Nanomaterials

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Two-Way Nanopore Sensing of Sequence-Specific Oligonucleotides and Small-Molecule Targets in Complex Matrices Using Integrated DNA Supersandwich Structures**

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Recent advances in molecular science and nanotechnology offer unprecedented opportunities to miniaturize chemical analysis systems into nanofluidic devices that mimic ion channels and have single-molecule sensitivity, target-specific selectivity, and reduced consumption of materials.[1] When analytes are present in the nanofluidic sensing system, they temporarily or permanently block the pathway for ion conduction, yielding characteristic changes in background current that serves as a signature for target identification, or concentration qualification.[2] Although the sensitivity approaches a relatively high level,[3] this technique is still challenging for treating multi-component or complex clinic samples.[4]

In recent years, the sequence-specific and label-free detection of DNA targets associated with many crucial pathogenic diseases has attracted a broad interest.[5] To meet the requirements of this application, these nanofluidic devices have been endowed with chemical selectivity by integration with nucleic-acid-based sensing elements.[6] For example, simple-structured DNA components were used for oligonucleotide detection using the hybridization technique.[7] Intermolecular DNA duplexes, forming T-Hg^{2+}-T structures, have been used for nanopore-based sensing of mercury.[8] We used DNA molecular motors, showing a conformational change in response to external stimuli, to construct adaptive DNA-nanopore switches.[9] In conventional DNA-based nanopore sensors, a single capture DNA hybridizes to a single target strand or binds to a single molecular target (Figure 1, insert), which restricts their performance. On the other hand, although there are already some nanofluidic sensors for DNA and proteins,[10] quite a few of them are capable of sensing small molecules,[11] let alone detect both nucleic acids and small molecules within one sensing device.

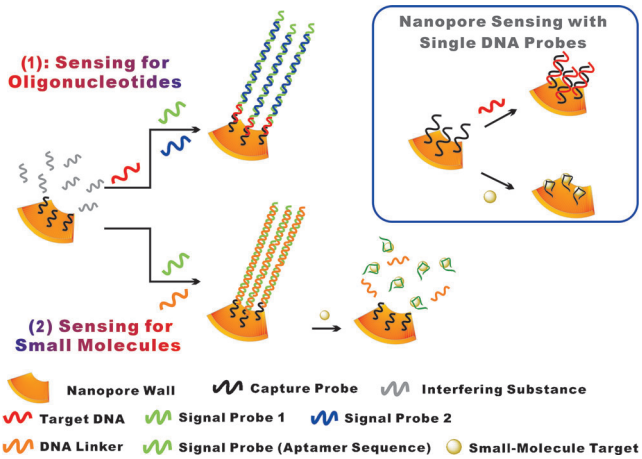

Figure 1. In conventional DNA-based nanopore sensors, a single capture DNA hybridizes to a single target strand or binds to a single molecular target (inset). Here we improved this sensing strategy by integrating a more complex DNA nanostructure within the nanopores. To detect oligonucleotides (1), a capture probe (CP) is grafted onto the nanopores. When the target DNA is present, it is first captured by the CP. To amplify this hybridization event, repeated units of signal probes hybridize to each other from the free end of the target DNA, creating long concatamers (supersandwich). To detect small molecules (2), such as ATP, pre-designed signal probes containing an aptamer sequence for ATP are used. The disassembly of the DNA nanostructures can therefore be used for sensing.

Herein, we show improvement of this sensing strategy by integrating a more complex DNA nanostructure within the nanopores that contains multiple target-binding sites on each of its long concatamers and provides a built-in amplification mechanism (Figure 1). This leads to enhanced signal strength, a better detection limit, and anti-interference capability. The principle for this sensor is based on turning off or turning on a smart nanofluidic switch by self-assembly and disassembly of supramolecular DNA nanostructures in nanopores (two-way sensing). The target oligonucleotide is from the HIV-

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1 U5 DNA long terminal repeats (LTRs), which is highly conserved in HIV genomes and thus provides important targets for the inhibition of viral infection.[12] Initially, a predesigned capture DNA probe (CP) was anchored onto the nanopores. When target DNA is present, it is first captured by the CP through hybridization with the complementary regions. To amplify the hybridization event, alternating units of signal probes (S1 and S2) were successively hybridized to each other starting from the free 3′-end of the target DNA, creating long concatamers, termed supersandwich structures.[13] Formation of complex DNA nanostructures inside the nanopores efficiently blocks the pathway for ion conduction. Thus, the presence of target DNA could be identified by monitoring the change in nanopore conductance. Alternatively, if the DNA probes contained an aptamer sequence for a specific small molecule, for example ATP, the disassembly of the preformed supersandwich structures could be used to detect ATP. We achieved a reliable detection limit of 10 fM for oligonucleotides and 1 nM for ATP. Particularly, these nanopore sensors could also be used in complex matrices with a high concentration of interfering substances in the buffer solution or even directly in serum.

A cylindrical nanopore array was prepared through UV treatment and chemical etching of irradiated poly(ethylene terephthalate) (PET) membranes.[14] The mean diameter of the nanopores for the sensor was 79 ± 7 nm and the pore density was approximately 5×10^8 cm^{-2} (Supporting Information, Figure S2). The nanopore membranes were chemically modified with capture DNA (20-mer) through a two-step chemical reaction (see the Supporting Information). For the assembly of the DNA supersandwich structures, two kinds of signal probes (S1 and S2, 33-mers) were added in advance to the reaction vessel. In the presence of the target DNA (38-mer), concatenated DNA sandwich structures grow from the CP. The formation of the proposed supersandwich structures was confirmed by gel electrophoresis (Figure 2 A). The analysis shows a ladder of different lengths of the DNA concatamers, the maximum of which approaches approximately 1000 base pairs (bp). In contrast, the length of a traditional DNA sandwich structure is less than 50 bp, in which a target hybridizes to merely one signal probe. The successful assembly of DNA supersandwiches in nanopores was verified by laser scanning confocal microscopy (LSCM, Figure 2 B, C) using fluorophore-labeled signal probes (S1-FITC). The membrane thickness seen in the fluorescent image quantitatively agrees with its calculated thickness.

To perform the sensing task, the modified PET membrane was mounted in an electrochemical cell with Tris buffer solution (10 mM, pH 7.4, 500 mM NaCl, 1 mM $MgCl_2$) on each side.[15] A scanning transmembrane electrical potential was applied to measure the resulting ionic current. For DNA detection, capture-probe-modified PET films were immersed in a solution containing signal probes (S1 and S2, 1 μM) and the target probe at a predetermined concentration (from 1 fM to 1 nM) for one hour before electrical measurements were done. The current–voltage (I–V) curves show that the nanopore conductance is very sensitive to the presence of target DNA (Figure 3 A). The addition of 10 fM target DNA leads to

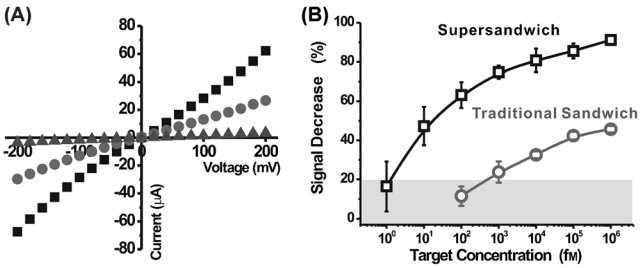

Figure 3. Detection of DNA as monitored by nanopore conductance. A) I–V curves before (■) and after treatment with 10 fM (●) or 1 nM (▲) target DNA. A distinct reduction in pore conductance was found following DNA assembly. B) Dose-response of the DNA sensor. The percent signal decrease is defined as $(I_0 - I(c))/I_0$, where $I(c)$ and I_0 were the intensity measured after DNA assembly at a concentration of c and without DNA assembly, respectively. A threshold of more than 20% change in conductance is defined for effective sensing events (above the gray area). Traditional sandwich-modified nanopores show lower signal gain and a poorer detection limit.

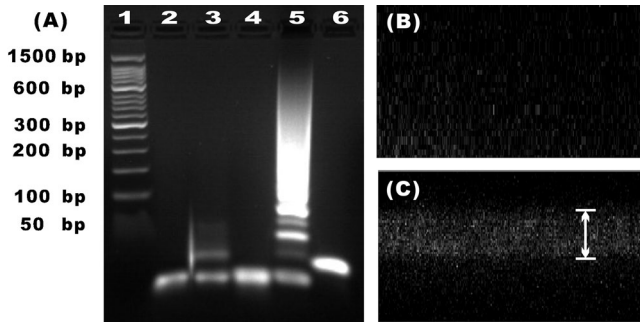

Figure 2. Characterization of the DNA assemblies in nanopores. A) Agarose gel electrophoresis: 1) DNA marker; 2) S1; 3) S2; 4) target; 5) target + S1 + S2 (supersandwich structures); 6) traditional sandwich structures. LSCM observation of the nanoporous membrane before (B) and after (C) DNA assembly. A fluorescent signal from the cross-sectional view shows the successful assembly of DNA structures inside the nanopores. The membrane thickness is ca. 12.8 ± 0.5 μm, as shown by the double-headed arrow.

a nearly 50% decrease in the conductance of the nanopore. When the target concentration was increased to 1 nM, the ionic conductance drops to less than 10% of the initial value. The current blockades are due to the formation of high-molecular-weight DNA nanostructures inside the nanopores. We have defined a change in conductance more than 20% as an effective sensing event. Below this threshold, the signal could stem from some non-specific interactions (see the following discussion). In this way, we obtained a reliable detection limit of 10 fM (Figure 3 B). As a control experiment, traditional DNA sandwich-modified nanopore devices showed much lower sensitivity (two orders of magnitude poorer in their detection limit). The signal gain was only one third to one half of that obtained in the supersandwich modified devices. These improvements can be attributed to the complex molecular structure and the increase in signal gain.

Another important feature of our nanofluidic sensing system is the specificity. To verify this, the sensing device was

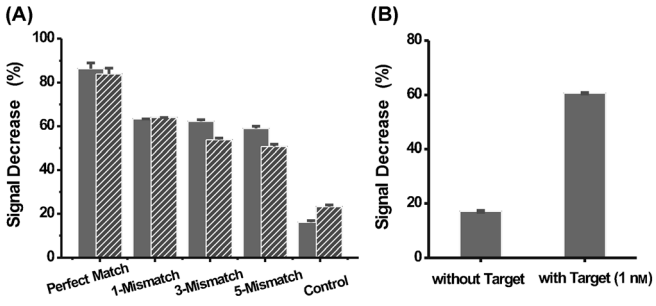

Figure 4. A) The nanofluidic oligonucleotide sensing system is highly selective in both pure buffer (solid) and in complex mixtures containing a high concentration (1 μM) of non-complementary sequences (meshed). The concentration of target DNA in the mixture was 1 nM (Supporting Information). B) The sensing device was also tested in 10% fetal bovine serum.

challenged with mismatched target sequences (here at 1 nM, Figure 4). A sharp contrast was observed between the perfectly complementary target from the non-specific mismatched ones. Single-base mismatch resolution was readily achieved. Notably, the conductance change for a non-complementary sequence is approximately 17%, which probably results from physical adsorption or other non-specific interference. Thus, we set a threshold of more than 20% change in conductance to identify an effective and reliable sensing event. Moreover, the nanopore sensor performed well when challenged in complex mixtures, for example, in a buffer solution containing a high concentration of non-specific sequences (1 μM, 1000-fold higher than the target) or even in 10% fetal bovine serum. The non-specific DNA strands and interfering substances in the serum were effectively precluded by the sequence-specific DNA nanostructures (Figure 4).

Alternatively, if the DNA probes contain aptamer sequences for small-molecule targets, the disassembly of the preformed DNA nanostructures could be used to detect the molecular targets.[16] To realize the detection of ATP, we designed an improved DNA supersandwich structure by introducing an aptamer sequence for ATP into the capture and signal probes, and connecting them with a DNA linker (Table S2), in which multiple ATP-binding sites were involved on each concatamer chain. Before the sensing task, high concentrations of signal probes and a DNA linker (1 μM each) were used to create sufficient DNA nanostructures to block the nanopores. A significant drop in nanopore conductance is therefore observed (Figure S5). After the addition of ATP (1 nM) to the blocked nanopores, a nearly 100% increase in conductance was found (Figure 5A). The ionic conductance further increases with ATP concentration from 1 nM to 1 μM (Figure 5C). The binding of ATP to its aptamer sequence leads to a structural transformation from a DNA/DNA duplex to a DNA/ATP complex,[17] leading to the disassembly of the hybridized DNA nanostructures that frees the path for ion conduction. Notably, this aptamer-based sensing strategy showed higher selectivity towards its target (ATP) than the other three types of nucleoside triphosphate (NTP), CTP, GTP, and UTP (Figure 5D). Excellent ATP discrimination was also found when the sensors were challenged with an NTP mixture (in which the concentration of the competing NTPs was 100-fold higher than ATP) and also in serum (Figure 5E,F). The high specificity can be attributed to the target-specific change in supramolecular structure; this was confirmed by gel electrophoresis (Figure 5B) and confocal microscopy (Figure S6).

Compared with conventional DNA sensors, in which a single capture probe hybridizes with a single target,[18] the dynamically integrated DNA superstructures provide a built-in amplification mechanism to promote the signal gain and thus sensitivity.[19] Herein, we adopt a "multiple-signal-amplification" mechanism for the signal-off sensing of oligonucleotides (decreasing the conductance of the nanopore) and a "multiple-target-binding-site" structure for the signal-on sensing of ATP (increasing the conductance of the nanopore) to optimize the analyte capture process. This means that minor changes in the supramolecular structures can be efficiently converted to detectable changes in nanopore conductance. In addition, the target specificity and anti-interference feature of these sensors is important for simple

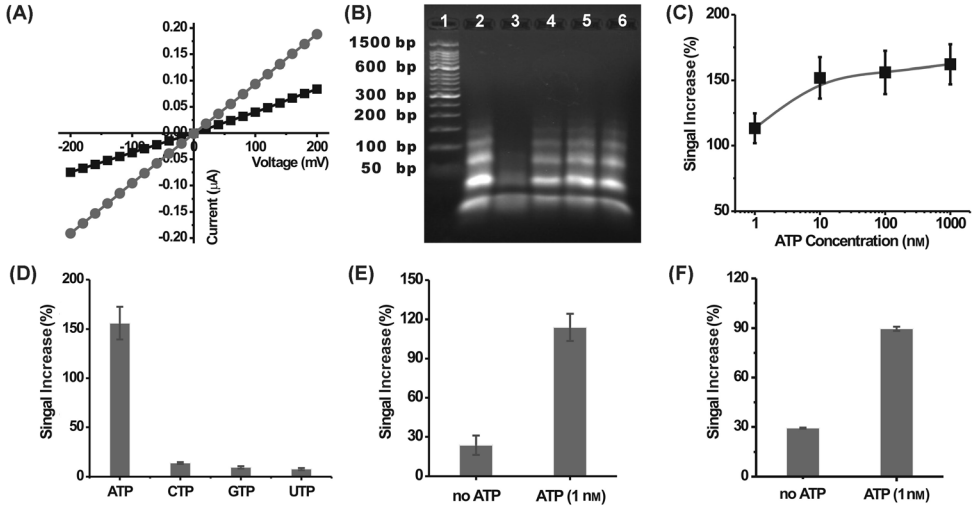

Figure 5. Detection of ATP using DNA supersandwich-modified nanopores. A) *I–V* curves before (■) and after 1 nM ATP treatment (●). B) Agarose gel electrophoresis: 1) DNA marker; 2) signal + linker (supersandwich structure); 3) signal + linker + ATP; 4) signal + linker + CTP; 5) signal + linker + GTP; 6) signal + linker + UTP. C) Dose-response curves. The signal increase is defined as $(I(c)-I_0)/I_0$, where fluorescence intensities $I(c)$ and I_0 were measured after ATP treatment at the concentrations shown c and without ATP treatment. D) The nanofluidic sensor was highly selective for ATP, in contrast with other NTPs. The sensors were also tested in an NTP mixture (E) and in 10% fetal bovine serum (F). The concentration of ATP was 1 nM, while the concentration of competing NTPs was 100 nM (Supporting Information).

sensing in complex mixtures, or even biological samples, save the purification process before the assay. Therefore, the present nanofluidic sensing device can compete well with existing sensors and takes a step forward toward real-world applications.

To date, numerous DNA aptamers have been selected for a broad range of targets.[20] With rationally designed DNA nanostructures involving aptamer sequences, in principle, this nanofluidic sensing strategy could be used for many other molecular targets and has the potential to be developed into a live nanopore assay (Supporting Information). In the next step, a series of dynamic DNA structures, such as molecular machines and DNAzymes, could be used to construct powerful and easy-to-use nanofluidic devices.[21]

In conclusion, we demonstrated a highly sensitive and selective nanofluidic sensing device that detects both sub-nanomolar DNA and sub-micromolar ATP, using the modulation of supramolecular DNA nanostructures in nanopores. We achieved a reliable detection limit of 10 fM for target DNA and 1 nM for ATP. Single-base mismatch resolution and discrimination between different types of nucleoside triphosphates was achieved. The biohybrid nanopore sensor worked well in complex mixtures and in serum. In the future, this sensing strategy could be developed into a live assay for disease-related molecular targets, with many practical applications in biotechnology and life science.

Experimental Section

Nanopore fabrication: Poly(ethylene terephthalate) films (PET, approximately 12 μm thick) were irradiated with a Au ion beam (11.4 MeV/u at GSI, Darmstadt). The track density is approximately 5×10^8 cm^{-2}. Cylindrical nanopores were fabricated through a previously published method.[14] Briefly, the PET films were pre-treated with UV light (approximately 4.2 mW cm^{-2}) for 1 h on each side before etching with NaOH (2 M) at 50 °C for 8 min (Supporting Information). After that, the films were thoroughly washed with and restored in distilled water. The pore diameters were evaluated by SEM (JSM-6700F).

Chemical modification: The capture DNA was immobilized onto the PET surface through a two-step reaction (Supporting Information).[22] *N*-hydroxysulfosuccinimide (NHSS) esters were initially formed by exposure of the PET film to a solution containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 15 mg) and NHSS (3 mg) for 8 h. Then, the PET-NHSS ester reacted with 5′-aminated capture DNA (1 μM) in 1 mL of Tris buffer solution (10 mM, pH 7.4, 500 mM NaCl, 1 mM $MgCl_2$) for 10 h. The reactions were conducted at 25 °C. Detailed sequences are listed in Tables S1 and S2.

For DNA detection, the capture-probe-modified PET film was immersed in another solution containing signal probe (1 μM) and target probe at different concentrations (1 fM–1 nM) for 1 h. DNA sensing was also performed in complex mixtures containing 1 μM non-complementary sequences or 10% fetal bovine serum (FBS, Supporting Information).

For ATP detection, the capture-probe-modified PET films were first put into a Tris buffer solution containing signal probe and DNA linker (1 μM each) for 10 h to allow sufficient assembly of DNA nanostructures. The resulting films were immersed in Tris buffer solution with different concentrations of ATP (1 nM–1 μM) for 1 h before electrical measurements. ATP sensing was also performed in an NTP mixture containing CTP, GTP, and UTP (100 nM each) and in 10% FBS (Supporting Information).

Characterization of the DNA supersandwich structures: The DNA concentrations for gel electrophoresis were 10 μM. For the disassembly of the DNA nanostructures, ATP, CTP, GTP, and UTP (100 nM) were separately added to the DNA samples and kept for 3 h at room temperature before running on a gel. 3% agarose gels containing 0.1 μL Gel-Red dye per milliliter were prepared using 1 × TBE buffer. The gel was run at 90 V for 35 min.

Fluorescein isothiocyanate labeled signal probes (S1-FITC) were employed for LSCM observation (Olympus FV1000). The chemical modification process was similar to that mentioned above.

Electrical measurements: The sample was mounted in a two-compartment electrochemical cell that was described in our previous work.[23] Ionic current was measured by a Keithley 6487 picoammeter. Ag/AgCl electrodes were used to measure the resulting ionic current. The electrolyte was Tris buffer solution (10 mM, pH 7.4, 500 mM NaCl, 1 mM $MgCl_2$). The effective area for ion conduction was approximately 7 mm^2.[24]

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