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Ultrasensitive Electrochemical Biosensor for HIV Gene Detection Based on Graphene Stabilized Gold Nanoclusters with Exonuclease Amplification

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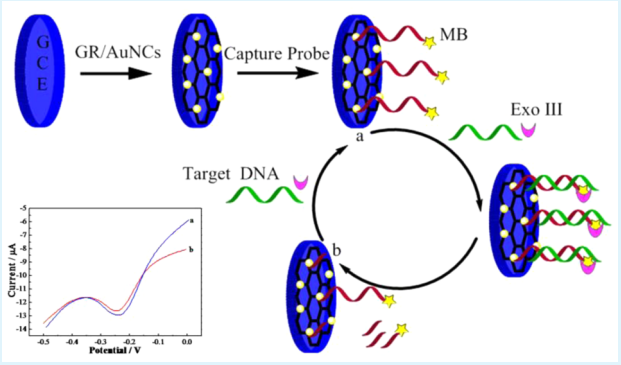
Supporting Information

ABSTRACT: Because human immunodeficiency virus (HIV) has been one of the most terrible viruses in recent decades, early diagnosis of the HIV gene is of great importance for all scientists around the world. In our work, we developed a novel electrochemical biosensor based on one-step ultrasonic synthesized graphene stabilized gold nanocluster (GR/AuNC) modified glassy carbon electrode (GCE) with an exonuclease III (Exo III)-assisted target recycling amplification strategy for the detection of HIV DNA. It is the first time that GR/AuNCs have been used as biosensor platform and aptamer with cytosine-rich base set as capture probe to construct the biosensor. With the combination of cytosine-rich capture probe, good conductivity and high surfaces of GR/AuNCs, and Exo III-assisted target recycling amplification, we realized high sensitivity and good selectivity detection of target HIV DNA with a detection limit of 30 aM (S/N = 3). Furthermore, the proposed biosensor has a promising potential application for target detection in human serum analysis.



KEYWORDS: *graphene stabilized gold nanoclusters, cytosine-rich capture probe, exonuclease III-assisted target recycling, HIV gene, electrochemistry detection*

INTRODUCTION

Since 1983, when human immunodeficiency virus (HIV) was first discovered in America, HIV has caused great horror all over the world with the emergence of one the most deadly diseases—acquired immune deficiency syndrome (AIDS). The genes of the HIV group are two of the same strands of RNA, which can transcribed into DNA for further gene expression in host cells through reverse transcription. It attacks the host's immune system and causes the destruction of T4 lymphocytes, which results in the breakdown of the human immune system, making the human body unable to defend itselg against many diseases and leading to death. Thus, the detection of HIV biomarker or gene is of great importance for the early diagnosis and clinical therapy of AIDS and the prevention of virus's propagation. At present, the most commonly used approach for the early diagnosis of AIDS is the detection of the HIV antibody in the host.[1−6] However, it takes a few weeks to several months from infection to the production of HIV antibodies, which is called the HIV window period. During this time, AIDS cannot be detected because not enough antibodies have been produced. In order to realize the early and quick diagnosis of AIDS in humans, some scientists detected the DNA of HIV in host cells with immediate detection after infection without considering the window period.

For decades, many methods have been developed for target HIV gene detection, including surface enhanced Raman scattering (SERS),[7,8] fluorescence method,[9,10] colorimetric approach,[11] light scattering,[12] and electrochemical techniques.[13−15] Among them, electrochemical techniques have caused wide public concern over decades for good sensitivity and lower cost. To improve the sensitivity and selectivity for target detection, a large amount of biological amplification methods can be employed in this area.[16−23] Exonuclease III is a kind of exonuclease that degrades dsDNA from blunt ends, 5′-overhangs or nicks, releasing 5′- mononucleotides from the 3′-end of DNA, and producing stretches of single-strand DNA. It exhibits good catalytic activities for the digestion of duplex DNA even with 3′-end modified with a signal molecule.[24,25] Thus, it can be used for the digestion of capture probe and the recycling of target and Exo III in electrical biosensor.[26−28]

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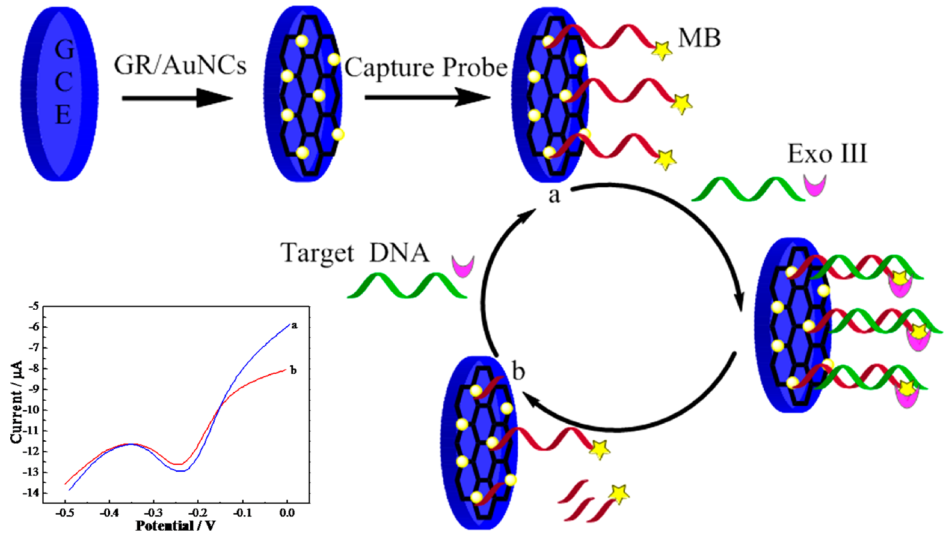
Scheme 1. Schematic Illustration of the Proposed Biosensor Fabrication Process



[a]The initial signal obtained from capture probe. [b]The final signal after incubation with target DNA and Exo III

Metal nanoclusters, which are composed of several to dozens of metal atoms, have attracted great interest in recent years. Because the continuous density of states breaks up into discrete energy levels and comparable size to the Fermi wavelength of electrons, metal nanoclusters exhibit excellent optical and electrical properties and good biocompatibility compared to larger metal nanoparticles. Bovine serum albumin (BSA),[29] glutathione (GSH),[30] polyamidoamine dendrimers (PAMAM)[31] or DNA[32] are the most commonly used template for the synthesis of noble metal nanoclusters and their application in electrochemistry biosensor have been reported before.[33−36] However, most of those materials are non-conducting and will hinder the electron transfer of electrical biosensor while binding to electrode surfaces. Tang and co-workers synthesized Au cluster/graphene hybrids with green ultrasonic method for fuel cell.[37] The Au cluster/graphene hybrids have much higher electrocatalytic activity and greatly enhanced electrocatalytic stability for oxidation reduction reaction (ORR) for the increased charge transfer from the clusters to the graphene. Thus, we predict that taking advantage of those hybrids as an electrochemical biosensor platform can promote an electrochemical response in the biosensor system. In their work, Zhu et al. made use of Au cluster/graphene hybrids for H_2O_2 electrogenerated chemiluminescence detection with direct electrochemistry method.[38] However, at present, rarely has an electrochemical biosensor taken advantage of this hybrid as a platform for the construction of electrical biosensor.

To realize the quick and sensitive detection of HIV DNA in a host with lower cost and simple strategy, we developed an electrochemical biosensor based on GR/AuNCs platform with Exo III-assisted DNA recycling amplification based on a C-rich capture probe. GR/AuNCs set a good conductive platform with super high specific area, which provides more fixed sites for C-rich capture probes, and good electrochemical results was obtained. Signal decreased for the C-rich probe being digested in the presence of target DNA, an ultrasensitive signal-off electrical biosensor was proposed with high sensitivity and good selectivity toward HIV DNA detection with low detection limit (30 aM). It can also be applied in human blood sample for recovery detection with good application prospect.

EXPERIMENTAL SECTION

Reagents and Materials. Graphene powder (G250) was purchased from Sinocarbon Materials Technology Co., Ltd. (Taiyuan Shanxi). $HAuCl_4·3H_2O$ was purchased from Sigma Chemical Co. (St. Louis, MO). $NH_3·H_2O$ (W%: 25−28) and *N*,*N*-dimethylformamide (DMF) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Exo III and 10 × NEBuffer1 (1 × NEBuffer 1:10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM DL-Dithiothreitol, pH 7.0 at 25 °C) were purchased from New England Biolabs (Beijing, China). Tris-HCl (20 mM, pH 7.4) containing 100 mM NaCl and 5 mM $MgCl_2$ was used to prepare DNA. All solutions were prepared with ultrapure water (18.25 MΩ·cm) produced by Aquapro water purification system.

The HPLC-purified DNA was synthesized by Shanghai Sangon Biotechnology Co., Ltd. (China). The sequences of oligonucleotides employed in this work were as follows:

Capture DNA: 5′-CCCCCTAGAAAAATCTCTAG-MB-3′

Target HIV DNA: 3′- TACACCTTTTAGAGATCGTCA-5′

Single-base mismatched DNA: 5′-ACAGCTAGAGATTTTCCA-CAT-3′

Four-base mismatched DNA: 5′-ACAGCCACAGGTTTTCCA-CAT-3′ unmatched DNA: 5′-CAGTAGCTGTCGGGGATAAGC-3′

Apparatus. Electrochemical determination was carried out using a CHI660C electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., China). A three-electrode electrochemical system which consists of saturated calomel electrode (SCE) as reference electrode, platinum electrode as the auxiliary electrode and GR-AuNCs modified glass carbon electrode (GCE) as working electrode was used in this experiment. Cyclic voltammetry (CV) was carried out in the 1 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl, while differential pulse voltammetry (DPV) scans were conducted in 20 mM tris-HCl (pH 7.4) from 0 to −0.5 V, electrochemical impedance spectroscopic (EIS) in 0.5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.5 M KNO_3. Transmission electron microscopic (TEM) images were obtained on FEI Tecnai G20 (America) with an accelerating voltage of 200 kV. Dynamic light scattering (DLS) measurement was performed by Malvern ZS90 (British), X-ray photoelectron spectroscopy (XPS) on Thermo Scientific Escalab 250Xi (British).

Preparation of GR/AuNCs. The GR/AuNCs were prepared according to the one-step ultrasonic method with minor revision.[37,38] In a typical experiment, 100 μL 50 mM $HAuCl_4$ was add to 0.17 mg/mL graphene solution which had been dispersed in 5 mL $NH_3·H_2O$ and 5 mL DMF with continuous ultrasonic procedure at 25 °C for 10 min, the resulting products were collected by centrifugation and washed twice to neutral with pure water to remove excess $NH_3·H_2O$

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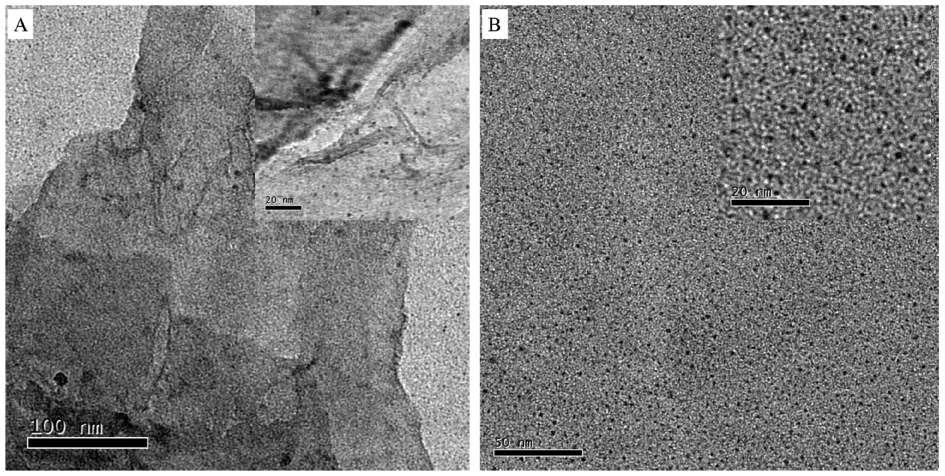

Figure 1. Typical TEM image of as-prepared graphene stabilized gold nanoclusters from (A) lower magnification of GR/AuNCs to (B) higher magnification of gold nanoclusters in solution.

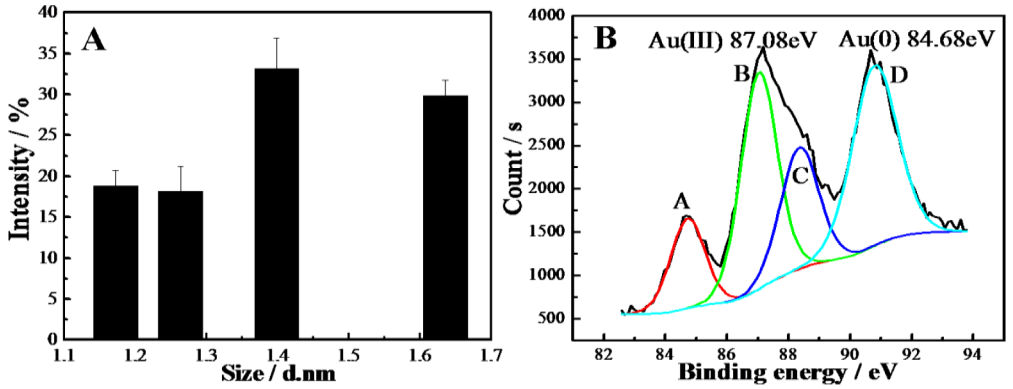

Figure 2. (A) The hydrodynamic sizes distribution of AuNCs measured by DLS and (B) XPS spectrum of Au element in GR/AuNCs composite: (peak A) Au (0) $4f_{7/2}$ peak; (peak B) oxidation state of Au $4f_{7/2}$ peak; (peak C) Au (0) $4f_{5/2}$ peak; and (peak D) oxidation state of Au $4f_{5/2}$ peak.

and finally dispersed in 10 mL DMF. The product obtained was stored in darkness at 4 °C and with intermittent ultrasonic for 30 min before first use.

Fabrication of the Proposed Biosensor. The bare GCE was thoroughly polished with 0.05 μm of gamma-alumina powder and successfully ultrasonicated in ultrapure water and ethanol for 5 min, respectively. After drying under N_2, the pretreated bare GCE electrode was further dropped with 5 μL GR/AuNCs dispersion and dried in thermostat at 37 °C to construct GR/AuNCs based biosensor. After that, 10 μL 100 nM capture probe was dropped onto GR/AuNC-modified electrode surfaces covered by plastic caps at room temperature for 12 h. Finally, the electrode was immersed in different concentrations of 50 μL target solution which contains 3 μL 10 U/μL Exo III and incubated at 37 °C for 1 h. After each step, the electrode was rinsed with tris-HCl (pH 7.4) to remove the nonspecific adsorption. The whole preparation process is outlined in Scheme 1.

RESULTS AND DISCUSSION

Design of Proposed Biosensor. We designed GR/AuNCs as biosensor platform and aptamers with MB labeled on 3′-end and C-rich base on 5′-end as capture probe to construct biosensor for the detection of target DNA. Instead of taking advantage of aptamers modified by −SH or $-NH_2$ as capture probe,[39,40] or stem−loop conformation to bind to gold electrode or gold nanoparticles covered GCE,[41,42] we make use of C-rich base capture probe binding to GR/AuNCs platform for Cytosine (C)-rich aptamer can strictly bind to gold atom through Au−N bond[32,43] and intertwined with gold nanoclusters, which is time-saving for no need of preparation time comparing with stem−loop design and makes MB near the electrode. Besides, stem−loop conformation capture probe fixed to GR/AuNCs modified GCE have stereohindrance effect in our case for the reason that double strand DNA have approximately 2 nm size which is bigger than the size of nanoclusters and it can prevent more capture probe from being captured. With the binding of C-rich capture probe on GR/AuNCs platform, an obvious methylene blue (MB) signal was obtained. In the presence of target DNA, a capture probe bound to the target can fold into a duplex DNA structure, which directly leads to the digestion of the capture probe by Exo III from its 3′-end with a MB molecule released from the electrode surface and target DNA recycling. In this way, an ultrasensitive signal-off electrical biosensor was realized.

Characterization of GR/AuNCs. Direct images of the assembled products by TEM further confirmed the obtained GR/AuNCs composite synthesized successfully. According to Figure 1A, the cluster is formed well in composite solution on the surface of graphene and around the template edge. The inset shows the surface of graphene sheet loaded a lot of gold nanoclusters. AuNCs are tightly attached to the graphene, which gives good evidence that GR/AuNCs composite is synthesized successfully. To more closely observe the black dots on the edge of graphene sheet, we examined a high-magnification TEM image (Figure 1B and inset). AuNCs are found separately with a uniform distribution approximately

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below 2 nm compared with the scale bar. According to Figure 1 and Figure S1 in Supporting Information, we predict gold nanocrystal is well-formed and can be a good sensing platform for electrical biosensor's construction.

The dynamic light scattering (DLS)-based analysis of the synthesized gold nanoclusters is shown in Figure 2A. The size distribution of gold nanoclusters is between 1.1 and 1.7 nm, the average size is 1.4 ± 0.3 nm obtained from 3 times average of size distribution multiplied by intensity percentage which is in good accordance with the TEM images. As for the X-ray photoelectron spectra (XPS) of gold nanoclusters (Figure 2B), An Au (0) $4f_{7/2}$ peak could be seen at 84.68 eV (peak A) compared with bulk Au at 83.8 eV, which is typical for small gold nanocluster.[36,37] peak C represent Au (0) $4f_{5/2}$, while B and D refer to the oxidation state of Au $4f_{7/2}$ and Au $4f_{5/2}$, respectively, which account for the oxidability of Au (0) to Au^{1+} and the excess Au^{3+} of ingredients. As for the whole XPS spectroscopy of graphene stabilized gold nanoclusters (Figure S2, Supporting Information), the C 1s, N 1s, and O 1s peaks are obvious, which proved the participation of those elements during the synthesize process of graphene-stabilized gold nanoclusters.

Amplification Effect of the Proposed Biosensor. DPV responses were investigated to evaluate the design principle of the proposed biosensor. As shown in Figure 3, when there were

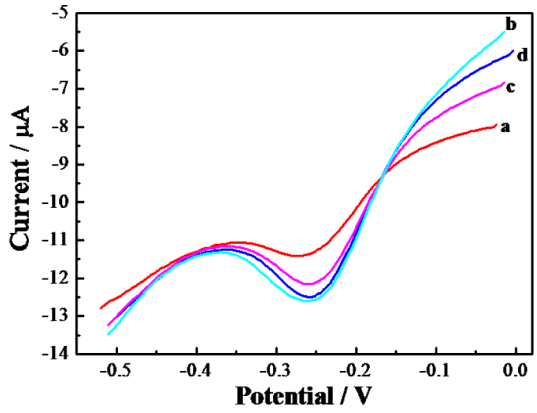

Figure 3. DPV of (a) 100 nM capture probe/GR/GCE; (b) 100 nM capture probe/GR/AuNCs/GCE; (c) 100 nM capture probe/GR/AuNCs-modified GCE after incubation with 10 nM target DNA with 30 U Exo III; and (d) without Exo III in 20 mM Tris-HCl (pH 7.4).

no AuNCs, the MB signal on capture DNA binding with graphene (curve a) is relatively low, but with the AuNCs formed on the graphene sheet, the initial signal is twice larger (curve b) for the high specific area of AuNCs and more capture probes being fixed on electrode. Besides, in the absence of Exo III, the capture DNA cannot be digested and the target do not recycling with target DNA, a small signal decrease was collected (curve c) for some double strand DNA being released by graphene for noncovalent absorption. But after Exo III digestion and target DNA recycling, the signal decrease is amplified significantly (curve d), which is indicated in the figure for the signal of curve c is lower than curve d, and thus, the signal decrease ($I_a - I_{c/d}$) in the presence of Exo III is greater than in the absence of Exo III, which proved that Exo III-assisted target recycling amplification is successfully realized. These results proved that the combined employment of AuNCs and Exo III enhanced the signal change apparently and an ultrasensitive electrochemical biosensor is constructed successfully.

Electrochemical Characterization of the Proposed Biosensor. In order to characterize the modification process of the biosensor, CV and EIS were performed. The CV of different electrode construction process has been normalized in Figure 4A, current density is obtained from the I/A (A refers to electrode surface areas which is obtained through the Randles–Sevcik equation $I_{pa} = 2.69 \times 10^5\, n^{3/2}\, c_0\, AD_R^{1/2} \nu^{1/2}$ with CVs of different electrode modified GCE in 1.0 mM $K_3Fe(CN)_6$ at different scan rates from 10 to 250 mV s^{-1}). In 1 mM $K_3[Fe(CN)_6]$ solution, the bare GCE electrode have relatively low peak current density value scaned from −0.2 V to 0.6 V with a scan rate of 100 mV s^{-1}. Upon decorating with GR/AuNCs, the peak current density of probe was significantly increased attributing to the increased specific surface area and good conductivity of the GR/AuNCs. After binding with the capture DNA, the peak current density decreased significantly for the repulsion of negatively charged redox probes ($[Fe(CN)_6]^{3-/4-}$) from the DNA phosphate backbone on electrode. After incubation with target DNA and Exo III, the peak current density increased for capture probe being digested and target DNA recycling.

In the terms of EIS, the results were in line with the CV measurements (Figure 4B). Taking advantage of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ as the redox probe, the semicircle diameter was equal to electron–transfer resistance (R_{et}). According to the impedance simulation of the inset figure in Figure 4B, the R_{et} of bare GE was 501 Ω, R_{et} decreased to nearly 0 Ω with the dropping of GR-AuNCs onto bare GCE electrode, and a straight line was obtained for GR/AuNCs can significantly promote electron transfer. With the immobilization of 100 μM capture probe on the electrode, the R_{et} increased to 1834 Ω. After incubation with 100 μM target DNA and 30 U Exo III, the R_{et} decreased again for capture probe being digested.

Optimization of Experimental Condition. To verify the optimal biosensor conditions, we optimized two experimental details. Because the concentration of capture DNA is of great importance for choosing the biggest signal change percentage ($\Delta I/I_0$), four different concentrations, from 50 nM to 1 μM, were employed to test the optimal condition (Figure 5A). When the capture probe's concentration increased from 50 to 100 nM, the signal change percentage increased because more capture probes have been absorbed on the electrode and are digested. But after the continuous increase of the capture probe from 100 nM to 1 μM, the signal change percentage decreased for 100 nM reached the saturated digest concentration of the Exo III with quantitative target DNA. Thus, we chose 100 nM as the optimal concentration to construct this biosensor.

The incubation time of Exo III with HIV target DNA complex had great effect on Exo III-assisted target recycling process. To investigate the optimal incubation time, 30 U Exo III was added into the solution (100 nM capture DNA and 10 nM HIV target DNA) and incubated at 37 °C for different duration. As shown in Figure 5B, with the increasing incubation time, the DPV current change was enhanced; it reached maximum at 60 min and kept a plateau after due to the decreased activity of Exo III after 60 min. In this case, we selected 60 min as the optimal incubation time.

Proposed Biosensor's Ability to Detect Target DNA. In the case of sensitivity of the proposed biosensor, under the optimal experimental conditions, different DNA concentrations were investigated following this method. The initial current, I_0,

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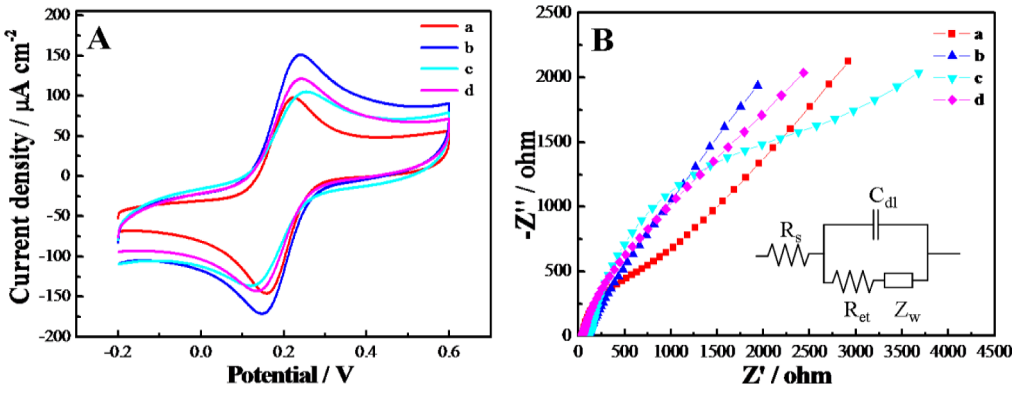

Figure 4. (A) Typical cyclic voltammograms and (B) Nyquist diagrams of electrochemical impedance spectra recorded for the electrode construction process: (a) bare GCE; (b) GR/AuNCs/GCE; (c) 100 nM capture DNA bound GR/AuNCs/GCE before and (d) after incubation with 50uL 100 nM target DNA and 30 U Exo III in 0.5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.5 M KNO_3 with the frequency range from 0.1 Hz-10000 Hz, signal amplitude at 0.005 V, formal potential at 0.2 V.

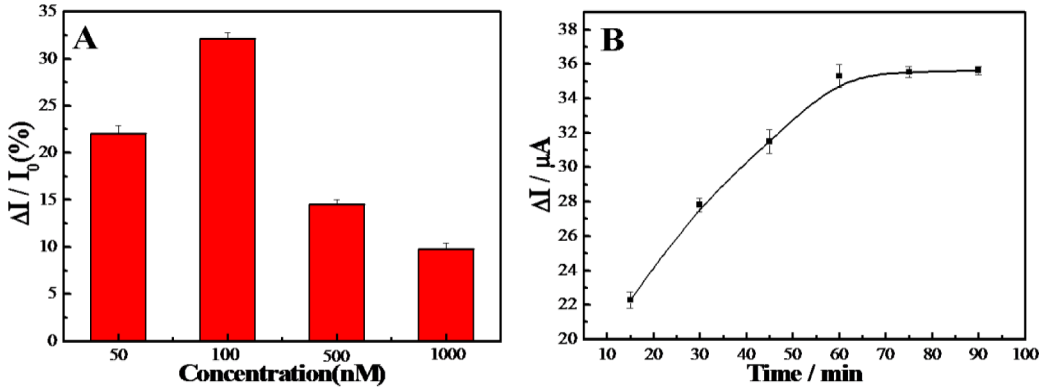

Figure 5. (A) Effect of concentration of capture probe and (B) incubation time of target recycling and Exo III digestion to the signal change percentage of the proposed biosensor.

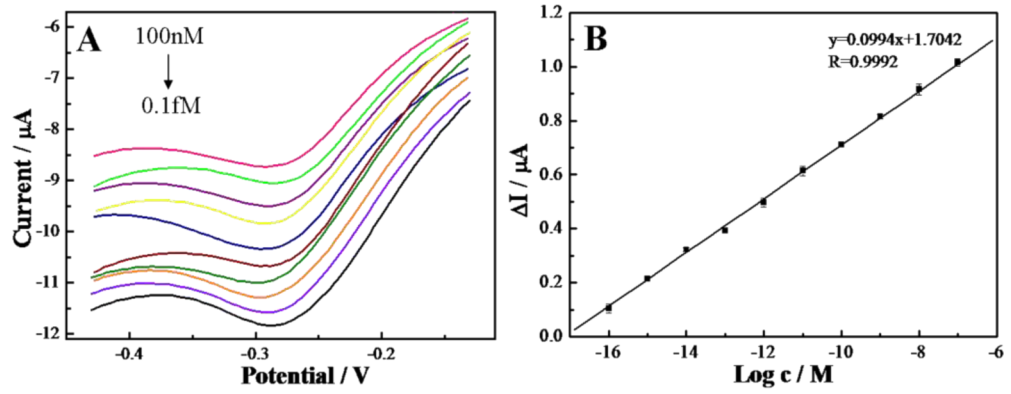

Figure 6. (A) Current change response of capture probe/AuNCs/GR/GCE after incubation with different concentration of target HIV gene and 10 U Exo III in 50 μL of 20 mM tris-HCl (pH 7.4), target concentrations: 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, 1 pM, 100 fM, 10 fM, 1 fM, and 0.1 fM. (B) The linear relationship of the current change versus logarithm value of the target DNA concentration. (S/N = 3).

was recorded for capture probe binding to GR/AuNCs modified electrode. After incubation with target DNA and Exo III, the current decreased to I_1, and the current reduction value ($\Delta I = I_0 - I_1$) was calculated. With the increasing of target DNA, the current reduction value is increased. As shown in Figure 6, The current reduction value (ΔI) exhibited a good linear relationship with the logarithm value of target DNA concentration within the range from 0.1 fM to 100 nM, the linear regression equation was ΔI (μA) = 0.0994 lg$c$ + 1.7042 (nM) (R = 0.9992) with a limit of detection (LOD) of 30 aM (S/N = 3), which is lower than other methods reported previously for the detection of target DNA with GR/Au NPs or GR/Au NRs as sensing interface (Table 1).[44−46]

To monitor whether the fabricated biosensor could detect target in actual sample, we performed recovery experiments in real sample. With standard addition method, three different concentrations of HIV target DNA (10 nM, 10 pM, 10 fM) were added into 100-fold-diluted human serum samples and detected for 8 times. As shown in Table 2, the proposed electrochemistry biosensor exhibited good sensitivity and a high

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Table 1. Comparison of Different DNA Biosensors with GR, GR/Au NPs or GR/Au NRs as Sensing Interface without Exonuclease Amplification

methods	techniques	dynamic range	detection limit	ref
rGO/GCE	EIS	10 fM − 1 μM	10 fM	44
AuNPs/rGO/GCE	DPV	0.1 pM − 10 nM	35 fM	45
AuNRs/rGO/GCE	DPV	10 fM − 1 nM	3.5 fM	46
Exo III/AuNCs/GR/GCE	DPV	0.1 fM − 100 nM	30 aM	this work

Table 2. Detection of Target HIV DNA Added in Human Serum with the Proposed Biosensor (n = 8)

sample	added	found	recovery (%)	RSD (%)
1	10 nM	9.34 nM	93.4	5.04
2	10 pM	9.71 pM	97.1	8.67
3	10 fM	9.98 fM	99.8	4.10

percentage recovery in real blood samples. The recoveries and relative standard deviation values were in the range of 93.4−99.8% and 4.10−8.67%, respectively, which proved the proposed sensor has good potential for target DNA detection in real blood samples.

Selectivity, Reproducibility and Stability of the Proposed Biosensor. The selectivity and reproducibility of this biosensor for HIV target DNA detection were investigated through interference experiments and repeated trials. Four types of DNA sequences, full matched target HIV DNA, single-base mismatched DNA, four-base mismatched DNA and unmatched DNA, were detected under the same condition respectively for specificity detection with the interference DNA's concentration 10 times larger than full matched target DNA. As shown in Figure 7A, neglectable DPV responses were observed with the addition of interfering DNA compared with the obvious decrease of electrochemical signal caused by target sequence, which manifested the developed biosensor had high specificity to target DNA detection for the reason that amplification of GR/AuNCs and Exo III cleavage activity ensured the excellent selectivity of proposed sensor.

Five different electrodes were employed to detect HIV target DNA (10 nM) three times under the same condition (Figure 7B) to test the reproducibility of the proposed sensor. Similar electrochemical signal decrease, 0.894, 0.949, 0.847, 0.888, 0.886 μA, was collected and relative standard deviation (RSD) was below 3.89%. These experimental results indicated that the proposed sensor has good reproducibility.

CONCLUSION

In summary, a simple and sensitive electrochemical biosensor was developed for HIV target DNA detection based on GR/AuNCs modified GCE with C-rich capture DNA and Exo III-assisted target recycling amplification strategy. The synthesized GR/AuNCs with uniform distribution were characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS), and X-ray photoelectron spectroscopy (XPS). This hybrid is a good sensing platform with high catalytic capability for sensitive and selective detection toward HIV DNA over other interfering DNA sequences combined with Exo III amplification strategy in our work; besides, taking advantage of cytosine-rich base capture probe to bind with gold nanoclustes on graphene sheet, we obtained good electrochemical results for the cytosine (C)-rich aptamer, which can strictly bind to gold atom through Au−N bond and intertwined with gold nanoclusters, which makes the signal molecule near the electrode surface. Last but not least, our detection limit for HIV gene detection is as low as 30 aM, which allows for the early detection of AIDS in human blood once the patient is infected with HIV. This work is significant in medical science applications as well. For these advantages, the proposed biosensor showed good potential for sensitive determination of other targets, including small molecules and proteins in future.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b05857.

XPS and TEM patterns of gold nanoclusters (PDF)

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Notes

The authors declare no competing financial interest.

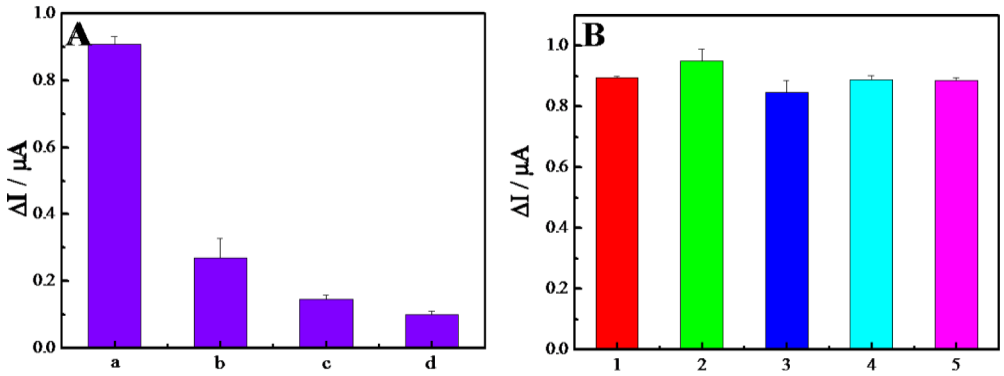

Figure 7. (A) Selectivity investigation of the proposed biosensor for (a) HIV target DNA (10 nM), (b) single-base mismatched DNA (100 nM), (c) four-base mismatched DNA (100 nM), (d) unmatched DNA (100 nM) detection. (B) Reproducibility of the biosensor for target detection with five different electrodes under the optimal experiment conditions with 10 nM target.

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