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# A novel electrochemiluminescence biosensor for the detection of 5-methylcytosine, TET 1 protein and β-glucosyltransferase activities based on gold nanoclusters-$H_2O_2$ system

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ABSTRACT

5-Methylcytosine (5 mC) is considered to play a key regulating role in gene transcription and can be oxidized by ten–eleven translocation (TET) dioxygenases to 5-hydroxymethylcytosine (5 hmC). In order to better understand the function of 5 mC in gene transcription and the mechanism of demethylation, sensitive and selective methods must be developed for detecting 5 mC, the TET 1 protein and T4 β-glucosyltransferase (β-GT). In this work, a novel electrochemiluminescence (ECL) biosensor was fabricated for the quantification of 5 mC, TET 1 protein and β-GT activities, as well as inhibitor screening, based on the interaction of chemically excited gold nanoclusters (AuNCs) with $H_2O_2$. The ECL biosensor was constructed on an Au nanoparticle (AuNP) decorated glassy carbon electrode, to which BSA coated AuNCs and thiol-containing probe DNA was subsequently attached. Hybridization with target 5 mC-DNA, was followed by demethylation by TET 1 and glycosylation by β-GT. After reaction of the glycosyl group with 4-carboxyphenylboronic acid, horseradish-peroxidase was then grafted to the DNA backbone to catalyze $H_2O_2$ reduction and the oxidation of hydroquinone to benzoquinone. The ECL of the chemically excited AuNCs was attenuated in the presence of $H_2O_2$, with more $H_2O_2$ being decomposed in the presence of 5 mC DNA, TET-1 or β-GT, leading to an enhanced ECL response. The proposed assay demonstrated high selectivity for 5 mC-DNA detection in the concentration range 0.01 nM–50 nM, with a detection limit of 3.46 pM. The applicability of this biosensor was confirmed by the evaluation of blood serum, with the developed biosensor having great potential in drug discovery.

## 1. Introduction

The process of DNA methylation and demethylation occurrs at the 5-position of cytosine and is responsible for a broad array of biological functions, especially gene transcriptional regulation and gene silencing, thereby significantly impacting gene expression in eukaryotic species [1]. Aberrant DNA methylation is associated with various diseases, such as cancers and diabetes [2]. It was recently discovered that ten–eleven translocation (TET) dioxygenases can further catalyze the conversion 5-methylationcytosine (5 mC) to 5-hydroxymethylcytosine (5 hmC) in the presence of iron(II) and α-ketoglutarate (α-KG) [3], providing the first strong evidence of the demethylation pathway [4]. That study established that TET dioxygenases are clearly important regulators of developmental gene expression processes. Subsequently, the oxidation products of 5 hmC could be glycosylated by T4 β-glucosyltransferase (β-GT), where the glucose molecule specifically attaches at the hydroxyl group of 5 hmC formed after the demethylation reaction [5]. A previous study suggested that 5 hmC participates in nuclear reprogramming and the levels of this compound are greatly reduced in diseased human tissues [6]. This indicates that 5 hmC levels can serve as a crucial marker for cancer diagnosis. The studies above motivate the development of sensitive technologies for 5 mC and 5 hmC quantification, whilst also allowing the mechanism of 5 mC demethylation to be explored.

To date, a number of molecules specific methods have been proposed for global 5 mC detection, including single-molecule real-time (SMRT) sequencing [7], high-throughput tag sequencing [8], electrochemical technology [9], bisulfite sequencing [10] and electrochemiluminescence (ECL) [11]. Among these, ECL is attracting a lot of attention owing to its versatility, simple operation, low sample size

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needed for analysis, high sensitivity and high efficiency [12]. For instance, Jiang et al. constructed an ECL immunosensor for carbohydrate antigen 15-3 detection based on polyamidoamine (PAMAM)-functionalized ZnO nanorods as the signal platform [13]. Li et al. presented a signal-off ECL biosensor for the ultrasensitive detection of lead ions based on a gold nanodendrite modified electrode and electrochemiluminescent quenching of CdS quantum dots by electrocatalytic silver/zinc oxide coupled structures. Adsorption of lead ions resulted in a decrease in the ECL emission [14]. Xu et al. synthesized a redox-active metal-organic framework for cocaine detection in the serum samples based ECL system [15].

Gold nanoclusters (AuNCs), as the bulk metal, display unique size-dependent electronic and optical properties [16], leading to catalytic activity [17], fluorescence [18] and ECL [19]. These properties making AuNCs promising materials for biological imaging [20], metal ions detection [21], and optoelectronic devices [22]. Further, due to their low toxicity, eco-friendly synthesis and excellent biocompatibility, AuNCs are finding increasing utilization in analytical applications as a new-type label [23]. For example, Yu et al. constructed a ECL resonance energy transfer (ERET) system, where a CoAl layered double hydroxide promoted ERET between AuNCs and $Ru(bpy)_3^{2+}$ [24]. Wu et al. presented an ECL sensor for $H_2O_2$ detection by using AuNCs-silica nanoparticle as luminophore [25]. $H_2O_2$ was found to destroy the structure of AuNCs@BSA, causing ECL quenching [26]. These studies serve as a platform for the development of novel ECL sensors based on AuNCs-$H_2O_2$ interactions.

Herein, we aimed to fabricate an ECL biosensor for the quantitative detection of 5 mC-DNA, TET 1 and β-GT based on the catalytic action of TET 1 and the β-GT glycosylation of 5 hmC. The underlying mechanism of the ECL biosensor is depicted in Scheme 1. Firstly, DNA 1 modified with thiol was captured on the surface of a glass carbon electrode (GCE) by gold nanoparticles (AuNPs) via Au-S bonding [27,28]. Subsequently, the target 5 mC-DNA then assembled on the modified electrode by hybridization, and then modified by the stepwise action of TET 1 proteins and β-GT. The glycosyl group was then reacted with 4-carboxyphenylboronic acid (CPBA) which acted as a covalent linkage for the binding of horseradish-peroxidase (HRP). BSA-AuNCs were specifically used here as the luminophore and to react with the coreactant $K_2S_2O_8$ to generate the ECL signal. Meanwhile, $H_2O_2$ was employed for its ability to quench the excited state of AuNCs*. As the 5 mC-DNA concentration is increased, more $H_2O_2$ is decomposed by HRP, thus affording a stronger ECL signal. This proposed strategy proved successful, allowing stable detection of 5 mC, TET 1 and β-GT with good selectivity. Further, the biosensor was able to monitor TET 1 and β-GT activities and evaluate inhibition of TET 1and β-GT, thus offering a new platform for the discovery of anticancer drugs.

## 2. Experimental

### 2.1. Reagents and apparatus

See Supplementary materials.

### 2.2. Preparation of BSA-AuNCs

BSA–AuNCs were prepared according to a literature method [29] and described in Supplementary materials.

### 2.3. Fabrication of AuNPs/GCE

Before the fabrication of ECL biosensor, AuNPs/GCE was prepared. First, a glassy carbon electrode (GCE) was polished with 30 nm alumina slurry following by rinsing ultrasonically with ethanol and deionized water for 3 min. After the pretreatment, the GCE was immersed into 10 mL of a 3.0 mM $HAuCl_4$ solution containing 0.1 M $KNO_3$ and the electrodeposition of gold nanoparticles (AuNPs) achieved via the amperometric method at −0.2 V for 200 s under constant stirring. Subsequently, the modified electrode (AuNPs/GCE) was washed with deionized water and dried in a nitrogen atmosphere.

### 2.4. Fabrication of the biosensor

See Supplementary materials.

### 2.5. Inhibition studies

To investigate the effect of inhibitor on the activity of TET 1, different concentrations of fumaric acid were introduced into the TET1 reaction buffer and incubated for 30 min at 37 °C. The ECL biosensor was then fabricated according to the procedure described in Section 2.4. To study the effect of inhibitors on the activity of β-GT, various concentrations of 4-phenylimidazole and uridine 5-diphosphate sodium salt, as two model inhibitors, were employed in the assay and incubated for 1 h at 37 °C. The procedures were similar to the assay of TET 1.

### 2.6. Measurement procedure

For ECL detection, the fabricated electrode was immersed in 8 mL of detection buffer containing 0.1 M $K_2S_2O_8$, 0.1 M KCl, 20 mM $H_2O_2$, 20 mM hydroquinone, and studied with an ECL analyzer between −2 to 0 V at 100 mV $s^{-1}$. Electrochemical impedance spectroscopy (EIS) measurements were performed in a 5 mM solution of $Fe(CN)_6^{3-/4-}$ (1:1) containing 0.1 M KCl (pH 7.4) on CHI660E electrochemical workstation.

## 3. Results and discussion

### 3.1. Characterization of AuNCs

The BSA–AuNCs detailed morphology information of the AuNCs possessed a spherical shape and a mean size in the range 2–3 nm as revealed by TEM (Fig. 1A), which manifested that the product has been successfully prepared.

### 3.2. EIS and ECL characterization of the biosensor

The stepwise fabrication of the ECL biosensor was monitored by electrochemical impedence spectroscopy (EIS). As shown in Fig. 1B, the bare GCE gave a small semicircle (curve a) in the Nyquist plot, indicating a reasonably low electron-transfer resistance of 125 Ω ($R_{et}$). After deposition of AuNPs and BSA–AuNCs on the surface of GCE electrode (curve b, c), even smaller semicircles were observed, consistent with the excellent electrical conductivity of AuNPs and AuNCs. After the probe DNA was self-assembled on the modified electrode, $R_{et}$ increased to 304.2 Ω (curve d) due to the probe DNA being non-conductive as well as electrostatic repulsion between phosphate skeleton of DNA probe and $Fe(CN)_6^{3-/4-}$. When 5 mC-DNA was captured on the electrode (curve e), the $R_{et}$ resistance further increased to 411.8 Ω, confirming the successful hybridization of target DNA. $R_{et}$ increased further (531.6 Ω) when double-stranded DNA was contacted by TET 1 (curve f), owing to enlargement of polarity caused by introduction of hydroxy. After 5 hmC-dsDNA/AuNCs/AuNPs/GCE glycosylation by β-GT (curve g), the $R_{et}$ value further increased to 671.3 Ω, suggesting the glycosyl group provided additional steric hindrance to the $Fe(CN)_6^{3-/4-}$ probe. The steric-hindrance resulted from introduction of HRP (curve h), resulting in an even larger semicircle in the EIS plot, which indicates the Ret value of 832.7 Ω. According to change of the $R_{et}$ value, the successful preparation of the electrode can be confirmed. Inset of Fig. 1B shows the equivalent circuit, which are best fitted the experiment data for different electrodes. In the circuit, $CPE_{dl}$ is the constant phase element of the electrical double layer, $R_{et}$ is the charge transfer resistance, $Z_w$ is the Warburg impedance and $R_s$ is the solution