Talanta 170 (2017) 546–551

Contents lists available at ScienceDirect

Talanta

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

A sensitive signal-off electrogenerated chemiluminescence biosensing method for the discrimination of DNA hydroxymethylation based on glycosylation modification and signal quenching from ferroceneboronic acid

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ARTICLE INFO

Keywords:
Electrogenerated chemiluminescence
5-hydroxymethylcytosine
T4 β-glucosyltransferase
Ferroceneboronic acid

ABSTRACT

In this study, a new and sensitive signal-off electrogenerated chemiluminescence (ECL) biosensing method for the quantification of 5-hydroxymethylcytosine in DNA (5-hmC-DNA) was developed. The method achieved simple and sensitive detection of 5-hmC-DNA based on the glycosylation of 5-hmC, combining both the amplification function of gold nanoparticles (AuNPs) and the high quenching efficiency of the tris(2, 2′-ripyridine) dichlororuthenium(II) ($Ru(bpy)_3^{2+}$)-ferrocene (Fc) system. First, the electrode modified with a mixture of Nafion and AuNPs was utilized as the platform for electrostatically adsorbing $Ru(bpy)_3^{2+}$(an ECL-emitting species) and assembling 5-hmC-DNA. The 5-hmC-DNA was glycosylated by T4 β-glucosyltransferase, yielding β-glucosyl-5-hydroxymethyl-cytosine in DNA (5-ghmC-DNA). Finally, quencher-FcBA was further covalently bound to 5-ghmC-DNA through formation of boronate ester covalent bonds between boronic acid and *cis*-diols of 5-ghmC, resulting in a decrease in ECL intensity. The results indicated that the decreased ECL intensity was directly linear to the concentration of 5-hmC-DNA in the range from 1.0×10^{-8} to 5.0×10^{-11} M with a low detection limit of 1.63×10^{-11} M. In addition, this ECL method was demonstrated to be useful for the quantification of 5-hmC in clinical serum samples. Moreover, the method allowed good discrimination among cytosine (5-C), 5-methylcytosine (5-mC), and 5-hmC in DNA.

1. Introduction

5-Hydroxymethylcytosine (5-hmC) is a recently rediscovered deoxyribonucleic acid (DNA) base. 5-hmC exists in mammalian tissues and cells and is abundant in neurons and embryonic stem cells. Moreover, 5-methylcytosine (5-mC) can be used to generate 5-hmC through the catalytic effects of ten-eleven translocation (TET) proteins. Similar to 5-mC, 5-hmC is also an important type of epigenetic modification. 5-hmC plays key roles in nuclear reprogramming, regulates gene activity, and initiates DNA demethylation in mammals [1–3]. In addition, the level of 5-hmC is dramatically reduced in liver, lung, brain, kidney, prostate, skin, colon, and breast cancers [4–8]. Lack of 5-hmC may also be a useful indicator in cancer diagnosis [5,9,10]. Therefore, the development of analytical technologies is critical for the detection of 5-hmC in the context of sequences or genes.

Owing to the similar structures of 5-hmC and 5-mC, most analytical techniques for detection of 5-mC, such as bisulfite sequencing [11] and 5-mC-sensitive restriction assays [12,13], fail to distinguish the rare 5-hmC from the abundant 5-mC. Interestingly, 5-hmC but not 5-mC can be selectively glucosylated by T4 phage β-glucosyltransferase (T4 β-GT) to form β-glucosyl-5-hydroxymethylcytosine (5-ghmC) [14]. This discovery established a novel paradigm for distinguishing and detecting 5-hmC from 5-mC. Subsequently, many methods of 5-hmC detection based on T4 β-GT catalytic glycosylation modification of 5-hmC were developed. For example, Szwagierczak and coworkers reported a method based on specific transfer of a glucose moiety of radiolabeled glucose to the hydroxyl group of 5-hmC by T4 β-GT. The method achieved the quantification of genomic 5-hmC [14]. However, the radioactive approach is harmful to technicians and the environment. Additionally, Song et al. presented an approach based on specific transfer of an engineered glucose moiety containing an azide group to the hydroxyl group of 5-hmC by T4 β-GT. The azide group can be chemically modified with biotin for detection, affinity enrichment, and sequencing of 5-hmC-containing DNA fragments in mammalian genomes [15]. However, this method is expensive and requires costly equipment and highly trained technicians. Pastor et al. proposed a strategy based on glucosylation, periodate oxidation, and biotinylation (GLIB). By this method, 5-hmC is first glucosylated to 5-ghmC. The glucose moiety of 5-ghmC is then oxidized by sodium periodate, and the vicinal hydroxyl groups are converted to aldehydes and further

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http://dx.doi.org/10.1016/j.talanta.2017.04.051
Received 22 January 2017; Received in revised form 11 April 2017; Accepted 21 April 2017
Available online 22 April 2017

modified with an aldehyde-reactive probe, which adds two biotin molecules to each 5-hmC. Finally, the 5-hmC content is assayed using a HeliScope Single Molecule Sequencer [16]. However, similar to the previous method, this method is also expensive. Additionally, several methods based on glycosylation modification of 5-hmC by T4 β-GT coupled with restriction endonuclease analysis can effectively distinguish 5-hmC from 5-mC at cleavable sites of restriction endonucleases. However, these methods are only applicable to certain loci or sequences and cannot identify 5-hmC in noncleavable DNA regions [17–20]. The limitations of these methods based on T4 β-GT catalytic glycosylation modification of 5-hmC restrict their wide application in 5-hmC detection. Therefore, the development of simple, inexpensive, environmentally friendly, time-saving, sensitive technologies that do not have restriction assay-like sequence limitations is needed for the detection and discrimination of 5-hmC from 5-mC.

Electrogenerated chemiluminescence (ECL) is a type of chemiluminescence generated by an electrochemical reaction. ECL has been extensively applied in many biochemistry-related applications because of its inherent features, such as high sensitivity, low background, wide linear range, convenient operation, and simplicity [21–25]. However, to date, the ECL method has not been commonly used for discrimination of 5-hmC [26,27]. In a previous work, our group proposed an ECL biosensing method for detecting T4 β-GT activity and recognizing 5-hmC in DNA (5-hmC-DNA) based on the inhibitory effect of glucosylated 5-hmC-DNA towards the activity of *Msp*I [26]. This method could facilitate the qualitative determination of 5-hmC at cleavable sites of restriction endonucleases for specific sequences (CCGG).

Accordingly, in this study, we aimed to quantify of 5-hmC in cleavable and noncleavable DNA regions. To this end, a novel signal-off ECL biosensing method was established for the quantification of 5-hmC-DNA based on T4 β-GT catalytic glycosylation modification of 5-hmC. In this work, tris(2, 2′-bipyridine) dichlororuthenium(II) ($Ru(bpy)_3^{2+}$) was utilized as an ECL-emitting species because of its chemical stability and higher luminescence yield [23]. Ferroceneboronic acid (FcBA) was utilized as a marker of glycosylated 5-hmC to quench the ECL signal, because FcBA has been extensively exploited to capture *cis*-diol-containing biomolecules through the formation of boronate ester covalent bonds between boronic acid and *cis*-diol-containing biomolecules [28–32]. In addition, ferrocene and its derivatives show more efficient quenching of ECL than known quenchers, such as 1,1-dimethyl-4,40-bipyridine dication and phenol [33–35]. Gold nanoparticles (AuNPs) were used to enhance the ECL signal because of their large surface area, outstanding electrocatalytic activity, stability, and conductivity [36]. Fig. 1 shows a schematic diagram of the fabrication process of the biosensing electrode for the detection of 5-hmC-DNA. Briefly, the electrode modified with a mixture of Nafion and AuNPs was first utilized as the platform for electrostatically adsorbing $Ru(bpy)_3^{2+}$ and assembling 5-hmC-DNA. With this platform, a strong ECL signal could be obtained based on the enhancement function of AuNPs on the ECL signal. After 5-hmC-DNA assembled on the electrode was glycosylated, quencher-FcBA of the ECL signal could be tagged directly onto the glycosylated 5-hmC through the formation of boronate ester covalent bonds between boronic acid and *cis*-diols of glycosylated 5-hmC. The strong ECL signal was quenched by quencher-FcBA. 5-hmC-DNA could be quantified based on the decrease in ECL intensity. Based on these findings, we also discussed the design and characteristics of the ECL biosensing method and demonstrated the analytical performance of this technique for the quantification of 5-hmC in DNA to distinguish 5-hmC from 5-mC.

2. Experimental

2.1. Reagents and apparatus

Tris(2-carboxyethyl) phosphinehydrochloride (TCEP, 98%), $Ru(bpy)_3^{2+}$, dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), Nafion (perfluorinated ion-exchangeresin, 5 wt% solution in a mixture of lower aliphatic alcohols and water), FcBA, and chloroauric acid ($HAuCl_4$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tri-*n*-propylamine (TPA) and sodium citrate were purchased from Sinopharm Chemical Reagent Co., Ltd. T4-β-Glucosyltransferase (β-GT) and UDP-glucose (UDP-Glu) were purchased from Takara (China). The sequences of DNA purchased from Takara were designed according to a previous study [37], and their base sequences were as follows: 5′-HS-$(CH_2)_6$-TAGAGTATGATGCGCTGACCCACAACATCCG-3′ (probe DNA, S1); 5′-CGGATGTTGTGGGTCAGC^{hm}GCATCATACTCTA-3′ (hydroxymethylated DNA, 5-hmC-DNA, S2); 5′-CGGATGTTGTGGGTCAGC^{m}GCATCATACTCTA-3′ (methylated DNA, 5-mC-DNA, S3); and 5′-CGGATGTTGTGGGTCAGCGCATCATACTCTA-3′ (untreated DNA, 5-C-DNA, S4). Millipore Milli-Q water (18 MΩ cm) was used throughout. Probe DNA immobilization buffer, DNA hybridization buffer, β-GT storage buffer, and β-GT reaction buffer were prepared as previously described [38].

ECL measurements were performed with an MPI-E ECL detector (Xi'an Remax Electronics, China). The experimental set-up for ECL measurement was described previously [39]. ECL emissions were detected with a photomultiplier tube (PMT) that was biased at −700 V. All electrochemical measurements, including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and square wave voltammetry (SWV), were conducted using a CHI 660 potentiostat (Chenhua Instruments, Shanghai). A traditional three-electrode configuration was employed, in which a GCE electrode (φ=3.0 mm) functioned as the working electrode, a Ag/AgCl (sat. KCl) electrode functioned as the reference electrode, and a platinum sheet functioned as the counter electrode. The transmission electron microscopy (TEM) images were obtained with a JEM-2100 transmission electron microscope (JEOL, Japan). Scanning electron microscopy (SEM) images were obtained with a Carl Zeiss Sigma scanning electron microscope (Carl Zeiss, Germany). Fluorescence images were obtained using an Olympus IX-51 inverted microscope (Olympus Corporation, Tokyo, Japan). XPS spectra were obtained with an Axis Ultra photoelectron spectrometer (Kratos Analytical ltd, UK). The UV–vis absorption spectra were recorded using a UV–vis spectrophotometer (Model UV-2450; Shimadzu Corporation, Japan).

2.2. Preparation of the biosensing electrode

An AuNPs suspension was prepared as described previously [39]. AuNPs-Nafion modified GCE (AuNPs-Nafion/GCE) was prepared as described previously [26]. AuNPs-Nafion/GCE was immersed in 50 μL of 1 mM $Ru(bpy)_3^{2+}$ for 30 min and rinsed thoroughly with 10 mM phosphate buffer (PB, pH 7.4) to form an ECL layer of $Ru(bpy)_3^{2+}$/AuNPs-Nafion/GCE. The biosensing electrode was fabricated by dropping 10 μL of 1 μM S1 solution on $Ru(bpy)_3^{2+}$/AuNPs-Nafion/GCE for 4 h at 37 °C and then washed with 10 mM PB (pH 7.4) thoroughly. Next, the electrode was blocked with 1 mM MCH solution for 0.5 h. The resulting MCH/S1/$Ru(bpy)_3^{2+}$/AuNPs-Nafion/GCE was then thoroughly washed with 10 mM PB (pH 7.4) and used as an ECL-biosensing electrode.

2.3. ECL measurements

The fabricated ECL-biosensing electrodes were coated with 10 μL of different concentrations of DNA S2 and incubated at 37 °C for 4 h to form the hydroxymethylated S2 and S1 hybrid. Subsequently, the hydroxymethylated S2 and S1 hybrid-modified electrode was coated with 10 μL of 1×β-GT reaction buffer containing 30 μM UDP-glucose and 50 U/mL β-GT at 37 °C for 2 h to form the glucosylated hydroxymethylated S2 and S1 hybrid. Finally, the glucosylated hydroxymethylated S2 and S1 hybrid-modified electrode was coated with 10 μL of 10 mM PBS containing 0.5 mM FcBA at 37 °C for 1 h. The electrode