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Simultaneous detection of CMPA and PMPA, hydrolytes of soman and cyclosarin nerve agents, by nanopore analysis

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ABSTRACT

A sensitive nanopore-based analytical method was developed for the detection of cyclohexyl methylphosphonic acid (CMPA) and pinacolyl methylphosphonate (PMPA), hydrolytes of nerve agents soman and cyclosarin, respectively. The method uses a multi-functionalized α-hemolysin protein ion channel as the sensing element, with a host molecule β-cyclodextrin lodged in the lumen of the channel as a molecular adapter. The capture and release of CMPA/PMPA by the β-cyclodextrin host caused current modulations in the nanopore. Since the event residence times and amplitudes were significantly different, CMPA and PMPA could conveniently be differentiated and simultaneously quantitated.

1. Introduction

Nanopore-based stochastic sensors can detect analytes at the single-molecule level, offering the potential as a highly sensitive, rapid, and multi-functional sensing platform. By monitoring the ionic current modulations induced by the passage of the analyte of interest through a single nanopore at a fixed applied potential, information about the identity and concentration of the analyte could be revealed, the former from the mean residence time (τ_{off}) of the analyte coupled with the extent of current blockage (amplitude), while the latter from the frequency of occurrence ($1/\tau_{on}$) of the recorded blockage events [1]. In addition to the development of ultrasensitive sensors for a wide variety of substances at the single-molecule level [2–11], these nanometer-sized pores have been used to investigate covalent and non-covalent bonding interactions [12–14], probe enzyme kinetics [15], study biomolecular folding and unfolding [16,17], and analyze DNA molecules [18–29].

Organophosphorus chemical agents, commonly known as nerve gases, are the most toxic group in chemical warfare agents. These compounds were developed just before and during World War II and are chemically related to the organophosphorus insecticides. They disrupt the nervous system by irreversibly binding to acetylcholine esterase, an enzyme that relaxes the activity of the neurotransmitter acetylcholine. Thus far, although various methods have been developed for the detection of these nerve agents or their simulants [30–42], most of them cannot satisfy our needs in terms of sensitivity, selectivity, portability, low cost, ease of use, and rapid response. A good overview of the current analytical approaches was recently provided by Jenkins and Bae [36]. Whereas techniques such as HPLC and GC/MS offer the requisite sensitivity, they are relatively expensive and not easily portable. Additionally, the complex procedures involved are not conducive to their deployment as early warning detectors. In contrast, real time detection methods, such as those involving QCM and SAW technologies, lack specificity thus generating false positive signals such as those created by chemically similar, but far less toxic compounds (e.g., many common pesticides). Biologically based sensors involving enzymes and antibodies, while providing generally high sensitivity and good specificity, also suffer from time delays as well as stability and one-time use concerns. More recent advances involving Ion Mobility Spectrometers (IMS) represent an interesting approach and one certainly worthy of further evaluation. The IMS approach is sensitive, selective, and portable. However, it has a

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limited linear dynamic range and poor resolution, and is subject to interferences from complex matrices although recent studies suggest that these weaknesses or limitations could potentially be overcome [42].

In our previous study [43], we have developed a stochastic sensing system to detect nerve agent simulants CMPA and PMPA, which are hydrolysis products of GD and GF [44], respectively. The sensing element was an engineered α-hemolysin (αHL) $(M113F/K147N)_7$ protein pore, and the host molecule βCD was used as a molecular adapter. Although this sensing system was sensitive (capable of detecting nanomolar PMPA/CMPA) and highly selective (without interference from other organophosphates), PMPA and CMPA produced current modulation events with very similar mean residence times (0.83 ms versus 0.62 ms) and blocking residual currents (0.08 pA versus 0.31 pA), and hence were difficult to be differentiated. It should be noted that, although poisoning by various nerve agents has relatively the same type of symptoms and receives the same emergency treatments (e.g., atropine or oximes), the capability to the rapid differentiation of one nerve agent against another is highly important. It has been reported that some antedotes are ineffective for certain nerve agents. For example, obidoxime is effective with sarin and VX, but ineffective against cyclosarin. Oxime HI 6 has been shown to be able to reactivate acetylcholine esterase inhibited by cyclosarin, but is ineffective with tabun [45]. Recently, we found that by using a multi-functionalized heteroheptameric αHL protein $(M113K)_3(M113Y\text{-}D8)_4$ pore, the difference between the residence times and amplitudes of PMPA and CMPA events became much larger, permitting the convenient differentiation between PMPA and CMPA [46]. In this work, we investigate whether the enhanced sensor resolution of the multi-functionalized heteroheptameric αHL protein nanopore approach could be utilized for the simultaneous detection and quantification of CMPA and PMPA mixture.

2. Experimental

2.1. Materials and reagents

n-pentane was purchased from Burdick & Jackson (Muskegon, MI). 1,2-Diphytanoylphosphatidylcholine was obtained from Avanti Polar Lipids (Alabaster, AL). Teflon film (25 μm thick) was bought from Goodfellow (Malvern, PA). Except for CMPA, which was obtained from Cerilliant Corporation (Round Rock, Texas), all other reagents, including PMPA, were purchased from Sigma-Aldrich (St. Louis, MO). Both PMPA and CMPA were dissolved in HPLC-grade water (ChromAR, Mallinckrodt chemicals) with the concentrations of all the stock solutions were 10 mM each. Both hexadecane [10% (v/v)] and 1,2-diphytanoylphosphatidylcholine (10 mg/mL) were dissolved in *n*-pentane.

The production of the mutant αHL genes and the assembly of the heteroheptameric αHL pores have been described elsewhere [43,46]. Briefly, M113K and M113Y-D8 genes were constructed by site-directed mutagenesis with a WT αHL gene in a T7 vector (pT7-αHL). The heteroheptameric M113K/M113Y-D8 pores were obtained by co-assembling M113K and M113Y-D8 αHL subunits in various ratios (from 1:6 to 6:1) on rabbit erythrocyte membranes. The resulting heteroheptamers, $(M113K)_1(M113Y\text{-}D8)_6$, $(M113K)_2(M113Y\text{-}D8)_5$, $(M113K)_3(M113Y\text{-}D8)_4$, through to $(M113K)_6(M113Y\text{-}D8)_1$, were separated and purified by SDS-polyacrylamide gel electrophoresis based on their different gel shifts, which were caused by the C-terminal extensions of eight aspartate residues (the "D8" tail). The αHL proteins obtained were stored in aliquots at −80 °C.

2.2. Single-channel recording and data analysis

Bilayer experiments have been described previously at 22 ± 1 °C [45]. Briefly, the experiments were carried out in a chamber which was divided by a Teflon septum into two compartments, *cis* and *trans*. A bilayer of 1,2-diphytanoylphosphatidylcholine was formed on an aperture in the septum by using the Montal-Mueller method [47] after pretreating the aperture with hexadecane. The experiments were performed under a series of symmetrical conditions with a 2.0 mL electrolyte solution comprising 1 M NaCl and 10 mM NaH_2PO_4, with the pH of the solution adjusted to 3.0 using hydrochloric acid unless otherwise stated. The αHL protein (with the final concentration at 0.2–2.0 ng mL^{-1}) was added to the *cis* compartment. The host compound β-cyclodextrin (βCD) and the analyte PMPA/CMPA were added to the *trans* compartment. The *cis* compartment was connected to "ground". Unless otherwise noted, the applied potential was −120 mV by using a patch-clamp amplifier and head-stage (Axopatch 200B, Molecular Devices; Sunnyvale, CA, USA). A negative potential indicates a lower potential in the *trans* chamber of the apparatus. The resultant ionic currents were sampled at 25 kHz and the amplified signals were filtered at 5 kHz by a computer equipped with a Digidata 1440 A/D converter (Molecular Devices). For data acquisition and analysis, the pClamp 10.0 software package (Molecular Devices) were used. All the results were reported as mean values ± standard deviation.

2.3. Molecular graphics

The software SPOCK 6.3 was used for the derivation of the model of the $(M113K)_3(M113Y\text{-}D8)_4$ αHL pore. Briefly, the structure of the $(M113K)_3(M113Y\text{-}D8)_4$ protein was obtained by reading the new amino acids from the library in the $SP_AALIB directory, and superimposing the Cα_Cβ bonds onto the residues of the wild-type αHL (PDB: 7AHL) based on Mackay's quaternion method. The image in Fig. 1 was displayed using PyMol (DeLano Scientific, Palo Alto, CA).

3. Results and discussion

3.1. The effect of solution pH and applied voltage bias on the nanopore sensor resolution

In nanopore analysis, typically a buffer solution containing 1 M NaCl or 1 M KCl at or near physiological pH (i.e., pH 7.4) is used to produce the open channel current which is monitored [43]. However, in a separate study, we found that a reduction in the pH of the electrolyte solution could enhance the nanopore resolution to DNA analysis [48]. To examine whether a low pH value of the electrolyte solution could also provide a better resolution to PMPA/CPMA differentiation than a higher pH solution, initial experiments were performed to detect PMPA/CPMA using the $(M113K)_3(M113Y\text{-}D8)_4$ pore (Fig. 1a) and βCD host at −120 mV in 1 M NaCl solutions at pH 3.0 and pH 6.5. In such nanopore sensing systems, βCD's binding to the αHL pore would result in partial blockade of the channel. The followed capture and release of PMPA/CMPA by the βCD host would cause further current modulations (Fig. 1b–d). Our experimental result showed that, at pH 3.0, the PMPA and CPMA events had amplitudes of 26.8 ± 0.5 and 20.7 ± 0.8 pA, respectively, and residence times of 1.18 ± 0.17 and 0.17 ± 0.01 ms, respectively (Figs. 1 and 2). In contrast, at pH 6.5, the event mean blockage amplitudes of PMPA and CPMA were 20.3 ± 0.1 pA, and 18.3 ± 0.8 pA, respectively, while their event mean residence times were 1.40 ± 0.02 ms, and 0.38 ± 0.01 ms, respectively (data not shown). Therefore, both the amplitude difference (∼2 pA) and residence time difference (∼3.7 folds) of PMPA/CPMA events at pH