"ALEXANDRU IOAN CUZA" UNIVERSITY OF IAȘI

The Doctoral School of the Faculty of Physics

PhD thesis summary

PHYSICAL DETERMINANTS OF CYTOTOXIC PEPTIDES ACTIVITY IN RECONSTITUTED LIPID SYSTEMS

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The Ph.D. Dissertation Defense of **Sorana Elena Iftemi**, entitled "***Physical determinants of cytotoxic peptides activity in reconstituted lipid systems***", will be held on **30th September 2015**, at 10 AM in "Ferdinand" conference hall.

The Ph.D. Dissertation Committee consist of:

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We invite you to attend this public Ph.D. Dissertation Defense.

CONTENT

I. The motivation of the study of interaction between membrane-active peptides and the lipid-protein systems

The membrane-active peptides are involved in the immun defense against microbial infections, they are found at plants, insects and superior vertebrates. These natural peptides contain 12-50 amino acids in the basic structure, today there are 800 of such compounds in the database. Due to their amino acid composition, amphypaticity, cationic charge and dimension, the peptides attach and they insert into the lipid membranes forming pores[1,2].

II. The membrane-active peptides and their interaction with the artificial lipid membranes

II.1. Structural and functional properties of the artificial lipid membranes

The biological cell membranes are structures with the role of cell isolation environment by maintaining key differences between the extracellular and intracellular composition. The cell membrane controls the entry process nutrients into the cell and the disposal of unused product, generates differences in ionic concentrations between external and internal environment of the cell and participate in the process of cellular communication signals acting as a sensor of external cells[3].

II.2. General characteristics of the membrane-activ peptides

The important structural characteristics of the membrane-active peptides are: net electric charge, helicity, intrinsec hydrophobicity, hydrophobic moment and the effect of the permeability of membranes on the dimension of the polar and hydrophobic domains.

II.3. Destabilization mechanism of the artificial lipid membranes induced by active-membrane peptides

In solution the peptides are generally random coil , they are absorbed at the lipid/aqueous interface forming α-helix structures.

When it reaches a critical concentration of absorbed peptides they are inserted in the bilayer forming transient pores leading to leakage of intracellular material and ultimately to cell death[4]. Figure II.3.1 shows a schematic representation of this mechanism.

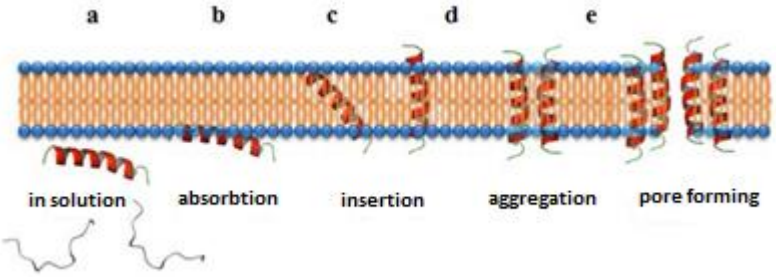

Figure II.3.1. Schematic representation of the steps of interaction between the peptide and the lipid bilayer membrane. With red are represented the peptides with a α-helix secondary structure (adaptation [5]).

So far scientists discovered and characterized two types of transmembrane pores: classics pores and toroidal pores.

The classic pores named „*barrel-stave*" has the core structure formed only by polar parts of the peptides. In the case of the toroidal pores the core structure contains in addition the polar domains of the membranes lipids.

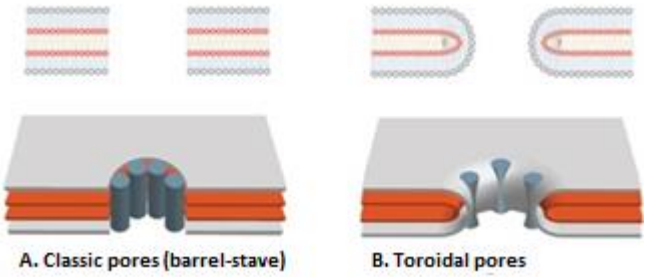

Figure II.3.2. Representative scheme of the transmembrane pores models, barrel-stave (A) and toroidal pores (B) (adaptation after **http://hwhuang.rice.edu/**).

III. Amiloid β peptides roles in human nervous system neurophysiology

III.1. General characteristics of the amiloid β peptides

The abnormal folding of the amiloid-β peptides leads to functions alterations so they interact with each other or with other compounds forming insoluble fibrillar structures. Such structures have been associated with the pathology of many disease.

Accumulation of abnormal amounts of amyloid peptides fibrils lead to amyloidosis. This fibrilar accumulations of amyloid proteins plays an important role in the development of neurodegenerative diseases as Alzheimer's disease, Parkinson etc.

In Alzheimer's disease the tau proteins are abnormal, the microtubules are affected and so the neurons can not function normally (see figure III.2.2).

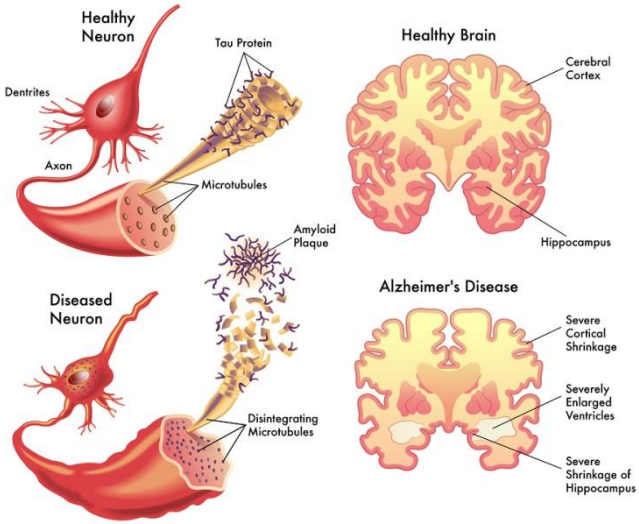

Figure III.2.2. Schematic representation of healthy neuron (top left) and Alzheimer disease affected neuron (left bottom) [adaptation after https://arcpointelkgrovevillage.wordpress.com].

III.3. The effect of the transition metals on the conformation of amiloidic peptides

Previous studies has shown that the implication of the amyloid β peptides in the development of the neurodegenerative diseases involve the ion channels formations of the amyloid oligomers[5].

One of the existing hypotheses that attempt to explain the occurrence of neurodegenerative diseases focus on abnormal accumulation at neuronal level of metals like Cu^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+} which give rise to toxic protein aggregates[6,7].

IV. Structural and functional details of the peptides used in the experimental studies carried out

IV.1. *Characteristics of the antimicrobial peptide trichogin GAIV*

Trichogin GA IV is the main component of the lipopeptaibol family and was first isolated from the soil fungus *Trichoderma longibrachiatum*.

The trichogin GAIV peptide has 11 amino acids:

Oct-Aib[1]-Gly[2]-Leu[3]-Aib[4]-Gly[5]-Gly[6]-Leu[7]-Aib[8]-Gly[9]-Ile[10]-Lol[11], where Oct is n-octanoyl and Lol is leucinol.

Previous studies has shown that similar as the alamethicin (Alm) peptide which is from the same family, the trichogin GAIV peptide forms classic pores (barrel-stave)[8].

It is believed that the presence of the acyl chain from the N-terminal plays an essential role in activity [9].

IV.2. Characteristics of the amiloid β peptide fragment 1-16 secreted by human and rat

Herein I used the human amyloidic fragment $A\beta_{(1-16)}$ which has the following amino acid sequence:

Asp^{1}-Ala^{2}-Glu^{3}-Phe^{4}-Arg^{5}-**His^{6}**-Asp^{7}-Ser^{8}-Gly^{9}-Tyr^{10}-Glu^{11}-Val^{12}-**His^{13}**-**His^{14}**-Gln^{15}-Lys^{16}

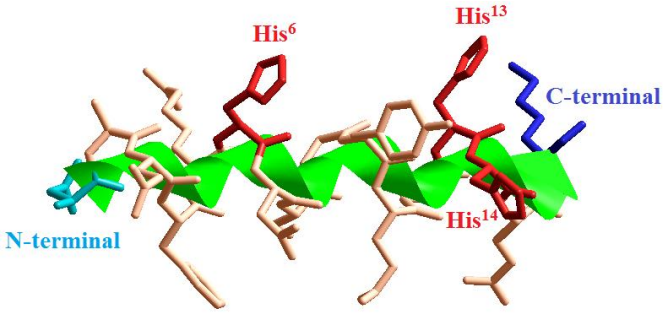

Figure IV.2.1. The secondary idealized structure of the human fragment $A\beta_{(1-16)}$ peptide [modeled in HyperChem].

V. Techniques and metods of investigation of the interaction between membrane-active peptides and lipid-protein systems

V.1. Formation of the artificial lipid membranes using Montal-Mueller method

Herein the lipid bilayers were made using the Montal-Mueller method[3].

The Montal-Mueller method it is schematic represented in the figure V.1.1.

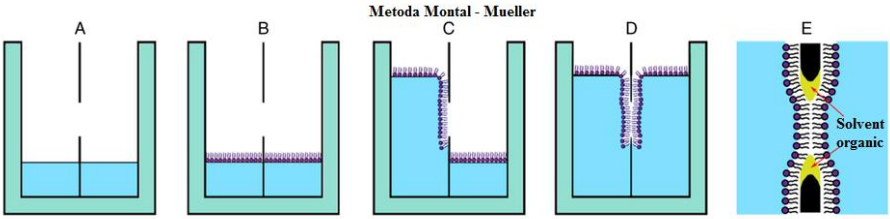

Figure V.1.1. Montal-Mueller method used for recontituition of the artificial lipid bilayers. (a) The electrophysiology solution is added, the solution level remain under the teflon film aperture which separates the two BLM cavities. (b) The lipids are added in the BLM cell; (c) and (d) The electrophysiology solution is added to cover the teflon film aperture; (e) The artificial lipid bilayer is formed at the level of the teflon film aperture which was threated previously with hexadecan-pentan (10%, v/v) used to increase the hydrophoicity of the film [10].

VI. Experimental results

VI.1. The membrane activity study of the antimicrobial peptide trichogin GAIV

In this experiments was used the single molecule electrophysiology tehniques to elucidate the interaction mechanism of the trichogin GAIV peptide with the planar lipidic membrane formed from zwitterionic lipids.

VI.1.1. Hydrophobic mismatch of trichogin GAIV peptide and planar lipid bilayers

The ionic current fluctuations due to the trichogin GAIV peptide interactions with the lipidic bilayer, at an applied potential shows the reversible changes of the membrane conductance from „*close*" state (C) to different „*open*" states (O_1 and O_2) (figure VI.1.1.1).

Conclusions

The uni-molecular analysis of the ionic current records asociated with the trichogin GAIV peptide membrane activity shows the presence of a subsequent conductive state denoted with O_2, which is almost double in the amplitude as compared to the first conductive state O_1. This may be an indication that substate O_2 originates from the superposition of two oligomers present in the membrane in their conductive substate O1.

The minimum number of trichogin GAIV monomers needed to form a single channel is N=3.

Unlike other peptides (for example alamethicin), the trichogin GAIV peptide activity it is observed at the negative and positive applied potential.

The electrically activity of the trichogin GAIV peptide pores is voltage-dependent, meaning that the power density of the ionic current fluctuations induced in the reconstituted lipidic membrane is quantitatively correlated to the voltage applied.

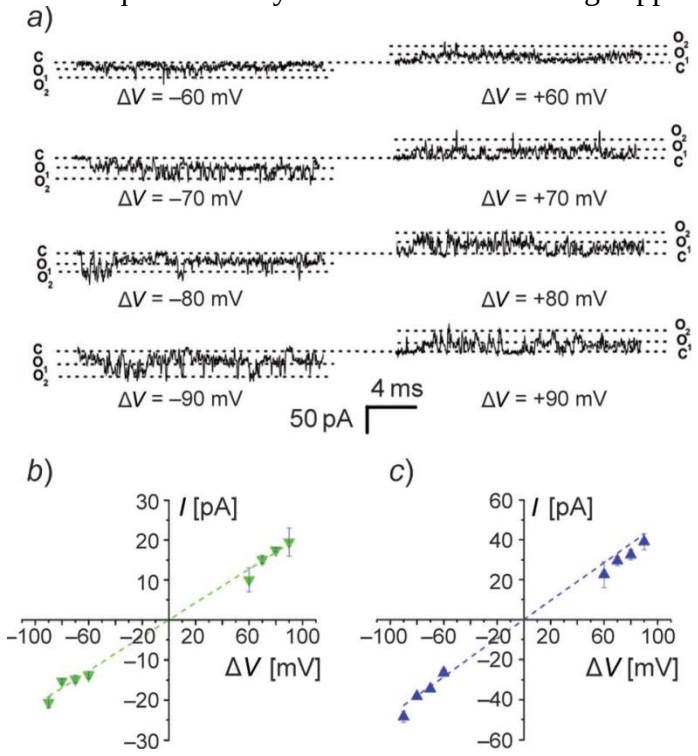

Figure VI.1.1.1. (a) Fluctuations of the ionic current recorded after added a 5 μM of trichogin GAIV peptide in *cis* side of the lipidic membrane. Diagrams of the ionic current corresponding to the first conductive state O_1 (b) and the second conductive state O_2 (c) showing the ohmic behavious of such ionic channels [11].

VI.2. Single molecule investigations of the interactions between the human $A\beta_{(1-16)}$ peptides and transition metals

VI.2.1. The use of α-hemolysin nanopore as a molecular detector of the human $A\beta_{(1-16)}$ peptides conformational changes induced by metal ions (Cu^{2+}, Fe^{2+}, Zn^{3+}, Al^{3+})

Herein, the α-HL protein pore was used as a molecular detector to investigate the conformational changes of the human peptide fragment $A\beta_{(1-16)}$ induced by various metal ions such as: Cu^{2+}, Fe^{2+}, Zn^{3+}, Al^{3+}.

Experimental results and discussions

In the first set of experiments I investigate the human peptide $A\beta_{(1-16)}$ unimolecular interactions with the de α-HL in the presence and absence of metal ions.

After the successfully insertion of the α-HL protein pore and apllying a negative potential the electroforetic transport of the human peptide $A\beta_{(1-16)}$ through the protein pore is facilitated.

The interactions between the peptide and the protein pore are observed by the ion current fluctuations associated with the protein pore blockage by the peptide.

During the course of reversible interactions between the human $A\beta_{1-16}$ peptide and a single α-HL pore, the *trans*-chamber addition of Cu^{2+} , Zn^{2+}, Al^{3+} or Fe^{3+} leads to distinct alterations in the kinetic fingerprint of blockage currents induced by the peptide from those in the absence of metals (figure VI.2.1.2, panel b-d).

We observed that average time of blockage events that reflect dwell times while single peptide resides on the protein pore (τ_{OFF}) (figure VI.2.1.4, panel a), and values of times in-between consecutive peptide-induced blockages events (τ_{ON})

(figure VI.2.1.4, panel b) increased monotonically with the concentration of added Cu^{2+} and Zn^{2+}, while little change ensued following Fe^{3+} and Al^{3+} addition.

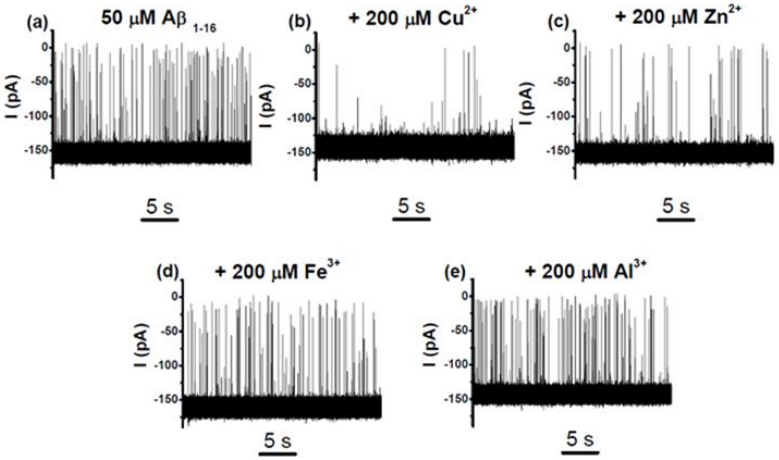

Figure VI.2.1.2. Fluctuations of the ionic current induced by the reversible interactions between the human peptide $A\beta_{(1-16)}$ and the α-HL pore in the absence of the metalic ions (a) and the presence of Cu^{2+} (b), Zn^{2+} (c), Fe^{3+}(d) și Al^{3+} (e) ions [12].

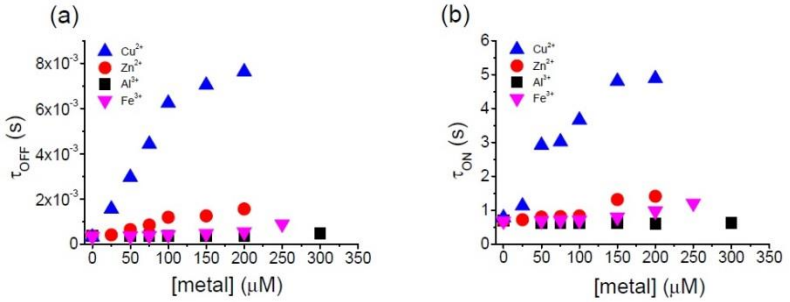

Figure VI.2.1.4. Dwell times τ_{off} and τ_{on} dependence of the metal ions concentration added in the system [13].

Conclusions

Our results demonstrate the potential of the single-molecule level kinetic analysis of metals–Aβ interaction, based on the unique current signatures through the α-HL protein immobilized in a lipid membrane which ensue following the reversible docking of 'metal-free' and 'metal-bound' $A\beta_{1-16}$ with the α-HL protein.

The kinetic analysis of the frequency of blockage events entailed by $A\beta_{1-16}$ - α-HL interactions in the presence of metals with augmented affinity towards the peptide (e.g., Cu^{2+} and Zn^{2+}), allowed us to quantify dissociation constants of Cu^{2+} and Zn^{2+} binding to the peptide.

VI.2.2. Comparative study of human and rat $A\beta_{(1-16)}$ peptides interactions with Cu^{2+} ions

Herein, we used the truncated more soluble $A\beta_{1-16}$ isoforms derived from human and rat amyloid peptides to explore their interaction with Cu^{2+}, by employing the membrane-immobilized α-hemolysin (α-HL) protein as a nanoscopic probe in conjunction with single-molecule electrophysiology techniques.

Experimental results

When added on the *trans* side of a planar lipid membrane containing a single α-HL protein inserted, and subjected to a negative (*trans*) potential, the human $A\beta_{1-16}$ is electrophoretically driven into the lumen of the protein pore and produces reversible blockages of the protein-mediated ionic current.

The additional kinetic analysis demonstrated that average times corresponding to in-between current blockage events (τ_{ON}), caused by either a 'Cu^{2+} - free' or a 'Cu^{2+} - complexed' peptide trapped within the protein lumen (figure VI.2.2.1), as well as the average duration of blockage events (τ_{off}), increased as the concentration of Cu^{2+} was being raised in the *trans* chamber.

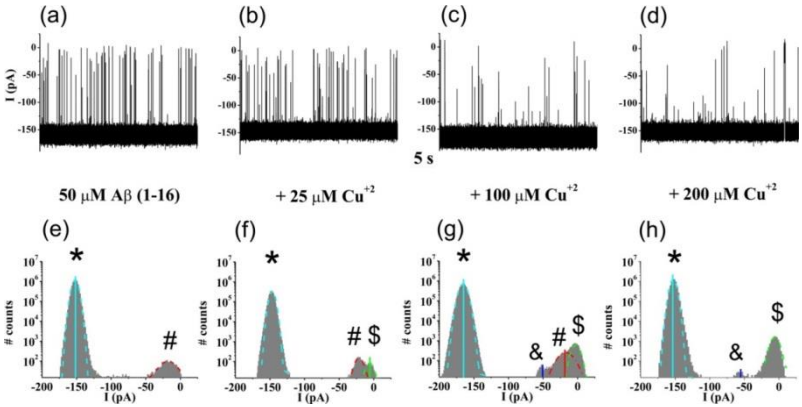

Figure VI.2.2.1. Typical single-channel current traces reflecting the uni-molecular interaction between the human $A\beta_{1-16}$ peptide with a α-HL pore, in the absence (panel a) and presence of *trans*-added Cu^{2+} at various concentrations (panels b, c and d) [13].

Data corresponding to control experiments (no Cu^{2+} added) have revealed that both τ_{ON} and τ_{OFF} can be fitted to exponentially decaying functions with increasing negative potential values (figure VI.2.2.4, panel a și b).

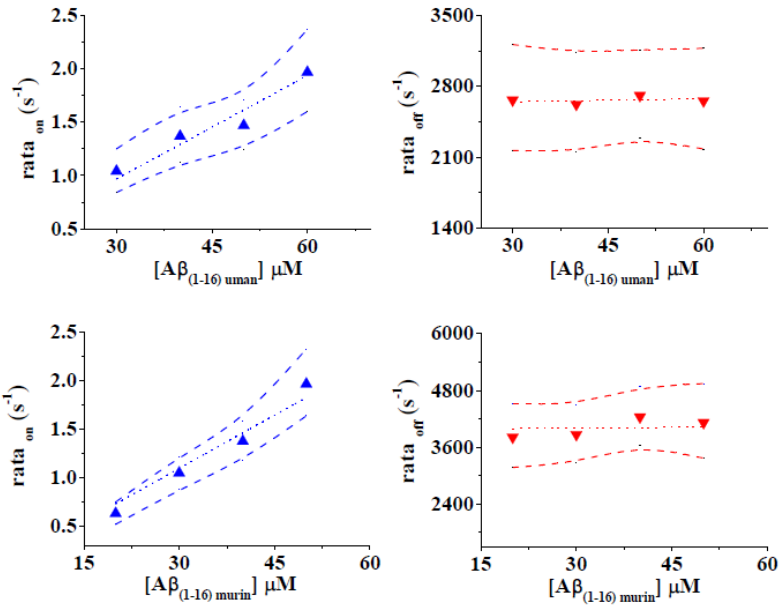

Figure VI.2.2.4. $Rate_{on}$ and $rate_{off}$ dependence of the increasing concentration of human and rat $A\beta_{(1-16)}$ peptides[13].

In contrast with the experimental data obtained with the human $A\beta_{(1-16)}$ peptide (figure VI.2.2.1), an increase concentration of Cu^{2+} ions added in *trans* side of the lipidic membrane, leads to a higher frequency of the blockages events associated with the interaction between the rat $A\beta_{(1-16)}$ peptide and the proteic pore.

Conclusions

The association reaction constant of the Cu^{2+} - free rat $A\beta_{1-16}$ fragment is almost double as compared to the human $A\beta_{1-16}$ peptide, and this may be indicative of a more flexible, random-coil-like conformation of the rat $A\beta_{1-16}$ fragment, which allows it

to find faster its optimal spatial configuration that allows is to enter the protein pore.

From the experimental data obtained here it is clear that the Cu^{2+} ions has a higher influence on the interactions between human $A\beta_{(1-16)}$ peptide with the α-HL pore as compared with the rat $A\beta_{(1-16)}$ peptide.

This study demonstrates the potential of the α-HL protein nanopore-based approach as a molecular detector of the conformational changes of amyloidic peptides induced by various metal ions.

Based on such peptides, the results from this experiments may contribute to the development of a easy detection and recognition method of ions or other small molecules (for example inhibitors of oligomerized Aβ peptide), based on proteic nanopores.

VI.3. The use of α-hemolysin proteic pore in stereoselective interactions detection of Cu^{2+} and histidine amino acid enantiomer

VI.3.1. Single molecule investigation of the conformational changes of $A\beta_1$, $A\beta_4^{H13dH}$, $A\beta_5^{H6dH}$ mutants induced by Cu^{2+} ions

Herein, we used the α-HL proteic pore as a nanosensor for the detection of the interactions between mutants of the amyloidic peptides and Cu^{2+} ions, in order to investigate the effect of the Cu^{2+} ions on the biding sites formed with the L,D-histidine amino acids from the peptides sequency.

The peptide added in the system leads to ionic currents blockages of the proteic pore, the value of the ionic current can be

determined by analysing the amplitude of the ionic current associated with the interactions between the peptides and the proteic pore.

By analysing the average time of blockage events (τ_{off}) and values of times in between of consecutive peptide-induced blockage events (τ_{on}) the asociating rate ($rate_{ON}$) and disociating rate ($rate_{OFF}$) (figure VI.3.2) can be determined. This rates describe the reversible interactions between the peptides and the proteic pore.

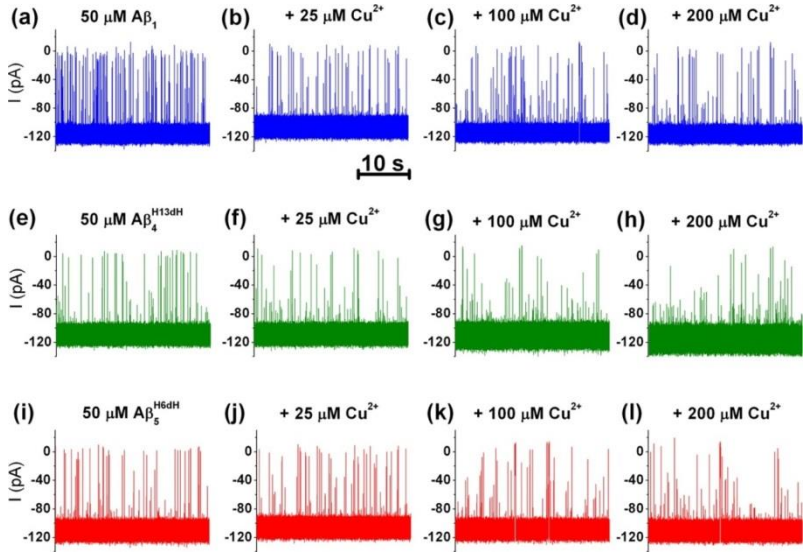

Figure VI.3.1 Representative electrophysiology data showing the unimolecular reversible interactions between the tested peptides and the α-HL pore, in the absence (panels a, e, and i) and presence of increasing concentrations of trans-added Cu2+ [14].

The Cu2+-induced changes of peptide-induced blockade events are fully reversible, since addition of excess EDTA on

the trans side of the membrane fully recovers the activity seen in the absence of Cu2+.

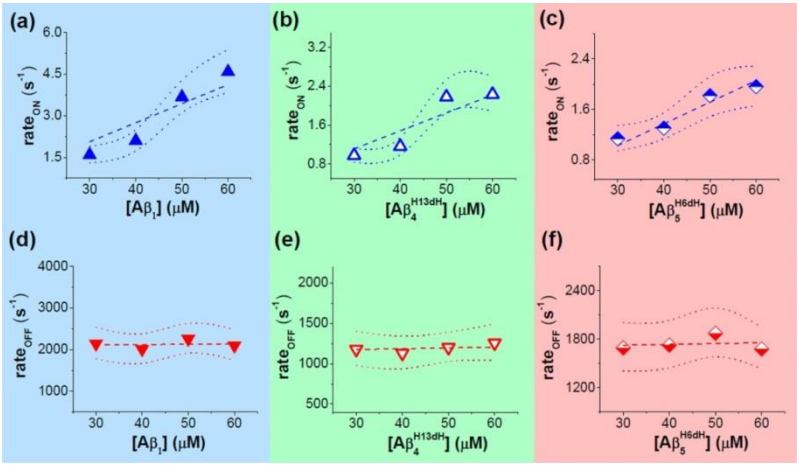

Figure VI.3.2. Rate$_{ON}$ and rate$_{OFF}$ dependence of the mutants peptides concentration (Aβ$_1$, Aβ$_4^{H13dH}$, Aβ$_5^{H6dH}$) [14].

Conclusions

This is the first report which demonstrates the proof-of-concept of a protein nanopore based, single-molecule sensing platform potential to assess the metal affinity to distinct chiral environments provided by the amino acid enantiomers.

The relative orientation of the L/D imidazole rings and the metal coordination bond length act as a conformational switch to induce and stabilize certain conformations of the peptide metal-binding pocket, and thus alter the metal affinity toward peptides.

The Cu^{2+} affinity for the tested peptides increase as L-His is replaced with its D-enantiomer, with the effect being more prevalent when such changes were inflicted on the His-6 residue.

These results underlines the potential of the α-HL pore to detect different peptides which has the identical chemical composition but with different biding sites containing amino acids enantiomers. This detection is possible by analysing the blockages events asociated with the interaction of the peptides with the α-HL pore.

The presence of the metal ions can be detected through the conformational changes of the peptide-complexed in the interaction with the α-HL pore.

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