analytical chemistry

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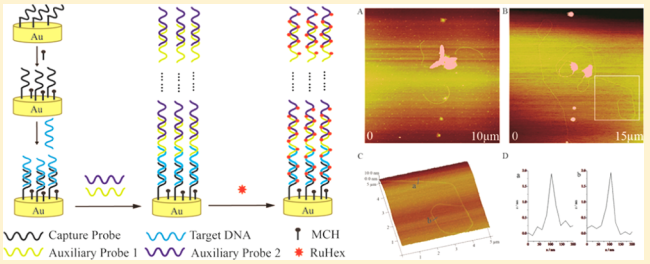
Enzyme-Free and Label-Free Ultrasensitive Electrochemical Detection of Human Immunodeficiency Virus DNA in Biological Samples Based on Long-Range Self-Assembled DNA Nanostructures

Xian Chen,[†] Cheng-Yi Hong,[†] Ya-Hui Lin,[†] Jing-Hua Chen,*[,†,‡] Guo-Nan Chen,[†] and Huang-Hao Yang*[,†]

[†]The Key Lab of Analysis and Detection Technology for Food Safety of the MOE, Fujian Provincial Key Laboratory of Analysis and Detection Technology for Food Safety, College of Chemistry and Chemical Engineering, Fuzhou University, Fuzhou 350108, China

[‡]Department of Pharmaceutical Analysis, Faculty of Pharmacy, Fujian Medical University, Fuzhou 350004, China

ABSTRACT: Biosensors based on nanomaterials have been used for detection of various biological molecules with high sensitivity and selectivity. Herein, we developed a simple and ultrasensitive electrochemical DNA biosensor using long-range self-assembled DNA nanostructures as carriers for signal amplification, which can achieve an impressive detection limit of 5 aM human immunodeficiency virus (HIV) DNA even in complex biological samples. In this study, we designed two auxiliary probes. A cascade of hybridization events between the two auxiliary probes can lead to long-range self-assembly and form micrometer-long one-dimensional DNA nanostructures. In the presence of target DNA, each copy of the target can act as a trigger to connect a DNA nanostructure to a capture probe on the electrode surface. Then, a great amount of redox indicator $[Ru(NH_3)_6]^{3+}$ can be electrostatically bound to the DNA nanostructures and eventually result in significantly amplified electrochemical signals.



DNA detection methods have attracted considerable interest in the past few years due to their broad applications in virus detection, transgenic detection, and early diagnosis of diseases.[1−5] Due to the low-abundant DNA biomarkers linked to the diseases in biological samples, it is highly desirable to develop ultrasensitive methods for DNA detection. Up to now, numerous amplified DNA detection methods, based on DNA amplification technology, have been developed for the ultrasensitive sequence-specific detection of DNA, such as polymerase chain reaction (PCR),[6] rolling circle amplification (RCA),[7−9] and nucleic-acid sequence-based amplification (NASBA).[10] Although these methods can be used for detection of exceedingly low levels of DNA, some inherent issues still can not be avoided, for example, complex operations, costly polymerase, tedious labels, and dedicated instrumentation. Electrochemical methods,[1,11] being simple, portable and low-cost, are particularly attractive for DNA detection.[12−16] However, the sensitivity is one of the most important limiting factors for the development of electrochemical DNA biosensors. Many efforts have been devoted to realizing the ultrasensitive electrochemical DNA detection by signal amplification. By means of nuclease,[17,18] some novel paradigms for the amplified detection of DNA even can rival PCR for sensitivity. However, these enzyme-based electrochemical DNA biosensors suffer from the complexity of the experimental system. Moreover, false-positives may happen during the amplification process sometimes. Therefore, it is highly desirable to develop the ultrasensitive electrochemical DNA biosensors without the need of nuclease.

As a vital part of modern nanotechnology, DNA self-assembly has attracted widespread interest since Seeman proposed the possibility of the use of DNA as a structural material for self-assembly in 1982.[19] Nowadays, many self-assembled DNA nanostructures have been fabricated[20−23] and applied in biosensing, such as sensing platform based on tetrahedron-structured probes[24] and DNA-detection system based on hybridization chain reaction (HCR).[25−27] The fabrication of these biosensors depends on the precise and complex design of the DNA probes that can self-assemble into specific structures. However, there is still a need to further improve the sensitivity of these sensors. One dimensional nanostructures[28] are the smallest dimension structures that can be used for biosensor fabrication and efficient transport of electrons, so a self-assembled one-dimensional DNA nanostructure is worth a try for ultra highly sensitive detection of DNA.[29−31]

In this study, we fabricated a simple and ultrasensitive electrochemical DNA biosensor using long-range self-assembled DNA nanostructures as carrier for signal amplification. We designed two auxiliary DNA probes, named AP1 and

Received: June 27, 2012
Accepted: September 6, 2012
Published: September 6, 2012

   dx.doi.org/10.1021/ac3017828 | *Anal. Chem.* 2012, 84, 8277−8283

Table 1. Sequence of Synthesized Oligonucleotides Probes Used in This Work[a]

oligonucleotide	sequence (from 5′ to 3′)
capture probe (CP)	CAGTGTGGAAAATCTCTAGC-$(CH_2)_6$-SH
complementary target DNA (TD)	GCTAGAGATTTTCCACACTG*ACTAAAAGGGTCTGAGGG*
auxiliary probe 1 (AP1)	**TACTCCCCCAGGTGC***CCCTCAGACCCTTTTAGT*
auxiliary probe 2 (AP2)	**GCACCTGGGGGAGTA***ACTAAAAGGGTCTGAGGG*
single-base mismatch target (1MT)	GCTAGAGATTGTCCACACTGACTAAAAGGGTCTGAGGG
two-base mismatch target (2MT)	GCTAGAGATTGGCCACACTGACTAAAAGGGTCTGAGGG
noncomplementary sequence (NC)	CCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGC

[a]CP is completely complementary with the 5′ end of TD. The underlined bases are the mismatched bases of 1MT and 2MT.

AP2. Long-range self-assembly of the two auxiliary probes can create one-dimensional DNA nanostructures. In the experiment, we found that the length of the self-assembled DNA nanostructures can even be more than 20 μm. This scale has exceeded that of RCA product.[32,33] Moreover, the length of the DNA nanostructures can be controlled by changing the concentration of the two auxiliary probes when self-assembled. On the basis of these characteristic findings, we constructed an enzyme-free and label-free electrochemical biosensor for ultrasensitive detection of DNA. The 38-base target DNA for detection is selected from the human immunodeficiency virus (HIV)-1 U5 long terminal repeat sequence. Hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$, RuHex) is used as the electrochemical indicator. Even in complex samples such as cell lysates and human serum, the target DNA concentration as low as 5 aM can be detected. To the best of our knowledge, our detection system is the most sensitive in biological samples, compared with other reported electrochemical DNA biosensors.

EXPERIMENTAL SECTION

Materials. All the oligonucleotides used in the present study were synthesized and purified by HPLC by Shanghai Shenggong Biotechnology Co. (Shanghai, China) and used without further purification. Hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$, RuHex), 6-mercapto-l-hexanol (MCH), disodium ethylene-diaminetetraacetic acid (Na_2EDTA), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. Other chemicals employed were all of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All solutions were prepared with ultrapure water obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA) with resistivity of 18.2 MΩ·cm. The buffers and solutions involved in this work were as follows: DNA immobilization buffer (I-buffer; 10 mM Tris-HCl + 1 mM EDTA + 500 mM NaCl + 10 mM TCEP, pH 7.4), DNA hybridization buffer (H-buffer; 10 mM Tris-HCl + 1 mM EDTA + 500 mM NaCl + 1 mM $MgCl_2$, pH 7.4), electrochemistry buffer (E-buffer; 10 mM Tris-HCl, pH 7.4), MCH solution (2 mM MCH in water), and RuHex solution (5 μM $[Ru(NH_3)_6]^{3+}$ in 10 mM Tris-HCl, pH 7.4).

The HeLa cell and human serum samples were used in this study. Both cell lysates and serum samples were prepared according to the reported procedure.[34−36] Cells were grown in RPMI-1640 (Gibco) plus 10% fetal bovine serum (FBS, Gibco). Cells were removed from the substrate by trypsinization, washed twice with 0.1 M PBS, and pelleted at 2000 rpm for 10 min at 4 °C. The cells were resuspended in a cold 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5% CHAPS, and 10% glycerol) at a concentration of 5×10^6 cells/mL by pipetting at least three times and incubated in ice for 30 min to thoroughly lyse the cells and then centrifuged for 20 min (12 000 rpm, 4 °C). The supernatant was flash frozen and stored at −80 °C. Human serum samples were supported by the Hospital of Fujian Medical University. The serum samples were centrifuged about 10 min at 1000 rpm. Then, the supernatants were collected. In order to detect the target DNA in complex biological samples, cell lysates or serum samples were analyzed after mixing with an equal volume of target DNA.

Electrode Pretreatment. The gold electrode (2 mm diameter) was cleaned by immersion in a freshly prepared piranha solution (a 3:1 v/v mixture of concentrated H_2SO_4 and 30% H_2O_2) for 20 min, followed by a thorough rinse with ultrapure water. *CAUTION: piranha is a vigorous oxidant and should be used with extreme caution.* Then, the electrode was polished on a microcloth (Shanghai Chenhua Inc., China) with 0.05 μm alumina slurry to obtain a mirror surface, followed by sonication in ethanol and ultrapure water for 5 min each, to remove residual alumina powder. The electrode was electrochemically cleaned by cycling the potential between −0.3 and +1.5 V (at a scan rate of 0.1 V/s) in a fresh 0.5 M H_2SO_4 solution until a stable cyclic voltammogram was obtained.[37−39] The electrochemically cleaned gold electrode was rinsed with a copious amount of ultrapure water, and the electrode was blow-dried with nitrogen.

DNA Self-Assembly on Gold Electrode. At the beginning of assembly, the cleaned gold electrode was incubated for 180 min in 6 μL of I-buffer which contained 1 μM thiolated capture probes. The DNA surface density on gold electrode was about 2.7×10^{12} molecule/cm^2, which was quantitatively measured with chronocoulometry (CC) as previously described.[40,41] The electrode was then thoroughly rinsed with ultrapure water and dried under a stream of nitrogen gas. The electrode was incubated in the MCH solution (2 mM) for 90 min to remove nonspecific DNA adsorption on the gold surface. Then, the electrode surface was rinsed thoroughly and dried in nitrogen. H-buffer (6 μL) containing target DNA was pipetted to the electrode surface and incubated for 90 min. The electrode was rinsed and dried. Afterward, 10 μL of a freshly prepared H-buffer containing 1 μM AP1 and 1 μM AP2 was dropped on the surface of the electrode and incubated for 90 min to complete the long-range self-assembly. When the assembly finished, the resulting electrode was again thoroughly rinsed and dried before electrochemical characterization.

Detection Method and Apparatus. All electrochemical measurements were carried out on a CHI 660C electrochemical working station (CH Instrument Company, USA). A three-

dx.doi.org/10.1021/ac3017828 | *Anal. Chem.* 2012, 84, 8277−8283

electrode cell (capacity, 10 mL; diameter, 25 mm) consisting of working electrode (the assembled gold electrode, d = 2 mm), Ag/AgCl reference electrode, and platinum counter electrode was used for all electrochemical measurements. Differential pulse voltammetry (DPV) was performed using a potential window of 0.2 to −0.6 V (versus Ag/AgCl) in 3 mL of RuHex solution, which was degassed with nitrogen for 15 min. Atomic force microscopic (AFM) images of the DNA nanostructures were taken out using a Nanoscope IIIa multimode atomic force microscope (Veeco Instruments, USA) in tapping mode to simultaneously collect height and phase data.

RESULTS AND DISCUSSION

Design Principle of the Sensor. In this study, we designed two auxiliary probes, AP1 and AP2. As shown in Table 1, both AP1 and AP2 included two fragments. The italic fragment at the 3′ end of AP1 is complementary with the italic fragment of AP2. The bold fragment at the 5′ end of AP2 can hybridize with the bold fragment of AP1. As a result, an AP1 molecule can hybridize to two regions of an AP2 molecule, and then, a single AP1 hybridizes more readily to complementary regions on each of two AP2 molecules than to two regions on a single AP2 molecule. Thus, a cascade of hybridization events between the two auxiliary probes can lead to long-range self-assembly and form micrometer-long DNA nanostructures. In addition, the italic fragment at the 3′ end of AP1 can also hybridize with the italic fragment of target DNA to link the DNA nanostructures (made from alternating AP1 and AP2 probes) to target DNA.

Figure 1 shows the schematic representation of our electrochemical DNA biosensor. In the presence of target

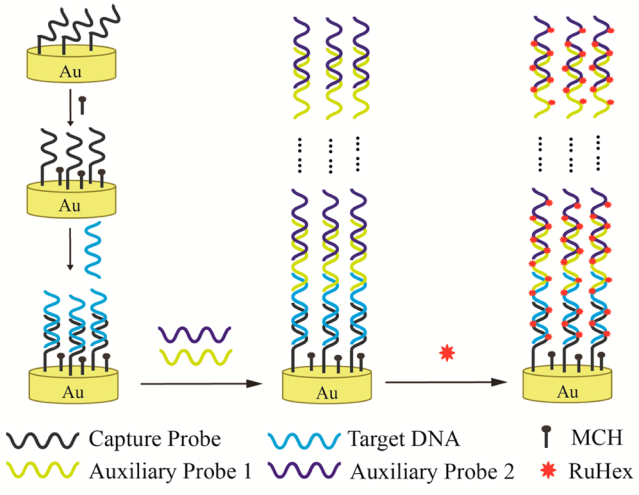


Figure 1. Schematic representation of the enzyme-free and label-free ultrasensitive electrochemical DNA biosensor based on long-range self-assembled DNA nanostructures.

DNA, one terminus of a target DNA can hybridize with a capture probe immobilized on the working electrode, and the other terminus can hybridize with a nicked DNA nanostructure via AP1. Thus, even one copy of the target can trigger the connection of a long DNA nanostructure to a capture probe on the electrode surface. Since the DNA backbone is negatively charged, myriad RuHex can bind to the DNA nanostructures through electrostatic interaction. Numerous RuHex can be gathered to the working electrode via DNA nanostructures and eventually result in significantly amplified electrochemical signals. In the absence of target DNA, although the added AP1 and AP2 can self-assemble to form DNA nanostructures in the solution, the DNA nanostructures can not link to the modified electrode.

Electrochemical Characterization. RuHex cations can quantitatively bind to the phosphate backbone of DNA via electrostatic interaction. The more that RuHex binds to DNA, the greater is the signal. In this study, the capture probes (CP) were immobilized on the gold electrode by gold−thiol chemistry at the beginning of fabricating the electrochemical DNA biosensor. In the absence of target DNA, there was only a small reduction peak observed (Figure 2a), due to the small

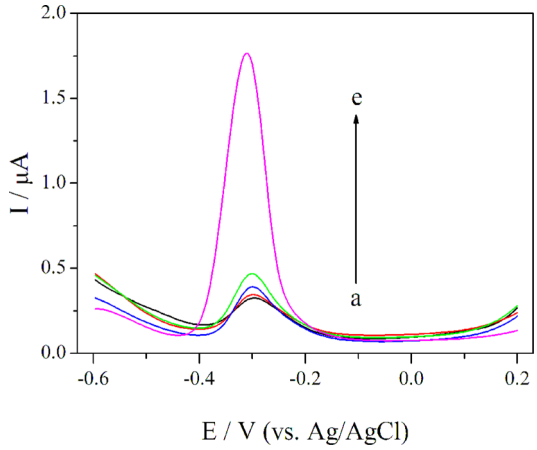


Figure 2. Differential pulse voltammetry (DPV) responses of the gold electrode modified with various oligonucleotides: (a) CP, (b) CP+TD, (c) CP+AP1+AP2, (d) CP+TD+AP1, and (e) CP+TD+AP1+AP2. The concentration of TD is 10 pM. The concentrations of AP1 and AP2 are both 1 μM.

amount of RuHex electrostatically binding to CP. After being hybridized with target DNA (TD), some more RuHex cations were binding to CP and TD, and a slightly larger peak was obtained (Figure 2b). Although auxiliary probe 1 (AP1) and auxiliary probe 2 (AP2) can self-assemble to be DNA nanostructures, the DNA nanostructures can not link to the electrode surface in the absence of target DNA. Thus, another small reduction peak was observed (Figure 2c), due to a small amount of nonspecific adsorption. In the presence of target DNA, if there was only AP1 and no AP2 in the solution, still only a small reduction peak was observed (Figure 2d). When the solution containing 1 μM AP1 and 1 μM AP2 was dripped on the surface of the electrode, the DNA nanostructures, self-assembled by numerous AP1 and AP2, were firmly immobilized on the gold electrode via target DNA and CP. Thus, the large number of RuHex binding to DNA nanostructures produced a remarkable amplified electrochemical signal (Figure 2e).

Optimization of Self-Assembled DNA Nanostructures. The electrochemical DNA biosensor proposed here is built based on long-range self-assembled DNA nanostructures, so the products of self-assembly play an important role in the sensing process. According to the electrochemical characterization described above, the peak currents would be remarkably amplified after self-assembly. Meanwhile, we found that the peak currents were closely related to the concentrations of the auxiliary probes and the hybridization time of the self-assembly,

dx.doi.org/10.1021/ac3017828 | Anal. Chem. 2012, 84, 8277−8283

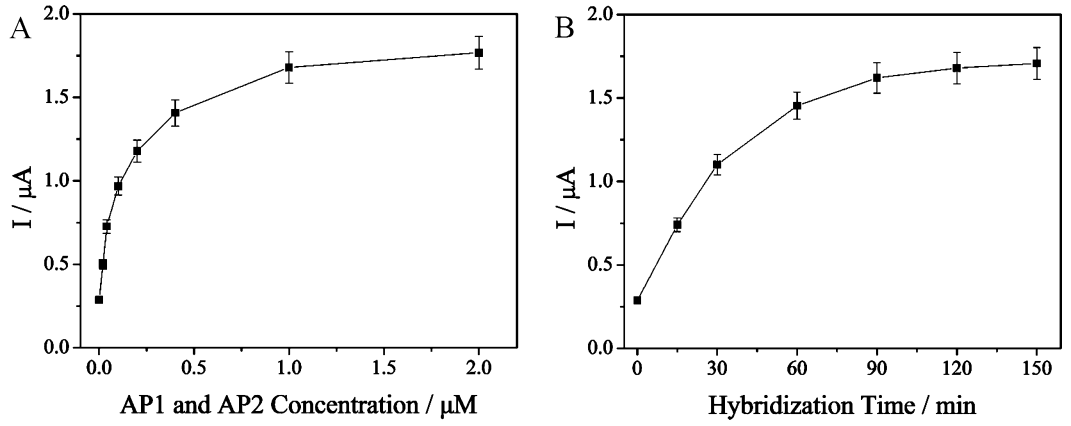


Figure 3. (A) Effects of the concentrations of AP1 and AP2 for detection of 10 pM target DNA in RuHex solution. The hybridization time for long-range self-assembly was 90 min. Both AP1 and AP2 were of the same concentration. Different concentrations of AP1 and AP2 were used (0, 0.02, 0.04, 0.1, 0.2, 0.4, 1.0, and 2.0 μM). (B) Effects of hybridization time for detection of 10 pM target DNA in RuHex solution. Both auxiliary probes were of the same concentration (1.0 μM). The measurements were performed after 0, 15, 30, 60, 90, 120, and 150 min of hybridization time for long-range self-assembly. The illustrated error bars represent the standard deviation of five repetitive measurements.

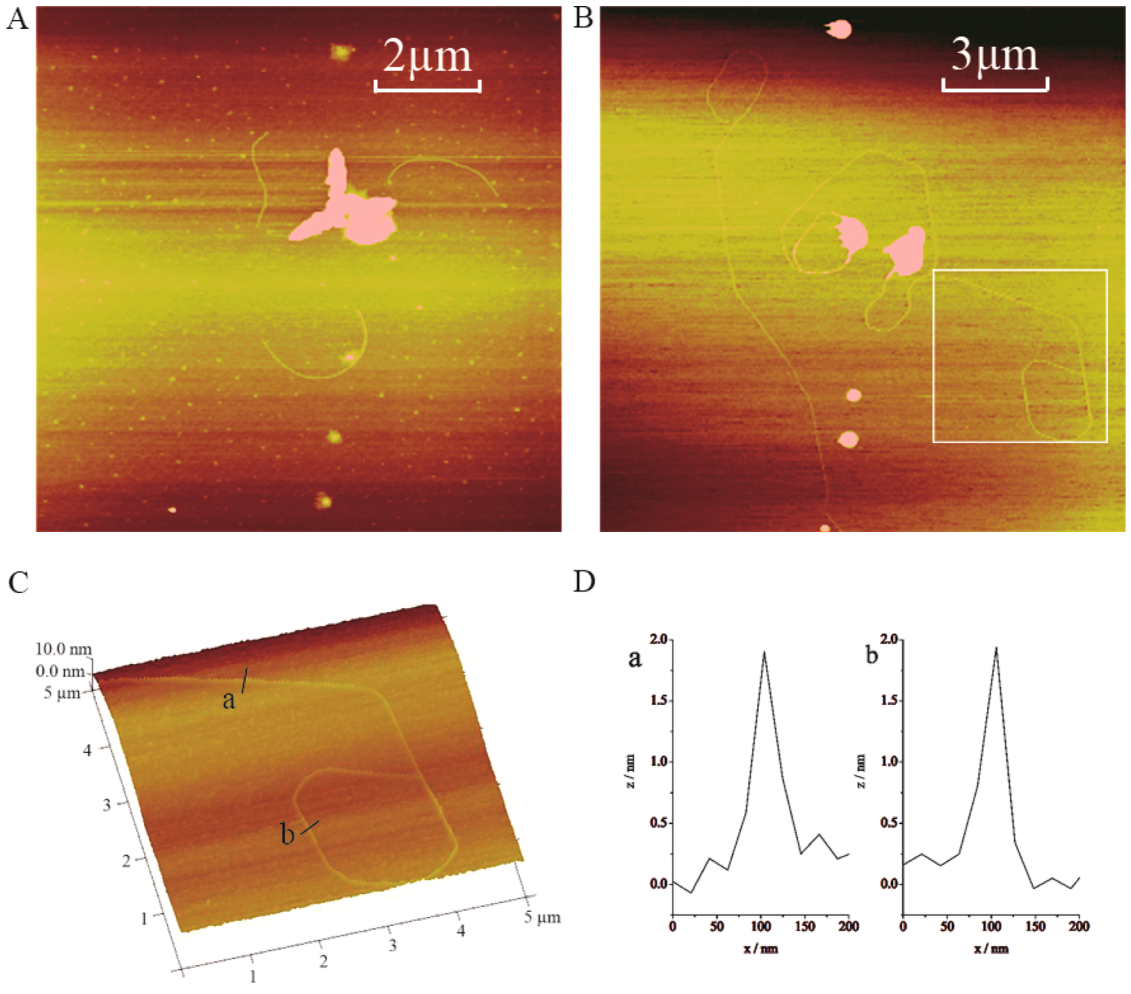


Figure 4. AFM height images of the long-range self-assembled DNA nanostructures on mica substrate. The self-assembly time is 90 min. The concentrations of both auxiliary probes are 0.1 μM (A) and 1 μM (B), respectively. (C) The partial enlargement of (B). Line charts in (D) are associated height profiles.

so the optimal concentrations and the hybridization time were investigated.

Figure 3A shows the effect of concentrations of AP1 and AP2 (from 0 to 2.0 μM) on the electrochemical signal for 10 pM target DNA. Both AP1 and AP2 were of the same concentration. The hybridization time for self-assembly was 90 min. The signal increased significantly as the concentrations increased up to 1.0 μM, suggesting that the DNA

dx.doi.org/10.1021/ac3017828 | *Anal. Chem.* 2012, 84, 8277−8283

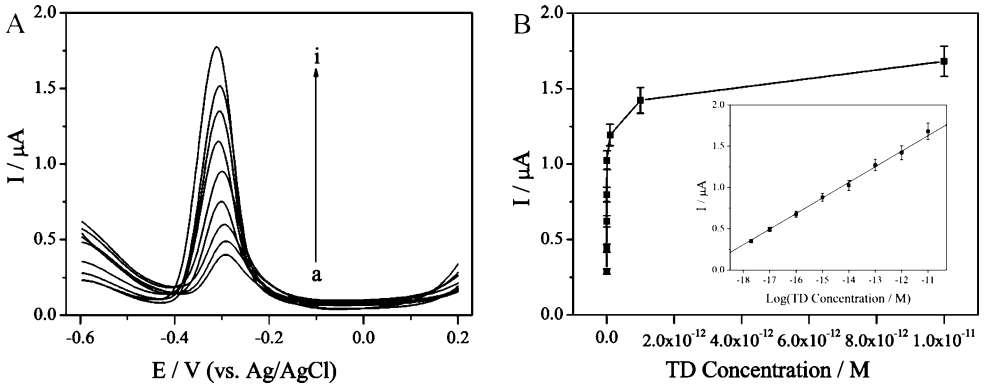


Figure 5. (A) DPV responses for different target DNA concentrations: (a) 0 M, (b) 2 aM, (c) 10 aM, (d) 100 aM, (e) 1 fM, (f) 10 fM, (g) 100 fM, (h) 1 pM, and (i) 10 pM. (B) The plots of peak currents vs the target DNA concentrations. Inset is the linear relationship between the peak current and the logarithm of the target DNA concentration. The illustrated error bars represent the standard deviation of five repetitive measurements.

nanostructures would be longer in the high concentration of AP1 and AP2. However, the signal did not further increase when the concentrations of AP1 and AP2 were up to 2.0 μM. The effect of hybridization time for self-assembly was also examined, as shown in Figure 3B. The peak current increased as the hybridization time changed from 0 to 90 min. Then, the peak current did not increase obviously as the hybridization time extended to 150 min. Therefore, 1.0 μM and 90 min were chosen as the optimum auxiliary probe concentration and hybridization time for long-range self-assembly, respectively.

Atomic force microscopy (AFM) was also employed to characterize the DNA nanostructures. Ten microliters of a freshly prepared H-buffer containing equal concentration of AP1 and AP2 was dripped on mica substrate and incubated for 90 min in a confined environment to form the self-assembled DNA nanostructures. As shown in Figure 4A, short DNA nanostructures of 2−3 μm were observed on mica substrate when both auxiliary probes were of the same concentration of 0.1 μM. When 1 μM AP1 and 1 μM AP2 were used, longer and curved DNA nanostructures with the length more than 20 μm can be observed (Figure 4B). This scale has exceeded that of RCA product.[32,33] The associated height profiles (Figure 4C,D) show that the average height of the DNA nanostructures is about 2 nm, which is matched with the diameter of dsDNA helix.[42] Moreover, according to the geometrical parameters of dsDNA, the axial separation of adjacent base pairs is 0.34 nm. It can be estimated that more than 60 000 base pairs were involved in the DNA nanostructures longer than 20 μm. These results confirm that the long-range self-assembly of the two auxiliary probes can create very long DNA nanostructures.

Electrochemical Detection of Target DNA. The sensitivity of the sensor was investigated by DPV measurement. As shown in Figure 5A, it is clear that the addition of target DNA at different concentrations to the sensor induced different increases in peak current, associated with the quantity of RuHex cations electrostatically binding to the DNA nanostructures on the electrode. Figure 5B shows that the peak current *i* increased as the target DNA concentration changed from 0 to 10 pM. The inset of Figure 5B shows that the peak currents of the sensor exhibited a linear correlation to the logarithm of the target DNA concentration range from 2 aM to 10 pM with a linear correlation coefficient of 0.9972. The directly measured detection limit is as low as 2 aM. Considering a sample volume of 6 μL (target DNA), an electrochemical detection of about 12 yoctomoles or about 7 copies of oligonucleotides is therefore afforded.

Selectivity of the Biosensor. Compared with other electrochemical methods for DNA detection, this biosensor is really superior in sensitivity with a wide linear range. Moreover, the sensor achieves its remarkable detection limit without sacrificing its specificity. In this work, four kinds of DNA sequences (see Table 1 for details) including complementary target DNA (TD), single-base mismatch target (1MT), two-base mismatch target (2MT), and noncomplementary sequence (NC) were chosen to study the selectivity of the biosensor. Figure 6 shows the peak currents of the blank and the four

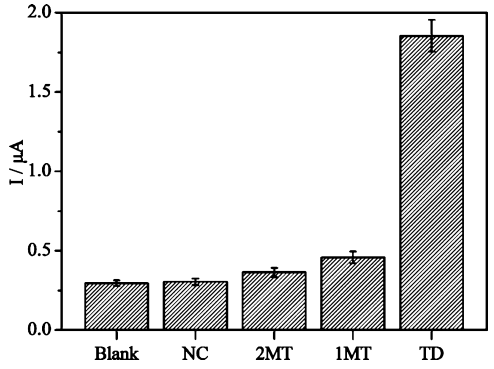


Figure 6. Bar chart of the DPV responses of the blank and the four various DNA sequences. The concentration of TD was 10 pM. The concentrations of 1MT, 2MT, and NC were 1 nM. The illustrated error bars represent the standard deviation of five repetitive measurements.

kinds of DNA sequences. The concentration of TD was 10 pM, while the concentrations of three other DNA sequences were 1 nM. Despite of the large concentrations of disrupting DNA, the sensor provides 4:1 differentiation between TD and 1MT, suggesting that the DNA nanostructure-based biosensor was able to discriminate the target with high specificity and sensitivity.

Challenged with Complex Samples. We applied this sensor to detect the target DNA in cell lysates and human serum. Both are the most challenging mediums containing many complex components. The measurements were conducted in 1:1 dilution of HeLa cell lysates and serum samples. Compared with the peak currents observed in the H-buffer under the same experimental condition (Figure 5A), signals

dx.doi.org/10.1021/ac3017828 | *Anal. Chem.* 2012, 84, 8277−8283

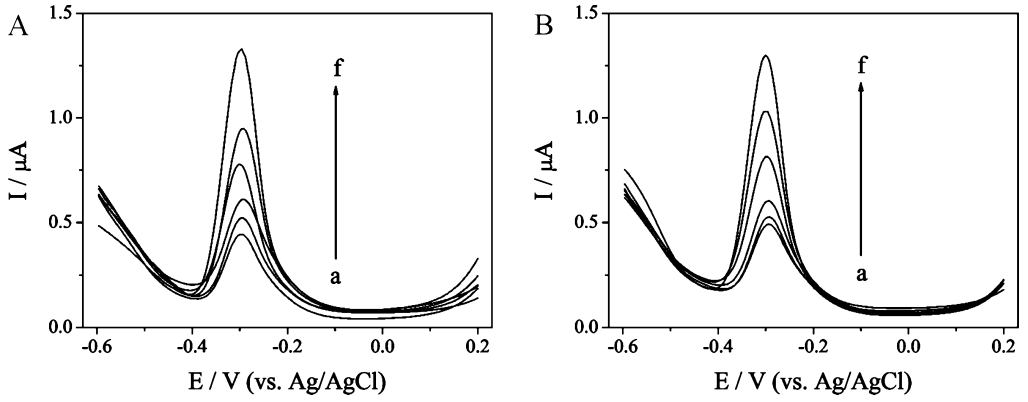


Figure 7. DPV of the biosensor in 1:1 dilution of HeLa cell lysates (A) and human serum (B) at different target concentrations: (a) 0 M, (b) 5 aM, (c) 10 aM, (d) 100 aM, (e) 1 fM, and (f) 100 fM.

were decreased a little when the sensor was introduced to cell lysates and human serum samples (Figure 7), which may be related to the interference of the sample matrix during the process of hybridization between target DNA and capture probe. In addition, signals of the blank were slightly larger in both biological samples than in H-buffer, due to more nonspecific adsorption brought by complex components in biological samples. However, the DPV curves show that, despite multicomponent nature of biological samples, the DNA nanostructure-based biosensor still performed excellently. As shown in Figure 7, even in 1:1 dilution of human serum, a target DNA concentration as low as 5 aM (about 30 yoctomoles or about 18 copies of oligonucleotides in 6 μL) can be detected. To the best of our knowledge, compared with other reported electrochemical DNA biosensors, our detection system is the most sensitive in biological samples, which indicates the feasibility of our proposed sensor in real physiological media.

Here, we consider that there are at least two reasons contributing to the surprising ultrasensitivity of the protocol. First, the ultrasensitivity comes from the significant length of the self-assembled DNA nanostructures. Compared with other signal probes, the DNA nanostructures longer than 20 μm can accumulate many more electrochemical indicators and produce a much larger signal. Second, compared with other nanomaterials, such as polythiophene,[43] gold nanoparticals[44,45] and carbon nanotubes,[46] DNA nanostructures are more biocompatible, more hydrophilic, and thus less prone to nonspecific adsorption onto the electrode surface. Both of these may contribute to the amazing ultrasensitivity of the biosensor.

CONCLUSIONS

We have developed an enzyme-free and label-free ultrasensitive electrochemical DNA biosensor for specific detection of HIV DNA in complex biological samples. Using long-range self-assembled DNA nanostructures as carrier for signal amplification, this newly proposed biosensor can detect as low as 5 aM target DNA even in cell lysates or human serum, with a sensitivity similar to that of PCR. Furthermore, this DNA biosensor, with no need of any kind of enzyme, label, or sophisticated equipment, is really simple and inexpensive. In view of these advantages, this ultrasensitive electrochemical DNA biosensor has a great potential in the area of DNA diagnostics and clinical analysis.

AUTHOR INFORMATION

Corresponding Author

*E-mail: hhyang@fio.org.cn (H.-H.Y.); cjh_huaxue@126.com (J.-H.C.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support from the National Basic Research Program of China (No. 2010CB732403), the National Natural Science Foundation of China (No. 21125524, No. 21105012), the Program for New Century Excellent Talents in University of China (09-0014), the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT1116), and the National Science Foundation of Fujian Province (No. 2010J06003).

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