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An ultrasensitive supersandwich electrochemical DNA biosensor based on gold nanoparticles decorated reduced graphene oxide

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ABSTRACT

In this article, a supersandwich-type electrochemical biosensor for sequence-specific DNA detection is described. In design, single-strand DNA labeled with methylene blue (MB) was used as signal probe, and auxiliary probe was designed to hybridize with two different regions of signal probe. The biosensor construction contained three steps: (i) capture DNA labeled with thiol was immobilized on the surface of gold nanoparticles decorated reduced graphene oxide (Au NPs/rGO); (ii) the sandwich structure formation contained "capture–target–signal probe"; and (iii) auxiliary probe was introduced to produce long concatamers containing signal molecule MB. Differential pulse voltammetry (DPV) was used to monitor the DNA hybridization event using peak current changes of MB in phosphate-buffered saline (PBS) containing 1.0 M $NaClO_4$. Under optimal conditions, the peak currents of MB were linear with the logarithm of the concentration of target DNA in the range of 0.1 μM to 0.1 fM with a detection limit of 35 aM (signal/noise = 3). In addition, this biosensor exhibited good selectivity even for single-base mismatched target DNA detection.

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As is well known, DNA biosensors have played an important role in early cancer diagnosis and gene mutation detection [1–3]. To date, various DNA biosensors based on different strategies have been developed. Among them, electrochemical DNA biosensors have attracted considerable attention [4–6] due to their highly sensitive and selective advantages [7,8]. During recent years, sandwich-type biosensors have become a mainstay in the DNA sensing field [9,10]. This type of biosensor generally contains capture probe, target sequence, and signal probe labeled with redox molecules [11,12]. Among them, capture probe and signal probe have complementary regions with target sequence. In the presence of target DNA, it can specifically hybridize with one region of capture or signal probe, resulting in longer DNA strand formation. However, the sensitivity of the biosensor is limited because each target sequence hybridizes to only a single copy of signal probe. To improve the sensitivity, multi-copy signal probes are needed to be introduced into the sandwich-type biosensor. Based on this assumption, Plaxco and coworkers [13] designed a signal probe that can hybridize to two different regions of target DNA. Thus, target DNA can hybridize many times with signal probe, resulting in long DNA concatamers containing multi-copy signal probes being attached to the electrode surface. This biosensor was called a supersandwich biosensor and showed high sensitivity (the detection limit is 100 fM). Based on the same principle, our group also developed a surpersandwich biosensor for the detection of *Escherichia coli* DNA sequence based on gold nanoparticles decorated reduced graphene oxide (Au NPs/rGO)[1] as sensing platform, and a detection limit of 0.35 fM was obtained [14]. In our experiment, we found that the formation of longer DNA concatamers cost multi-copy target DNA; thus, the sensitivity of the biosensor is limited. If the formation of longer DNA concatamers needs only one-copy target DNA, the sensitivity of the biosensor would be further improved. Based on this motivation, Yang and coworkers [15] designed another novel supersandwich-type DNA biosensor. In that design, auxiliary probe, which has complementary sequence with two different areas of signal probe, was introduced to hybridize many times with signal probe to create long DNA concatamers. This biosensor also showed higher sensitivity.

Inspired by Yang and coworkers' work, we also hoped to fabricate an ultrasensitive DNA biosensor for the detection of the K-ras gene because it is the most frequently mutated member in human

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[1] *Abbreviations used:* Au NPs/rGO, gold nanoparticles decorated reduced graphene oxide; $HAuCl_4{\cdot}4H_2O$, chloroauric acid; PBS, phosphate buffer saline; Ag/AgCl, silver/silver chloride; GCE, glassy carbon electrode; SDS, sodium dodecyl sulfate; DPV, differential pulse voltammetry; MB, methylene blue.

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tumors [16]. Thus, detection of the K-ras mutation gene would provide useful information in early diagnosis and in monitoring the disease. As is well known, graphene is a two-dimensional nanomaterial with a large surface area (2630 m^2/g) and extraordinary electronic properties [17], and Au NPs have excellent biocompatibility. In this study, rGO and Au NPs were used to immobilize capture DNA labeled with thiol, and the auxiliary probe was employed for long DNA concatamer fabrication. In the presence of signal probe and target DNA, a supersandwich structure containing multi-copy signal probes was created. Thus, the response signal was obviously amplified in contrast to the traditional sandwich assay, and sensitivity was clearly improved.

Materials and methods

Reagents

Chloroauric acid ($HAuCl_4{\cdot}4H_2O$) was obtained from Shanghai Chemical Reagent (Shanghai, China). Reduced graphene oxide was obtained from Sinocarbon Materials Technology (Taiyuan, China). K-ras gene sequence fragments were designed according to the literature [18]. These sequence fragments were purchased from Shanghai Sangon (Shanghai, China), and base sequences are listed as follows:

Capture probe: 5′-SH-$(CH_2)_6$-ATG-GTG-GTA-GTT-GGA-GCT-G-3′.
Complementary sequence: 5′-ACT-CTT-GCC-TAC-GCC-ACC-AGC-TCC-AAC-TAC-CAC-3′.
Signal probe: 5′-TGG-CGT-AGG-CAA-GAG-TCT-GTA-GAA-CGA-TCT-G-MB-3′.
Auxiliary probe: 5′-ACT-CTT-GCC-TAC-GCC-ACA-GAT-CGT-TCT-ACA-G-3′.
Single-base mismatch sequence: 5′-ACT-CTT-GCC-TAC-GCC-AXC-AGC-TCC-AAC-TAC-CAC-3′ (where X stands for T, A, or G).
Non-complementary sequence: 5′-GTC-ACG-TAA-CGT-ATA-GAT-CTA-CTT-CGT-CCA-GCT-3′.

Various sequence stock solutions were prepared with 0.01 M phosphate buffer saline (PBS, pH 7.4) and stored in a refrigerator. The buffers employed in this study are listed as follows: hybridization solution (0.01 M PBS + 0.1 M NaCl, pH 7.4) and electrochemical test solution (0.1 M PBS + 1.0 M $NaClO_4$, pH 7.4). All solutions were prepared with twice-quartz-distilled water.

Apparatus

All electrochemical measurements were performed on a CHI 650C electrochemical workstation (Shanghai Chenhua Instruments, Shanghai, China) with a conventional three-electrode system composed of a platinum wire as auxiliary, a silver/silver chloride (Ag/AgCl) as reference, and a glassy carbon electrode (GCE) or the modified electrode as working electrode. All potentials in this study are referenced to the Ag/AgCl.

Preparation of supersandwich-type biosensor

Prior to modification, the bare GCE (3 mm in diameter, CH Instruments) was polished to a mirror-like surface with 1.0-, 0.25-, and 0.05-μm gamma alumina suspensions, followed by rinsing thoroughly with water. After successive sonication in 95% ethanol and water for 5 min, the electrode was electrochemically cleaned in 0.50 M H_2SO_4 using a repetitive cyclic potential scan for 20 cycles between −0.3 and +1.50 V to remove any possible impurities on the surface. The GCE was allowed to dry at room temperature.

Next, 5.0 μl of 0.5 mg ml^{-1} rGO was dropped onto the surface of the fresh pretreated GCE and dried naturally to form a thin film at room temperature. The electrode obtained was then subjected to electrochemical deposition for 20 s at −200 mV in 0.1 M $NaNO_3$ solution containing 5.0 mg ml^{-1} $HAuCl_4$. Thus, Au NPs/rGO modified electrode was obtained and denoted as Au NPs/rGO/GCE.

Then, 5.0 μl of 1.0×10^{-6} M capture probe was dropped onto the surface of Au NPs/rGO/GCE and kept for 3 h in a refrigerator. During this process, capture probe was covalently linked onto the surface of the Au NPs. After that, the modified electrode was immersed in 0.1% sodium dodecyl sulfate (SDS) solution for 5.0 min to remove the unbound capture probe. Thus, the capture probe modified electrode was obtained and denoted as cDNA/Au NPs/rGO/GCE.

After that, 5.0 μl of the hybridization solution containing target DNA was dropped onto the surface of cDNA/Au NPs/rGO/GCE and incubated for 1 h at room temperature. Subsequently, it was incubated with 1.0 μM signal probe for 1 h. Finally, 10.0 μl of probe solution containing 1.0 μM signal and 1.0 μM auxiliary was dropped onto the surface of the modified electrode above and incubated for 2 h at room temperature. The resulting electrode was thoroughly rinsed with distilled water and dried under a nitrogen atmosphere.

Electrochemical measurements

Differential pulse voltammetry (DPV) was performed in a 10.0-ml electrochemical cell with 3.0 ml of 0.1 M PBS containing 1.0 M $NaClO_4$ (pH 7.4), which was deoxygenated with nitrogen bubbling for 10 min, and a nitrogen atmosphere was kept over the solution in electrochemical measurements. The experimental parameters were as follows: initial potential, −0.2 V; final potential, −0.650 V; pulse amplitudes, 0.05 V; pulse width, 0.05 s; sample width, 0.0167 s.

Results and discussion

Design strategy of biosensor

The fabrication protocol of the biosensor is shown in Fig. 1. In the design, Au NPs/rGO was employed to improve the electrode surface area and immobilization of capture probe. In the presence of target DNA, one terminus of target DNA can hybridize to one region of capture probe and the other terminus hybridizes with one region of signal probe, forming a "capture–target–signal probe" sandwich structure. When auxiliary probe is introduced, the 5′ terminus of auxiliary probe can hybridize to the 3′ terminus of signal probe, whereas the other terminus of auxiliary probe hybridizes to the 5′ terminus of signal probe, and continuous hybridization leads to supersandwich structure formation. The DNA hybridization event was investigated by measuring the changes in peak current intensity of the methylene blue (MB) in DPV before and after hybridization. The signal intensity from the supersandwich biosensor was obviously higher than that of the traditional sandwich-type biosensor.

Investigation of assembled process of biosensor

The assembled process of the biosensor was investigated through the changes in peak current intensity of signal molecules, and the results obtained are shown in Fig. 2A. From this figure, it can be seen that only a small signal could be observed in the presence of signal probe (curve b) and a very strong signal was

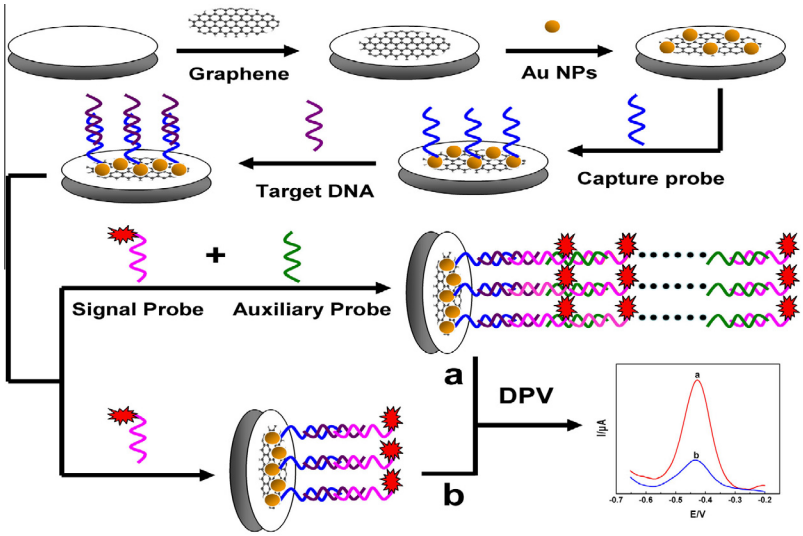

Fig.1. Schematic representation of the fabrication procedure of the DNA biosensor. (a) DPV cures from the supersandwich biosensor; (b) DPV cures from the sandwich biosensor.

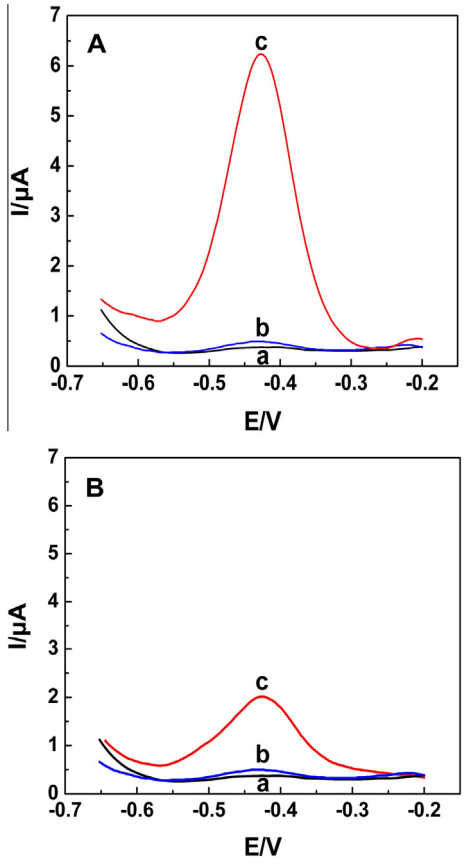

Fig.2. (A) DPV curves of the different assembled stages of the DNA biosensor in the presence of 0.1 nM target DNA: (a) capture probe; (b) capture probe + signal probe; (c) capture probe + target DNA + signal probe + auxiliary probe + . . . + signal probe + auxiliary probe (supersandwich type). (B) DPV curves of the traditional sandwich biosensor: (a) capture probe; (b) capture probe + signal probe; (c) capture probe + target DNA + signal probe. The concentration of both capture probe and signal probe is 10 μM. The supporting electrolyte is 0.10 M PBS containing 1.0 M $NaClO_4$ at pH 7.4.

observed in the presence of target DNA and auxiliary probe (curve c), indicating the formation of a supersandwich structure containing long DNA concatamers. The signal intensity was higher than that of the traditional sandwich assay under the same conditions (as seen in Fig. 2B).

Optimization of experimental conditions

In this study, the effects of the experimental conditions on the response signal (e.g., hybridization time, amounts of auxiliary probe and signal probe) were investigated. The supersandwich fabrication contains two steps of DNA hybridization. The first hybridization was used for traditional sandwich formation, and the second hybridization was used for the formation of longer DNA concatamers. When the concentration of target DNA and signal probe was kept constant (the hybridization experiment was performed at room temperature), the effect of the first hybridization time on signal intensity was as shown in Fig. 3A. It can be observed that the signal intensity of MB increased gradually as the hybridization time increased from 15 to 75 min and reached a maximal value at 60 min. So, the first hybridization time was selected as 60 min. Fig. 3B shows the influence of the amounts of auxiliary and signal probes on the response signal. It can be observed that the signal intensity of MB increased as the volume of the mixture solution containing 1.0×10^{-6} M auxiliary and 1.0×10^{-6} M signal probe increased from 2.0 to 10.0 μl. After that, it almost remained constant, so 10 μl of the solution was employed. Similarly, the second hybridization time was also investigated (as seen in Fig. 3C). It can be observed that the signal intensities of MB increased dramatically as the hybridization time increased from 0.5 to 2 h and remained constant after 2 h, indicating that the hybridization reaction was completed at 2 h. Therefore, the second hybridization time was selected for 2 h in this study.

Analytical performance

In the experiment, we selected the oxidation peak current of MB as the analytical signal to investigate the relationship between the signal intensity and concentration of target DNA. The results obtained are shown in Fig. 4. It can be observed that the signal intensities of MB increased as the concentration of the target DNA increased (as seen in curves a–j of Fig. 4A), and the signal intensity was linear with the logarithm of the concentration of target DNA from 0.1 fM to 0.1 μM with a detection limit of 35 aM (signal/noise = 3) (as seen in Fig. 4B). The linear regression

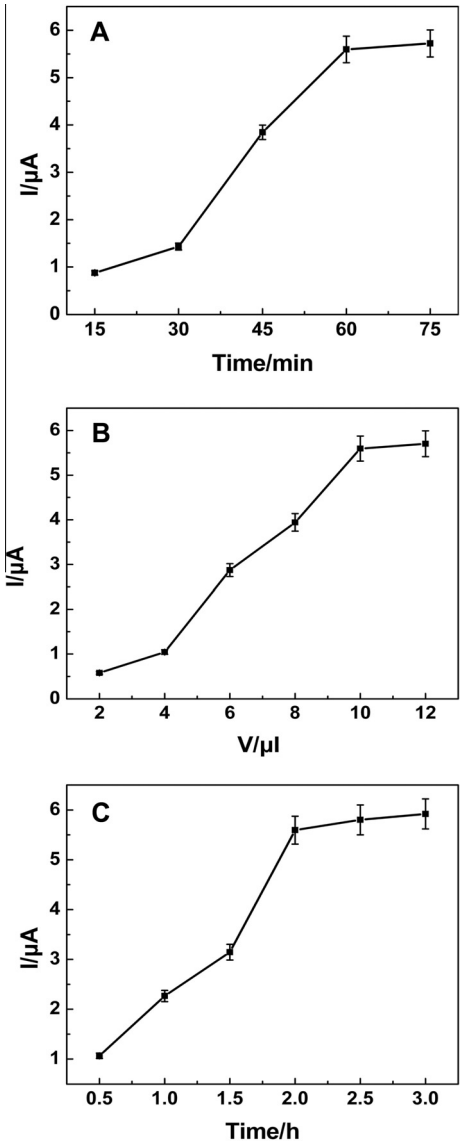

Fig.3. Optimization of experimental conditions. (A) Dependence of the peak current of MB on the hybridization time between DNA targets hybridized with capture probe and signal probe. (B) Dependence of the peak current of MB on the amount of auxiliary probes and signal probes The concentration of signal probe and auxiliary probe is 1.0×10^{-6} M, and the ratio is 1:1. (C) Dependence of the peak current of MB on the hybridization time between signal probe and the solution containing auxiliary probes and signal probes. The concentration of the target DNA is 1.0×10^{-10} M.

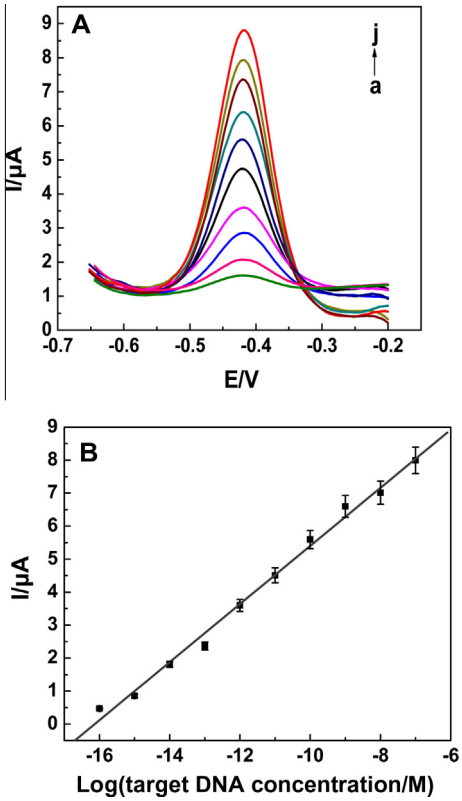

Fig.4. (A) DPV curves of the biosensor in the presence of different target DNA concentrations in the 0.10-M PBS solutions containing 1.0 M $NaClO_4$ (pH 7.4): (a) 0.1 fM; (b) 1 fM; (c) 10 fM; (d) 100 fM; (e) 1 pM; (f) 10 pM; (g) 100 pM; (h) 1 nM; (i) 10 nM; (j) 100 nM. (B) Linear relationship between peak current and the logarithm of the target DNA concentration. The illustrated error bars represent the standard deviation of five repetitive measurements at each concentration.

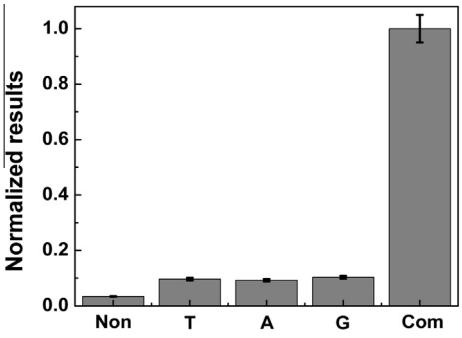

Fig.5. Normalized histograms of the DPV signals (I/I_{COM}) of the biosensor-recognized different DNA sequences. Non, non-complementary; Com, complementary.

equation was I (μA) = 14.21 + 0.88 lg c (unit of c is M), and the correlation coefficient was 0.9964. The sensitivity of the biosensor is higher than those reported by Plaxco and coworkers [13] and Yang and coworkers [15] and is almost consistent with our previous work [19]. We believed that the reason involves two aspects: (i) the Au NPs/rGO provides larger surface areas (0.093 cm^2, where bare electrode is 0.071 cm^2) and good conductivity; (ii) auxiliary probe hybridizes many times with signal probe, resulting in longer DNA concatamer formation containing multi-copy signal molecules.

Selectivity of DNA biosensor

The selectivity of the DNA biosensor was investigated by using the non-complementary, single-base mismatch (T, A, and G), and complementary DNA sequences. The experimental results obtained are shown with histograms in Fig. 5. It can be observed that the complementary sequence obtained the largest signal intensity, the non-complementary sequence showed no signal, and the single-base mismatch sequence showed only small signal. It obtained only 9.7% signal intensities of the complementary sequence. These results indicate that this biosensor has good selectivity that can distinguish single-base mismatch DNA sequence.

Reproducibility and stability of DNA biosensor

The reproducibility of the biosensor was also investigated. In this study, five DNA biosensors (independently fabricated under the same conditions) were used to detect 0.1 nM target DNA, and their respective signal intensities were recorded. The signal values

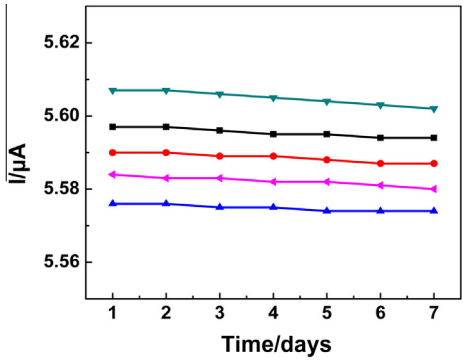

Fig.6. Signal intensities of five biosensors obtained within 1 week.

obtained were 5.597, 5.590, 5.576, 5.607, and 5.584 μA. The relative standard deviation was 0.21%, indicating that the DNA biosensor had good reproducibility. Similarly, the stability of the biosensor was investigated using five biosensors independently to detect 0.1 nM target DNA (one time daily) and recorded the signal intensity of MB within 1 week. These results are shown in Fig. 6. It can be observed that the values obtained show no distinct changes, indicating that the biosensor has excellent stability.

Conclusions

A supersandwich electrochemical DNA biosensor has been designed. Auxiliary probe was introduced to create long DNA concatamers. The biosensor design is novel and exhibits high sensitivity and selectivity.

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