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

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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,

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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

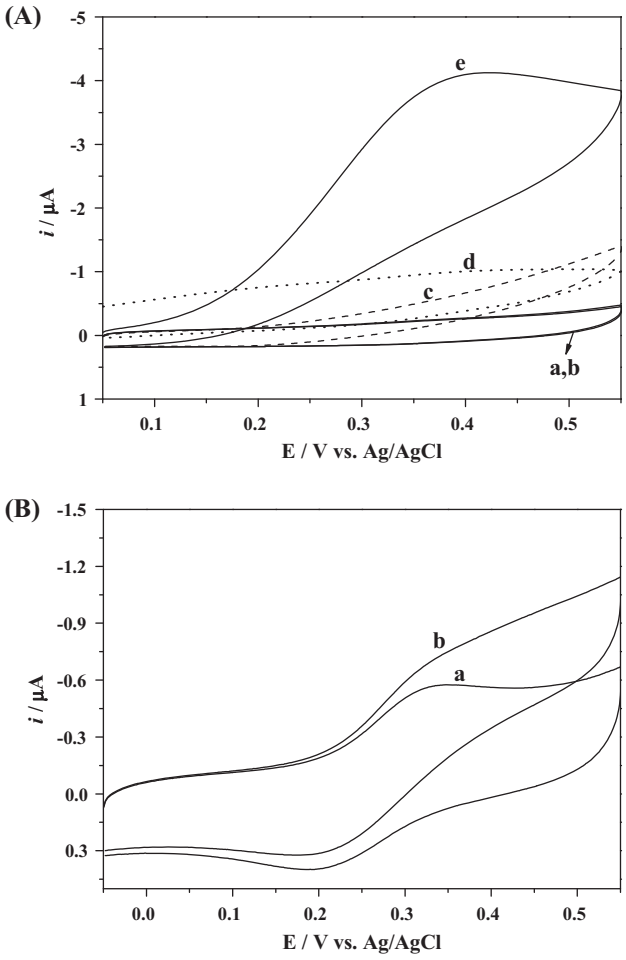

Fig. 1. (A) CVs of the Aβ(1–16)Cys-modified electrode in different solutions (curve a: blank; curve b: TCEP; curve c: Na_2SO_3; curve d: $NaBH_4$; curve e: hydrazine). The concentrations of all of the reducing reagents are 0.5 mM. (B) CVs of Aβ(1–16)Cys-modified electrode in the solution of 0.2 mM $Fe(CN)_6^{4-}$ in the absence (curve a) and presence (curve b) of 0.5 mM TCEP.

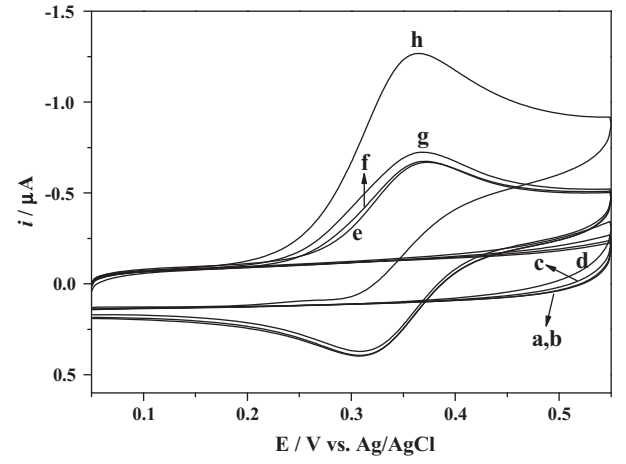

Fig. 2. CVs of the Aβ(1–16)Cys-modified electrode in different solutions (curve a: blank; curve b: TCEP; curve c: AA; curve d: AA/TCEP; curve e: FcA; curve f: FcA/TCEP; curve g: FcA/AA; curve h: FcA/TCEP/AA). The concentrations of TCEP, AA and FcA are 0.5, 0.02, and 0.2 mM, respectively. The scan rate was 20 mV/s.

decreased after the addition of AA to the FcA/TCEP solution (curve h). The electrode reaction is characteristic of an electrochemical–chemical reaction mechanism, indicating that FcA was regenerated from its oxidation product FcA^+. Furthermore, we validated the role of TCEP in the detection system by conducting a control experiment in which TCEP was not introduced. As a result, a smaller increase in the anodic current of FcA was observed (curve g). The results indicated that the electrochemical–chemical reaction between FcA and AA was promoted by the chemical–chemical reaction between AA and TCEP.

3.2. Principle for Aβ detection

To demonstrate the application and sensitivity of AA-triggered ECC redox cycling in biosensing, Aβ peptides were tested in a competitive-type immunoassay format. In conventional competitive-type immunoassays, antibodies are immobilized on a solid support surface for the capture of antigens. The disadvantages of the format are that the random orientation of antibodies decreases their immunoaffinity capacity and the instability of antibodies limits the regeneration and long-time storage of the sensing electrode. Thus, the competitive immunoassays used in this work were carried out by immobilizing Aβ peptides (antigen) on the electrode surface. The schematic representation of the method is illustrated in Fig. 3. Aβ(1–16) peptide appended with a cysteine residue (Aβ(1–16)Cys) was immobilized on a gold electrode through the formation of the Au–S bond. The ALP-conjugated mAb(1–16) (mAb(1–16)–ALP) specific to the amino acids 3–8 of Aβ can be captured by the electrode through the strong and specific antibody–Aβ interaction (Sample I). After the addition of the AAP substrate, the enzymatic reaction from AAP to AA proceeds. The produced AA will trigger the ECC redox cycling. If the mAb(1–16)–ALP bound to the native Aβ in the samples (Sample II), it would be incapable of anchoring onto the electrode. Consequently, the AA-triggered ECC redox cycling reaction would not occur due to the lack of mAb(1–16)–ALP on the electrode surface.

3.3. Feasibility for Aβ detection

Curve a in Fig. 4A shows the CV of the Aβ(1–16)-modified gold electrode after the capture of mAb(1–16)–ALP and the follow-up incubation with the FcA/TCEP-containing AAP substrate. The anodic current of FcA increased as its cathodic current decreased. The result demonstrated that mAb(1–16)–ALP promoted the produce of AA, thus initiating the ECC redox cycling. When the electrode was incubated with the mixed solution of mAb(1–16)–ALP and Aβ(1–16), the anodic current of FcA almost dropped to the background level (curve b), indicating that Aβ(1–16) in the sample held back the binding of mAb(1–16)–ALP to the immobilized Aβ(1–16) on electrode. The native Aβ peptides in human CSF are proteolytic cleavage product from amyloid precursor protein (APP) by β- and γ-secretase. Among various Aβ species, Aβ(1–40) (60–70%) and Aβ(1–42) (5–15%) are the two predominant cleavage product [32]. To demonstrate the feasibility and specificity of the method to total Aβ detection, other Aβ species was tested. It can be seen that the presence of Aβ(1–40) (curve c) and Aβ(1–42) (curve d) but not Aβ(22–42) (curve e) restricted the increase of the anodic current of FcA. The results are understandable since mAb(1–16) can capture all the native Aβ species by binding to the amino acids 3–8 (Glu–Phe–Arg–His–Asp–Ser) in the N-terminus. Thus, Aβ(22–42) lacking this sequence could not prevent the attachment of the mAb(1–16)–ALP to the sensing electrode. Amperometry is a simple, sensitive and classical electrochemical technique that has been successfully applied in the electrochemical immunassays. We also found that the results of amperometry are in agreement with those obtained by cyclic voltammetry (Fig. 4B). Furthermore, we found that the amperometric current increased with the increase of the mAb(1–16) concentration and began to level off beyond 0.05 μg/mL in the absence of any Aβ

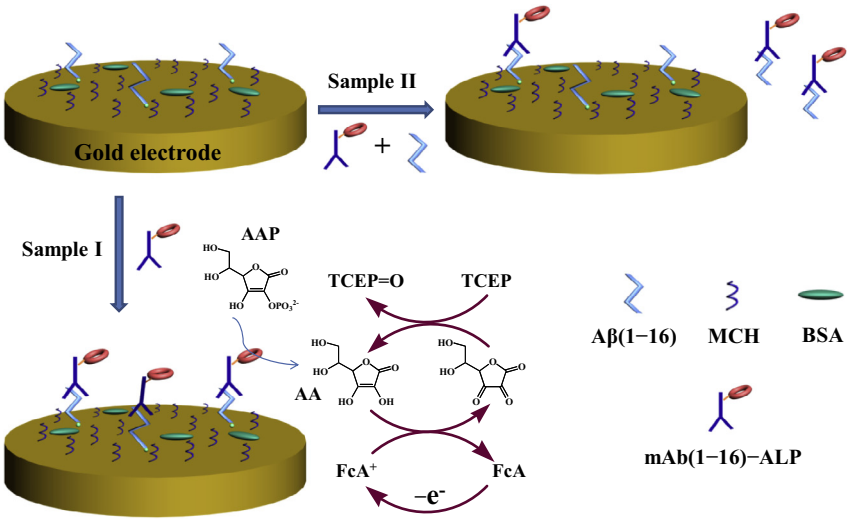

Fig. 3. Schematic representation for Aβ detection by ALP-based signal amplification combined with AA-triggered "outer-sphere to inner-sphere" ECC redox cycling on a SAM-covered gold electrode.

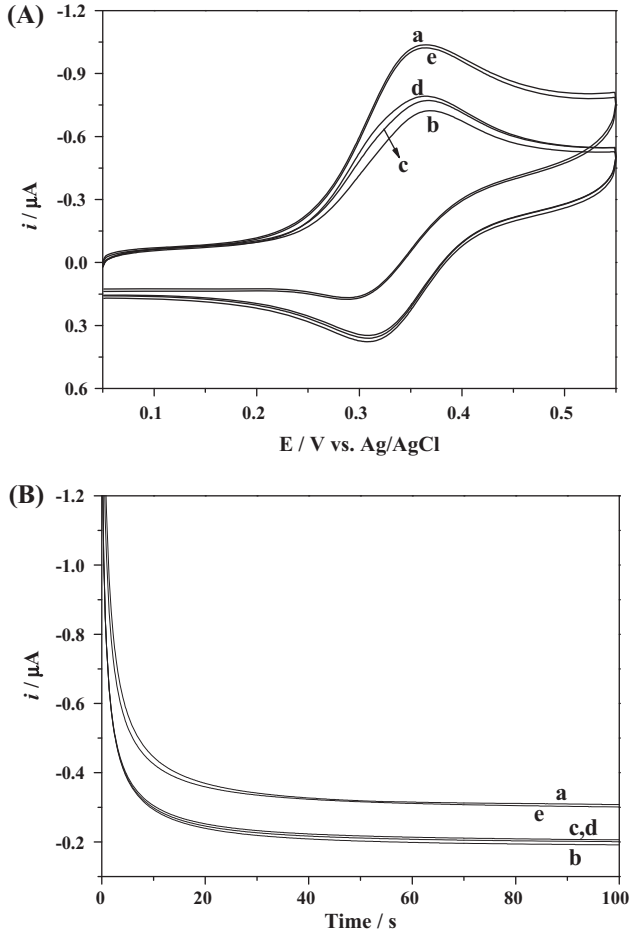

Fig. 4. CVs (A) and amperometric responses (B) acquired at the Aβ(1–16)Cys-modified electrode after incubation with mAb(1–16)–ALP in the absence (curve a) and presence of 0.2 nM Aβ(1–16) (curve b), Aβ(1–40) (curve c), Aβ(1–42) (curve d) or Aβ(22–42) (curve e) and follow-up incubation with AAP/TCEP/FcA.

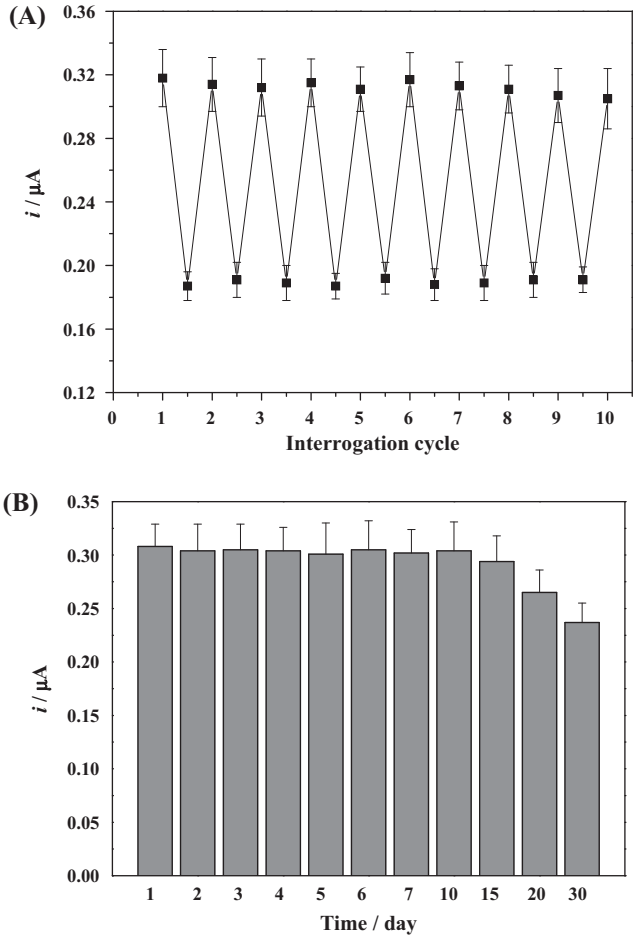

Fig. 5. (A) Amperometric current for ten regeneration/assay cycles and (B) the storage stability of the modified electrode. The experimental conditions are the same as those in Fig. 4A.

species, indicating that the electrode surface has been saturated by the mAb(1–16)–ALP molecules. Thus, in the following competitive detection assays, mAb(1–16) at the concentration of 0.05 μg/mL was used.

The accumulation, polymer formation and poor dissolution of the enzyme substrates/products on the electrode surface may lead to the passivation of electrode in electrochemical immunoassays. Thus, analytical signals generated by the sensor may be unreliable and unreproducible. Previously, the results from surface plasmon resonance (SPR) indicated that the Aβ peptides binding to the antibody can be desorbed by NaOH solution [32]. Herein, we also found that the electrode can be conveniently regenerated by immersion in 10 mM NaOH solution. As a result, no apparent change in the catalytic oxidation current of FcA was observed after 10 regeneration/assay cycles (Fig. 5A). Thus, multiple samples can be determined using one electrode, dramatically increasing the sample throughput and reducing the analysis time and cost. We also investigated the stability of the sensor and found that the electrode remains stable for almost two weeks and only loses 23% of its capture efficiency after one month (Fig. 5B).

3.4. Sensitivity for Aβ detection

With the regeneration and stability of the sensing electrode, we assessed the sensitivity and reproducibility of the method. Aβ samples containing Aβ(1–40), Aβ(1–42) and Aβ(1–16) in the ratio of 6:1:3 were tested because (1) the Aβ(1–40)/Aβ(1–42) ratio of 6:1 is close to the real ratio in human CSF [32], (2) all the natural Aβ species contain the common N-terminus of Aβ [33], and (3) mAb(1–16) binds to the same N-terminus of different Aβ species with similar binding affinities [22,32]. As a result, we found that the amperometric currents decreased with the increasing Aβ concentrations in the range of 0–1 nM (Fig. 6A). The current variation Δi ($\Delta i = i - i_0$, where i_0 and i represent the amperometric current in the absence and presence of Aβ, respectively) was used here to evaluate the analytical merits. The dependence of Δi on Aβ concentration is presented in Fig. 6B. It can be observed that Δi increased linearly with Aβ concentration varying from 1 pM to 0.2 nM. The linear regression equation is Δi (μA) = 0.003 + 0.551 [Aβ] (nM) (R^2 = 0.99). The detection limit estimated from 3 s of the baseline signals was 0.2 pM. This value is lower than that achievable by SPR (3.5 pM) [32], ALP-based electrochemical–chemical redox cycling (5 pM) [22], resonance light scattering (0.8 ng/mL) [34] and gold nanoparticle-based dot-blotimmunoassay (50 pg/mL) [35]. The low detection limit demonstrated that the sensor with AA-triggered ECC redox cycling is sensitive and high-effective.

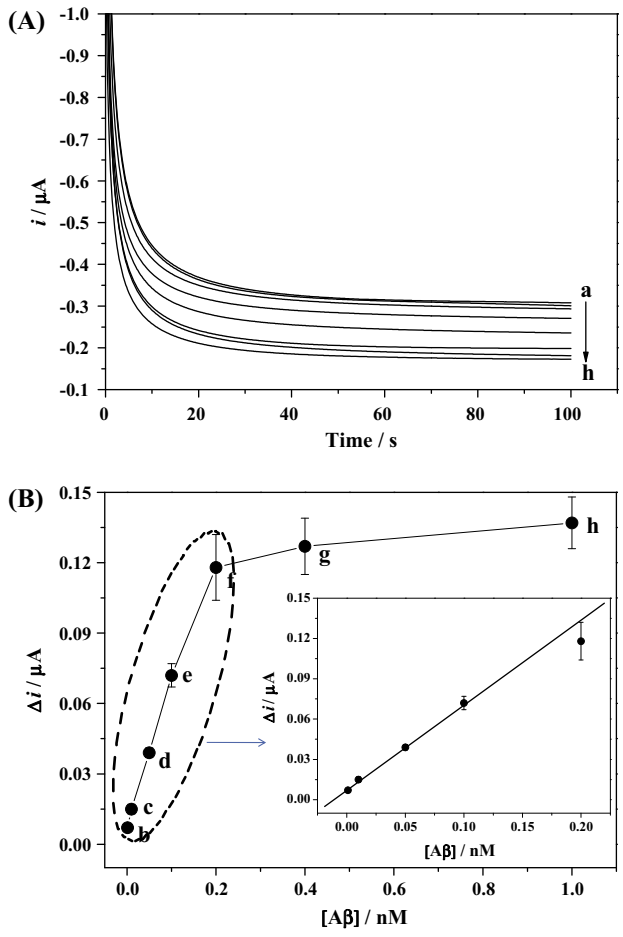

Fig. 6. (A) Amperometric responses of the electrode after incubation with mAb(1–16)–ALP in the presence of different concentrations of Aβ (from a to h: 0, 0.001, 0.01, 0.05, 0.1, 0.2, 0.4 and 1 nM) and follow-up incubation with AAP/TCEP/FcA. (B) Dependence of Δi on total Aβ concentration. The amperometric currents were collected at 100 s. Each point was averaged from three replicates, and the error bars show the absolute standard. The concentrations of AAP, TCEP and FcA are 1, 0.5, and 0.2 mM, respectively. The concentration of mAb(1–16) is 0.05 μg/mL.

3.5. Detection of Aβ(1–42)

One of the pathological hall marks of AD is the deposition of Aβ in the brain, while Aβ(1–42) exhibits greater tendency to form

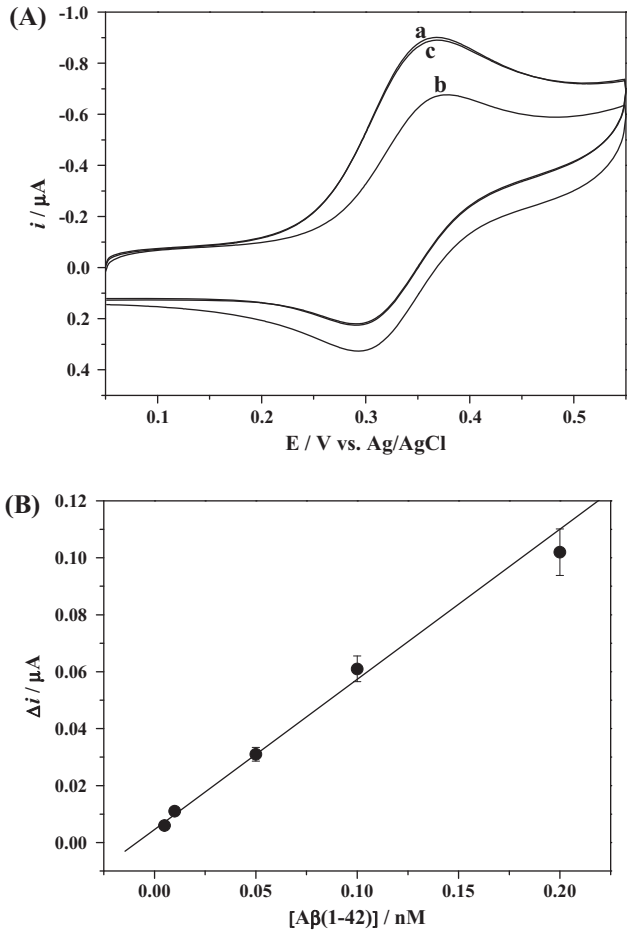

Fig. 7. (A) CVs acquired at the CysAβ(22–42)-modified electrode after the incubation with mAb(1–42)–ALP in the absence (curve a) and presence of Aβ(1–42) (curve b) or Aβ(1–40) (curve c) and the follow-up incubation with AAP/TCEP/FcA. The other experimental conditions are the same as those in Fig. 4A. (B) Linear dependence of Δi on Aβ(1–42) concentration in the range of 5–0.2 pM.

amyloid fibrils than other Aβ species. At present, clinical practice of AD diagnostics is based on the detection of Aβ(1–42) monomer/oligomer or total Aβ species [33,36–38]. However, assay of Aβ(1–42) or Aβ only might be unable to discriminate between AD and health or other types of dementia because the levels of Aβ(1–42) or total Aβ may differ by gender and age. Thus, assay of the relative level of Aβ(1–42) will be more precise for diagnosis and prognosis of AD. In this term, the same procedure with total Aβ detection was implemented for Aβ(1–42) detection with the ALP-conjugated mAb(1–42) (mAb(1–42)–ALP). The CysAβ(22–42) peptide containing the C-terminus of Aβ(1–42) and a cysteine residue was immobilized onto the gold electrode for the capture of mAb(1–42)–ALP. As a result, the presence of Aβ(1–42) in the mAb(1–42)–ALP solution reduced the occurrence of the ECC redox cycling reaction, whereas no apparent change was observed in the case of Aβ (1–40) (Fig. 7A). The result is acceptable since the anti-Aβ(1–42) antibody can selectively bind to Aβ(1–42) but not Aβ(1–40). Thus, the method is selective to Aβ(1–42). Moreover, we also found that the Δi increased linearly with the Aβ(1–42) concentration in the range of 5 pM–0.2 nM (Fig. 7B). The linear regression equation is Δi (μA) = 0.004 + 0.531 [Aβ(1–42)] (nM) (R^2 = 0.99).

4. Conclusions

In summary, we, for the first time, demonstrated that electrochemical biosensors with AAP as the enzyme substrate on SAM-covered gold electrodes can be designed. This method is based on AA-triggered "outer-sphere to inner-sphere" ECC redox cycling using FcA and TCEP as the redox mediator and the reducing reagent, respectively. The chemical–chemical reaction between AA and TCEP promoted the electrochemical–chemical reaction between FcA and AA, which led to an increase in the anodic current of FcA. The keys to success for the strategy are that AA and TCEP showed no electrochemical signal in the potential scanning range and that TCEP facilitated the regeneration of AA but not FcA. To demonstrate the applications and analytical merits of this method, Aβ peptides were tested as the model targets. The low detection limit (0.2 pM) and simple operation procedure of the method indicated that our work would be valuable for extending the application of enzyme amplification and developing sensitive electrochemical biosensors on SAM-covered electrodes.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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