Biosensors and Bioelectronics 25 (2010) 1088–1094

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Label-free electrochemical DNA biosensor array for simultaneous detection of the HIV-1 and HIV-2 oligonucleotides incorporating different hairpin-DNA probes and redox indicator

Dongdong Zhang, Yage Peng, Honglan Qi, Qiang Gao, Chengxiao Zhang*

Key Laboratory of Applied Surface and Colloid Chemistry (Shaanxi Normal University), Ministry of Education, School of Chemistry and Materials Science, Shaanxi Normal University, Xi'an 710062, PR China

ARTICLE INFO

Article history:
Received 17 July 2009
Received in revised form 4 September 2009
Accepted 22 September 2009
Available online 1 October 2009

Keywords:
Biosensor array
DNA hybridization
Electroanalysis
Simultaneous detection
Human immunodeficiency virus

ABSTRACT

A label-free electrochemical DNA biosensor array was developed as a model system for simultaneous detection of multiplexed DNAs using microlitres of sample. A novel multi-electrode array was comprised of six gold working electrodes and a gold auxiliary electrode, which were fabricated by gold sputtering technology, and a printed Ag/AgCl reference electrode was fabricated by screen-printing technology. The DNA biosensor array for simultaneous detection of the human immunodeficiency virus (HIV) oligonucleotide sequences, HIV-1 and HIV-2, was fabricated in sequence by self-assembling each of two kinds of thiolated hairpin-DNA probes onto the surfaces of the corresponding three working electrodes, respectively. The hybridization events were monitored by square wave voltammetry using methylene blue (MB) as a hybridization redox indicator. The oxidation currents of MB accumulated on the array decreased with increasing the concentration of HIVs due to higher affinity of MB for single strand rather than double strands of DNA. Under the optimized conditions, the peak currents were linear over ranges from 20 to 100 nM for HIV-1 and HIV-2, with the same detection limits of 0.1 nM (S/N = 3), respectively. The biosensor array showed a good specificity without the obvious cross-interference. Furthermore, single-base mutation oligonucleotides and random oligonucleotides can be easily discriminated from complementary target DNAs. This work demonstrates that different hairpin-DNA probes can be used to design the label-free electrochemical biosensor array for simultaneous detection of multiplexed DNA sequences for various clinical applications.

1. Introduction

A rapid, inexpensive and easy-to-perform detection assay of multiplexed pathogenic viruses DNA from a single solution and a small amount of sample is of great demand in biomarker profiling and clinical diagnostics (Stoeva et al., 2006; Lin et al., 2007). Microarray is a powerful device for multiplexed analysis and high-throughput screening (Thompson et al., 2005). Numerous biosensor arrays have been developed for the detection of DNAs (Vet et al., 1999; Cao et al., 2002; Wang et al., 2003; Stoermer et al., 2006; Stoeva et al., 2006; Levine et al., 2009), proteins (Dai et al., 2006; Lao et al., 2006) and carbohydrates (Stevens et al., 2006; Liang et al., 2007). In recent years, DNA biosensor array was shown to be a powerful tool for high throughput measurements of gene expression and genomic diagnosis, such as cancer, allergy and autoimmunity (Lockhart and Winzeler, 2000; Staudt and Brown, 2000; Staudt, 2002). The DNA biosensor arrays reported mostly use optical transducers such as fluorescence (Vet et al., 1999; Stoermer et al., 2006; Stoeva et al., 2006), Raman scattering spectroscopy (Cao et al., 2002), chemiluminescence (Mallard et al., 2005). Although DNA biosensor array using optical readout has many advantages such as high sensitivity and high-throughput (hundred thousands of probes) (Dufva, 2005), it needs much expensive instrument for optical imaging, laser light and labeled probes. Furthermore, the detection limit of optical biosensor array depends on the thickness of the test solution. Compared to optical biosensor array, electrochemical biosensor array has many advantages such as inexpensive instrument, low detection limit and simplicity due to ease of obtaining electrical signal.

Extensive efforts have been devoted to design novel electrochemical DNA biosensor array to improve the sensitivity, to reduce the cost and to simplify the assay steps. They include employment of different electrode materials and electrode configurations; development of efficient immobilization methods; exploitation of novel redox probe and label-free strategies. Two methods in the design of electrochemical DNA biosensor array

* Corresponding author. Tel.: +86 29 85303825; fax: +86 29 85307567.
E-mail address: cxzhang@snnu.edu.cn (C. Zhang).

0956-5663/$ – see front matter.
doi:10.1016/j.bios.2009.09.032

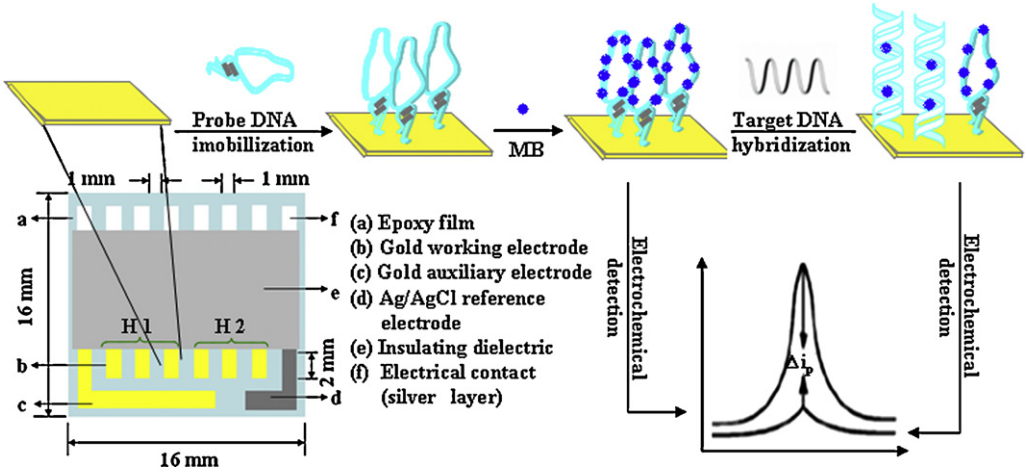

Fig. 1. Schematic diagrams of multi-electrode array and representation of biosensor array with fabrication steps and performance.

have been exploited, including label and label-free approaches. In labeled approach, the DNA probe or target sequence, which was tagged with the redox label such as quantum dots (Wang et al., 2003), ferrocene (Levine et al., 2009) or horseradish peroxidase (Wan et al., 2009) has been reported. Wang et al. (2003) demonstrated a multi-target electrochemical DNA detection using three encoding quantum dots (zinc sulfide, cadmium sulfide, and lead sulfide) to tag the three DNA targets in connection with a sandwich hybridization assay and obtained different signals from stripping voltammetry of the corresponding heavy metals. Levine et al. (2009) expatiated that the multiplexed detection of DNA hybridization using the complementary metal-oxide-semiconductor biosensor array by functionalizing the chip with two distinct probes and then hybridizing each with its complementary target DNA which was covalently attached with ferrocene redox molecule label. Wan et al. (2009) reported an electrochemical protocol for the detection of multiple DNA targets with an electrode array, which relied on the "sandwich-type" detection strategy and horseradish peroxidase-based amplification. Recently, Henry et al. (2009) reported a label-free strategy for the exploitation of methylene blue (MB) as an electrochemical hybridization indicator of simulated post-PCR long single-stranded DNA (ss-DNA) sequences. By comparison with label-free strategy, label strategy requires a complicated labeling procedure that can affect the bioaffinity of the probe. Thus, increasing efforts were focused on developing a label-free electrochemical DNA biosensor array.

Compared to sensors for single analyte detection, DNA sensor array for multiplexed analytes detection requires the probes to have higher sensitivity and specificity (Liu et al., 2007). One such promising probe for quantitative genomic studies is hairpin-DNA, which was at first described by Tyagi and Kramer (1996), a ss-DNA molecule composed of a hairpin shaped oligonucleotide that possess a stem-and-loop structure. Fan et al. (2003) first reported a strategy for transduction of DNA hybridization into an electrochemical signal based on an electroactive ferrocene-tagged hairpin-DNA probe by means of a conformational change built on an electrochemical DNA (E-DNA) sensor. Jin et al. (2007) reported an E-DNA sensor for the highly selective detection of the p53 gene by immobilizing hairpin-DNA on a gold electrode and using MB as an external redox indicator. And recently, our group developed a highly selective electrogenerated chemiluminescence biosensor for the detection of target DNA using hairpin-DNA as the recognition element and ruthenium complex as the signal-producing compound (Zhang et al., 2008). Hairpin-DNA probe has been found to exhibit extraordinary stability, better selectivity, and higher specificity than similar assays performed using linear ss-DNA (Williams and Hall, 1996; Riccelli et al., 2001). In addition, different hairpin-DNA probes can be designed by choosing loop sequence and size, in other words, we could design multiplexed hairpin-DNA probes as different recognition elements of biosensor array for the detection of multiple DNA analytes.

The World Health Organization (WHO)/Joint United Nations Program on HIV/AIDS (UNAIDS) estimates that 40 million people are living with human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) infection to date (McMichael, 2006). Current estimates suggest that less than 1 in 10 people are aware of their HIV sero-status (Pant Pai, 2007). The standard diagnostic tests for HIV infection are blood tests: enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA), and Western blot test. In all of these tests, check for the presence of antibodies to HIV, they do not check for the virus itself (http://www.ehealthmd.com). Compared to immunoassay in which antibodies need a long period to generate, genetic testing can be detected with real-time once the patient was infected. Therefore, highly specific, rapid, and convenient HIV detection systems are required to facilitate identification of infected individuals, preventing them from further infecting others, monitoring disease progression of infected individuals, and investigating clinical trials of new drugs or vaccine candidates (Kim et al., 2009).

The aim of the present work is to design and fabricate a simple, inexpensive and stable multi-electrode array and to develop a label-free electrochemical DNA biosensor array for the simultaneous detection of HIV-1 and HIV-2 as a model system of multiplexed DNA sequences-specific analysis using microlitres of sample. Schematic diagram of the multi-electrode array designed is shown in Fig. 1. A multi-electrode array was comprised of six gold working electrodes and a gold auxiliary electrode, which were fabricated by gold sputtering technology, and a printed Ag/AgCl reference electrode was fabricated by screen-printing technology. The DNA biosensor array for simultaneous detection of HIV-1 and HIV-2 was fabricated in sequence by self-assembling each of two kinds of thiolated hairpin-DNA probes onto the surfaces of the corresponding three working electrodes, respectively. The hybridization events were monitored by square wave voltammetry using MB as a hybridization redox indicator. The oxidation currents of MB decreased with increasing the concentration of HIVs due to higher affinity of MB for single strand rather than double strands of DNA. In this paper, the fabrication and the performance of biosensor array were presented. Up to date, based on two different hairpin-DNA probes label-free biosensor array for the quantization of multiplexed DNA sequences-specific analysis has not been reported.

2. Materials and methods

2.1. Materials and reagents

BY-2100 silver paste was purchased from Shanghai Bao Yin Electronic Materials Ltd. (Shanghai, China). The base substrate for multi-electrode array was an epoxy film (0.5 mm thickness). 6-Mercapto-l-hexanol (MCH) was obtained from Sigma (USA). Methylene blue (MB) was obtained from Xi'an Chemical Reagent Company (Xi'an, China). All DNA oligonucleotides including probes (H1 and H2) and targets (HIV-1 and HIV-2), which were adopted from Vet et al. (1999), HIV-1 M1 and HIV-2 M2, which were designed according to Jin et al. (2007), were synthesized by Shanghai Sangon Biological Science & Technology Company (Shanghai, China).

H1 GCGAGCCTGGGATTAAATAAAATAGTAAGAATGTATAGCGCTCGC-$(CH_2)_6$-SH
H2 GCGAGCAAAGGACCAGGCGCAACTAAATTCAGCTCGC-$(CH_2)_6$-SH
HIV-1 (H1 complement) GCTATACATTCTTACTATTTTATTTAATC-CCAG
HIV-2 (H2 complement) TGAATTTAGTTGCGCCTGGTCCTTT
HIV-1 M1 (single mismatch of H1) GCTATACATTCTTAATATTT-TATTTAATCCCAG
HIV-2 M2 (single mismatch of H2) TGAATTTAGTTGCACCTGGTC-CTTT

Underline means the binding sequence of the probe. The oligonucleotide stock solutions and more dilute solutions of targets (10 μM) were prepared with 20 mM Tris–HCl containing 100 mM $MgCl_2$ (pH 8.00) and kept frozen. 50 mM sodium phosphate buffer saline (PBS, pH 7.00) containing 100 mM KCl was used as electrolyte buffer and washing solution. All reagents were of analytical grade, and Millipore Milli-Q water (18 MΩ) was used throughout.

2.2. Apparatus

All electrochemical measurements were performed using a CHI 1030A multi-channel electrochemical workstation or a CHI 660B electrochemical workstation (Shanghai Chenhua Instrument Co. Ltd., Shanghai, China). Gold sputtering was performed using SCD-005 sputter coater instrument (Quanta 200, FEI, Holand).

2.3. Fabrication of base multi-electrode array

Schematic diagram of the multi-electrode array designed in this study is shown in Fig. 1. A base substrate (*a*, an epoxy film, 16 mm × 16 mm × 0.5 mm) was rinsed with water, cleaned with acetone, ethanol and dried in air. A gold multi-electrode strip (*b, c, d* and *f*) was deposited onto an cleaned epoxy film using the gold sputtering (Bal-tec, 80 mA during 240 s, sample distance: 5 cm) at 0.05 mbar Ar plasma pressure through a designed pattern (16 mm × 16 mm, 100-μm thick), and then the papery pattern was peeled off and the gold multi-electrode strip was hard-baked at 120 °C for 60 min. The Ag/AgCl reference electrode (*d*) and electrical contact (*f*) were made by printing silver paste onto the surface of the gold strip and then soft-baking at 80 °C for 60 min, and finally electrochemically chloridizing Ag/Au reference strip in a 0.10 M HCl (Shen and Liu, 2007). In order to form small-area working electrodes, an insulating layer (*e*) was made by hand-coating a thick film of hydrophobic epoxy adhesive.

2.4. Fabrication of biosensor array

2.0 μL of 1.0×10^{-6} M H1 (H2) solution was only dropped onto the surface of the corresponding working electrodes numbered channels CH1, CH2 and CH3 (CH4, CH5 and CH6) of multi-electrode array, allowed to self-assemble for 6 h in the moist environment of culture disk in the water bath (kept at 37 ± 0.1 °C), and then rinsed with washing solution to remove the weakly adsorbed H1 (H2). Finally, 20.0 μL of 1.0 mM MCH was dropped onto the surfaces of channels from CH1 to CH6 for 30 min to block the un-assembled surfaces, and rinsed with washing solution thoroughly. It should be noted that a cross-flowing of the immobilizing solution must be avoided in fabrication of the biosensor array.

2.5. Electrochemical measurement

The biosensor array fabricated with above-described protocol was immersed in a stirred solution containing 25 μM MB–50 mM PBS–100 mM KCl (pH 7.00) for 5 min to accumulate MB and then rinsed with washing solution. 20.0 μL of a fixed concentrations of target DNAs mixture solution containing HIV-1 and HIV-2 was dropped onto the working electrodes region, allowed to hybridize for 60 min in the moist environment of culture disk in the water bath (kept at 37 ± 0.1 °C) and then the biosensor array was rinsed with washing solution. The square wave voltammetric (SWV) measurement was performed in the potential range from −0.5 to −0.1 V with a amplitude of 0.05 V and a frequency of 15 Hz in 100 μL of electrolyte buffer that the electrolyte buffer was dropped onto the area of working electrodes, counter electrode and reference electrode of the biosensor array and formed a subminiature electrolytic cell. The concentrations of HIV-1 and HIV-2 were quantified by decreased average of oxidation peak currents at three channels ΔI ($\Delta I = I_0 - I$), where I_0 and I was the oxidation peak current before and after hybridization, respectively. All measurement experiments were carried out at room temperature (20 ± 1 °C).

3. Results and discussion

3.1. The electrochemical characteristics of biosensor array

The multi-electrode array freshly fabricated was electrochemically characterized by cyclic voltammetry in 0.1 M H_2SO_4 (see Supplementary material, Fig. S1). The characteristic single sharp reduction peaks located at ~0.6 V and multiple overlapping oxidation peaks appeared in the range of 1.0–1.3 V. The average reduction peak potential obtained at six electrodes was 0.585 ± 0.003 V, RSD = 0.18%, and the average reduction peak current obtained at six electrodes was $(1.417 \pm 0.059) \times 10^{-5}$ A, RSD = 4.2%. This indicates that the multi-electrode array fabricated has a same surface morphology and area of six working electrodes (Zhang et al., 2007). The real working electrodes area was estimated to be 0.030 ± 0.002 cm^2, respectively (Trasatti and Petrii, 1991). The thickness of the gold film sputtered was estimated to be ~30 nm by the time of gold sputtering. The good reproducibility was obtained when the cyclic scans were more than 50 cycles between 0 and 1.5 V. This suggests that the multi-electrode array fabricated has a good reproducibility and can be used as working electrodes. Furthermore, it was found that the sputtered gold film on epoxy substrate without an adhesion underlayer was more stable than that on glass or silica substrate.

Fig. 2 shows the Nyquist plots of faradic impedance spectroscopy (FIS) at immobilization steps of probe H1 (Fig. 2, H1) and H2 (Fig. 2, H2). From Fig. 2, it can be seen that the multi-electrode array exhibits a very small semicircle at high frequencies (curves of a and a′). When H1 and H2 were self-assembled onto the array, the R_{et} increased to 771 Ω (curve b) and 652 Ω (curve b′), respectively. This is attributed to fact that self-assembled hairpin-DNA probe having a single strand nucleic acid with negative charges on its phosphate backbone makes an electrostatic repulsive force to

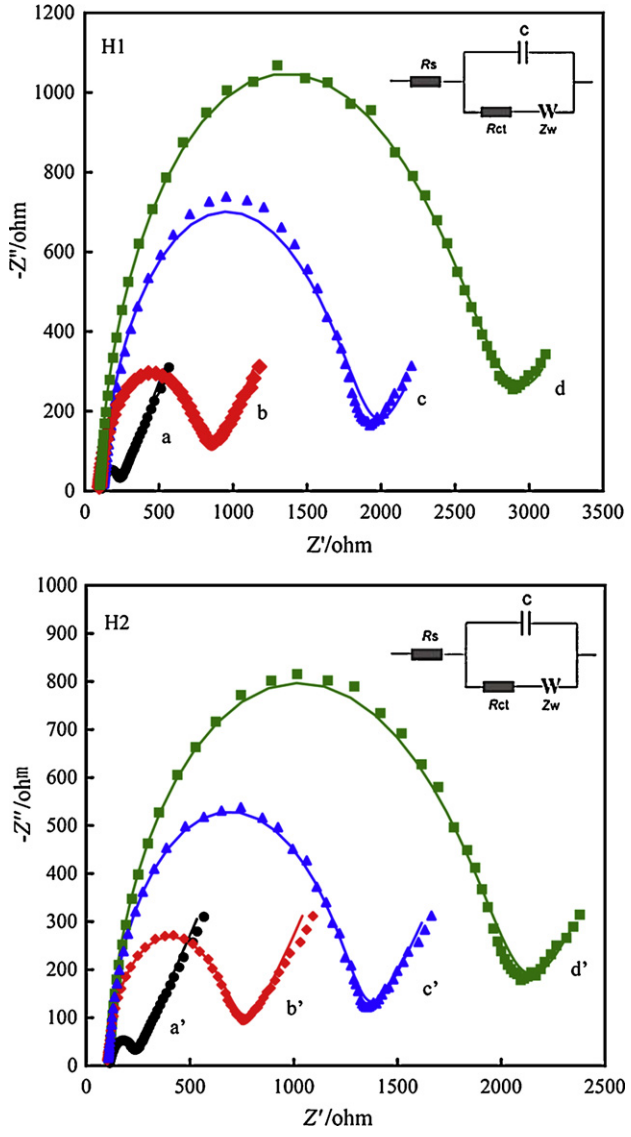

Fig. 2. Faradic impedance spectra (FIS) of multi-electrode array in each immobilization step and binding step in 100 μL of 0.1 M PBS solution (pH 7.40) containing 10 mM $K_3Fe(CN)_6$–10 mM $K_4Fe(CN)_6$. H1: (a) Bare Au; (b) H1/Au; (c) H1/MCH/Au; (d) HIV-1/H1/MCH/Au; H2: (a′) bare Au; (b′) H2/Au; (c′) H2/MCH/Au; (d′) HIV-2/H2/MCH/Au. Fitted data (solid line). The frequency was from 100 kHz to 0.1 Hz and the amplitude was 5.0 mV. The inset shows the equivalent circuit applied to fit the impedance spectroscopy.

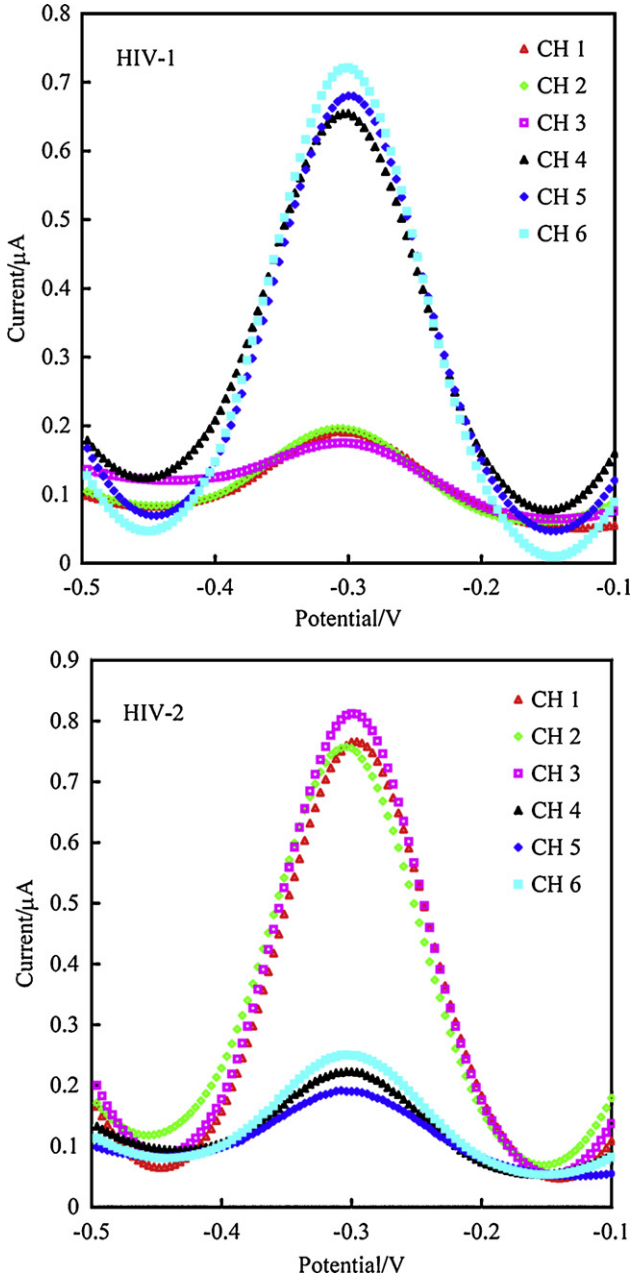

Fig. 3. Cross-interference assays of the biosensor array for HIV-1 and HIV-2 in the 100 μL of electrolyte buffer solution. The concentrations of H1 and H2 were 1.0×10^{-6} M, the concentrations of HIV-1 and HIV-2 were 2.5×10^{-7} M, respectively. Accumulation concentration of MB was 25 μM, accumulation time was 5 min and hybridization time was 60 min. CH representation of different channel (biosensor).

$[Fe(CN)_6]^{3-/4-}$. This indicates that the probes have been assembled onto the array. Additionally, the R_{et} of channels of H1 immobilized was bigger than that of H2 immobilized, attributed to the fact that the length of loop base sequence of the H1 (33 base) is longer than that of the H2 (25 base). When a short-chain alkyl molecule MCH was employed to occupy the left bare sites on the array for eliminating the non-specific adsorption, the R_{et} further increased to 1849 Ω (curve c) and 1240 Ω (curve c′), respectively. After HIV-1 and HIV-2 were hybridized onto the surface of the array, the R_{et} greatly increased to 2832 Ω (curve d) and 2014 Ω (curve d′), respectively. This is probably attributed to the fact that a double strands of DNA on the surface of the array is formed and the transfer of redox couple $[Fe(CN)_6]^{3-/4-}$ to the surface of gold electrode is then prohibited. After target DNAs (HIV-1 and HIV-2) were hybridized, the increased ratio of the R_{et} was 53% for HIV-1 and 62% for HIV-2, respectively. This also suggests that a fabricated DNA biosensors array can detect the HIV-1 and HIV-2 in FIS approach.

The DNA biosensor array was also checked by square wave voltammetry for the detection of HIV-1 and HIV-2. Fig. 3 shows the square wave voltammograms of fabricated biosensor array with 20.0 μL of 2.5×10^{-7} M HIV-1 or HIV-2. From Fig. 3 HIV-1, it can be seen that when the sample solution only contains HIV-1, much smaller peak currents appear only at the channels CH1∼CH3, in which H1 is immobilized, indicating that the channels CH1∼CH3 only have distinct responses for HIV-1. Much large peak currents appear at the channels CH4∼CH6, in which H2 is immobilized, indicating that the channels CH4∼CH6 have not obvious responses. The $\Delta I/I_0$ were 16.2%, 12.1% and 8.5% (RSD = 3.9%); 86.3%, 84.7% and 88.7% (RSD = 2.0%) for CH1∼CH6, respectively. This demonstrated that the H1 channels of the array had fine responses for

HIV-1 target. From Fig. 3 HIV-2, it can be also seen that when the sample solution only contains HIV-2, much smaller peak currents appear only at the channels CH4∼CH6, indicating that the channels CH4∼CH6 have distinct responses for HIV-2. Much larger peak currents appear at the channels CH1∼CH3, indicating that the channels CH1∼CH3 have not obvious responses. The $\Delta I/I_0$ were 77.6%, 81.7% and 74.3% (RSD = 3.7%); 21.3%, 23.6% and 15.7% (RSD = 4.1%) for CH1∼CH6, respectively. It is concluded that: (1) The biosensor array showed a good specificity without the obvious cross-interference, which is essential in the performance of the biosensor array. (2) A fabricated DNA array can simultaneously detect the HIV-1 and HIV-2 and the sensitivity of the biosensor array in proposed approach is higher than that in FIS approach. Therefore, square wave voltammetry was used in following experiments.

3.2. Optimization of the self-assembly time, MB concentration and accumulation time

The self-assembly time, MB concentration and accumulation time were optimized to obtain high sensitivity and reproducibility of the biosensor array. The dependence ΔI on the self-assembly time for H1 and H2 showed that the ΔI increased with increasing self-assembly time from 2 to 6 h, attributed to the fact that the surface density increased from 3.8×10^{12} to 7.4×10^{12} molecules cm^{-2} of 1.0×10^{-6} M H1 and from 4.2×10^{12} to 7.9×10^{12} molecules cm^{-2} of 1.0×10^{-6} M H2, which was measured by chronocoulometry in 10 mM Tris–HCl containing 50 μM $[Ru(NH_3)_6]^{3+}$ according to the method employed in our previously work (Li et al., 2008), and is in good agreement with that reported previously (Steel et al., 1998; Li et al., 2008). The ΔI reached a maximum at 6 h (see Supplementary material, Fig. S2). With further increase of the self-assembling time, however, the ΔI decreased, attributed to steric and electrostatic hindrance arising from the more tightly packed probe and some interstitial space between the probes, which is necessary for high hybridization efficiency. Therefore, the self-assembly time of 6 h was employed in following experiments.

Both the concentration of MB and accumulation time affected the quantity of MB accumulated on ss-DNA probe and ds-DNA, led to a much effect on the sensitivity of the biosensor array. The effect of the concentration of MB on ΔI was studied when accumulation time was fixed as 5 min. The ΔI at both H1 channels and H2 channels increased rapidly with increasing the concentration of MB from 2.0 to 20 μM, and then reached a constant value (see Supplementary material, Fig. S3). Therefore, 25 μM MB was chosen as the concentration of MB used in following experiments.

The effect of the accumulation time on the ΔI was also studied when the concentration of MB was fixed as 25 μM MB. The result showed that the ΔI increased with increasing accumulation time up to 5 min and then reached a constant value (see Supplementary material, Fig. S4). Therefore, 5 min was chosen as the optimum accumulation time.

3.3. Performance of biosensor array

3.3.1. Simultaneous detection of HIV-1 and HIV-2

Under the optimized condition, the dependence of the ΔI on the fixed concentrations of target HIV-1 and HIV-2 mixture solutions in the range from 1 to 250 nM were examined (Fig. 4). The result showed that the oxidation peak currents were linear over ranges from 20 to 100 nM for HIV-1 and for HIV-2, with the same detection limits of 0.1 nM (S/N = 3), respectively. The linear regression equations were ΔI (μA) = −0.0045C (nM) + 0.60 (R = 0.9974) for HIV-1 and ΔI (μA) = −0.0036C (nM) + 0.47 (R = 0.9989) for HIV-2, respectively. The obtained linear ranges and detection limits of label-free electrochemical DNA biosensor array in this work are

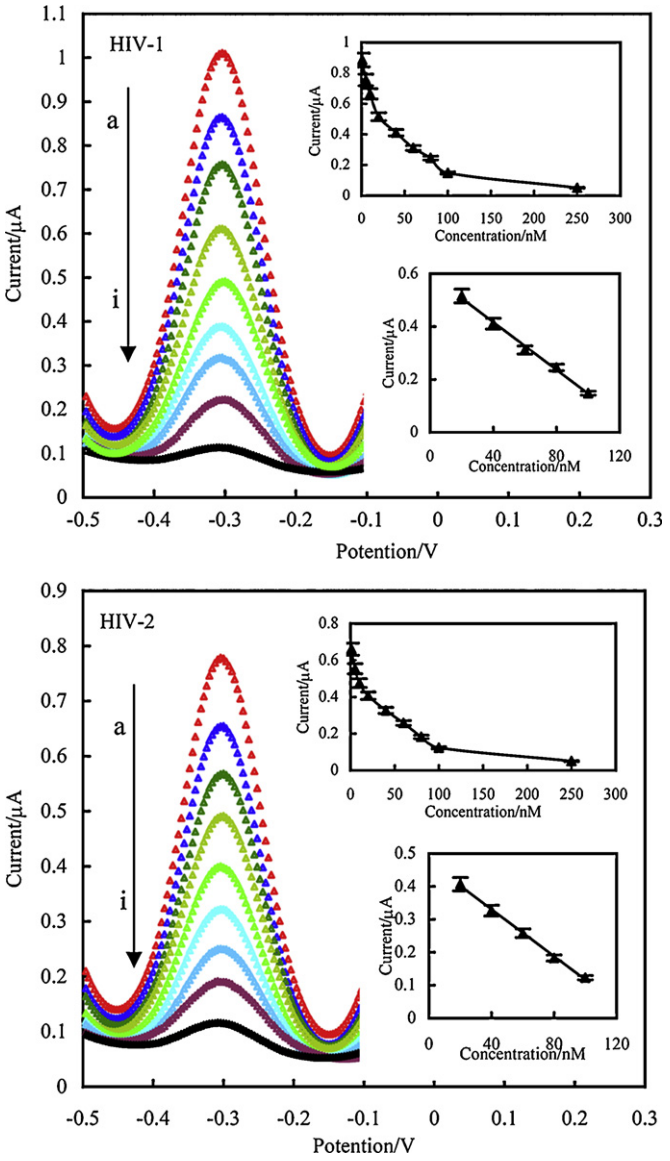

Fig. 4. Simultaneous detection of the biosensor array for HIV-1 and HIV-2 in the 100 μL of electrolyte buffer solution. Curve a: 0 nM, curve b: 1 nM, curve c: 10 nM, curve d: 20 nM, curve e: 40 nM, curve f: 60 nM, curve g: 80 nM, curve h: 100 nM, and curve i: 250 nM. (Inset) Plot of peak current vs. the concentration of HIV. The relative standard deviations (percentage of RSD) are all less than 5%. Other conditions were same as Fig. 3.

similar to that (25–100 nM, 0.2 nM for the p53 gene) of the label-free single biosensor reported by Jin et al. (2007) and excelled that (6.5–50 nM, 17.5 nM for the BRCA1 gene) of the label-free biosensor array reported by Henry et al. (2009). Therefore, a highly sensitivity of label-free electrochemical DNA biosensor array designed was obtained.

3.3.2. Specificity of the biosensor array

To evaluate the specificity of the biosensor array fabricated using H1 and H2 as probes, special species HIV-1 and HIV-2 for complementary targets, and HIV-1 M1 and HIV-2 M2 for single mismatch targets were investigated. It is noted that the location of mutation selected in the center of target DNAs duo to its greatest effect on the hybridization according to Jin et al. (2007). The detailed data on specificity assays of biosensor array for different target DNAs are listed in Table 1. Before the fabricated array

Table 1
Specificity assays of biosensor array for different target DNAs.

Hairpin probe/combined bases	Target DNA/bases	ΔG (kcal/mol)[a] (37 °C)	Current from SWV (μA)[f]	$\Delta I/I_0$ (%)
H1/33[b]-6[c]	No	−3.1	0.885 ± 0.048	0
	HIV-1/33[d]	−36.1	0.148 ± 0.005	83.3%
	HIV-2/25[d](4[e])	−7.3	0.693 ± 0.030	21.7%
	HIV-1 M1/33[d] (32[e])	−33.2	0.504 ± 0.017	43.1%
	HIV-2 M2/25[d] (4[e])	−4.7	0.744 ± 0.036	13.0%
H2/25[b]-6[c]	No	−4.2	0.661 ± 0.028	0
	HIV-1/33[d](3[e])	−5.2	0.587 ± 0.025	11.2%
	HIV-2/25[d]	−32.7	0.123 ± 0.006	81.4%
	HIV-1 M1/33[d] (3[e])	−5.2	0.622 ± 0.027	5.9%
	HIV-2 M2/25[d] (24[e])	−27.1	0.321 ± 0.015	51.4%

[a] ΔG means the free energy of duplex formation.
[b] The number in superscript is the number of loop base.
[c] The number in superscript is the number of length base.
[d] The number in superscript is the number of base.
[e] The number in superscript is the number of complementary base.
[f] H1, H2: 1.0×10^{-6} M; T1, T2, T1M1, T2M2: 2.5×10^{-7} M.

was hybridized with target DNAs, the obtained average current of channels CH1∼CH3 (0.885 ± 0.048) μA (RSD = 5.4%) was bigger than that of channels CH4∼CH6 (0.661 ± 0.028) μA (RSD = 4.2%). This is attributed to the fact that the guanine percentage content (*G*%) of H1 (24.5%) is bigger than that of H2 (21.3%) (Yang et al., 2002). The $\Delta I/I_0$ of H1-immobilized, channels CH1∼CH3 for different target DNAs were 83.3% for HIV-1, 43.1% for HIV-1 M1, 21.7% for HIV-2 and 13.0% for HIV-2 M2, respectively. This is accord with the order of the values of ΔG for the possible duplex formed between H1 and their targets, H1∼HIV-1 (−36.1 kcal/mol) < H1∼HIV-1 M1 (−33.2 kcal/mol) < H1∼HIV-2 (−7.3 kcal/mol) < H1∼HIV-2 M2 (−4.7 kcal/mol), as predicted with the computer program RNA Structure v. 4.5 (SantaLucia, 1998; Mathews et al., 1999). The biggest $\Delta I/I_0$ of H1-immobilized, channels CH1∼CH3 for HIV-1 was obtained, indicting that the CH1∼CH3 of fabricated array has a good specificity for respected target HIV-1. As the same way, the $\Delta I/I_0$ of H2-immobilized, channels CH4∼CH6 for different target DNAs were 81.4% for HIV-2, 51.4% for HIV-2 M2, 11.2% for HIV-1 and 5.9% for HIV-1 M1, respectively. This also is accord with the order of the values of ΔG for the possible duplex formed between H2 and their targets, H2∼T2 (−32.7 kcal/mol) < H2∼HIV-2 M2 (−27.1 kcal/mol) < H2∼HIV-1 (−5.2 kcal/mol) = H2∼HIV-1 M1 (−5.2 kcal/mol), as predicted. It was found that the biggest $\Delta I/I_0$ of H2-immbilized, channels CH4∼CH6 for HIV-2 was obtained, indicting that the CH4∼CH6 of fabricated array has a good specificity for respected target HIV-2. The result indicates that single-base mutation oligonucleotides and random oligonucleotides can be easily discriminated from complementary target DNAs and the biosensor array fabricated can be used for multiplexed DNA sequences-specific analysis. The result suggests that the specificity of the biosensor array mainly depends on the ΔG of the possible duplex formed between the probe and their targets. It is noted that the specificity of the biosensor array designed in this work for single mismatch targets should be improved.

4. Conclusion

The present study described a fabrication of a simple, inexpensive, and stable multi-electrode array. A label-free electrochemical DNA biosensor array for the simultaneous detection of specific multiplexed DNA sequences HIV-1 and HIV-2 as a model system using microlitre of sample has developed. The biosensor array showed good specificity without obvious cross-interference for the simultaneous analysis of multiplexed DNA sequences. Furthermore, single-base mutation oligonucleotides and random oligonucleotides can be easily discriminated from complementary target DNAs. This work demonstrates that different hairpin-DNA probes can be used to design the label-free electrochemical biosensor array for simultaneous detection of multiplexed DNA sequences for various clinical applications, furthering the application in proteomics research.

Acknowledgements

Financial supports from the National Science Foundations of China (No. 90607016) and excellent PhD program of Shaanxi Normal University (No. S2008YB03) are gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.09.032.

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