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Signal amplification of streptavidin–horseradish peroxidase functionalized carbon nanotubes for amperometric detection of attomolar DNA†

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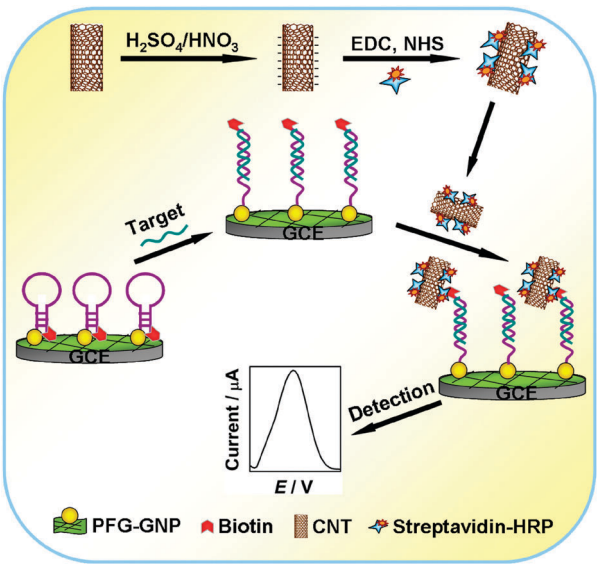
A novel biosensing strategy for selective electrochemical detection of DNA down to the attomolar level with a linear range of 5 orders of magnitude was developed by the specific recognitions of target DNA and streptavidin to biotin labelled molecular beacon and signal amplification of streptavidin–horseradish peroxidase functionalized carbon nanotubes.

The specific sequence detection of DNA has attracted considerable interest due to its broad applications in molecular diagnostics, genetics therapy, and early screening of cancers.[1] It is highly desirable to develop an ultrasensitive detection method for a specific DNA sequence, especially for low-abundant DNA. Some signal amplification strategies have been designed for the detection of DNA at a low level. For example, an ultrasensitive DNA sensor has been constructed at a silane copolymer modified indium–tin oxide electrode by the electrocatalysis of Pd nanoparticles toward oxidation of $NaBH_4$;[2] and several enzyme-based sensors have been developed for the detection of femtomolar DNA targets.[3] In order to improve the specificity of DNA detection, molecular beacon (MB), a single-stranded oligonucleotide hybridization probe with a stem–loop structure, has also been introduced for design of a novel DNA sensing strategy by combining with some signal amplification strategies.[4] This work designed a new interfacial MB nanoprobe for target DNA and a novel signal amplification method by using streptavidin–horseradish peroxidase (HRP) functionalized carbon nanotubes (CNTs) as a detection probe to amplify the detectable signal.

The interfacial nanoprobe was prepared by immobilizing MB labelled with biotin and thiol at 5′ and 3′ ends on a glassy carbon electrode (GCE) modified with gold nanoparticles (GNP) and 1-pyrenebutyrate functionalized graphene (PFG) (Scheme 1), and the detection probe was synthesized by using a covalent method (ESI†). The nanoprobe was expected to be in the "closed" state of MB, in which the detection probe was blocked for the interaction with the immobilized biotin end due to the large steric effect. After hybridization with target DNA, the loop sequence (11-base, ESI†) formed a rigid duplex with the target, which broke the relatively shorter stem duplex (6-base). Consequently, the end biotin was "activated" by a force away from the electrode for easy access to the detection probe, streptavidin–HRP labelled CNTs. The introduction of detection probe to a sensor surface led to a significantly amplified current signal produced from an enzymatic cycle. Thus an enzyme-based signal-on DNA sensor was developed for the specific detection of target DNA down to the attomolar level.



Scheme 1 Schematic representation of functionalization of CNTs with streptavidin–HRP and detection of DNA hybridization with MB as a switch.

Here, multi-walled carbon nanotubes (MWCNTs) were used as carriers for loading numerous enzyme tags and accelerating electron-transfer after an enzymatic catalytic reaction of HRP due to the unique electronic, chemical, and mechanical properties.[5] The pristine MWCNTs were firstly functionalized and shortened by sonication in 3 : 1 H_2SO_4/HNO_3 for 4 h to introduce hydrophilic carboxylate groups.[6] The carboxylated MWCNTs showed a homogeneous surface and good dispersion (Fig. 1A). Then, streptavidin–HRP was covalently linked on the carboxylated MWCNTs, the latter became synechia with the increase in their average diameter of

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† Electronic supplementary information (ESI) available: Experimental details and optimal conditions for the volume ratios of GNP : PFG. See DOI: 10.1039/c1cc10840a

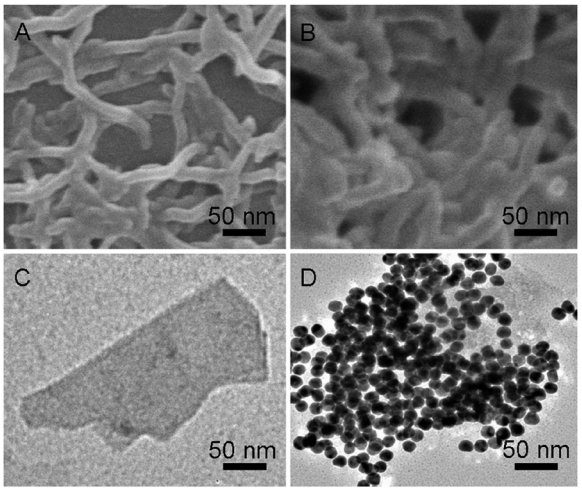

Fig. 1 SEM images of (A) MWCNTs and (B) streptavidin–HRP–CNTs bioconjugate and TEM images of (C) graphene and (D) GNP–PFG composite.

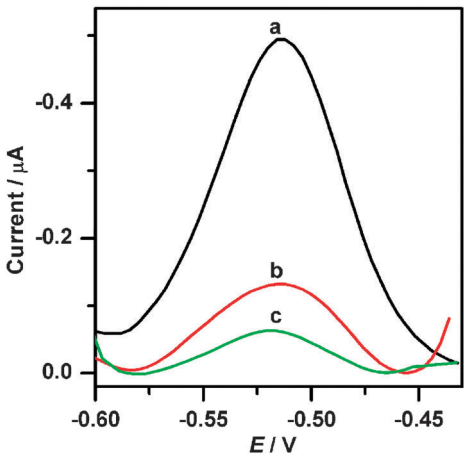

Fig. 2 DPV responses for streptavidin–HRP–CNTs probe (a) and streptavidin–HRP probe (b) in the presence of 1 fM target DNA and streptavidin–HRP–CNTs probe in the absence of target DNA (c).

27 nm (Fig. 1B), indicating that the streptavidin–HRP molecules were loaded onto the carboxylated MWCNTs to form streptavidin–HRP–CNTs bioconjugate.

In order to immobilize a large amount of MB on the electrode surface, the composite of GNP and PFG was prepared. Compared to other carbon structures, graphene with a two-dimensional structure and good conductivity provided a larger surface to support the substrate, and the assembly of 1-pyrenebutyric acid on graphene became easy *via* π–π stacking interaction,[7] which greatly improve the dispersion of graphene in water. Further, the GNP–PFG composite was formed *via* the interaction between GNP and the carboxylic group attached on PFG. Transmission electron microscopic (TEM) images revealed few-layer flexible wrinkled sheets of the graphene situated on the top of a copper grid (Fig. 1C). Benefiting from the high specific surface of PFG sheet, it showed an efficient capacity of loading GNP. As shown in Fig. 1D, the colloidal GNP with a diameter of 14 nm were distributed on the whole surface of PFG sheets. Due to this ingenious conformation, the presence of the GNP could well separate the PFG sheets from aggregating by the π–π stacking interaction.

The ratio of GNP to PFG sheets ($\gamma_{G/P}$) affected greatly the electrochemical response of the proposed sensor for detection of DNA target (Fig. S1 in ESI†). At low GNP loadings ($\gamma_{G/P} < 10:1$), the increase of GNP loading enhanced the capability for immobilizing MB, thus increased the electrochemical response to target DNA. Contrarily, when the $\gamma_{G/P}$ was higher than 10 : 1, the electrochemical response decreased due to the slightly increased electronic resistance resulting from the citrate moieties on GNP. Thus, 10 : 1 was chosen as the optimal $\gamma_{G/P}$ for preparation of the composite.

After the biosensor hybridized with the target oligonucleotide, it was rinsed with phosphate buffered saline (PBS) and then incubated with 6 μL of streptavidin–HRP–CNTs at 37 °C for 30 min. Afterwards, the biosensor was rinsed with PBS and subjected to differential pulse voltammetric (DPV) measurement in PBS containing *o*-phenylenediamine (*o*-PD) and hydrogen peroxide (H_2O_2). The detection solution exhibited a stable DPV peak at −0.514 V (Fig. 2a), which corresponded to the reduction of 2,2′-diaminoazobenzene, the enzymatic product.[8] The electrochemical signal was directly related to the coverage of the HRP, which could be used to monitor the DNA hybridization. As a contrast, an experiment only using streptavidin–HRP as the detection probe instead of streptavidin–HRP–CNTs bioconjugate was performed. As shown in Fig. 2b, the signal produced from streptavidin–HRP probe was only 26% of that from streptavidin–HRP–CNTs at 1 fM target DNA. MWCNTs obviously played a role of dual signal amplification in both the aggregation of enzyme and the electronic transduction event.

In the absence of target DNA, only 12% of DVP response was observed compared with that in the presence of target DNA (Fig. 2c). This result should be due to the fact that the immobilized MB probe was in the "closed" state in the absence of the target, which shielded biotin from being approached by the streptavidin–HRP–CNTs bioconjugate due to the steric effect. The small response resulted from the nonenzymatic reduction of H_2O_2 or nonspecific adsorption of a small amount of streptavidin–HRP–CNTs on the sensor.

The DPV signal was intensified in a concentration-dependent manner. With the increasing target DNA concentration, the DPV peak current increased. The response was logarithmically related to the target concentration across a range of 10 aM to 1 pM (Fig. 3). The detectable concentration range of 5 orders of magnitude was relatively wide. At the current signal of 3 times standard deviation (SD) above the mean at a zero standard, the limit of detection was 2.8 aM, which is much lower than 10 fM for enzyme-based electrochemical DNA sensors[3a] and 0.52 fM for an streptavidin/CdTe tagged-polybead labelled electrochemical DNA biosensor.[9] This high sensitivity highlighted the importance of using MWCNTs to carry numerous streptavidin–HRP, that is, the multiple enzymatic turnovers significantly amplified the current. This improved analytical performance should also originate from the steric effect of the large size of MWCNTs, which could reduce background current, and increase the ratio of signal to noise.

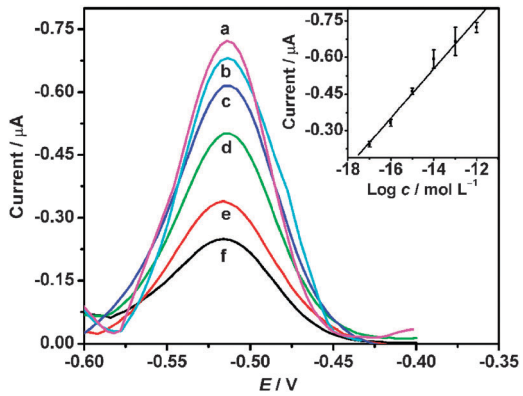

Fig. 3 DPV curves at target concentrations of 1 pM (a), 100 fM (b), 10 fM (c), 1 fM (d), 100 aM (e), 10 aM (f). Inset: linear relationship between peak current and the logarithm of target DNA concentration.

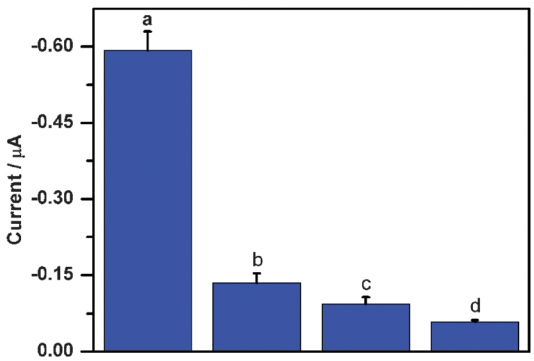

Fig. 4 Histograms of peak currents for complementary sequence (a), single-base mismatched sequence (b), three-base mismatched sequence (c) and blank (d).

The specificity of the interfacial nanoprobe of MB (oligo 1) was studied by hybridizing with the same concentration of perfect complementary target (oligo 2), single-base mismatched oligonucleotide (oligo 3) and three-base mismatched oligonucleotide (oligo 4) (Table S1 in ESI†). As shown in Fig. 4, the probe presented good performance to discriminate oligo 2 and the bases mismatched oligonucleotides. The signals of oligo 3 and oligo 4 were 4.5 and 6.2 times lower than that of oligo 2 and closed to the response of blank, respectively, indicating good selectivity. This high specificity arose from the conformational constraint of the stem–loop structure of MB; that is, the presence of the stem made it thermodynamically unfavorable for the binding of the mismatched sequence to the loop.[10] Due to its signal-on nature, this sensor was also resistant to the non-complementary sequences and could not give the detection signal.[11] These results demonstrated that the proposed approach was able to effectively detect the target with specificity. In addition, the responses of five freshly prepared biosensors to 10 fM of target DNA showed a relative standard deviation of 3.7%, indicating good fabrication reproducibility.

This work has demonstrated a highly sensitive and specific DNA detection platform based on interfacially immobilized MB as a switch and the streptavidin–HRP–CNTs bioconjugate for signal amplification. In the closed conformation, the biotin label is sterically shielded and thus inaccessible to the reporter enzyme. Upon target binding, the disruption of the stem–loop and the formation of the duplex make the streptavidin–HRP–CNTs bioconjugate accessible for binding, which catalyzes the oxidation of *o*-phenylenediamine by hydrogen peroxide to produce electrochemical active 2,2′-diaminoazobenzene, leading to the greatly enhanced detecting signal compared with free streptavidin–HRP. To shift positively the applied potential for amperometric detection, other co-substrates of peroxidase like hydroquinone may be also used in this system. Moreover, the background current is small due to the steric effect, which shields biotin from being approached by the large-sized streptavidin–HRP–CNTs bioconjugate. This novel method can detect target DNA down to the attomolar level with excellent selectivity to differentiate single-base mismatched and three-base mismatched sequences of DNA, which makes it promising for genetic target analysis in biomedical and bioanalytical applications.

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