



A novel electrochemical biosensor based on dynamic polymerase-extending hybridization for *E. coli* O157:H7 DNA detection

Lijiang Wang^a, Qingjun Liu^b, Zhaoying Hu^b, Yuanfan Zhang^b, Chunsheng Wu^b, Mo Yang^c, Ping Wang^{b,*}

^a Zhejiang California International Nanosystems Institute, Zhejiang University, Hangzhou 310027, PR China

^b Biosensor National Special Laboratory, Key Laboratory of Biomedical Engineering of Education Ministry, Department of Biomedical Engineering, Zhejiang University, Hangzhou 310027, PR China

^c Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

ARTICLE INFO

Article history:

Received 21 October 2008

Received in revised form

26 November 2008

Accepted 1 December 2008

Available online 6 December 2008

Keywords:

Electrochemical biosensor
Polymerase-extending DNA hybridization
Nanopore membrane
E. coli O157:H7

ABSTRACT

A novel biosensor based on single-stranded DNA (ssDNA) probe functionalized aluminum anodized oxide (AAO) nanopore membranes was demonstrated for *Escherichia coli* O157:H7 DNA detection. An original and dynamic polymerase-extending (PE) DNA hybridization procedure is proposed, where hybridization happens in the existence of Taq DNA polymerase and dNTPs under controlled reaction temperature. The probe strand would be extended as long as the target DNA strand, then the capability to block the ionic flow in the pores has been prominently enhanced by the double strand complex. We have investigated the variation of ionic conductivity during the fabrication of the film and the hybridization using cyclic voltammetry and impedance spectroscopy. The present approach provides low detection limit for DNA (a few hundreds of pmol), rapid label-free and easy-to-use bacteria detection, which holds the potential for future use in various ss-DNA analyses by integrated into a self-contained biochip.

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1. Introduction

Deoxyribonucleic acid (DNA) analysis has become an important tool for genetic diagnostics; identification of disease-causing microorganisms in human body [1], food [2], or environment [3,4], and the assessment of medical treatment [5]. The core of DNA sensing is detecting target ss-DNA fragments by utilizing their hybridization with complementary probe sequences. Traditional methods for identification of the DNA hybridization event, such as membrane blots or gel electrophoresis, are time-consuming and labor intensive. As alternative to the conventional methods, biosensor technique has triggered strong interests for its simpler nucleic acid assays and faster, cheaper process. The transducing elements reported in the literature include optical [6], microgravimetric [7] and electrochemical (EC) devices [8]. Among these methods, EC-transducers have their unique advantages in the detection of DNA hybridization and have arisen considerable interests [9,10]. These sensitive EC devices could be integrated into existing detection schemes, so the EC detection is desirable to realize miniaturization and portability. Moreover, low cost, minimal power requirements, and independence of optical pathway, all make it excellent can-

didate for DNA diagnostics [11]. In addition, electrochemistry provides innovative routes for interfacing the nucleic acid recognition system with signal generating and amplifying elements, which inspired us initially.

Aluminum anodized oxide (AAO) membranes can be synthesized via anodization of metal aluminum [12]. Precise control of pore diameter and length can be achieved [13,14]. Due to these fairly well-defined nanopores, AAO membranes have found popular applications in many areas such as nanostructured materials preparation [15], biological and chemical separations [16], solution flow regulation [17,18], and biosensors [19]. In the studies of Vlasouk's group, AAO filters modified with covalently linked DNA can be used to detect or separate/purify the target ss-DNA [16,20,21]. They investigated the variation of ionic conductivity in nanopores caused by probe immobilization at the pore walls and its subsequent hybridization with complementary DNA strands. As the target ss-DNA was captured by the DNA probes immobilized on the pore walls, the hybridization events would further block the ionic flow through the pores, which would increase the impedance. This analysis provides a basis for articulating specific recommendations for this DNA biosensor's future use as a convenient combination of detection and separation/purification for unmodified target ss-DNA (and RNA). Compared with other porous membranes adopted in DNA sensors [22,23], the use of AAO filter as a sensing membrane offers several advantages. For instance the fabrication process is

* Corresponding author. Tel.: +86 571 87952832; fax: +86 571 87951676.
E-mail address: cnpwang@zju.edu.cn (P. Wang).

correspondingly inexpensive and highly reproducible. In addition, the three-dimensional structure of the AAO membrane provides a much higher surface area available for probe immobilization for the same spot diameter as compared to flat surfaces. Foremost, this matrix is a suitable and protective environment for biomolecule immobilization and can transduce a biorecognition event such as DNA hybridization into an electrical signal.

The *Escherichia coli* O157:H7 is selected as the target bacteria because it is one of the most threatening pathogens in the world. On average, *E. coli* O157:H7 is responsible for 73,000 illnesses, 2100 hospitalizations and 60 deaths annually in the United States alone, and as of September 2006, there was a multistate outbreak of *E. coli* O157:H7 infections associated with consumption of fresh spinach in the country [24,25]. So the exploitation of new methods for rapid and sensitive detection of such microorganism predicates significance to the safety in food or water supplies [26–28]. Especially to the selectivity, DNA is an ideal target for specific detection of pathogenic bacteria, and there is a patent need for more direct and inexpensive sensing of bacteria DNA. In this “proof of concept” report, a novel electrochemical biosensor is proposed for bacteria DNA detection using AAO nanopore membranes (73 ± 7 nm diameter) by means of cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). This original biosensor could detect the DNA hybridization free of labels, and offer a promising approach for rapid, sensitive and real-time solution monitoring of *E. coli* O157:H7. The critical process in fabricating this DNA biosensor is the specific single strand probe immobilization, while the immobilization efficiency depends on the chemical functionalization in the nanopores. The procedure of probe immobilization comprises three steps: silanization with aminosilane, activation by glutaraldehyde, and covalent attachment of 5'-aminated DNA probe (20-mer in our case), which is specific to the *E. coli* O157:H7 gene *eaeA* [16,29,30]. Commonly, the immobilized probes should hybridize with the target ss-DNA in the sample to induce changes in the electrochemical parameters (e.g. resistance and conductivity). But in this paper, the hybridization process is dynamic and rapid based on the polymerase-extending (PE) method. Once the hybridization event has happened, the probe strand can be extended according to the principle of complementary base pairing in the presence of Taq DNA polymerase and deoxyribonucleotide triphosphates (dNTPs) with the control of reaction temperature. The variation of impedance induced by the complete double strands DNA (ds-DNA) would be much more distinct than that by the routine hybridization complex. To our best knowledge, a dynamic design for DNA hybridization has not been demonstrated, nor has an AAO membrane assisted electrochemical biosensor for bacterial DNA detection ever been reported.

2. Materials and methods

2.1. Materials and instruments

Free standing AAO nanopore membranes, with a uniform 73 ± 7 nm pore diameter with $50 \mu\text{m}$ thickness and a high pore density ($1 \times 10^9/\text{cm}^2$), were purchased from Synkera Technologies, Inc. 3-aminopropyltrimethoxysilane (APS) and glutaraldehyde were obtained from Sigma–Fluka. E.Z.N.A.™ Bacterial DNA Kit (Omega Bio-Tek) was used for the bacteria DNA extraction. All other chemicals, such as toluene, propylamine, potassium ferrocyanide, and potassium ferricyanide were analytical pure grade or better quality. Deionized water ($18.2 \text{ M}\Omega/\text{cm}$) produced by a Milli-Q system (Bedford, MA) was used throughout. Electron micrographs of fresh and silanized AAO membranes were taken using Scan Electron Microscope (SEM, Hitachi S-4700). Electrochemical cyclic voltammetric experiments and impedance spectroscopy measurements

were performed using CH Instruments 660A electrochemical analyzer.

2.2. Oligonucleotides and PCR amplification

All the oligonucleotides used in sensor development were purchased from TAKARA Biotechnology Co., Ltd. A 20-base oligonucleotide modified at 5' end with $\text{C}_6\text{-NH}_2$ was used as an ssDNA probe ($5'\text{-NH}_2\text{-(CH}_2\text{)}_6\text{-CCAAGAGTTGCAGTTCCTGA}$), which was specific for *E. coli* O157:H7 *eaeA* gene. A 120-base ss-DNA with 3' end complementary sequence of probe ($5'\text{-TTCGGCTAAA GCGGATAACGCCGATACCATTACTTATACCGCGACGGTGAAAAAGAATGGGGTAGCTCAGGCTAATGTCCCTGTTTCATTTAATATTGTTTCAGGAAGTCAACTCTTGG}$), was acted as positive control to characterize the sensor performance, while the negative control with complementary sequence of positive one ($5'\text{-CCGCC GATTTGCGCTATTGCGGCTATGGTAATGAATATGGCGCTGCCACTTTTCTTACCCATCGAGTCCGATTACAGGGACAAA-GTAAATTATAACAAAGTCCTTGACGTTGAGAACC}$) and the single base mismatch oligonucleotide ($5'\text{-TTCGGCTAAAGCGGATAACGCCGATACCATTACTTATACCGCGACGGTGAAAAAGATGGGGTAGCTCAGGCTAATGTCCCTGTTTCATTTAATATTGTTTCAGGAAGTCAACTCTTGG}$) were adopted to test the specificity of the probe. A short fragment (120 bases) of the *E. coli* O157:H7 *eaeA* gene with the same sequence as the positive control was amplified by asymmetric PCR (forward primer: $5'\text{-TTCGGCTAAAGCG GATAACG}$, reverse primer: $5'\text{-CCAAGAGTTGCAGTTCCTGA}$).

Asymmetric PCR, a PCR procedure that predominantly produces ssDNA was utilized to amplify the target DNA for the direct hybridization detection. The concentration ratio of forward primer to reverse primer was 50:1, and the lower concentrated forward primer played a “limiting primer” role. After the limiting primer was consumed, the remaining reverse primer continued to extend the single-stranded *eaeA* gene fragments. The whole asymmetric PCR reaction was executed in a Bio-Rad Thermal Cycler with a temperature profile of 95°C for 5 min, followed by 40 cycles of 30 s denaturation at 95°C , 30 s annealing at 55°C , 45 s extension at 72°C , and 10 final extension. Regular PCR was also carried out to prove the successful amplification of *eaeA* gene, which was examined by electrophoresis in 2% agarose gel.

2.3. Nanopore biosensor setup and data acquisition

The experimental apparatus is illustrated in Fig. 1. A membrane, with an active area of 0.03 cm^2 , was placed in the middle of two equal volume chambers, which are clamped by four screws. Both chambers were L-shaped and filled with 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in phosphate buffered saline (PBS, pH 7.0), which was selected to indicate the efficiency of ion blockage. Au electrode was the working electrode, while Pt wire electrode and Ag/AgCl (in saturated KCl) electrode were used as the counter electrode and the reference electrode, respectively. Au and reference electrode were placed in one upward hole of the chamber, and the counter electrode was in the opposite side. The CV rate was 50 mV/s and the impedance spectrum was obtained at the equilibrium voltage 0.20 V vs. Ag/AgCl by applying 5 mV ac voltage.

2.4. ss-DNA probe immobilization

After evaluating different methods [21], we found that the most reliable approach which ensures the nanopores unsealed and a high density of covalently linked DNA inside the nanopores is achieved by a glutaraldehyde linker, which joins the amino groups of 5'-aminated DNA and the terminal amino group of aminosilane. Briefly, a fresh AAO filter was washed with 15 min sonication in DI water and dried in oven, then immersed into a 0.5% toluene solution

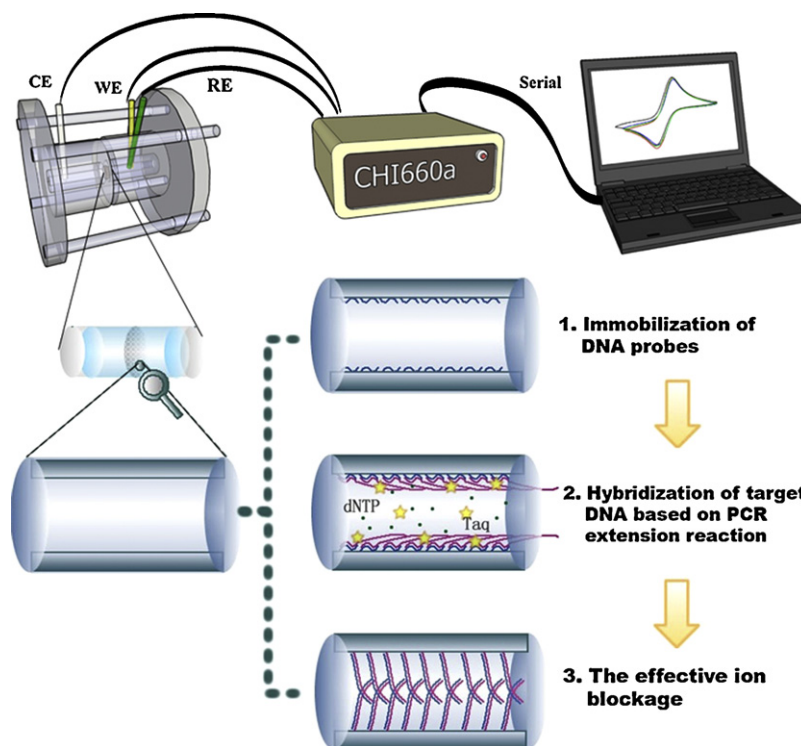


Fig. 1. The sketch map of the electrochemical DNA biosensor system and the mechanism of the novel polymerase-extending hybridization method (WE: working electrode; RE: reference electrode; CE: counter electrode).

of APS for 6 h. After sonication washing in toluene (15 min, 3 times) and baking at 120 °C for 4 h, the aminated membrane was activated in an 8% aqueous solution of glutaraldehyde for 12 h. In order to neutralize the unreacted glutaraldehyde, the activated membrane was left overnight in 10^{-5} M aqueous solution of propylamine. Following thorough washing with DI water and drying, the filter was immersed in 50 μ L of aqueous solution of 5'-aminated ss-DNA (20 μ M) and kept at high humidity overnight. Finally, the filter was thoroughly washed by DI water and stored at 4 °C for use.

2.5. Dynamic polymerase-extending hybridization

Routine hybridization reaction was carried out by immersing the nanopore membrane immobilized with specific probes in 50 μ L hybridization buffer containing the complementary target ss-DNA for at least 2 h at room temperature [21]. In this study, a novel hybridization process is proposed to improve the effect of ion blockage caused by the hybridization complex. The functionalized AAO filter was dipped into an Eppendorf tube loading 50 μ L reaction buffer mainly including Taq DNA polymerase, target ss-DNA (5 μ M), dNTPs, Mg^{2+} and PCR buffer, and then the whole tube was subjected to the thermal treatment in water bath: 55 °C for 10 min. After the hybridization based on the PE method, the membrane was removed from the EP tube and rinsed with DI water. Finally, CV and EIS measurements were performed to evaluate the efficiency of ion blockage. The scheme of this novel and effective polymerase-extending hybridization method is illustrated in Fig. 1.

3. Results and discussion

3.1. Biosensor fabrication

Similar to the methods of glass and silica surface modification, we modified the surface of nanopore by the aminosilane-

glutaraldehyde chemistry and found the most reliable concentration of APS was 0.5% after considerable evaluations. From the SEM micrographs of the fresh and silanized membranes (Fig. 2), we can see the open-through nanopore membrane without any treatment contains parallel circular pores with a very tight pore size distribution (about 73 ± 7 nm). This geometry makes it an ideal experimental tool for fundamental studies of ion transfer phenomena in porous support (Fig. 2A). Furthermore, modified by the aminosilane-glutaraldehyde layer, the pores were not occluded, and even the aperture remained the same (Fig. 2B), through which the ss-DNA probes could diffuse into and immobilize on the nanopore wall by condensation reaction.

CV in Fig. 3A clearly shows that both reductive and oxidative current decrease after aminosilane-glutaraldehyde modification of the nanopores, however, after probe immobilization the current increased greatly. When the nanopore wall was covered with a layer of activated aminosilane, the inside diameter of pores should be reduced slightly, which resulted in a lower ion mobility through the pores. As expected, a noticeable current increase was observed after the probe immobilization. It can be explained as a consequence of the increased surface charge density from the negatively charged DNA backbone and possible partial dissolution during the multistep procedure of probe immobilization. Accordingly, the resistance of the pores after immobilization of ss-DNA probes decreased compared to the fresh alumina membrane, which confirmed the results of Takmakov's work [21]. The impedance in Fig. 3B also illustrates the changes accompanying the stepwise modification process.

3.2. Target ss-DNA detection by CV and EIS

Synthesized positive control DNAs were applied to hybridize with the probes with PE method originated by ourselves, while another group of hybridization trial was carried out with the routine method. It can be clearly observed that the reductive and oxidative

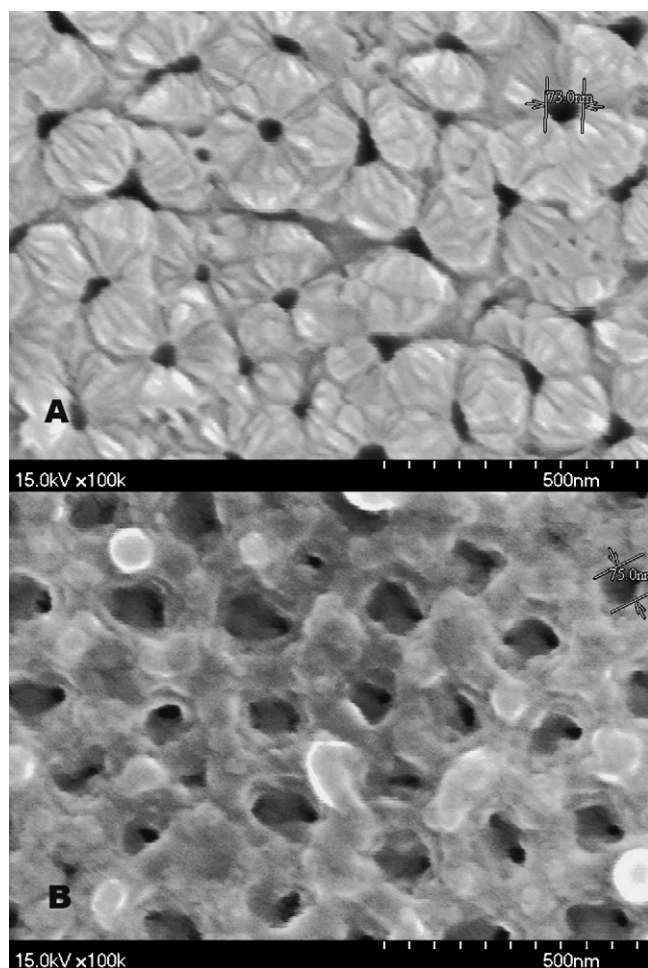


Fig. 2. SEM micrographs of (A) untreated nanoporous alumina membrane (top surface) and (B) the aminosilane-glutaraldehyde modified membrane.

currents decreased after these two DNA hybridization methods. However, an obvious current diminishment appeared when PE hybridization method was adopted (Fig. 4A), which verified the PE hybridization more effective for ion blockage. Impedimetric measurement was most sensitive to the ion flow changes accompanying hybridization inside the nanopores. Fig. 4B shows the impedance bode plot of the nanopore membrane based DNA biosensor for the target DNA hybridization with different methods: probe immobilization, PE hybridization and the routine hybridization in the frequency ranging from 1 Hz to 100 kHz with the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple in PBS.

To understand and quantitatively analyze the EIS data, it is crucial to construct an equivalent circuit, which must address all relevant physical phenomena and incorporate them correctly. In this study, experiments were conducted with bare gold electrode, solution of ferro/ferricyanide and supporting electrolyte. In this case the equivalent circuit is composed of two parts: the classic Randles circuit and circuit of AAO. The classic Randles circuit contains four elements: the double layer capacitance C_{dl} , charge transfer resistance of the electrode reaction R_{ct} , solution resistance R_s and diffusion impedance Z_w of the unstirred layer. Circuit of AAO consists of two elements: the resistance of the pores R_p and the membrane capacitance C_m . In general, the reaction kinetics is represented by a charge transfer resistance R_{ct} connected in series with diffusion impedance Z_w , and C_{dl} is related to the dielectric features at the electrode/electrolyte interface. Since the changes of ionic flow do not affected by electrode–electrolyte interface in

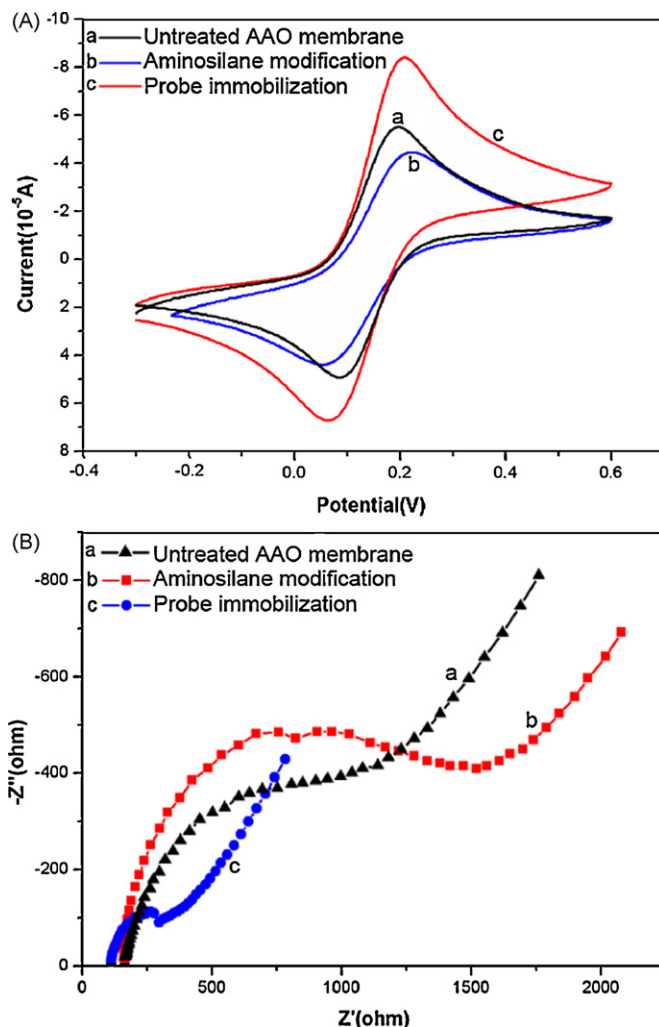


Fig. 3. (A) CV in the region for $[\text{Fe}(\text{CN})_6]^{3-/4-}$ oxidation/reduction for ss-DNA probe immobilization and (B) Nyquist plot (Z_{im} vs. Z_{re}) for membrane impedance at 0.20 V for ss-DNA probe immobilization: a–untreated AAO membrane; b–aminosilane modification; c–probe immobilization.

this study, no further discussion about the Randles circuit would be done here. As shown in Fig. 4B, in the frequency ranging from 1 Hz to 1000 Hz, compared to the probe immobilization (a), the impedance increased after the PE hybridization (b) and the routine hybridization (c), suggesting that the hybridization events happened on the nanopore wall can change the values of these electrical elements in the equivalent circuit.

The results of the fitting of the experimental data to the equivalent circuit by model fitting software are summarized in Table 1, which mainly lists these two pivotal parameters, R_p and C_m . Some scatter of the parameter values reflects the inherent irregularities of the membranes enhanced by the small size of the samples and fitting errors. With the routine hybridization method, hybridiza-

Table 1

Fitting parameters for the curves in Fig. 4B using the equivalent scheme.

	Pore resistance $R_p(\Omega)$	Membrane capacitance $C_m(\mu\text{F})$
Blank membrane	297.40 ± 12.77	17.40 ± 0.57
Probe immobilization	99.20 ± 8.62	2.76 ± 0.20
Routine hybridization	127.80 ± 5.34	1.66 ± 0.10
PE hybridization	540.76 ± 25.55	12.03 ± 0.23

The value shows means and standard deviation, which is derived from at least 3 independent detections.

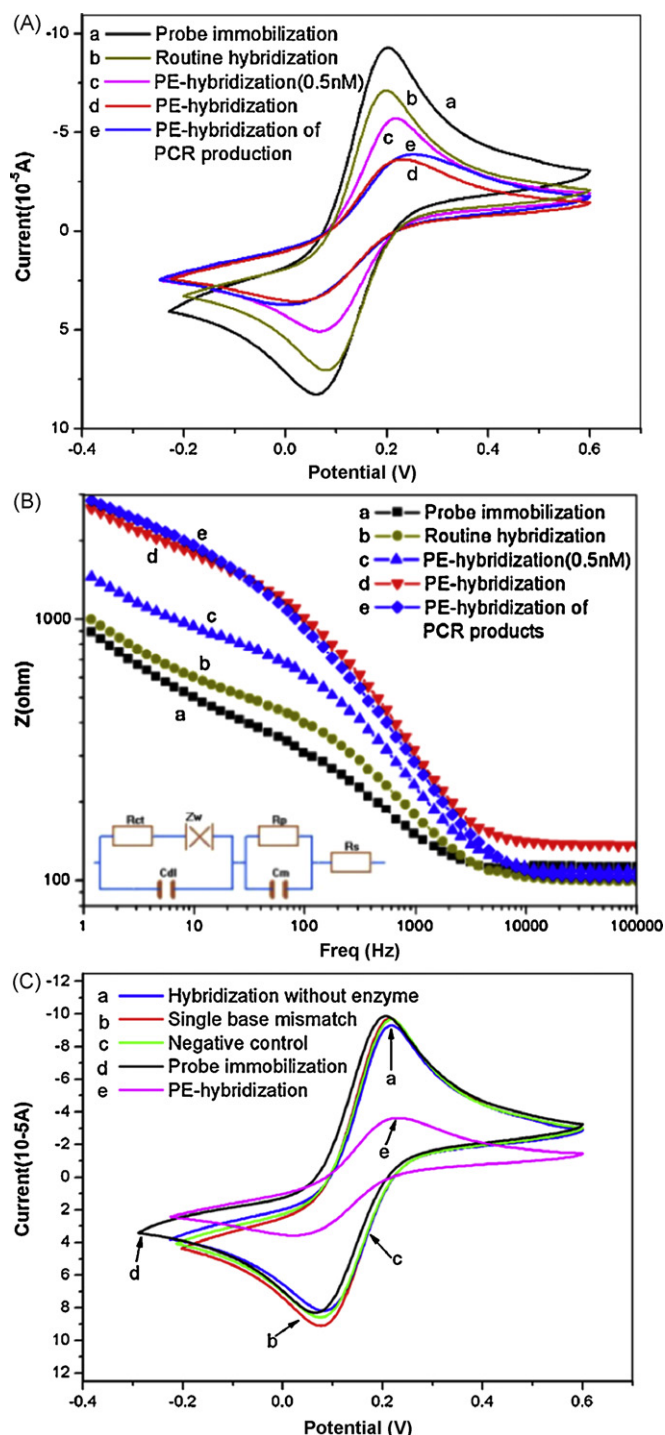


Fig. 4. (A) CV in the region for $[\text{Fe}(\text{CN})_6]^{3-/4-}$ oxidation/reduction for the target DNA hybridization with different methods; (B) impedance bode plots for the target DNA hybridization with different methods; (C) CV in the region for $[\text{Fe}(\text{CN})_6]^{3-/4-}$ oxidation/reduction for detection of biosensor sensitivity by different control groups.

tion complexes led to an increased R_p from 99 to 127, which nevertheless was lower than the blank membrane. The effect was similar to the previous report [20], whereas the PE hybridization method changed the nanopore resistance from 99 to 540 Ω with a net increase of 413 Ω compared to that by routine hybridization method.

This change was the most significant one among all the electrical elements in the equivalent circuit, indicating that the ion flow was assuredly blocked by the DNA hybridization inside the

nanopores, and the blockage effect was enhanced by PE hybridization method. The ionic conductance through nanopores, which is the pivot of the whole exploration and the foundation of this DNA biosensor development, has a complex nature with contributions from: (a) Volume exclusion due to additional DNA upon hybridization. When analyte binds to the nanopore walls, it will decrease the effective cross-section of the channel, so the current through the nanopores reduces. Particularly, this effect is probably strengthened by a change in DNA orientation with respect to the surface, namely stiffer dsDNA would lift up away from the surface and hinder the ion diffusion to a greater extent. This mechanism is the basis for most DNA detection studies with AAO membrane by routine hybridization method [20]. In our work, we have adopted the Taq DNA polymerase to complete a dynamic and steady hybridization process. The whole course of PE method was similar to the extending step of PCR, and the probe acted as the role of primer. Once the complementary ss-DNA was combined with the immobilized probe, the 3' end of probe would be extended by the polymerase to form a rigid hybridization complex of 120 bp, whereas in routine hybridization, there were only 20 bp stiff dsDNA standing inside the nanopores, and the other soft ss-DNA (100-base) belonging to the target DNA would still be floating in the electrolyte. Therefore, the capability of hindering the ionic current was greatly improved by our PE method (Fig. 4A and C, Table 1). (b) The change in surface charge [31]. Binding of charged analytes to the channel walls in this case affects the ion concentration in the pore and thus the resulting conductance, which reveals the reason for the initial decrease of R_p and C_m after immobilization of DNA probes inside the nanopores. In the routine hybridization, the C_m became smaller than that of probe immobilization (Table 1), which attributes to more negative charge of the floating single strand with 100 bp. However, it is not the case in PE hybridization; the C_m was increased as a result of the formation of "complete" hybridization complexes [32]. (c) The distribution of pore diameters. It is critical that the pore diameter is comparable to the size of an analyte molecule (for biological molecules it is often nm-grade) in order to have a substantial ion blockage. In our case, the pore diameter (73 nm) was almost double the DNA length (nearly 42 nm for a hybridization complex of 120 bp), thus the PE hybridization was expected to affect the conductance the most.

The asymmetric PCR and regular PCR for *E. coli* O157:H7 *eaeA* gene were carried out in parallel, and the PCR products were first tested by the gel electrophoresis. In Fig. 5, electrophoresis testified the successful amplification of regular PCR products, while single-stranded asymmetric PCR products could not be effectively confirmed on the agarose gel due to the low EtBr staining efficiency for ssDNA [33]. Based on PE hybridization method, the asymmetric PCR product was tested by our DNA biosensor, and showed the same effect on the resistance with the positive DNA control (Fig. 4C). This successful detection is very important for our future work because it indicates that this DNA biosensor has the potential to be integrated into a handheld device for on-site detection of microorganism. This biosensor also shows a good specificity, the CV curve did not change when the negative control or single base mismatch sequences were used, but agreed with the curve of probe immobilization. Based on the results of these control groups, it could be seen that there was no hybridization event happening in the absence of target complementary ss-DNA. To confirm the function of Taq DNA polymerase, the PE hybridization procedure was performed just without the polymerase. As shown in Fig. 4C, there was no obvious variation from the original value before hybridization. It could be explained that if there was no existence of Taq DNA polymerase in the reaction system, the hybridization between the target ss-DNA and probe inside the nanopores was impossible to be completed within 10 min [21]. At present, the sensitivity limit we have reached was 0.5 nM for

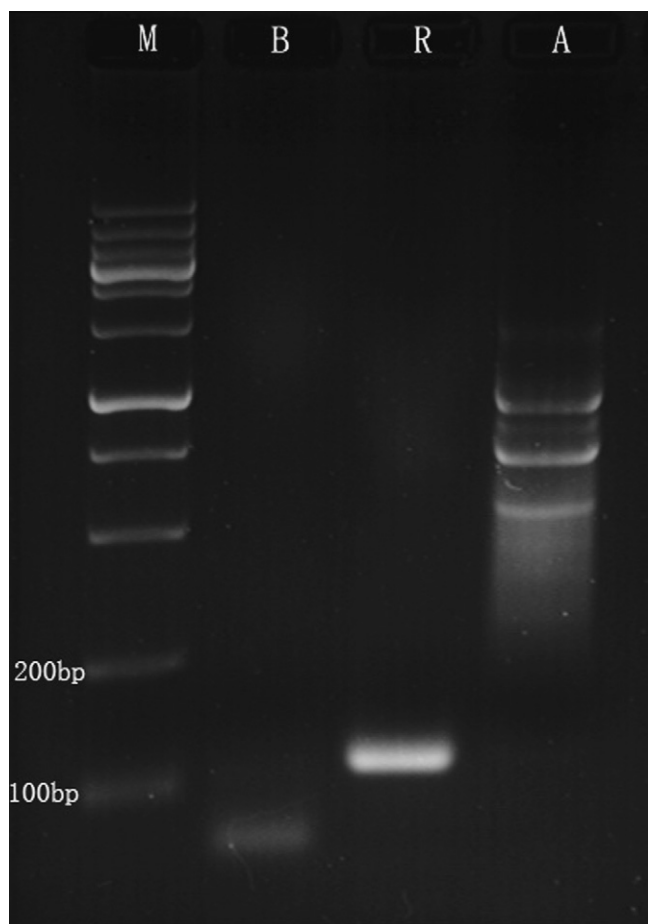


Fig. 5. Gel electrophoresis detection of *E. coli* O157:H7 *eaeA* gene PCR products. M, DNA marker; R, regular PCR products; B, blank control (purified water as PCR template); A, *eaeA* asymmetric product.

complementary target DNA by the PE method with this AAO membrane based biosensor (Fig. 4A and B). More efforts are being exerted to achieve better sensitivity.

At present, it is impractical to utilize a DNA biosensor to directly detect foodborne pathogen with real sample, such as milk, fruit juice and ground beef, because the target is bacteria DNA not the membrane antigen. Many works should be completed before DNA detection, for instance bacteria separation and enrichment, DNA extraction and PCR amplification. It is very difficult to accomplish all these steps in single DNA biosensor now, and an ideal DNA sensor for bacteria detection would be the one that can integrate all of functions mentioned above and can detect the real food or water sample. To further the application of our biosensor, we are proposing a self-contained and fully integrated biochip for sample-to-answer DNA analysis [34]. Sample preparation (including magnetic bead-based cell capture, cell pre-concentration and purification, and cell lysis), polymerase chain reaction, DNA hybridization, and electrochemical detection would be performed in this fully automated and miniature device. In the part of DNA detection, the AAO membrane based DNA biosensor we have studied and demonstrated has been adopted because of its convenience, sensitivity and label-free. In particular, the EC technology will make this biosensor easily integrated into the future handheld nucleic acid testing device for on-site food and water monitoring or point-of-care testing of infectious diseases.

4. Conclusions

In this study, we have shown that the nanopore AAO membrane can be successfully employed to immobilize specific ss-probes by aminosilanes and glutaraldehyde linker, and then be used for electrical detection of complementary target bacteria DNA without additional modification. During the course of hybridization, a novel PE method has been applied to enhance the ion blockage in nanopores by the whole double strand complex, and has greatly improved the sensitivity of the DNA biosensor and makes this system very attractive for further development of various DNA/RNA detections. This new-style AAO membranes based biosensor provides a sensitivity limit of 0.5 nM for complementary target DNA by PE method, which has the potential to be a powerful analytical tool for diagnostic, forensic analysis, and pathogen detection in near future. We are progressing toward experimental realization of this conceivability and hope to achieve even better sensitivity.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 60725102), the Natural Science Foundation for the Zhejiang Province of China (Grant No. R205505).

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