



“DNA-Dressed Nanopore” for complementary sequence detection

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ABSTRACT

Single molecule electrical sensing with nanopores is a rapidly developing field with potential revolutionary effects on bioanalytics and diagnostics. The recent success of this technology is in the simplicity of its working principle, which exploits the conductance modulations induced by the electrophoretic translocation of molecules through a nanometric channel. Initially proposed as fast and powerful tools for molecular stochastic sensing, nanopores find now application in a range of different domains, thanks to the possibility of finely tuning their surface properties, thus introducing artificial binding and recognition sites. Here we show the results of DNA translocation and hybridization experiments at the single molecule level by a novel class of selective biosensor devices that we call “DNA-Dressed Nanopore” (DNA^2), based on solid state nanopore with large initial dimensions, resized and activated by functionalization with DNA molecules. The presented data demonstrate the ability of the DNA^2 to selectively detect complementary target sequences, that is to distinguish between molecules depending on their affinity to the functionalization. The DNA^2 can thus constitute the basis for the design of integrable parallel devices for mutation DNA analysis, diagnostics and bioanalytic investigations.

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1. Introduction

Nanopore sensors were proposed as versatile and powerful single molecule analytical devices about a decade ago (Hinterdorfer and Oijen, 2009), stimulating, in the last years, an intense and interdisciplinary debate about alternative fabrication techniques and possible advanced applications. The device working principle is extremely simple, being based on ionic current measurements during electrophoretic translocation of molecules through a nanometric channel, without the need for target labelling and optical detection. By applying a voltage over the nanopore, negatively charged biopolymers are drawn through it, and their passage produces a detectable conductance modulation correlated with their dimension and electrostatic interaction with the charge present on the pore walls.

Actually, after the first papers on protein pores (Kasianowicz et al., 1996; Vercoutere et al., 2003) demonstrating their possible use as a low cost, fast processing and high throughput alternative to current DNA analysis and sequencing techniques (Deamer and Akeson, 2000; Stoddart et al., 2009), much work has been done to realize engineered devices, with specific characteristics and sensing abilities (Hall et al., 2010; Howorka et al., 2001; Iqbal et al., 2007;

Hou et al., 2011). The spectrum of proposed nanopore applications is now quite large, the focus being gradually moved from the simple molecular stochastic sensing, to the introduction of novel biological and chemical functionalities (Gyurcsanyi, 2008; Kim et al., 2007; Wanunu and Meller, 2007; Yusko et al., 2011), able to confer selectivity to the sensing process (Bayley and Cremer, 2001; Howorka and Siwy, 2009).

In this context, we recently demonstrated that silicon nitride, SiN, nanopores can be efficiently and stably functionalized with both single strand and double strand DNA probe molecules. Nanopores with a 20–80 nm diameter produced on a free-standing SiN membrane by focused ion beam (FIB) milling, have been, in fact, chemically functionalized and electrically characterized (Mussi et al., 2010a). As an example, SEM images of the same pore before and after the functionalization with 45-mer oligonucleotides are reported in Fig. 1a (up and down, respectively). The functionalization layer, which appears as a grey shadow in the pore region, produces a diameter reduction from 65 nm to about 40 nm, related to the 15.3 nm contour length of the probe molecules. In this case, the initial diameter of the pore, which is emphasized by a dotted circle in both images to better appreciate the induced resizing, was chosen sufficiently large to distinguish the presence of the probe molecules along the pore edge in the SEM image acquired after the chemical modification (Fig. 1a, down). However, it is possible to select a nanopore with a smaller initial diameter, so that the functionalization procedure allows to simultaneously obtain the

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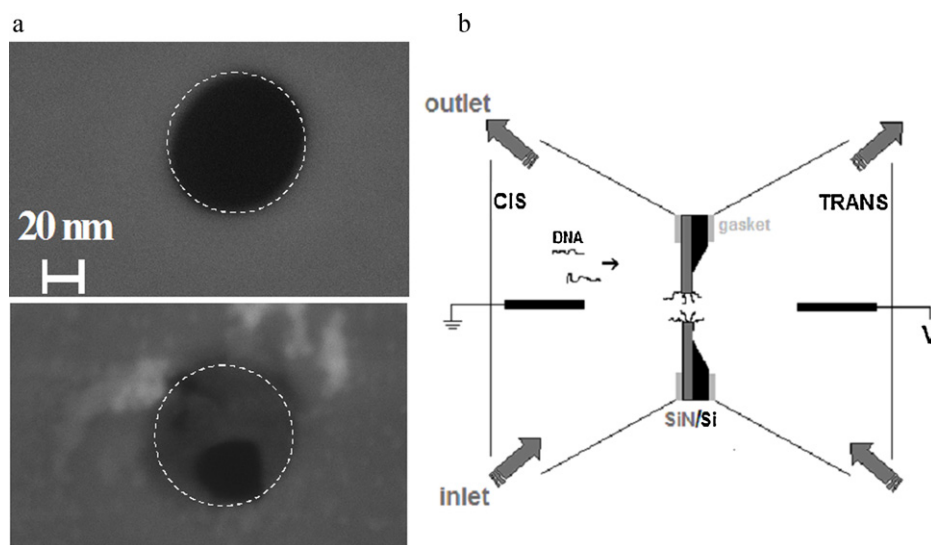


Fig. 1. Description of the device. (a) SEM images of the nanopore before (top) and after (bottom) the functionalization with 45-mer oligonucleotides, 15.3 nm long. The functionalization layer, which appears as a grey shadow in the pore region, produces a diameter reduction from 65 nm to about 40 nm. The initial diameter of the nanopore is emphasized by a dotted circle in both images, to better appreciate the resizing induced by the chemical modification. The original SEM images are reported in [Supplementary Information](#). (b) Sketch of the experimental set-up. A voltage is applied across the bio-functionalized nanopore realized on a SiN membrane deposited on a Si substrate (SiN/Si structure). The pore is inserted between two facing chambers filled with ionic solution by means of a microfluidic system (inlet, outlet). Rubber gaskets are used for the mechanical seal. The molecular target (DNA) is added on the cis side, and electrophoretically driven through the nanopore, towards the trans side.

size reduction needed for single molecule sensitivity (Mussi et al., 2010a,b), and the selective activation of the device. Here we show how this kind of “DNA-Dressed Nanopores” (DNA^2) is successfully used in single molecule translocation and hybridization experiments, thus proving its versatility and effectiveness for biosensing.

The translocation experiment is realized using long λ -DNA target molecules, that do not interact selectively with the probes (dsLNA) covalently attached to the nanopore surface. Typical current drops associated with the transient channel obstruction due to the passage of the molecules are revealed, demonstrating the single molecule sensitivity of the device.

The hybridization experiment is performed with target molecules both non-complementary and perfectly complementary to probe oligonucleotides. The experimental data prove the ability of the chemically modified nanopore to selectively detect sequences which are complementary to those of the used probe molecules, that is to distinguish between molecules based on their affinity to the functionalization.

Next sections separately describe translocation and hybridization experiments, highlighting the striking different appearance of current recordings during the selective interaction of probe and target molecules with respect to those corresponding to simple translocation events. In particular, the ionic current registered during hybridization experiments shows a typical “toggling” fluctuation, that we ascribe to the dynamic specific interaction of target and probe molecules, causing discrete jumps between different nanopore conductance levels.

Even if the full understanding of the hybridization mechanism requires further theoretical modelling, our findings prove that DNA^2 is an effective biosensor in both translocation and hybridization experiments.

2. Materials and methods

2.1. Pore fabrication and imaging

Pores are produced on a free-standing SiN membrane using a CrossBeam workstation 1540XB model by Zeiss combining an ultra high resolution scanning electron microscope (UHRSEM) and

a focused ion beam (FIB). The membrane is obtained by depositing a thin (20 nm nominal thickness) SiN film on a 300 μm thick Si substrate, and anisotropically etching the Si side of the chip till exposing an area 80 $\mu\text{m} \times 80 \mu\text{m}$ large. The pore drilling is made by exposing the membrane to a resting and finely focused ion beam (spot size ~ 8 nm) of 2 pA for 1 s. The SEM images are obtained using an in lens detector and an accelerating voltage of 20 kV. In order to obtain a good signal to noise ratio, the image is taken at scan rate with a dwell time of 6.4 μs , averaging over 10 line scans.

2.2. DNA^2 preparation

The functionalization procedure of the pore consists of three steps. The entire membrane is activated for 1 h with 3-aminopropyltriethoxysilane (APTES) (20% V/V in ddH₂O). The APTES links to the –OH groups of the native silicon oxide layer present on the silicon nitride surface. The chip is then treated with 1,4-phenylenediisocyanate (0.5% P/V in dimethyl-sulfoxide) cross-linker for 5 h followed by two washes in dimethyl-sulfoxide and two washes in double distilled water. Finally, an over-night treatment at 37 °C with 100 nM amino-modified DNA in ddH₂O pH 8 is performed, followed by two washes (30 min each) with 28% ammonia solution and by two washes with ddH₂O (15 min each) to de-activate the substrate. To improve the resizing control, the first step is slightly different in the case of the chip used for the hybridization experiments: the pore chip is treated with Oxygen Plasma (60 s, 30 W) and exposed for 5 min to 20 μl APTES in vapour phase inside a low vacuum chamber ($T = 25$ °C, $P = 30$ kPa).

The nanopore used for the translocation experiment is functionalized with dsLNA, a decoy oligodeoxynucleotide (ODN) containing locked nucleic acids with the consensus sequence for NF- κ B, a transcription factor involved in numerous cellular function with sequence 5′-TA aga ggg aaa ttc cgg gaa att cct ac AT-3′ (Sigma-Proligo). For the hybridization experiment, the probe is an amino-modified 45-mer oligonucleotide (Eurofins MWG Operon, Germany) corresponding to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 5′-GCC AAA AGG GTC ATC ATC TCT GCC CCC TCT GCT GAT GCC CCC ATG-3′ sequence.

2.3. Electrical measurements

The DNA² is mounted between two facing Plexiglas chambers filled by means of a microfluidic system with 300 μ l of buffer solution. Rubber gaskets are used for the mechanical seal. Two Ag/AgCl pellet electrodes (Warner Instruments, E206) properly inserted and screwed in the dedicated cylindrical lodgings of the microfluidic cell, apply a voltage bias and collect the ion current by means of a patch-clamp amplifier (Axopatch 200B, Axon Instruments). Signals are acquired at a 250 kHz sampling rate. The amplifier internal low-pass fourpole Bessel filter is set at 5 kHz. The entire apparatus is placed in a double Faraday cage enclosure on an anti-vibration table.

2.4. Translocation experiment

A filtered KCl ionic solution (conductivity ~ 10 S/m) buffered at pH 8 with 10 mM HEPES was used for the translocation experiment (0.01 M on the cis-side, 1 M on the trans side). The target is 48.5 kbp λ -DNA (0.6 μ g/ μ l in KCl), Wako Chemicals GmbH (Neuss, Germany).

2.5. Hybridization experiment

The hybridization experiment was realized using a buffer solution (HB) which contains: 50% formamide, 5 $\times$ SSC (0.75 M sodium chloride, 75 mM sodium citrate) 0.5% SDS, 5 mM potassium phosphate, 2 μ l antifoam A (Sigma–Aldrich, St. Louis, MO, USA) pH 7.2. It has a measured conductivity of 6 S/m. The non-complementary target, NC (0.17 nM), is an oligonucleotide with the same sequence of the probe. The perfectly complementary target, PC, is 5'-CAT GGG GGC ATC AGC AGA GGG GGC AGA GAT GAT GAC CCT TTT GGC-3' (0.17 nM). The experiment is performed at T_{amb} .

3. Results and discussion

3.1. Translocation experiment

To demonstrate the single molecule sensitivity of the device, a translocation experiment is realized using long λ -DNA target molecules that do not selectively interact with the probe oligonucleotides attached to the nanopore surface. The experiment is performed with a pore having an *effective* diameter reduced to about 7 nm by the functionalization. The *effective* diameter of the nanopore is defined here as $d_{eff} = 2\sqrt{l/c \cdot \pi \cdot R}$, where c is the conductivity of the used ionic solution, l is the SiN membrane nominal thickness, and R the measured electrical resistance (Mussi et al., 2010a,b). This definition corresponds to making a constant diameter approximation for the nanometric channel, both before and after the chemical modification. This kind of “electrical determination” is not geometrically accurate, because it does not take into account the asymmetry of the FIB drilled pore, the presence, on the channel walls, of a not negligible surface charge (including that carried by the probe molecules) and the fact that the functionalization layer does not form a uniform hard shell around the periphery of the pore. Nevertheless, the estimation of the d_{eff} value is a useful way to compare results obtained with different DNA², and to rapidly analyse the effects of the bio-functionalization on the nanopore electrical properties. Fig. 1b presents a sketch of the set-up used in the experiment. A voltage is applied across the bio-functionalized nanopore, which is inserted between two facing chambers filled with salt solution, and an ionic current is established. Target molecules are then inserted on the cis-side and electrophoretically driven through the nanometric channel. A typical current trace registered by applying a voltage bias of 400 mV

is reported in Fig. 2a. Each of the appearing spikes corresponds to the pore conductance modulation induced by the passage of a single DNA molecule. The current variations have characteristic amplitude, ΔI , and duration, Δt , as shown in the magnification of Fig. 2c, presenting a single blockade event with $\Delta I = 272$ pA and $\Delta t = 334$ μ s. Two current levels are identified by looking at the all-point current histogram reported in Fig. 2b, obtained as normalized current counts. The higher level (0) corresponds to the open pore current, and the lower level (1) is associated with blockade events having the dwell time distribution shown in Fig. 2d, obtained by considering many similar current traces. The histogram of Fig. 2d shows the presence of two classes of events, fast collisions with the pore entrance, associated with unsuccessful threading attempts of the DNA molecules, giving rise to lower and shorter current reductions, and full translocations, producing longer and deeper reductions (Wanunu et al., 2008). By fitting the dwell time histogram with Gaussian curves (solid lines), we obtained the peak values $\Delta t_1 = 57$ μ s and $\Delta t_2 = 283$ μ s. Δt_1 estimation is affected by the used Bessel filter (5 kHz), which alters the shape of the short current modulations due to molecule bumping with the pore. Differently, Δt_2 and the corresponding amplitude ΔI_2 of the longer blockade events characterize the translocation process, and strongly depend on the specific experimental conditions. The value of ΔI_2 can be similarly obtained by the Gaussian fit of the amplitude distribution, which gives $\Delta I_2 = 275$ pA. By considering the asymmetric salt concentrations condition (0.01 M on the cis-side, 1 M on the trans side) used here to enhance the nanopore capture efficiency (Wanunu et al., 2010), the approximation made in the determination of the nanopore dimension, and the presence of the functionalization layer altering its conductive properties, the estimated dwell time and amplitude, $\Delta t_2 = 283$ μ s and $\Delta I_2 = 275$ pA at 400 mV, are actually in agreement with those reported in the literature for similar experimental conditions (Storm et al., 2005a,b). We also found that a change in the voltage bias consistently affects the blockades duration and amplitude that are, in fact, mainly determined by the linear dimension, charge and conformation of the target molecules, the channel geometry, the solution conductivity and the applied voltage (Fologea et al., 2007; Li et al., 2003; Wanunu et al., 2008; Ramachandran et al., 2011), as well as by the approach used to statistically analyse the current traces (see supplemental material for details on the home-made software utilized to obtain the histogram of Fig. 2d). A detailed analysis of the translocation process through the DNA² is indeed out of the scope of this paper. Anyhow, the presented data clearly demonstrate that DNA² can be used as single molecule bioanalytical device, finding applications in many different fields, from the study of protein unfolding, to, in principle, DNA sequencing.

3.2. Hybridization experiment

The stable functionalization with oligonucleotides also imparts selectivity to the detection mechanism, and allows to identify complementary target sequences.

To establish this capability, we performed hybridization experiments on a chemically modified chip using target molecules non-complementary (NC), and perfectly complementary (PC) to the probe ones. The experiment uses a large nanopore with an initial diameter of 60 nm, functionalized with amino-modified 45-mer oligonucleotides, and the apparatus sketched in Fig. 1b, where the KCl solution is substituted by a buffer solution (HB) to maximize hybridization probability and specificity. Fig. 3a, top trace, shows the open pore current recorded by applying a voltage bias of 200 mV (I open). The voltage/current curve collected before the target insertion is reported in Fig. 3b (black squares) and allows to evaluate the chip initial electrical resistance, $R \sim 3.8$ M Ω . This value corresponds to $d_{eff} = 33$ nm (Mussi et al., 2010a), as deduced

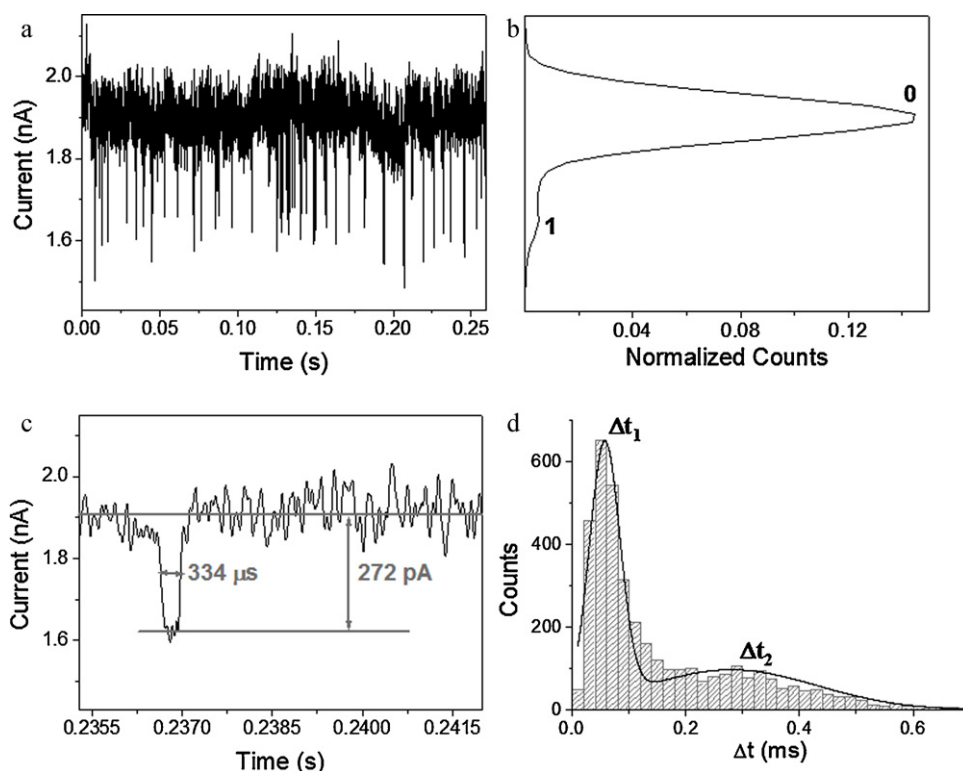


Fig. 2. Translocation experiment (a) Typical current trace registered during the translocation of 48.5 kbp λ -DNA molecules ($0.1 \mu\text{g}/\mu\text{l}$ in KCl solution) through a DNA^2 at 400 mV voltage bias. (b) All-point current histogram of the trace of (a) obtained as normalized current counts showing the presence of two current levels, 0 (open pore) and 1 (blockade). (c) Expanded view of the trace of (a) containing a spike associated with the translocation of a single molecule which modulates the pore conductance. The blockade has a duration of about $334 \mu\text{s}$ and an amplitude of 272 pA . (d) Dwell time distribution of the blockade events obtained by considering many current traces (~ 100) similar to that of (a). The histogram is fitted by Gaussian curves (solid lines), obtaining the peak values $\Delta t_1 = 57 \mu\text{s}$ and $\Delta t_2 = 283 \mu\text{s}$.

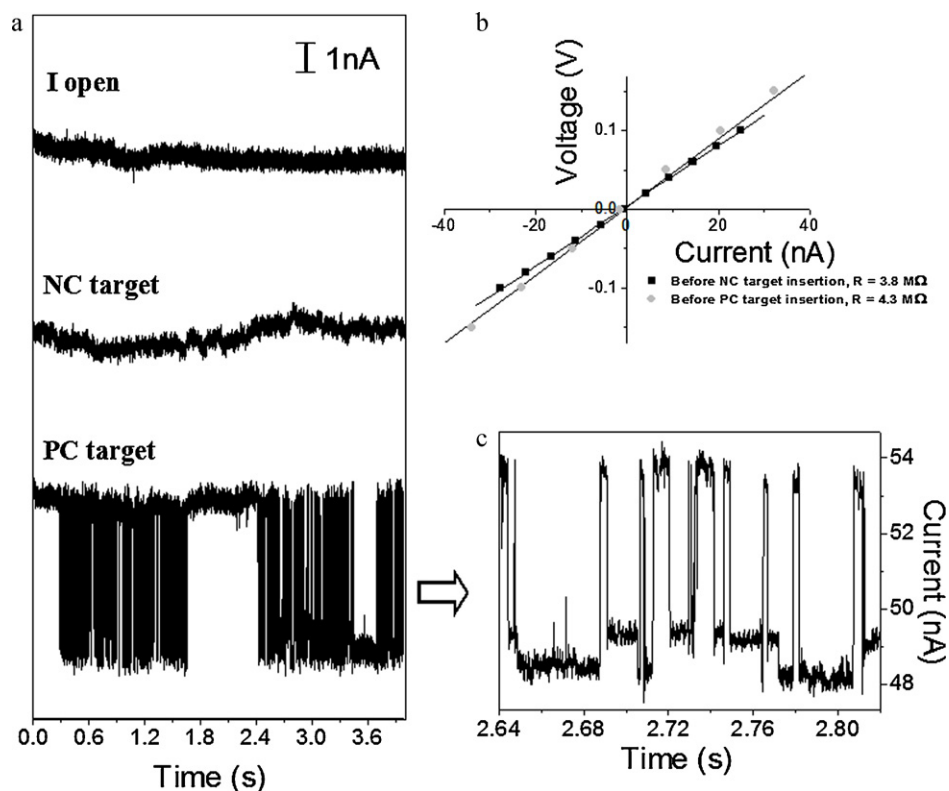


Fig. 3. Hybridization experiment. (a) Current trace registered through a DNA^2 by applying a voltage bias of 200 mV in HB solution: without target (I open), with a non-complementary target consisting of the same molecules used for the functionalization (NC target, middle), with a perfectly complementary target (PC target, bottom). (b) Voltage/current curves measured for the DNA^2 before the insertion of the NC target (black squares) and, after the cell washing, before the subsequent PC target insertion (grey circles). The electrical resistances are, respectively, $R = 3.8 \text{ M}\Omega$ and $R = 4.3 \text{ M}\Omega$, demonstrating no residual contamination of the nanopore due to the passage of the NC molecules. (c) Enlargement of the current trace of the PC target (a) in the range between 2.64 s and 2.82 s, which evidences the presence of many different current levels.

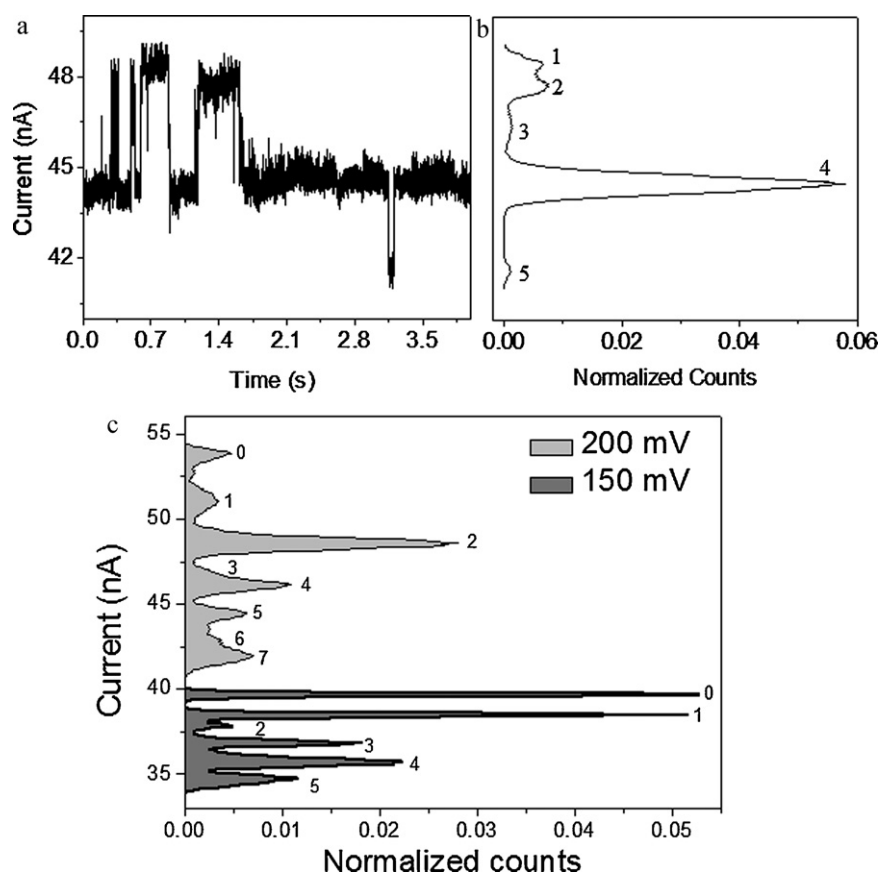


Fig. 4. Analysis of the “toggling” behaviour during probe-target interaction. (a) Single current trace (HB solution, 200 mV voltage bias) registered during the electrophoretic passage of PC target through the DNA². (b) All-point current histogram of the trace of (a) obtained as normalized current counts, showing number and position of the appearing current levels. (c) All-point current histograms summed up over all the traces (~200) registered in the same conditions at different applied voltages (150 and 200 mV). Six levels, labelled by numbers 0–5, are identified at 150 mV, while eight levels, labelled by numbers 0–7 are identified at 200 mV.

by considering the nominal membrane thickness of 20 nm and the measured HB conductivity of 6 S/m. The control experiment is performed by adding, on the cis side, an HB solution containing, as target molecules, oligonucleotides with the same sequence of those used for the functionalization procedure (0.17 nM). This NC target is left 6 h in the cell before starting the electrical measurements, in order to favour the diffusion of the molecules. Afterwards, the ionic current is continuously recorded for 15 min, but, even if the current signal appears more noisy than the open pore one, no translocation events are detected. A typical trace registered at 200 mV is shown in Fig. 3a, middle signal. The passage of such short target molecules is, in fact, too fast to be detected as a current modulation, as can be expected by taking into account the results of the translocation experiment reported in Section 3.1, and the quite large dimension of the functionalized nanopore.

Afterwards, the cell is washed several times with water and HB solution. The voltage/current curve collected after washing is reported in Fig. 3b (grey circles). The deduced electrical resistance $R = 4.3 \text{ M}\Omega$, $d_{\text{eff}} = 31 \text{ nm}$, demonstrates no residual contamination of the nanopore due to the previous passage of the NC molecules.

The experiment is then repeated with PC target molecules left 6 h in the cell before starting the electrical measurements. The used target concentration is sufficiently high to guarantee that, despite the hybridization with the probes attached on the entire SiN membrane, enough unhybridized strands remain in the cis chamber, and can be driven towards the nanopore when a voltage bias is applied. The result appears remarkably different in this case. One of the traces measured at 200 mV is shown at the bottom of Fig. 3a. Many different current levels can be distinguished, as evi-

dent in the enlargement of Fig. 3c. An analogous trend is obtained when performing the experiment at 150 mV (see [Supplementary Information](#)). We ascribe this multi-level “toggling” behaviour to a complex probe-target binding–unbinding and conformation kinetics, similar to that revealing the adsorption–desorption history and configurational changes of vestibule-captured molecules interacting with protein pores (Winters-Hilt, 2006), binding events

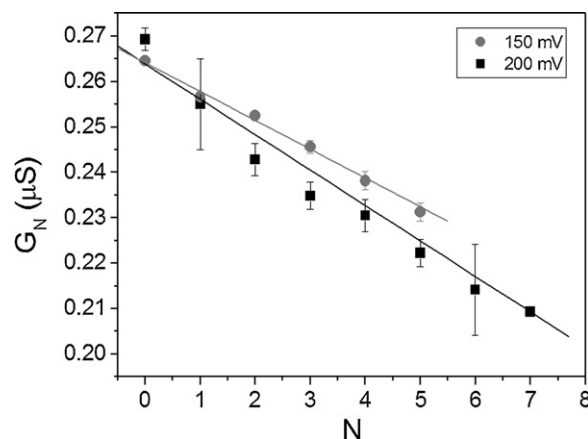


Fig. 5. Analysis of the conductance. Values of the levels conductance, G_N obtained by the Gaussian fit of the all-point current histogram of Fig. 4c (peak values), as a function of the level index N , for 150 and 200 mV. Data errors are set as HWHM of the Gaussian bands. A linear fit of the data estimates: ΔG (150 mV) = $(6.5 \pm 0.5) \text{ nS}$ and ΔG (200 mV) = $(8 \pm 1) \text{ nS}$.

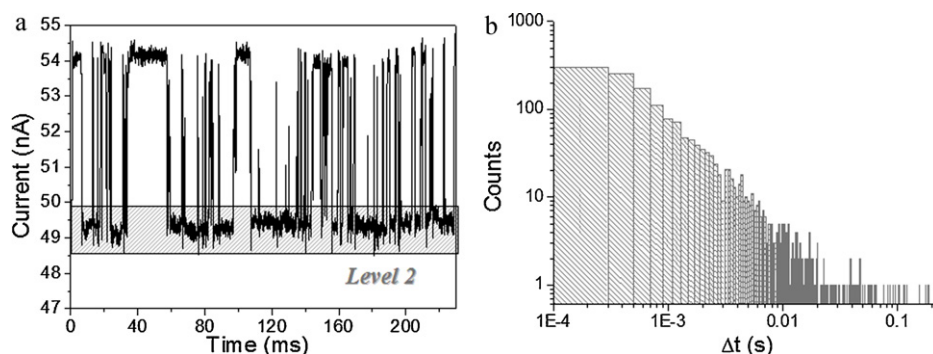


Fig. 6. Analysis of the dwell time distribution. (a) Current trace registered at 200 mV during the electrophoretic passage of PC target through the DNA², showing the variable duration of “events” corresponding to the same current level. (b) Dwell time distribution obtained by summing up over all the traces (~200) for the level which is named 2 in Fig. 4c (~49 nA), and shaded in (a).

between organic analytes and protein pores containing a molecular adapter (Astier et al., 2006; Gu et al., 1995), or, as recently observed, single molecule hybridization events on carbon nanotubes field effect transistors (Sorgenfrei et al., 2011). To analyse the data, we computed the all-point current histogram of the current traces. As an example, one of the traces is reported in Fig. 4a. The corresponding all-point current histogram presented in Fig. 4b shows the appearance of 5 current levels. When summing up over all the traces registered at the same voltage (~200), the number of levels appears similar for different biases, as shown by the resulting current histograms reported in Fig. 4c for 150 mV and 200 mV. To determine the position of the current levels, the histograms are fitted by multi Gaussian curves (see Supplementary Information). This procedure allows to identify 6 levels at 150 mV, indicated by numbers 0–5 in Fig. 4c, and 8 at 200 mV, indicated by numbers 0–7 in Fig. 4c. Each current level can be tentatively assigned to a different “occupation state” of the DNA², as produced by a change in the discrete number of molecules interacting with the nanopore, and in the conformation of the organic layer along the channel surfaces (i.e., binding–unbinding events, entering–exiting of target molecules, organization and morphology of the grafted chains, mechanical gating effects). In particular, since, as supposed in calculating the reduced effective diameter, the probe molecules are likely to be also attached along the interior nano-channel surfaces, the revealed time evolution of the current depends closely on the probe–target configuration in the whole nanopore region. In this picture, the higher current level (level 0) can be described as an “open pore configuration”, while the appearance of more levels at 200 mV with respect to 150 mV is due to the fact that, by increasing the voltage, more target molecules can enter and interact simultaneously with the functionalized channel. The probability associated with the identified levels, i.e., the peak values for the normalized counts (Fig. 4c), also changes with the applied voltage. The most probable occupation states are the first one at 150 mV (level 0), and the third one at 200 mV (level 2), indicating, again, an increase in the DNA² capture efficiency with increasing voltage bias.

Intriguingly, adjacent levels appear to be almost equally spaced. Fig. 5 reports the values of the levels conductance, G_N obtained by the Gaussian fit of the all-point current histogram of Fig. 4c (peak values), as a function of the level index N , for 150 and 200 mV. By making a linear approximation, the conductance of the N th level can be written as:

$$G_N = G_0 - N\Delta G$$

where G_0 is the open pore conductance, and ΔG corresponds to the current jump between adjacent levels. The linear fit of the data estimates ΔG (150 mV) = (6.5 ± 0.5) nS and ΔG (200 mV) = (8 ± 1) nS, that give a similar percentage $\Delta G/G$ (150 mV) = 2.5% and $\Delta G/G$

(200 mV) = 3%. This result supports the hypothesis that the current levels correspond to a discrete set of possible conformations assumed by the molecules inside the nanochannel. In particular, we speculate that an important role is played by the number and grafting position of the probe molecules attached at the pore entrance and along the channel surface. Anyhow, a complete explanation of this complex behaviour will require further theoretical modelling and investigation of competing interactions in confined geometries. In this regard, the approach very recently proposed by Peleg et al. (2011) appears particularly promising and appropriate to explain the present results.

Finally, the peculiar dynamics associated with the interaction of a PC target with the DNA² can be also characterized by analysing the dwell time distribution for the identified levels. As shown by the current trace reported in Fig. 6a, there is a large variability in the duration of different events corresponding to the same level. An example of dwell time distribution obtained by summing up over all the traces (see SI) is reported in Fig. 6b for the level which is named 2 in Fig. 4c (~49 nA), and shaded in Fig. 6a. The dwell times span the range between 100 μ s and 0.1 s, indicating that the signal fluctuations cannot be associated with simple translocation processes, but are rather an effect of the rich interaction dynamics and conformation variability of molecules inside the functionalized channel.

4. Conclusions

In summary, we demonstrated that the “DNA-Dressed Nanopore”, prepared by stably bio-functionalizing a solid state nanopore, is successfully used as an effective biosensor in single molecule translocation experiments and, moreover, is able to recognize a target sequence perfectly complementary to that of the used probe molecules. The translocation experiment demonstrates that the functionalization procedure allows to reduce the diameter of the nanopore till a dimension compatible with single molecule resolution. The hybridization experiment allows to verify the possibility to use the device to electrically analyse the interaction between probe and target molecules. The present results relate to hybridization experiments between oligonucleotides, but the method can be extended to study different kind of molecular interactions (DNA–DNA, DNA–protein, protein–protein). In particular, the great potentiality of the introduced approach resides in the possibility to use a simple chemical procedure to simultaneously resize and selectively activate a large solid state nanopore, without the need for demanding nano-fabrication techniques. We prospect that such biosensing units could constitute the basis of integrable parallel devices for mutation DNA analysis, diagnostic and bioanalytic investigations (Mussi et al., 2009).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bios.2011.08.005](https://doi.org/10.1016/j.bios.2011.08.005).

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