Journal of Electroanalytical Chemistry 731 (2014) 78–83

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

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

Ascorbic acid-triggered electrochemical–chemical–chemical redox cycling for design of enzyme-amplified electrochemical biosensors on self-assembled monolayer-covered gold electrodes

Ning Xia [a], Lin Liu [a,b,*], Ruijuan Wu [a], Huiping Liu [a], Su-Juan Li [a,*], Yuanqiang Hao [b]

[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, Anyang, Henan 476000, People's Republic of China

ARTICLE INFO

Article history:
Received 18 June 2014
Received in revised form 9 August 2014
Accepted 14 August 2014
Available online 27 August 2014

Keywords:
Electrochemical biosensors
Alkaline phosphatase
Ascorbic acid
Self-assembled monolayer
Redox cycling
Ferrocenecarboxylic acid

ABSTRACT

L-Ascorbic acid 2-phosphate (AAP) is an optimal substrate for alkaline phosphatase (ALP) in electrochemical bioassays because of its low cost, good water solubility, less electrode passivation and high signal-to-background ratio. However, developing of electrochemical sensors with AAP as the enzyme substrate on self-assembled monolayer (SAM)-covered electrode is limited because the insulating SAM hinders the electron transfer between the electrode and ascorbic acid (AA, the enzymatic product of AAP). In this work, we first reported a strategy for developing AAP-based electrochemical biosensors on SAM-covered gold electrode. The method is based on AA-triggered "outer-sphere to inner-sphere" electrochemical–chemical–chemical (ECC) redox cycling with ferrocenecarboxylic acid (FcA) as the redox mediator. Specifically, AA produced from AAP facilitated the regeneration of FcA from its electrochemical-oxidation product (referred to as FcA^+ in the text), leading to an increase in the anodic current of FcA. Electrochemically inert tris(2-carboxyethyl)phosphine (TCEP) was used as a chemical reducing reagent to regenerate AA from its oxidation product, thus amplifying the electrochemical signal. The applications and performances of the proposed method were demonstrated in the competitive assays of β-amyloid (Aβ) peptides. The theoretical simplicity and high sensitivity indicated that our work would be valuable for developing simple and sensitive electrochemical biosensors.

1. Introduction

Electrochemical bioassays have attracted growing attention in clinical applications due to their intrinsic advantages, such as high sensitivity, fast response time, simple instrumentation, and low cost [1–4]. For the ultrasensitive detection of analytes with the electrochemical techniques, a popular approach is driving the enhancement of sensitivity with signal amplification [5,6]. Among kinds of amplified strategies, enzyme amplification is still the most commonly employed. However, single amplification by enzyme labels is not sufficient for detecting an ultra-low analyte concentration. Thus, multienzyme report probes are prepared by bioconjugating large amounts of enzymes on various materials, such as nanoparticles, carbon nanotubes, magnetic beads and graphene [7–13]. These methods are sensitive and reliable, but their practical applications are limited because of the denaturation and leakage of enzymes, the high cost of multienzyme report probes and their complicate preparation. Thus, developing new strategies for signal amplification is beneficial for the practical applications of electrochemical biosensors.

Alkaline phosphatase (ALP) is one of the most used enzymatic labels for the design of electrochemical biosensors [14,15]. It can remove a phosphate group from the substrate by hydrolyzing phosphoric acid monoesters into a phosphate ion and an electroactive molecule with a free hydroxyl group. Recently, the strategy for signal amplification using an ALP-based enzymatic reaction plus a redox-cycling reaction has been particularly popular in electrochemical immunoassays since it only requires the addition of more chemicals to the electrolyte solution and not a change in the detection procedure of conventional enzyme-based immunoassays [16–24]. In the system, the enzymatic product is regenerated after its electrochemical oxidization by a chemical reducing reagent, thus amplifying the electrochemical signal. Moreover, the reducing reagent can also prevent the auto-oxidation of the enzymatic product. Among the commonly used ALP substrate/product couples,

* Corresponding authors. Address: College of Chemistry and Chemical Engineering, Anyang Normal University, Anyang, Henan 455000, People's Republic of China (L. Liu). Tel.: +86 732 3300925.
E-mail addresses: liulin@aynu.edu.cn (L. Liu), lisujuan1981@gmail.com (S.-J. Li).

http://dx.doi.org/10.1016/j.jelechem.2014.08.021
1572-6657/

including 4-aminophenyl phosphate (p-APP)/4-aminophenol (p-AP), hydroquinone diphosphate (HQDP)/hydroquinone (HQ), L-ascorbic acid 2-phosphate (AAP)/L-ascorbic acid (AA), 4-amino-1-naphthyl phosphate (ANP)/4-amino-1-naphthol (AN) and 1-naphthyl phosphate (NPP)/1-naphthol (NP), AAP is better because of its low cost, the easy dissolution of AAP and AA in aqueous solutions, the high formal potential of AAP and low formal potential of AA and the high signal-to-background ratio [20]. Self-assembled monolayer (SAM) on gold has been used frequently for controlling the adsorption of biomolecules and developing electrochemical biosensors [1,25–27]. SAM is a more convenient (and more effective) choice for modifying electrodes in electrochemistry than Langmuir–Blodgett films or nonspecific physisorbed films because it forms spontaneously, is easy to handle mechanically, and does not desorb readily [28]. However, AAP as the enzyme substrate has never been employed on SAM-covered gold electrodes because the insulating SAM hinders the electron transfer between the electrode and AA [29].

Recently, Yang's group reported a simple and ultrasensitive sensing method for the detection of troponin I and *E. coli* O157:H7 by employing "outer-sphere to inner-sphere" electrochemical–chemical–chemical (ECC) redox cycling on indium-tin oxide (ITO) electrodes. In their work, $Ru(NH_3)_6^{3+}/Ru(NH_3)_6^{2+}$ was used as the redox mediator. The ECC redox cycling is particularly suitable for the HQDP/HQ couple considering the negligible side reaction and high signal-to-background ratio [30,31]. However, the method is unsuitable for the AAP/AA couple because AA shows no (or slow) reaction to $Ru(NH_3)_6^{3+}$ in the cycling system, thus penalizing the regeneration of $Ru(NH_3)_6^{2+}$ after its electrochemical oxidation. In the present work, we reported a new AA-triggered "outer-sphere to inner-sphere" ECC redox cycling with ferrocenecarboxylic acid (FcA) as the redox mediator on a SAM-covered gold electrode. To demonstrate the feasibility and sensitivity of the method, β-amyloid (Aβ) peptides serving as reliable molecular biomarkers of Alzheimer's disease (AD) were tested as the model targets.

2. Experimental

2.1. Chemical and reagents

Streptavidin-conjugated alkaline phosphatase (SA–ALP), tris(2-carboxyethyl)phosphine (TCEP), AAP, 6-mercapto-1-hexanol (MCH), bovine serum albumin (BSA) and tris-(hydroxymethyl) aminomethane hydrochloride (Tris–HCl) were purchased from Sigma–Aldrich. Biotinylated monoclonal antibody (mAb) of Aβ(1–16) (clone 6E10, mAb(1–16)) specific to the common N-terminus of Aβ and biotinylated Aβ(1–42) mAb (clone 12F4, mAb(1–42)) specific to the C-terminus of Aβ(1–42) were obtained from Covance Inc. (Dedham, MA, USA). All of Aβ peptides were synthesized and purified by ChinaPeptides Co., Ltd (Shanghai, China). Their sequences are DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA (Aβ(1–42)), DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV (Aβ(1–40)), DAEFRHDSGYEVHHQK (Aβ(1–16)), DAEFRHDSGYEVHHQKC (Aβ(1–16)Cys) and CEDVGSNKGAIIGLMVGGVVIA (CysAβ(22–42)). The Aβ stock solutions (0.1 mM) were prepared daily, as in the previous report [32]. Their concentrations were determined using a Cary50 spectrophotometer with the extinction coefficient (ε) of 1410 M^{-1} cm^{-1} at 276 nm. The mAb solutions were diluted with phosphate-buffered saline (PBS buffer, pH 7.4, 10 mM) before use. The ALP-conjugated mAb(1–16) and mAb(1–42) (denoted as mAb(1–16)–ALP and mAb(1–42)–ALP, respectively) are prepared by mixing the biotinylated mAb and the excess concentration of SA–ALP (1 μM) through the strong streptavidin–biotin interaction. The formation of the conjugates has been demonstrated in our previous report [32]. All aqueous solutions were prepared with deionized water treated with a Millipore system.

2.2. Procedure for Aβ detection

The cleaned gold electrode was immersed in a solution of Aβ(1–16)Cys or CysAβ(22–42) containing 50 μM TCEP and kept overnight. This step was followed by washing the electrode thoroughly with water and soaking it in a 0.1 mM MCH solution for 15 min. To minimize nonspecific adsorptions in the assay, the electrode was further soaked in PBS containing 1% BSA for 30 min. After the peptide-modified electrode had been washed with 1 mM EDTA solution, 10 μL of PBS containing a given concentration of Aβ peptides and ALP-conjugated antibodies was cast onto the electrode surface for 30 min. Again, the electrode was rinsed with water to remove any non-specifically adsorbed substance. Then, the electrode was incubated with 30 μL of Tris buffer (10 mM, pH 8.0) containing AAP, TCEP, FcA, 1 mM $MgCl_2$ and 50 mM Na_2SO_4. After incubation for 30 min, voltammetric and amperometric detection was carried out on a CHI 660E electrochemical workstation electrochemical workstation (CH Instruments, Shanghai, China) in the homemade plastic electrochemical cell. A platinum wire and a Ag/AgCl electrode were used as the auxiliary and reference electrodes, respectively.

After each assay, the electrode surface was regenerated with 10 mM NaOH (desorbing the target ALP-conjugated antibodies), followed by rinsing the electrode with water/ethanol (1:1) and EDTA solution. To test the stability, the sensing electrode was stored in the dark, at room temperature in a sealed container. This allowed long-term stability testing for up to one month.

3. Results and discussion

3.1. AA-triggered ECC redox cycling

In electrochemical–chemical (EC) and ECC redox cycling, the reducing reagent should be electrochemically inactive to obtain a high signal-to-background ratio. Chemical modification of an electrode with SAM for electrochemistry makes it possible to generate barrier layers that prevent free diffusion of electroactive species to the surface of the electrode. By examining different reducing reagents (e. g., TCEP, $NaBH_4$, hydrazine and Na_2SO_3) that can reduce quinone (including oxidized AA) at a fast rate, we found that these reducing reagents except TCEP showed high background current on the SAM-covered gold electrode in the potential scanning range (Fig. 1A). Thus, TCEP was used as the reducing reagent for AA-triggered "outer-sphere to inner-sphere" ECC redox cycling. Additionally, in this system, the redox mediator should be relatively stable in air and not be regenerated after its electrochemical oxidation by TCEP and the reaction between the redox mediator and AA should be very fast. Among the commonly used redox mediators, such as $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$, $Ru(NH_3)_6^{3+}/Ru(NH_3)_6^{2+}$ and FcA/FcA^+, we found that $Fe(CN)_6^{3-}$ generated from the electro-oxidization of $Fe(CN)_6^{4-}$ was reduced by TCEP (Fig. 1B), whereas AA shows no (or slow) reaction to $Ru(NH_3)_6^{3+}$ even in the presence of TCEP [30]. Thus, FcA was chosen as the redox mediator for AA-triggered ECC redox cycling on the SAM-covered gold electrode.

Fig. 2 shows the CVs of the SAM-covered electrode in various solutions. It can be observed that the background current of TCEP (curve b) is close to that of the control (curve a) and AA shows no redox peaks in both the absence (curve c) and presence (curve d) of TCEP. The addition of TCEP to the FcA solution did not induce an apparent change in the CVs of FcA (cf. curves e and f). Interestingly, the anodic current of FcA increased as its cathodic current