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A novel DNA sensor using a sandwich format by electrochemical measurement of marker ion fluxes across nanoporous alumina membrane

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ABSTRACT

A sandwich strategy for highly sensitive DNA analysis has been proposed by measuring the hindered diffusion flux of $Fe(CN)_6^{3-}$ caused by DNA hybridization in nanopores of a porous anodic alumina (PAA) membrane. The flux of $Fe(CN)_6^{3-}$ passing through the PAA nanopores is recorded using an electrochemical detector made with Au film sputtered on one face of PAA membrane. To achieve target DNA analysis, the PAA membranes are functionalized with capture DNA and followed by the hybridization event that gives rise to the pore blocking due to the synergetic effect of steric and electrostatic repulsion in the confined nanopores. Both of these two effects can be amplified by using 12-nm Au nanoparticle tags labeled with single strand DNA to perform a sandwich assay, which further amplified detection signals evidenced from the decreased flux of $Fe(CN)_6^{3-}$. Based on this strategy, the proposed DNA biosensor gives a detection limit of 1 pM. This thin piece of membrane sensor, coupled with the simple electrochemical system, fast response and high sensitivity, show potentials to be successfully used for bioanalysis.

1. Introduction

The utilization of the properties of nanopores to control over the ionic and molecular transport across biological or solid state membranes represents a promising strategy in the development of sensitive biosensors [1–4]. There are different approaches for constructing a nanopore-based biosensor, in which the detection signal would be based on modulations of ionic and molecular flux. The first approach is based on measuring transient changes of ion current [5,6], which occur when target molecules pass through a single nanopore. In the second approach, the nanopore walls are decorated with recognition sites for a specific target, the target species function as a stimulus, actuating (opening or closing) ion channels and thus modulating the flux of ions to be detected. Generally, changes in the current-voltage curves before and after introduction of analyte into the nanopores are always monitored for single nanopores [7,8]. However, for nanopores/nanochannel array sensors, the ion conductance [9,10] and electrochemical impedance spectroscopy [11] is the common used detection technique, in addition, the ion-channel type amplification mechanism by using the markers is a promising alternative method for highly sensitive detection of the target species [12–16]. Amplification is achieved because the concentration of markers exceeds the concentration of the target species by many orders of magnitude. The flux change of marker through nanopores converts the analyte binding event into a versatile and quantifiable signal, either by optical or electrochemical response.

In our previous work, we have focused on marker ion flux modulations induced by DNA-Morpholino hybridization in nanopore arrays as the basis for label-free DNA biosensing [17]. In confined nanopores, presence of target analyte can cause two effects to occur: i) volume exclusion (steric effect) [7,18] when the size of analyte is large enough compared to the pore diameter to cause a partial or complete occlusion of a pore, and ii) modification of the surface charge occurring upon binding of the target to the pore walls [19,20]. Considering the influence of those two described cases, we selected the highly negative charged ferricyanide as marker ion for DNA analysis due to the steric and electrostatic effect produced by hybridization event both directing to reduced marker flux. In this case, the presence of target DNA in nanopores can be sensitively and quantifiably detected from the decreased flux of ferricyanide passing through functionalized nanopores.

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In this work, we developed a novel DNA sensor using a sandwich format by electrochemical measurement of the transported fluxes of ferricyanide across nanoporous membrane. The nanoporous membrane has arisen much interest since the pioneering work of Martin [21–23]. Here, the porous anodic alumina (PAA) membrane was selected as nanopore arrays due to its well-defined nanopores [24], tailorable surface properties [25], wide-ranging applications [26–28] and commercial availability. Measuring the flux-related electrochemical signal of transported ferricyanide was achieved by sputtering an Au film on PAA membrane surface. Based on this device, a label-free electrochemical detection strategy makes use of the ion-channel type amplification mechanism by immobilizing capture DNA on the inner wall of nanopores whose selective binding of target DNA reduces the flux of ferricyanide and, thus, the electrochemical signal due to the occurrence of steric and charge repulsion effect. However, this strategy exhibited a high detection limit for target DNA (only 1 nM) and had limitations to satisfy the requirement for bioanalysis. To further improve the detection sensitivity, a sandwich assay using gold nanoparticle tags with amplified steric and charge repulsion effect was introduced into the above system, and a highly sensitive DNA sensor with low detection limit of 1 pM was constructed.

2. Experimental

2.1. Materials and reagents

PAA filter membrane (catalogue number: 6809–7003, 13 mm diameter; 60 μm thick containing 200 or 20 nm pores of 1×10^9 cm^{-2} density) was purchased from Whatman International Ltd. (England). The morphology of PAA membrane was characterized by scanning electron microscopy (SEM, Hitachi, S-4800, 15 kV). Gold (III) tetrachloride trihydrate ($HAuCl_4 \cdot 3H_2O$) and 3-Aminopropyltriethoxysilane (APTES) was from Sigma-Aldrich. 25% glutaraldehyde (AR) aqueous solution was from Sinopharm Chemical Reagent Co., Ltd. To avoid its self-polymerization by aldol condensation, the stock solution of glutaraldehyde was stored at 4 °C in the dark. Phosphate buffer solutions (PBS, pH 7.4) were prepared using analytical grade from the chemicals of Na_2HPO_4 and KH_2PO_4. DNA was purchased from Sangon Biotech (Shanghai) Co.,Ltd and its corresponding solutions were prepared with 10 mM pH 7.4 PBS. The DNA sequence is as follows:

Capture DNA: 5′-NH_2-GCA GTA ACG CTA TGT GAC-3′

Target DNA: 5′-GGG CTC AAG ACT ACA AAT CGC GTC ACA TAG CGT TAC TGC-3′

Single strand DNA (ssDNA) immobilized on gold nanoparticles: 5′-ATT-TGT-AGT-CTT-GAG-CCC-SH-3′

All reagents were analytical grade and solutions were prepared using ultrapure water (specific resistance of >18.2 MΩ, Millipore, France).

2.2. Preparation of ssDNA labeled gold nanoparticles (ssDNA-AuNPs)

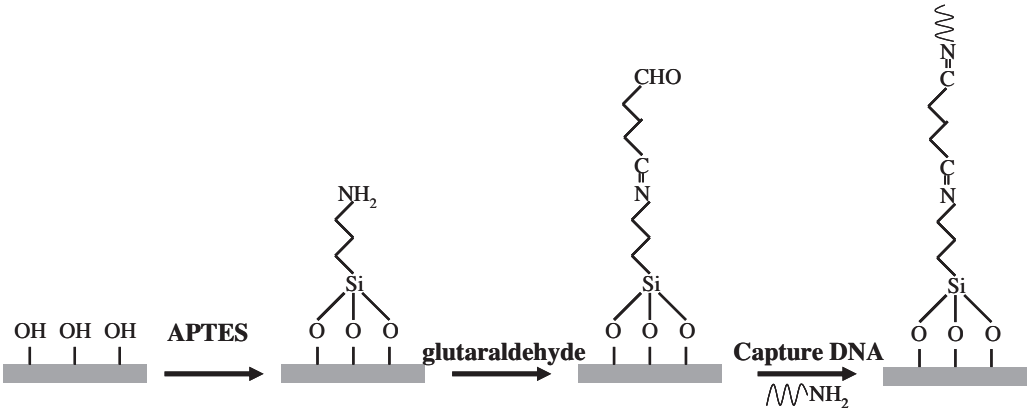
AuNPs with an average diameter of 12 ± 1 nm were prepared with the citrate reduction method [29]. Concentration of the as-prepared AuNPs was determined by UV–vis spectroscopy using the Lambert-Beer's law (molar extinction coefficient of 12 nm AuNPs is $2.7 \times 10^8\ M^{-1}\ cm^{-1}$ at λ_{520}). The as-prepared AuNPs were further modified with the thiolated ssDNA. Briefly, 56 μL thiolated ssDNA (100 μM) was incubated with 1 mL of AuNPs (1.25 nM) for 16 h at room temperature. The mixture was slowly brought up to a final salt concentration of 0.1 M NaCl and 10 mM PBS (pH 7.4) and allowed to age for 40 h. After that, the ssDNA-AuNPs suspension was subject to centrifugation in order to remove excessive DNA. The precipitate was washed with 10 mM PBS (pH 7.4) containing 0.1 M NaCl, recentrifuged, and finally dispersed in 1 mL 10 mM PBS (pH 7.4) solution containing 0.1 M NaCl for further use.

2.3. Surface modification of PAA membrane

The process for functionalization of the inner walls of PAA nanopores was described in scheme 1. PAA membrane was first cleaned as described elsewhere [30]. The cleaned membrane was then immersed into a 10 mL acetone solution containing 2.5% APTES for about 1 h with grafting aminopropyl functional groups. After that, excess silane solution was removed from the PAA nanopores by rinsing with copious amounts of acetone, followed by deionized water wash. The aminopropyl-grafted PAA membrane was further functionalized by overnight treatment with 2.5% glutaraldehyde, followed by copious amount of rinsing and subsequent drying. After the above treatments, glutaraldehyde was successfully covalently coupled onto the amine functional groups on PAA. The remaining modifications (originating from the surface-bound aldehydes) were performed after deposition of gold electrode on the membrane and after placing it inside the cell.

2.4. Fabrication of Au film working electrode and electrochemical measurements

100 nm thickness of Au film was sputtered only on one face of the above modified PAA membrane with nanopore diameter of



Scheme 1. . Functionalization of the inner walls of PAA nanopores with aminated capture DNA.