

Nanopore Detection of Nerve Agent Degradation Products

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ABSTRACT

Due to the terrorist attacks in recent years, the fight against terrorism has become an international priority. One of the urgent needs in combating terrorism is to improve the current capability of detecting and identifying terrorist agents accurately and rapidly. Here, we report a stochastic nanopore sensing method for the detection of nerve agent degradation products. A multi-functionalized α -hemolysin protein ion channel embedded in a planar lipid bilayer, with a host molecule β -cyclodextrin lodged in the lumen of the channel as a molecular adapter, was used as the sensing element. The soman and cyclosarin degradation products, i.e., cyclohexyl methylphosphonic acid (CMPA) and pinacolyl methylphosphonate (PMPA), could be identified and quantified at nanomolar level. In addition, the significantly different signatures (e.g., residence times and amplitudes) of CMPA and PMPA events permitted their convenient differentiation and even the potential for simultaneous detection in aqueous environments.

Keywords: nanopore, stochastic sensing, nerve agents, α -hemolysin, degradation products

1 INTRODUCTION

Stochastic sensing can successfully detect analytes at the single-molecule level by using a single nanopore, which can be obtained from a biological ion channel by self-assembly or artificially fabricated in a solid-state membrane. Nanopore detection is achieved by monitoring the ionic current modulations induced by the passage of analytes of interest through the nanopore at a fixed applied potential. Individual binding events are detected as transient blockades in the recorded current. In this way, the concentration of the analyte can be obtained by the frequency of occurrence ($1/\tau_{\text{on}}$) of the recorded blockage events, while the mean residence time (τ_{off}) of the analyte coupled with the extent of current blockage (amplitude) allows to determine its identity [1]. Nanopore stochastic sensors have been developed as an effective platform to detect a wide variety of substances, including organic molecules [2], cations [3], anions [4], enantiomers [5], proteins [6], and terrorist agents [7-10]. In addition, it has been clearly established that these nanometer-sized pores offer exciting new possibilities for studying biomolecular folding and unfolding [12], investigating covalent and non-covalent bonding interactions [13-15], probing enzyme kinetics [16], as well as analyzing and even sequencing DNA molecules [17, 18].

Recently, our group developed a stochastic sensing strategy to detect organophosphorus chemical agents (i.e. nerve gases), which are the most toxic group in chemical warfare agents. They can irreversibly bind to acetylcholine esterase, thus disrupting the nervous system. In this sensing system [18], an engineered α -hemolysin (α HL) (M113F/K147N)₇ protein pore, with the host molecule β CD lodged in the lumen of the channel as a molecular adapter, was used as the sensing element, while CMPA and PMPA, hydrolysis products of GD and GF [19], respectively, were employed as nerve agent simulants. Using this (M113F/K147N)₇ protein pore / β CD sensing system, PMPA and CMPA could be identified with detection limits of 53 nM and 102 nM, respectively. Furthermore, the nanopore sensor was very selective to PMPA and CMPA; sarin, tabun, and VX hydrolysis products, as well as other common pesticides, would not interfere with detection of these analytes. However, PMPA and CMPA were difficult to be differentiated in the (M113F/K147)₇ pore because they produced current modulation events with very similar mean residence times and blocking amplitudes. In this work, a nanopore sensor using a multi-functionalized α HL protein was successfully developed for the differentiation of CMPA and PMPA. Note that the development of a sensor with an exceptionally low false alarm rate, i.e., with the ability to detect terrorist agents rapidly and accurately in the presence of common interferences is of high importance in combating terrorism.

2 EXPERIMENTAL SECTION

2.1 Materials and Reagents

1,2-diphytanoylphosphatidylcholine was purchased from Avanti Polar Lipids (Alabaster, AL), while *n*-pentane was obtained from Burdick & Jackson (Muskegon, MI). Teflon film (25 μ m thick) was bought from Goodfellow (Malvern, PA). CMPA was purchased from Cerilliant Corporation (Round Rock, Texas). PMPA was obtained from Sigma (St. Louis, MO). All these analytes were dissolved in HPLC-grade water (ChromAR, Mallinckrodt chemicals) with the concentrations of all the stock solutions were 10 mM each. All other reagents were purchased from Sigma (St. Louis, MO).

2.2 Mutagenesis

Mutant α HL genes (M113K, and M113Y-D8) were constructed by side-directed mutagenesis with a WT α HL

gene in a T7 vector (pT7- α HL), which has been described elsewhere [12].

2.3 Formation of Protein Pore

M113K, and M113Y-D8 monomers were first synthesized by coupled *in vitro* transcription and translation (IVTT) using the *E. Coli* T7 S30 Extract System for Circular DNA from Promega (Madison, WI). Subsequently, rabbit red cell membranes were mixed with mixtures of M113K and M113Y-D8 monomers with different ratios. This allows different heteroheptamers to be assembled by bias assembly after incubating for 2 h. The heteroheptamers were purified by SDS-polyacrylamide gel electrophoresis based on their different gel shifts caused by the C-terminal extensions of eight aspartate residues (the “D8” tail) and stored in aliquots at -80°C .

2.4 Planar Bilayer Recording

Single-channel recordings were carried out as described at $22 \pm 1^{\circ}\text{C}$ [9]. The *cis* and *trans* compartments of the chamber were separated by a Teflon septum. An aperture (150 μm) in the septum was pretreated with 10% (v/v) hexadecane in *n*-pentane. A bilayer of 10 mg/mL 1,2-diphytanoylphosphatidylcholine in *n*-pentane was formed on the aperture. The formation of the bilayer was achieved by using the Montal-Mueller method [20], and monitored by using a function generator (BK precision 4012A; Yorba Linda, CA). The experiments were performed under symmetrical buffer conditions with each compartment containing a 2.0 mL solution of 1 M NaCl and 10 mM Na_2HPO_4 (pH 7.5). Unless otherwise noted, the α HL protein was added to the *cis* compartment, which was connected to “ground”, while β -cyclodextrin (β CD) and PMPA (or CMPA) were added to the *trans* compartment. In such a way, after insertion of a single α HL channel, its mushroom cap would be located in the *cis* compartment, while the β -barrel of the α HL would insert into the lipid bilayer and connect with the *trans* of the chamber device. The final concentration of the α HL protein was 0.2–2.0 $\text{ng}\cdot\text{mL}^{-1}$. The transmembrane potential, which was applied with Ag/AgCl electrodes with 3% agarose bridges (Sigma) containing 3 M KCl (EMD Chemicals Inc; Darmstadt, Germany), was -120 mV, unless otherwise noted. A negative potential indicates a lower potential in the *trans* chamber of the apparatus. Currents were recorded with a patch clamp amplifier (Axopatch 200B, Molecular Devices; Sunnyvale, CA, USA). They were low-pass filtered with a built-in four-pole Bessel filter at 5 kHz and sampled at 25 kHz by a computer equipped with a Digidata 1440 A/D converter (Molecular Devices). To shield against ambient electrical noise, a metal box was used to serve as a Faraday cage, inside which the bilayer recording amplifying headstage, stirring system, chamber, and chamber holder were enclosed.

2.5 Data analysis

Data were analyzed with the following software: pClamp 10.0 (Molecular Devices) and Origin 7.0 (Microcal, Northampton, MA). Conductance values were obtained from the amplitude histograms after the peaks were fit to Gaussian functions. τ_{on} and τ_{off} values for the analytes were obtained from dwell time histograms by fitting the distributions to single exponential functions by the Levenberg-Marquardt procedure.

2.6 Molecular Graphics

The model of $(\text{M113K})_3(\text{M113Y-D8})_4$ was derived from the structure of the wild-type α HL pore (PDB: 7AHL) with the “mutate” function of SPOCK 6.3. Mutations were performed by reading the new amino acid from the library in the $\$$ SP_AALIB directory, and superimposing the $\text{C}\alpha\text{-C}\beta$ bond onto the wild-type residue based on Mackay’s quaternion method.

3 RESULTS AND DISCUSSION

3.1 Nanopore Sensing Element

The initial experiment was performed in a buffer solution containing 1 M NaCl and 10 mM Na_2HPO_4 (pH 6.5) in the presence of 40 μM β CD at an applied voltage bias of +120 mV. Various α HL heteroheptamer pores, including $(\text{M113K})_1(\text{M113Y-D8})_6$, $(\text{M113K})_2(\text{M113Y-D8})_5$, $(\text{M113K})_3(\text{M113Y-D8})_4$, $(\text{M113K})_4(\text{M113Y-D8})_3$, $(\text{M113K})_5(\text{M113Y-D8})_2$, and $(\text{M113K})_6(\text{M113Y-D8})_1$, were tested with CMPA and PMPA. The experimental results demonstrated (data not shown) that, among these six different mutant heteroheptamer protein pores, the $(\text{M113K})_2(\text{M113Y-D8})_5$ and $(\text{M113K})_3(\text{M113Y-D8})_4$ pores provided the potential for the differentiation and even simultaneous detection of CMPA and PMPA since these two nerve agent hydrolytes produced events with significantly different residence times and/or current blockage amplitudes in these two channels. The $(\text{M113K})_3(\text{M113Y-D8})_4$ pore (Fig. 1A) was chosen as the stochastic sensing element in the remaining experiments since the difference in the event signatures between CMPA and PMPA in the $(\text{M113K})_3(\text{M113Y-D8})_4$ pore was larger than that in the $(\text{M113K})_2(\text{M113Y-D8})_5$ pore. It should be noted that there are four possible permutations for the $(\text{M113K})_3(\text{M113Y-D8})_4$ pore (Figs. 1B–1E). However, both the values of the open channel currents and the residence times of CMPA and PMPA events did not change significantly in six replicate experiments, which suggests that either the $(\text{M113K})_3(\text{M113Y-D8})_4$ pore has only one dominant permutation under our experimental condition, or the four possible permutations would not affect the binding of CMPA / PMPA to the host β CD molecule significantly.

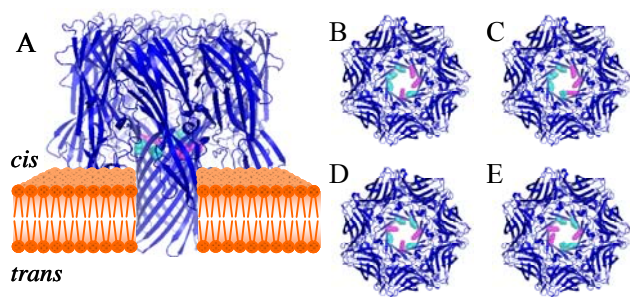


Figure 1: Molecular graphics representation of the staphylococcal α HL protein. (A) Side view of the $(M113K)_3(M113Y-D8)_4$ pore; and (B-E) View into the $(M113K)_3(M113Y-D8)_4$ pore from the *cis* side of the lipid bilayer, highlighting the position 113 (purple and cyan), where the naturally occurring Met residues have been substituted with Lys (purple) and Tyr (cyan). β CD was not displayed in the figure for better viewing of the four possible different permutations of the mutant α HL $(M113K)_3(M113Y-D8)_4$ pore.

3.2 Voltage Effect on Sensor Resolution

Using the $(M113K)_3(M113Y-D8)_4$ pore, the voltage effect on the detection of $10 \mu\text{M}$ PMPA was investigated in the presence of $40 \mu\text{M}$ β CD with an applied potential ranging from -40 mV to -200 mV . With an increase in the applied voltage, the mean residence times of both the host molecule β CD and PMPA events decreased, while both their event frequencies increased (data not shown). Furthermore, the amplitude of the analyte PMPA events increased. For the sensitive and convenient detection of PMPA, large values of the event frequency and mean blockage amplitude for PMPA as well as large values of the event residence time and frequency for the host molecule β CD are desired. Considering that the lipid bilayer used in the experiments would become less stable with the increase of the applied potential, -120 mV was used as the optimum applied voltage for our sensor system since all the three parameters (i.e., dwell time, event frequency, and amplitude) had relatively large values and also the sensor is quite stable at this potential.

3.3 pH Effect on Sensor Resolution

To investigate whether the pH can be used as an effective approach to significantly increase the resolution and sensitivity for the stochastic detection of PMPA and CPMA, PMPA detection with the $(M113K)_3(M113Y-D8)_4$ pore was performed in a series of NaCl solutions at different pH values, including 3, 4, 5, 6.5, and 7.5, in the presence of $40 \mu\text{M}$ β CD at -120 mV . Our experiments demonstrated (data not shown) that, although the residence time and frequency of the PMPA events did not change significantly with an increase in the pH of the solution, both the residence time and frequency of the host β CD events

increased drastically. Furthermore, a larger open channel value and hence a larger current blockage amplitude for the PMPA events were observed in a buffer solution with a lower pH value. This suggests that a lower pH buffer solution offers the potential for a higher nanopore resolution. Note that an increase in the residence time and frequency of β CD events also facilitates data analysis. To this end, the feasibility of utilizing the $(M113K)_3(M113Y-D8)_4$ pore to differentiate and even simultaneously detect PMPA and CMPA was then carried out at pH 3. Our experimental results showed that the event residence time and amplitude for PMPA were $1.4 \pm 0.1 \text{ ms}$, and $26.0 \pm 2.0 \text{ pA}$, respectively, while those were 0.20 ± 0.10 , and $22.0 \pm 1.0 \text{ pA}$, respectively for CMPA (Fig. 2), thus suggesting that CMPA and PMPA could be conveniently differentiated. In addition, a mixture of CMPA and PMPA solutions produced two types of events in the pore with amplitudes at 21.5 pA and 25 pA , respectively, suggesting that PMPA and CMPA could be potentially simultaneously detected and even quantified.

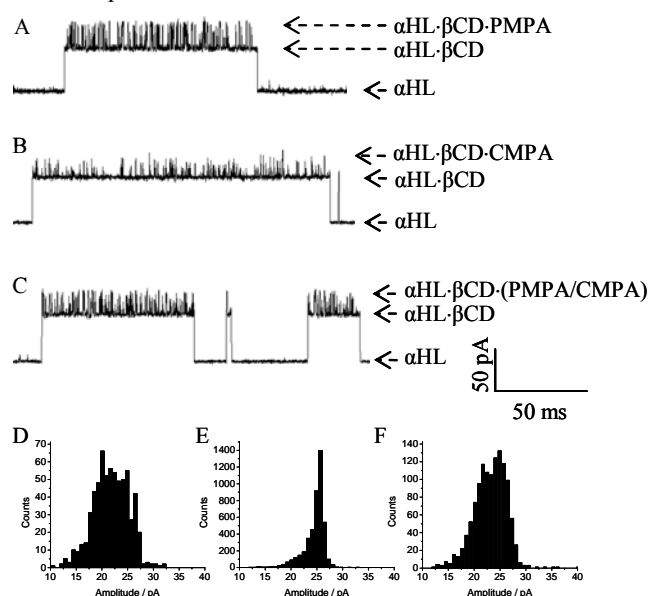


Figure 2: Differentiation of PMPA and CMPA. A-C) Single-channel traces of $10 \mu\text{M}$ PMPA, $30 \mu\text{M}$ CMPA, and a mixture of $30 \mu\text{M}$ CMPA and $10 \mu\text{M}$ PMPA, respectively. D-F) The corresponding event amplitude histograms for the traces shown in Figs. 2A-2C. The experiments were performed in the $(M113K)_3(M113Y-D8)_4$ pore in a solution containing 1 M NaCl and 10 mM Na_2HPO_4 (pH 3).

3.4 Salt Effect on Nanopore Sensitivity

It has been reported that the resolution and sensitivity of the nanopore stochastic sensor could be significantly enhanced by employing the salt effect [12]. For this purpose, PMPA detection in the $(M113K)_3(M113Y-D8)_4$ pore was carried out in a series of NaCl solutions at different concentrations, including 1 M , 2 M , and 3 M . Our experiments demonstrated (data not shown) that, with the

increase of NaCl concentration, the mean residence time of the host β CD events decreased so significantly that a drastic decrease in the frequency of PMPA events was observed, and hence a reduced sensor resolution was obtained. Note that employing a very low concentration of salt as the supporting electrolyte is also not a feasible approach to increase the nanopore resolution. In this case, with a decrease in the salt concentration, a smaller open channel current and hence a smaller event blockage amplitude will result in a smaller difference in the signatures between PMPA and CMPA.

3.4 Dose Response

To demonstrate the nanopore sensor to be of analytical use, the effect of the concentration of PMPA on the event frequency ($1/\tau_{on}$) was investigated. Since the event frequency ($1/\tau_{on}$) was linearly related to the concentration of PMPA (Fig. 3), this nanopore sensor has a potential to be developed as a rapid and sensitive method to detect organophosphates in an aqueous environment at the single-molecule level.

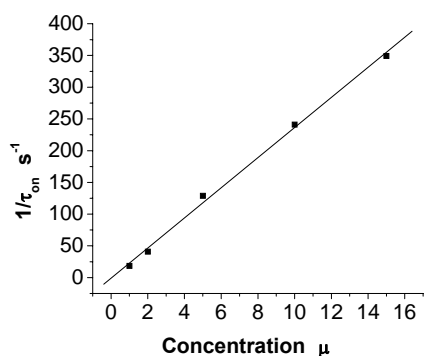


Figure 3: Plot of $1/\tau_{on}$ as a function of PMPA concentration.

4 CONCLUSIONS

A multi-functionalized α HL protein pore was employed successfully for the rapid detection and differentiation of organophosphorus nerve agent hydrolytes PMPA and CMPA. This is the first time to use a multi-functionalized α HL protein pore as the stochastic sensing element in the nanopore field. This approach opens up new strategies for future nanopore sensor design.

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