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Sequence-Specific Recognition of DNA Oligomer Using Peptide Nucleic Acid (PNA)-Modified Synthetic Ion Channels: PNA/DNA Hybridization in Nanoconfined Environment

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ABSTRACT Here we demonstrate the design and construction of a simple, highly sensitive and selective nanofluidic sensing device, based on a single synthetic conical nanochannel for the sequence specific detection of single-stranded DNA oligonucleotides. The biosensing performance of the device depends sensitively on the surface charge and chemical groups incorporated on the inner channel wall that act as binding sites for different analytes. Uncharged peptide nucleic acid (PNA) probes are covalently immobilized on the channel surface through carbodiimide coupling chemistry. This diminishes the channel surface charge, leading to a significant decrease in the rectified ion current flowing through the channel. The PNA-modified channel acts as a highly specific and selective device for the detection of a complementary single-stranded DNA sequence. Upon PNA/DNA hybridization, the channel surface charge density increased due to the presence of the negatively charged DNA strand. The changes in the surface charge-dependent current—voltage (*I*—*V*) curves and rectification ratio of the channel confirm the success of immobilization and PNA/DNA hybridization within a confined space at the nanoscale. In addition, a control experiment indicated that the biosensor exhibits remarkable specificity toward a cDNA strand and also has the ability to discriminate single-base mismatch DNA sequences on the basis of rectified ion flux through the nanochannel. In this context, we envision that the single conical nanochannels functionalized with a PNA probe will provide a biosensing platform for the detection and discrimination of short single-stranded DNA oligomer of unknown sequence.

KEYWORDS: solid-state nanopores · PNA/DNA hybridization · biosensor · polymer membrane · heavy ion irradiation · track-etching

In living organisms, ion channels regulate the flow of ions or particular analytes across the cell boundaries, providing the basis for communication with each other, for example, for metabolic and signaling purposes.[1] Biological ion channels offer precisely controlled structures and interfacial chemistry. However, they are not suitable for practical applications due to the fragility and sensitivity of the embedding lipid bilayer induced by external parameters such as pH, temperature, salt concentration, *etc.* To overcome these difficulties, various routes have been explored to meet the challenge of fabricating channels in solid-state materials with the ability to mimic functions of biological ion channels.[2−6] Solid-state synthetic nanochannels present several advantages over their biological counterparts such as robustness, control over shape and size, and tailorable surface charge properties. Furthermore, synthetic ion channels have attracted a considerable interest due to their unique transport properties and potential applications in areas such as biosensing,[7−12] separation,[13−16] and integration into nanofluidic devices.[17−20]

Up to now, detection and analysis of DNA oligonucleotides have been achieved by using a resistive-pulse sensing technique.[21−31] In this strategy, DNA molecules were driven through a single biological/synthetic nanochannel under the influence of an applied voltage, and by measuring the changes in the ionic current/electrical signal, one can be able to detect individual molecules. In addition, the degree and duration of ion current reduction provide information about structure and length of translocating nucleotide. Fast DNA translocation and concomitant electronic noise make this technique limited for more practical applications, such as signal resolving and detecting, sizing, or quantifying short DNA oligonucleotides.

In recent years, nucleic acid hybridization technique has proven to be a potential platform to create biosensing devices for rapid and low-cost detection of DNA sequences.[32−35] In this approach, a single-stranded DNA probe was immobilized onto

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the sensor surface and subsequently, recognition of a target DNA strand with sequence complementary to the tethered nucleotide was achieved *via* hybridization. Upon DNA/DNA duplex formation, the sensor directly tranduces the hybridization events into a measurable electronic read-out signal. The hybridizing capabilities of these devices strongly depend on several external parameters such as temperature, ionic strength, and probe length. In this framework, the design and construction of robust and inexpensive nanofluidic devices for highly sequence selective detection of short single-stranded DNA oligomers is still a remaining challenge in materials science and biophysics.

Currently, single conical nanochannels have been proven a novel biosensing platform on the basis of ion transport, governed by their surface charges, that is, ion current rectification and permselectivity.[36−38] In these channels, both rectification and permselectivity depend sensibly on surface charge density and effective diameter, especially at the tip opening of the channel.[39−41] Any minor change in the surface charge or effective cross-section can lead to a significant change in electronic read-out originated from the ionic transport through the channel. To achieve this goal, different methods have been developed to modify the surface charge properties of nanochannels. One of the most widespread and sophisticated approach was developed by Martin and co-workers for generating gold nanotubes with modifiable surface properties within the channels fabricated in different templates.[42,43] This strategy is based on covering the channel walls with a gold film by electroless plating, followed by the chemisorption of end-functionalized thiol molecules for various potential applications such as permselective ionic transport, biosensing, and bioseparation. However, this gold plating procedure is multistep, complex, and time-consuming. We and others have developed a simple and straightforward alternative strategy to achieve nanoscale control over surface properties by directly exploiting the carboxyl (−COOH) groups generated during the track-etching process.[20,44−47] Furthermore, these inherent groups can be used as a starting point to introduce various recognition elements on the inner wall of the channel for the integration of highly sensitive, selective, miniaturized, and cost-effective sensing devices.

In the present work, we describe the design and construction of a peptide nucleic acid (PNA)-modified nanofluidic device which shows the potential for the sequence-selective detection of single-stranded DNA nucleotides and also has the potential to discriminate DNA strands with single base differences in the sequence. To date, various strategies have already been developed for sequence-specific DNA detection, using a variety of PNA-functionalized solid-state materials, such as nanowires,[48,49] nanotubes,[50−52] nanoparticles,[53−55] electrodes,[56−58] and ion-sensitive field-effect transistors.[59] To the best of our knowledge, DNA sensing on the basis of rectified ion flux with single conical track-etched nanochannels in polymer membranes is still unexplored. Since the PNA probes incorporated on the inner wall of the channel, act as binding sites for the cDNA sequence, the peptide nucleic acid is a structural DNA analogue with a backbone of repeating neutral *N*-(2-aminoethyl) glycine units linked by peptide bonds, and bearing nucleotide bases. The PNA has an efficient and sequence-specific binding affinity to its complementary single-stranded DNA oligomer.[60−62] To accomplish this, an amine-terminated PNA probe is covalently immobilized on the inner wall of a single conical nanochannel *via* carbodiimide coupling chemistry. Upon exposing the PNA-modified channel to a solution of cDNA strands, hybridization occurred inside the confined geometry. The hybridization process boosted the charge density on the channel surface due to the negatively charged phosphate backbone of DNA oligomer. The success of immobilization of the PNA probe and sequence-specific PNA/DNA duplex formation on the inner walls is monitored *via* the change in the rectified ionic flux and permselectivity displayed by the single conical nanochannels.

RESULTS AND DISCUSSION

Single conical nanochannels were fabricated in heavy ion irradiated polyimide (PI) membranes of 12 μm thickness by selective chemical etching of the damage trails caused by the swift heavy ions along their trajectories.[63,64] The chemical etching of ion tracks in polymer membranes leads to the generation of −COOH groups on the channel surface. The covalent coupling of an amine-terminated PNA probe with the −COOH groups (Figure 1) was achieved by the use of water-soluble reagents *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS).[65] The EDC reagent first activated the −COOH groups into the highly reactive *o*-acylisourea intermediate. This intermediate is further converted into a more stable sulfo-succinimidyl amine-reactive ester in the presence of excess sulfo-NHS molecules. Subsequently, the succinimidyl intermediate was covalently coupled with the terminal amine on PNA (5′-H_2N-O-Lys-TAGTCGGAAGCA) probe. At the N-terminus of the PNA probe, an amino acid (lysine) was attached through peptide linkage to the backbone followed by ethylene glycol unit (O linker), bearing primary amine used for covalent coupling with surface −COOH groups. The solubility of PNA in water is enhanced by the conjugation of lysine residue in order to stay with aqueous reaction conditions.

Figure 2 shows current−voltage (*I*−*V*) curves of a single conical nanochannel used for the hybridization of DNA with immobilized PNA probes on the channel surface. The transmembrane ionic current was recorded under symmetrical electrolyte conditions using 0.1 M KCl (pH 7.6) solution in both halves of the conductivity

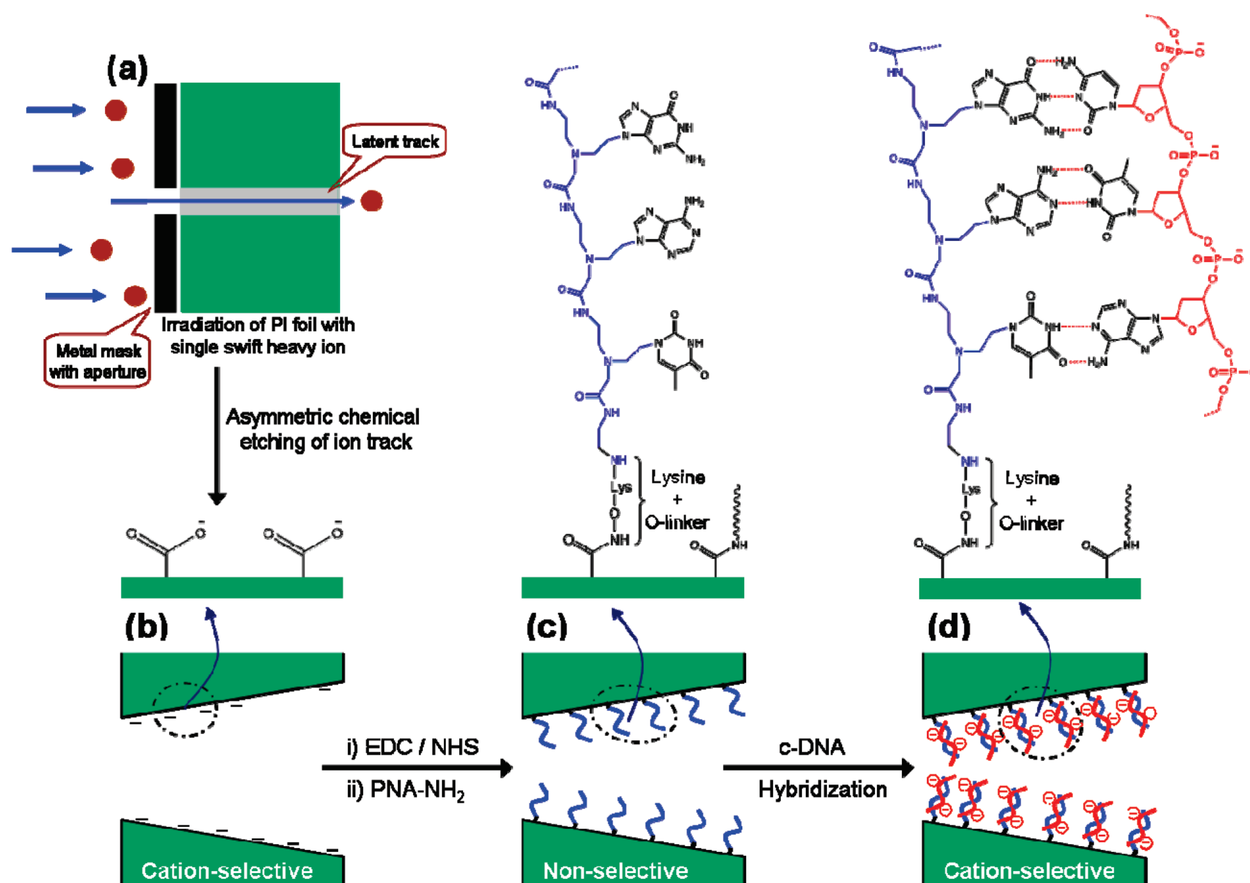

Figure 1. Schematic representation of (a) formation of latent track in polymer membrane after irradiation with single swift heavy ion, (b) fabrication of a single conical nanochannel by asymmetric track-etching technique, (c) covalent attachment of amine-terminated PNA-probe with carboxyl groups *via* carbodiimide coupling chemistry, and (d) subsequent hybridization of cDNA oligonucleotide with the tethered PNA probe.

cell. In conical channels, the surface charge governs the direction of ion current rectification. At neutral and basic pH, conical channels with inherent $-COO^-$ groups are cation selective and rectify the ionic current in such a way that the preferential direction of cation flow is from the narrow toward the wide channel opening. Decorating the inner wall of the channel with an uncharged PNA probe led to a drastic change in the rectified ionic current. Before modification, permselective transport of ions across the membrane measured at a potential difference of +1 V resulted in a rectified ion current of 53.6 ± 2.7 nA. Upon immobilizing PNA, a significant decrease in the rectified ionic current (16.7 ± 0.3 nA) was observed under the same applied bias. This means that uncharged PNA molecules diminish the negative channel surface charge, leading to a ~69% decrease in the rectified ionic current, displayed by the corresponding $I-V$ curve as shown in Figure 2. The changes in the $I-V$ characteristics prior to and after modification confirmed the success of the PNA immobilization on the inner channel wall.

The next step is to show the functioning of a PNA-modified channel as a biosensor for the detection of single-stranded DNA oligomer 5′-TGCTTCCGACTA-3′ (c-DNA) with sequence complementary to the immobilized PNA probe. It is known that the PNA oligomer is an analogue of DNA, used as a DNA hybridization partner due to its chemical robustness. The spacer arm (lysine residue and O linker) introduced at the N-terminus, used to distance the PNA hybridization por-

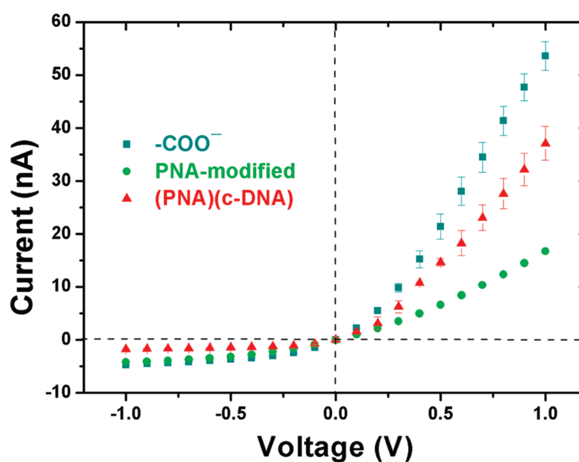

Figure 2. $I-V$ characteristics of a single conical nanochannel (A) with tip d = 20 nm and base D = 1.68 μm, bearing carboxylate groups (■), immobilized PNA probe (●), and PNA/DNA duplexes (▲) on the channel surface, respectively. $I-V$ curves are measured in 0.1 M KCl (pH 7.6) solution.

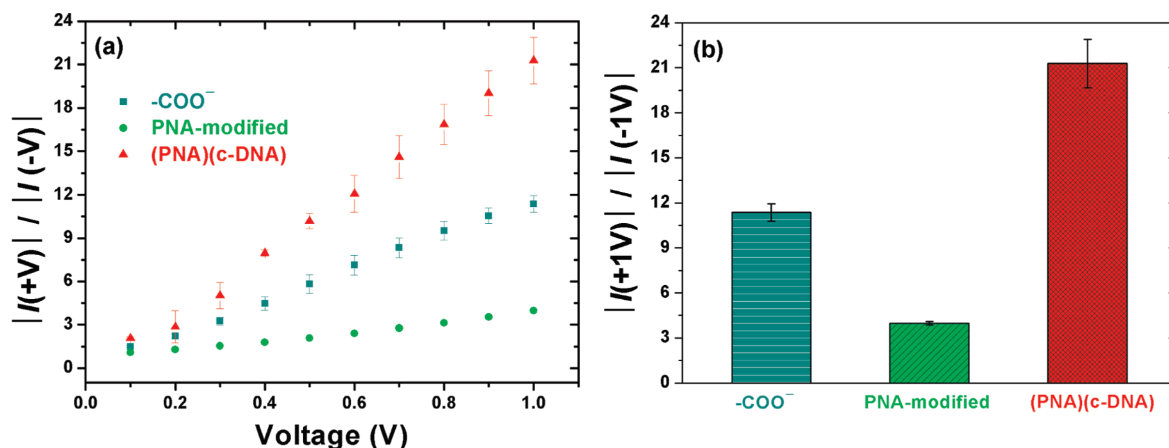

Figure 3. (a) Rectification ratio calculated from the respective $I-V$ curves shown in Figure 2; (b) rectification ratio measured at a potential difference of ±1 V.

tion from the surface, is sufficiently long for the successful binding of cDNA oligonucleotides.[56,59]

To perform the hybridization process, PNA-modified single-channel membrane was mounted between the two halves of the conductivity cell. The PNA-modified channel was exposed to an aqueous solution of c-DNA for a preset period of time. Subsequently, the physically adsorbed DNA molecules were removed by thoroughly washing the channel with PBS solution. The hybridization of c-DNA strand on the sensor surface can be recognized from the changes in the rectified ion flux through the channel. It is worth mentioning that upon PNA/DNA hybridization, negative charges were generated on the inner channel wall due to the negatively charged phosphate backbone of the DNA nucleotide. Therefore, the presence of these negative charges triggered the ion current rectification, and the value of ionic current measured at +1 V was increased from 16.7 to 37.0 ± 3.2 nA as shown in Figure 2. From the $I-V$ curve, indicating pronounced rectification, we conclude that the DNA recognition *via* PNA/DNA hybridization rendered the surface negatively charged, thus restoring the rectification characteristics displayed by the channel with the preferential transport of cationic species.

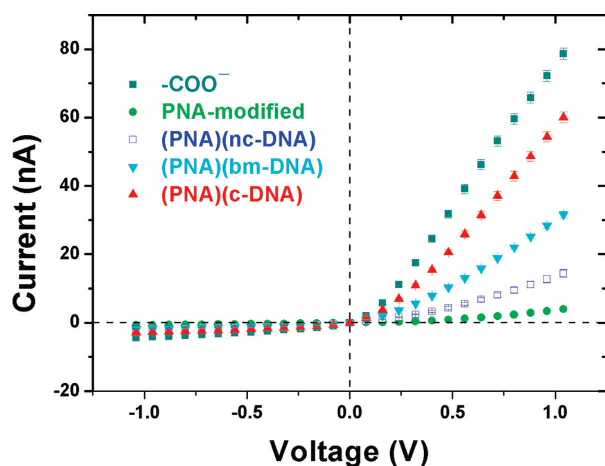

Figure 4. $I-V$ characteristics of a single conical nanochannel (B) with tip d = 26 nm and base D = 1.26 μm, bearing $-COO^-$ groups (■), immobilized PNA probe (●), and subsequent hybridization of nc-DNA (□), bm-DNA (▼) and c-DNA (▲) on the channel surface, respectively. $I-V$ curves are measured in 0.1 M KCl (pH 7.6) solution.

The extent of rectification (permselectivity) of conical nanochannels is described by the rectification ratio, r, and is defined as the absolute value of the ionic current measured at positive potential divided by the current at negative potential. The value of r is sensibly correlated with the surface charge, and consequently a slight increase/decrease in surface charge can trigger a marked increase/decrease in rectification ratio of the nanochannel.[39−41,66]

Figure 3 displays the rectification ratio of the channel, studied for the sequence-specific detection of c-DNA oligomer. From Figure 3a, it was obvious that dressing the inner channel wall with neutral PNA probes led to a marked diminution of surface charge which in turn resulted in a significant decrease in the rectification ratio. Upon PNA/DNA hybridization, the negatively charged phosphate backbone of DNA molecules boosted the surface charge density on the inner channel wall, and as a result a marked increase in r value was observed. It is evident from Figure 3b that modification of carboxyl groups with PNA can lead to a ~4-fold decrease in rectification ratio of the channel from 11.4 ± 0.6 to 3.9 ± 0.1, measured at a potential difference of ±1 V. After hybridizing DNA onto the PNA-modified channel, an increase in the rectification ratio from 3.9 to 21.3 ± 1.6 (~8-fold) is observed. Compared with the carboxylated channel, the rectification ratio is almost doubled in the case of a channel bearing PNA/DNA duplexes (Figure 3). This clearly shows that the magnitude of surface charges is increased upon hybridization because of the negative character of DNA molecules. Martin and co-workers have also demonstrated that the DNA-functionalized single conical gold nanotubes embedded in the polymer membranes rectify the ion current, and that the rectification ratio increases by

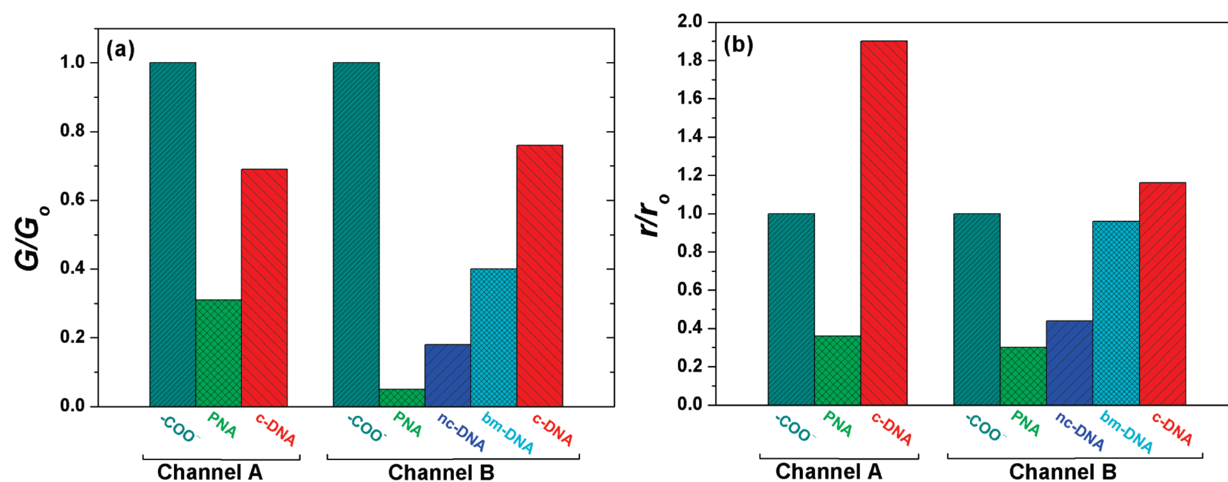

Figure 5. Comparison of (a) normalized conductance (G/G_o) and (b) rectification ratio (r/r_o) of single conical channel A (Figure 2) and channel B (Figure 4), prior to and after hybridization with respect to a carboxylated surface at a potential of +1 V, respectively.

extending the DNA chain length to be attached on the surface of nanotubes.[42] Recently, Fu *et al*. demonstrated a nanopore DNA sensor based on modified nanopipettes. They have showed that an electrostatically attached single-stranded DNA oligomer selectively hybridizes with a cDNA oligomer, resulting in an increase in the rectification ratio displayed by the nanopore.[67] This clearly supports our findings that introducing negatively charged DNA strands, *via* hybridization onto the PNA surface, led to a significant increase in the rectification ratio.

However, another important aspect of biosensing platforms relies on the reproducibility as well as selectivity of the detection and transduction of specific events upon the binding of DNA molecules. In other words, in order to show that this approach is valid to create a DNA-sensing platform inside the nanoconfined geometry, it is mandatory to demonstrate that the changes in the rectified current are solely due to the formation of PNA/DNA duplex, and not to the physical adsorption of the DNA oligomer on the PNA surface. To verify the selectivity/specificity of the PNA-modified channel, we repeated the same experiments using completely noncomplementary 5′-ACTAATTCTTAT-3′ (nc-DNA), single-base mismatch 5′-TGC$\underline{A}$TCCGACTA-3′ (bm-DNA), and fully complementary 5′-TGCTTCCGACTA-3′ (c-DNA) oligomers for hybridization, respectively. Figure 4 shows the variations in the $I-V$ plots after exposing the PNA-modified nanochannel to nc-DNA, bm-DNA, and c-DNA strands, respectively. We did not observe any significant change in the $I-V$ characteristics upon exposing the PNA-modified channel to nc-DNA oligomer. This confirmed the lack of hybridization of nc-DNA, leaving the original PNA surface undisturbed. When the same channel was exposed to a single-base mismatch DNA strand (bm-DNA), hybridization takes place to some extent as exhibited by an increase in the rectified ion flux from 14.4 ± 1.2 to 31.6 ± 1 nA, measured at +1 V. Subsequently, treating the same sensor with a c-DNA strand resulted in a further increase in the rectified ion flux to 60 ± 1.6 nA. From the variations in the $I-V$ plots, we can conclude that the sensor exhibits a remarkable selectivity and specificity toward the fully complementary sequence compared to noncomplementary and single-base mismatch ones.

Figure 5 gives the comparison of normalized conductance (G/G_0) and rectification ratio (r/r_0), where G and r are the conductance and rectification ratio of the channel bearing $-COO^-$ groups while G_0 and r_0 are the channel conductance and rectification ratio after treating with PNA and selected DNA strands, respectively. It is worth mentioning that upon PNA/DNA hybridization, the overall conductance was decreased, while the rectification ratio of the channel was increased compared with that of those displayed by the channel bearing $-COO^-$ groups. In fact, these properties are solely dependent on the surface charge density as well as the effective cross-section, especially at the narrow opening of the conical nanochannels. As is demonstrated in the above experimental results, neutral PNA molecules attached to the channel surface drastically decrease the conductance due to the loss of surface charges. Subsequently, the binding of cDNA leads to a significant increase in the channel conductance. This conductance value is still lower than the carboxylated channel because PNA/DNA duplex formation also resulted in a decrease of the effective diameter at the tip opening which governs the ionic transport across the membrane.

Here, we would like to mention the recent work by Gyurcsányi and co-workers in which PNA-functionalized gold nanotubes embedded in polymer membranes were applied for the label-free detection of cDNA sequences.[50] These authors have demonstrated that the flux of anionic dye through the nanotubes decreased upon PNA/DNA hybridization due to charge repulsion. Smirnov and co-workers have also demonstrated a de-

crease in ionic flux/conductance through the nanoporous membranes, upon binding cDNA with single-stranded DNA covalently attached to the channel walls.[68,69] Howorka *et al*. have reported the duplex formation inside the lumen of single protein nanopore α-hemolysin.[70,71] They showed the covalent binding of a single DNA oligonucleotide to a cysteine residue located at the cis opening of the pore. The hybridization of cDNA strand causes changes in the ionic current flowing through the nanopore. On the contrary, our technique is straightforward and requires a very simple instrument (picoammeter) for the measurement of the electronic readout in the form of transmembrane ionic current. Furthermore, the presented sensor allows the recognition and discrimination of single-base mismatch *via* hybridization on the PNA-modified surface at the nanoscale level, based on the charge-dependent rectified ion flux through the single conical nanochannels.

CONCLUSIONS

In conclusion, here we have described the construction of a DNA sensing device based on a single conical nanochannel fabricated in a polyimide membrane. We presented a very simple and straightforward method to incorporate a PNA-probe into the channel by directly exploiting the carboxyl groups generated during the track-etching process. The PNA-modified channel provides a novel sensing platform which can discriminate between complementary and single-base mismatch DNA strands on the basis of rectified ion flux *via* hybridization in nanoconfined spaces. Surface-charge dependent $I-V$ characteristics exhibit the success of PNA immobilization as well as the hybridization process within the constrained geometry of the nanofluidic device. Furthermore, it is observed from the control experiment that the sensor displays a good selectivity and specificity to a DNA strand with a sequence complementary to the attached PNA-probe. In this context, we believe that on the basis of these findings one would be able to construct a setup for the detection and identification of an unknown DNA oligomer by using specific PNA-sequences.

EXPERIMENTAL SECTION

Membranes of polyimide (PI) (Kapton 50 HN, DuPont) having 12 μm thickness were irradiated at the linear accelerator UNILAC (GSI, Darmstadt) with single swift heavy ions (Pb, U, or Au) of energy 11.4 MeV/nucleon. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 98%, Fluka), *N*-hydroxysulfosuccinimide (sulfo-NHS, 98.5+ %, Aldrich), and phosphate buffered saline (PBS, pH 7.6, Sigma) were purchased from Sigma-Aldrich. The PNA probe and single-stranded DNA oligonucleotides were purchased from Eurogentec (Köln, Germany). The PNA used for functionalization was amino-terminated at its N-terminal. The 12-mer PNA probe employed was 5′-H_2N-O-Lys-TAGTCGGAAGCA (O and Lys denotes an ethylene glycol linker and lysine molecule covalently attached at 5′ position, respectively). The 12-mer DNAs were fully complementary 5′-TGCTTCCGACTA-3′ (c-DNA) and noncomplementary 5′-ACTAATTCTTAT-3′ (nc-DNA) to the PNA probe, respectively. The single-base mismatch was a DNA strand with sequence 5′-TGC*A*TCCGACTA-3′ (bm-DNA), where the italic underline character (*A*) represents the location of the mismatch base.

The fabrication of single conical nanochannels in PI membrane was accomplished by asymmetric chemical etching of single-ion tracked membranes.[63,64] Briefly, the polymer membrane irradiated with a single energetic heavy ion was placed in a conductivity cell in which it served as a dividing wall between the two compartments. Sodium hypochlorite (13% active chlorine content) was used as etching solution, while a stopping solution (1 M KI) was filled in on the other side of the membrane. The etching process was carried out at 50 °C. During etching, a potential of −1 V was applied across the membrane in order to observe the current flowing through the nascent nanopore. The current remains zero as long as the channel is not yet etched through, and after the breakthrough an increase of current is observed. The etching process was stopped when the current reached a certain value, and the channel was washed first with 1 M KI in order to neutralize the etchant, followed by rinsing with deionized water. After etching, the diameter of the large opening (D) of the channel was determined by field emission scanning electron microscopy (FESEM). For this purpose a PI sample containing 10^7 channels cm^{-2} was etched simultaneously with the single channel under the same conditions. The diameter of the small opening (d) was estimated by assuming the conical geometry of the channel from its conductivity using the following relation[63]

$$d = 4LI/\pi D\kappa U$$

where L is the length of the pore which could be approximated to the thickness of the membrane, d and D are the small and large opening diameter of the channel, respectively, κ is the specific conductivity of the electrolyte, U is the voltage applied across the membrane, and I is the measured current. The channel conductance is measured by using 1 M KCl as an electrolyte solution at pH 3.0. At this acidic pH value, the linear $I-V$ behaviour indicated that the channel is uncharged and the above relation is valid using κ as the bulk conductivity of the electrolyte solution.

The solutions used for chemical modification of the channel surface were prepared in 0.1 M MES buffer [2-(*N*-morpholino) ethanesulfonic acid], pH = 5.5. Functionalization of the nanochannel wall with PNA was carried out in the same conductivity cell used for the chemical etching of tracked polymer membranes. The carboxylic (−COOH) groups, generated on the channel surface during the track-etching process, were modified with amine-terminated PNA probe by the following procedure. The carboxyl groups were first activated into amine-reactive ester molecules by using a solution of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 10 mM) and *N*-hydroxysulfosuccinimide (sulfo-NHS, 20 mM) for 30 min. After activation, the foil was washed with the same buffer solution. Subsequently, the sulfo-NHS-ester molecules were covalently coupled with the amine group present at the N-terminus of the PNA probe (5 μM) for overnight. Finally, the PNA-modified channel was washed thoroughly with buffer solution.

The membrane containing the single conical channel was mounted between the two halves of the conductivity cell, and both halves were filled with phosphate buffer saline (pH = 7.6) prepared in 0.1 M KCl solution. Ag/AgCl electrode was placed into each half-cell solution, and the picoammeter/voltage source (Keithley Instruments type 6487, Cleveland, OH) was used to provide the desired transmembrane potential in order to measure the resulting ion current flowing through the channel. A scanning triangle voltage from −2 to +2 V was applied on the tip side, while the base side of the channel remained connected to the ground electrode.

The aqueous solutions of complementary c-DNA (1 μM), noncomplementary nc-DNA (2 μM), and single-base mismatch bm-DNA (2 μM) sequences were prepared in phosphate buffered saline (PBS) (pH = 7.6). A polymer membrane containing a

single PNA-modified channel was mounted in the conductivity cell. To achieve PNA/DNA hybridization, the PNA-modified channel was exposed to c-DNA, nc-DNA, and/or bm-DNA solutions for 5 h at 30 °C. After hybridization, the channel was washed thoroughly with PBS buffer solution. After washing, the $I-V$ curves were measured using 0.1 M KCl prepared in PBS (pH = 7.6) as an electrolyte for the examination of successful hybridization.

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