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ARTICLE *in* ANALYTICAL CHEMISTRY · AUGUST 2007

Impact Factor: 5.64 · DOI: 10.1021/ac070639m · Source: PubMed

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Electrochemical DNA Biosensor Based on Conducting Polyaniline Nanotube Array

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Most of the recent developments in ultrasensitive detection of nucleic acid are based on the gold nanoparticles and carbon nanotubes as a medium of signal amplification. Here, we present an ultrasensitive electrochemical nucleic acid biosensor using the conducting polyaniline (PANI) nanotube array as the signal enhancement element. The PANI nanotube array of a highly organized structure was fabricated under a well-controlled nanoscale dimension on the graphite electrode using a thin nanoporous layer as a template, and 21-mer oligonucleotide probes were immobilized on these PANI nanotubes. In comparison with gold nanoparticle- or carbon nanotube-based DNA biosensors, our PANI nanotube array-based DNA biosensor could achieve similar sensitivity without catalytic enhancement, purification, or end-opening processing. The electrochemical results showed that the conducting PANI nanotube array had a signal enhancement capability, allowing the DNA biosensor to readily detect the target oligonucleotide at a concentration as low as 1.0 fM (~300 zmol of target molecules). In addition, this biosensor demonstrated good capability of differentiating the perfect matched target oligonucleotide from one-nucleotide mismatched oligonucleotides even at a concentration of 37.59 fM. This detection specificity indicates that this biosensor could be applied to single-nucleotide polymorphism analysis and single-mutation detection.

The accomplishment of The Human Genome Project has stimulated our interest in identifying diseases based on the genetic information even before the abnormal symptoms can be noticed. Thus, detection of particular gene mutations, copy number variation, and single-nucleotide polymorphism becomes extremely important for diagnosis and treatment of the gene-related diseases. Therefore, there is a great desire for sensitive, accurate, rapid, cost-effective, and user-friendly approaches for gene analysis and detection.

Recent developments in nanomaterials and nanotechnologies create many opportunities to advance biomolecular sensing[1] and gene detection.[2,3] Particularly, use of nanomaterial as a medium of signal amplification attracts a great deal of attention. Mirkin and co-workers found oligonucleotide-labeled gold nanoparticle substantially altered the melting profiles. After being processed with a signal amplification method based on nanoparticle-promoted reduction of silver, this approach offered a sensitivity 100 times higher than an analogous fluorescence system.[4] They also developed gold nanoparticle-based electrical DNA detection with catalytically deposited silver as an enhancing element.[5] Wang et al. proposed a new strategy for amplifying a hybridization signal using an electroactive microsphere with redox marker inside.[6] The electroactive microsphere was found to be a good signal amplification medium for hybridization events. Carbon nanotube coat, aligned array, or composite was also used in nucleic acid detection after purification and end-opening processing.[7−9]

Polyaniline (PANI), a conducting polymer with good electrochemical activity and chemical stability, has been widely used in the DNA biosensor.[10,11] Compared with a gold nanoparticle- or carbon nanotube-based DNA biosensor, the conducting PANI-based DNA biosensor has some advantages: low-temperature synthesis, tunable conductivity, and no need for purification, end-opening, or catalytic deposition processing. Unfortunately, PANI is usually less favorable as the element in biosensor construction because of its relative lower conductivity than the carbon nanotube as well as their nonoriented nanofiber morphology, leading to low detection sensitivity. However, when a PANI nanotube array of well-organized orientation is fabricated on electrodes using the

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10.1021/ac070639m CCC: $37.00
Published on Web 05/27/2007

well-designed synthesis approach, an enhanced detection sensitivity can be achieved, which is similar to the gold nanoparticle- and carbon nanotube-based detecting system for DNA hybridization.

Recently, we have developed an approach to integrate a PANI nanotube array of well-organized orientation on electrodes. The highly organized nanostructure allows us to achieve an enhanced detection sensitivity for DNA hybridization. Controlled fabrication of the PANI nanotube array on the graphite electrode is illustrated schematically (Supporting Information Figure S-1). The PANI nanotubes are synthesized on a graphite electrode through a thin nanoporous layer as the template. Using well-controlled approaches, we can manipulate the morphology and orientation of the PANI nanotubes. After removing the template, these PANI nanotubes form an array structure with a good alignment and orientation. Oligonucleotide probes are then immobilized on the surface of inner walls and outer walls of PANI nanotubes, making each PANI nanotube work like a signal amplification nanodevice for hybridization events. Thus, the PANI nanotube array modified electrode provides a high-efficiency route for ultrasensitive DNA hybridization detection.

EXPERIMENTAL SECTION

An aluminum layer of 2.0-μm thickness was deposited on a graphite electrode using a magnetron sputtering method, followed by two-step anodization in oxalic acid at 40 V and 4 °C to create an alumina template of nanopore array. To exhaust a thin barrier at the interface of the alumina layer and the electrode, the alumina template was overetched slightly. The electrode was then immersed in aniline solution for 48 h and then transferred to the solution of a mixture of 1 M *p*-toluenesulfonic acid (TSA) and 0.5 M ammonium persulfate for polymerization. The aniline polymerization process lasted 10 s at room temperature. The PANI nanotubes formed on the wall of these nanopores of the alumina template. A PANI film was also formed on the template surface. Subsequently, the PANI film on the template surface was mechanically polished with alumina powder, and the alumina template was dissolved in H_2SO_4 solution. Thus, a PANI nanotube array was fabricated on the electrode. The alumina template and the PANI nanotube array were characterized with a scanning electron microscope (JSM-T300, Jeol).

The oligonucleotides used in this experiment were purchased from Takara Biotech. (Dalian) Co. Ltd. (Dalian, China), and their stock solutions were prepared with Tris-HCl + EDTA buffer (pH 7.6). The nucleotide used as the probe in this experiment was a 21-mer oligonucleotide having the sequence 5′-COOH-CTC ACC GCC TGC CCA CTC ATT-3′. The COOH groups at the 5′-end was for immobilization on the PANI tubes. The intended target had the sequence complementary to the probe, which was 5′-AAT GAG TGG GCA GGC GGT GAG-3′. Two oligonucleotides having one mismatched nucleotide were designed as 5′-AAT GAG TGG GCA GG***T*** GGT GAG-3′ and 5′-AAT GAG TGG GC***C*** GGC GGT GAG-3′. The letters in italic boldface type were the mismatched bases.

The DNA biosensor was prepared by covalently linking oligonucleotide probes to the PANI nanotube array by immersing the electrode in the 22.5 μM oligonucleotide probe solution with 0.1 M 1-ethyl-3-(3-dimethylaminopropy-1)-carbodiimide (Merck) for 12 h at room temperature. For electrochemical measurements, the DNA biosensor was immersed in a 300-μL nucleotide solution with different concentration targets at 55 °C for 2 h. After washing off the unhybridized oligonucleotides, the DNA biosensor was immersed in the 22.5 μM daunorubicin (DNR; Sigma) aqueous solution for 15 min. The electrochemical signals of DNR bound to the hybrids were measured with an electrochemical analyzer (CHI 620C, CH Instruments, Inc.) in a three-electrode system in which the PANI nanotube array electrode, Ag/AgCl electrode, and platinum wire served as the working electrode, the reference electrode, and the counter electrode in PBS buffer solution (pH 7.4), respectively. To examine the ability of the DNA biosensor to differentiate the mismatch hybridization and the complementary hybridization, the DNA biosensor was also incubated with different oligonucleotide solutions at 59.7 and 61.7 °C, respectively. The chemicals without specific note were purchased from Sinopharm Chemical Reagent Co.. The data processing was conducted with software Origin 7.5 (OriginLab Co.). For more details, see methods and measurements in Supporting Information.

RESULTS AND DISCUSSION

Fabrication of PANI Nanotube Array on the Electrode. The PANI nanotube array was fabricated on the graphite electrode through an alumina template, which was prepared by two-step anodization process of a thin aluminum layer on the electrode.[12] The nanoporous template formed on this electrode has a homogeneous diameter of 40 nm (Figure 1a). Due to the solvophobic effect and electrostatic effect, aniline monomers started to polymerize on the inner wall of the nanopores initially, and PANI film is formed on the surface of the alumina template.[13] After removing this PANI film, scanning electron micrography was used to characterize the composite of the PANI tubular structure and the alumina template. Figure 1b shows many black spots, which are the open ends of the PANI nanotubes, indicating that the aniline polymerization generated nanotubes inside the nanopores when a 10-s polymerization process was applied. This result was consistent with the observation that aniline polymerization formed nanotubes rather than nanofibers in a short polymerization period.[13] If a longer polymerization period was applied, however, nanotubes would become nanofibers which fill up the nanopores, causing these black spots to disappear. After removal of the PANI film on the template surface, the electrode was immersed in an acid solution to dissolve the alumina template, leaving the nanotube array on the electrode with a defined orientation (Figure 1c).

Electrochemical Characterization of the PANI Nanotube Array Synthesized on an Electrode. We used cyclic voltammetric (CV) measurement to characterize the PANI nanotube array synthesized on the electrode. This PANI nanotube array modified electrode was immersed in 1 mM $K_3Fe(CN)_6$ solution containing 0.1 M KCl, and the CV measurements were conducted at the scanning rate of 0.1 V/s. The PANI particle modified electrode and a bare electrode were also characterized under the same conditions for comparison. The CV curves showed that the PANI nanotube array modified electrode (Figure 2c) had the largest CV area compared with the bare electrode (Figure 2a, Supporting Information Figure S-2a) and PANI particle modified electrode (Figure 2b), indicating that the PANI nanotube array

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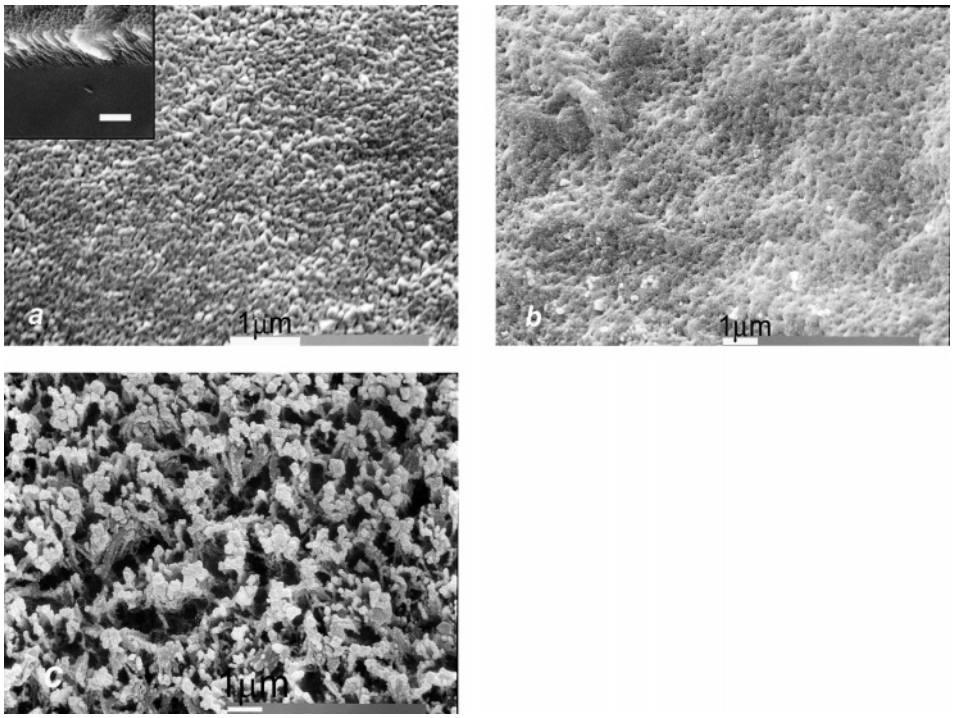

Figure 1. Scanning electron micrographs of PANI nanotube array formation on the graphite electrode. (a) The alumina template with nanopore array on the electrode. The inset shows an aluminum layer of ~2.0 μm (top) deposited on the electrode (bottom). The scale bar is 2 μm. (b) The composite of PANI nanotube and alumina template on the electrode. (c) PANI nanotube array on electrode after removal of the alumina template.

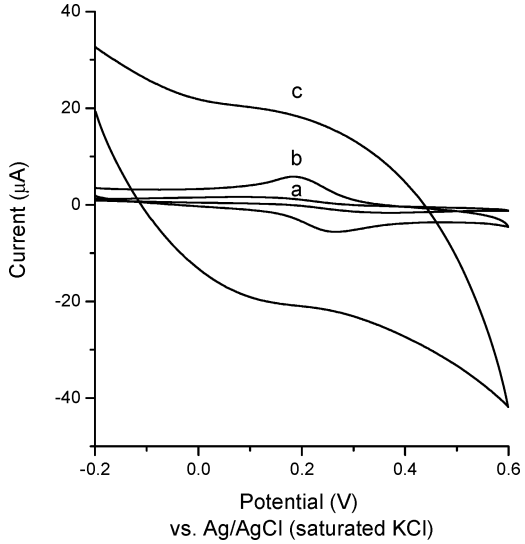

Figure 2. Cyclic voltammograms of three kinds of electrodes measured in 1 mM $K_3Fe(CN)_6$ solution with 0.1 M KCl. (a) Bare graphite electrode, (b) PANI particle modified graphite electrode, (c) PANI nanotube array modified graphite electrode. Scan rate: 0.1V/s.

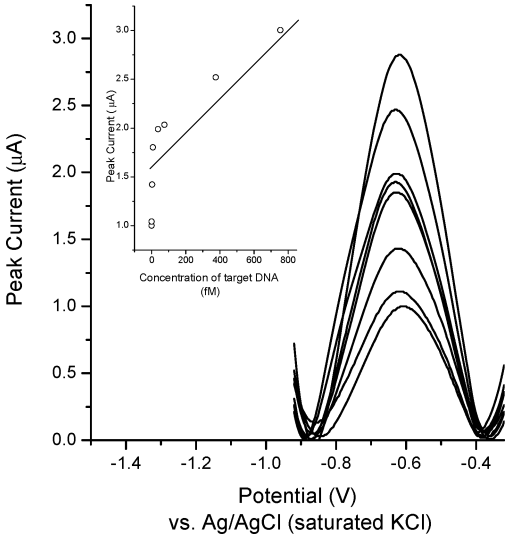

Figure 3. Background-subtracted differential pulse voltammograms of PANI nanotube array DNA biosensor after hybridizing with the target nucleotide at different concentrations of 0, 0.756, 3.759, 7.557, 37.59, 75.57, 375.9, and 755.7 fM (from bottom to top) at 55 °C. Inset is the calibration curve of peak currents versus the target concentrations with a correlation coefficient of $R = 0.94$. This DNA biosensor shows a linear response over three orders of target nucleotide concentration (concentration of DNR 22.5 μM; hybridization time 2 h; labeling time 15 min).

modified electrode had a much larger effective surface area on the electrode due to the tubular structure as well as the highly organized array morphology.

Detection of the PANI Nanotube Array-Based DNA Biosensor. Oligonucleotide probes were covalently immobilized on the PANI nanotube array, converting the PANI nanotube array modified electrode into a DNA biosensing element. To examine its detection sensitivity, we measured the peak currents of the biosensor after hybridization with the target DNA at different concentrations, in which daunorubicin was an indicator for specific binding with duplexes (Please refer to the electrochemical signal of daunorubicin in Supporting Information Figure S-3.). Figure 3 shows an increase of the electrochemical signal of daunorubicin with the increase of target nucleotide concentration. These peak currents in differential pulse voltammograms demonstrate a good linear relationship with the target nucleotide concentration in the range of 3.759−755.7 fM (Supporting Information Table S-1), and

the correlation coefficient R in this range was calculated to be 0.94. Considering the least detectable concentration of 0.756 fM, the detection limit of 1.0 fM was estimated using the 3σ method (σ is the standard deviation of blank solution, $n = 11$). The sample volume used in these measurements was only 300 μL, which was equivalent to absolute detection limit of ~300 zmol of target DNA molecules. The detection sensitivity of this biosensor is much higher than that of the PANI particle DNA biosensor and PANI nanofiber DNA biosensor at the level of picomolar[10,11] and was similar to the carbon nanotube-based DNA biosensor.[9]

The ultrasensitive behavior of our current DNA electrochemical biosensor was interpreted in terms of a couple of signal-enhancing mechanisms. First, the PANI nanotube array demonstrates a collective effect due to the large effective surface area, similar to the reservoir effect in the signal amplification of the carbon nanotube electrochemical biosensors.[14] It was estimated that more than 10^5 probes could be immobilized on each PANI nanotube, yielding a significantly higher surface density of the nucleotide probes than any other DNA biosensors. Thus, when this PANI biosensor was incubated in the sample solution, it behaved like a collector to attract the targets even at very low concentration. Second, it has been reported that the PANI nanotubes synthesized using a template-based method show not only a well-organized nanostructure but also a good degree of alignment of polyaniline chain, and the good alignment enhances the PANI conductivity.[13] In addition, based on knowledge about the effect of doped acid on the PANI conductivity[15] and our previous study,[16,17] we used TSA as the dopant in the current PANI nanotube fabrication. The TSA-doped PANI was very difficult to reduce or deprotonate due to the steric effect of the sulfonic group. Furthermore, it has been documented that the well-organized nanostructures of biosensors always provide faster kinetics for the analyte than the disordered structures.[2,18] In the case of nucleotide hybridization, the well-spaced and organized nanotube array allowed the target nucleotides in solution to access the probes much easier and faster than the disordered counterparts, leading to the enhancement of the hybridization.

Regeneration of the PANI Nanotube Array-Based DNA Biosensor. The reusability of any biosensor is extremely important to practical applications such as clinical diagnoses and biological monitoring and depends strongly upon the stability of the PANI electric property. In our test, one DNA biosensor was first incubated in 755.7 fM target oligonucleotide solution at 55 °C for 2 h and then labeled in DNR aqueous solution for 15 min. After the electrochemical measurement, the PANI nanotube array modified electrode was intentionally left in air for 24 h. This DNA biosensor was regenerated by incubating in a buffer solution at 85 °C twice, 5 min each time, and was used for the next electrochemical measurement. This regeneration procedure was repeated more than three times for one DNA biosensor. The relative standard deviation of these measurements was only 3.00%, indicating the robust behavior of the PANI nanotube array and the stability of the immobilized nucleotide probes.

Table 1. Comparison of Hybridization Efficiencies of One Mismatch and Perfect Match Hybridizations at Two Different Hybridization Temperatures

	61.7 °C		59.7 °C	
	peak current (nA)[a]	hybridization (%)[b]	peak current (nA)[a]	hybridization (%)[b]
TC mismatch[c]	61	22	166	47
TG mismatch[c]	147	54	211	60
perfect match[c]	274	100	354	100

[a] The peak currents were corrected by subtracting the background of the biosensor with DNR 118 ± 7.51% nA ($n = 10$). [b] The hybridization efficiencies were calculated using the peak currents of the mismatched hybridizations against that of the perfect hybridization. [c] The concentrations of the target oligonucleotide in three hybridizations were 37.59 fM.

Detection Specificity of the PANI Nanotube Array-Based DNA Biosensor. Nucleotide detection is based on the principle of complementary hybridization whose stability is characterized by the melting temperature (T_m). Usually, the hybridization process is carried out at a temperature of 5−10 °C below the melting temperature to ensure duplex formation. However, many mismatches such as point mutations are present and need to be identified. To examine the detection specificity of this DNA biosensor, we measured the DPV responses of the PANI nanotube array-based DNA biosensor after hybridization with one TC-mismatch, one TG-mismatch, and perfect match oligonucleotides incubated at 61.7 and 59.7 °C, respectively (Supporting Information Figure S-4). In all these measurements, the mismatched oligonucleotides and perfect matched target were at a concentration of 35.79 fM.

Table 1 lists the measured peak currents and the calculated hybridization efficiency for three oligonucleotides at two different temperatures. Please notice that, as the incubation temperature increases, the absolute peak currents for one kind of oligonucleotide decreases, since the duplexes are destabilized more at higher temperature. (Please also refer to the peak currents of hybridization at 55 °C.) Referring to the complementary hybridization at each temperature as 100% hybridization, the hybridization efficiencies of one TC mismatch and one TG mismatch at 59.7 °C were calculated to be 47 and 60%, respectively (Table 1). When the hybridization temperature was raised to 61.7 °C, these hybridization efficiencies dropped to 22 and 54%, respectively. In comparison with the Cu/Au alloy nanoparticle-based biosensor, which had hybridization efficiency for one AC mismatch in a 24-mer oligonucleotide at 5 nM was still over 80%,[19] our PANI nanotube array-based DNA biosensor demonstrated a much better detection specificity. The data in Table 1 also show that TC mismatch is much less stable than the TG mismatch hybridization at the selected hybridization temperature, indicating that our DNA biosensor have a great potential to identify single-nucleotide polymorphisim. Additionally, the results in Table 1 show clearly that the PANI nanotube array-based DNA biosensor had good

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hybridization specificity with the target even at the ultralow concentration of 35.79 fM.

CONCLUSIONS

We developed one conducting polyaniline nanotube array-based ultrasensitive biosensor for DNA hybridization detection. This approach does not need catalytic deposition, end-opening, or purification processes, which are usually required in gold nanoparticle or carbon nanotube applications. This DNA electrochemical biosensor demonstrated an extremely high sensitivity that could detect the presence of a target nucleotide at a concentration as low as 1.0 fM, which corresponded to 300 zmol of target DNA molecules in 300 μL of sample and was much higher than other PANI biosensor with sensitivity of about 1 pM. This ultrasensitive property was interpreted in terms of the collective effect of PANI nanotubes, the enhanced PANI conductivity, and the faster hybridization kinetics of oriented nanostructure. This biosensor also demonstrated good hybridization specificity even at the ultralow concentration of 37.59 fM, demonstrating the possibility of this biosensor for single-nucleotide polymorphism or single-mutation analysis. The PANI nanotube array-based detection system will also have a great potential in other enzyme- or protein-based applications.

ACKNOWLEDGMENT

This work was partly supported by National Foundation of Sciences of China (NFSC, Grant 30470451). The authors thank Jun Gong (Institute of Metal Research, Chinese Academy of Sciences) and Guojin Liao (Northeastern University, China) for aluminum sputtering and Yuehua Liu and Dongjuan Liu (China Medical University) for microscope characterization.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review April 1, 2007. Accepted May 21, 2007.

AC070639M