Sensors and Actuators B 216 (2015) 343–348

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

An electrochemical method to assay the activity of NAD(P)H: Quinone oxidoreductase 1

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ARTICLE INFO

Article history:
Received 15 December 2014
Received in revised form 8 April 2015
Accepted 19 April 2015
Available online 24 April 2015

Keywords:
Electrochemical method
NQO1
Inhibitor screening

ABSTRACT

A simple and rapid electrochemical method is reported in this paper to assay the activity of NAD(P)H: quinone oxidoreductase 1 (NQO1), a potential target for anticancer therapy. Firstly, a quinone derivative, the substrate of NQO1, is designed to be covalently immobilized on the surface of gold electrode. In the presence of the enzyme, the quinone derivative transforms to the corresponding hydroquinone derivative, followed by the expected cyclizative cleavage reaction via the *gem*-dialkyl effect. So, the hydroquinone derivative species are released from the electrode surface into the bulk solution. As the electrochemical probe, quinone derivative can generate a pair of redox waves in aqueous buffer and its peak current is correlated with the activity of NQO1, so an electrochemical method to determine the enzyme activity can be proposed. Under the optimized conditions, NQO1 activity can be assayed in the range from 0 to 6 U/mL with a detection limit of 0.22 U/mL. Moreover, resveratrol, an antitumor compound, is evaluated with the maximum inhibition rate of 95% and IC_{50} value of 23.70 μM. Therefore, with wide detection range, high sensitivity, acceptable reliability and rapid response, the established method can be used for not only the investigation of NQO1 activity but also the screening of the enzyme inhibitor.

1. Introduction

NAD(P)H: quinone oxidoreductase 1 (NQO1, EC 1.6.99.2) is a cytosolic homodimericflavoprotein which contains one flavin-adenine dinucleotide prosthetic group per monomer. NQO1 is an obligatory two electron reductase enzyme. It catalyzes the direct two-electron reduction of a broad range of quinone substrates to the corresponding hydroquinone utilizing the co-factors NADH and NADPH equally well as the electron donor [1,2]. Moreover, NQO1 diverts quinone eletrophiles from participating in reactions, which can lead to one-electron reductions and the generation of highly reactive semiquinone radical intermediate and various reactive oxygen species [3].

Elevated levels of NQO1 expression have been documented in a variety of human tumors, including pancreas, colon, breast, lung, liver, stomach, kidney, thyroid and ovary, with levels 2- to 50-fold greater when compared with normal tissues of the same origin [4–6]. The differentiated expression of NQO1 between tumors and normal tissues suggests that NQO1 targeted drugs would be highly selective in killing tumor cells while saving normal tissues. Furthermore, the inhibition of NQO1 has been proposed to result in cell death through the accumulation of increased intracellular superoxide [7,8]. Thus, NQO1 has been considered as an ideal target for anticancer therapies.

Up to now, spectroscopic methods have been proposed to detect NQO1 activity and screen its inhibitor. Buffinton et al. have evaluated the NQO1 activity by measuring the NADPH oxidation at 340 nm, because NQO1 catalyzes its substrate at the expense of reducing equivalents from NADPH [9]. Moreover, NQO1 activity can be measured by the reduction of the enzyme substrate 2,6-dichlorophenol-indophenol at 600 nm [10–14]. Recently, Silvers et al. designed a fluorescent reporter for the detection of cancer cells based on NQO1 activity [15]. The disadvantages of these methods are the requirement of the coloration of the test solution and fluorescent labels.

As a simple, rapid, and convenient technique, especially for the qualitative and quantitative studies of redox species, electrochemical method has not been reported for the assay of NQO1 activity. On the other hand, as one of important examples of organic redox couples, quinone-hydroquinone has been well studied, and they have played important role in the development of current

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http://dx.doi.org/10.1016/j.snb.2015.04.059
0925-4005/

understanding of organic redox chemistry. The electrochemical behavior of quinones both in aqueous and aprotic solvents has been extensively studied [16–19]. Meanwhile, quinone-based polymers have been applied for the detection of proteins, antibodies, and pesticides, in view of the very stable electroactivity of quinones in neutral aqueous medium [20]. So, in this paper, we have proposed an electrochemical method for the detection of NQO1 activity and the screening of its inhibitor. This method is based on the reduction and cyclizative cleavage of one quinone derivative, the enzyme substrate, while the quinone derivative can be successfully designed to be covalently immobilized on the surface of gold electrode. So, in the presence of NQO1, the quinone substrate converts to its corresponding hydroquinone derivatives [21,22]. Subsequently, the expected cyclizative cleavage reaction of the hydroquinone derivatives via the *gem*-dialkyl effect occurs [23,24]. Consequently, the obtained peak current at the modified electrode will be changed as a result of the departure of quinone derivatives from the electrode surface to the bulk solution, thus a simple, rapid, direct and easily-operated method is developed in this work.

2. Experimental

2.1. Chemicals

NQO1 (human, 100 U/mg protein), cysteamine (CA, >98%), β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH, 98%), 2-mercaptoethanol (99%), 4-(dimethylamino) pyridine (DMAP) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodimide hydrochloride (EDC) were purchased from Sigma–Aldrich Co. (Shanghai, China). 3-Methyl-3-(2,4,5,-trimethyl-3,6-dioxo-cyclohexa-1,4-dienyl)-butyric acid (DCDBA) was synthesized by Hangzhou Yuhao chemical technology Co., Ltd. (Hangzhou, China). Resveratrol was obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). $NaClO_4$, $HClO_4$ and acetonitrile (97%) were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Other chemicals were all of analytical grade.

The buffer solutions used in this work are as follows: NQO1 buffer, 25 mM Tris–HCl (pH 7.4) containing 0.2 mM NADH; washing buffer, 0.1 M phosphate buffer saline (pH 7.4) containing 0.9% NaCl and 0.05% Tween 20; electrolyte solution for cyclic voltammetry and differential pulse voltammetry, 0.99 M $NaClO_4$solution containing 0.01 M $HClO_4$; electrolyte solution for electrochemical impedance spectroscopy, 5 mM $[Fe(CN)_6]^{3-/4-}$ with 0.1 M KCl. All buffers were prepared with double-distilled water, which was purified with a Milli-Q purification system (Branstead, USA) to a specific resistance of 18 MΩ cm.

2.2. Modification of the gold electrode

The gold electrode was firstly cleaned as described in detail in Ref. [25]. Then the pretreated electrode was immersed into 2 mM CA solution for 12 h at room temperature, so that CA self-assembly monolayer could be formed on the electrode surface. Afterwards, the electrode was washed thoroughly to remove any unbound CA. Subsequently, the enzyme substrate was covalently attached to the electrode by incubating CA-modified gold electrode with 50 mM DCDBA acetonitrile solution containing 2 mM DMAP and 35 mM EDC for 12 h at room temperature. After that, the modified electrode was dipped in 1 mM 2-mercaptoethanol aqueous solution for 10 min, followed by rinsing with washing buffer to remove any remaining substances. Finally, the electrode was immersed in 1% Tween 20 for 1 h. After being rinsed with water, it could be used for the following experiments.

2.3. Reduction and cyclizative cleavage of quinone derivative

The quinone derivative modified electrode was incubated with 500 μL NQO1 solution with different concentrations at 32 °C for 0.5 h. Afterward, the electrode was rinsed thoroughly with double-distilled water to remove non-specific enzymes absorbed on the electrode surface. Subsequently, the electrode was used for the following electrochemical measurement.

2.4. Inhibition effect

500 μL 8 U/mL NQO1 solution was firstly mixed with 50 μL resveratrol aqueous solution with different concentrations. Then the mixed solution was pre-incubated at 32 °C for 0.5 h. Afterward, the quinone derivative modified electrode was immersed into the mixed solution for 0.5 h. Finally, the electrode was rinsed thoroughly and used for electrochemical measurement.

The inhibition efficiency of resveratrol can be calculated as follows:

$$\text{Inhibition ratio}\,(\%) = \frac{(I_0 - I_2)}{(I_1 - I_2)} \times 100$$

where I_0 was the peak current obtained in the absence of NQO1 and resveratrol, I_1 was the peak current with both NQO1and resveratrol, and I_2 was the peak current obtained in the presence of NQO1.

2.5. Electrochemical measurements

Electrochemical measurements were performed on a model 660c Electrochemical Analyzer (CH Instruments) with a conventional three-electrode system, in which the gold electrode, an Ag/AgCl electrode and a platinum electrode were adopted as the working, reference and counter electrodes, respectively. Cyclic voltammetry (CV) was performed over a range from 0.4 to −0.5 V with a scan rate of 100 mV/s. Differential pulse voltammetry (DPV) was performed in the potential range from 0.05 to 0.30 V at the same scan rate. The electrolyte solution was deoxygenated for 0.5 h by purified N_2 before the electrochemical detection every time and the system was covered with purified N_2 during the detection procedure. Electrochemical impedance spectroscopy (EIS) was carried out by applying a bias potential of 0.224 V vs. SCE and 10 mV amplitude in the frequency range from 1 Hz to 10 kHz.

3. Results and discussion

The principle of the electrochemical method for the detection of NQO1 activity is illustrated in Scheme 1. At the beginning, the gold electrode is covalently modified with CA, the smallest stable ω-functionalized alkanethiol containing two functional groups, —SH and —NH_2, which separately locate at each end of the two-carbon chain. Then, DCDBA, the substrate of the enzyme, is immobilized on the electrode surface by the covalent bonding between the primary amine group of CA and the carboxylic group of DCDBA. It has been reported that quinone derivatives can be used as electrochemical probe [26] and the electrochemical behavior can be investigated at the DCDBA modified electrode. In the presence of NQO1, DCDBA, the quinone derivative is firstly reduced and changed to be its corresponding hydroquinone derivative [21,22]. Subsequently, the hydroquinone derivative is further cyclized and released from the electrode surface to the electrolyte solution, resulting in the decrease of electrochemical signal [27–30]. So, the redox peak current can be correlated with the enzyme catalytic reaction and NQO1 activity can be assayed using the proposed electrochemical method.