Journal of Electroanalytical Chemistry 731 (2014) 133–138

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

journal homepage: www.elsevier.com/locate/jelechem

Electrogenerated chemiluminescence biosensing method for methyltransferase activity using tris(1, 10-phenanthroline) ruthenium-assembled graphene oxide

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ARTICLE INFO

Article history:
Received 18 May 2014
Received in revised form 8 August 2014
Accepted 14 August 2014
Available online 27 August 2014

Keywords:
Electrogenerated chemiluminescence
Graphene oxide
Tris(1, 10-phenanthroline) ruthenium
Methyltransferase activity

ABSTRACT

A novel electrogenerated chemiluminescence (ECL) biosensing method for highly sensitive detection of DNA methylation and assay of the methyltransferase (MTase) activity was developed by using the methylation-sensitive restriction endonuclease Dpn I and methylation-responsive hairpin-capture DNA probe to improve selectivity and by employing signal amplification of graphene oxide (GO) to enhance the assay sensitivity. The ECL biosensing electrode was fabricated by self-assembling a design of 5′-thiol modified methylation-responsive hairpin-capture DNA probe on the surface of a gold electrode. When DNA adenine methylation (Dam) MTase and *S*-adenosylmethionine were introduced, all adenines in the symmetric tetranucleotide 5′-GATC-3′ of hairpin-capture DNA probe on the biosensing electrode were methylated. After the methylated biosensing electrode was treated by Dpn I endonuclease, the methylated adenines were cleaved, methylation-induced scission of hairpin-capture DNA probe would displace the hairpin section and remain the "capture DNA probe" section on the gold electrode. Subsequently, the remained "capture DNA probe" on the gold electrode can hybridize with the 5′-amino modified DNA probe. Finally, tris(1, 10-phenanthroline) ruthenium ($Ru(phen)_3^{2+}$)-assembled GO composites were conjugated to the electrode surface via EDC–NHS coupling, a strong ECL response was electrochemically generated. The increased ECL intensity was proportion to Dam MTase activity in the range from 0.05U/mL to 40 U/mL with a detection limit of 0.02 U/mL. The present work demonstrates that the combination of the enzyme-linkage and GO as a platform for signal probe is a great promising approach for MTase activity and evaluation of the capability of inhibitors for the MTase.

1. Introduction

DNA methylation [1], a critical process existing in both prokaryotes and eukaryotes, is achieved by DNA methyltransferase (MTase) catalyzing covalent addition of a methyl group to cytosine or adenine in the presence of *S*-adenosylmethionine (SAM). DNA methylation plays an important role in many biological processes including transcription, genomic imprinting, cellular differentiation, chromatin structure and embryogenesis [2–4]. A number of human diseases have been found to be associated with aberrant gene methylation. Moreover, DNA MTase is a novel family of pharmacological targets for the treatment of tumors [5]. The aberrant DNA MTase activity was reported to be related to pathogenesis of cancer, such relationship provides a potential target in disease diagnosis and therapy [6]. Therefore, it is critical to develop sensitive methods for the detection of MTases activity, analysis of DNA methylation process and inhibitor screen of DNA MTase in the field of life science research and early cancer diagnosis [7].

Some methods for the detection of DNA methylation and assay of MTases activity have been established including radioactive labeling of [methyl-^{3}H]-SAM, polymerase chain reaction (PCR) [8,9], capillary electrophoresis [10], high-performance liquid chromatography (HPLC), colorimetric [11–13], fluorescence [14–16], light scattering [17], surface enhanced Raman spectroscopy (SERS) [18], and electrochemical methods [19–22]. The above methods usually challenged with some shortcomings, such as time-consuming, DNA-consuming, laborious treatment and lower sensitivity.

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http://dx.doi.org/10.1016/j.jelechem.2014.08.022
1572-6657/

Recently, the design of a hairpin DNA probe as a substrate of MTase has demonstrated a potential application in MTase activity detection [14,22–24], which may overcome these limitations. It is still highly required to develop sensitive, simple biosensing methods for MTase quantification and activity assay by taking the advantage of hairpin DNA probe.

Electrogenerated chemiluminescence (ECL) method exhibiting many advantages such as simplicity, high sensitivity, rapidity and easy controllability has been proved to be very useful in immunoassay, DNA hybridization assays and enzymatic biosensors [25–27]. Recently, Li developed an ECL biosensing method for highly sensitive detection of DNA methylation and assay of the CpG methyltransferase activity on the basis of enzyme-linkage reactions and ruthenium complex served as an ECL tag [28]. In order to amplify signals in DNA methylation detection, supersandwich amplification technique has been introduced [29]. Moreover, the sensitivity of ECL biosensors can be further enhanced by using various nanomaterial-based amplifications [30]. Recent years, graphene oxide (GO), a novel one-atom thick and two-dimensional graphitic carbon system with abundant oxygen functional groups, has attracted increasing attentions due to unique physical and chemical properties [31–33]. Applications of thionine-assembled GO composites in the amplification of DNA methylation detection were also reported [21]. GO, is negatively charged and has many π-conjugated aromatic domain in its basal plane [34]. Thus it is a remarkable material for strongly immobilizing substances that are positively charged and have aromatic structure through both electrostatic interaction and π–π stacking interaction [35]. GO can immobilize tris(1, 10-phenanthroline)ruthenium ($Ru(phen)_3^{2+}$) with excellent long-term stability through strong electrostatic interaction and π–π stacking interaction [36]. Based on its high $Ru(phen)_3^{2+}$ loading ability, good biocompatibility and physiological stability, GO can be used as efficient carriers in ECL biosensor.

The aim of present work is to develop a highly sensitive ECL biosensing method for the detection of DNA methylation and assay of the Dam MTase based on Dpn I endonuclease and methylation-responsive hairpin-capture DNA probe for successively selectivity improvement and to employ signal amplification of GO for assay sensitivity enhancement. In this paper, the fabrication of the ECL biosensing electrode and the analytical performance for the detection of DNA methylation and assay of the Dam MTase were presented.

2. Experimental section

2.1. Reagents and apparatus

Graphite powder (99.998%, 325mesh, Alfa Aesar), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%), 6-mercaptohexanol (MCH), tris(1, 10-phenanthroine) ruthenium, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), gentamycin, 5-fluorouracil, mitomycin, benzylpenicillin, and N-hydroxysuccinimide (NHS, 98%) were purchased from Sigma–Aldrich. The Dam MTase (Escherichia coli), S-adenosyl-L-methionine (SAM), Dpn I endonuclease, and the corresponding buffer solution were provided by New England Biolabs In (Beijing, China). Other reagents with analytical grade were used without further purification. Millipore Milli-Q water (18 ΩM cm) was used throughout. The synthetic oligonucleotides were purchased from Shenggong Bioengineering Co. Ltd. (Shanghai, China).

Hairpin-capture DNA probe **S1**: 5′-HS-$(CH_2)_6$-TAC TGA TTG C***GA TC***G AGA ATG CTT TTG CAT TCT C***GA TC***G CAA T-3′.

Complementary probe **S2**: 5′-NH_2-$(CH_2)_6$-TCG CAA TCA GTA-3′.

Control DNA probe **S3**: 5′-HS-$(CH_2)_6$-TAC TGA TTG C***GA CT***G AGA ATG CTT TTG CAT TCT C***GA CT***G CAA T-3′.

A MPI-E ECL analysis system (Xi'an Remex Analytic Instrument Co., Ltd) was employed for ECL detection. A commercial cylindroid glass cell was used as an ECL cell, which contained a conventional three-electrode system consisting of either a biosensing electrode or a gold electrode (ϕ = 2.0 mm) as working electrode, a platinum plate as the counter electrode, and an Ag/AgCl (saturated KCl) as the reference electrode, respectively. ECL emissions were detected with a photomultiplier tube (PMT) that was biased at −900 V unless otherwise stated. Atomic force microscopy (AFM) images were performed on an SHIMADZU SPM-9500 system with touching mode. The transmission electron microscope (TEM) image was carried out by Tecnai G2 F20 S-TWIN (FEI, USA).

2.2. Synthesis of GO sheets and $Ru(phen)_3^{2+}$/GO composites

GO was synthesized by a modified Hummers method [37], starting from graphite powder. The synthesized GO sheets are rippled and resemble crumpled silk veil waves as viewed from TEM images (Fig. S1A). The thickness of the GO sheet is about 1.4 nm as indicated by AFM images (Fig. S1B). For assembly of $Ru(phen)_3^{2+}$ on GO surface, 3 mg sample of GO was added to 0.5 mg of $Ru(phen)_3^{2+}$ dissolved in 5 mL aqueous solution, and the reaction mixture was stirred for overnight at room temperature after being sonicated for 15 min. The resulting mixture was sonicated for 5 min at room temperature and centrifugated at 8000 rpm for 10 min to remove the excess of $Ru(phen)_3^{2+}$. Further purification was carried out by washing thoroughly with deionized water and absolute ethanol. Finally dried under vacuum at ambient temperature overnight to obtain $Ru(phen)_3^{2+}$-assembled GO ($Ru(phen)_3^{2+}$/GO) composites.

2.3. Fabrication of biosensing electrode

A gold disk electrode was cleaned according to the reported protocol [26]. The cleaned electrode was thoroughly rinsed with water and then was immersed in 500 uL of probe immobilization buffer (10 mM Tris–HCl, 10 mM TCEP, 0.1 M NaCl, pH 7.40) containing 2 μM S1 for 4 h at 37 °C. After that the modified electrode was washed twice with water to remove the physically adsorbed hairpin-capture DNA probe and then immersed in 1 mM MCH for 1 h to block uncovered surface of the electrode and finally rinsed thoroughly with Milli-Q water. This electrode was used as an ECL biosensing electrode.

2.4. ECL measurements

The Dam MTase assay solution was prepared by mixing 1× Dam buffer with SAM solution and Dam MTase solution to a final concentration 50 mM Tris–HCl pH 7.5, 10 mM NaCl, 10 mM EDTA, 5 mM 2-mercaptohexanol, 160 mM SAM and different activity (concentration) of Dam MTase. For the detection of Dam MTase, an ECL biosensing electrode fabricated was immersed in 500 μL of the solution prepared above and incubated at 37 °C for 60 min to form the methylated hairpin-capture DNA probe. Subsequently, the methylated hairpin-capture DNA probe modified electrode was immersed in 500 μL of buffer solution containing 1× NEB buffer 4 (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 1 mM DTT, pH 7.9) and 80 U Dpn I for 2 h at 37 °C to cleave the methylated adenines, washed with the 10 mM Tris–HCl buffer (pH 7.4). After methylation-induced scission of hairpin-capture DNA probe being completed, a 5 μL of 2.0 μM S2 DNA probe solution (in 10 mM Tris–HCl buffer, pH 7.4) was dropped on the cleaved electrode at room temperature for 2 h. The capture DNA probe section which remained on the modified electrode was then hybridized with the S2 DNA probe. The hybridized electrode was transferred into 1 mg/mL the activated $Ru(phen)_3^{2+}$/GO solution 180 min. The