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Nanopore Back Titration Analysis of Dipicolinic Acid

Yujing Han, Shuo Zhou, Liang Wang, and Xiyun Guan*

Department of Biological and Chemical Sciences, Illinois Institute of Technology, Chicago, Illinois 60616, United States

Abstract

Here we report a novel label-free nanopore back titration method for the detection of dipicolinic acid, a marker molecule for bacterial spores. By competitive binding of the target analyte and a large ligand probe to metal ions, dipicolinic acid could be sensitively and selectively detected. This nanopore back titration approach should find useful applications in the detection of other species of medical, biological, or environmental importance if their direct detection is difficult to achieve.

Keywords

Back titration; Dipicolinic acid; Label-free; Nanopore; Terbium

Nanopore stochastic sensing has attracted substantial interest as an emerging label-free and amplification-free technique for measuring single molecules [1–4]. One challenge to develop nanopore sensors is to slow down the translocation of target analytes in the nanopore so that analyte events could be captured by the current recording technique (usually, ~100 μ s event residence time is required). In addition to taking advantage of the experimental conditions to regulate molecular and ionic transport [5–8], two major approaches have been utilized in the nanopore sensor design: one involves the structural modification of the nanopore interior to construct binding sites [9–12], while the other relies on a host or probe molecule to form host-guest or probe-analyte complexes [13–18]. Although a wide variety of substances have been detected, nanopore detection of many small molecules, especially anions, by these two approaches is not successful. Herein, we report a novel nanopore back titration approach to detect small molecules using pyridine-2,6-dicarboxylic acid (DPA, also known as dipicolinic acid, see Supporting Information, Figure S1, for its structure) as a model analyte. DPA, a unique component of bacterial endospores such as *Bacillus anthracis* that composes approximately 10% of their dry weight [19], is an important useful marker molecule in the detection of anthrax spores. Thus far, numerous techniques have been developed for the highly sensitive detection of DPA, including gas-liquid chromatography, surface-enhanced Raman spectroscopy, lanthanide ion-based fluorescence, and so on [20–25]. However, most of these methods require use of instruments relatively expensive and not easily portable.

*Correspondence: Dr. Xiyun Guan, Department of Chemistry, Illinois Institute of Technology, 3101 S Dearborn St, Chicago, IL 60616, USA, Tel: 312-567-8922; Fax: 312-567-3494, xguan5@iit.edu.

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The principle of nanopore back titration analysis of DPA is shown in Scheme 1, where a large metal chelating agent serves as a ligand probe. The interaction of this molecular probe with a nanopore will produce a characteristic current blockage event. After addition of appropriate metal ions to the electrolyte solution containing the ligand probe, these chelating agent molecules and metal ions will form stable metal chelate complexes. Due to the difference in the net negative charge and/or conformation, these complexes will produce a new type of events with different residence times and/or blockage amplitudes from those of the chelating agent molecules, which allows the complexes and the chelating agent to be readily differentiated. It could be visualized that, with a fixed concentration of the ligand probe, as the concentration of the added metal ions increases, we would observe a decrease in the event frequency of the ligand probe but an increase in the event frequency of the complex molecules. Ideally, if all the ligand probe molecules can be complexed with the metal ions, no free ligand probe events could be observed. In contrast, upon addition of the target analyte such as DPA in this work, since the competitive binding between DPA and the metal ions can release the ligand probe molecules from the metal ion-ligand probe complexes, an increase in the frequency of the free ligand probe events while a decrease in the frequency of the metal ion - ligand probe complex events could be expected. Consequently, the change in the frequency of the ligand probe events can be utilized to determine the concentration of DPA molecules.

To demonstrate this concept of nanopore back titration analysis of DPA, the initial experiment was performed with 2 μM diethylenetriamine penta(methylene phosphonic acid) (DTPMPA, see Supporting Information, Figure S1, for its structure) in an engineered α -hemolysin (M113K)₇ protein pore. Note that the mutant (M113K)₇ pore contains seven Lysine amino acid residues near the constriction of the α -hemolysin channel, and has been utilized for the successful detection of negatively charged analyte molecules [26]. However, direct detection of DPA by this pore is not successful (data not shown). One likely interpretation is that DPA has a much smaller molecular size than the nanopore cavity and its interaction with the nanopore is very weak so that no current modulation events could be observed when DPA passes through the channel. Our experimental results showed that, with DTPMPA alone in the electrolyte solution, only one major type of events with a blockage residual current at 2.6 ± 0.1 pA and a mean residence time of 6.1 ± 0.2 ms could be observed (Figure 1a, and Supporting Information, Figure. S2). Similar phenomenon was observed with the interaction of DTPMPA and the (M113R)₇ pore under the same physical condition [27]. In contrast, after addition of 2 μM Tb^{3+} (a well-known metal ion to chelate with DPA [28]) to the solution, in addition to the long-lived events, a new type of events possessing a significantly larger residual current at 11.3 ± 0.1 pA and shorter mean residence time of 0.28 ± 0.01 ms appeared (Figure 1b, and Supporting Information, Figure S2), suggesting the formation of Tb^{3+} -DTPMPA complexes. Furthermore, with the increase in the concentration of added Tb^{3+} , the frequency of the new type of short-lived events increased (Figure 1c). The results are not unreasonable considering that DTPMPA is negatively charged, while the α -hemolysin (M113K)₇ pore contains a binding site for negatively charged species, so that electrostatic interaction is the dominant force between DTPMPA and the α -hemolysin pore. The electrostatic interaction nature of the mutant α -hemolysin pore containing positively charged amino acid residues such as (M113K)₇ and

(M113R)₇ has been well documented [26, 27]. It is apparent that the produced Tb³⁺-DTPMPA complexes have smaller net negative charges than the DTPMPA species, thus resulting in smaller residence time events due to the weaker electrostatic interaction between the complex molecules and the nanopore. Note that the wide spread blockage amplitude of the short-lived complex events might be attributed to multi-chelation states, which are consistent with the relevant chelation kinetics studies documented previously [29, 30]. On the other hand, when DPA was further added to the electrolyte solution, the frequency of the long-lived and large blockage amplitude DTPMPA events increased, while that of the smaller residence time complex events decreased (Figure 1d and 1e), suggesting that DPA can compete with DTPMPA to form stable Tb³⁺-DPA complexes, thus releasing DTPMPA. Since the concentration of added DPA is related to the change in the frequency of the DTPMPA events, DPA could be quantitated.

It should be noted that, two factors would significantly affect the sensitivity and detection limit of the nanopore back titration sensor. First, since the detection of DPA is based on the competitive binding of DPA and DTPMPA to Tb³⁺, a much larger binding affinity between DPA and Tb³⁺ over that between DTPMPA and Tb³⁺ is desired. Second, due to the relatively weaker interaction between DTPMPA and Tb³⁺, complete removal of DTPMPA events before the back titration step is extremely difficult if the concentration of Tb³⁺ added in the solution is not significantly larger than that of DTPMPA. However, an excess amount of Tb³⁺ in the solution will lead to the direct chelation between DPA and Tb³⁺ without releasing DTPMPA from the Tb³⁺-DTPMPA complexes, thus affecting the detection limit for DPA detection. Therefore, a feasible approach to achieve sensitive detection of DPA is to use low concentrations of the ligand probe DTPMPA. For example, if the back titration experiment was performed with the initial concentrations of both DTPMPA and Tb³⁺ at 100 nM each, DTPMPA could be detected with a detection limit (defined as the concentration corresponding to three times the standard deviation of a blank signal) at as low as 34.6 nM (Figure 2). A further improvement in the detection limit of our nanopore back titration analysis of DPA could be achieved if lower initial concentrations of DTPMPA and terbium are employed. Note that DTPMPA could be detected with a detection limit as low as ~10 nM (Supporting Information, Figure S3). Although this detection limit is not impressive compared to the fluorescence assay, which could detect terbium monodipicolinate at ~2 nM [31], our nanopore back-titration method has the advantage of not requiring use of fluorescent reagents, and hence could be utilized as a useful strategy to develop sensors for a variety of species in the situations that development of their fluorescent probes is expensive and/or difficult to achieve.

To demonstrate the selectivity of the nanopore DPA sensor, four structurally similar organic compounds were examined with this nanopore back titration system. These organic ligands include 2,3-pyridinedicarboxylic acid, also known as quinolinic acid (QA); benzoic acid (BA); ethylenediaminetetraacetic acid (EDTA); and phthalic acid. Our experimental results (Figure 3) showed that at least 5-fold more DTPMPA events were produced in the case of DPA than other ligands, suggesting this nanopore sensor responded selectively toward DPA. Our observation was consistent with that made by Lee et al, where detection of DPA was achieved using a screen-printed fluorescent assay [32]. Further calculation (Supporting

Information) found that the metal chelate formation constants [$\log(K_f)$ values] were 6.62, 4.89, 4.82, 5.15, and 5.26 for DPA, BA, EDTA, PA, and QA, respectively. With the exception of BA and EDTA, our experimental results agreed very well with the literature values [30, 33–35]. Although our experimental K_f value for the Tb^{3+} -BA complex was two orders larger, while that for the Tb^{3+} -EDTA complex was twelve orders smaller than their corresponding literature values, our results are not unreasonable considering that different experimental conditions were used in the experiments; furthermore, our experiments were performed real-time and the chemical equilibrium might not have been reached.

In summary, a real-time, label-free nanopore stochastic sensor for the detection of dipicolinic acid was successfully developed with good sensitivity and selectivity. As far as we aware, this is the first time for the back titration analytical method to be introduced into the nanopore stochastic sensing. This nanopore back titration analysis approach should find useful applications in the detection of other substances of medical, biological, or environmental importance if their direct detection is difficult to achieve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Tb³⁺	Terbium
QA	Quinolinic acid
BA	Benzoic acid
DPA	Dipicolinic acid
DTPMPA	Diethylenetriamine penta(methylene phosphonic acid)

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Figure 1.

Nanopore back titration analysis of DPA. (*Left*) Typical single-channel current recording trace segments, and (*Right*) the corresponding amplitude histograms. (a) 2 μM DTPMPA, (b) 2 μM DTPMPA + 2 μM Tb^{3+} , (c) 2 μM DTPMPA + 4 μM Tb^{3+} , (d) 2 μM DTPMPA + 4 μM Tb^{3+} + 2 μM DPA, and (e) 2 μM DTPMPA + 4 μM Tb^{3+} + 8 μM DPA. The experiments were performed with the α -hemolysin (M113K)₇ pore at +40 mV in a solution containing 1 M NaCl and 10 mM Tris (pH 7.5). Traces were filtered at 1000 Hz for display purpose. Dashed lines represent the levels of zero current.

Figure 2.

Characteristics of the nanopore DPA sensor. (a) Dose-response curve; and (b) Selectivity study. The experiments were performed with the α -hemolysin (M113K)₇ pore at +40 mV in a solution containing 1 M NaCl, and 10 mM Tris (pH 7.5). The electrolyte solution used in the experiment shown in Fig. 2a contained additional 100 nM DTPMPA, and 100 nM Tb³⁺, while that in the experiment shown in Fig. 2b contained additional 2 μ M DTPMPA, and 2 μ M Tb³⁺. The concentrations of all the analytes shown in Fig. 2b, including DPA, QA, BA, EDTA, and PA, were 2 μ M each. Only the long-lived DTPMPA events were utilized in the analysis of event frequency.

Scheme 1.

Schematic representation of the principle of nanopore back titration. Before addition of the target analyte, the ligand probe molecules and the metal ions in the electrolyte solution form stable metal chelate complexes, which would produce characteristic current modulation events in the nanopore. After addition of the analyte to the solution, the competitive binding between the analyte and the metal ions could release the ligand probe molecules from the metal ion-ligand probe complexes. The events of these freed ligand molecules could be differentiated from those of the metal ion-ligand complexes based on their significantly different signatures such as residence times and/or amplitudes due to the difference in the net negative charge and/or conformation between the ligand and metal-ligand complex. By monitoring the change in the frequency of the ligand probe events, the concentration of the analyte could be quantitated.