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Novel label-free electrochemical strategy for sensitive determination of ten-eleven translocation protein 1

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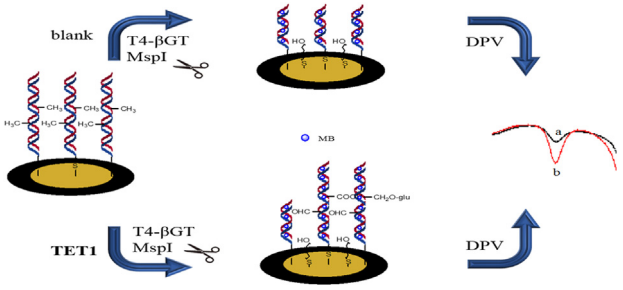
HIGHLIGHTS

- A novel electrochemical biosensor was developed for TET1 detection and its inhibitor screening.
- This biosensor shows a wide linear range from 0.0042 to 0.0210 μg μL $^{-1}$ with a low detection limit of 0.00098 μg μL $^{-1}$.
- This method is an attractive candidate for construction platform for monitoring DNA methylation and demethylation..

GRAPHICAL ABSTRACT



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ABSTRACT

A label-free electrochemical method was developed for sensitive determination of ten-eleven translocation protein 1 (TET1) which can mediate the demethylation of DNA. This strategy is mainly based on MspI-mediated restriction endonuclease reaction. Current response difference of the biosensor before and after cleavage by MspI was dependent on the activity and concentration of TET1. With the aid of Au nanoparticles, this method shows a good linear range from 0.0042 μg μL^{-1} to 0.0210 μg μL $^{-1}$ with a correlation coefficient of 0.9350 and a low limit of detection 0.00098 μg μL $^{-1}$. Finally, this method was used to investigate the effects of n-oxalylglycine (NOG) and taxol on activity of TET1. The results indicated that NOG could inhibit TET1 activity but taxol could not. So this electrochemical biosensor could be applied to TET activity evaluation and inhibitor screening in field of biomedicine and clinical diagnosis.

1. Introduction

DNA methylation as one of the best-characterized epigenetic modifications plays an important role in embryogenesis [1], cellular differentiation [2], genetic imprinting [3] and regulation of gene expression [4]. The most common methylation of DNA occurs at the fifth position of cytosine to give 5-methylcytosine (5 mC) at the CpG

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dinucleotide site [5]. In 2009, TET1 was first discovered to convert 5 mC to 5-hydroxymethylcytosine (5hmC) and 5hmC was observed in neurons and embryonic stem cells [6,7], which bring a new blossom on DNA demethylation research. The mammalian ten-eleven translocation proteins (TET) are a family of 2-oxoglutatrate- and Fe(II)-dependent dioxygenases and contains three members: TET1, TET2 and TET3. In 2010, TET2 and TET3 were also identified to catalyze a similar reaction [8,9]. Subsequent research shows that 5hmC can be further oxidized to be 5-formylcytosine (5 fC) and 5-carboxylcytosine (5caC) in DNA [10]. Moreover, 5 fC and 5caC can be further excised by thymine-DNA glycosylase (TDG) and replaced by unmodified cytosine through the base excision repair (BER) pathway [11,12]. Following research on crystal structure of TET2-DNA complex proved the TET-mediated DNA demethylation in terms of molecular structure [13]. The discovery of TET protein family and the oxidized 5 mC derivatives serve as true milestones for epigenetic research and show increasingly importance in various biological and pathological processes. Studies in the past few years have emphasized the finding of aberrant DNA methylation as a hallmark of cancer [14]. Growing evidences indicate that altered TET expression and 5-hmC levels have been observed for numerous cancers [15]. However, most of the mechanisms for participation in carcinogenesis remain unknown. The research on TET protein expression levels and activities in cancer has major implications for understanding the potential link between TET proteins and cancer.

As far as we know, methods for TET activity analysis are quite limited. A few cases are involved in the present reports using ELISA-based commercially available colorimetric kits and 2D-TLC or mass spectrometry (MS)-based techniques [8–10,16–18]. Other methods rely on detecting levels of 5hmC via immunoblotting. Recently, a fluorescence polarization biophysical assay for TET1 and an electrochemiluminescence biosensor for the detection of 5-mC and TET1 have been reported [19,20]. Clearly, new assays for TET activity or concentration and their binders screening are needed to expand our understanding of the complexities of DNA-modified epigenetics and the applications of TET determination for both research and pharmaceutical purposes.

Electrochemical techniques, compared with other methods, have advantages of low cost, portability, and high sensitivity. It has been widely used for enzyme activity analysis and biomarker detection [21,22]. Herein, a label-free electrochemical strategy is introduced for TET1 detection. Full-methylated DNA (GG5mCC/C5mCGG) was used as target and methylene blue (MB) as electrochemical signal probe. The motif of (GG5mCC/C5mCGG) can be recognized and cleaved by endonuclease enzyme MspI [23]. After treatment with TET1, 5 mC was oxidized to 5hmC, 5 fC and 5caC. Evidences show that MspI cannot cleave 5 fC and 5caC [24,25]. At the same time, glycosylation of 5hmC residues by T4 Phage β-glucosyltransferase can make CCGG residues insensitive to digestion by MspI. So, the difference of MB currents can indicate the activity and concentration of TET1. Furthermore, nanoparticles were introduced into the fabrication process of biosensor to enhance sensitivity of the biosensor. Before the immobilization of DNA, glassy carbon electrode was modified by film of Au nanoparticles. The results showed that Au nanoparticles could apparently improve performance of this sensor because it has special electrical properties and high specific surface area.

2. Experimental

2.1. Materials and apparatus

TET1 was purchased from Epigentek Group Inc. (Cat.No. E12002, USA) and stored at −80 °C. Restriction endonuclease MspI (Cat.No. R0106L) and 10 × cutsmart buffer (Cat.No. B7204S) were purchased from New England Biolabs (NEB) Inc. T4 phage β-glucosyltransferase (T4-βGT) was purchased from Thermo scientific company. Tris (2-carboxyethyl) phosphine (TCEP), methylene blue (MB), α-ketoglutaric acid (α-KG), ammonium iron (II) sulfate ($Fe(NH_4)_2(SO_4)_2$), D/L-dithiothreitol (DTT), 1-hexanethiol (1-HT) were all purchased from Aladdin reagent (Shanghai, China). Sodium phosphates, Tris-HCl, bovine serum albumin (BSA), sodium chloride (NaCl), potassium chloride (KCl), ethylenediaminetetraacetic acid disodium salt (EDTA), concentrated hydrochloric acid (HCl), sodium hydroxide (NaOH), chloroauric acid ($HAuCl_4$), HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), ascorbic acid, potassium ferrocyanide ($K_4Fe(CN)_6$), potassium ferricyanide ($K_3Fe(CN)_6$) and other chemicals were purchased from Sinopharm Group (Shanghai, China). N-oxalylglycine (NOG) was obtained from Sigma–Aldrich (MO, USA). The oligonucleotides were purchased from Takara (Dalian, China). The DNA sequences were as follows:

S1: 5′–SH–$(CH_2)_6$ –GACTGAGTACTC^{m}CGGACACTGATAGC-3'.

S2: 3′-CTGACTCATGAGG^{m}CCTGTGACTATCG-5'.

The synthesized oligonucleotides were dissolved in 10 mM Tris, 1 mM EDTA, 1 M NaCl and 1 mM TCEP (pH 7.4). The stock solutions were used after annealing and then stored at −20 °C according to description of products. MB was dissolved in phosphate buffered saline (PBS, 10.0 mM Na_2HPO_4–NaH_2PO_4 containing 0.1 M NaCl, pH 7.4) to form a concentration of 1.0 mM. The water was obtained from a Millipore filtration system. All reagents were of analytical reagent grade.

Electrochemical experiments were performed on a CHI660D electrochemical workstation (Shanghai Chenhua Instrument Co., China) with a conventional three-electrode system, a glassy carbon electrode (GCE, 3 mm in diameter), a platinum wire and a saturated calomel electrode (SCE), used as the working electrode, counter electrode and reference electrode, respectively. All the potentials were controlled versus SCE.

2.2. Preparation of the modified electrode surfaces

The bare glassy carbon electrode (GCE, A = 0.071 cm^2) was mechanically polished with polishing micro-cloth containing 0.05 μm Al_2O_3 slurry to a mirror finish, and then carefully cleaned in HNO_3–H_2O (v/v, 1:1) and ethanol, deionized water in turn via ultrasonication each for 2 min. Gold nanoparticles (AuNPs) were electrodeposited at −0.20 V for 200 s in 1.0 mL of solution containing 3 mM $HAuCl_4$ solution and 0.1 M KNO_3. The electrode was signed as AuNPs/GCE. It was firstly incubated with 1 μM annealed DNA probe for 12 h at room temperature, and then rinsed with 2.0 mL of washing buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM NaCl, pH 7.4) and 1.0 mL of deionized water successively. The electrode was named as S1–S2/AuNPs/GCE. After that 5.0 μL of a certain concentration of TET1 was dripped on the electrode surface and incubated for 3 h with reaction buffer containing 50 mM HEPES, 100 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM α-KG, 2 mM ascorbic acid, 2.5 mM DTT, 100 mM NaCl, 1.2 mM ATP. After that, it was rinsed with buffer and then incubated with 5.0 μL of T4-βGT for 15 min in 37 °C water bath. The electrode was subsequently immersed in 0.5 mL of 1-HT for 30 min at room temperature. Finally, 1U MspI with 1x cutsmart buffer (50 mM K-acetate, 20 mM Tris-acetate, 10 mM Mg-acetate, 100 μg mL^{-1} BSA, pH 7.9) was dripped on the electrode surface and incubated for 3 h in 37 °C water bath, and then rinsed with washing buffer for electrochemical detection.

2.3. Electrochemical detection

The fabricated electrode was immersed in MB (1.0 mM) under slightly stirring for 10 min. After it was rinsed and dried under air

condition, current response of MB was detected by differential pulse voltammetry (DPV) in PBS (pH 7.4). The differential pulse voltammograms (DPVs) were recorded between −0.8 V and −0.3 V.

As for application of this sensor on TET1 inhibitors screening, the modified electrode was fabricated as previous procedure and electrochemical detection was performed as the same as above description. The difference is that the reaction buffer for TET1 incubation contained a certain concentration of inhibitors such as NOG.

2.4. Gel electrophoresis

In gel electrophoresis assay, S1 and S2 were firstly annealed to form dsDNA (S1−S2 duplexes, 5 mC-DNA). Then one of dsDNA sample was treated with 2.5 mM DTT. The other dsDNA sample was incubated with 1 U MspI. Finally, all sample were analyzed by 20% native polyacrylamide gel electrophoresis (PAGE) in 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2.0 mM EDTA, pH 8.0) at a 350 V constant voltage for 15 min, 400 V for 10 min, 500 V for 90 min at room temperature. Then, the gel was stained with Gel Red nucleic acid gel stain for 10 min. The gels were imaged using a Gel Documentation system (Huifuxingye, Beijing, China).

3. Result and discussion

3.1. Design strategy of electrochemical assay

Herein, we proposed a sensitive electrochemical method for TET1 detection based on T4-βGT catalytic glycosylation reaction and MspI-mediated restriction endonuclease reaction. The principle of design strategy is illustrated in Scheme 1. The dsDNA (S1−S2 duplexes) was designed containing the preferred TET1 recognition sites (methylated cytosine) and the duplex complementary sequence of 5′-C^{m}CGG-3′ which can be identified by MspI. Firstly, the thiolated 5 mC-DNA was self-assembled onto the AuNPs/GCE via Au−S bounding. In absence of TET1, 5 mC-DNA was selectively digested by MspI and only a small amount of MB could be absorbed on the modified electrode (curve a). When the modified electrode was incubated with TET1 in the presence of α-KG and $Fe(NH_4)_2(SO_4)_2$, 5 mC was oxidized to 5hmC and further to 5caC or 5 fC. Under the catalysis of T4-βGT, glucosyl group in UDP-glucose can be transferred to the hydroxymethyl [6], preventing 5hmC-DNA from cleavage by MspI. Then 1-HT was used to blocking the electrode surface. After treated with MspI, only few 5 mC-DNA which was not oxidized by TET1 can be cleaved and a relatively larger electrochemical signal of MB was achieved (curve b). As an organic dye, MB has excellent electrochemical response and can interact with deoxyribose-phosphate backbone in DNA by electrostatic interaction. That means, the electrochemical response of MB in this assay depends on the length of the dsDNA at the electrode surface. Fig. 1 shows the DPVs of biosensor after and before the treatment by TET1. In the absence of TET1, the peak current of biosensor is small (curve a). However, the current response of biosensor has been significantly enhanced after the treatment by

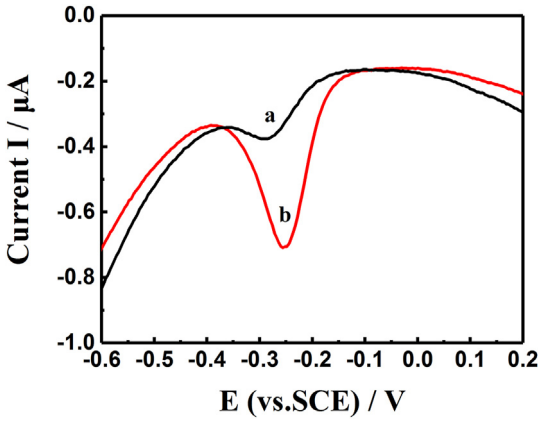
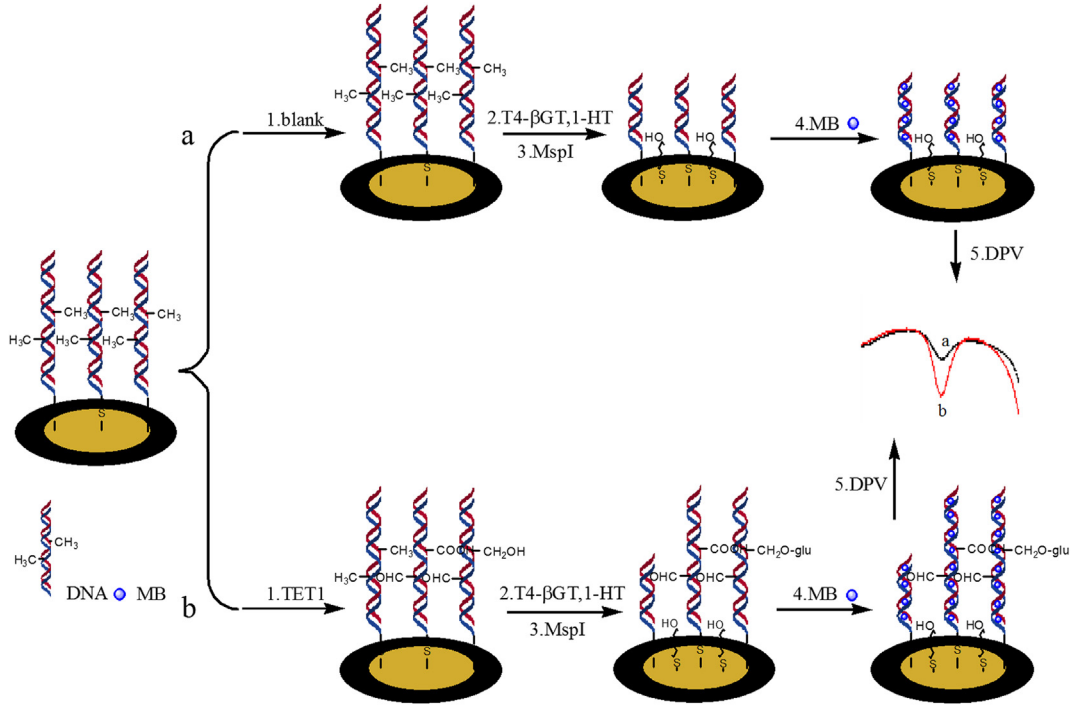


Fig. 1. DPVs of biosensor before (curve a) and after (curve b) the treatment by TET1.



Scheme 1. The principle of biosensor for TET1 determination.

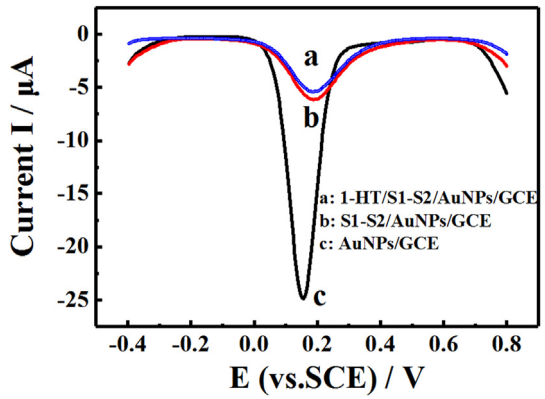


Fig. 2. DPVs of different modified electrodes in 1.0 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$.

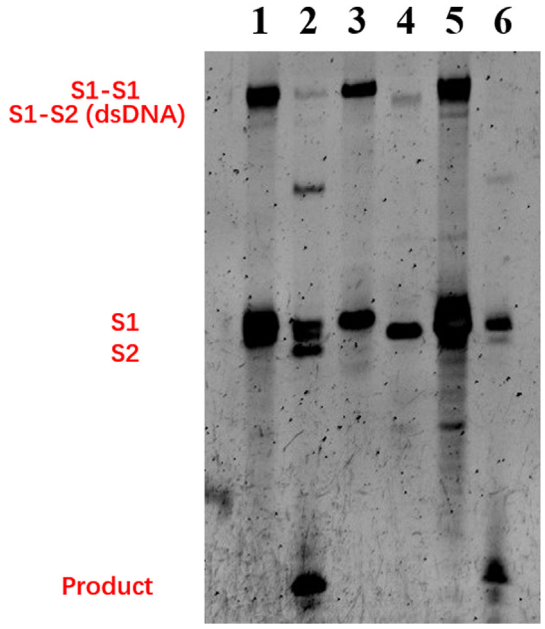


Fig. 3. Gel electrophoresis of MspI-treated dsDNA. Lane 1, dsDNA; Lane 2, dsDNA incubated with MspI; Lane 3, DNA S1; Lane 4, DNA S2; Lane 5, dsDNA incubated with DTT; Lane 6, dsDNA incubated with DTT and MspI.

TET1 (curve b). So, the electrochemical signal is related to the TET1 concentration. It was also used to screen the inhibitors of TET1, which might be applied to the discovery of anticancer drugs.

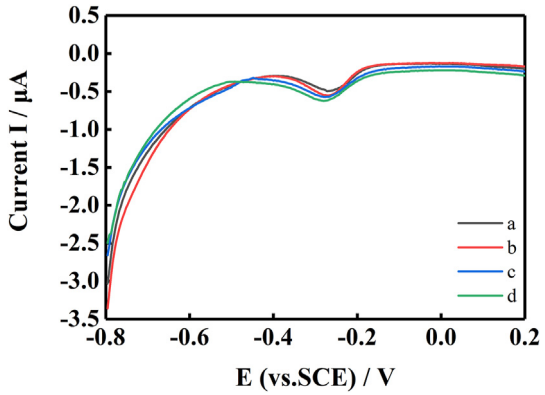


Fig. 5. DPVs of the modified electrodes before (curve d) and after stored at 4 °C for 10 days (curve a, b, c) in the treatment of 0.0126 μg μL^{-1} TET1.

3.2. Characterization

DPVs of different modified electrodes during biosensor preparation are recorded in 1.0 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ as shown in Fig. 2. The oxidation peak current of Fe^{2+} is the largest and a well-defined peak can be observed at AuNPs/GCE (curve c). After mobilization of S1–S2 duplexes (curve b) and 1-HT (curve a), the peak current decreased greatly due to the blocking electron transfer by DNA and 1-HT. This result suggested that the thiolated 5 mC-DNA was successfully immobilized onto the electrode surface.

To verify the feasibility of this strategy, native PAGE experiments were used to characterize the renaturation of dsDNA and MspI-induced reaction. The results were shown in Fig. 3. Land 1 represents hybridization products between S1 and S2. Land 3 and land 4 are S1and S2, respectively. In contrast with land 3 and land 4, land 1 proved that S1 and S2 have been successfully hybridized. Since S1 is labeled with a thiol group, it may lead to the formation of S1–S1 polymers as presented in land 1 and land 3 (top band). After the treatment of dsDNA by MspI (land 2), an obvious band of product appears at the lower part of land (bottom band), suggesting the success in cleavage event. When dsDNA was incubated with DTT, the thiol group of S1 would be kept in reduced form and less S1–S1 polymers was obtained (comparing land 5 to land 1). When dsDNA was incubated with both DTT and MspI (land 6), the original band of dsDNA disappears and the band of product with higher electrophoretic mobility can be observed at bottom of land 6. These PAGE results indicate the MspI could cleave the methylated dsDNA efficiently, and DTT also plays an important role in preventing from formation of disulfide bonds.

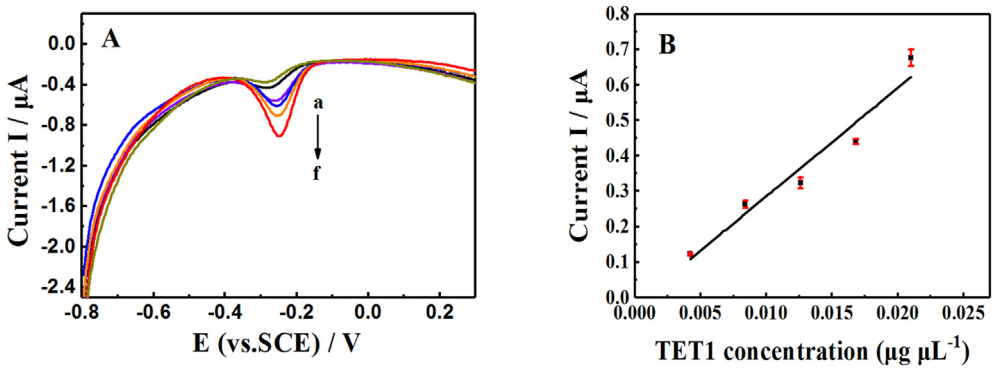


Fig. 4. (A) The dependence of biosensor responses on TET1 concentrations, (a) 0 (control); (b) 0.0042 μg μL^{-1}; (c) 0.0084 μg μL^{-1}; (d) 0.0126 μg μL^{-1}; (e) 0.0168 μg μL^{-1}; (f) 0.0210 μg μL^{-1}. (B) Calibration curve for TET1 detection.

Table 1
Comparison of different methods for TET1 detection.

Method	Linear range	Detection limit	Reference
LC-MS	10–30 μg mL^{-1}	not mentioned	[16–18]
TET activity assay kit (fluorometric)	1–10 ng μL^{-1}	1 ng μL^{-1}	Epigentek Group Inc.
Electrochemiluminescence biosensor	1–10 μg mL^{-1}	0.37 μg mL^{-1}	[20]
Electrochemical biosensor	0.0042–0.021 μg μL^{-1}	0.00098 μg μL^{-1}	this work

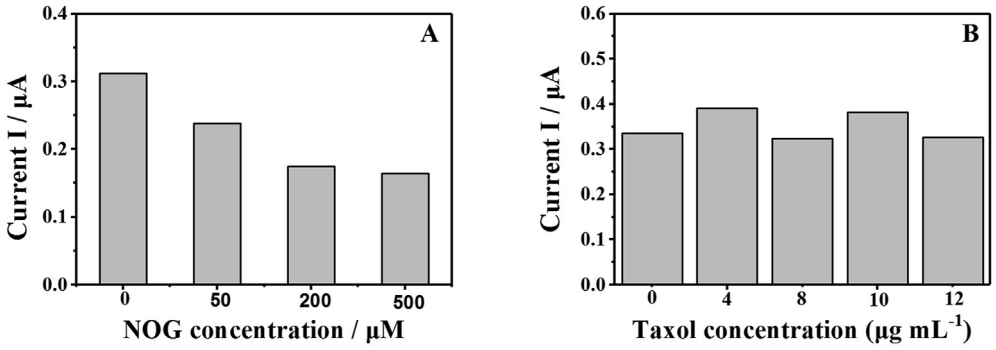


Fig. 6. The effects of (A) NOG and (B) taxol on activity of TET1 (0.0042 μg μL^{-1}).

3.3. TET1 detection

Under optimized conditions, performance of this biosensor has been investigated by electrochemical techniques and the dependence of peak current on TET1 concentration is illustrated in Fig. 4. Fig. 4A shows the DPVs of this biosensor after treatment by TET1 with different concentrations. The peak current increases gradually with increase of the concentration of TET1. This is consistent with our expectations. Furthermore, the relationship between DPV response and TET1 concentration from 0.0042 μg μL^{-1} to 0.0210 μg μL^{-1}could be described as a linear regression equation of $Y = 30.6X - 0.0199$ with a correlation coefficient of 0.9350 (where Y represents the peak current and X represents the concentration of TET1). The low limit of detection was calculated to be 0.00098 μg μL^{-1}. The corresponding calibration curve is drawn in Fig. 4B. Stability of the biosensor was tested too. Three modified electrodes were stored at 4 °C for 10 days and the percentage decrease in current was smaller than 6.22% (0.0126 μg μL^{-1} TET1), indicating good stability of the biosensor. The obtained DPV curves were shown in Fig. 5. The constructed biosensor was also compared with other methods in terms of linear range and detection limit for TET1 detection (Table 1). The results show that the performance is satisfactory because of wide linear range and low detection limit. Moreover, all three TET proteins (TET1, TET2, TET3) belong to a family of dioxygenase enzymes. They share a common core catalytic domain at their C termini and identical catalytic activity to successively oxidize the methyl group of 5 mC, yielding three distinct forms of oxidized methylcytosines [26,27]. So, this method could be also used to evaluation the activity of TET2 and TET3.

Finally, this method was used to research the effects of NOG and taxol on the activity of TET1. The results are listed in Fig. 6. Fig. 6A shows that the peak current of MB decreases with increase of NOG concentration, and but remains within a limited range when taxol concentration is increased (Fig. 6B). TET1 is an α-KG and Fe^{2+}-dependent dioxygenase. NOG is an α-ketoglutarate analogue and inhibits α-KG-dependent hydroxylases [6]. The data indicated that this electrochemical biosensor could be used for TET1 inhibitor screening.

4. Conclusions

In conclusion, a label-free and cost-effective electrochemical method was developed for sensitive determination of TET1. High sensitivity was achieved by employing restriction endonuclease and AuNPs. This method shows a good linear range from 0.0042 μg μL^{-1} to 0.0210 μg μL^{-1} with a correlation coefficient of 0.9350 and a low limit of detection 0.00098 μg μL^{-1}. NOG and taxol were finally selected to demonstrate inhibitor screening capability of this method. Since TET1 protein mediates DNA demethylation and DNA methylation plays important roles in many biological processes, this method could be used for TET1 activity evaluation and inhibitor screening in field of biomedicine and clinical diagnosis. At the same time, it is an attractive candidate for construction platform for monitoring DNA methylation and demethylation.

CRediT authorship contribution statement

Zhenya Yu: Investigation, Methodology, Data curation. **Xue Chen:** Investigation, Methodology, Data curation. **Ying Cheng:** Investigation, Writing - original draft, Data curation. **Hongmei Yang:** Investigation, Data curation. **Fang Wang:** Supervision, Funding acquisition, Project administration, Conceptualization, Writing - review & editing. **Zilin Chen:** Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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