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Short communication

# Construction of highly ordered polyaniline nanowires and their applications in DNA sensing

Yuanqiang Hao [a,1], Binbin Zhou [a,b,1], Fangbin Wang [b], Juan Li [a], Liu Deng [a,*], You-Nian Liu [a,c,*]

[a] College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, PR China
[b] Hunan Institute of Food Quality Supervision Inspection and Research, Changsha, Hunan 410111, PR China
[c] Key Laboratory of Resources Chemistry of Nonferrous Metals, Central South University, Ministry of Education, Changsha, Hunan 410083, PR China

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ABSTRACT

A novel electrochemical active polyaniline (PANI) nanowire was fabricated and utilized for the construction of a highly sensitive and selective electrochemical sensor for hepatitis B virus gene. The uniform PANI nanowire was prepared by the enzymatic polymerization of aniline monomers on the amyloid-like nanofiber (AP nanowire), which was self-assembled from an aniline-attached nonapeptide, aniline-GGAAKLVFF (AP). The prepared PANI nanowires were characterized by electron microscopy, UV–vis absorption spectra, and cyclic voltammetry (CV). These ultra-thin nanowires displayed high electrochemical activity. Then the nucleic acid biosensor was constructed by modifying a glass carbon electrode with AP nanowires which were functionalized by a designed hair-pin loop DNA. Upon the presence of target nucleic acid and horseradish peroxidase (HRP) labeled oligonucleotide, the HRP will catalyze the polymerization of aniline monomers conjugated in AP nanowires, leading to the formation of PANI nanowires which can bring about a dramatical increase in the current response of the biosensor. The dynamic range of the sensor for hepatitis B virus gene is 2.0–800.0 fM with a low detection limit of 1.0 fM ($3\sigma$, $n=10$). The biosensor also displayed highly selectivity and stability. All these excellent performances of the developed biosensor indicate that this platform can be easily extended to the detection of other nucleic acids.



## 1. Introduction

Conducting polymers have promising applications in various fields including biosensors, electronic devices, integrated circuits, batteries etc., due to their excellent mechanical, photonic and electronic properties (Das and Prusty, 2012; Gerard et al., 2002; Hatchett and Josowicz, 2008). In particular, one-dimensional nanostructured polyaniline (1D nano-PANI), a most promising candidate as a structural element of nanoscale devices, has attracted enormous interest because of its reversible switching of electrical conductivity, facile process ability, mechanical flexibility and high environmental stability (Lu et al., 2011; Tran et al., 2009). Many strategies have been developed for the fabrication of 1D nano-PANI, such as hard template synthesis, soft-template synthesis and self-assembly method without any external templates (Zhang and Wang, 2006). Among the above-mentioned methods, soft template methods have been drawn extensive attention, because of its facile and low cost of preparation, and excellent environmental stability. Due to the intermolecular hydrogen bonds and specific secondary structures, peptides have been recognized as very useful soft materials as templates for the construction of controllable nanostructures, such as gold nanoparticles, platinum and silver nanowires (Kim et al., 2012; Reches and Gazit, 2003; Scheibel et al., 2003; Zhang et al., 2012; Zhou et al., 2013). The special advantages of programmable sequence, the unique self-assembly properties and the easy access for chemical functionalization also allow peptide-based nanostructures to act as favorable templates for creating nanoscale conducting polymers (Castelletto et al., 2009; Liu et al., 2011; Ryu and Park, 2009; Zhang et al., 2012).

This work aimed to develop an effective route to fabricate a novel PANI nanowire by using a peptide-assembled nanowire as the template. And then based on the excellent electrochemical activity of the formed PANI nanowire, we tried to construct an electrochemical biosensor for nucleic acids, specifically hepatitis B (HBV) gene. Hepatitis B virus (HBV) infection is one of the most common infectious diseases worldwide which may lead to chronic hepatitis, cirrhosis, various cancer diseases and even death (Beasley, 1988; Wang et al., 2009). Due to the clinical reason, accurate and sensitive diagnosis of HBV during the early stages of infection is quite crucial. Various techniques have been applied to achieve sensitive detection of DNA, such as molecular biology

* Corresponding authors at: College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, PR China. Tel./fax: +86 731 8883 6964.
*E-mail addresses:* dengliu@csu.edu.cn (L. Deng), liuyounian@csu.edu.cn (Y.-N. Liu).
[1] These authors contributed equally to this work.

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(Kuang et al., 2011), electrochemistry (Hsieh et al., 2011; Liu et al., 2013), spectroscopy (Cheng et al., 2011), and colorimetry (Xia et al., 2010). The electrochemical approach has the advantages of high sensitivity, low-cost, fast response and simple instrumentation (Yang and Zhang, 2011). As concentrations of many DNA biomarkers in the human fluidic system are ultra-low, different signal amplification strategies have been introduced into these detection tools to achieve the sensitive detection of DNA. One of the most useful ways is to amplify the signal by large loading of signal labels such as enzymes or nanomaterials (Gao et al., 2011; Ji et al., 2012). And the ultrasensitive electrochemical sensor for HBV gene developed here utilized the catalytic effect of horseradish peroxidase (HRP) for signal amplification.

We first systematically explored the synthesis and properties of the PANI nanowires to pave the way for their further analytical applications. By the covalent attachment of aniline monomer to the *N*-terminal of a designed nonapeptide GGAAKLVFF, aniline-GGAAKLVFF (AP) as a structural unit was obtained. And followed, AP readily self-assembled to ultra-thin and uniform amyloid-like fibril (AP nanowire). Then aniline monomers on the AP nanowire can be enzymatically polymerized into PANI nanowire in the presence of HRP and $H_2O_2$. Due to the covalent linkage and the ordered template self-assembled from the peptide, aniline monomers were forced to uniformly polymerize in the head-to-tail orientation on the AP nanowire. Based on the strategy for the fabrication of PANI nanowire and the high electrochemical activity of the formed PANI, a sensitive electrochemical biosensor for HBV gene was developed. AP nanowire as the substrate was functionalized by designing hair-pin loop DNA (S1) and then deposited on a carbon glassy electrode. Upon the presence of target nucleic acid (S2) the DNA loop will be opened due to the formation of heteroduplex between S2 and complementary DNA sequence of S1. Then a HRP-labeled oligonucleotide probe (S3) was introduced, and S3 can hybridize with the remaining base sequence on S1 which brought the labeled HRP adjacent to the aniline monomers on the AP nanowire. HRP can effectively catalyze the polymerization of aniline in the presence of $H_2O_2$. Then the formed PANI can generate a stable electrochemical signal, which allows the ultrasensitive quantification of nucleic acids. Taking HBV gene as a model, the developed electrochemical biosensor displayed wide linear range, high sensitivity and selectivity, and good stability, implying that this promising strategy can be extended to the detection of a wide range of nucleic acids.

## 2. Experimental section

### 2.1. Materials

Wang resin, Fmoc-protected amino acids, triisopropylsilane (TIS), diisopropylcarbodiimide (DIC), 1-hydroxylbenzotriazole, trifluoroacetic acid (TFA) and piperidine were purchased from GL Biochem (Shanghai, China). 3-Aminophenylacetic acid was obtained from Aladdin (Shanghai, China). A solution of 30% hydrogen peroxide ($H_2O_2$) was purchased from Beijing Chemical Co. (Beijing, China). Horseradish peroxidase (HRP) type II (200 units/mg solid) was received from Sigma-Aldrich (St. Louis, MO). Oligonucleotide sequences were purchased from Sangon Co., Ltd. (Shanghai, China). Organic solvents were obtained from Hengxing Co. (Tianjin, China). All aqueous solutions were prepared with deionized water (18 MΩ $cm^{-1}$) treated with a water purification system (Simplicity Plus, Millipore Corp., Billerica, MA).

### 2.2. Peptide synthesis

Aniline conjugated GGAAKLVFF peptide (AP) was synthesized by solid phase (Fmoc) chemistry on a peptide synthesizer from CS Bio Co. (Menlo Park, CA), and was purified by semi-preparative reversed-phase HPLC (Shimadzu 6AD, Columbia, MO) equipped with a C18 column (dimension of 250 × 4.6 mm i.d.) (detailed information shown in *Experimental S1.1*). The molecular weight of the AP was verified by electrospray ionization mass spectrometry (ESI-MS). ESI-MS for AP: *m*/*z*: calcd. for $C_{53}H_{75}N_{11}O_{11}$ $[M+H]^+$: 1043.23; found 1042.91.

### 2.3. Sample preparation

Water (1 mL) was added to 4.5 mg of lyophilized peptide, and the sample was sonicated at 25 °C for 10 min in a bath sonicator. The solution was then mixed for 1 h in an IKA Vortex mixer, followed by incubation for 24 h. Then the as treated sample (50 μL) was pipetted out and followed by adding of 50 μL phosphate buffer (pH 4.5). The aniline monomers were polymerized enzymatically by adding HRP (1 mg $mL^{-1}$ in 50 mM phosphate buffer at pH 4.5) and 50 μL 30% $H_2O_2$ successively. To avoid the inactivation of HRP by excess $H_2O_2$, $H_2O_2$ was added dropwise over 15 min. Then, the sample was incubated for 2 h at room temperature to form the PANI.

### 2.4. Atomic force microscopic measurements

The morphology of AP nanowires and PANI nanowires were characterized with a MFP-3D-SA microscope (Asylum Research, Santa Barbara, CA) using the tapping mode in air. For imaging, aliquots of samples were cast onto these mica sheets that had been pretreated with 10 mM $NiCl_2$ for 15 min, afterwards, the mica sheets were gently rinsed with water to remove salt, and then dried with nitrogen.

### 2.5. Electron microscopy measurements

For electron microscopy characterization of AP nanowires and PANI nanowires, 5 μL of the polymerized or nonpolymerized sample was placed on the grid which was covered by a carbon-stabilized formvar film. After 2–3 min, excess fluid was removed with a filter paper. The specimens were finally examined and photographed in a T20 FEI TECNAI $G^2$ electron microscope at 80 kV.

### 2.6. Preparation of DNA sensor based on AP nanowire

Firstly, 500 μL AP nanowire solution was added with 200 μL of 100 μM capture probe (S1) in 0.1 M HEPES buffer (pH 7.5) in the presence of EDC (0.1 M) and NHS (0.1 M). The mixture was allowed to equilibrate for 2 h with constant stirring. The S1 functionalized AP nanowires were collected by centrifugation, followed by washing three times with PBS. Then, five-microliter solution of the S1/AP nanowire was cast on the glass carbon electrode surface and then dried at 4 °C. Then 2 μL hybridization solution containing the target nucleic acid (HBV) was uniformly spread onto the sensing interface. After hybridizing for 60 min, the electrode was rinsed thoroughly with the PBS buffer at 50 °C, and further hybridized with 5.0 μL of 100 μM of HRP labeled oligonucleotide detection probes (S2) at 25 °C for 30 min. Finally, after thorough rinsing with hybridization buffer (pH 9.5), the biosensor was incubated with 10 mM $H_2O_2$ in phosphate buffer (0.10 M, pH 4.0) for 30 min. The electro-oxidation of PANI was recorded by square-wave voltammetry (SWV) at pH 4.0 in 0.10 M phosphate buffer. In the case of lower nucleic acid concentrations, smoothing was applied after each measurement to remove random noise and electromagnetic interference.

## 3. Results and discussion

### 3.1. Preparation and characterization of the PANI nanowires

The procedure for the preparation of PANI nanowire was depicted in Fig. 1A. Aniline monomer was first conjugated to a special peptide which can self-assemble into nanostructures. AAKLVFF, derived from the sequence of β-amyloid (16–20), was chosen as the construction unit. This heptapeptide has very interesting self-assembly properties, and in aqueous solution it can form very well ordered fibrils (Castelletto et al., 2008). In order to preserve the self-assembling properties of AAKLVFF and the polymerization ability of aniline, two glycine residues were attached to the *N*-terminal of AAKLVFF. Then, 3-aminophenylacetic acid, as the aniline monomer, was conjugated to the *N*-terminal of GGAAKLVFF, leading to the formation of aniline-GGAAKLVVF (AP). AP can readily self-assemble into amyloid-like fibrils (AP nanowires) in aqueous solution spontaneously. And PANI nanowires were successfully obtained by enzymatic polymerization of aniline monomers on the self-assembled AP nanowires in the presence of HRP and $H_2O_2$ (shown in Fig. 1A). Considering both the activity of the enzyme and the protonation of the aniline monomer, the polymerization was carried out at pH 4.5 (Liu et al., 1998). The formation of PANI was first confirmed by a dramatic color change of the solution from colorless (AP monomers) to light green (PANI) (Fig. S1).

The morphology of PANI nanowires was characterized by AFM and TEM measurements. Fig. 1B and C shows tapping-mode AFM images of AP nanowires and PANI nanowires, respectively. The self-assembled AP nanowires displayed a smooth surface with apparent heights of about 5 nm and lengths of more than several micrometers, corresponding to aspect ratios of $\sim 10^3$. After the formation of PANI on the AP nanowires, no apparent change was observed in the morphology of PANI nanowires. This is due to that the aniline monomers were located on the surface of the AP nanowires before the polymerization and there were no additional aniline monomers introduced during the polymerization. In addition, the morphology of the as-prepared paptide-PANI nanowire is quite uniform and no individual PANI agglomerates were observed. Similar results were also obtained by TEM measurements. The above morphological studies indicate that the proposed strategy is able to obtain homogeneous AP nanowire templates as well as PANI nanowires with small sizes ( $< 10$ nm) and high aspect ratios, which are of great importance for their further applications.

UV–vis spectra were also used to determine the formation of PANI. Before polymerization, no obvious absorption in the visible region was observed for AP nanowire. On the other hand, after the polymerization, the UV–vis spectrum of the PANI nanowires showed the characteristic absorption at about 660 nm (Fig. S3), attributing to an intermolecular and/or intramolecular charge-transfer process from the benzenoid to quinoid ring (Alber et al., 2013). In addition, no absorption band at approximately 460 nm appears in the spectrum, indicating that PANI was primarily formed by the desired head-to-tail coupling without branched structures, which make these PANI nanowires retained the full electrochemical activity of PANI (Liu et al., 1999).

To construct effective a biosensor based on the electrochemical activity of the formed PANI nanowire, we investigated the electrochemistry of the PANI nanowires. The cyclic voltammogram of the PANI nanowire-modified electrode in 0.1 M of $H_2SO_4$ displays two pairs of redox peaks with the formal potential ($E^{o\prime}$) of ca. 0.31 and 0.55 V, respectively (as shown in Fig. S4), similar to the reported results (Ryu and Park, 2009). The redox pair with $E^{o\prime}$ of 0.31 V corresponds to the transition between the fully reduced leucoemeraldine and the partially oxidized emeraldine salt, and the other redox pair with $E^{o\prime}$ of 0.55 V can be ascribed to the transition from the emeraldine salt to fully oxidized pernigraniline.

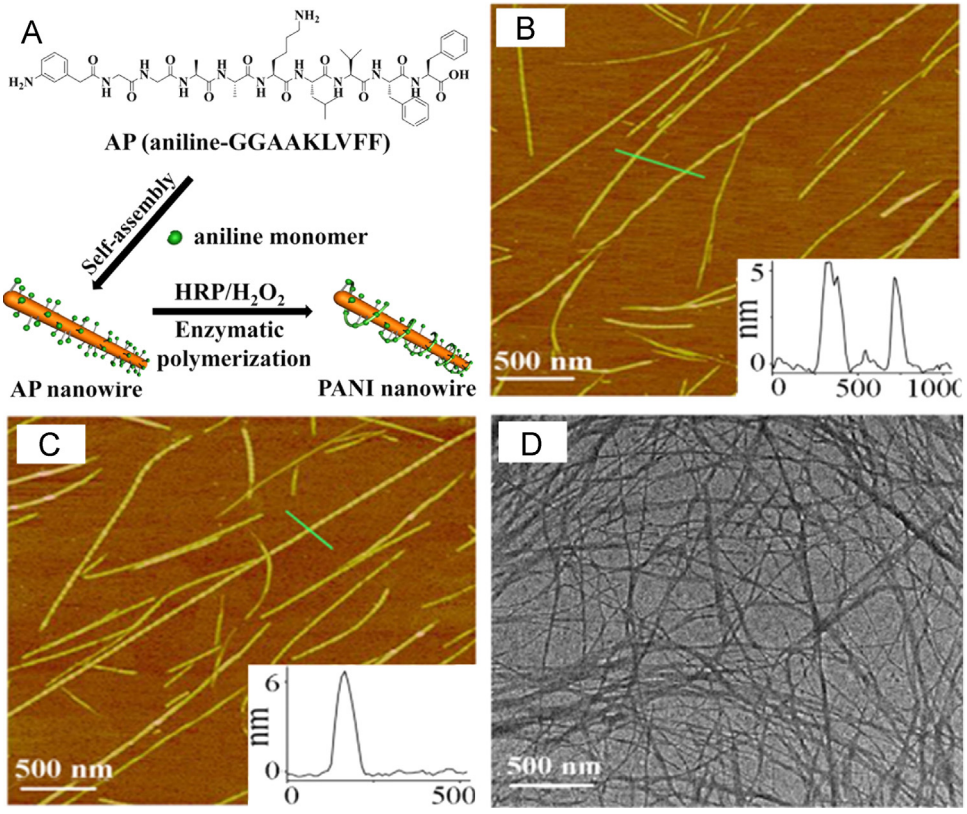


**Fig. 1.** (A) Schematic illustration of the steps for synthesis of PANI nanowires, (B) AFM image of AP nanowires, (C) AFM and (D) TEM image of PANI nanowires.

### 3.2. Construction and condition optimization of the electrochemical biosensor

Based on the uniform structure and high electrochemical activity of these prepared PANI nanowires, we constructed a highy sensitive and selective electrochemical biosensor. As shown in Fig. 2A, the prepared AP nanowires with covalently labeled nucleic acid capture probe (S1) was firstly deposited on a glassy carbon electrode. Then S1 hybridized with the target nucleic acid forming a heteroduplex. After that, S1 could further hybridize with HRP-labeled oligonucleotide (S2), which brought HRP adjacent to the aniline monomers on the AP nanowires. HRP can catalyze the polymerization of aniline in the presence of $H_2O_2$. Then the formed PANI can generate a stable electrochemical signal, which allows the ultrasensitive quantification of nucleic acids.

The biosensor is based on the electrochemical signals generated by the PANI nanowires which were formed by the HRP-catalyzed polymerization of anilines in the presence of $H_2O_2$. Thus polymerization time and $H_2O_2$ concentration are crucial for the performance of the DNA biosensor, and the influences of these two parameters on the amperometric response of the biosensor were examined (Fig. 2B and C). The current response of the biosensor enhanced with the polymerization time increasing from 5 min to 50 min, demonstrating that there was a little activity loss of HRP during the reaction process. Then 30 min was chosen to achieve a balance between high sensitivity and assay time. The electrochemical signal increased with the concentration of $H_2O_2$ from 0.05 mM to 10 mM. The increase was attributed to more enzymatic active sites in the higher $H_2O_2$ concentration (Jin et al., 2001). When the $H_2O_2$ concentration exceeded 10 mM, electrochemical signal decreases were observed which is probably due to the inhibition of HRP activity by high concentration of $H_2O_2$ (Chance, 1949).

### 3.3. Quantitative measurements of HBV DNA

As a very sensitive and very fast voltammetric method, square wave voltammetry (SWV) was used for the quantification of the target nucleic acid. In the presence of target DNA (taking 29 mer HBV DNA as a model), an obvious cathodic peak at 0.26 V appears for the prepared electrode, corresponding to the transition of leucoemeraldine/emeraldine for PANI. Fig. 3A shows the current response of the biosensor to different concentrations of 29 mer HBV DNA. The dynamic range of the sensor is 2.0–800 fM (Fig. 3B) with a limit of detection (LOD) of 1.0 fM ($3\sigma$, $n=10$) (Long and Winefordner, 1983), and the linear equation can be expressed by $I=0.01178\times[\mathrm{DNA}]/\mathrm{fM}+0.627$ ($R^2=0.998$). The LOD is lower than those achieved by previously reported DNA sensors, such as fluorescence biosensor based on graphene oxide (He et al., 2010), colorimetric biosensor based on hemin-graphene intrinsic peroxidase (Guo et al., 2011). And the LOD is similar to the value obtained from electrochemical biosensor which was based on the DNA-guided deposition of PANI (Ryu and Park, 2009).

More importantly, the constructed DNA sensor shows excellent selectivity even towards single-nucleotide mismatches. As shown in Fig. 3C, when the target DNA was replaced by mutated DNA, the current dropped by at least 81% for single-base mismatches, by ca. 90% for two-base mismatches, and the current became indistinguishable from the background when three bases in the sequence did not match the basic unit. The relative standard deviation of six different freshly prepared DNA sensors was 3.7%, implying a good fabrication reproducibility of the DNA sensor. Moreover, it was found that the peak current of the as-prepared electrode retained 96% of the initial value after the electrode was stored in distilled water at 4 °C over 15 days. The high stability of the modified electrode can be attributed to the extraordinary stable structure of

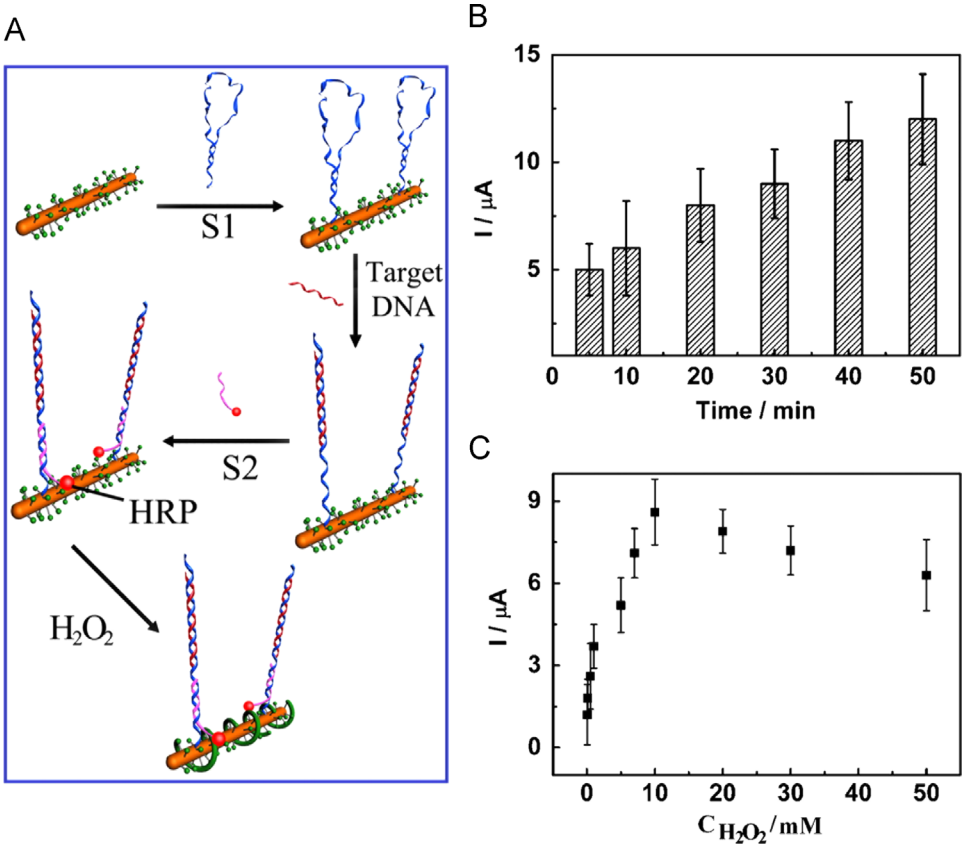


**Fig. 2.** (A) The protocol for the fabrication of the DNA sensor. Influence of polymerization time (B) and $H_2O_2$ concentration (C) on the response current of the prepared DNA biosensor in the presence of 700 fM HBV.

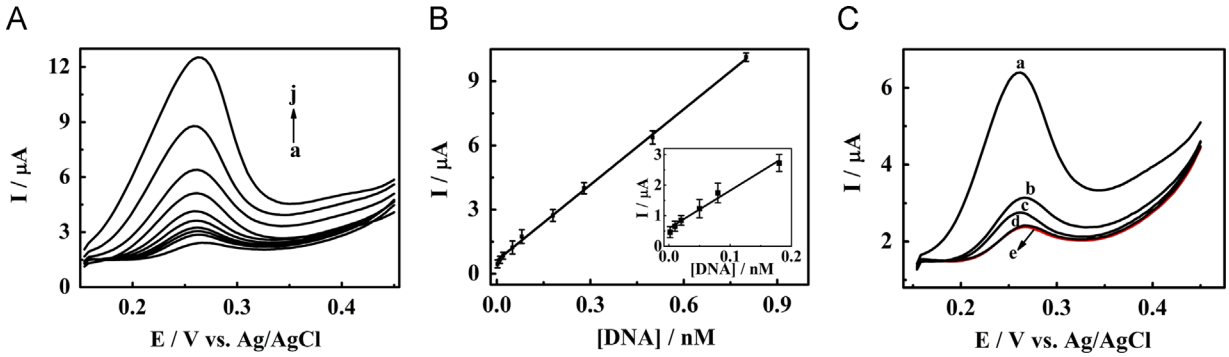


**Fig. 3.** (A) SWV response of the DNA biosensor with different concentrations of target DNA, from a to j, 0.0, 2.0, 10.0, 20.0, 50.0, 80.0, 180.0, 280.0, 500.0, and 800.0 fM. The supporting electrolyte was 0.10 M phosphate buffer (pH 4.5); the amplitude was 25 mV; the step was 3 mV; and the frequency was 15 Hz. (B) Calibration curve of *I* vs. DNA concentrations. Error bars represent the standard deviation of three measurements. The insert is part of the calibration curve at low DNA contrations. (C) SWV response in the presence of different types of HBV (300 fM): complementary (a), one-(b), two-(c), and three-base mismatched (d) DNA sequence. Line (e) indicates the background.

the nonapeptide self-assembled nanowire and the designed covalent linkage of the PANI to the peptide-based template. Besides, due to the ordered arrangement of the peptide, aniline monomers on the peptide nanowire surface are prone to uniform polymerization in the head-to-tail orientation. This orientation leads to the excellent electrochemical activity of PANI nanowire, allowing the ultrasensitive electrochemical quantification of the nucleic acid. And the HRP catalysis-dependent polymerization of the aniline can lower the non-hybridization-related background noise and interference which guarantees a high selectivity for the constructed DNA sensor.

## 4. Conclusions

In summary, a novel strategy was proposed for the construction of PANI nanowire. And based the electrochemical activity of the formed PANI, a highly sensitive and selective electrochemical sensor was constructed for the detection of nucleic acid. The structural unit of the self-assembled nanowire, aniline-GGAAKLVFF (AP), was synthesized by attaching the aniline monomer to GGAAKLVFF nonapeptide. After the self-assembly of AP into a highly ordered amyloid-like nanofiber (AP nanowire) and upon the treatment of HRP/$H_2O_2$, the aniline monomers located on the surface of AP nanowire can be polymerized to form the PANI nanowires. During the process, the as-assembled AP nanowires, acting as a template, guide the polymerization in the desired head-to-tail orientation. The prepared PANI nanowire inherits the merits of the AP nanowires, such as the uniform morphology, small cross-section (less than 10 nm), high aspect ratio (about 1000). More importantly, the formed PANI nanowire showed excellent electrochemical activity, implying the great potential applications for the construction of highly sensitive electrochemical biosensors. Based on these unique properties of the AP nanowires, we developed a novel assay for nucleic acid detection. Taking HBV gene as a model, the dynamic range of the sensor is 2.0–800 fM with a low limit of detection (LOD) of 1.0 fM ($S/N=3$). And the electrochemical biosensor displayed good fabrication reproducibility and high stability, as well as high selectivity for it can discriminate the single-nucleotide mismatched DNA sequence.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.09.023.

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