SCIENTIFIC REPORT

IInd STAGE

2022

Multiplex detection, with molecular sensitivity and selectivity, of physiologically relevant miRNAs, using xeno nucleic acids
-RNANANODETECT-

2 - Detection of the specific fingerprint of poly(Arg)-PNA, miRNA molecules, and PNA-miRNA duplexes using the α-HL nanopore (part II). Highlighting the specificity of the technique based on the use of the α-HL nanopore in the detection of miRNA molecules. Elaboration of the dielectrophoretic mechanism for capturing the poly(Arg)-PNA – miRNA complex by a single nanopore.

During the global COVID-19 pandemic, the need to use pathogen diagnostic technologies in a timely manner to detect and prevent illness has become clearer than ever. Current techniques for detecting DNA or RNA sequences (such as PCR) present a number of challenges because they are complex, expensive and require skilled personnel. Nucleic acid detection using nanopores is a real-time, cheaper, easy-to-implement and easy-to-operate method.

In our study we tested a single-molecule detection method using the α-hemolysin (α-HL) protein nanopore, which does not require labeling or amplification, based on the complementarity between the target nucleic acid sequence and the sequence of a synthesized sample nucleic acid. Thus, we recorded and analyzed signals given by poly(Arg)-PNA sample molecules (synthetic analogues of DNA molecules, in which the backbone is replaced by a peptide structure), target DNA similar to miRNA sequences and duplexes poly(Arg)-PNA–DNA similar to miRNA, formed following hybridization of complementary nucleic acid sequences. The presence or absence of the hybridized complex indicates whether or not the sample contains the sought DNA similar to the miRNA, demonstrating the specificity of the technique based on the use of the α-HL nanopore in the detection of DNA molecules similar to miRNA.

Act 2.1 - A.2.3 Selection and testing of PNA molecules functionalized with polyarginine tails of different lengths in order to obtain a specific signal, dependent on the length of the tail, upon interaction with a single pore of α-HL (part a-II).

The sensitivity and analytical performance of the technique were tested on nucleic acid sequences similar in composition and length to specific sequences for the nucleocapsid phosphoprotein (N-gene) of the SARS-CoV-2 virus, representative for Covid-19 testing (Table 1). Peptidonucleic acids, PNA, complementary to DNA sequences, conjugated with arginine amino acid sequences of different lengths, poly(Arg), were designed as probe molecules. The poly(Arg)-PNA sequences, denoted here PA5, PA9, and PA13, are functionalized with 5, 9, and 13 C-terminal Arg amino acids (Table 1). To test PNA molecules functionalized with polyarginine tails of different lengths for a specific signal dependent on tail length, we analyzed the interaction between a single α-HL nanopore and poly(Arg)-PNA probe molecules added at a concentration of 4 μM of the cis and trans side, respectively, at a concentration of 3M KCl of the recording solution and pH=7.

Table 1. Primary structures and corresponding molecular masses of poly(Arg)-PNA probe molecules and miRNA-like single-stranded DNA target molecules. Red rectangles depict hybridized poly(Arg)-PNA-DNA duplexes. *(Mereuță et al., Anal. Chem 2022, 94)*

	(Poly-Arg)-PNA (N→C)	Mw (g/mol)	ssDNA (5'→3')	Mw (g/mol)
PA5	GTTTGTTCTG-Arg5	3520.5	CAGAACAAACCCAAGGAAAT (cDNA(PA5)) CAGAACAAACCCAAGGAAAT Arg5 -GTCTTGTTTG	6122
PA9	CTTTTGGTGT-Arg9	4145.3	ACACCAAAAGATCACATTGG (cDNA(PA9)) ACACCAAAAGATCACATTGG Arg9 -TGTGGTTTTC	6104
PA13	GTTTGTTCTG -Arg13	4770	CAGAACAAACCCAAGGAAAT (cDNA(PA13)) CAGAACAAACCCAAGGAAAT Arg13 -GTCTTGTTTG	6122

For the poly(Arg)-PNA segments added to the cis side of the α-HL nanopore, negative transmembrane potential differences were applied (Fig. 1. I. a, b, c). From the statistical analysis we observed that the average time of the intervals between successive capture events (τ_{on}) depends inversely proportionally on the length of the poly(Arg) fragment. This is because the electrophoretic force acting on the poly(Arg)-PNA molecules increases with their net positive electrical charge, resulting in more frequent electrophoretic trapping of the sample molecules in the nanopore.

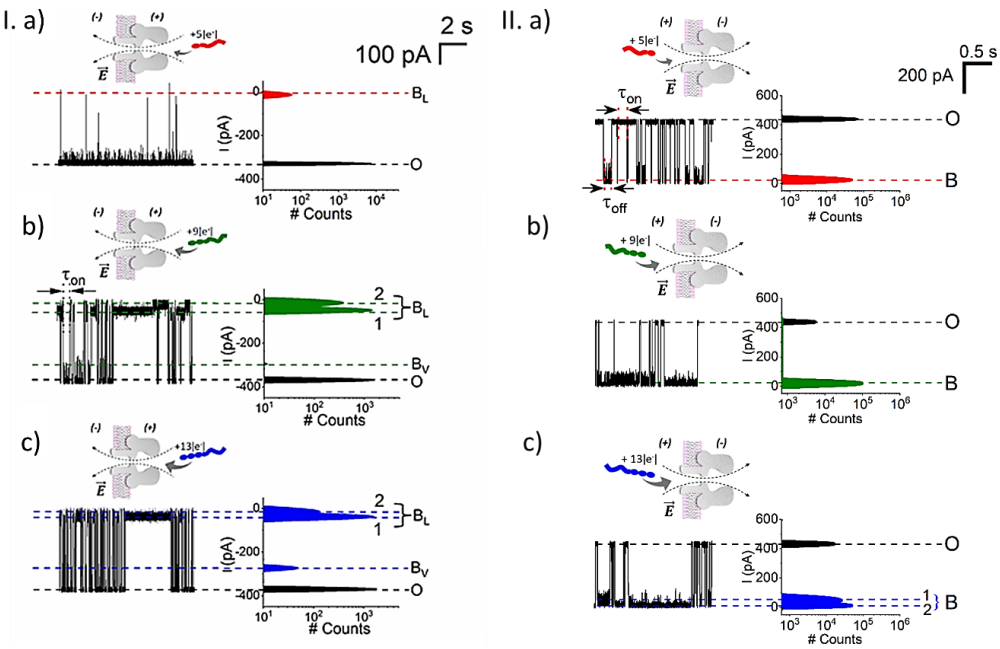

Fig. 1 Detection of poly(Arg)-PNA probe molecule: I. at the cis entrance into the vestibule of the α-HL nanopore, II. at trans entry into the lumen of the α-HL nanopore. Typical experiments showing the reversible interaction between the α-HL nanopore and 4 μM PA5 (a), PA9 (b), or PA13 (c), at $\Delta V = -140$ mV, in an electrolyte containing 3 M KCl, pH = 7. *(Mereuță et al., Anal. Chem 2022, 94)*

The B_V and B_L substates (Fig. 1) were attributed to analyte translocation within the vestibule and lumen of the nanopore, with different internal volumes. The poly(Arg)-PNA molecules adopt different conformations within the lumen, resulting in the appearance of B_{L1} and B_{L2} sublevels. Thus, we confirmed that the probe molecules PA5, PA9, and PA13 elicited a distinct response while reversibly interacting with the nanopore, and the longer the poly(Arg) sequence, the better such interactions are seen.

To further refine the discrimination of molecules, we performed experiments with analytes added to the trans side of the nanopore (Fig. 1, II). In this case, the trans-positive potential differences favor the reversible capture of sample molecules by the nanopore. Due to the net negative electric charge at the

entrance to the nanopore lumen, the capture of cationic poly(Arg)-PNA molecules is enhanced due to electrostatic attraction interactions. Statistical analysis showed that the length of the poly(Arg) sequences facilitates trapping events at the lumen of the protein nanopore.

Act 2.2 - A3.1 Determination of the potential of poly(Arg)-PNA molecules in the detection of uni- and multi-nucleobases, as well as the discrimination of miRNA target molecules

Next we tested the potential of poly(Arg)-PNA molecules in discriminating miRNA-like single-stranded DNA sequences. Knowing the specific fingerprints for each poly(Arg)-PNA sample molecule, we added the complementary miRNA-like single-stranded DNA target molecules, named DNAc(PA5), DNAc(PA9), and DNAc(PA13) to the system, leading to the formation of poly(Arg)-PNA-DNA molecular duplexes hybridized through hydrogen bonds between Watson-Crick base pairs (Fig. 2). The presence of these molecular duplexes is detected and differentiated according to the length of the poly(Arg) sequence attached to the probe molecules PA5, PA9 and PA13.

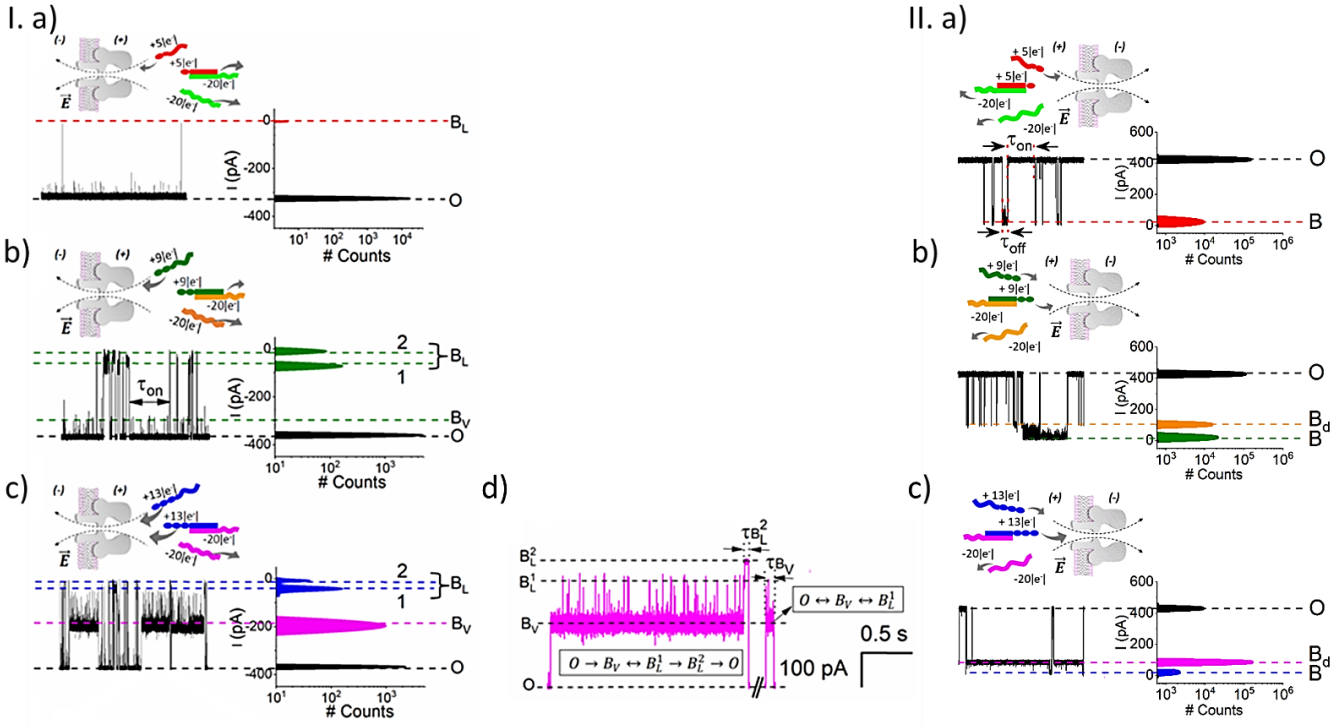

Fig. 2 Detection of miRNA-like DNA target molecules following hybridization with poly(Arg)-PNA sample molecules using the protein nanopore of α-HL, when the molecules of a) PA5 and cDNA(PA5), b) PA9 and cDNA(PA9) , c) PA13 and cDNA(PA13) were added I. to cis or II. to trans, in concentrations of 4 μM poly(ARG)-PNA and 8 μM miRNA-like cDNA. d) Event type and associated sublevels occurring when PA13 and cDNA(PA13) molecules are added in cis, for 3M KCl, pH = 7. *(Mereuță et al., Anal. Chem 2022, 94)*

In the case of short probe molecules PA5 and PA9 and complementary target molecules added at cis, at negative polarities, the recorded signal is given only by the cationic probe molecules of poly(ARG)-PNA, which are attracted to the nanopore in the electric field, while target DNA molecules and anionic duplexes move in the opposite direction of translocation (

Fig. 2, I. a, b). For the probe molecule PA13 and the target molecule cDNA(PA13), the hybridized duplex is channeled toward the vestibule of the nanopore and will produce in the ionic current the new B_V blocking level (Fig. 2, I.c), but will not be able to translocate through the constriction zone (~1.4 nm).

However, the reversibly locked duplex can partially occlude the constriction zone (B_{L1}) and can follow two pathways: i) either electrophoretic forces acting on the two oppositely charged sequences lead to unzipping of the duplex and promote the translocation of the PA13 probe molecule through the lumen (B_{L2}), ii) either fail to unfold it and thus the duplex returns to the cis environment (Fig. 2, I.d).

Subsequent addition of complementary miRNA-like cDNA molecules to the trans region containing the poly(Arg)-PNA probe molecules resulted in probe-type-dependent blocking events. The B_d level is likely generated by the partial blocking of ion flux by the poly(Arg)-PNA-cDNA duplex (~2.3 nm) trapped at the mouth of the nanopore (~2 nm), before being released back into solution in the tras side.

Act 2.3 - A3.2 Implementation of the nanopore-based protocol for the detection of uni- or multi-nucleobase non-complementarities in miRNA sequences of similar lengths, based on the analysis of the residence and unzipping times of the PNA-miRNA complex inside α-HL.

We tested this nanopore-based method to detect non-complementary nucleic acid sequences and obtained promising results. The data demonstrate that the poly(Arg) sequence of variable length in the structure of the PNA probe molecule can form the basis of a miRNA-like single-stranded DNA molecule detection system based solely on the ability to discriminate the blocking substates given by the hybridized duplex in a heterogeneous mixt.

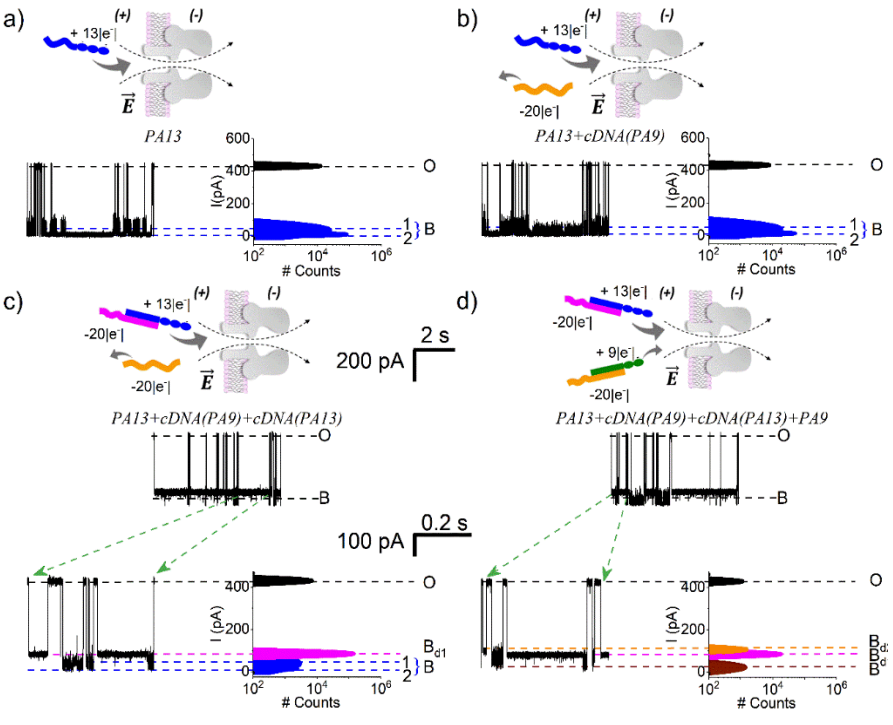

Fig. 3 Multiplex detection of target DNA molecules by α-HL nanopore capture of poly(Arg)-PNA-cDNA hybridized duplexes. (a) The signal given by the interaction of the probe molecules PA13−α-HL measured at ΔV= +140mV (b) remains unchanged in the presence of the non-complementary target molecule cDNA(PA9). (c) Addition of target complementary molecules cDNA(PA13) caused the level of B_{d1} blocking to appear, suggesting capture of PA13-cDNA(PA13) hybridized duplexes. (d) Subsequent pipetting of the PA9 probe molecule, complementary to the cDNA(PA9) already present, generated hybridization of PA9-cDNA(PA9) duplexes producing the Bd2 level. The concentrations of poly(Arg)-PNA probe molecules and DNA target molecules were 4 and 8 μM, respectively, in 3M KCl, pH = 7. *(Mereuță et al., Anal. Chem 2022, 94)*

Act 2.4 - A3.3 Single-Molecule Investigation of the Temperature Dependence of PNA-miRNA Stability, for Non-Complementarity Domains of Different Lengths. Correlation with thermodynamic values of hybridization energies.

The stability of miRNA-like PNA-DNA duplexes is influenced by several factors, including temperature, pH, concentration of the electrophysiological solution KCl, and the length of the constituent sequences. Taking into account that at small temperature variations the system is stable, we analyzed the influence of the length of the poly(Arg) sequence on the detection process of miRNA-like single-stranded DNA target molecules when the molecules are added in the trans side, for 3M KCl, pH = 7, and we calculated the related thermodynamic reaction constants. We observed that the longer the length of the poly(Arg) sequence, the faster the unzipping process takes place due to the increased electric force acting on the complex. In other words, the hybridization energy is higher for the PA9-cDNA(PA9) duplex with the shortest poly(Arg) sequence which experiences a lower electrophoretic unfolding force.

Act 2.5 - A3.4 Determination of the detection limit of miRNA and the dynamic range of the nanosensor, the dose-response curves and the reversibility of the sensor, in solutions compatible with biological environments.

Following the experiments in this study, it was observed that the α-HL biosensor used provides correct results even at very low analyte concentrations, which makes the detection limit of miRNA-like DNA to be of the micromolar order.

In order to perform the detection in solutions compatible with biological environments, we performed experiments in which the electrophysiological solution of KCl has different concentrations: 3M, 1M and 0.5M, the latter being the closest to the concentration of K+ and Cl- ions in the body. We observed that the hybridization and stability of the complexes varied with the concentration of the KCl salt used. *(Asandei et al., Chem. Asian J. 2022, 17)*

It was observed that as the concentration of ions in the solution decreases and the electrical charges are less and less shielded, the number of hybridized duplexes decreases due to the interaction between the positive electrical charge of the poly(Arg) sequence in the PNA structure and the negative electrical charge of the sequences of DNA, preventing hybridization of the complex. Following this stage of the study we concluded that the sample poly(Arg)-PNA molecule containing a larger number of amino acids is suitable for a wide range of concentrations of the recording solution, providing the system with high stability.

We were thus able to fulfill the objectives we proposed and to demonstrate the multiplex detection capacity of the α-HL biosensor, with sensitivity and molecular selectivity, of DNA molecules similar to physiologically relevant miRNAs, following that in the next stage we will optimize this

Results and dissemination of results.

At this stage, a number of **3 articles** with an impact factor were published, 2 of them being in the red zone (Q1):

1. *A Nanopore Sensor for Multiplexed Detection of Short Polynucleotides Based on Length-Variable, Poly-Arginine-Conjugated Peptide Nucleic Acids*, Loredana Mereuta, Alina Asandei, Isabela Dragomir, Jonggwan Park, Yoonkyung Park, and Tudor Luchian, **Analytical Chemistry 2022** 94 (24), 8774-8782, DOI: 10.1021/acs.analchem.2c01587

2. *A Single-Molecule Insight into the Ionic Strength-dependent, Cationic Peptide Nucleic Acids-Oligonucleotides Interactions*, Alina Asandei, Loredana Mereuta, Ioana C. Bucataru, Yoonkyung Park, Tudor Luchian, **Chemistry An Asian Journal 2022**, e202200261, DOI: 10.1002/asia.202200261

3. Probing the Hepatitis B virus e-antigen with a nanopore sensor based on collisional events analysis, Ioana C. Bucataru, Isabela Dragomir, Alina Asandei, Ana-Maria Pantazica, Alina Ghionescu, Norica Branza-Nichita, Yoonkyung Park4, Tudor Luchian, **Biosensors 2022**, 4;12(8):596. DOI: 10.3390/bios12080596.

The results obtained during this stage were presented at **1 international conference** and **1 national conference**:

1. Sixth Edition of International Conference on Analytical and Nanoanalytical Methods for Biomedical and Environmental Sciences, "IC-ANMBES 2022", June 8-10, 2022, Brasov, Romania

- *Detection of nucleobases on short functionalized peptide-nucleic acid sequences using nanopore-tweezing method*, Isabela S. Dragomir, Alina Asandei, Irina Schiopu, Ioana C. Bucataru, Loredana Mereuta, Tudor Luchian
- *Protein nanopore-based method for sequence specific detection of single-stranded DNA using gold nanoparticles and peptide nucleic acids*, Ioana Cezara Bucataru, Loredana Mereuta, Alina Asandei, Isabela Dragomir, Tudor Luchian

2. XVII-th National Conference of Biophysics with International Participation, CNB 2022, 23-25 Septembrie 2022, Târgu Mureș, România

- *A tug-of-war between electric forces: The nanopore-tweezing method applied in molecular sensing*, Isabela S. Dragomir, Alina Asandei, Irina Schiopu, Ioana C. Bucataru, Loredana Mereuta, Tudor Luchian

Date 1.11.2022

Project director,

Prof. Univ. Dr. Tudor LUCHIAN