Microchimica Acta (2018) 185: 549
https://doi.org/10.1007/s00604-018-3087-9

ORIGINAL PAPER

Voltammetric determination of the Alzheimer's disease-related ApoE 4 gene from unamplified genomic DNA extracts by ferrocene-capped gold nanoparticles

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Received: 24 August 2018 / Accepted: 6 November 2018 / Published online: 14 November 2018

Abstract

A sensitive method is described for detection of the apoE 4 gene detection which is important for early diagnosis of Alzheimer's disease. It is based on signal amplification by using ferrocene (Fc) capped gold nanoparticles modified with streptavidin. The immobilized oligonucleotide probe captures complementary apoE 4 gene. This is followed by the specific recognition of the GCGC sequences which are hydrolyzed by the restriction enzyme HhaI. Cleavage only occurs at the complementary apoE 4 duplex, while mismatches prevent enzymatic cleavage. Thus, the apoE 4 sequence can be discriminated against other apoE sequences. Benefitting from amplified signal by Fc-capped nanoparticle/streptavidin and the recognition of HhaI, the detection limit is as low as 0.1 pM of the ApoE 4 gene. Four genomic DNA samples extracted from blood were analyzed for the presence of the apoE 4 gene. The approach presented here will provide viable proof-of-principle for an enzyme-assisted electrochemical assay for the apoE 4 gene in genomic DNAs.

Keywords ApoE 4 gene · Restriction enzyme HhaI · Cyclic voltammetry · DNA extract · Biosensor

Introduction

Alzheimer's disease (AD) is one of the common neurodegenerative disorders, characterized by the accumulation of amyloid-beta (Aβ), the phosphorylation of tau and the formation of neurofibrillary tangles in brain [1, 2]. Aβ peptide is considered as the major component of the senile plaques and its accumulation plays a vital role in the pathogenesis of AD and is one of the biomarkers of AD, detection of the concentration of Aβ in CSF can provide useful information of diagnosis of AD. [3–6] However, studies reveal that the concentration change of Aβ is not only related with AD [7], new biomarkers for AD needs to be discovered. Apolipoprotein E(apoE) forms complex with Aβ thus regulates Aβ metabolism, aggregation, and deposition [8]. The three apoE isoforms (E2, E3 and E4) contain two polymorphic codon sites (112 and 158) of the gene located on chromosome [8]. Among the three apoE isoforms, apoE 4 increase the cytotoxicity of Aβ peptides oligomers, and the binding between apoE 4 and Aβ-degrading enzyme inhibit the clearance of Aβ aggregations [9]. The presence of the apoE 4 gene not only increases the risk for AD but also lowers the age of onset [10]. Individuals carrying apoE 4 gene presents higher AD risk compared to those carrying apoE 2 gene or apoE 3 gene. The discrimination and the detection of the apoE 4 gene may assessing the risk factor of AD and provide useful information for the early diagnosis and the precise medication for the treatment of AD.

Assays for apoE gene detection are typically based on the amplification strategy, including polymerase chain reaction (PCR) [11], primer extension [12, 13]. A collaborative single nucleotide polymorphism mapping study around apoE in PCR products has been established by Martin et al. [14], strong evidence indicate the apoE 4 polymorphism associate with AD. Based on the cleavage of HhaI at the GCGC sites on apoE, restriction isotyping of human apoE gene has been achieved by PCR amplification [15]. By sophisticate design

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and modification, microarray offers a multiplexed parallel identification of gene polymorphisms but suffers from low sensitivity and expensive chips [16, 17]. A microarray analysis was designed to investigate the relationship of AD pathology and apoE genotype [18]. Based on allele-specific PCR methodology by TaqMan probe, apoE genotyping has been demonstrated and the risk of apoE gene variation has been assessing [19]. Combined with surface invasive cleavage reaction, ligation and rolling circle amplification, apoE genotyping can be achieved directly from unamplified DNAs, high temperature was used to achieve a higher cleavage efficiency [20]. However, these methods normally relies on tedious assay processes, expensive equipment, skilled operations thus limit their practical use. In addition, above assay can not direct discriminate apoE 4 gene from all apoE genotypes or genomic DNAs which is restricted by their sensitivity unless with the aid of the amplification techniques or increased temperature. Great efforts have been devoted for developing simple and rapid method for genotyping with high sensitivity and better reliability.

Electrochemical methods are simple, sensitive, and do not involve complicated instrumentation [21–23]. DNA electrochemical biosensors have been developed for the detection of apoE genotypes in PCR-amplified DNA extracted from human blood [24–26]. Marrazza et al. reported a DNA electrochemical biosensor for apoE genotyping by coupled with PCR, the assay can discriminate the six genotypes of apoE [26]. An electrochemical DNA biosensor using a disposable electrochemical printed chip for the detection of apoE 4 from unpurified PCR amplicons has been demonstrated by Ahmed et al. [24]. Based on the modified Randles equivalent circuit, discrimination of the single-nucleotide mismatch in apoE alleles has been performed by comparing the difference in the charge transfer resistance before and after Ni^{2+} addition [25]. However, the direct and effective quantification of the gene from unamplified DNA is still under challenge.

In this work, an electrochemical assay for apoE 4 gene discrimination and quantification by the recognition of enzyme and the amplification of Fc-capped streptavidin/gold nanoparticle was demonstrated. By immobilizing the allele specific probe onto the electrode to recognize the corresponding target, the existence of apoE 4 gene can be discriminated by the sequence specific restriction enzyme. To achieve sufficient sensitivity for the detection of unamplified DNA from real samples, the cleavage of the duplex was detected by the signal amplification of Fc-capped streptavidin/gold nanoparticle. The large number of Fc attached on the gold nanoparticle significantly increase the detection signal. The successful apoE 4 gene detection was demonstrated with diluted genomic DNA samples. Current assay is rapid, easy operation, label-free thus offers a potentially approach with high sensitivity and applicable to perform an amplification-free apoE 4 gene detection.

Experimental section

Materials

NaCl, KCl, MgCl, K_2HPO_4, KH_2PO_4 and $K_3Fe[CN]_6$ were acquired from Sangon Biotechnology Co., Ltd. (Shanghai, China, http://www.sangon.com/). Gold nanoparticle/streptavidin conjugates, 6-mercaptohexanol (MCH), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 6-(ferrocenyl)hexanethiol were purchased from Sigma-Aldrich (St. Louis, MO, http://www.sigmaaldrich.com). The biotinylated probe possesses sequence of 5′-biotin-CAG GCG GCC GCG CAC GTC TTT TT-$(CH_2)_6$-SH-3′, which is complementary to that of the fragment of apoE 4 gene at codon 112 (5′-GAC GTG CGC GGC CGC CTG-3′). The single-base mismatched sequence is 5′-GAC GTG TGC GGC CGC CTG-3′(mismatching sequence underlined), being a fragment of apoE 2/3 gene (apoE 2 or apoE 3 gene) at codon 112. All the oligonucleotides were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China, http://www.sangon.com/). The restriction enzyme HhaI was obtained from New England Biolabs (Ipswich, MA, http://www.neb-china.com/). All the reagents were of analytical grade and used without further purification. The biotinylated probe and the targets were prepared with phosphate buffered saline (PBS, 10 mM phosphate with 0.1 M NaCl, pH = 7.4) containing 1 mM TCEP, and PBS containing 5 mM $MgCl_2$, respectively. Unless otherwise stated, all the stock solutions were prepared daily with deionized water treated with a water purification system (Simplicity185, Millipore Corp, Billerica, MA). The preparation and characterization of Fc-capped gold nanoparticle/streptavidin conjugates have been reported previously [27].

Procedures

Gold electrode was polished with 1.0 and 0.05 μm alumina slurry on a polishing cloth, followed by sonication in ethanol and water. For immobilization of the biotinylated probe, the electrode was immersed in 1.0 μM probe solution for 16 h at 37 °C, followed by blocking the empty sites with 1 mM MCH. The hybridization reaction was carried out by covering the probe-modified electrode with the target solutions for 3 h at 37 °C. For the enzymatic cleavage reaction, the duplex DNA-modified electrode was dipped in 100 U/mL of HhaI solution for 2 h at 37 °C. Finally, the electrode was exposed to 10 μL of Fc-capped gold nanoparticle/streptavidin conjugates for 1 h. After each step, the electrode was rinsed with saline and dried under N_2.

DNA extraction

The unamplified genomic DNA extracts were obtained from the blood of four donors using the TIANamp

Genomic DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) based on the manufacturer's instructions. The volume of the obtained DNA extracts was 50 μL and that of the blood samples used was 2 mL.

Electrochemical characterization

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were performed on a CHI 620E electrochemical workstation (CH Instruments, Shanghai, China) and PalmSens4C potentiostat (PalmSens, Netherlands), respectively. A three-electrode setup was employed in which a gold electrode with a diameter of 2 mm served as the working electrode, a platinum wire and a Ag/AgCl electrode were used as the counter and reference electrodes, respectively. The CV characterization was carried out in 0.1 M $KClO_4$ via scanning the electrode potential between −0.1 and 0.6 V. The EIS measurement was performed in an aqueous solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) and 0.1 M KCl at the frequency range from 100 kHz to 100 mHz. The DC/AC values are 0.24 V/ 0.01 V respectively.

Results and discussion

Principle of the electrochemical assay

As illustrate in Fig. 1, the biotinylated probe with sequence complementary to apoE 4 gene at codon 112 was first immobilized on the Au electrode via Au-S bond. To eliminate nonspecific adsorption of the conjugates, the electrode was blocked with MCH. After hybridization of the anchored probe to apoE 4 gene, the duplex with GCGC sequence can be recognized and cleavage by HhaI, and the detachment of the biotinylated fragment from the electrode hinders the attachment of Fc-capped gold nanoparticle/streptavidin conjugates, resulting in tiny anodic peak currents (top). In the presence of apoE 2/3 gene with a single base mismatch relative to apoE 4 gene, the mismatch sequence (GTGC) prevented the cleavage reaction, and the attachment of the conjugates leads to larger anodic peaks (bottom). Because each gold nanoparticle was covered with a large number of Fc tags [27–30], the electrochemical signal was significantly enhanced.

EIS characterization

The stepwise modification of the Au electrode was characterized by EIS (Fig. 2). R_{et} was estimated from the diameter of the semicircle in the Nyquist plot. In comparison with the small semicircle in the impedance plot for the bare gold electrode (R_{et} = 107 Ω, curve a), modification of the electrode with the negatively charged biotinylated probe increased the R_{et} to 4862 Ω (curve b). Blocking of the electrode by MCH and then hybridization of the probe to apoE 4 gene further increased R_{et} to 6430 Ω (curve c) and 6947 Ω (curve d), respectively. However, cleavage of the duplex by HhaI leads to decreased R_{et} of 838 Ω (curve e). Interestingly, the attachment of Fc-capped gold nanoparticle/streptavidin conjugates further decreased R_{et} to 366 Ω (curve f), which might be ascribed to the incomplete cleavage reaction or the tiny nonspecific adsorption of the conjugates. Without the cleavage reaction, the attachment of the conjugates on the duplex yields R_{et} of 6051 Ω

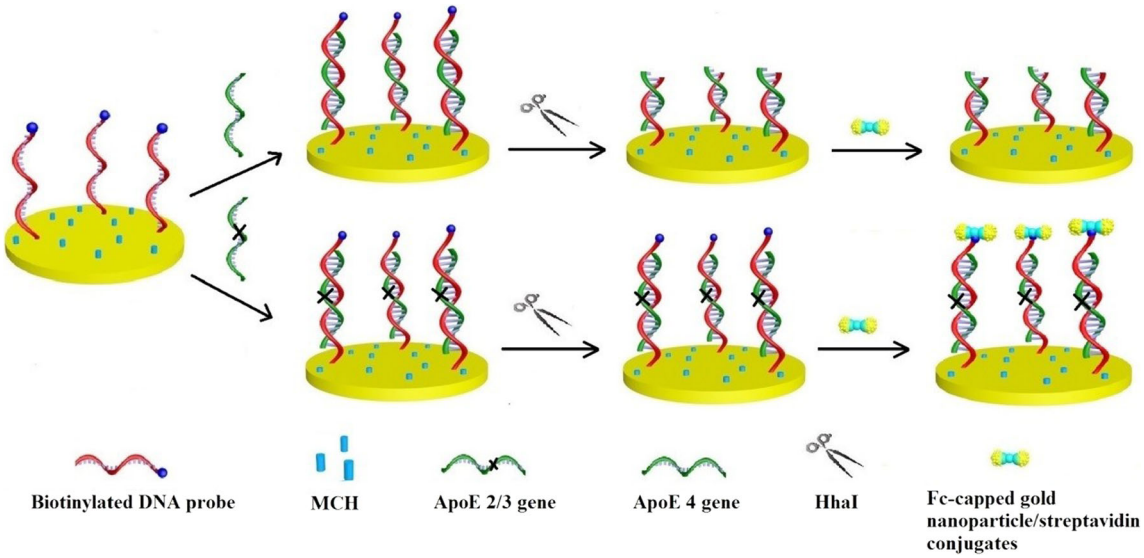

Fig. 1 Schematic of electrochemical detection of apoE 4 gene. The Au electrodes were covered with biotinylated DNA probes, followed by hybridization with apoE 4 gene at codon 112 (top) or apoE 2/3 gene with a single base mismatch relative to apoE 4 gene (bottom), enzymatic cleavage by HhaI and then attachment of Fc-capped gold nanoparticle/streptavidin conjugates

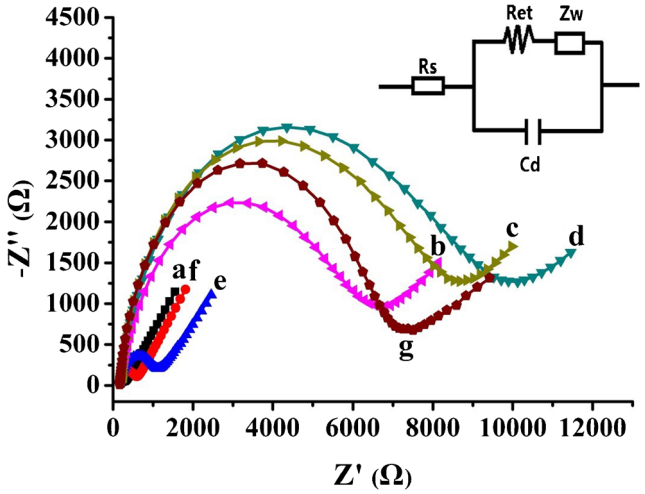

Fig. 2 Electrochemical impedance spectra acquired at a bare Au electrode (**a**) and the electrodes assembled with biotinylated probe (**b**), biotinylated probe/MCH (**c**), biotinylated probe/MCH /apoE 4 gene (**d**), biotinylated probe/MCH/apoE 4 gene /HhaI (**e**), biotinylated probe/MCH/apoE 4 gene/HhaI/Fc-capped gold nanoparticle/streptavidin conjugates (f) and biotinylated probe/MCH/apoE 4 gene/Fc-capped gold nanoparticle/streptavidin conjugates (**g**). The electron transfer resistance (R_{et}) was attained by the inset Randles equivalent circuit model. The EIS measurement was performed in an aqueous solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) and 0.1 M KCl at the frequency range from 100 kHz to 100 mHz. The DC/AC values are 0.24 V/ 0.01 V respectively

(curve g), and the smaller R_{et} than those in curves c and d indicates that the attached conjugates make $[Fe(CN)_6]^{3-/4-}$ molecules more accessible to the electrode surface.

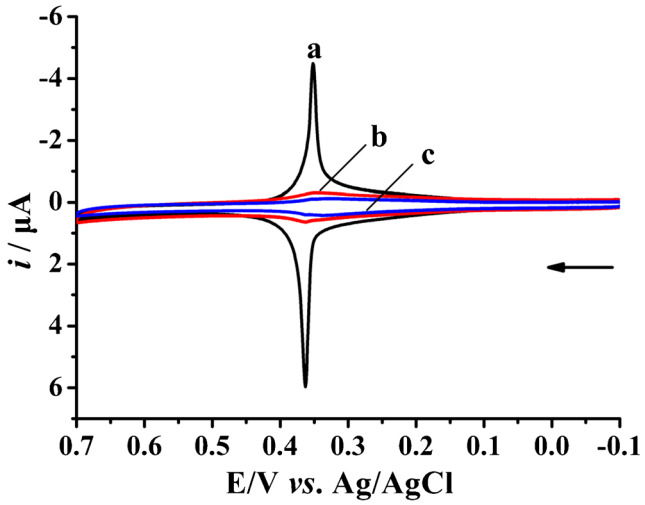

Fig. 3 CV responses acquired at the electrode covered with (**a**) biotinylated probe upon hybridization with 50 pM apoE 4 gene, (**b**) biotinylated probe upon hybridization with 50 pM apoE 4 gene, followed by cleavage by HhaI, (**c**) unbiotinylated probe upon hybridization with 50 pM apoE 4 gene. In the three cases, the attachment of Fc-capped gold nanoparticle/streptavidin conjugates was performed. The scan rate was 0.1 V/s, and the arrow indicates the scan direction

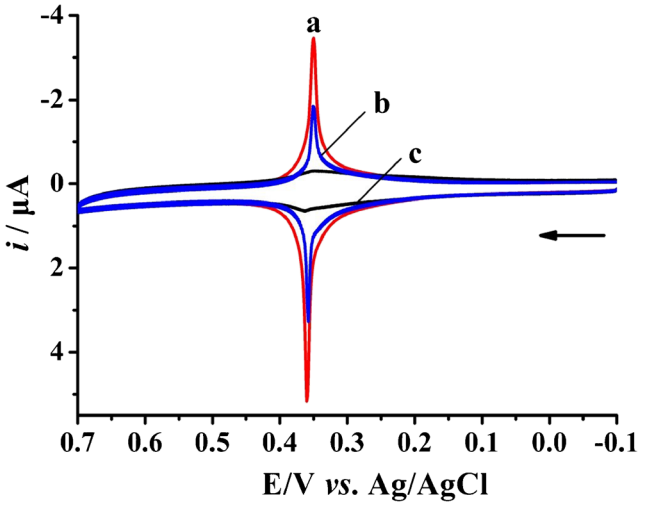

Fig. 4 CV responses acquired at the electrode upon hybridization of the biotinylated probe to (**a**) 50 pM of apoE 2/3 gene with a single base mismatch relative to apoE 4 gene, (**b**) 5 pM of apoE 4 gene, and (**c**) 50 pM of apoE 4 gene, followed by the cleavage reaction and the conjugates attachment. Other experimental conditions as those in Fig. 3

Feasibility of apoE 4 gene assay

The voltammetric response acquired at the biotinylated probe-covered electrode after hybridization with apoE 4 gene, followed by the conjugates attachment is shown in curve a of Fig. 3. A pair of well-defined redox waves with enhanced peak currents was observed. However, after the cleavage reaction, the detachment of the biotinylated fragments from the electrode leads to much smaller voltammetric peaks (curve b, Fig. 3). The smaller signals in Fig. 3b might be caused by the incomplete cleavage reaction or the tiny nonspecific

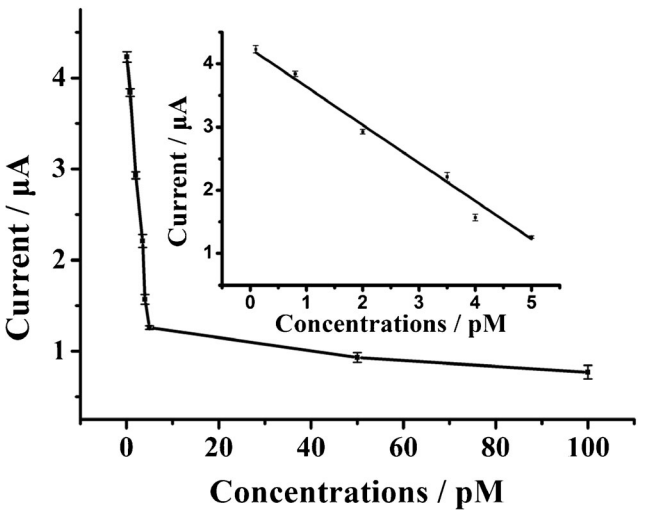

Fig. 5 Dependence of the anodic peak currents on the concentrations of apoE 4 gene (0.1 pM, 0.8 pM, 2 pM, 3.5 pM, 4 pM, 5 pM, 50 pM and 100 pM). The absolute errors deduced from three replicate measurements are shown as the error bars. The inset shows the linear portion of the curve between 0.1 and 5 pM. The anodic peak currents were acquired at the voltage correspond to the oxidation peak at voltage of 0.363 V

Table 1 Comparison of AuNPs-based electrochemical biosensors for DNA detection

Signal probe	Linear range (pM)	Detection limit (pM)	Reference
Thionine-capped DNA/gold nanoparticle conjugates	1.0–100	0.5	[31]
HRP modified AuNPs	10–1000	0.6	[32]
AuNP modified electrode	0.3–13.2	0.09	[33]
DNA functionalized AuNPs	100–10,000	33	[34]
Fc-capped nanoparticle/streptavidin	0.1–5.0	0.1	This work

adsorption of the conjugates, being consistent with the EIS characterization (curve f, Fig. 2). As expected, the hybridization of the probe without a biotin tag to apoE 4 gene, followed by the conjugates attachment (curve c, Fig. 3) results in a featureless voltammogram, indicating the negligible nonspecific adsorption of the conjugates. The sensing protocol thus provides a viable means for apoE 4 gene assay.

Selectivity of the assay

The selectivity of the method was demonstrated by assaying apoE 4 gene and apoE 2/3 gene with a single base mismatch relative to apoE 4 gene (Fig. 4). The enzymatic cleavage of the complementary duplex in the case of 50 pM apoE 4 gene results in a smaller anodic peak current of 0.23 μA (curve c), suggesting that most of the DNA duplexes have been cleaved by HhaI. When the concentration of apoE 4 was decreased to 5 pM, much increased anodic peak current of 1.25 μA was observed (curve b), suggesting the capability of the method for apoE 4 gene assay. The hybridization of the biotinylated probe with apoE 2/3 gene prevented the cleavage reaction due to the presence of the single-base mismatch on the cleavage sites (curve a), thus, the biotin tag was left intact and almost the same anodic peak current with that in the case of apoE 4 gene without the cleavage reaction was attained. The method is capable of distinguishing apoE 4 gene and apoE 2/3 gene with a single base mismatch and holds great promise for apoE 4 gene assay in complex sample media.

Quantitative apoE 4 gene assay

The dependence of the anodic peak currents on the concentrations of apoE 4 gene is depicted in Fig. 5. The hybridization of the pre-immobilized biotinylated probe to apoE 4 gene with higher concentrations leads to the formation of more DNA duplexes and the detachment of more biotinylated fragments via enzymatic cleavage reaction results in smaller voltammetric signals. A linear concentration range of apoE 4 gene between 0.1 and 5 pM was obtained (the inset of Fig. 5) and the linear regression equation is expressed as i (μA) = −0.602 [apoE 4 gene] (pM) + 4.24 (R^2 = 0.99). The relative standard deviations from three replicate measurements are all below 7%, indicating that the method is highly reproducible. The detection level of 0.1 pM, such a detection limit is much lower than that other AuNPs-based electrochemical biosensors for DNA detection (Table 1).

ApoE 4 gene assay in genomic DNA extracts

The feasibility of the method for the assay of apoE 4 gene in unamplified genomic DNA extracts from serum samples has

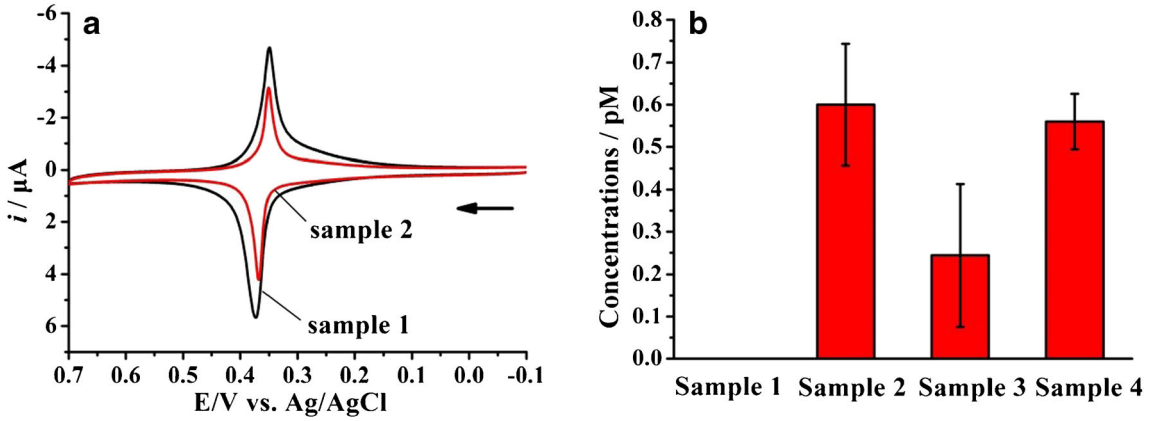

Fig. 6 **a** CVs acquired at the biotinylated probe-covered electrode upon hybridization with 10-fold diluted serum sample 1 or 2. **b** ApoE 4 gene assay from four genomic DNA extracts. The absolute errors deduced from three replicate measurements are shown as the error bars

been demonstrated. The biotinylated probe possesses a sequence complementary to that of apoE 4 gene at codon 112. The duplex formed is recognized and cleaved by HhaI. The detachment of the biotinylated segment prevented the conjugates from attaching to the electrode surface, leading to lower electrochemical signals. However, the existence of the mismatched sequence relative to that of apoE 4 gene hindered the cleavage reaction, thus yielding higher electrochemical signals. As shown in Fig. 6a, the anodic peak current corresponding to sample 1 (5.26 μA) is comparable with that acquired upon hybridization of the pre-immobilized biotinylated probe to apoE 2/3 gene with a single base mismatch relative to apoE 4 gene in Fig. 4a, which suggests the absence of apoE 4 gene in sample 1. The anodic peak current in the case of sample 2 (4.18 μA) is much lower than that in the case of sample 1, indicating the existence of apoE 4 gene in sample 2. To further demonstrate the amenability of the method for real sample analysis, assay of two more genomic DNA extracts from serum samples was carried out and the concentrations of apoE 4 gene in these samples were also determined (Fig. 6b). The decreased anodic peak currents corresponding to samples 2, 3 and 4 indicate the existence of one or two apoE 4 alleles, which presents higher risk for AD. The absence of the apoE 4 gene does not exclude the presence of apoE 2/3 allele which is neutral or even protective for AD. The above results were consistent with those by the single-base extension assay carried out by KingMed Diagnostics Inc. (Tianjin, China). The method is highly sensitive, selective, and quantitative, serving as a viable means for apoE 4 gene assay in AD-related clinical samples. However, the electrode surface with the modification of the DNA probes can not be regenerated, a novel electrode sensors which can be regenerated still need to be develop.

Conclusion

Proof-of-principle demonstration of apoE 4 gene quantification has been performed by the cleavage of HhaI and signal amplification by Fc-capped gold nanoparticle/streptavidin. The restriction enzyme HhaI can selectively cleave the GCGC base pairs in the apoE 4 gene duplex. The mismatched sequences prevent the enzymatic cleavage reaction which lead to different voltammetric signals. The method is amenable to the quantification of the apoE 4 sequences directly from diluted genomic DNAs. Four genomic DNAs from serum samples were assayed and three samples contains apoE4 gene and the concentration was accurately measured. The limitation of the assay is the reproducibility of the assay needs to be improved. The capability of the method for quantification of apoE 4 gene in unamplified genomic DNA extracts provides a viable means for point-of-care diagnosis of neurodegenerative diseases.

Acknowledgments The authors thank the financial support of this work by the National Natural Science Foundation of China (Nos. 21876208, 21705166, 21575166).

Compliance with ethical standards The author(s) declare that they have no competing interests.

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