SCIENTIFIC REPORT
STEP III
2023

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

<u>Step 3</u> - Multiplex analysis of the profiles of different miRNAs molecules in electrolytic solutions. Evaluation of the capability of the α-HL-based nanosensor for the direct multiplex detection of miRNAs in biological samples.

Act 3.1 - A4.1 Optimizing the nanopore capture of the PNA-miRNA duplex by adjusting the length of the arginine polypeptide attached to the PNA molecules and the subsequent determination of the sensitivity to identify the duplex from a solution containing different miRNA molecules.

To optimize the capture of the miRNA-like DNA-PNA duplex by the nanopore, we tested two probe molecules, functionalized with a polypeptide consisting of 5 amino acids, PA5, respectively 9 amino acids of arginine, PA9. Thus, in the *trans* zone of the nanopore, the PA5 sample molecule (**Fig. 1, a**) was initially added, then the non-complementary cDNA target molecule (PA9) **(Fig. 1, b)**, obtaining a specific signal for these nucleic acid sequences (level B in the amplitude histogram), due to the fact that the two are not complementary, so they do not hybridize. Upon addition of the target molecule complementary to the PA5 sequence, no shift occurred in the signal (**Fig. 1, c**), indicating that the molecular duplex PA5-cDNA(PA5) does not interact with the nanopore. A possible explanation is given by the large negative electric charge of the duplex, so that the molecule experiences an electrophoretic force that moves it away from the the nanopore. When PA9 sample molecules are added to the system, an additional Bd level appears (**Fig. 1, d**), specific to the PA9-cDNA (PA9) hybridized complex.

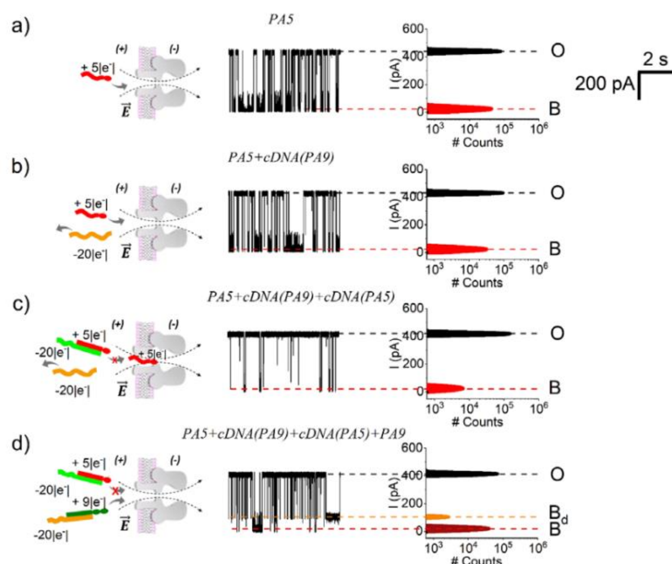

Fig. 1. An indirect method for multiplex detection of short nucleic acid sequences. a) Reversible interactions of PA5 molecules (4 μM) with the α-HL nanopore measured at ΔV = +140 mV and b) upon addition of non-complementary target sequences cDNA(PA9) (8 μM), respectively, c) upon addition of complementary target molecules cDNA(PA5) (8 μM). d) Subsequent addition of PA9 probe molecules (4 μM), complementary to the cDNA(PA9) already present generated an additional blocking state corresponding to hybridized PA9-cDNA(PA9) complexes (Bd). The electrophysiological solution is 3M KCl, pH = 7. *(Mereuţă et al., Anal. Chem 2022, 94)*

Therefore, the method of detecting single-stranded DNA target molecules similar to miRNA using the α-HL nanopore is selective, with a high sensitivity, with micromolar concentrations (8 μM) as the lower limit of detection. We can also say that the sensitivity to identify the duplex in a solution containing different DNA molecules similar to miRNA depends on the number of amino acids in the sequence attached to the probe molecule, so detection occurs when the polypeptide has at least 9 amino acids.

Act 3.2 - A4.2 Quantitative analysis of the capture of poly(Arg)-PNA – miRNA duplexes and their residence times in the nanopore.

To provide additional information, useful in the detection process, we analyzed kinetic parameters that govern the interaction process of the molecules of interest with the nanopore. The poly(Arg)-PNA – DNA similar with miRNA duplexes have an electric dipole structure given by the negative charge of DNA backbone and positive charge of the poly(Arg) sequence, which differ according to the poly(Arg) sequence. We observed that the capture time of the duplexes and their residence times in the nanopore is influenced by the length of poly(Arg) sequence, allowing us to identify them.

Act 3.3 - A4.3 Determination of the main parameters describing the PNA-miRNA duplex association interactions with the α-HL nanopore, in order to quickly and precisely characterize distinct miRNA molecules

One of the main parameters describing the association interactions of the PNA-miRNA duplex with the α-HL nanopore is the reaction constant. The interaction process of molecules with a single nanopore is a stochastic process, which can be modeled as a stationary Markov reaction. Therefore, following the calculations made with the help of the Markov formalism, we were able to determine the reaction constants specific to the interaction process, k1, k2 and k3.

Another essential parameter in the discrimination of miRNA-like DNA molecules is the relative blockage produced by the molecule of interest ($\Delta I_{block}/I_0 = (I_0 - I_{block})/I_0$). We observed that each type of PNA-DNA similar with miRNA duplex has a specific blockage level that can lead to target DNA discrimination.

Act 3.4 - A5.1 Testing the feasibility of the miRNA detection strategy from human sera ('spiked') in which the target miRNA was added.

We tested the detection strategy of miRNA-like single-stranded DNA molecules that is based on protein nanopores and the hybridization property between a PNA probe sequence and a DNA target sequence, using the alamethicin protein pore, together with the pair: target DNA molecule and probe PNA molecule. Alamethicin pores can change the number of monomers in the composition (nanopores have between 4 and 12 monomers), so they can have several conductive states that can be observed in the ion signal (**Fig. 4. Ia**). When alamethicin monomers are functionalized with a PNA nucleic acid probe molecule, the conductance states are altered due to channel blocking by PNA (**Fig. 4. Ib, IIa**). Upon addition to the system of complementary miRNA-like DNA target molecules, the two nucleotide sequences will hybridize, reducing the mobility of alamethicin monomers, and this process will be observed in the recorded ion signal, allowing detection (**Fig. 4. Ic, IIb**).

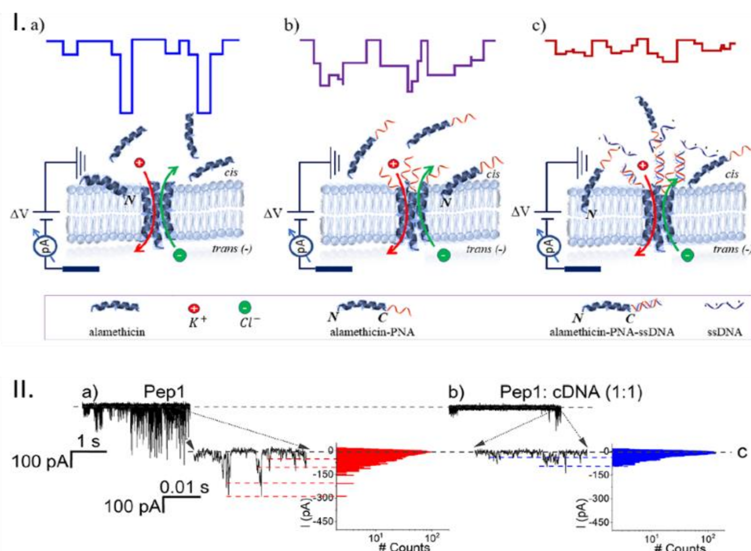

Fig. 4. Detection of target molecules with probe molecules and alamethicin nanopore. I. Schematic representation of the detection principle. II. Original recordings and the related histograms. (*Mereuță et al., ACS Appl. Mater. Interfaces 2023, 15*)

Act 3.5 - A5.2 Testing the reproducibility of the nanopore-based miRNA expression profiling and detection strategy, as well as data correlation with qRT-PCR assays.

qRT-PCR assays are expensive and require extensive processing time, so we turned to a faster and easier to use method, namely UV-VIS spectroscopy. It is known from the literature that hybridized nucleic acid duplexes have an absorption maximum at 260 nm, at neutral pH, which we also observed in the spectrum analysis. Thus, the formation profile of PNA-DNA nucleic acid duplexes as a key point in the detection of miRNA-like DNA molecules can be observed both by electrophysiology experiments and by analyzing UV-VIS spectra.

Act 3.6 - A5.3 Multiplex analysis of the detection profiles of miR-N21, miR-148b, miR-221 and miR-155 in human serum.

Using the hybridization process between the PNA sample molecule and the target DNA molecule, observable with the help of protein nanopores, we were able to determine the multiplex detection profiles of some DNA sequences similar to miRNA molecules (such as miR-N21, miR-148b, miR- 221 and miR-155 from human serum). We started from the signal given by alamethicin monomers functionalized with the probe molecule, Pep1, in a KCl solution with a concentration of 3 M, close to the concentration of KCl in human serum. When the complementary cDNA target molecules are added to the system (molar ratio of 1:1), the signal generated by the alamethicin nanopores is reduced due to the fact that the action of the alamethicin monomers is restricted by the nucleic acid complex formed. Upon addition of a non-complementary target sequence, nDNA, we observe that the resulting signal resembles with the one given by alamethicin monomers functionalized with the probe molecule, indicating that the two nucleic acids do not hybridize.

We thus managed to fulfill the objectives we proposed and to demonstrate the possibility of multiplex analysis of the profiles of different DNA molecules similar to miRNAs, in electrolytic solutions and similar biological solutions.

Results and manner of dissemination of results.

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

1. *Synthetic Receptor Based on a Peptide Antibiotic-Functionalized Chimera for Hybridization-Based Polynucleotide Detection*, Mereuta, Loredana; Asandei, Alina; Schiopu, Irina; Park, Jonggwan; Park, Yoonkyung; Luchian, Tudor, ACS APPLIED MATERIALS & INTERFACES 2023, 15 (27), 33159-33168, DOI:10.1021/acsami.3c06086

2. *Considerable slowdown of short DNA fragment translocation across a protein nanopore using pH-induced generation of enthalpic traps inside the permeation pathway*, Mereuta, Loredana; Asandei, Alina; Andricioaei, Ioan; Park, Jonggwan; Park, Yoonkyung; Luchian, Tudor, NANOSCALE 2023, 15(36), 14754-14763. DOI: 10.1039/d3nr03344a.

Date Project director,

15.11.2023 Prof. Univ. Dr. Tudor LUCHIAN