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Investigation of MTH1 activity *via* mismatch-based DNA chain elongation

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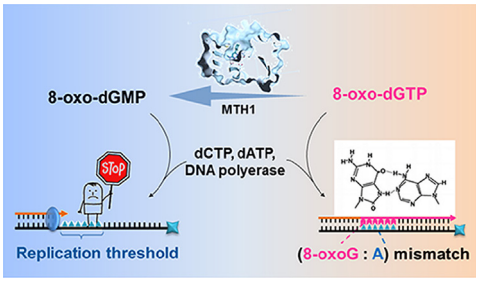
HIGHLIGHTS

- Mismatch-based DNA chain elongation strategy (MB-DCE) is firstly proposed.
- MutT Homolog 1 (MTH1) is critical important for biomedical cancer research.
- An electrochemical method is developed for MTH1 activity based on MB-DCE.
- This method can reveal MTH1 activity in normal and breast cancer cell lines.

GRAPHICAL ABSTRACT



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ABSTRACT

Accumulation and misincorporation of oxidative damaged 8-oxo-7,8-dihydroguanine triphosphates (8-oxo-dGTP) in genomic DNA may cause serious cellular function disorders. MutT Homolog 1 (MTH1), a protein enzyme that can help to prevent 8-oxo-dGTP misincorporation, plays critical roles in oxidative stress neutralization, oncogene-associated tumor malignancy, and anticancer therapies. So, in this work, a simple and function-oriented method is developed for the assay of MTH1 activity. Specifically, a mismatch-based ("8-oxoG: A" mismatch) DNA chain elongation strategy (MB-DCE) is firstly proposed to reveal the misincorporation efficiency of 8-oxo-dGTP. Then, further coupled with the inherent activity of MTH1 to prevent 8-oxo-dGTP misincorporation, a relationship can be established to reveal the activity of MTH1 through MB-DCE. As the method is designed directly towards the cellular function of MTH1, activity of MTH1 in different breast cancer cell lines has been detected, implying the potential application of this assay method for biomedical research and clinical diagnose in the future.

1. Introduction

Dysfunctional redox regulation in cell can produce reactive oxygen species (ROS), which could damage genomic DNA and free deoxynucleoside triphosphates (dNTPs) [1,2]. Among the damaged dNTPs, 8-oxo-7,8-dihydroguanine triphosphates (8-oxo-dGTP) is the most abundant due to the low oxidative potential of guanine [3,4]. 8-oxo-dGTP could be incorporated into DNA by mispairing with adenine (A), forming the "8-oxoG: A" mismatched base pair. This would induce the guanine (G) to thymine (T) transversion mutation, causing cell function disorder and cell death [2,5]. Besides, such mutation often occurs in the coding sequence of

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oncogenes and tumor suppressor genes, revealing the carcinogenic ability of 8-oxoGTP in tumorigenesis [6–9]. So, accumulation of 8-oxo-dGTP in cellular pool may elevate potential risk of cell damage and cancer development [10,11].

MutT Homolog 1 (MTH1) is an 8-oxo-dGTPase that can convert 8-oxo-dGTP to 8-oxo-dGMP. This means MTH1 can reduce the oxidative damages caused by 8-oxo-dGTP misincorporation [12]. Recently, MTH1 has been further validated as a novel and critical component in oncogene-associated tumor malignancy [6] and cancer phenotypic lethal, which renders it as a potential target for anticancer treatment [13–15]. So, the investigation of MTH1 activity is not only essential for evaluating the susceptibility of cancer, but also important for predicting tumor malignancy and evaluating curative effect in clinical practice [13]. Nevertheless, although the current techniques for MTH1 analysis include quantitative reverse transcription-polymerase chain reaction (RT-PCR) at the gene transcription level, western blotting (WB) and enzyme-linked immune-sorbent assay (ELISA) at the protein expressing level [13,14], these methods are used to detect the relative abundance of mRNA or protein. Obviously, the assay of MTH1 activity is more important since it closely relates to its cellular function to prevent 8-oxo-dGTP misincorporation. So far, to our knowledge, there is still no method to investigate MTH1 activity through 8-oxo-dGTP misincorporation. Liquid chromatography–mass spectrometry (LC-MS) may be a potential useful technique, but it cannot be used to determine the 8-oxo-dGTP misincorporation owing to the generation of oxidized bases during MS detection [16,17]. Besides, LC-MS is limited by time-consuming procedures with high cost. So, simple, cost effective and function-oriented method for the assay of MTH1 activity is highly desirable. In this work, we have proposed the mismatch-based ("8-oxoG: A" mismatch) DNA chain elongation strategy (MB-DCE) for the first time. Then, coupled with the inherent activity of MTH1, the MB-DCE can be well developed into a simple and effective electrochemical method to investigate MTH1 activity (Scheme 1).

2. Materials and methods

2.1. Materials and reagents

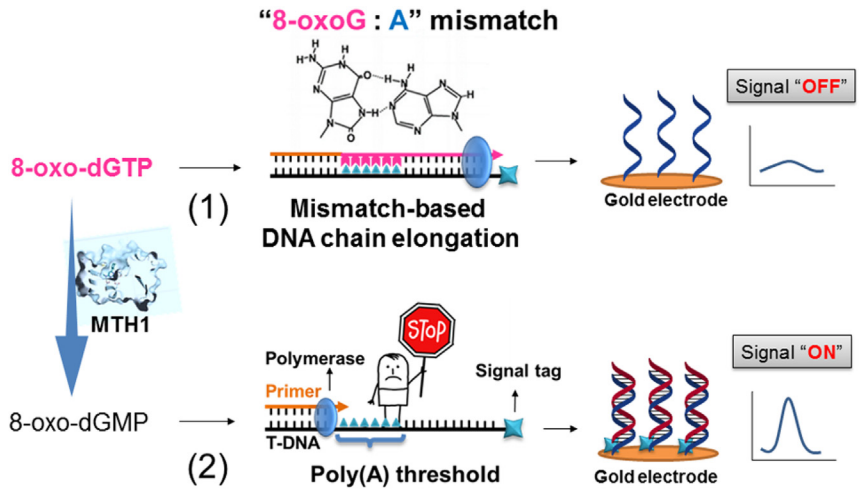
Bst 2.0 DNA polymerase (supplied with 10 × reaction buffer) and human 8-oxoguanine DNA glycosylase 1 (hOGG1) were purchased from New England Biolabs Ltd. 8-oxo-2'-deoxyguanosine-5'-triphosphate (8-oxo-dGTP) was purchased from Bioscience GmbH (Jena, Germany). The MTH1 inhibitor, TH588, was purchased from Selleck Chemicals. dATP (100 mM), dCTP (100 mM), 6-mercapto-1-hexanol (MCH), tris(2-carboxyethyl)phosphine (TCEP), and ethylene diamine tetraacetic acid (EDTA) were from Sigma–Aldrich. The fetal calf serum (FCS) was obtained from Biological Industries (Beth Haemek, Israel). Protein extraction kit (P0027) and BCA protein assay kit (P0010S) were purchased from Beyotime Biotech. Co. Ltd. (Shanghai, China). Other regents were all of analytical grade and were used without further purification. DNA oligonucleotides were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). Oligonucleotides used in the experiment were listed in supporting information.

Scheme 2. Schematic illustration to show the oligonucleotide sequences immobilized on the gold electrode surface.

Buffer solutions used in the experiments were as follows. Stock solution of DNA oligonucleotides was 10 mM Tris–HCl, 1 mM EDTA, and 10 mM TCEP, pH 8.0. DNA immobilization buffer was 10 mM Tris–HCl, 1 mM TCEP and 1 M NaCl, pH 7.4. DNA hybridization buffer was 10 mM phosphate buffer containing 0.25 M NaCl, pH 7.4. MTH1 reaction buffer was 20 mM Tris–HCl, 4 mM $MgCl_2$, 40 mM NaCl, 0.5% tween 20 and 8 mM DTT, pH 7.4. All solutions were prepared with deionized water, which was purified to a resistance of 18.2 MΩ with a Milli-Q purification system (Bedford, MA).

2.2. Preparation of C-DNA modified gold electrode

DNA modified electrode was prepared with the previously method with slightly modification [18]. Briefly, the gold electrode (3 mm in diameter) was first cleaned with piranha solution (98% H_2SO_4: 30% H_2O_2 = 3: 1, v/v) for 10 min. Then, it was polished to a mirror sheen with alumina powder. After successive sonication in ethanol and deionized water for 5 min, the electrode was immersed in 50% nitric acid for 30 min. After which, it was electrochemically



Scheme 1. Schematic illustration to show principle of the mismatch-based DNA chain elongation (MB-DCE) strategy for the design of an electrochemical assay method to detect MTH1 activity.

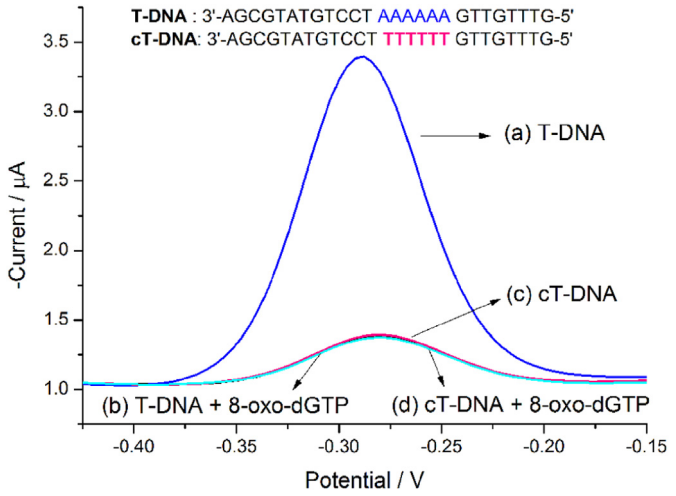


Fig. 1. Square wave voltammograms of C-DNA modified gold electrodes after incubation with different reaction solutions in the MB-DCE strategy. Curve (a) and (c) are the cases separately by using T-DNA and cT-DNA as the template in the absence of 8-oxo-dGTP. Curve (b) and (d) are the cases separately by using (b) T-DNA and (d) cT-DNA as the template in the presence of 20 μM 8-oxo-dGTP.

cleaned with 0.5 M H_2SO_4 followed by drying with high purity nitrogen. The above cleaned gold electrode surface was immediately used for C-DNA immobilization by incubating the electrode with 0.5 μM C-DNA at 25 °C for 12 h. The C-DNA modified electrode was further dipped in 1 mM MCH for 30 min to passivate the surface and obtain a well-aligned C-DNA monolayer. Finally, the electrode was rinsed with sufficient deionized water before the following experiments.

2.3. Cell culture and MTH1 protein extraction

Cells were cultivated in DMEM medium supplemented with 10% FBS and 10 U ml^{-1} penicillin/streptomycin at 37 °C with 5% CO_2. All cells were cultivated and collected to an amount of more than 2×10^7 cells for protein extraction. Cytosol that contain MTH1 was extracted by protein extraction kit. To inhibit protease activity during these procedures, 1 mM PMSF, 10 μg mL^{-1} Aprotinin and 10 μg mL^{-1} Leupeptinto was added. 200 μl of the extract was filtered through a centrifugal filter (Amicon Ultra-0.5, 30000 NMWL, Millipore) at 14000 g until complete passage of the sample to remove large molecular weight proteins. Protein concentration of the resulting through-fraction was determined by the protein assay kit with the BCA method [19]. Obtained extract that contained MTH1 was diluted to 4.0 mg mL^{-1} in a buffer solution containing 10% glycerol, 20 mM Tris–HCl, 2 mM DTT, 100 mM NaCl, pH 8.0. The protein were frozen at −80 °C for later usage.

2.4. MTH1 assay

10 μL extracted MTH1 sample (1.0 mg mL^{-1}) was incubated with 10 μL 8-oxo-dGTP (40 μM) in the MTH1 reaction buffer at 37 °C for 30 min. After thermal deactivation at 65 °C for 10 min and cooled to room temperature, the mixture was filtered through a centrifugal filter (Amicon Ultra-0.5, 3000 NMWL, Millipore) by centrifugation at 14000 g until complete passage of the sample. 4 μL of the flow through that containing 8-oxo-dGTP was then incubated with reaction solution containing primer/template hybrids (0.5 μM), dATP (50 μM), dCTP (50 μM), *Bst* 2.0 DNA polymerase (0.1 U μL^{-1}) for 45 min at 37 °C in the polymerase reaction buffer. The total reaction volume was 20 μL. Afterwards, 5 μL of the above reaction solution was incubated with the C-DNA modified gold electrode for 1 h at 35 °C. The electrode was rinsed with sufficient buffer before the following electrochemical measurements.

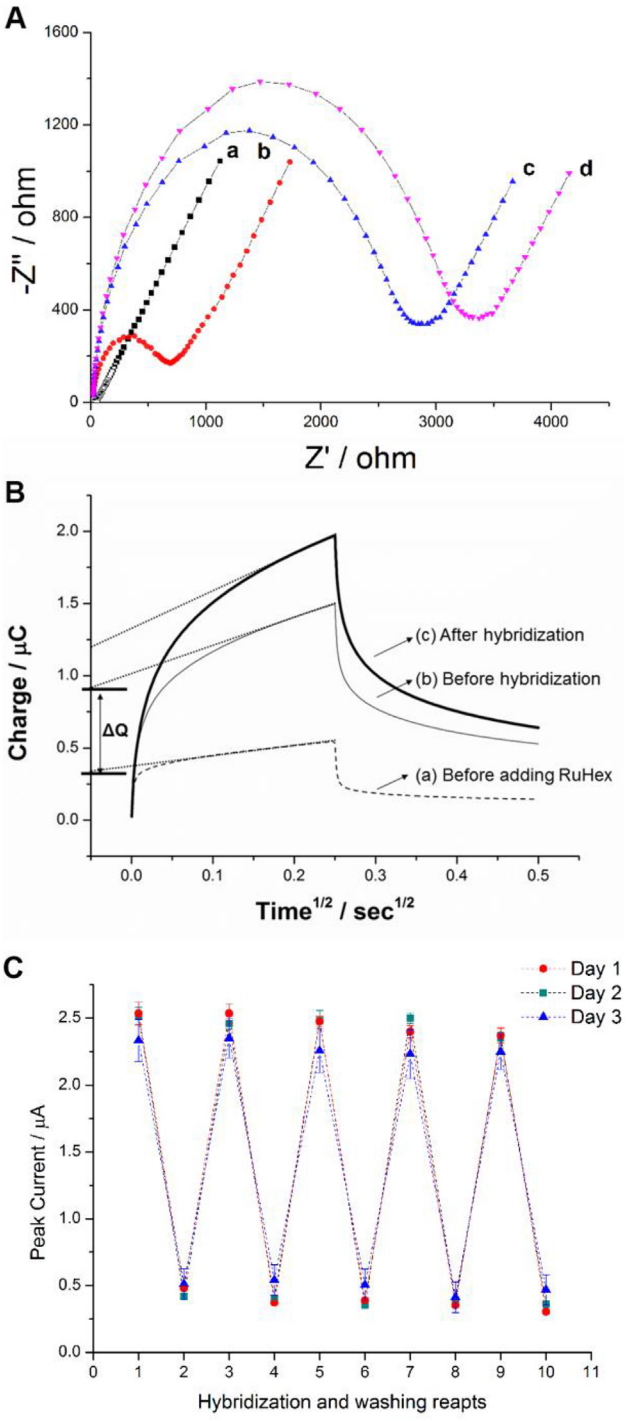


Fig. 2. (A) Nyquist plots corresponding to (a) bare gold electrode, (b) C-DNA modified electrode, (c) MCH modified electrode, and (d) the electrode after incubation with T-DNA. (B) Chronocoulometric response curves for C-DNA/MCH monolayer (a, b) and T-DNA/C-DNA/MCH (c) modified electrodes before and after the addition of 50 μM RuHex. The lines represent the fit to the data used to determine the intercept at $t = 0$. (C) Peak current values of the C-DNA modified electrode by repeated hybridization and release of T-DNA. The experiments were conducted five times a day for three independent days at the same C-DNA modified electrode.

2.5. Electrochemical measurements

All electrochemical measurements were carried out on a CHI660D Potentiostat (CH Instruments) working station with a three-electrode system, which included the above C-DNA modified gold electrode as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum electrode as the counter electrode. 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 1 M KCl was used as the electrolyte solution for electrochemical impedance spectroscopy (EIS) tests. EIS was measured with an amplitude of 5 mV, over the frequency ranging from 0.01 Hz to 10 kHz. Cyclic voltammetry (CV) was measured at a scan rate of 100 mV s^{-1} over the potential range from −0.24 V to −0.60 V. 10 mM Tris–HCl buffer (pH 7.4) containing 200 mM NaCl was used as the electrolyte solution for CV. Square wave voltammetry (SWV) experimental parameters were 20 mV amplitude signal at a frequency of 100 Hz, over the potential range from −0.5 V to −0.1 V. Experiment parameters for chronocoulometry (CC) were 250 ms pulse period and 0.25 ms sample interval. The electrolyte solution was 10 mM Tris–HCl containing 50 μM RuHex, pH 7.4.

3. Results and discussion

Scheme 1 shows the principle of MB-DCE. In the system, the DNA replication template (T-DNA) contains a poly(A) region in the middle and a signal molecule (methylene blue) at 5′ end, which respectively act as the threshold of DNA chain elongation (DCE) and electrochemical signal tag. By further introducing primer DNA and DNA polymerase, with 8-oxo-dGTP, dCTP and dATP as the DCE substrates, 8-oxo-dGTP becomes the key factor to extend through the poly(A) region by "8-oxoG: A" mismatch, thus the MB-DCE strategy is developed. Furthermore, MTH1 activity can be determined based on this strategy. Specifically, as shown in Scheme 1B, in the absence of MTH1, DNA polymerase can extend through the poly(A) region smoothly by "8-oxoG: A" mismatch, thus T-DNA is locked in the double-stranded DNA. Consequently, the locked T-DNA cannot hybridize with the single-stranded capture DNA (C-DNA) that is immobilized on the electrode surface. Therefore, signal production is inhibited (the "signal off" state). However, in the presence of MTH1, 8-oxo-dGTP is converted to 8-oxo-dGMP, which cannot be incorporated into DNA during DCE. Elongation of the DNA chain is prohibited. Consequently, the single-stranded T-DNA can hybridize with C-DNA on the electrode surface. Electrochemical signals can be produced (the "signal on" state). Therefore, a positive relationship can be established between MTH1 activity and electrochemical signals. The oligonucleotide sequences used in this work have been shown in Scheme 2.

To demonstrate the feasibility of MB-DCE strategy, a control DNA replication template (cT-DNA) has been introduced, in which the poly(A) region is replaced by poly(T). Curve a in Fig. 1 shows the experimental result by using T-DNA as the template, which gives a significant electrochemical signal (curve a). So, DNA chain elongation is prohibited in the absence of 8-oxo-dGTP. However, the control group by using cT-DNA as the template produces a small signal (curve c), indicating that DNA chain elongation can proceed through poly(T) region, even if 8-oxo-dGTP is absent in the system. We have further conducted experiments by using both poly(A) and poly(T) in the presence of 8-oxo-dGTP. As is shown by curve b and d, both cases result in small signals. So, the above results indicate that the poly(A) region of T-DNA is the key point for the achievement of MB-DCE, and the MB-DCE strategy is based on "8-oxoG: A" mismatch.

The stepwise modification of the electrode surface has been investigated with electrochemical impedance spectroscopy (Fig. 2A). Before modification, the Nyquist plot of the bare electrode shows nearly a straight line, indicating the resistance to interface electron transfer is small. The sequential modification of C-DNA and MCH induces a semicircle and subsequent semicircle enlargement (curve b, c), which is due to the augmented resistance to interface electron transfer, revealing the formation of C-DNA/MCH monolayer on the electrode surface. The DNA coverage density is calculated to be 3.96×10^{12} molecules cm^{-2} by chronocoulometry technique and the Cottrell equation (see details in supporting information) [20,21]. The semicircle diameter further increases after the capture of T-DNA, which has also been confirmed by chronocoulometry (Fig. 2B). The hybridization efficiency between T-DNA and C-DNA has been investigated by a day-to-day hybridization repeatability assay. The experiments are conducted five times a day for three independent days at the same C-DNA modified electrode. The low coefficient of variability (CV) (the "signal on" state: CV_{on} = 4.5%; the "signal off" state: CV_{off} = 16.6%) and high signal to noise ratio have revealed good hybridization efficiency between T-DNA and C-DNA. Further experimental results indicate that high stability and reusable ability of the fabricated

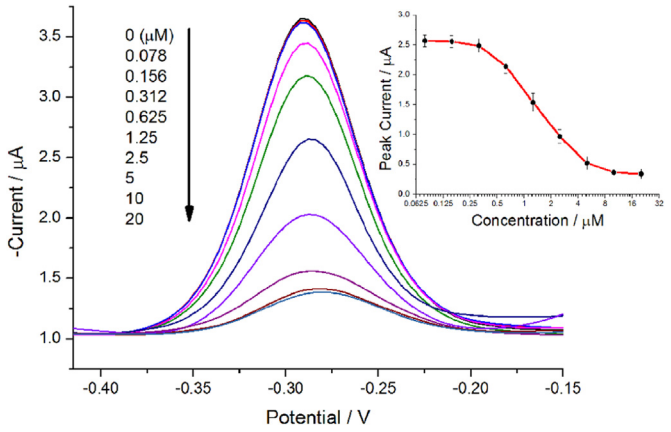


Fig. 3. Square wave voltammograms of the C-DNA modified gold electrode. The electrodes were incubated with various reaction solution that contains different concentration of 8-oxo-dGTP: 0, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10 and 20 μM. The inset shows the calibration curve corresponding to the peak value for various concentration of 8-oxo-dGTP.

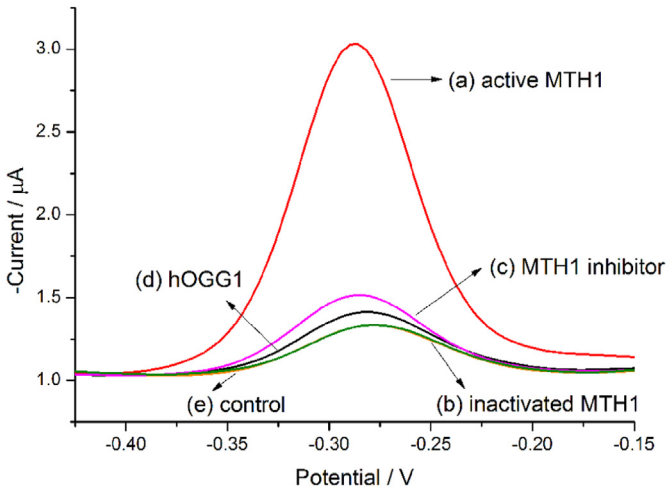


Fig. 4. Square wave voltammograms of C-DNA modified gold electrodes after incubation with different reaction solution that contains (a) active MTH1 (1.0 mg mL^{-1}), (b) inactivated MTH1 (1.0 mg mL^{-1}), (c) active MTH1 (1.0 mg mL^{-1}) with 10 μM TH588 inhibitor, and (d) hOGG1 (5.0 units).

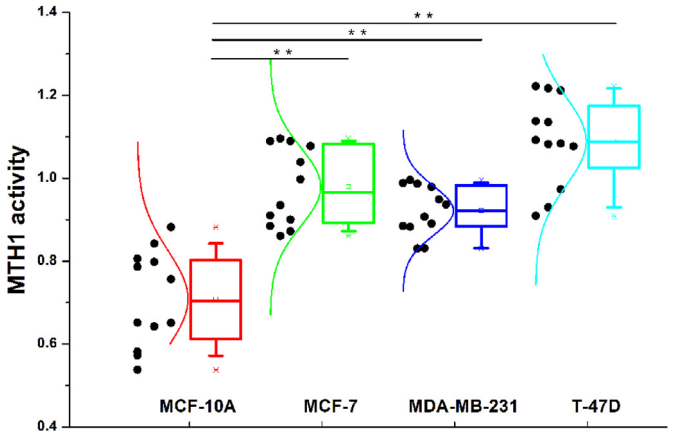


Fig. 5. Box plots shows MTH1 activity (nmol min^{-1} mg^{-1}) in different breast cell lines. From left to right: MCF-10A, MCF-7, MDA-MB-231 and T-47D. Black dots represent independent activity tests for MTH1 (n = 12). $p < 0.01$.

method are shown in electronic supplementary material. Activity changes of MTH1 in cancerous cell lines are statistically significant compared to normal breast cell line (MCF-10A). These malignancy cell lines show an increase (ranging from 30% to 54%) in enzyme activity (Table 1). Therefore, this method may be useful to determine some minor changes of MTH1 activity in different cell samples, which may help to reveal the role of MTH1 in cancer cells and cancer malignancy in the field of cancer research and clinical diagnose [6,23,24].

4. Conclusions

In conclusion, the MB-DCE strategy has been proposed in this work for the first time, which has been developed as a method to reveal 8-oxo-dGTP incorporation efficiency. Based on this strategy, a simple and effective method has been further established for the assay of MTH1 activity. As the design is focused on the catalytic activity of MTH1 to prevent 8-oxo-dGTP misincorporation, its cellular function can be revealed directly. The MB-DCE may be used for the analysis of 8-oxo-dGTP incorporation efficiency to reveal cellular oxidative stress. So, this study may not only have great potential to help evaluate the intracellular oxidative stress in biomedical research, but also be important to predict the curative effect of MTH1-targeted drugs in clinical practice in the future.

Table 1
MTH1 Activity in different breast cell lines.

Cell line	Type of cancer	Original tissue	ER/PgR[a] (+/−)	MTH1 activity (nmol min^{-1} mg^{-1})
MCF-10A	Normal	Epithelium	−/−	0.71 ± 0.12
MCF-7	Invasive ductal carcinoma	Pleural effusion	+/+	0.98 ± 0.09
MBA-MB-231	Invasive ductal carcinoma	Pleural effusion	−/−	0.92 ± 0.06
T-47D	Invasive ductal carcinoma	Pleural effusion	+/+	1.09 ± 0.11

[a] Estrogen receptor-negative (ER); Progesterone receptor-positive (PgR) [25].

electrochemical sensing surface can be obtained (Fig. 2C).

Experimental conditions that may influence the MB-DCE has been optimized (Figure S1 and S2). Based on the influence of primer concentration on SWV signals, 2.5 μM has been chosen in the following experiments to ensure the hybridization efficiency between T-DNA and primer DNA. And, 45 min is the optimal reaction time to complete DNA chain elongation. To reveal 8-oxo-dGTP incorporation efficiency during MB-DCE, different concentrations of 8-oxo-dGTP are added. Fig. 3 shows that the peak currents of the square wave voltammogram (SWV) decrease with the increased concentrations of 8-oxo-dGTP, indicating that higher concentration of 8-oxo-dGTP would facilitate MB-DCE, but signals tend to level off when the concentration is above 10 μM. The inset shows the calibration curve corresponding to the obtained peak value for various concentration of 8-oxo-dGTP. A detection range from 0.078 to 10 μM can be obtained.

Under the optimized conditions, feasibility of the MB-DCE strategy for the development of a method to assay MTH1 activity has been investigated. Control experiments are conducted by introducing thermal deactivated MTH1, MTH1 inhibitor (TH588) [13] and 8-oxoguanine DNA glycosylase 1 (hOGG1) [22]. SWV curves in Fig. 4 show that active MTH1 can produce obvious electrochemical signal (curve a), while the inactivated MTH1 (curve b) and inhibited MTH1 (curve c) produce small signals as that of background (curve e). Curve d shows that the influence of hOGG1 on this strategy is also limited. So, signal production arises from MTH1 activity, and this method can be used for MTH1 assay based on the MB-DCE strategy.

With the established method, MTH1 activity in normal and breast cancer cell lines are tested. The normal cell line is MCF-10A, and the cancer cell lines are MCF-7, MDA-MB-231 and T-47D, which have representative characteristics in breast cancer. As indicated by the plots in Fig. 5, MTH1 activity in cancerous cell lines are much higher than that in normal breast cell line. Details of the calculation

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2015.12.011.

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