SCIENTIFIC REPORT
Ist STAGE
2021
Multiplex detection with molecular sensitivity and selectivity, of some physiologically relevant miRNAs, using xeno nucleic acids

-RNANANODETECT-

1 - Macroscopic characterization of PNA-miRNA base-paired constructs. Proof-of-concept of reproducible signature detection of poly(Arg)-functionalized PNAs, miRNAs and PNA-miRNA duplexes with the α-HL nanopore. (Ist part).

One of the main goals of biotechnology and nanotechnology is to design molecular genome detection protocols without the use of molecular markers, to discriminate and manipulate individual DNA / RNA molecules in aqueous solutions. The single-molecule molecular detection technique using nanopores allows direct and real-time detection of a wide variety of molecules, with low cost and low material consumption.

The working principle of this approach generally follows this sequence of events: (a) an electric field conducts a macromolecule of interest to the nanopore inserted in the lipid membrane, (b) the transient capture of the macromolecule inside the nanopore leads to the displacement of a corresponding solvent volume in the system, which involves changes in the electrical resistance of the nanopore, (c) nanopore blocking events occur when a potential difference is applied on each side of the nanopore, causing reversible changes in the ionic current along the nanopore. Statistical analysis of the amplitude of such blocking events, blocking duration and frequency of events provides important information about the physical and chemical characteristics of the studied macromolecule, with high temporal (microsecond) resolution and spatial resolution (nanometer scale).

In order to implement this stage, we employed the approach of selective detection and discrimination of specific sequences of short polynucleotide molecules, using the α-HL nanopore as a molecular nanosensor. We studied the changes in the ionic current blockages given by the translocation through the protein nanopore of poly(Arg)-PNA molecules, miRNA-like DNA and miRNA-like PNA-DNA duplexes, where PNA molecules are peptide nucleic acids, synthetic analogs of DNA molecules in which the phosphate-deoxyribose structure is replaced by a polypeptide chain. The specific signature of poly(Arg)-PNA and short polynucleotide molecules molecules was first investigated. It was found that the electrically neutral PNA molecules do not interact with the nanopore, regardless of the polarity applied (**Figure 1.a, b**). On the other hand, the PNA molecules functionalized with a chain formed by arginine aminoacids, positive charged at neutral pH, namely poly(Arg)-PNA, added in the *cis* side, are captured at the nanopore's entrance by the electric field generated when a transmembranar negative potential is applied and then they translocate to *trans* side (**Figure 1.c, d**).

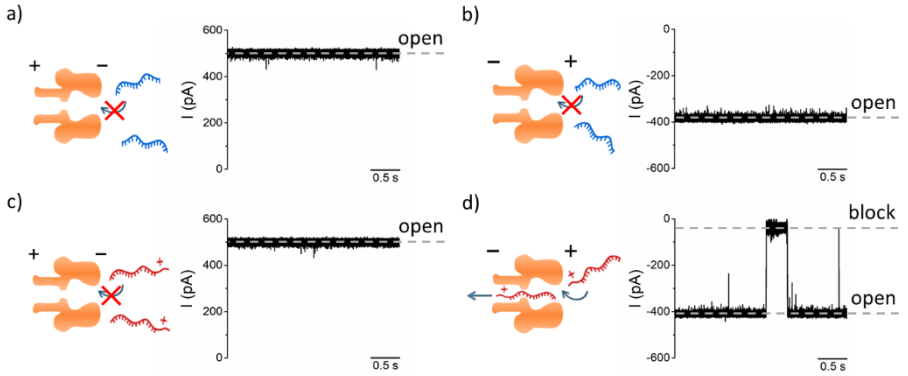

Figure 1. Distinct interactions of neutral (a, b) and cationic (c, d) PNAs with a single α-HL nanopore, depending on the applied potential: **a), c) trans-positive** (ΔV = + 180 mV), or **b), d) trans-negative** (ΔV = - 180 mV). (*Dragomir et al., Anal.Chem, 2020, 92*)

It has been observed that short polynucleotide molecules molecules translocating through the nanopore from the *cis* side to the *trans* side give a blockage with a particular form and a duration on the order of 10^{-4} s for applied potential between 180-200 mV, as we can see in **Figure 2. I**.

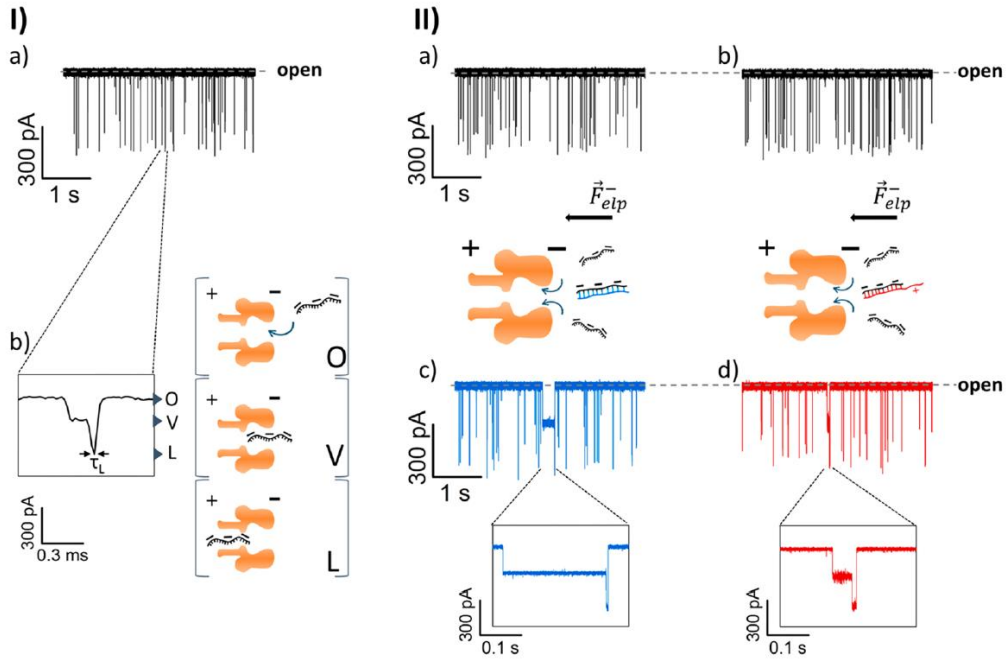

Figure 2. I. Interaction with a single α-HL nanopore of the target short polynucleotide molecules: **a)** reversible fluctuations of the ionic current given by the target molecules cDNA passing through the nanopore, b) zoom-in of a typical blockade event induced by cDNA. **II. Interaction with a single α-HL nanopore of the duplex formed between the probe PNA molecule and the target DNA: a), b)** reversible fluctuations of the ionic current given by cDNA; **c), d**) the addition of complementary neutral (BPNA), respectively cationic (RPNA) PNA sequences produces specific blockade signatures, where the zoom-in shows the duplex blocking the nanopore. (*Dragomir et al., Anal.Chem, 2020, 92*)

According to previous findings (*Asandei A. et al., ACS Sens. 2019, 4 (6)*), the blocking signal specific to the translocation of a single short polynucleotide molecule through the nanopore has two levels: the first is the molecule's entry into the wider vestibule (substate V), and the second is the molecule's passage through the constriction region into the nanopore's lumen (substate L), followed by the escape to the *trans* side of the nanopore.

In order to study the specific signature of DNA-PNA duplexes, the PNA probe molecules are added in the experimental setup where the short polynucleotide molecules are present. If the target molecules are complementary to the probe molecules, PNA-DNA hybridization will occur, with a signal characteristic to hybridized complexes that will be immediately recognizable (**Figura 2.II. c, d**). If the two types of molecules aren't complementary, we'll only see the distinct fingerprints of poly (Arg) -PNA molecules or short polynucleotide molecules in the recorded signal. In this way, the detection with molecular sensitivity and selectivity of DNA similar to physiologically relevant miRNAs is performed with the help of the protein nanopore system made by α-HL - PNA molecules.

Act 1.1 - A1.1 Selective design of target miRNAs and probe PNAs sequences, to achieve optimal hybridization in salt buffers. Spectroscopic and isothermal calorimetry (ITC) assessment of stability and discrimination of double-stranded PNA-miRNAs (mis)matches.

To obtain an efficient hybridization in the electrophysiological solution, we tested a target short polynucleotide molecules (cDNA) similar in structure to miRNA, complementary to two types of sample PNA molecules, namely BPNA, containing complementary nucleobases and RPNA, which also has a polypeptide chain $(R)_{10}$ conjugate at the 5 'end. This polypeptide chain gives the RPNA molecule a net positive electrical charge given by the arginine amino acids (electrophysiological solution 3 M KCl, and 10 mM HEPES, pH = 7.3).

Table 1. Primary sequences of the molecules of interest used in this study. The N-terminal of the PNA is referred to as the 5′-end of the PNA, according to established convention.

Primary sequences	
cDNA	3’- CACTATATGCCACTA - 5’
RPNA	Ac-$(R)_{10}$-5’- GTGATATACGGTGAT - 3’
BPNA	5’- GTGATATACGGTGAT - 3’

The cDNA molecules at a concentration of 0.5 μM were added to the *cis* side of the protein nanopore, after which the PNA molecules at a concentration of 5 μM were added. Once added to the *cis*, the PNA sample molecules will hybridize to the short polynucleotide target molecules, the process being visible in the ionic current mediated by the protein nanopore. The hybridized BPNA-cDNA complex has a negative net electrical charge given by the phosphate groups of the cDNA of -15 $|e^-|$, the BPNA molecule being neutral. The RPNA-cDNA complex has an electric dipole structure, given by the positive polypeptide sequence $(R)_{10}$ with an electric charge of 10 $|e^-|$ and

the negative charge of the phosphate groups of the cDNA of -15 |e⁻|, the complex having a net electric charge of -5 |e⁻|. The complexes with negative net electric charges will be directed in the *cis-trans* direction by the electrophoretic force $\vec{F}_{elp}^{-}$ and we will be able to discriminate them according to the specific signature of each in the ionic current mediated by the nanopore.

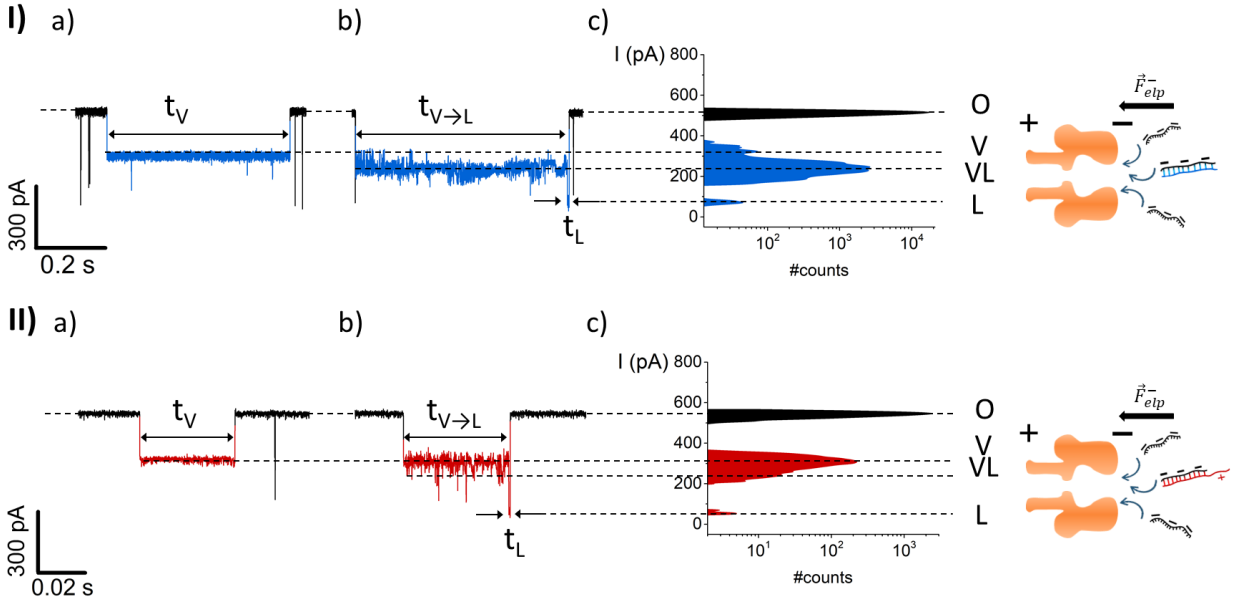

Figure 3. Types of events resulted from the interaction between the α-HL proteic nanopore and the molecular complexes: I. BPNA-cDNA, respectively **II.** RPNA-cDNA. **I.a)** and **II.a)** the complex is captured inside the nanopore's vestibule, then it returns back in the *cis* side; **I.b)** and **II.b)** after capture, the complex dissociates and the cDNA molecule translocates to *trans* side through the nanopore's lumen, while the BPNA, respectively RPNA molecules return to *cis*. The amplitudes of blockage events caused by either cDNA-BPNA (**I,c**) or cDNA-RPNA (**II,c**) duplexes trapped within the nanopore from the vestibule entrance are shown in the all-points histograms. (*Dragomir et al., Anal.Chem, 2020, 92*)

Due to steric and volume reasons, the PNA-cDNA complex with a diameter of ~ 2 nm enters the vestibule with an inner diameter of 2.6 nm but cannot translocate further through the constriction zone measuring 1.4 nm in diameter. Thus, it was observed that the interaction between the PNA-cDNA molecular complexes and the α-HL protein nanopore results in two types of events: those corresponding to the return of the complex situated in the vestibule to the *cis* side, without the complex dissociation, as in **Figure 3. Ia)** and **II.a)**, respectively the events in which the complex dissociated in the cDNA molecule that will translocate further through the lumen to *trans* and the BPNA molecules (**Figure 3. Ib**), respectively RPNA (**Figure 3. II. b**), which return to the *cis* side.

It was observed that the mean time of a BPNA-cDNA block (τ_{off} BPNA-cDNA = 0.196 ± 0.033 s at ΔV = + 200 mV) is longer than that given by RPNA-cDNA (τ_{off} RPNA-cDNA = 0.078 ± 0.004 s at ΔV = + 200 mV), because in the case of the macrodipole molecular structure BPNA-

cDNA acts two electrophoretic forces in opposite directions on the charged components of the complex, leading to its unzipping in a shorter time, unlike the negative BPNA-cDNA complex, on which a single electrophoretic force acts, towards *trans*.

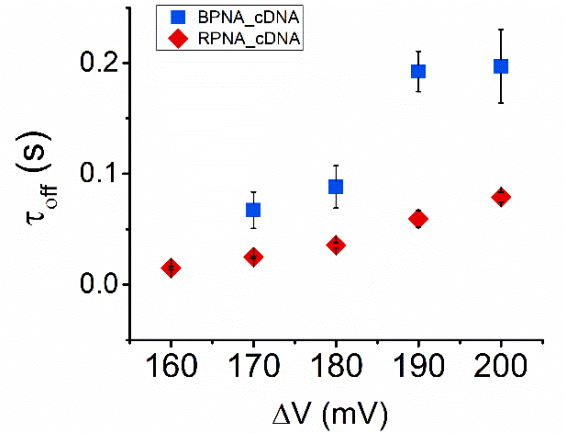

Figure 4. Voltage dependence of the dwell time of blockage events associated to BPNA-cDNA (■) and RPNA-cDNA (♦) interactions with α-HL. The values of dwell time corresponding to blockade events given by the BPNA-cDNA complex are greater than the ones corresponding to RPNA-cDNA complex. In both cases, the duration of blockages increases with the applied transmembrane potential.

Analyzing the average interaction time of the complexes with the α-HL protein nanopore (**Figure 4.**) we can investigate the stability of PNA-DNA complexes similar to miRNAs without using techniques such as spectroscopy and isothermal titration calorimetry (ITC), which require a much higher concentration of molecules. The data obtained from electrophysiology experiments (reaction constants) are correlated with the results we would have obtained using the mentioned above techniques. We notice that the BPNA-cDNA complex is more stable than the RPNA-cDNA complex with macrodipole structure.

Act 1.2 - A1.2 Thermodynamic investigation of the influence played by variable sized poly(Arg) tails attached to PNAs, to the base-pairing energy of PNA-miRNAs.

Based on the obtained results, a kinetic model that describes the stochastic interaction between the α-HL protein nanopore and the PNA- miRNA-like, short polynucleotide molecular complexes was developed (**Figure 5.**), that is useful in the analysis of their association energy.

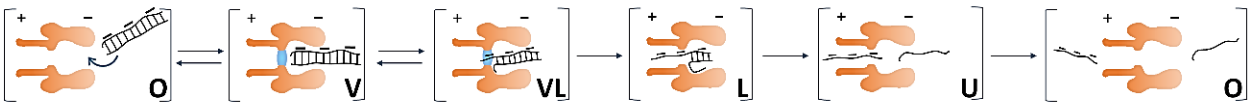

Figure 5. The kinetic model describing the interaction between the α-HL proteic nanopore and PNA-cDNA molecular complexes: the ”O” state represents the open pore; the ”V” state is given by the duplex captured inside the nanopore’s vestibule; the unzipping process starts with the cDNA fragments from the duplex that is captured in the lumen, described by the ”VL” state; as the cDNA molecule translocates through the lumen to the “L” state, the complex gradually unzips until the two molecules completely detach from each other, in the ”U” state; the cDNA molecule translocates to *trans* side and the PNA molecule diffuses in the *cis* side, the system returning to the ”O” state. (*Dragomir et al., Anal.Chem, 2020, 92*)

Analyzing the complete dissociation constant of PNA-cDNA molecular complexes in the nanopore vestibular zone (k_{LU}), it was observed that the dissociation of RPNA-cDNA macrodipole complexes is faster than that of BPNA-cDNA complexes (**Figure 6, a**), which means that the binding energy is higher in the case of the BPNA-cDNA complex without the conjugated poly(Arg) chain. The presence of the polyarginine tail in the case of the RPNA-cDNA complex contributes to the existence of two opposite-direction electrophoretic forces acting on the complex (F_{el} acting on the positively charged poly(Arg) and F_{elp} acting on the negatively charged cDNA molecule), as in **Figure 6, b)**, resulting from the application of a transmembrane electrical potential, ΔV. This configuration leads to a decrease in the free energy of the RPNA-cDNA duplex ($\Delta G_{\blacklozenge}$) compared to that of the BPNA-cDNA duplex ($\Delta G_{\blacksquare}$), giving the latter a higher stability (**Figure 6. b**).

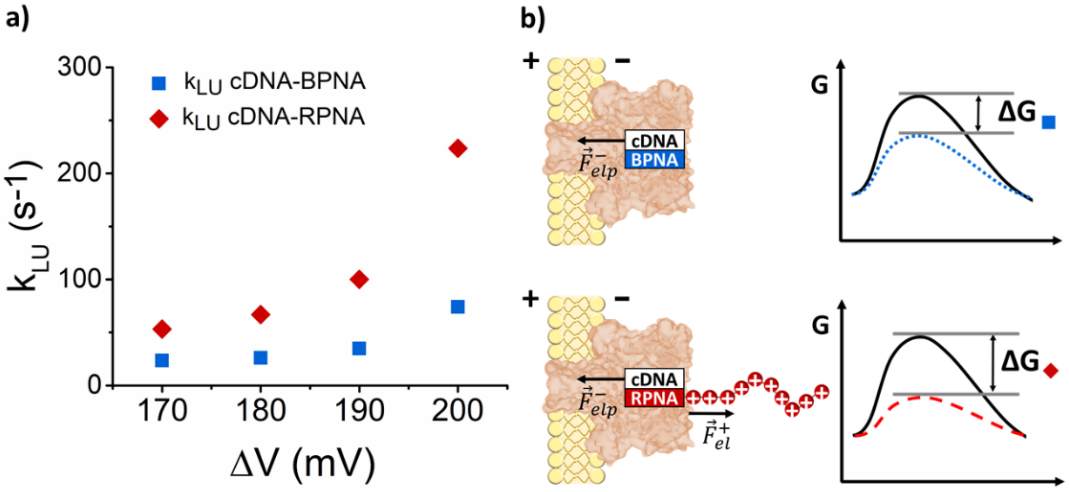

Figura 6. a) Qualitative estimation of the reaction constant k_{LU} that characterizes the complete unzip kinetics of PNA-cDNA complex; **b)** Representation of the free-energy profiles characteristic for the unzip process of BPNA-cDNA(■) and RPNA-cDNA(♦) molecular complexes. (*Dragomir et al., Anal.Chem, 2020, 92*)

Act 1.3 - A.2.1 Concentration- and voltage-dependent signature of target (miRNA) and probe (poly(Arg)-functionalized PNA's) reversible capture on a single α-HL nanopore.

After several experiments, it was found that the use of a molar ratio of 1:10 cDNA:PNA is more appropriate because it increases the probability of hybridization, PNA molecules being in excess. Thus, cDNA molecules in a concentration of 0.5 µM and PNA molecules in a concentration of 5 µM were added to the *cis* side of the α-HL protein nanopore.

We analyzed the voltage dependance of the time intervals required for lumen translocation of cDNA fragments added to the *cis* side of the nanopore and those resulting from the dissociation of BPNA-cDNA and RPNA-cDNA complexes in the vestibule (**Figure 7. a**) and it was observed

that the translocation time decreases with the increase of the applied potential, as we would have expected, due to the increase of the electric force acting on the molecules.

We can also see that the translocation time of an individual cDNA molecule is two orders of magnitude shorter than that of a cDNA molecule from the PNA-cDNA duplex in the vestibule of the α-HL nanopore. Another observation is that the translocation time of cDNA from the more stable BPNA-cDNA complex is approximately double compared to the translocation time of cDNA from the cDNA-RPNA duplex, due to the additional electrical force felt by the poly(Arg) in the RPNA-cDNA complex, which leads to faster dissociation of the complex, so to a faster translocation of the cDNA molecule through the lumen.

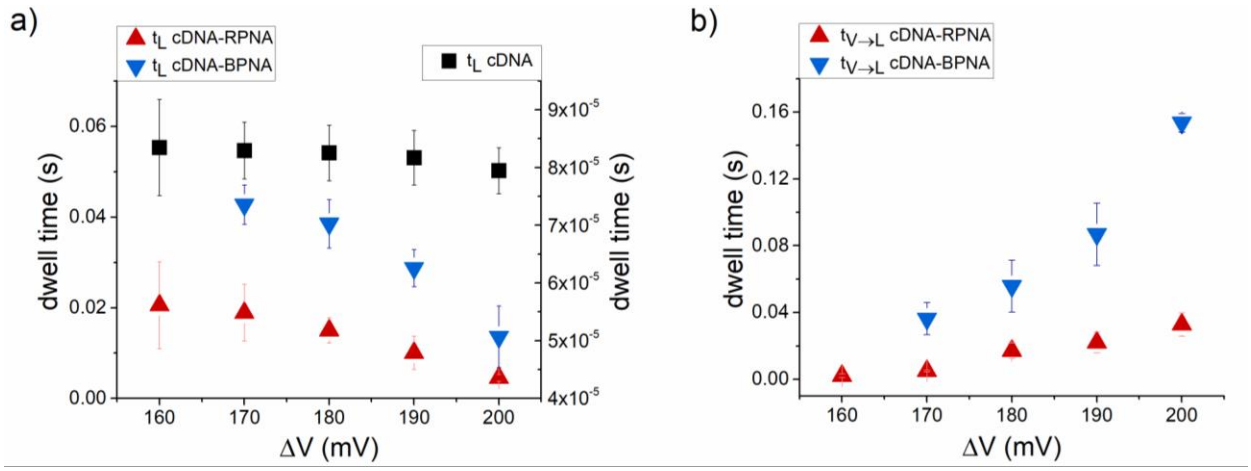

Figura 7. Voltage dependence of the time intervals associated with the interaction of cDNA-PNA duplexes with the α-HL. (a) The average times corresponding to the molecular states whereby unzipped cDNA fragments from the vestibule-captured cDNA-BPNA (▼), cDNA-RPNA (▲) duplexes, as well as cDNAs added alone in the *cis* chamber (■), dwell inside the nanopore's lumen. (b) Average durations corresponding to the vestibule-to-lumen transitions undertaken by cDNA-BPNA (▼) or cDNA-RPNA (▲) duplexes, initially captured inside the nanopore's vestibule. (*Dragomir et al., Anal.Chem, 2020, 92*)

The average durations corresponding to the vestibule-lumen transitions of the BPNA-cDNA and RPNA-cDNA complexes (**Figure 7. b**) increase with the applied potential, which is surprising. One possible explanation is that at high potential differences, the interaction between the complex and the constriction zone is stronger and for the cDNA molecule is more difficult to reach a conformation favorable to insertion in the 1.5 nm constriction zone, and then to translocate through the lumen. Thus, the complex lingers a longer time $t_{V\rightarrow L}$ in the vestibule at high potentials, until the cDNA molecule finds a favorable position to start the unzipping process.

Therefore, in the interaction between the α-HL nanopore and the complexes of PNA-cDNA molecule similar to miRNA, the opposite electrophoretic forces, directly proportional to the applied potential, lead to the decrease of the RPNA-cDNA complex unzipping time, due to its macrodipole structure given by the negative cDNA backbone and the poly(Arg)-functionalized PNA.

Act 1.4 - A2.2 Identification of optimal conditions to enhance the stochastic capture and subsequent identification sensitivity of PNA-miRNA duplexes

In the studies described above, in which cDNA was used as the target molecule and BPNA and RPNA as probe molecules, we chose *cis* as the addition side to better serve the purpose of the experiment, and positive potentials were applied due to the fact that the cDNA molecule similar to miRNA has a negative net electric charge and thus will move from *cis* to *trans* in the electric field derived from the transmembrane voltage, facilitating interaction and translocation through the protein nanopore.

We tested two other types of target (DNA_i) and probe (pb_i) molecules to determine the optimal conditions for this sort of experiment and we used various experimental configurations in which we modified the molecules of interest side of addition (*cis* or *trans*) and the polarity of transmembrane potential applied (+ or -).

Tabel 2. Primary sequences of the molecules used herein: the probe molecules pb3 and pb4 are complementary to the target molecules, DNA1 and DNA2

pb3	**CTT TTG GTG T** RRRRRRRRR
pb4	**GTT TGT TCT G** RRRRR
DNA1	**ACA CCA AAA G** ATCACATTGG (5 → 3)
DNA2	**CAG AAC AAA C** CCAAGGAAAT (5 → 3)

The capture of molecules of interest by the nanopore is governed by several competing effects, the most important being: electrophoresis, entropic penalty given by physical limitation, enthalpy contribution associated with specific interactions between peptides and pore entry and electroosmosis. The heptameric α-HL protein nanopore is geometrically and electrically asymmetric, so that the four effects mentioned above will alter the analyte capture rate differently on each side of the nanopore.

Following the experiments, it was found that in order to achieve the proposed objective, is necessary to add the molecules of interest in the *trans* side of the molecular nanopore and to apply positive transmembrane voltages. In the case of other experimental configurations, the complex could not be detected.

A possible explanation is given by the electrostatic attraction between positively charged molecules in the constituency of molecular duplexes (pb_i) and negatively charged amino acids from the lumen mouth ($q_{ring} = |-7e^-|$). Another explanation is given by the fact that the electrophoretic force is more intense on the *trans* side of the nanopore, at the entrance to the β-barrel lumen, than on the entrance to the vestibule of the nanopore, on the *cis* side. At first approximation, considering the nanopore and membrane perfect insulators, in the case of stationary, the electric field is more intense in the narrower area of the ion channel. Consequently,

the electric field at the entrance to the lumen is larger than that at the entrance to the pore vestibule, which leads to an increased probability of capturing the complex in the *trans* area.

Act 1.5 - A.2.3 Testing and selection of distinct length poly(Arg)-conjugated PNAs, to optimally provide a specific, length-dependent signature of events while interacting with a single α-HL. (Ist part).

To increase the efficiency of the method and to control various parameters such as the stability of the PNA-miRNA duplex, we tested PNA molecules functionalized with polyarginine tails of different lengths and analyzed the signals given by them after interaction with α-HL protein nanopore. The PNA molecules used are: **pb3** - **CTT TTG GTG T** - $(R)_9$ containing 9 arginine amino acids and **pb4** - GTT TGT TCT G - $(R)_5$ with 5 arginine amino acids.

PNA molecules were added to the *trans* compartment, at a concentration of 4 μM and positive transmembrane voltages were applied, so that the molecules diffused in the lumen side are captured electrophoretically by the nanopore and translocated through the ion channel, reaching finally in the *cis* compartment. The mode of interaction with the α-HL nanopore (τ_{on}) and the translocation time (τ_{off}) differ for pb3 and pb4 as seen in **Figure 8**.

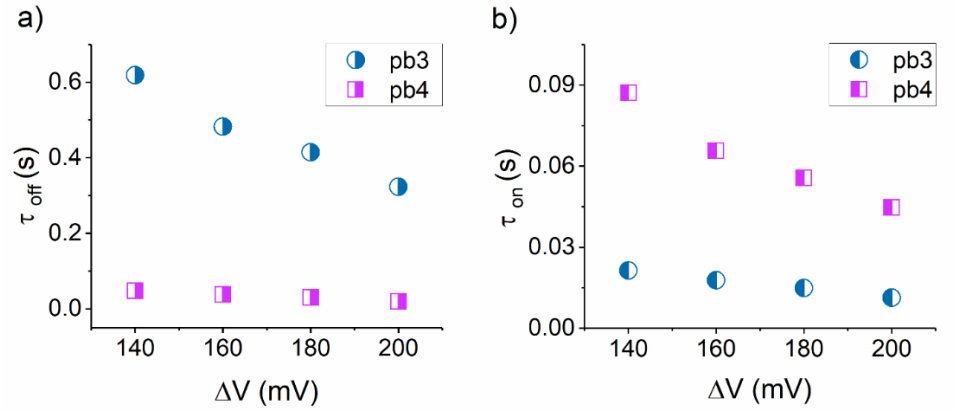

Figure 8. Interaction kinetics of the α-HL protein nanopore and the sample molecules, pb3 and pb4, added in a concentration of 4 μM in the *trans* compartment: a) τ_{off} is the translocation time of the molecule through the nanopore, b) τ_{on} represents the interval of time between two successive events.

We can see that the nanopore translocation time, τ_{off}, is longer in the case of pb3 - $(R)_9$ than in pb4 - $(R)_5$, and also the variation with the applied potential is faster. With a higher number of positively charged amino acids, the electrostatic attraction interaction between them and the negative electrical charges of the amino acid residues on the inner surface of the nanopore`s ion channel leads to a slower translocation of the pb3 molecule through the nanopore. As the applied transmembrane potential increases, the polyarginine chain in pb3 will feel a stronger electrophoretic force, which will lead to linearization along the electric field and to an increasingly rapid translocation. In the case of the time interval between two successive events, τ_{on}, we observe that the interaction between pb3 - $(R)_9$ and the protein nanopore is faster than that with pb4 - $(R)_5$,

due to the increased electrostatic attraction between the nine arginines and the negative amino acids from the entry into the lumen and the fact that pb3 - $(R)_9$ is linearized to a greater extent than pb4 - $(R)_5$ in the electric field felt by molecules.

To study the behavior of the two PNA sample molecules functionalized with polyarginine tails of different lengths inside the ion channel of the α-HL protein nanopore, we calculated the relative amplitude of the blockade corresponding to the residual ion current I_{block} as follows:

$$\frac{\Delta I_{block}}{I_{open}} = \frac{I_{block} - I_{open}}{I_{open}}$$

where I_{open} represents the intensity of the ionic current measured through the free α-HL pore and I_{block} corresponds to the blockage intensity given by the interaction of the molecule with the protein nanopore.

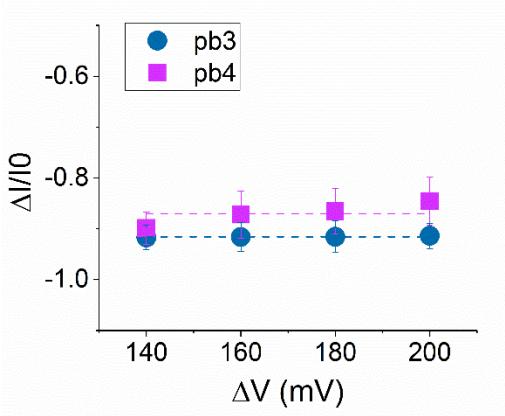

Figure 9. Variation of relative blockade amplitude given by the interaction of PNA molecules with α-HL protein nanopore. The values for pb3 molecules are represented in blue and the values for pb4 molecules are represented in magenta. Dotted lines represent the linear fit of values with a constant slope set to 0.

Considering that the relative blockage amplitude in the ionic current has negative values, in **Figure 9.** we observe that the pb3 - $(R)_9$ molecules block the ion channel to a greater extent ($\left| \Delta I_{block} / I_{open} \text{ pb3} \right| = 0.9156 \pm 0.0007$) compared to the molecules of pb4 - $(R)_5$ ($\left| \Delta I_{block} / I_{open} \text{ pb4} \right| = 0.8705 \pm 0.0109$), possibly due to their larger size and the interaction of the 9 arginines with the amino acids on the inner surface of the nanopore.

We thus managed to meet the objectives we proposed and to demonstrate the viability of the technique, and in the following stages we want to streamline the multiplex detection, with sensitivity and molecular selectivity, of DNA molecules similar to physiologically relevant miRNAs, using the system composed by biological nanopore and poly (Arg) -PNA molecules.

Results and dissemination of results.

At this stage, a number of 4 articles with impact factor were published, three of them being in the red zone (Q1):

1. Schiopu Irina, Asandei Alina, Mereuta Loredana, Dragomir Isabela, Bucataru Ioana Cezara, Luchian Tudor.Single-molecule detection and manipulation with biological nanopores. *Studia Universitatis Babes-Bolyai, Chemia* . **2021**, 66 161-174.
2. Alina Asandei, Loredana Mereuta, Irina Schiopu, Yoonkyung Park, Tudor Luchian. Teaching an old dog new tricks: A lipid membrane-based electric immunosensor for real-time probing of the spike S-1 protein subunit from SARS-CoV-2. *Proteomics*, **2021**, e2100047.
3. Tudor Luchian, Loredana Mereuta, Yoonkyung Park, Alina Asandei, Irina Schiopu. Single-molecule, hybridization-based strategies for short nucleic acids detection and recognition with nanopores. *Proteomics*, **2021**, e2100046.
4. Isabela S Dragomir, Alina Asandei, Irina Schiopu, Ioana C Bucataru, Loredana Mereuta, Tudor Luchian. The Nanopore-Tweezing-Based, Targeted Detection of Nucleobases on Short Functionalized Peptide Nucleic Acid Sequences. *Polymers*, **2021** 13, 1210.

15.11.2021

Project director,

Prof. Univ. Dr. Tudor LUCHIAN