Habilitation Thesis

Domain: Physics

Uni-molecular transport through protein nanopores used in the recognition, characterization, sequencing and detection of molecules of biological interest

Alina ASANDEI, Ph. D., Senior Research Scientist

'Alexandru Ioan Cuza' University of Iasi, Faculty of Physics,

Iași –2022

Summary of Habilitation Thesis

The habilitation thesis entitled "**Uni-molecular transport through protein nanopores used in the recognition, characterization, sequencing and detection of molecules of biological interest**" includes some of the most important scientific results published by the author since obtaining her PhD. The studies I have summarized demonstrate the remarkable innovation and great potential of the α-HL in the detection, characterization, sequencing and recognition of molecules of physiological interest such as: antibiotics, nucleic acids and small peptides. The thesis is divided into three sections. The first section presents the contributions and scientific results obtained by the authors. The second section presents the author's academic and professional career. And the third section includes the bibliography cited throughout the thesis.

Decades of advances in nanotechnology, biomolecular sciences and protein engineering ushered the introduction of groundbreaking technologies devoted to understanding how matter behaves at the single molecule level. Arguably, one of the simplest in concept is the nanopore-based paradigm, with deep roots in what is originally known as the Coulter counter, resistive-pulse technique. Historically, a nanopore system comprising the oligomeric protein generated by Staphylococcus aureus toxin α-hemolysin (α-HL) was first applied to detecting polynucleotides, as revealed in 1996 by John J. Kasianowicz, et al. Nowadays, a wide variety of other solid-state or protein-based nanopores have emerged as efficient tools for stochastic sensing of analytes as small as single metal ions, handling single molecules, or real-time, label-free probing of chemical reactions at single-molecule level. In our studies, we used the α-HL pore free or complexed with cyclodextrin molecules. A complex system formed by α-HL pore and γ-cyclodextrin (γ-CD) permits the detection of, and differentiation between three different antibiotics from the β-lactam family, based not only on the physical size of the antibiotic, but also of the polarity of the drug, which critically affects its ability to form transient complexes with the caged CD molecule. The data were also used to estimate the standard free energy of binding between ampicillin to γ-CDs binding. Furthermore, efficient measuring of binding thermodynamics of interactions between CDs and antibiotics remains a challenge in the fields of physical chemistry and pharmaceutics. We report on a single-molecule investigation of pH- and voltage-dependent reversible interactions

between ampicillin and γ-CDs, through monitoring of ionic current signatures across an α-HL protein entrapping a γ-CD molecule. Our data reveal that electric and electro-osmotic driving forces alter the reversible reaction rates of ampicillin interaction with γ-CDs, as well as free energy changes accompanying the interaction. We found that close to neutral pH values facilitate more unstable γ-CD ampicillin complexes, as well as a decreased affinity of the γ-CD ampicillin reversible interaction, as compared to acidic pH. We posit that a pH-dependent partial electric charge on the ampicillin molecule and anionic selectivity of the α-HL-γCD complex account for the antibiotic and γ-CD intracavity manifestations. This approach may provide unique alternatives for the characterization of CD guest interactions, useful for pharmaceutical formulations and tunable drug delivery systems.

Continuing the studies with free α-HL protein nanopore– without molecular adapter, it was observed that Achilles heel of this approach is he relatively short dwell time of the analytes inside the nanopore. This hinders the collection of sufficient data required to infer statistically meaningful conclusions about the physical or chemical state of the studied analyte. To mitigate this, two different approaches were successfully applied to slowdown analytes crossing the protein pore. First, we will reveal how the electroosmotic flow can be harnessed to control residence time, direction, and the sequence of spatiotemporal dynamics of a single peptide along the nanopore. This also allows one to identify the mesoscopic trajectory of a peptide exiting the nanopore through either the vestibule or β-barrel moiety. Second, we lay out the principles of an approach dubbed “nanopore tweezing”, enabling simultaneous capture rate increase and escape rate decrease of a peptide from the α-HL, with the applied voltage. At its core, this method requires the creation of an electrical dipole on the peptide under study, via engineering positive and negative amino acid residues at the two ends of the peptide. Concise applications of this approach are being demonstrated, as in proof-of-concept experiments we probed the primary structure exploration of polypeptides, via discrimination between selected neutral amino acid residues. Also, we employed the nanopore tweezing technique to capture amino acid-functionalized peptide nucleic acids (PNAs) with α-HL-based nanopores and correlated the ensuing stochastic fluctuations of the ionic current through the nanopore with the composition and order of bases in the PNAs primary structure. We demonstrated that while the system enables the 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 of single-molecule sequencing. The distinct interactions of the duplex at either end of the nanopore present powerful opportunities for introducing new generations of force-spectroscopy nanopore-based platforms, enabling from the same experiment duplex detection and assessment of interstrand base pairing energy. Thus, we used PNAs that were functionalized with polypeptide chains with a positive charge that, in the presence of complementary DNAs, form PNA-DNA duplexes. The opposite charged state at the polypeptide-functionalized PNA-DNA duplex extremities, facilitated unzipping of a captured duplex at the lumen entry of a voltage-biased nanopore, followed by monomers threading. By employing a kinetic description within the discrete Markov chains theory, we proposed a minimalist kinetic model to successfully describe the electric force-induced strand separation in the duplex. Afterwards single-channel current recordings were used to selectively detect individual ssDNA strands in the vestibule of the α-HL protein nanopore. The sensing mechanism was based on the detection of the intrinsic topological change of target ssDNA molecules after the hybridization with complementary PNA fragments. The readily distinguishable current signatures of PNA-DNA duplexes reversible association with the α-HL’s vestibule, in terms of blockade amplitudes and kinetic features, allows specific detection of nucleic acid hybridization.

Real-time and easy-to-use detection of nucleic acids is crucial for many applications, including medical diagnostics, genetic screening, forensic science, or monitoring the onset and progression of various diseases. In this context, an exploratory single molecule approach for multiplexed discrimination among similar-sized single-stranded DNAs (ssDNA) is presented. The underlying strategy combined (i) a method based on length-variable, short arginine (poly-Arg) tags appended to PNA probes, designed to hybridize with selected regions from complementary ssDNA targets (cDNA) in solution and (ii) formation and subsequent detection with the α-HL nanopore of (poly-Arg)-PNA-cDNA. We discovered that the length-variable poly-Arg tail marked distinctly the molecular processes associated with the nanopore-mediated duplexes capture, trapping and unzipping. This enabled the detection of ssDNA targets via the signatures of (poly-Arg)-PNA-cDNA blockade events. The ionic current blockade signature of free poly(Arg)-PNAs and their

corresponding duplexes with target ssDNAs interacting with a single α-HL nanopore is highly ionic strength dependent, with high salt-containing electrolytes facilitating both capture and isolation of such complexes. Our data illustrate the effect of low ionic strength in reducing the effective volume of free poly(Arg)-PNAs and augmentation of their electrophoretic mobility while traversing the nanopore. We found that unlike in high salt electrolytes, the specific hybridization of cationic moiety-containing PNAs with complementary negatively charged ssDNAs in a low salt concentration is dramatically impeded. We suggested a scenario in which reduced charge screening by counterions in low salt electrolytes enables non-specific, electrostatic interactions with the anionic backbone of polynucleotides, thus reducing the ability of PNA-DNA complementary association via hydrogen bonding patterns. We applied an experimental strategy with spatially-separated poly(Arg)-PNAs and ssDNAs, and present evidence at the single molecule level suggestive of the real-time, long-range interactions-driven formation of poly(Arg)-PNA-DNA complexes, as individual strands entering the nanopore from opposite directions collide inside a nanocavity.

Due to the pressing need to generate specific drugs or vaccines for COVID-19 and management of its outbreak, detailed knowledge regarding the SARSCoV-2 entry into host cells and timely, cheap, and easy-to-use detection methods are of critical importance for containing the SARS-CoV-2 epidemic. Although SARS-CoV-2 binding to ACE2 receptors is widely recognized to be the main step mediating the pathogenesis of COVID-19, nevertheless SARS-CoV-2 spike S1 protein functional properties when interacting with neutral reconstituted lipid membranes and lung epithelial cells triggers the formation of permeation pathways and ion passage across them. Unlike the oversimplified lipid membrane system employed herein, human cells possess a rich compositional and chemical diversity of membrane lipids; however, by virtue of their asymmetry and demonstrated ability to modulate protein function, one may not rule out additional infectivity pathways of the virus in biological cells, as described herein. While the physiological ramifications of our findings revealing the lipid membrane activity of the free SARS-CoV-2 spike S1 subunit are still debatable for SARS-CoV-2 pathogenesis, we stress that a particular characteristic of the SARSCoV-2 S monomer is the existence of a novel furin cleavage site between the S1 and S2 subunits, and the dissociation of the S1 subunit is reckoned to prime the spike for infection. By

employing the α-HL-based nanopore, in our proof-of concept design, we demonstrate the successful deployment of a molecular sensor enabling sensitive detection of the immunological reaction between SARS-CoV-2 spike S1 protein’s RBD and a specific antibody, which may be useful for diagnosing the positive presence of regions of the viral proteomic material and our results may offer new perspectives in understanding the pathogenesis of the SARS-CoV-2 infection, its treatment, and real-time detection. we established a simple to operate and effective setup to specifically detect in a time-resolved manner the SARS-CoV-2 S1 protein subunit in aqueous solution and presence of physiologically relevant molecules. The use of both monoclonal antibodies and the ACE2 receptor, as specific binding substrates, augmented the S1 detection. The method opens the perspective of fast and cheap detection of other S1 proteins containing receptor-binding residues mutations, artificial or natural antibodies in the aqueous sample (e.g., IgG and IgM), testing for efficacy of therapeutics-directed inhibitors (e.g., peptides) to the S proteins of SARS-CoV or related viruses, or enable alternatives for monitoring of the interaction of viral antigens with selected protein targets, relevant for the discovery of decoy therapeutic proteins.

In the second section, the author's academic and professional career is described, bringing into discussion the contributions related to the research and didactic activity. The author is involved in the guidance of students as a coordinator of undergraduate theses as well as a member of doctoral student guidance committees, using her ability to manage interdisciplinary research involving physics, chemistry and biology. Also, being the coordinator or key person of the research projects, she involved the doctoral students in achieving the proposed objectives.