydroxysuccinimide (NHS). Further, 100 μM NHS solution prepared freshly was injected into the microfluidic chip. We did not find any signal,

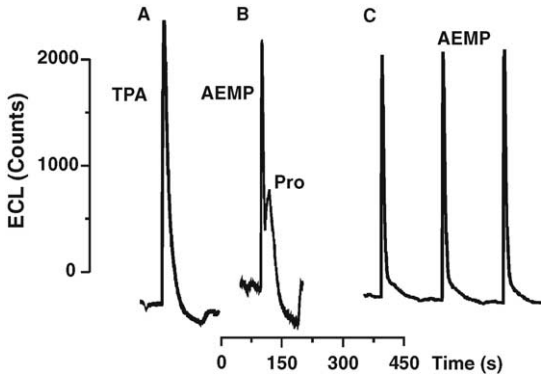

Fig. 2. The electropherograms of 40 μM TPA (A), the mixture of 40 μM AEMP and 100 μM proline (B), and three consecutive injection of 40 μM AEMP (C). Both injection and separation voltages were set 920 V. Phosphate buffer (10 mM) solution (pH 7.5) as separation electrolyte. $Ru(bpy)_3^{2+}$ (5 mM) solution as detection solution. detection potential, 1.25 V [vs. Ag/AgCl/KCl (sat)]. Time of a single running was set 150 s.

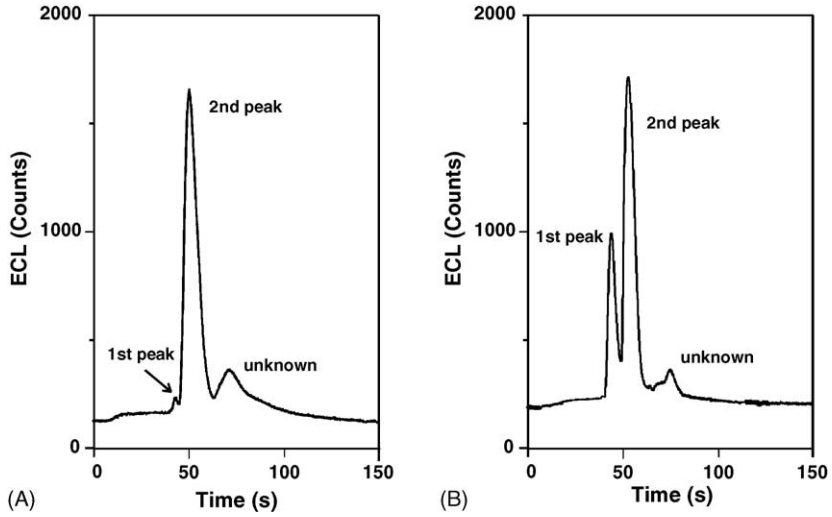

Fig. 3. The electropherograms of the product resulted from AEMP and NHS-LC-biotin, about 50 μM (A), the product spiked 15 μM AEMP (B). All other conditions as shown in Fig. 2.

but only a baseline was obtained. So, no peak in Fig. 3A was from NHS. We did not find the source of the third peak, so denoted an unknown peak, which did not interfere the determination of biotinlyated AEMP. The further works indicated that the unknown peak did not always appear, as shown in Fig. 4.

3.3. Interaction between biotin and avidin

The reaction between avidin and biotin was carried out as shown in Experimental section. Firstly, we injected directly the solution obtained from the reaction above and hoped to observe the peaks from AEMP, AEMP-LC-biotin and AEMP-LC-biotin-avidin. The results indicated that two peaks related AEMP and AEMP-LC-biotin were observed in the two runs. But the intensity of peak of AEMP-LC-biotin in the first running was higher than that in the second running significantly. After analysis, we can find that avidin, as a protein with molecule weight of ~67,000 Da, can be adsorbed on the surface of separation channel. The previous works indicated that protein adsorption can lead to band broadening [20], poor migration time reproducibility [21] in CE. Because the surface of channel in PDMS layer is more hydrophobic than that of electrophoresis capillary, the adsorption of avidin on channel is also higher than that in CE. While no peak of avidin–biotin–AEMP was observed in the present condition and it may be due to the physical adsorption of avidin on the surface of the separation channel. The difference of peak height of AEMP-LC-biotin was presumed from the unspecific adsorption between AEMP-LC-biotin and avidin on the surface of the channel.

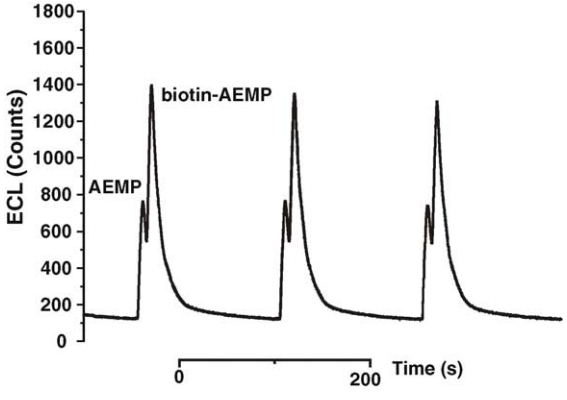

Fig. 4. The electropherograms of the three consecutive injections of the dialyzate from the product of biotin–avidin reaction. All other conditions as shown in Fig. 2.

To eliminate the effect of the adsorption of avidin on the determination of biotinylated AEMP, a dialysis described as Experimental section was carried out. The dialyzate was injected into microfluidic chip and the electropherograms of three consecutive injections were shown in Fig. 4. AEMP and AEMP-LC-biotin in the electropherograms had similar intention time to that in the product of reaction between AEMP and NHS-LC-biotin and had a good peak reproducibility.

3.4. Analytical performance

The present work indicated that AEMP was a sensitive ECL label. Combined with microfluidic chip separation, the detection limits based on 3 S/N of AEMP was 2.7 μM. Although tagging with NHS-LC-biotin resulted in a low sensitivity, AEMP-LC-biotin had detection limits of 5.2 μM, also. The precisions (RSD) of peak height for six replicate injections of 40 μM AEMP and 50 μM AEMP-LC-biotin were 5.7 and 3.1%, respectively. The calibration range and the calibration function for AEMP are 5–100 μM and $I = 52.95C - 87.53$ ($r^2 = 0.9939$), respectively, where I is the ECL intensity and C is the concentration of AEMP.

According to the Fig. 4, we can find the content of biotin–AEMP unreacted with avidin. The biotin–AEMP

reacted with avidin was calculated by the difference of the total biotin–AEMP added and that unreacted. Following the analysis above, the binding ratio between biotin and avidin is about 4.4, which is larger than the theoretic value of 4 because we presumed the complete reaction between AEMP and NHS-LC-biotin and no loss in the whole process.

4. Conclusion

AEMP was developed as ECL probe and used in microfluidic chip-ECL detection with investigation of the interaction between avidin and biotin as model. The preliminary results indicated that AEMP can be used as a potential alternative of traditional $Ru(bpy)_3^{2+}$ label with a high sensitivity and stability. The present work indicated that AEMP has a good reactivity to the analytes containing carboxyl group with a similar ECL efficiency. Microfluidic chip-ECL combined to AEMP labeling will be a promising approach with strong potential application.

Acknowledgements

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