The nanopore-tweezing-based, targeted detection of nucleobases on short functionalized peptide-nucleic acid sequences

Isabela S. Dragomir1,#, Alina Asandei1,#, Irina Schiopu1, Ioana C. Bucataru2, Loredana Mereuta2, Tudor Luchian2

1Interdisciplinary Research Institute, Sciences Department, “Alexandru I. Cuza” University, Iasi 700506, Romania
2Department of Physics, Alexandru I. Cuza University, 700506 Iasi, Romania

# These authors contributed equally

Abstract

Quantum leaps advances in the single-molecule investigative science have been made possible over the past decades through the implication of nanopores, as versatile components on dedicated biosensors. Here, we employed the nanopore-tweezing technique to capture amino acid-functionalized, peptide-nucleic acids (PNA) with α-hemolysin-based nanopores, and correlate the ensuing stochastic fluctuations of the ionic current through the nanopore with the composition and order of bases in the PNAs primary structure. We demonstrate that while the system enables detection of distinct bases on homopolymeric PNA or triplet bases on heteropolymeric strands, it also reveals rich insights into the conformational dynamics of the entrapped PNA within the nanopore, relevant for perfecting the recognition capability single-molecule sequencing.

Keywords: nanopore tweezer, peptide nucleic acid, sequencing, single molecule recordings

Introduction

Nucleic acid sequencing stands as the method of choice to revealing genetic variations at the molecular level, and it became undisputed in fundamental and clinical or forensic science, epidemiology, or biotechnology applications. Intrinsic limitations of approaches derived from or pertaining directly to the original Sanger sequencing method [1], include extensive and costly biochemical labelling, sample preparation and difficulty to achieve long read lengths. To overcome this, ‘label-free’ and relatively simple to operate and apply, single-molecule nanopore sequencing techniques, came to the rescue [2–11].

In the simplest embodiment, a single-stranded DNA (ssDNA) is uni-directionally driven through an isolated nanopore, and it determines a characteristic ionic current blockade signature that can be used to infer the correspondingly ssDNA sequence. In the late 1990s [12,13], the ‘proof-of-concept’ demonstration of the approach has been implemented with the α-hemolysin protein (α-HL) from Staphylococcus aureus, which forms large heptameric protein nanopores in lipid bilayers, resembling a mushroom-like assembly with a central channel approximately 10 nm long, with a diameter of 1.5 nm at the most constricted region [14].
Ever since, massive creative efforts and implication of other protein- or solid-state-based nanopores [7,9,15–22] have facilitated enormous leaps to the technique, bringing it closer to fulfilling the golden standards that would enable a mammalian-sized genome to be sequenced for $1000 or less [23].

A pressing handicap standing in front of accurate nanopore sequencing is that the ssDNA translocation is rapid, e.g. with measured rates of ~ 1 nt/μs at ∆V = 100 mV in the α-HL system [13,15], and this alone poses a serious challenge to the sensitive resolve of individual bases as they are driven through the nanopore. To time-extend the α-HL nanopore probing of individual single-stranded polynucleotide sequences, and increase the signal-to-noise ratio of ionic current blockades occurring during translocation, various strategies were devised, including: ssDNA ratcheting by the action of a DNA polymerase [24], a combination between exonuclease sequencing and an engineered α-HL pore equipped with a cyclodextrin molecular adapter [6,25], or immobilization of a ssDNA sequence within the nanopore via molecular ‘stoppers’ [20,26–30].

In a previous, related project from our lab, in order to enhance the time resolution of α-HL-based molecular detection and discrimination on polypeptides, we introduced a new method dubbed ‘the nanopore-tweezer approach’. In short, we used model polypeptides whose N- and C-termini were engineered to contain patches of glutamates and arginines rendering them as macrodipoles, and demonstrated that an increase in the transmembrane potential (ΔV) led to an increase of both the polypeptide capture rate by the nanopore and residence time inside the nanopore [31–33].

Since their discovery as structural DNA analogues containing an uncharged N-(2-aminoethyl)-glycine-based pseudopeptide backbone and mimicking DNA in forming Watson-Crick complementary duplexes with normal DNA [34,35], charge neutral peptide nucleic acids (PNAs) demonstrated tremendous potential to the growing field of nucleic acids nanotechnology, as well as medicinal chemistry and molecular biology [36–45], and constitute an excellent model substrate for devising innovative approaches directed at nucleic acids primary structure reading.

Herein we extended the α-HL nanopore-tweezer method and assessed the system’s ability to discriminate among distinct nucleobases on PNA sequences, from ionic current fluctuations measured in a single PNA-α-HL blockade event.

**Materials and Methods**

*Chemicals and Reagents*

The polypeptide-functionalized PNAs used in this study (Table 1) were designed by us and synthesized and purified by Panagene Inc., Republic of Korea. The 1,2-diphytanoyl-sn-glycero-3-phosphocholine lipid (DPhPC) was purchased from Avanti Polar Lipids, Alabaster, AL, USA, and the α-hemolysin monomeric protein (α-HL), potassium chloride (KCl), n-pentane, hexadecane, EDTA and buffers (Tris and HEPES) were procured from Sigma-Aldrich, Germany.
Table 1. The primary structure of the polypeptide-functionalized PNAs, and their corresponding generic names employed herein.

<table>
<thead>
<tr>
<th>AE6</th>
<th>Ac-(K)₁₁-5’-GGG-GGG-3’-(E)₁₁-NH₂</th>
</tr>
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<tbody>
<tr>
<td>AE7</td>
<td>Ac-(K)₈-5’-TTT-TTT-TTT-3’-(E)₈-NH₂</td>
</tr>
<tr>
<td>AE8</td>
<td>Ac-(K)₈-5’-AAA-AAA-AAA-AAA-3’-(E)₈-NH₂</td>
</tr>
<tr>
<td>AE9</td>
<td>Ac-(K)₈-5’-CCC-CCC-CCC-CCC-3’-(E)₈-NH₂</td>
</tr>
<tr>
<td>AE1</td>
<td>Ac-(K)₈-5’-GGG-TTT-GGG-TTT-3’-(E)₈-NH₂</td>
</tr>
<tr>
<td>AE4</td>
<td>Ac-(K)₈-5’-TTT-GGG-TTT-GGG-3’-(E)₈-NH₂</td>
</tr>
</tbody>
</table>

Buffer solutions and sample preparation

The 3 M KCl electrolyte solution used in the electrophysiology experiments was prepared in ultra-pure water, buffered with 10 mM HEPES at a pH = 7.4. Stock solutions of 200 µM from the polypeptide-functionalized PNAs were made in 1 M NaCl dissolved in ultra-pure water, buffered with TE (1 mM EDTA, 10 mM Tris) at a pH = 8.25, and were kept at -20°C before use. Preceding each experiment, the polypeptide-functionalized PNA solutions were heated to 95°C using an IKA Digital Block Heater (Cole-Parmer, USA) and slowly cooled down to ~ 23°C. All experiments were performed at a room temperature of ~ 23°C.

Electrophysiology experiments

The lipid membranes for electrophysiology experiments with nanopores were formed as described previously [46,47]. Insertion of a single α-HL protein nanopore in the bilayer membrane was achieved by adding small volumes of protein solution in the grounded, cis compartment of the bilayer chamber, followed by gentle stirring. The polypeptide-functionalized PNAs were then added in the trans compartment from the stock solutions, to achieve a final bath concentration of 9 µM. The PNAs-induced fluctuations in the ionic current through the nanopore were recorded by two Ag/AgCl electrodes connected to an Axopatch 200B amplifier (Molecular Devices, CA, USA) set in voltage-clamp mode, at various holding voltages. Data acquisition was undertaken with a NI PCI 6221, 16-bit card (National Instruments, Austin, TX, USA) at a sampling frequency of 50 kHz and low-pass filtered at 10 kHz, within the graphical programming environment LabVIEW 8.20 (National Instruments, Austin, TX, USA). The experimental set-up was shielded from the environmental electrical and mechanical noise with a Faraday cage (Warner Instruments, Hamden, CT, USA), and mechanically isolated with a vibration-free platform (BenchMate 2210, Warner Instruments, Hamden, CT, USA). The all-amplitudes analysis of the ionic current fluctuations associated with the reversible α-HL-PNA interactions and Gaussian fitting of the resulting amplitude histograms, was performed with Origin 6 (OriginLab, Northampton, MA, USA).
Results and Discussion

Encouraged by the successful application of the nanopore-tweezer technique for single-molecule interrogation of primary structure on model polypeptides [48,49], we embarked herein in a ‘proof-of-concept’ attempt to demonstrate bases recognition and discrimination on engineered PNAs with a similar approach (Fig. 1).

**Fig. 1** Over-simplified representation of the nanopore-tweezer technique aimed at the primary structure characterization of individual PNAs. (a) In the absence of (non)specific interactions, the ionic current through a single α-HL nanopore isolated in a lipid membrane, clamped at a constant potential difference (ΔV), remains constant (b). (c) Capture of an electrically charged analyte with the nanopore, and its journey across the nanopore, are seen as reversible changes of the ionic current through the nanopore between the open state (I_{open} – free nanopore) and blocked state (I_{blocked} – nanopore transiently occupied by the analyte) (d). (e) If the analyte under study (e.g., PNA) is decorated with oppositely charged segments at its ends turning it into a macro-dipole, during the capture events inside the voltage-biased nanopore, an electrostatic tug of war between opposite electric forces exerted at the sides of the analyte ensues, increasing the residence time of the analyte inside the nanopore. This allows visualization of characteristic ionic current fluctuations through the nanopore (f), whose features may correlate with the PNA’s primary structure.

Our strategy was twofold: firstly, we employed PNAs engineered with the lysine and glutamic acids segments at the N- and C-terminus (Table 1). Their combined length was chosen to ensure that while captured inside the α-HL nanopore in their unfolded form, such constructs fit inside the ~10 nm long α-HL pore, and the lysine and glutamic acids segments from the PNAs termini face the α-HL’s trans and cis openings. In doing so, we sought to increase the constructs mean residence time in the pore, due to an electrostatic tug of war between the charges on opposite sides of the construct and the applied potential (Fig. 1), as we demonstrated previously [31].

Secondly, all experiments shown herein were undertaken experiments with the constructs added on the trans side of the membrane, which in most cases was positively polarized with respect to ground. Hence, we achieved an increased capture rate of the constructs at the β-barrel entry of
the α-HL, as the net negative charges located at the nanopore entrance (∼ −7.3 e− at pH ∼ 7.3) [50], decrease the free energy barrier for capture through attractive electrostatic interactions manifested between the PNAs – guided towards the nanopore’s mouth with the lysine-containing terminus head on at such positive ΔVs - and the nanopore opening [31,51].

By virtue of previous geometrical considerations made within the frame of similar paradigms, whereby asymmetrically charge-tagged polypeptides were investigated with the α-HL nanopore [48,49], we posit that while captured inside the nanopore, the PNA’s middle domain bases most likely visit the nanopore’s constriction region (see schematics in Fig. 1, e). Taken into account constriction region dimensions (∼0.6 nm in length and 1.4 nm in diameter and an estimated volume of ∼924 Å³) [14], and assuming that the current amplitude fluctuations associated with the presence of a PNA fragment inside the nanopore (Fig. 1, f) are chiefly correlated with the reversible blockade events occurring while the PNA slides back and forth along the sterically most sensitive region (i.e., the α-HL’s constriction region), a theoretical readout spatial resolution of ∼1.6 bases on the PNA primary structure was proposed. Thus, central to the objective of reading the PNA sequence through such current recordings, is the expectation that distinct blockade levels corresponding to specific bases presented the nanopore’s constriction domain would permit their identification on a PNA sequence. It should be noted that such an approach has been previously validated by experimental results obtained with distinct protein nanopores [52,53].

**Use of homopolymeric PNAs, to investigate sequence recognition with the nanopore**

To examine the possibility of individual bases detection within a PNA chain, we designed distinct sequences comprising of homopolymeric guanine, cytosine, adenine, and thymine (Table 1). As longer polyG strands cannot be readily synthesized due to formation of secondary structures [54], the number of guanine bases was restricted to six.
Fig. 2. Discrimination of individual bases on homopolymeric PNAs. Selected traces illustrating the reversible blockades of the ion current through an open α-HL pore due to interactions with the (AE6) G$_6$ (a), (AE9) C$_{12}$ (b), (AE8) A$_{12}$ (c) and (AE7) T$_{12}$ (d) PNAs. The all-points histogram on the right hand side of each original trace illustrates the values of the average blockade level, needed to calculate the total relative blockade of the nanopore (see also Table 2). All such experiments were carried out with PNAs added to the trans side of the membrane [9 μM], in an electrolyte containing 3 M KCl, 10 mM HEPES, pH=7.5, and at a transmembrane potential of $\Delta V = +150$ mV. In certain instances, the nanopore remained stuck in the ‘blocked’ state upon capturing the PNA constructs, so a flip of the $\Delta V$ to 0 mV was required to dislodge the fragments from the nanopore. Close up inspections of the residual currents through PNA-blocked nanopores revealed supplementary blockade substates (zoomed-in excerpts on each panel), also quantified from the corresponding all-points histograms (see also Table 2).

In Fig. 2 we represent selected traces demonstrating the reversible changes in the open-pore currents carried by the nanopore following interactions with distinct PNAs. In the simplest scenario and as we argued above, an entrapped macro-dipole-like PNA would position itself
symmetrically around the constriction region of the nanopore. From symmetry considerations, we then posit that of the total of 6 (AE6 construct) to 12 (AE7, AE8 and AE9 constructs) bases present in the middle segment of all constructs, nearly half of them (3 ÷ 6 bases) most likely probed the constriction region and partly the adjacent halve of the nanopore corresponding to the β-barrel, which largely make up the first recognition site in the α–HL nanopore [55].

We sought base discrimination in terms of differences in relative changes of the open nanopore current following fragments capture to the averaged blocked substate (denoted by total relative blockade – see Table 2). As a first finding, we noted that the order of total relative blockades $\frac{I_{\text{blocked}}}{I_{\text{open}}}$ corresponding to the averaged ‘blocked’ substate shown in Fig. 2, was: $C_{12}$ PNA $\approx T_{12}$ PNA $> A_{12}$ PNA $> G_{6}$ PNA. These results are in line with previous data demonstrating that poly(dA)60 oligonucleotides blocked the α-HL nanopore to a lesser extent than poly(dC)60, while at a particular proposed recognition site inside the nanopore closest to the constriction region (R1) – as implicated herein, too - a single thymine gave a larger block as compared to adenine [55].

**Table 2** Relative blockage values $\frac{I_{\text{blocked}}}{I_{\text{open}}}$ calculated for the averaged ‘blocked substate’ (total relative blockade), as well as additional sub-states blockades seen in the zoomed-in traces in Figs 2-3, denoted by corresponding numbers, at $\Delta V = +150$ mV.

<table>
<thead>
<tr>
<th></th>
<th>AE6: $K_{11} - G_6 - E_{11}$</th>
<th>AE9: $K_8 - C_{12} - E_8$</th>
<th>AE8: $K_8 - A_{12} - E_8$</th>
<th>AE7: $K_8 - T_{12} - E_8$</th>
<th>AE1: $K_8 - (G_3 - T_3)_2 - E_8$</th>
<th>AE4: $K_8 - (T_3 - G_3)_2 - E_8$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total relative blockade</strong></td>
<td>$-0.746 \pm 0.007$</td>
<td>$-0.849 \pm 0.012$</td>
<td>$-0.830 \pm 0.023$</td>
<td>$-0.844 \pm 0.034$</td>
<td>$-0.839 \pm 0.021$</td>
<td>$-0.871 \pm 0.007$</td>
</tr>
<tr>
<td>1</td>
<td>$-0.735 \pm 0.002$</td>
<td>$-0.779 \pm 0.007$</td>
<td>$-0.736 \pm 0.005$</td>
<td>$-0.701 \pm 0.009$</td>
<td>$-0.742 \pm 0.004$</td>
<td>$-0.748 \pm 0.005$</td>
</tr>
<tr>
<td>2</td>
<td>$-0.756 \pm 0.007$</td>
<td>$-0.813 \pm 0.003$</td>
<td>$-0.791 \pm 0.005$</td>
<td>$-0.760 \pm 0.012$</td>
<td>$-0.809 \pm 0.003$</td>
<td>$-0.822 \pm 0.001$</td>
</tr>
<tr>
<td>3</td>
<td>$-0.779 \pm 0.003$</td>
<td>$-0.839 \pm 0.003$</td>
<td>$-0.82 \pm 0.002$</td>
<td>$-0.846 \pm 0.003$</td>
<td>$-0.858 \pm 0.003$</td>
<td>$-0.875 \pm 0.003$</td>
</tr>
<tr>
<td>4</td>
<td>$-0.800 \pm 0.002$</td>
<td>$-0.857 \pm 0.001$</td>
<td>$-0.872 \pm 0.004$</td>
<td>$-0.940 \pm 0.006$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>$-0.817 \pm 0.003$</td>
<td>$-0.873 \pm 0.002$</td>
<td>$-0.943 \pm 0.002$</td>
<td>$-0.97 \pm 0.014$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>$-0.862 \pm 0.011$</td>
<td>$-0.901 \pm 0.005$</td>
<td>$-0.973 \pm 0.001$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In should be reminded that herein, unlike in previous, related work, electrically-neutral N-2-aminoethylglycine repeating units in PNAs replace the net negative sugar-phosphate backbone found in DNAs, so that the residual ionic current measured across a α-HL-PNA system is carried out by both anions and cations. This is relevant, as it has been proven that while captured inside the α-HL, charged analytes (dendrimers or ssDNAs) alter the ion selectivity the α-HL nanopore [29,56]. It yet remains to be clarified the molecular mechanism through which bases recognition with the α-HL is modulated by the PNA/ssDNA backbone charge and steric differences.

In line with previous results from our laboratory, we noted the presence of additional PNA-induced conductance fluctuations of the α-HL, as the residual current measured across the α-HL-PNA system visits multiple substates. (Figure 2, panels d−f, zoomed-in traces in insets, and Table 1). Remarkably though, certain puzzling particularities still linger in the present work.

While probing homopolymeric peptides with the similar system [48,49], we observed that the residual ionic current flipped randomly between only two distinct blockade substates, indicative of a simple model in which the deeper blockade corresponded to a group of three amino acids centered on the constriction region of the nanopore, while the shallower one was assigned to the same group of residues shifting out of the constriction region, during the peptide passage across the nanopore.

Herein, such an oversimplified, relatively unambiguous interpretation lacks, since depending on the PNA studied, as many as 5 ÷7 blockade substates were seen in the recorded trace (Fig. 2). Although in stark contrast to our expectations (i.e., we were predicting a similar blockade pattern of current fluctuations for the studied homopolymeric PNAs), one possible explanation for our results may lie in stochastic nature of the disruptions in the conformational substates and structure of the PNA within the nanopore [57,58], as its experiences fluctuating electric forces exerted at its oppositely charged moieties. This in turn would cause sterically-related changes in the residual ionic current through the nanopore, seen as reversible fluctuations as reported herein.

**Triplet base recognition in a heteropolymeric PNA background**

To further probe the PNA recognition by the α-HL nanopore, constructs presenting alternated triplet bases in the middle domains were proposed. Based on their individual volumes ($V_C = 115\,Å^3$, $V_T = 138\,Å^3$, $V_A = 139.2\,Å^3$, $V_G = 145.9\,Å^3$) [59], and to generate heteropolymers able to affect with a greatest propensity the ionic current across the nanopore as a result of bases substitutions, we designed sequences containing in the middle section two consecutive alternating groups of guanine and thymine, namely AE1: K₈–G₃–T₃–G₃–T₃–E₈ and AE4: K₈–T₃–G₃–T₃–G₃–E₈, respectively, also flanked by oppositely charged amino acid patches (Table 2).
Fig. 3. Influence of the PNA’s primary structure on the ionic current fluctuations. Representative current recordings through a single α-HL nanopore clamped at $\Delta V = +150$ mV, displaying the transient pore blockades by a) AE1 ($K_8$–$G_3$–$T_3$–$E_8$) and b) AE4 ($K_8$–$T_3$–$G_3$–$T_3$–$E_8$) PNA, added in the trans side of the lipid membrane [9 μM], in an electrolyte containing 3 M KCl, 10 mM HEPES, pH=7.5. The additional fluctuations of the residual ionic current are presented in the zoomed-in excerpts, together with the corresponding all-points histograms showing the amplitudes distribution of the blockade substates.

Representative data shown in Fig. 3 indicate that the order of bases in the PNA’s primary sequence, influences the total relative blockade describing the averaged ‘blocked’ substate (Table 2), as well as residual current fluctuations seen within the ‘blocked’ substate, in terms of substates number, amplitude and relative occupancies, as judged qualitatively from the distribution of Gaussian peaks in the all-points histograms (see also Table 2). This in turn is unexpected, as in either case (i.e., the nanopore transiently blocked by AE1 ($K_8$–$G_3$–$T_3$–$G_3$–$E_8$) or AE4 ($K_8$–$T_3$–$G_3$–$T_3$–$G_3$–$E_8$) PNA), a similar heterogeneous frame of three bases out of the overall available pool, namely either GGT, GTT, TTG or TGG, present itself and gets ‘read’ at the α-HL’s constriction region at a given time. In other words, regardless of the PNA type (either AE1 or AE4), at least a similar number of blockade substates would have been predicted to ensue during a single PNA capture.

To account for the heterogeneity of blockade substate distributions recorded, one must recall one of the main features of the macro-dipole-like PNA detection principle implicated herein (vide supra). As before, we hypothesize that once entrapped inside the nanopore, either AE1 or AE4 construct presents with the largest likelihood only its middle section near the nanopore’s constriction region. Based on topological and spatial resolution considerations, the distinct triplet bases ‘read’ at the constriction region are: (TTG) or (TGG) (AE1 construct) or (GGT) or (GTT) (AE4 construct). This may serve as a plausible hypothesis correlating the uneven distribution of distinguishable blockade current levels within the residual ionic current in Fig. 3, as being determined the distinct base triplets read by the nanopore in either case.
The AE4 (K₈–T₃–G₃–T₃–G₃–E₈) PNA-induced conductance fluctuations in a single α-HL nanopore are voltage-dependent

In the previous chapter ‘Use of homopolymeric PNA's, to investigate sequence recognition with the nanopore’, we postulated that current fluctuations seen while a PNA fragment is lodged within the nanopore may reflect the dynamic unfolding of distinct conformational substates of the PNA within the nanopore. In a proof-of-concept attempt to verify this assertion, we recorded and analyzed the kinetics of such fluctuations seen with the AE4 (K₈–T₃–G₃–T₃–G₃–E₈) PNA heteropolymer entrapped inside the α-HL (Fig. 3, b, and Fig. 4) at two distinct ΔVs.

Fig. 4 Fluctuations kinetics of the residual current through the α-HL-PNA system are voltage-dependent. a) Excerpted segment from a recording made at ΔV = +120 mV, capturing a AE4 (K₈–T₃–G₃–T₃–G₃–E₈) PNA-induced blockade event on the ionic current mediated by the α-HL. The expanded trace in (b) displays the fluctuations making up the residual current through the α-HL while blocked by the PNA. (c) Representative, normalized power spectra of residual current fluctuations entailed by an AE4 PNA lodged inside the α-HL, recorded at two distinct potentials.

While the amplitudes distribution of the residual blockade ionic current recorded at ΔV = +120 mV suggests a similar number of six blockade substates (Fig. 4, b) as measured at +150 mV (Fig. 3, b), the kinetics of such fluctuations is faster on the latter case (Fig. 4, c). For brevity, we quantified the ‘corner frequency’ (fₖ) of power spectra generated at the two ΔV’s, fₖ(ΔV = +120 mV) = 76.2 ± 11.7 Hz, and fₖ(ΔV = +150 mV) = 132.2 ± 41.4 Hz, respectively.

In relation to the hypothesis made above regarding the molecular mechanism generating such blockade fluctuations, we suggest that a larger electric force acting on the entrapped PNA entails a pronounced disruption of stacking interactions [60], thus altering the kinetic behavior and folding conformations of the molecule within the nanopore. Alternatively, one could propose
additional contributions stemming from the voltage-dependent movement fluctuations of the studied PNA fragments inside the nanopore. For our case though this seems counter-intuitive, as the tug-of-war between the forces acting at the ends of the oppositely charged PNA not only stabilizes the entrapped molecule, but elevated forces – manifested at larger $\Delta V$’s - would deepen of the central minimum in the free-energy profile of the entrapped PNA and further stabilize it [31,32].

Clearly, an elevated level of understanding of these phenomena, extremely relevant for the task of polymers sequencing with nanopores, will need further experimental and theoretical refinement.

**Conclusions**

To further expand the paradigm of nanopores suitability for single molecule sequencing applications, we employed in the present work the $\alpha$-HL protein nanopore in conjunction with the nanopore tweezing technique and demonstrated its ability to provide base-specific readout on model PNAs. It was shown that the system pore can recognize bases in homopolymeric PNA, and the single molecule stretching experiments of PNA strands inside the nanopore revealed fluctuations of the residual current which may reflect that studied PNAs adopt multiple conformations as they translocate through the nanopore, thus altering distinctly the nanopore conductance. Although qualitative in nature, with further experimentation strategies (e.g., nanopore mutagenesis and by site-directed chemical modification, variable PNA composition, temperature and salt concentrations) our findings may provide powerful diagnostics for the coupling of stacking interactions with the elastic properties of individual nucleic acid fragments, complementary to established protocols [61]. We also discovered that the order of T and G bases in triplets probing the nanopore’s constriction region generated pronounced differences in residual current fluctuations through the nanopore. Although the total blockade currents measured in $\alpha$-HL arise from nucleotides are not uniquely attributable to an individual base in a specific position, our findings are consistent with recently published studies in which we demonstrated that $\alpha$-HL sensitivity to the molecular exclusion at the most constricted region provides the specificity needed to discriminate between distinct groups of amino acids [48–49].

**Author Contributions**


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**Conflicts of Interest**

The authors declare no conflict of interest.
References


