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Facilitated Alkyl Detection by a DNA Conjugate with an α-Hemolysin Nanopore

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Herein an α-hemolysin nanopore was utilized to detect a DNA conjugate, where a propyl linker was attached to the 20-mer oligonucleotides at each end of an individual strand. The studies showed the alkyl linker produced a clear current change when translocating through the protein pore, which could be used to sense and identify small organic molecule at single-molecule level.

Small organic molecules, such as alkyl and benzene, play increasingly important roles in human health and environment protection[1, 2]. Recently, many techniques were developed to detect small organic molecules, including electrochemistry[3], spectroscopy[4], chromatography, mass spectroscopy and microporous metal-organic frameworks[5]. However, these methods are generally slow, arduous and costly, and some of them have certain limits in aqueous solubility, selectivity and sensitivity. Thus, it is still a challenge to develop methods for real-time, sensitive detection of small organic molecules. The nanopore is developed into a powerful sensitive platform for single-molecule detection of nucleic acids[6-9], peptides[10], proteins[11, 12], ions[13], and small organic molecules[14-16], such as real-time monitoring an ATP-binding aptamer changing its molecular conformation[17]. The crystal structure revealed that the diameter of the narrowest section is at an approximate value of 1.4 nm for an α-hemolysin (α-HL) protein nanopore[18]. When an individual molecule with the right size translocating through the nanopore, it induces characteristic changes of the ionic currents through the pore. By analysing the characteristic conductance changes of events and their frequencies of occurrence, one can electrically elucidate the kinetic translocation process and quantify the target samples at single-molecule level.

In this study, we introduced a DNA conjugate for the analysis of a short alkyl linker with a single α-HL pore (Fig. 1). The propyl linker "-$CH_2CH_2CH_2$-" was attached to two 20-mer oligonucleotides (B20) at each end of the DNA conjugate, which was named B20-$(CH_2)_3$-B20. The B20 at the 5' end of B20-$(CH_2)_3$-B20 (B20-1) could form a four-base pair hairpin structure with the B20 at the 3' end (B20-2) (Figure S1 in the Supplementary Information Files), which aimed to slow down the translocation speed and enhance the temporal resolution of the alkyl linker[19, 20]. The B20-1 acted as a guide to pull the B20-$(CH_2)_3$-B20 conjugate into the vestibule of the α-HL pore. When the hairpin structure of the DNA conjugate entered into the *cis* entrance of nanopore, the vestibule (2.6-4.6 nm wide) of the α-HL pore provided accommodation for the hairpin structure and the β-barrel (1.4-2.0 nm wide) of the α-HL pore gave occupation for the B20-1 of the B20-$(CH_2)_3$-B20 molecule[18] (Fig. 1a). The events with a blockage close to the open pore current could be attributed to the voltage-driven translocation of an individual B20-$(CH_2)_3$-B20 molecule[21, 22]. One of the typical current traces appeared to be a three-level current blockage, which could be divided into three stages: SI (blue), SII (yellow) and SIII (wine), respectively (Fig. 1b). The sequence of the synthesized DNA conjugate B20-$(CH_2)_3$-B20 was showed in Fig. 1c. The experiment was measured in solution with 1.0 M KCl, which was buffered with 1 mM EDTA and 10 mM Tris (pH = 8.00). The potential across the bilayer with a value of + 150 mV was applied through a pair of Ag/AgCl electrodes from the *cis* side of the protein pore.

One typical signature for B20-$(CH_{2)3}$-B20 featured three-stage current blockages with different current levels: I, II and III (Fig. 2a). To find its translocation process in greater detail, we analyzed the current traces of B20-$(CH_2)_3$-B20 in this experiment. All of the stages show $i/i_0 > 0.6$, where i_0 was the open pore current and i was the blockage current with molecule staying in the pore (Fig. 2b). Hence, we assigned the current depths of the three stages as i_1/i_0, i_2/i_0 and i_3/i_0, respectively.

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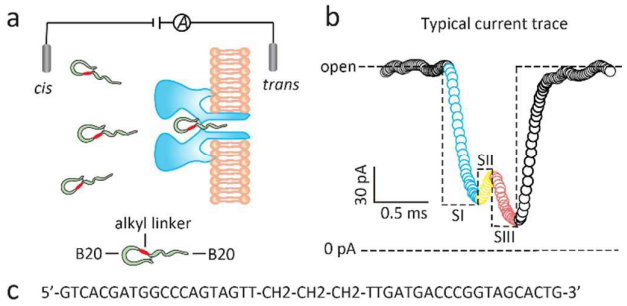

Fig. 1 (a) The translocation of a DNA conjugate through a single α-HL pore. A single protein α-HL pore was inserted in a lipid bilayer. A short propyl linker (red) was attached to two 20-mer oligonucleotides B20 (green) at each end of the DNA conjugate, which may form a four-base pair hairpin structure. The two chambers of the bilayer cell are named *cis* and *trans*; (b) The typical multi-level current trace for a translocation event of B20-$(CH_2)_3$-B20. The three-level current blockage was divided into three stages: SI (blue), SII (yellow) and SIII (wine), respectively; (c) The sequence of the synthesized DNA conjugate. The experimental data were acquired in 1.0 M KCl, 1 mM EDTA and 10 mM Tris (pH = 8.00). The potential across the bilayer was applied with a value of 150 mV through a pair of Ag/AgCl electrodes from the *cis* side

The Stage I (SI) had an i_1/i_0 of 0.72 ± 0.01. Previous studies had demonstrated that the four-base pair hairpin structure induced i/i_0 around 0.68[23, 24]. Therefore, SI was suggested for the leading part of the 20-base oligonucleotide together with the hairpin structure and the propyl linker occupying the β-barrel of α-HL. Then the current increased to Stage II (SII) with an i_2/i_0 of 0.64 ± 0.02. Previous studies have demonstrated that the signals for poly(dA)$_n$ and poly(dC)$_n$ shaped as a single-peak pulse without the intermediate bulge[22]. Thus, it was responsible for this transient blockage that the propyl linker sliding in the β-barrel of the α-HL pore toward the *trans* opening. Finally, the current dropped to Stage III (SIII) state with i_3/i_0 = 0.82 ± 0.01. SIII was almost fully reduced the pore current, which were consisted with previous work of ssDNA in its linear form translocating through the α-HL pore[21, 22]. The DNA oligomer was consistently unzipping during stage II, driven by the voltage. The molecular configuration of B20-$(CH_2)_3$-B20 in the α-HL pore, corresponding to each stage, was illustrated in Fig. 2c. Firstly, the DNA conjugate was captured in the vestibule of α-HL pore and underwent an unzipping process. Secondly, the propyl linker translocated the narrowest construction of the α-HL pore after completing the unzipping process of the hairpin structure, resulting in a clear current step as SII. Thirdly, the linear form of DNA conjugate rapidly transported through the α-HL pore, leading to a deep current blockage of SIII.

Except for the typical three-level current trace as Type I, we also found another two current traces (Type II and Type III) for B20-$(CH_2)_3$-B20. Type II showed similar SI and SII to Type I, with the current blockage of i_1/i_0 = 0.72 ± 0.01 and i_2/i_0 = 0.62 ± 0.02, respectively (Fig. 3a). However, the ionic currents of SIII, with i_3/i_0 =0.72 ± 0.01, was significantly lower than Type I. Interestingly, w e

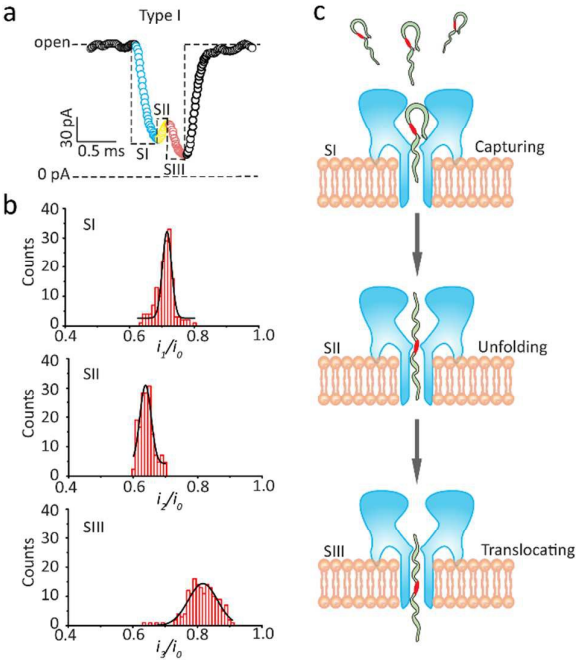

Fig. 2 (a) Representative three-stage level current trace for a translocation event of B20-$(CH_2)_3$-B20; (b) Histograms of blockage currents for SI, SII and SIII (from top to bottom), respectively. Each of the blockages was fitted into a Gaussian distribution; (c) Illustration of the translocation process of B20-$(CH_2)_3$-B20 through the α-HL pore. SI: trapping of the DNA conjugate in the pore, unfolding of the four-base pair DNA hairpin structure, and the translocation of the single-strand B20 at the 5' end. SII: translocation of the propyl linker. SIII: translocation of B20-$(CH_2)_3$-B20 molecule in its linear form

found that the SIII and SI of Type II had the same current level. Since the B20-$(CH_2)_3$-B20 had the same B20 structure at both ends (3' and 5'), Type II showed the same value of blocked current in SI and SIII when they went through the *cis* entry of the α-HL pore[25]. Thus, the current traces of Type II could be appointed to the trapped B20-$(CH_2)_3$-B20 returning to the *cis* solution without translocation. Type III showed a partial current blockage followed by a terminal spike with two current level states of i_1/i_0 = 0.71 ± 0.01 and i_2/i_0 = 0.85 ± 0.01, which were close to the SI and SIII of Type I (Fig. 3b). The duration time of propyl linker was too short to be measured by the apparatus due to the small size of propyl linker. Thus, these "step-spike" events should be attributed to the DNA hairpin structure of B20-$(CH_2)_3$-B20 translocating through the α-HL pore, rapidly[20]. In addition, we analyzed the frequencies of three typical blockage events caused by the translocation of B20-$(CH_2)_3$-B20. Because the concentration of the target molecules B20-$(CH_2)_3$-B20 could be considered a constant after a single protein pore was formed in the bilayer, the number of the signature events was linear to the measuring time[17]. The three lines had slopes of 0.31, 0.54 and 0.81 event s^{-1} for Type I (black), Type II (red) and Type III (blue),

respectively (Fig. 3c). The assay revealed a higher frequency of 0.85 for all the three-level events (Type I and II) compared to the "shoulder-spike" events (Type III) with a frequency of 0.81. Thus, it

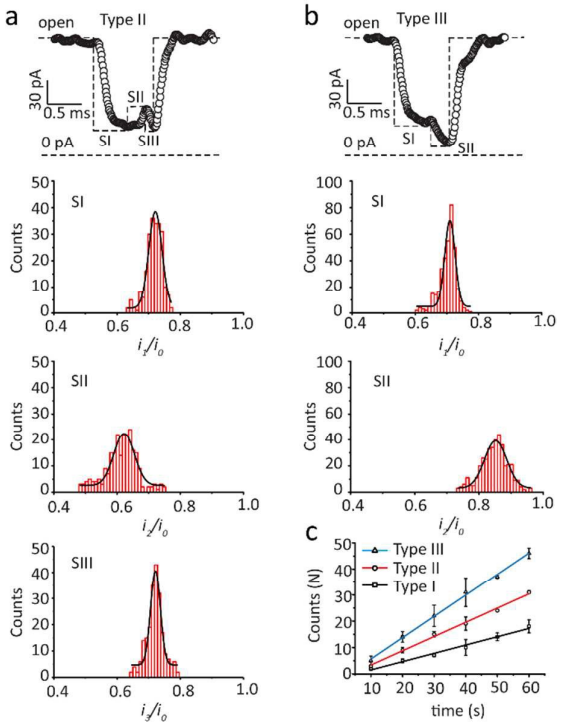

Fig. 3 (a) Top: typical current trace of Type II for B20-$(CH_2)_3$-B20 molecule. Bottom: histograms of blockage currents for SI, SII and SIII, respectively (from top to bottom); (b) Top: typical current trace of Type III for B20-$(CH_2)_3$-B20 molecule. Bottom: histograms of blockage currents for SI and SII, respectively (from top to bottom); (c) Number of the signature events versus the probing time for Type I (black), Type II (red) and Type III (blue), respectively.

indicated that the DNA hairpin structure might play a role in slowing down the translocation speed of the small alkyl linker.

Further evidence to support this translocation mechanism of B20-$(CH_2)_3$-B20 could be obtained by studying the lifetime of the multi-level events. As listed in Table 1, t_D, t_1, t_2 and t_3 were the entire lifetime, and the duration time for SI, SII and SIII of a blockage event, respectively. The entire lifetime t_D, corresponding to type I, II and III, was 1.38 ± 0.02 ms, 2.75 ± 0.02 ms and 1.12 ± 0.02 ms, respectively (Figure S2-4 in the Supporting Information Files). Type III had the shortest lifetime, which may provide evidence for its "shoulder-spike" current trace generated by the DNA conjugate translocating through the α-HL pore rapidly. The duration time of Type I are close to Type III, which could be appointed to the similar effect of B20-$(CH_2)_3$-B20 with the pore. Previous studies had demonstrated that the value of t_D became shorter when the applied potential increased and were linear with the number of bases (for 12-mer and above)[21, 26]. Therefore, we can estimate t_D of poly(dA)$_{20}$ at 150mv, from the average translocation rates of 1~20 μs/nucleotide for single-stranded polynucleotides, should be much shorter than the Stage I of all muti-level signals with t_1 = 0.41 ± 0.01 ms, 0.77 ± 0.02 ms and 0.78 ± 0.01 ms for Type I, Type II and Type III, respectively[22, 27, 28](Figure S2-4 in the Supplementary Information Files). It suggested that the unzipping process of the DNA hairpin structure resulted in a long duration time of SI. However, SII of Type I lasted for a comparably short time of 0.22 ± 0.01 ms, which agreed with our previous studies of the interaction between short organic linker and α-HL pore[23]. Additionally, the last Stage showed the duration time of 0.15 ± 0.02 ms and 0.21 ± 0.01 ms for Type I and III, respectively. Therefore, both the current depth and duration time for the last stage of Type I and III were consist with previous studies of ssDNA translocation through the α-HL nanopore, which illustrated that the final Stage was attributed to the translocation of B20-$(CH_2)_3$-B20 in its linear form[21]. Additionally, the possible reason for the distinguishable current changes could be the different interactions of nucleotides and the alkyl linker with the cation K^+. A recent work which employed α-HL for detecting a furan-containing double-strand DNA indicated the residence time of the DNA duplex inside of the pore was modulated by the specific interactions of the cations with the DNA and/or α-HL[9]. All these results further demonstrated the translocation mechanism of B20-$(CH_2)_3$-B20 molecule, which also ensured the clear temporal resolution of the alkyl linker.

In conclusion, we synthesized a DNA conjugate to generate a typical three-level signature for the detection of the target alkyl linker at single-molecule level. A four-base hairpin structure was introduced to enhance the temporal resolution of the alkyl linker. Also, we investigated the translocation process of B20-$(CH_2)_3$-B20 through a single α-HL pore by analyzing the current blockage and duration time of three type events. The results showed that the unfolding process of the hairpin structure was followed by the translocation of the short organic linkage. The translocation process of the alkyl linker produced an easily recognized increase of the ionic currents, which could be utilized for sensing and identifying small organic molecule. In conclusion, all these results of our experiments contributed to a deeper understanding of the translocation process of biomolecules through the protein pore and provided a potential probability of sensing small organics at the single-molecule level.

Table 1. Blockage currents and duration time of the multi-level signals. [a)]

Type	i_1/i_0	i_2/i_0	i_3/i_0	t_1 (ms)	t_2 (ms)	t_3 (ms)	t_D (ms)
I	0.72 ± 0.01	0.64 ± 0.02	0.82 ± 0.01	0.41 ± 0.01	0.22 ± 0.01	0.15 ± 0.02	1.38 ± 0.02
II	0.72 ± 0.01	0.62 ± 0.02	0.72 ± 0.01	0.77 ± 0.02	0.41 ± 0.01	0.43 ± 0.01	2.75 ± 0.02
III	0.71 ± 0.01	0.85 ± 0.01	N.A.	0.78 ± 0.01	0.21 ± 0.01	N.A.	1.12 ± 0.02

a) The histograms of duration time for Type I, II and III are shown in the Supplementary Files.

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Facilitated Alkyl Detection by a DNA Conjugate with an α-Hemolysin Nanopore

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Abstract Herein an α-hemolysin nanopore was utilized to detect a DNA conjugate, where a propyl linker was attached to the 20-mer oligonucleotides at each end of an individual strand. The studies showed the alkyl linker produced a clear current change when translocating through the protein pore, which could be used to sense and identify small organic molecule at single-molecule level.