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Nanopore biosensor for sensitive and label-free nucleic acid detection based on hybridization chain reaction amplification

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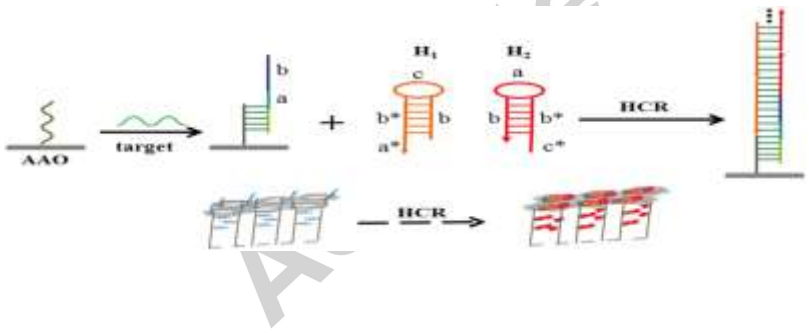
Abstract

A label-free nanopore biosensor for detection of DNA target is proposed utilizing hybridization chain reaction (HCR) strategy for signal amplification. The DNA target triggered HCR to form large DNA nanostructure inside the naopore and out the nanopore membrane, which inducing the ionic current decrease effectively due to the blockage of the nanopore. The developed method achieves a desirable sensitivity of 30 fM with a wide linear dynamic range from 0.1 to 10 pM and demonstrated good application for real sample analysis. This work has great potential to be applied in the early diagnosis of gene-related diseases and provide a new paradigm for label-free nucleic acid amplification strategy in ultrasensitive nanopore biosensor.

Graphical abstract

A label-free nanopore biosensor has been developed for the detection of DNA target with high sensitivity and specificity utilizing hybridization chain reaction as signal amplification strategy.



Keywords

Nanopore biosensor; hybridization chain reaction; label-free; DNA detection

1. Introduction

Nanopores have drawn increasing interest for constructing label-free biosensors in recent years [1-4].The sensing principle with nanopore is simple and straightforward, which is based on changes in ionic current when molecules pass or interact with the nanopore. One approach is to

measure current change pulse when single molecule passes a single nanopore, which has been successfully applied for single molecule analysis and DNA sequencing [5-9]. Measuring the permanent current change is another important type of nanopore biosensor, which is usually achieved via immobilizing molecule probe on the nanopore surface to interact with target molecule [10]. Biosensors utilizing different pore shapes (asymmetricn anopore, and symmetricna nopore),pore density (single nanopore and nanopore array) and materials (porous polyethylene terephthalate (PET) membrane, porous anodic aluminium oxide (AAO) membrane, porous polycarbonate (PC) membrane, glass nanopore and carbon nanotube) have been applied for detecting various targets [11-14]. Changes of pore size and surface charge properties can affect the conductance of nanopore [15, 16]. Biosensors based on pore size change often had restricted detection sensitivity due to the relative small size of target molecule compared with tens to hundreds nanometers of pore size. Considering the low abundance of nucleic acid in real samples, sensitive nanopore biosensors are urgently needed. Recently, a supersandwich structure has been designed to improve the detection sensitivity of nanopore biosensor [17, 18]. In this strategy, two DNA signal probes S1 and S2 were utilized to hybridize with target DNA to form a supersandwich nanostructure on the porous PET nanopore membrane. The detection sensitivity was significantly improved because of the large size nanostructure. Nevertheless, since the two DNA signal probes S1 and S2 could also hybridize in solution alternately with each other without DNA target, the supersandwich structure assembly might occur at a relatively low efficiency.

Nucleic acid-based signal amplification strategies have been widely used for optical and electrochemical biosensor construction [19-23]. Nevertheless, nanopore biosensors utilizing nucleic acid-based signal amplification strategy is rare. Herein, we proposed a novel label-free nanopore biosensor utilizing HCR as signal amplification strategy to improve the detection sensitivity. The enzyme-free and isothermal HCR enables in situ and highly efficient assembly of large size DNA nanostructure. In a HCR system, two DNA hairpin probes H1 and H2 are employed and they can stably coexist until triggered by DNA target sequence [24-26]. Several fluorescence and electrochemical biosensors based on HCR amplification have been developed [25, 27-33]. In this work, HCR enables in situ assembly of long dsDNA complex on nanopore membrane surfaces at a high efficiency to effectively reduce the pore size and ionic current. Porous anodic aluminum oxide (AAO) membrane is employed as nanopore membrane because of its uniform and tuneable pore sizes, high pore density and close pore distance [34].Moreover, the AAO nanopore membrane possess symmetric cylindrical pores, so we will focus on the biosensor development by tuning the pore size to induce ionic current decrease, while ion current rectification (ICR) phenomenon in asymmetricn anopores will not be discussed here. Survivin mRNA is chosen as model target due to its overexpression in many cancer cells [35].

2. Experimental section

2.1 Chemicals and Materials.

Oligonucleotides were synthesized and purified by Shanghai Sangon Biological Engineering Technology (China), the sequences are listed in Table S1[26], Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and sulfosuccinimidyl4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Aminopropyl triethoxy silane (APTES) was purchased from Adamas (shanghai, China).The Anodic aluminum oxide (AAO) membranes with 50, 150, 250 nm pore diameter were purchased from PuYuan Nano (Hefei, PR China). Silver wire (99%, 0.5 mm diameter) was purchased from SigmaAldrich (USA). All other reagents were of analytical grade. All the solutions were prepared in double-distilled water which was purified by a Milli-Q system (Millipore, Bedford, MA) and had an electric resistance >18.25 MΩ. For Ag/AgCl electrode preparation, the silver wire was polished using sandpaper and then the tip of wire was immersed in bleach solution overnight.

2.2 Electrochemical measurements.

The AAO nanopore membrane was mounted in a home-made two-compartment electrochemical cell. The electrochemical cell was made of teflon and the volume of each compartment was ~ 600 μL .So both compartments were filled with 400 μL 100 mM KCl solution as electrolyte. Measurements were recorded by a PGSTAT12Autolab (metrohm, Switzerland), using a linear sweep voltammetry setting between -0.2 V to 0.2V at scan rate of 50mV/s. Two Ag/AgCl electrodes were used for measurement. The diameter of the hole between two compartments cells was 2 mm; hence the effective AAO nanopore membrane area was estimated to be 3.14 mm^2. The AAO nanopore membranes were cut into circular pieces with diameter slightly larger than 2 mm.

2.3 Surface modification of the AAO nanopore membrane

The AAO nanopore membranes were cleaned by boiling in hydrogen peroxide for 30 min and washed with distilled water. The AAO membranes were then dried in an oven and immersed in APTES solution (5% in methanol) for 8 h at 25℃and followed by washing with methanol. The membranes were then subsequently cured in a hot oven at 110℃ for 1 h. The silanized AAO nanopore membranes were incubated with 1 mg/mL sulfo-SMCC in 10 mM phosphate buffer (500 mM NaCl, pH=7.4) for 6 h at 25 ℃, and then rinsed with distilled water. Finally, the membranes were incubated with 1 μM 5'-thiol DNA (capture DNA probe) in 10 mM Tris (500 mM NaCl, 10 mM $MgCl_2$, pH=7.4) for10 h at 25℃, rinsed with 10 mM Tris buffer. The membrane was functionalized on both sides since the membrane was put in a small beaker immersed with reagent solution. The modified AAO nanopore membranes were stored at 4 ℃ fridge before use.

2.4 HCR assembly on surface of AAO nanopore membrane

Firstly, the modified AAO nanopore membranes were incubated with 100 μL various concentrations of target mRNA in 10 mM Tris-HCl solution (500 mM NaCl, 10 mM $MgCl_2$, pH= 7.4) for 3 h at 25℃. The

membrane was put in a small beaker and the mouth of beaker was wrapped with plastic film to prevent water evaporation. Then the membranes were rinsed with 10 mM Tris-HCl and subsequently immersed in 10 mM Tris-HCl solution (500 mM NaCl,10 mM $MgCl_2$, pH= 7.4) containing1 μM hairpin DNA probe H1 and 1μM hairpin DNA probe H2 for 4 h at 37℃,and then rinsed with10 mM Tris buffer.

2.5 RNA extraction from cell and detection

HeLa cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100U mL^{-1} streptomycin. C166 cells (mouse endothelial cell line) were incubated in DMEM supplemented with 10% fetal bovine serum. All cell lines were maintained at 37℃ in a 100% humidified atmosphere containing 5% CO_2. The total RNA was extracted from cells using SanPrep Column microRNA Mini-Preps Kit (Sangon Biological Engineering Technology) according to the manufacturer's instructions. The extracted RNA was dissolved in 30 μL Rnase-free water and diluted with buffer solution containing 10 U mL^{-1} of Rnase inhibitor for detection.

3. Results and discussion

3.1 Principle of the detection strategy

The analytical principle of the label-free nanopore biosensor is illustrated in Figure 1. The approach is based on measurement of ionic current decrease of the AAO nanopore membrane due to the steric blockage of the large DNA nanostructure formed via HCR process. Firstly, the thiol-functionalized DNA capture probe was immobilized on surface the nanopore membrane according to previous literature (Figure S1, ESI) [12]. The designed DNA capture probe was used to capture target DNA via complementary hybridization. Then target DNA triggered a chain reaction by alternating hybridizing with the two hairpin DNA probes H1 and H2 to form a large DNA nanostructure complex. The HCR process formed a large nanostructure on the surface of nanopore membrane, which was expected to effectively block the nanopore and induce drastic current decrease. The nanopore membrane was mounted in a home-made two-compartment electrochemical cell for ionic current measurement (Figure S2, ESI).

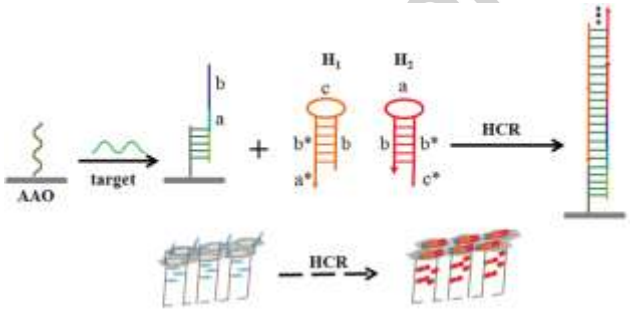


Figure 1. Illustration of the HCR assembly on the surface of AAO nanopore membrane.

3.2 Validation of the detection mechanism

Gel electrophoresis analysis was conducted to verify the formation of the HCR nanostructure triggered by target DNA (Figure 2A). The HCR experiment was performed in solution. When

using 100 nM target, 1μM DNA hairpin probe H1 and DNA hairpin probe H2, the bands appeared in different positions as shown in lane 2. When 100 nM target DNA was mixed with capture probe plus 1 μM H1 and H2, similar results were observed as in lane 3, suggesting the target could effectively trigger HCR. There was no new band appeared when H1 and H2 were mixed, indicating the two hairpin DNA probes were stably coexisted in the absence of target DNA (Lane 1). This result confirmed the success of HCR process and was consistent with previous reports [27, 28]. It also demonstrated HCR could only occur in the presence of target DNA, which was in favour of assembling long dsDNA complex on nanopore surfaces at a high efficiency. Then the surface modification processes of the nanopore membrane and biosensor response to mRNA target were characterized by measuring the ionic current. The resulting current-voltage (I-V) curves under different conditions were shown in Figure 2B. The ionic current only showed slight change for different surface modification steps (curve b, c and d), while it dramatically decreased by nearly 100% (from -5.26 $\times 10^{-5}$A to -3.05 $\times 10^{-11}$ A, measured at -0.2 V) after HCR process in the presence of 1nM target DNA, 1μM DNA hairpin probe H1 and 1μM DNA hairpin probe H2. This result suggested the formation of HCR product on the surface of
AAO nanopore membrane could effectively block the ionic current. The ionic current signal decrease was expressed as $(I_0-I)/I_0$, where I_0 was the current measured with bare AAO nanopore membrane, and I was the current for different surface modification processes or HCR processes.

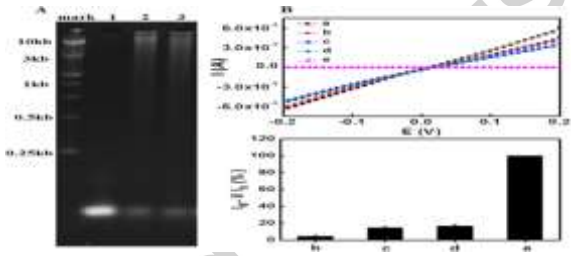


Figure 2. (A) Gelelectrophoresis images for different HCR conditions: marker; lane 1, 1μM DNA hairpin probe H1 + DNA hairpin probe H2; lane2, 100 nM target +1μM DNA hairpin probe H1 + DNA hairpin probe H2; lane 3, 100 nM capture probe +100 nM target +1μM DNA hairpin probe H1 + DNA hairpin probe H2. (B) I-V curves for ionic current measurements and the ionic current signal decrease expressed as $(I_0-I)/I_0$. a, bare AAO membrane; b, after silanization with APTES; c, after modification with sulfo-SMCC; d, after immobilization of capture DNA; e, after the HCR process.

Scanning electron microscope (SEM) and atomic force microscope (AFM) characterizat-ion were used to further verify the formation of large size DNA nanostructure (Figure 3). SEM images confirmed the HCR assembly of nanostructure on inside of pore and the outer membrane surface. AFM results also validated the assembly process. Formation of DNA nanostructure complex could be observed from the topographic image. Moreover, the section height profiles indicated the size of nanopore before HCR assembly was ~155 nm. While after HCR assembly, the size of DNA nanostructures formed on the surface could be observed and the size was ~140 nm, demonstrating the successful formation of DNA nanostructure. These results were consistent with previous research that the assembly occurred inside of the pore and outer membrane surface

[12, 16-18]. The HCR product had similar sizes with the nanopore so when the assembly process occurred inside the pore; the pore size was significantly reduced. Besides, distance between pores was around 20nm, so HCR product on the outer membranes could also effectively block the ionic current

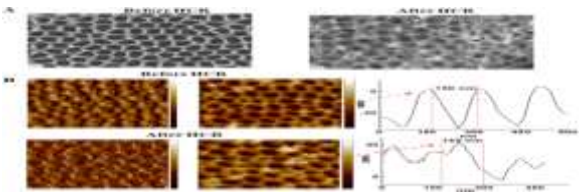


Figure 3. (A) SEM images of AAO nanopore membrane before and after HCR process. (B)The AFM topographic (left), height (middle) images and the section height profiles (right) of the AAO membrane before and after HCR process.

3.3 Analytical performance of the developed nanopore biosensor

We first investigated the pore size effect to the ionic current decrease. Three different pore sizes were studied and 150 nm diameter of pore size showed the strongest response for 1 nM target (Figure S3, ESI). Hence all the experiments were performed using AAO nanopore membrane with 150 nm pore diameter, which had pore density around $5x10^{-9}/cm^2$estimated by SEM images. Then the feasibility of the proposed biosensor was tested by running a series of samples with different target mRNA concentrations and recording the I-V curves. As shown in Figure 4, upon increasing the target mRNA concentration from 0.1 pM to 1nM, the current decreases monotonically. The current almost dropped 100% when 1nM target DNA was used. A good linear correlation between the percentage of the decreased current and the logarithm of target concentrations in the range from 0.1 to 10 pM was observed ($y=17.9+6.35x$, $R^2=0.93$, measured at -0.2V). The detection limit was estimated to be 30 fM according to the 3σ rule, showing effective improvement compared to conventional amplification-free nanopore biosensors [11-14]. The high sensitivity was due to the effective blockage of ionic current by HCR product inside the pore and outside the membrane. Based on the pores density of $5x10^9/cm^2$ estimate by SEM images, the effective AAO nanopore membrane area (~3.14 mm^2) was estimated to contain~$1.57x10^8$ pores. The area of one 150 nm diameter pore was $1.77x10^{-8}$ mm^2, so the total area of the pores was ~2.78 mm^2, which occupied 88.5% area of the AAO nanopore. This caclualtion was consistent with SEM image results that the pores were close to each other. Due to the small distance between pores, the HCR products at the outside of the membrane could still block ionic current. We estimated numbers of HCR product with 100 fM target; the number of target molecules in 100 μL volume would be ~$6x10^6$(10^{-13}mol/L*10^{-4}L*6.02*10^{23}). Considering efficiency of target capture and HCR process, the number of HCR product would be slightly less than ~$6x10^6$. The number of HCR products was estimated to be less than 3.4% of pore numbers, so the ionic current decrease signal for 100 fM target was expected to be detectable, which was in consistent with the experiment results. The signals for 10 fM targets showed unstable fluctuations and high error bars, hence the linear range was determined to be ranged from 0.1 pM to 10 pM. Since it employed a different

detection mechanism, the developed method for DNA detection demonstrated improved sensitivity compared to fluorescence based HCR amplification methods [30, 36, 37]. The desirable sensitivity enables detection of low abundance DNA in real samples.

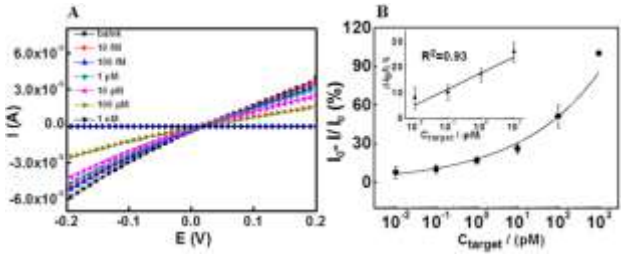


Figure 4. (A) Typical I-V curves of the HCR assay under different target mRNA concentrations. (B) The calibration curve of the biosensor using $(I_0-I)/I_0$ for ionic current decrease in the dynamic ranges from 0.1 pM to 1 nM, the insert shows the linear relationship in the range from 0.1 to 10 pM. Error bars are the SD of four repetitive experiments.

The selectivity of the proposed method was evaluated by challenging it with the target mRNA, single-base mismatched, three-base mismatched and random non-complementary DNA sequences, respectively (Figure 5[38]). For 1 nM analytes, only slight current decreases were observed upon response to the random non-complementary, three-base mismatched and single-base mismatched DNA sequences, respectively. On the contrary, significant current decrease (almost 100%) was observed when using 1 nM target. The results demonstrated a high selectivity of the proposed method toward target mRNA over other DNA sequences. The results could be attributed to the requirement of full complementarity between the trigger DNA and substrate hairpin H1 in the HCR system [28].

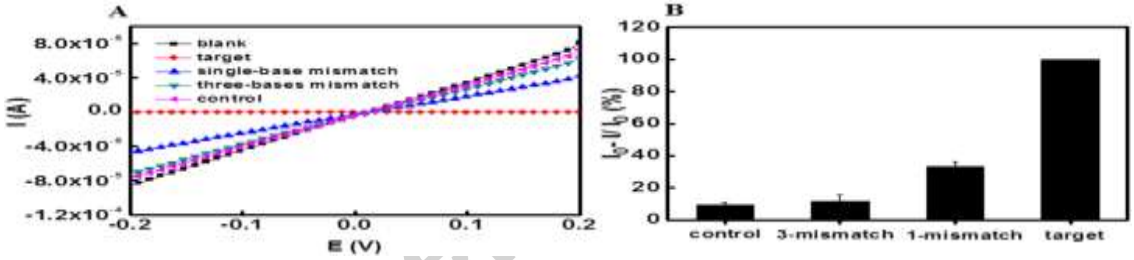


Figure 5. (A) Selective ionic current responses of the biosensor and (B) ion current signal decrease expressed as $(I_0-I)/I_0$ for 1 nM control probe, 1 nM three-base mismatch sequence, 1 nM one-base mismatch sequence and 1 nM target, respectively.

The reproducibility of the proposed method was investigated by analyzing 100 pM target mRNA with six capture DNA modified AAO nanopore membranes. The obtained current decrease were 60%, 43%, 58%, 48%, 46%, 54% respectively. The standard deviation (SD) was ~7%, a little bit high but acceptable and better than some nucleic acid amplification methods with complicated procedures such as quantative polymerase chain reaction (qPCR)[38]. The reason could be

attributed to differences of pore density from different AAO membranes and may be improved utilizing better AAO membrane products (Figure S4, ESI).

3.4 Real sample analysis of the developed nanopore biosensor

The proposed method was then applied to detect survivin mRNA in two different cell lines: Hela and C166 as shown in Figure 6. The HeLa cell extracts showed obvious response of current drop (22.4% for 1000 cell extracts). According to the calibration curve, the concentration of survivin mRNA for 1000 cell extracts was estimated to be 0.7±0.2 pM from three repetitive experiments). The obtained result showed a good agreement with the mRNA expression level in Hela cell [35]. On the other hand, a slightly current drop was observed for C166 cell extracts (6.0% for 1000 cell extracts), which was consistent with almost no expression of survivin mRNA in C166 cell [26, 39]. The results demonstrated that the developed method was applicable for DNA detection in biological samples.

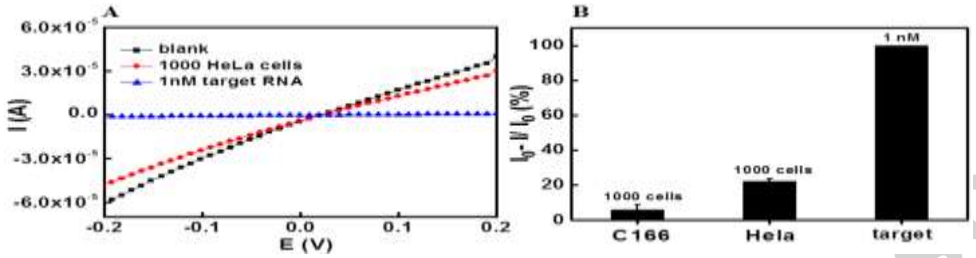


Figure 6. (A)The ionic current responses of the biosensor and (B) ion current signal decrease expressed as $(I_0-I)/I_0$ for RNA extracts from 1000 C166 cells, RNA extracts from 1000 Hela cells and 1nM target mRNA, respectively.

4. Conclusion

In conclusion, we have demonstrated a simple, label-free and sensitive nanopore biosensor for DNA detection. Highly efficient nanostructure assembly on surfaces of AAO nanopore membrane was achieved via target triggered HCR. The detection sensitivity was significantly improved by the enhancement of current blockage with large size of HCR product compare to conventional amplification-free nanopore biosensors. This method demonstrated desirable selectively and applicability for biological samples analysis using surviving mRNA as a model target. It is envisioned the developed method would be useful for DNA detection in clinic samples.

Acknowledgement

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Highlights

- Highly efficient assembly of large DNA nanostructure on porous anodic aluminum oxide nanopore membrane utilizing hybridization chain reaction.
- A label-free nanopore biosensor has been developed utilizing hybridization chain reaction as signal amplification strategy.
- The developed biosensor demonstrated significant improvement in detection sensitivity compared to conventional amplification-free nanopore biosensor.
- This work may provide a new paradigm for the design of label-free and ultra-sensitive nanopore biosensor utilizing nucleic acid-based amplification strategy.