Electrochimica Acta 139 (2014) 323–330

Contents lists available at ScienceDirect

Electrochimica Acta

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

Electrochemical-chemical-chemical redox cycling triggered by thiocholine and hydroquinone with ferrocenecarboxylic acid as the redox mediator

Lin Liu[a,b,*], Yanping Gao[a], Huiping Liu[a], Jimin Du[a], Ning Xia[a,*]

[a] College of Chemistry and Chemical Engineering, Anyang Normal University, Anyang, Henan 455000, People's Republic of China
[b] College of Chemistry and Chemical Engineering, Shangqiu Normal University, Shangqiu, Henan 476000, People's Republic of China

ARTICLE INFO

Article history:
Received 16 May 2014
Received in revised form 1 July 2014
Accepted 2 July 2014
Available online 18 July 2014

Keywords:
Electrochemical biosensors
redox cycling
thiocholine
hydroquinone
ferrocenecarboxylic acid

ABSTRACT

Enzyme amplification is most commonly used to develop electrochemical affinity biosensors. However, single amplification by enzyme labels is not sufficient for detecting an ultra-low analyte concentration. Moreover, some of enzymatic products, such as thiocholine and hydroquinone (HQ), show poor electrochemical signals and/or are unstable in air for long incubation periods. In this work, we reported two "outer-sphere to inner-sphere" electrochemical-chemical-chemical (ECC) redox cycling reactions triggered by thiocholine and HQ. By examining the effect of different redox mediators and reductants, we found that ferrocenecarboxylic acid (FcA) and tris(2-carboxyethyl)phosphine (TCEP) were the optimal redox mediator and reductant, respectively. In the redox cycling, FcA was regenerated by thiocholine or HQ after its electro-oxidization; thiocholine or HQ was also then regenerated by TCEP after its oxidation, thus enhancing the anodic current of FcA. To demonstrate the applications and analytical merits of the two redox cycling reactions in biosensing, thrombin was tested in a "sandwich" format with acetylcholinesterase (AChE) and alkaline phosphatase (ALP) as the enzyme labels. The detection limits were determined to be 5 ng L^{-1} and 0.5 ng L^{-1} with the thiocholine- and HQ-triggered redox cycling, respectively. The results will be valuable for developing enzyme-amplified electrochemical affinity biosensors.

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1. Introduction

In recent years, enzyme-amplified electrochemical biosensors including immunosensors, DNA sensors and aptasensors have attracted considerable attention and have emerged as viable alternatives to conventional spectrophotometric enzyme affinity assays [1–3]. The enzymatic reaction may follow two strategies: one in which the current is the electrocatalytic response of a redox couple serving as a substrate to a redox enzyme label and another in which an electrochemically active product of the enzyme label is detected [1]. Despite a number of advances in the design and application of enzyme-amplified electrochemical affinity biosensors, however, single amplification by enzyme labels is not sufficient for detecting an ultra-low analyte concentration with a sensitivity and multiplexation comparable to or better than those of optical biosensing methods. For example, simple sandwich immunoassays in which an enzyme-conjugated antibody is employed for the recognition of the target analyte and the generation of an electrochemical signal typically obtain detection limits at the picolmolar scale [4]. For this reason, a number of research groups have been exploring various methods to improve the sensitivity by the integrating nanomaterials or developing more advanced enzymatic approaches to further amplify the electrochemical response [5,6]. Among them, the signal amplification strategy using an enzymatic reaction plus a redox-cycling reaction is particularly popular in bioassays because it only requires the addition of more chemicals to the electrolyte solution and not a change in the detection procedure of conventional enzyme-based assays [7–9]. Numerous of electrochemical immunosensors and DNA sensors with enzymatic amplification plus electrochemical-chemical (EC) redox cycling have been reported [8,10,11]. In these systems, the enzymatic product is regenerated after its electrochemical-oxidization by a chemical reducing reagent, which results in amplification of the electrochemical signal. Very recently, Yang's group reported

* Corresponding author. Tel.: +86 732 330 0925.
E-mail addresses: liulin@aynu.edu.cn (L. Liu), xianing82414@csu.edu.cn (N. Xia).

http://dx.doi.org/10.1016/j.electacta.2014.07.043
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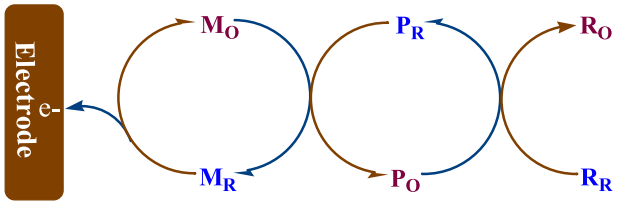

Fig. 1. Schematic of ECC redox cycling. M_R and M_O represent the reduced and oxidized forms of the redox mediator, respectively. P_R and P_O represent the reduced and oxidized forms of the enzymatic product, respectively. R_R and R_O represent the reduced and oxidized forms of the reductant, respectively.

the first ultrasensitive sensing method that employs "outer-sphere to inner-sphere" electrochemical-chemical-chemical (ECC) redox cycling on indium-tin oxide (ITO) electrodes [12,13]. In the work, $Ru(NH_3)_6^{3+}/Ru(NH_3)_6^{2+}$ was used as the redox mediator. $Ru(NH_3)_6^{2+}$ could be regenerated from $Ru(NH_3)_6^{3+}$ by the alkaline phosphatase (ALP)-enzymatic products, such as 4-aminophenol (AP) and hydroquinone (HQ). Subsequently, AP or HQ could be regenerated by tris(2-carboxyethyl)phosphine (TCEP) after its oxidation. The triple chemical amplification approach using the enzymatic reaction, CC redox cycling and ECC redox cycling permits low detection limits for troponin I (10 fg/mL) and E. coli O157:H7 (10^3 CFU/mL) [12,13]. In the "outer-sphere to inner-sphere" ECC redox cycling (Fig. 1), four decisive factors should be taken into account: (1) the redox mediator should be relatively stable in air and able to regenerate after its electrochemical-oxidation by the enzymatic product, (2) the reaction between the redox mediator and the enzymatic product and the reaction between the oxidized form of enzymatic product and the reductant should be fast, (3) the unwanted reaction between the redox mediator and the reductant should be very slow and (4) the reductant should be electrochemically inert in the electrochemical scanning window. In these terms, the ECC redox cycling with $Ru(NH_3)_6^{2+}$ as the redox mediator is reliable, but there still remains significant room to seek new redox mediators and develop novelty ECC redox cycling-based sensors because (1) $Ru(NH_3)_6^{2+}$ is less stable in air for long incubation times, (2) $Ru(NH_3)_6^{3+}$ is not completely reduced into $Ru(NH_3)_6^{2+}$ by HQ during the electrochemical scanning and (3) HQ could be oxidized during the electrochemical scanning, with its redox peak partly overlapping those of $Ru(NH_3)_6^{3+}/Ru(NH_3)_6^{2+}$.

Acetylcholinesterase (AChE) is a hydrolase that can hydrolyze acetylcholine (or acetylthiocholine) to choline (or thiocholine) with a high turnover frequency (each AChE molecule degrades ~25000 acetylcholine molecules per second) [14,15]. However, although thiocholine is a strong reducing reagent, its direct electrochemistry at electrodes often needs a large anodic potential to obtain an appreciable signal [16–18]. Thus, most of the reported AChE or AChE-based electrochemical techniques to date are based on the strong absorption of thiocholine to a soft metal through the formation of metal-thiol bonds [16,19–21]. The detection could then be achieved by monitoring the chemisorption/electrochemical desorption process of thiocholine. In view of the strong reduction ability of thiocholine and the large anodic potential for its oxidation, herein, we suggested that it is possible to develop thiocholine-triggered "outer-sphere to inner-sphere" ECC redox cycling. By examining the effect of different redox mediators and reductants on the redox cycling, we found that ferrocenecarboxylic acid (FcA) and tris(2-carboxyethyl)phosphine (TCEP) are the optimal redox mediator and reductant, respectively. Furthermore, we found that the FcA mediator was also suitable for HQ-triggered ECC redox cycling. Lastly, two electrochemical sensors based on thiocholine- and HQ-triggered redox cycling were developed, and their analytical merits were evaluated in assays of thrombin.

2. Experimental

2.1. Chemicals and reagents

AChE, TCEP, acetylthiocholine chloride, 6-mercapto-1-hexanol (MCH), streptavidin (SA), streptavidin-conjugated alkaline phosphatase (SA–ALP), sulfosuccinimidyl 4-(*N*-maleimidiomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), s-acetylmercaptosuccinic anhydride, bovine serum albumin (BSA) and tris-(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) were purchased from Sigma-Aldrich. The thiolated and biotinylated nucleic acid aptamers with the sequences 5'-HS-$(CH_2)_6$-TTT TTT TTT TGG TTG GTG TGG TTG G-3' (aptamer-1) and 5'-biotin-TTT TTT AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3' (aptamer-2) were obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). All other reagents were purchased from Beijing Chemicals, Ltd. (Beijing, China). The preparation of hydroquinone diphosphate (HQDP) followed the reported procedure [13]. The aptamer solutions were prepared using 10 mmol L^{-1} phosphate-buffered saline (PBS buffer, pH 7.4) and stored at 18 °C.

Streptavidin-conjugated AChE (SA-AChE) was synthesized by the thiol–maleimide coupling reaction, as in previous reports [20,22]. Briefly, maleimide-labeled SA was prepared by adding SA (0.1 g L^{-1}) and sulfo-SMCC (0.1 mg mL^{-1}) successively into 0.1 mol L^{-1} PBS (pH 8.0) at room temperature. After 1 h, the mixed solution was ultrafiltered to remove the excess sulfo-SMCC. The sulfhydrylation of AChE was conducted by incubating AChE (1 g L^{-1}) with *s*-acetylmercaptosuccinic anhydride (0.3 g L^{-1}) in the PBS for 10 min, followed by filtering the solution to remove the unreacted *s*-acetylmercaptosuccinic anhydride. To ensure that all of the SA molecules are labeled by AChE, the maleimide-labeled SA and thiolated AChE were mixed at a molar ratio of 1:4, and the mixture was incubated for 3 h to synthesize SA-AChE.

2.2. Optimization of redox mediator and reductant

The gold disk electrodes were polished sequentially with 0.3-μm and 0.05-μm alumina, followed by ultrasonic cleaning in ethanol and water. The electrode was then treated electrochemically by cycling the potential in the range of–0.3 to +1.5 V in 0.5 M H_2SO_4 solution. The cleaned gold electrode was immersed in a solution containing 5 μmol L^{-1} aptamer-1 and 50 μM TCEP and stored overnight at 4 °C. This step was followed by washing the electrode thoroughly with water and soaking it in a 0.1 mM MCH solution for 15 min. The electrode was further soaked in PBS (10 mmol L^{-1}, pH 7.4) containing 1% BSA and 0.1 mmol L^{-1} cysteamine for 10 min to minimize nonspecific adsorptions. Cyclic voltammograms (CVs) of the aptamer-1-modified electrodes were collected in the PBS solutions containing different types of reductants, redox mediators and/or enzymatic products (thiocholine or HQ) on a DY2013 electrochemical workstation (Digi-Ivy,Inc., Austin, TX). A platinum wire and a Ag/AgCl electrode were used as the auxiliary and reference electrodes, respectively.

2.3. Procedure for thrombin detection

For the capture of thrombin, 10 μL of the thrombin solution was cast onto the aptamer- 1-covered electrode surface for 30 min. The electrodes were then rinsed with water to remove any non-specifically adsorbed substance. To attach SA-AChE or SA-ALP, the electrodes were first allowed to react with 10 μL of aptamer-2 solution for 30 min and then rinsed and exposed to 10 μL of PBS containing 1 μg mL^{-1} SA-AChE or 2.33 U mL^{-1} SA-ALP for 10 min. After washing with water again, the electrodes were immersed in PBS buffer (0.1 mol L^{-1}, pH 8.0) containing 0.5 mmol L^{-1} acetylthiocholine, 0.5 mmol L^{-1} TCEP and 0.2 mmol L^{-1} FcA or in Tris buffer

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