Detection of a single enzyme molecule based on a solid-state nanopore sensor

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Abstract
The nanopore sensor as a high-throughput and low-cost technology can detect a single molecule in a solution. In the present study, relatively large silicon nitride (Si$_3$N$_4$) nanopores with diameters of $\sim$28 and $\sim$88 nm were fabricated successfully using a focused Ga ion beam. We have used solid-state nanopores with various sizes to detect the single horseradish peroxidase (HRP) molecule and for the first time analyzed single HRP molecular translocation events. In addition, a real-time monitored single enzyme molecular biochemical reaction and a translocation of the product of enzyme catalysis substrates were investigated by using a Si$_3$N$_4$ nanopore. Our nanopore system showed a high sensitivity in detecting single enzyme molecules and a real-time monitored single enzyme molecular biochemical reaction. This method could also be significant for studying gene expression or enzyme dynamics at the single-molecule level.

Keywords: nanopores, sensor, single enzyme molecule, horseradish peroxidase

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1. Introduction
Nanopores are considered to be the most promising technology for single-molecule detection in a solution with a high throughput, low cost, and free label, which can open novel paths to scientific discovery [1]. Nanopores have been used to detect nanometer scale objects, such as nanoparticles [2–4], viruses [5–7], DNA sequence [8–19], and protein molecules [20–24]. The principle is that a biased voltage is applied across a thin membrane containing a nanometer-sized pore. Charged molecules in the solution are driven into the pore. The appearance of the molecule in the pore apparently changes the pore’s resistance, thus, resulting in a sharp change in the current signal. The pulse frequency is related to the quantity of molecules. The change in the current signal not only provides the sizes and concentrations of the molecules, but also reveals the dynamics process of the molecule translocation behavior.

The nanopore mainly includes biological nanopores and solid-state nanopores. Single protein molecules have also been detected, and their properties have been investigated using biological nanopores, such as small and unfolded proteins [25–30], a study of the structure of peptides [31], peptide antibody interactions [32, 33], measured self-peptide aggregation [34], and enzymatic kinetic reactions [35–38], which have been shown to be very sensitive tools for protein molecule detection due to their small fixed pore diameters of $\sim$1.5 nm. Biological nanopores have the advantage of a well-defined geometry, but their real-time applications are limited by the fragility of the lipid membrane into which the protein pores are embedded. Besides, only polypeptides or denatured proteins are able to pass through, which limits protein pores being used in measuring the process of protein unfolding [39].

Solid-state nanopores with the development of micro–nanoprocessing technology present obvious advantages over...
their biological counterparts, such as very high stability, controllability of diameter and channel length, adjustable surface properties, and the potential for integration into devices and arrays [40]. Solid-state nanopores have been demonstrated to be capable of detecting and analyzing protein molecules including discriminating protein translocations [21, 41–44], protein complexes and protein kinetics [45], protein unfolding and folding [39, 42], and protein–protein interactions [24, 39, 46–49]. Currently, silicon nitride (Si₃N₄) nanopores have been proven to be a novel protein sensing platform with excellent physical and chemical stabilities. Many protein molecules have been studied by using solid-state nanopores including lysozyme [44], avidin [44], immunoglobulin G [44], β-lactoglobulin [44], ovalbumin [50], bovine serum albumin [24, 39, 44, 51], β-galactosidase [50], his-tagged proteins [52], mammalian prion protein [44], phi29 connector protein [53], and histidine-containing phosphocarrier protein [50]. Besides, the Meller group has demonstrated that ubiquitin and ubiquitin chains can be efficiently discriminated using Si₃N₄ nanopores [23]. Radenovic et al have used Si₃N₄ nanopore sensing to detect a single Escherichia coli (E. coli) RNA polymerase (RNAP)-DNA transcription complex and a single E. coli RNAP enzyme, which can discriminate and can identify between those two types of molecular translocations [54].

Horseradish peroxidase (HRP) is the most widely studied member of this peroxidase family. HRP is a monomeric heme-containing plant enzyme that has found enormous diagnostic, biosensing, and biotechnological applications [55]. So far, single enzyme molecule detection has been implemented successfully by using fluorescence detection and electrochemical cycling in rare cases [56–66]. The product of certain enzyme reactions is light, which can be detected by using a range of sensitive optical devices. Until now, individual enzyme molecules could not be monitored by chemiluminescence because they were able to diffuse freely in solution [56].

Herein, we fabricated a Si₃N₄ nanopore chip by means of focused ion beam (FIB) lithography and show, for the first time, solid-state nanopores to detect a single HRP molecule. After the successful detection of the single HRP, we further investigated the sensitivity of our nanopore system to real-time monitor a single enzyme molecular biochemical reaction and an enzyme catalytic substrate translocation in 0.1 M KCl and 10 mM phosphate-buffered saline (PBS) pH 7.0. Our approach could be significant for studying gene expression or enzyme dynamics at the single-molecule level, which provides the basis for the development of this technology as a novel method for single enzyme molecule detection.

2. Experiment

2.1. Chemicals and apparatuses

The HRP molecule (1 mg ml⁻¹, Enzyme Commission No. 1.11.1.7, 44 kDa) was obtained from Sigma-Aldrich (St. Louis, MO, USA), which was shown in scheme 1(a). The sample (HRP) was dissolved in 0.02 μM filtered 10 mM PBS, stored at 4°C, and used within two days of preparation. Potassium chloride (KCl) and 2,2’-Azino-bis(3-ethylbenzothiazolone-6-sulfonic acid) ((ABTS), 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide ((H₂O₂), 30%) was bought from Sinopharm Chemical Reagent Co., Ltd. All solutions were prepared with ultrapure water from a Milli-Q water purification system (resistivity of 18.2 MΩ cm, 25°C, Millipore Corporation, Billerica, MA, USA) and were filtered through 0.02 μM in a FEI Strata 201 FIB system (FEI Co., Hillsboro, OR, USA), a Zetasizer (Malvern Zetasizer Nano ZS), and an Axopatch 700B (Molecular Devices, Inc., Sunnyvale, CA, USA).

2.2. Nanopore sensor fabrication

Scheme 1(c) shows the structure of a nanopore chip. A free-standing membrane was obtained by depositing a thin
(100 nm nominal thickness) Si$_3$N$_4$ film on a 300 μm thick Si substrate. Fabrication of this membrane consisted of first depositing a layer of low-stress Si$_3$N$_4$ on a Si wafer using low pressure chemical vapor deposition (the deposition rate was 5 nm min$^{-1}$, the chamber pressure was about 4 mbar, and the substrate temperature was 810 °C) followed by photolithography (the opening window size for photolithography is 500 × 500 μm$^2$), deep reactive ion etching (DRIE), and tetramethylammonium hydroxide (TMAH) etching (the TMAH was used for Si etching, and the Si$_3$N$_4$ was used as an etch mask against the TMAH etching). The etch rate was about 40 μm h$^{-1}$, and the Si/Si$_3$N$_4$ etch selectivity was greater than 1000. DRIE was used to etch Si$_3$N$_4$ (500 × 500 μm$^2$), then, the 5% TMAH etching was used for the Si etching at 80 °C. The Si wafer was (100) type to form a 50 × 50 μm$^2$ membrane. A nanopore was drilled in the surface of the membrane by bombarding the surface with Ga$^+$ ions using a FEI Strata 201 FIB system (FEI Co., Hillsboro, OR, USA) at an acceleration potential of 30 kV, while the current was measured as 1 pA. The milling time was 1.5 s under a spot mode.

2.3. Electrical measurements

The chips were held in place using a custom built poly(carbonate) flow cell with polydimethylsiloxane gaskets to ensure that the only path of the ionic current was through the nanopore. Figure 1(a) displays Si$_3$N$_4$ chips. The cell was made of two facing Plexiglas chambers filled with filtered 0.1 M KCl and 10 mM PBS. Figures 1(b)–(d) show the fluid device used in the experiment. Electrodes (Ag/AgCl) were placed in both chambers and were connected to the headstage of a patch clamp amplifier (Axopatch 700B, Molecular Devices, Inc., Sunnyvale, CA, USA) which allowed the ionic current to be measured under constant voltage in scheme 1(b).

Figure 1. (a) Si$_3$N$_4$ chips, (b)–(d) detection of single molecules was carried out in the nanofluid developed in our laboratory to investigate their electrical properties.

The HRP (10 ng ml$^{-1}$) was added to the cis side. Signals were acquired at a 100 kHz sampling rate. The amplifier internal low-pass eight-pole Bessel filter was set at 10 kHz. The entire apparatus was placed in a double Faraday cage enclosure on an antivibration table.

3. Results and discussion

3.1. Characterization of the pore

We fabricated single cylinder Si$_3$N$_4$ nanopores by FIB. The Si$_3$N$_4$ nanopores were produced with small diameters of about 28 nm and membranes of thicknesses of ~100 nm. Figure 2(a) shows a FESEM image of a typical Si$_3$N$_4$ nanopore used in the experiments reported here. Figures 2(b), (c) show the FESEM of nanopore trans and an enlarged view FESEM of nanopore trans. Figure 2(d) shows a typical current–voltage (I–V) curve of a nanopore in 0.1 M KCl, buffered at pH 7 with 10 mM PBS. Two electrolyte reservoirs are separated by a Si-supported free-standing insulating 100 nm thick Si$_3$N$_4$ membrane, which contains a ~28 nm single cylinder nanopore. The samples were always added to this cis reservoir. In the absence of an analyte, a voltage was applied through the two electrodes, and the ionic current flowed through the nanopore and was recorded with a current amplifier. No ionic current change events were evident in the current trace. Upon addition of the analyte to the cis reservoir, spikes became apparent in the current trace, which reflected the passage of the analyte through the nanopore. Current blockage signals from individual molecular translocations can be characterized by the time duration ($t_b$) and the magnitude of the blockage current ($\Delta I_b$).
3.2. HRP ζ-potential and hydrodynamic diameter

Prior to translocation experiments, we analyzed the ζ-potential (ζ_{HRP}) and measured the hydrodynamic diameter (D_h) of the HRPs in different pH values and salt concentrations via dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS), which was used to evaluate the surface charge and to demonstrate that these conditions did not promote HRP aggregation. ζ_{HRP} was determined in low ionic strength solutions (0.1 M KCl, adjusted with HCl or NaOH to the desired pH). Figures 3(a), (b) show ζ_{HRP} and D_h versus different pH values. ζ_{HRP} is positive for pH \( \leq 4 \), then, drops rapidly, crosses 0 mV between pH 4.2 and 4.5, and becomes negative for pH \( \geq 5 \). Hence, the pI value is 4.3 ± 0.2. The D_h (∼8 nm) of HRPs was found in 0.1 M KCl pH 6 to 7, which indicated they did not aggregate in that solution. However, the D_h of HRPs (∼100 nm) was observed in 0.1 M KCl, pH 2.4, 5, 8, 10, and 12, which indicated they did aggregate in that solution. The D_h of HRPs in different pHs with 0.1 M KCl solutions was added to the supplementary material (see S1 figure S1 in the supplementary material). Besides, because electrical experiments with nanoparticles are usually performed with solutions of high conductance (high salinity), we studied the salt concentration dependence of ζ_{HRP} and the D_h of HRPs from 0.1 to 2 M KCl, pH 7. Figure 3(d) shows that ζ_{HRP} quickly decreases with the increasing in KCl concentration. A determination of the electrophoretic mobility at higher salt concentrations was impeded by experimental limitations (high voltages and high currents), but the absence of HRP aggregation as evidenced by DLS (see S2 figure S2 in the supplementary material), even at KCl concentrations up to 1 M, showed that these salt conditions do not promote HRP aggregation. However, we further investigated its D_h in 1.5–2 M KCl, pH 7 (repeated three experiments) and found that they aggregated in 1.5–2 M KCl, pH 7 solution. The results were added to the supplementary material (see S2 figure S2 in the supplementary material).

3.3. Detection of single enzyme molecules

Solid-state nanopores can serve as sensors to detect single molecules by their translocation characteristics. To demonstrate this option, we added HRP to the cis compartment of the nanopore. The size of the solid-state nanopore is controllable and is easy to process. We performed translocation experiments using ~28 nm nanopores. After addition of HRP to the nanopore reservoir, we performed translocation experiments using voltages from 500 to 1000 mV, and they were sampled at 100 kHz (figures 4(a)–(e)). We observed sparse submillisecond current blockades caused by HRP molecule translocation through the nanopore, which was conducted in 0.1 M KCl, 10 mM PBS (pH 7). HRP molecules translocating through nanopores are governed by the competing effects of electrophoresis and electro-osmosis [44, 47]. At pH 7, the Si_3N_4 nanopore is negatively charged. Therefore, there will be an electro-osmotic flow from the electrically grounded reservoir into the Si_3N_4 nanopore when a negative voltage is applied, and there will be an oppositely directed flow when a positive potential is applied. HRPs were negatively charged in 0.1 M KCl, 10 mM PBS, pH 7, and we observed translocations through the Si_3N_4 nanopore when a positive potential was applied. At the same time, we also found spikelike current increases. Subsequently, we measured translocation events at 1 M KCl 10 mM PBS, pH 7 (figures 4(g), (f)). We observed a spikelike current decrease. It has been reported that DNA translocation is shown to result in either a decrease (\( (KCl) > 0.4 \text{ M} \)) or an increase in the ionic current (\( (KCl) < 0.4 \text{ M} \)) [9]. Therefore, we deem that this phenomenon mainly depends on the salinity of the solution. At a lower 0.1 M KCl concentration, the spikelike current increases, and at a 1 M KCl concentration, the spikelike current decreases.

Besides, nanopores (diameters of ~28 nm) were used to further detect the translocation of HRPs in 1.5 M KCl, 10 mM PBS pH 7. \( \sigma (15.4 \text{ S m}^{-1}) \) is the conductivity of the 1.5 M KCl solution at 24 °C, and the conductance of the pore...
\( G = 90.6324 \text{ nS}, 100 \text{ mV} \) can be introduced in figure 5(a).

We observed many translocation events that showed large blockage current (figure 5(b)), and the enzyme molecules were stuck in the nanopore from time to time (figure 5(c)). Nanopores cannot work normally, and they often need to apply a reverse voltage or relatively large voltage to rescue the enzyme molecules that were stuck in the nanopore as shown in figure 5(d). Two dimensional scatter plots were fitted about the mean amplitude versus the event duration for each translocation of HRP in 1.5 M KCl, 10 mM PBS pH 7.

The scope of the blockage current from 0.5 to 3 nA applied voltage 200 mV and 0.5 to 6 nA applied voltage 300 mV (figure 5(e)). This phenomenon can be attributed to agglomeration of the enzyme molecule under high salt concentration, resulting in size inhomogeneity, which is consistent with test results of the hydraulic radius. In this case, the nanopores can be used to detect or to assess the distribution of particle uniformity. There is no statistical significance of enzyme molecule detection due to enzyme molecule agglomeration in high salt concentrations.

### 3.4. Statistical analysis of HRP translocation

Figure 6(a) shows histograms of the mean current amplitude of translocation events measured for HRP at various voltages. Based on the fitting curves, the peak values of the current blockage are 29.25 \pm 1.256, 52.33 \pm 2.552, 87.48 \pm 2.335, 110.9 \pm 1.424, 141.4, 181.4 \pm 2.887, 222.6 \pm 6.113, 258.8 \pm 4.118, and 298.7 \pm 4.472 pA at 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mV, respectively, which correspond to the most probable current drops induced by a single HRP through the nanopore at varied voltages. We analyzed the distribution of the current blockade and dwell times. We found that the current amplitude linearly increases with the voltages, which yields a slope of 0.313 and an intercept of \(-40\) in figure 6(b). An exponentially decaying function \( t_d \sim e^{-v/v_0} \) was employed to fit the dwell time dependent on the voltage (figure 6(c)). The current blockade values were fitted with a first-order polynomial function, indicating that conductance blockades increase at higher applied voltages. The transport duration values were fitted with an exponential decay function, indicating that the transport velocity is voltage dependent. In addition, we analyzed the transition time of HRP in our experiments. The current blockage duration \( t_d \) is regarded as the dwell time of a HRP from the entrance to the exit of the nanopore. We observed the majority of HRP quickly passes through the pore with less than 50 \pm 3 ms, typed as short-lived events at high voltage (figure 6(d)), at the medium-voltage region event durations of less than 100 \pm 5 ms (figure 6(e)). However, at low voltages, we observed long event durations (figure 6(f)). We think this may be caused by HRP adhering to the sides of the walls. We fitted two dimensional scatter plots of mean amplitude versus event duration for each HRP (see S3 in the supplementary material).
Figure 5. (a) A typical current–voltage (I–V) curve of a nanopore in 1.5 M KCl, buffered at pH 7 with 10 mM PBS. (b) Representative single-molecule transport events in 1.5 M KCl, buffered at pH 7 with 10 mM PBS. (c) The enzyme molecules were stuck in the nanopore at 300 mV. (d) Nanopores can work normally with relatively large voltages applied at 400 mV. (e), (f) Two dimensional scatter plots of mean amplitude versus event duration for HRP in 1.5 M KCl buffered at pH 7 with 10 mM PBS at 200, 300 mV.

Figure 6. (a) Histograms of the mean current amplitude at various voltages, (b) an increase in current blocking at higher bias voltages, (c) voltage dependency of transport duration, (d) HRP translocation events at 1000 mV, (e) HRP translocation events at 600 mV, (f) HRP translocation events at 300 mV.
3.5. Detection of HRP with pores of different diameters

We repeated the experiments using nanopores with pore sizes (~88 nm) in 0.1 M KCl and 10 mM PBS. The I–V curve and the SEM of the nanopores were put in the supplementary material (see figures S5-1 and S5-2 in the supplementary material). Representative translocation events are presented in figure 7(a). Compared to the nanopores with pore sizes of ~28 nm as shown in figure 7(b), we found that higher bias voltages were required to get clear signals for ~88 nm pores. Bias voltage is also a significant factor to control better results. We analyzed the translocation events at 500, 700, and 900 mV. The three voltages were chosen mainly because translocation events were not observed at low voltages in the current in the signal-to-noise ratio. Figure 7(c) expresses histograms of count versus translocation time. As for event duration, the peaks were obtained Gaussian fittings, which were 0.176 ± 0.009 s at 900 mV, 0.210 ± 0.009 s at 700 mV, and 0.291 ± 0.006 s at 500 mV. We found that the translocation time decreased with the increase in voltage. Figures 7(d, e) present histograms of count versus the conductive amplitude (ΔG) together with event scatter plots of translocation time versus ΔG. The peaks were obtained by Gaussian fitting. We observed ΔG values that were almost the same at three biased voltages (ΔG value 0.063 ± 0.0009 at 900 mV, 0.064 ± 0.0008 at 700 mV, and 0.068 ± 0.0004 at 500 mV). ΔG is foreign to the biased voltage in theory. This similar ΔG value proved the dependability of the results. Besides, we made a comparison of the translocation events for nanopores with ~28 and ~88 nm at 1000 mV. Figure 7(f) indicates histograms of count versus translocation time at bias voltages of 500 mV (diameters of ~28 nm) and 1000 mV (diameters of ~88 nm). As for event duration, the peaks were obtained by Gaussian fitting to the histograms. The translocation time (Δt) values are 0.0988 ± 0.0048 s (1000 mV, 88 nm) and 0.0758 ± 0.0006 s (500 mV, ~28 nm). It was believed that higher bias voltage would generate faster translocation events due to greater electrical forces. However, in our results, the translocation time was even longer through the nanopores (diameters of ~88 nm) at bias voltages of 500 mV. That value is 0.291 ± 0.006 s. We suppose this retardant was caused by the pore dimension and shape. As described before, the electric field density variation caused by pore shapes and dimensions could be part of the reason for longer translocation times with higher voltages [4]. Figures 7(g, h) indicate histograms of count versus ΔG at biased voltages of 500 mV (diameters of ~28 nm) and 1000 mV (diameters of ~88 nm) together with event scatter plots of the translocation time versus ΔG value. The peaks were obtained by Gaussian fitting to the histograms. The ΔG values are 0.0889 ± 0.000615 nS with nanopores (diameters of ~28 nm) and 0.1140 ± 0.00269 nS with nanopores (diameter of ~88 nm), which could easily be understood since the same particle would have a bigger influence in a more confined room.

3.6. Typical HRP events

The current blockage signals revealed the information of the size, conformation, and interactions of HRP passing
through the nanopore. We observed three typical current traces. For HRP event (I) is of short duration in figure 8(a), suggesting ballistic single enzyme molecule transport. Event (II) is several milliseconds in duration and has two levels in figure 8(b), suggesting transient adherence of two HRP molecules to the sides of the nanopore walls. Event (III) shows a longer duration sticking event in figure 8(c). Those typical current traces for the HRP events are similar to the previously measured mammalian prion protein [44].

3.7. Real-time detection of enzyme catalysis by the nanopore system

After the successful detection of a single HRP, we further investigated the sensitivity of our nanopore system (≈28 nm) to real-time monitoring of the single enzyme molecule biochemical reaction and enzyme catalytic substrate translocation in 0.1 M KCl, 10 mM PBS pH 7.0. Figure 9(a) shows a scheme of the catalyzed redox reaction of the HRP enzyme. In the presence of the HRP enzyme molecules, only a single
enzyme molecule translocation was observed in figure 9(a). However, after addition of the substrate (10 nM H2O2 and 1.5 mM ABTS) to the buffer solution with the HRP enzyme molecules, enzyme catalysis substrates produced new substances. Thus, new blockage events having residence times and amplitudes that differ from those of the enzyme molecules can be observed in figure 9(b). The response time of the enzyme catalysis substrates can achieve millisecond magnitude by the nanopores, which was put in the supplementary material (see figure S6-1 in the supplementary material). The representative translocation events of the single molecule were shown in figure 9(c) with blockage currents of 146 pA and duration times of 0.024 ms. We would like to mention the recent work by Ali and co-workers in which HRP-modified polymer single conical nanochannels were applied for detection of H2O2 [67]. These authors have demonstrated the function of the immobilized enzyme in a single nanochannel as a H2O2 sensor by studying products of the redox reactions occurring in the presence of the substrate ABTS.

However, in figure 9(d), we also noticed some translocation signals of an increase in the ionic current in the process of catalytic substrate translocation. The translocation signals were analyzed from 1 to 7 in figure 9(d). We believe that the translocation signals of the increase in the ionic current came from single enzyme molecule translocation. This phenomenon may be a translocation rate too fast to complete the catalytic reaction by applying a voltage driven enzyme molecule through the nanopore and some resting enzyme molecules by applying voltage driven resting enzyme molecules through the nanopore. For the translocation signals of the ionic current decrease, we attributed the current changes to the appearance of cationic products of the redox reaction that occurred in the presence of HRP, ABTS, and H2O2 [67].

It is also possible that electrostatic and steric effect of ABTS+ molecules can act in concert to produce the observed ion current changes. The scatter plot of current blockage versus translocation time was fitted for ABTS+ and HRP in figure 9(e). Outliers with long event durations are observable in most of the scatter plots. In addition, in figure 9(e), we observed ABTS+ and HRP can be discriminated with current blockage and translocation time. However, according to the theoretical calculation, a single ABTS+ cannot be detected. Therefore, we think that the decrease in the ionic current most probably constitutes a collective effect of many ABTS+ molecules being driven through the nanopore. We also expected that, for sufficiently small nanopores, one could potentially observe discrete current openings and closings due to the presence of a single ABTS+ blocking the nanopore. The product of enzyme catalysis substrates can be distinguished by the nanopore system.

The enzyme catalytic redox reaction is as follows: (1), (2), and (3). In the presence of H2O2, the HRP enzyme is rapidly converted into an oxidized peroxidase form known as compound I (reaction 1 below). Then, compound I accepts one electron from the reducing substrate molecule to generate compound II (reaction 2 below). Subsequently, compound II is reduced back to the resting enzyme via one electron transfer from another substrate molecule (reaction 3 below) [67],

\[
\text{HRP(Fe}^{3+})\text{Porp} + \text{H}_2\text{O}_2 \rightarrow \text{HRP(Fe}^{4+} = \text{O})\text{Porp}^{++} + \text{H}_2\text{O},
\]

(1)

\[
\text{Compound I}
\]

\[
\text{HRP(Fe}^{4+} = \text{O})\text{Porp}^{++} + \text{HA} \rightarrow \text{HRP(Fe}^{4+} = \text{O})\text{Porp} + \text{A'},
\]

(2)

\[
\text{Compound II}
\]

\[
\text{HRP(Fe}^{4+} = \text{O})\text{Porp} + \text{HA} \rightarrow \text{HRP(Fe}^{3+})\text{Porp} + \text{A'} + \text{H}_2\text{O}.
\]

(3)

Peroxidase catalytic cycle: HA is the substrate, and radical-cation A' is the product.

ABTS was used as a substrate, expecting the formation of the ABTS++ product.

The translocation of the product of enzyme catalysis substrates reveals the biochemical reaction in situ, catalytic kinetic behavior, molecular size shape, and so forth. All typical events of the translocation of the product of enzyme catalysis substrates were analyzed. We observed six typical events for the translocation of the product of enzyme catalysis substrates in figure 9(f). For event type I, the current signal has a typical slope spike shape with a deep intensity and a short dwell time suggesting ballistic transport. We think that the typical slope spike shape may be related to the molecular shape. For event type II, the current signal has a typical spike shape with a deep intensity and a short dwell time. This signal can be attributed to the molecular translocation rapidly reaching the bottom of the hole and, then, producing vibration and friction along the hole wall until through the nanopore. For event type III, the current signal has a specific platform for a long time. The other shape is similar to event type I. The typical platform exists for a long time, and we deem that the product of the enzyme catalysis substrates (ABTS++) is adsorbed in the pore wall, the current signal is blocked persistently, and it recovers until the enzyme catalysis product is desorbed and is impelled out the nanopore, displaying the long life. For event type IV, the current signal first appears to have a typical platform and, then, produces vibration and friction along the hole wall until through the nanopore, which may mainly be attributed to the molecular electrostatic adsorption and vibration. For event type V, the current signal has a typical slope spike shape with a deep intensity and a short dwell time and, then, appears to have a typical platform for a long time. The typical platform is similar to event type I, which is mainly attributed to the electrostatic adsorption. For event type VI, two slope spike shapes with deep intensities and short dwell times were observed, which we supposed was caused by two translocation of product of enzyme catalysis substrates in succession. Identification of the typical translocation events of product of enzyme catalysis substrates were put in the supplementary material (see figures S6–2 in the supplementary material).
4. Conclusions

To summarize, we successfully fabricated a Si$_3$N$_4$ nanopore using a FIB and exhibited the first solid-state nanopore measurements of HRP that provide a basis to study single enzyme molecules in a solution on the nanoscale level. The $\zeta$-potential ($\zeta_{\text{HHRP}}$) and size measurement of the HRP were analyzed via DLS in different pHs and salt concentrations. We optimized the detection conditions of a single HRP molecule by a nanopore: pH 6 to 7, salt concentration range: 0.1–1 M KCl, the threshold voltage 200 mV in the current signal-to-noise ratio. We analyzed single HRP molecular translocation events through solid-state nanopores (~28 nm). A linear dependence has been found between current blockades versus biased voltage. An exponentially decaying function ($I(t) \sim e^{-t/\tau}$) has been found between the duration time versus the biased voltage. We repeated the experiments using nanopores with various pore sizes. A comparison of the translocation events with ~28 and ~88 nm pores were investigated by analyzing histograms of $\Delta G$ and $\Delta t$. The results proved that the same particle would have a bigger influence in a more confined room. In addition, real-time monitoring of a single enzyme molecule biochemical reaction and enzyme catalytic substrate translocation were explored. We observed new translocation events having residence times and amplitudes that differ from those of the enzyme molecule. The product of the enzyme catalysis substrates and the enzyme molecule can be effectively distinguished by the nanopore system. We believe this approach offers the potential for further development as studying gene expression or enzyme dynamics at the single-molecule level.

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