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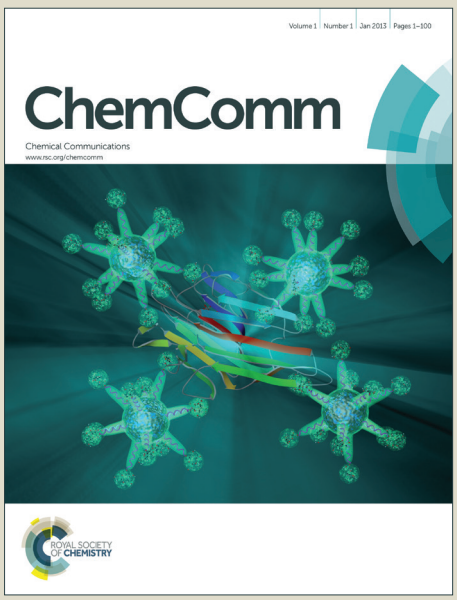

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One-by-one single-molecule detection of mutated nucleobases by monitoring tunneling current using a DNA tip†

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A DNA molecule was utilized as a probe tip to achieve single-molecule genetic diagnoses. Hybridization of the probe and target DNAs resulted in electron tunneling along the emergent double-stranded DNA. Simple stationary monitoring of the tunneling current leads to single-molecule DNA detection and discover base mismatches and methylation.

The electron transport through a single DNA molecule is of profound significance in technological and scientific perspectives. Recently, much effort has been devoted to investigating electron transport through a single molecule based on, e.g., scanning tunneling microscope (STM)-based break junction technique.[1-3] This methodology has been further applied to exploring the electron transport in DNA at the single-molecule level.[4-7] Such studies bring fundamental understanding of the charge transport property of relatively short DNA duplexes. The measurements further reveal that the alteration of a nucleobases in a double-stranded DNA (dsDNA) causes a significant change in its conductance.[5, 8] It is thus anticipated that the electrical conductance measurements enable to discover the mutation of a single DNA. To achieve this, in-depth understanding of the electron transport through mutated DNAs is crucial.

Recently, we developed a method to quantitatively investigate electron transfer from a single molecule to neighboring another single molecule through the use of an STM molecular tip. For example, this technique was successfully applied to exploring electron transfer mediated by a hydrogen bond interaction between single molecules,[9] which deepens the understanding of electronic functions of a single molecule.[1, 3, 10] Moreover, a DNA molecular tip constructed by immobilizing a single-stranded DNA (ssDNA) on a Au

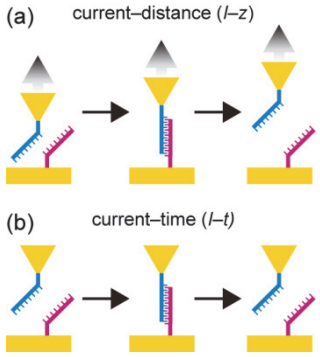

Fig. 1 Current measurements for the single-molecule DNA detection in (a) distance and (b) time domains.

STM tip was utilized to examine electron transport through a dsDNA of various lengths formed by hybridization of the DNA tip with the target ssDNA on a substrate (Fig. 1a).[11] In the present research, we demonstrate that the DNA tip allows for the direct detection of DNA mutations at the single-molecule level. The DNA tip was held stationary in close proximity to the target ssDNA, similar to the measurement of single-molecule conductance,[12, 13] to monitor the tunneling current for the mutation detection. As a proof of concept for single-molecule genetic diagnosis, the mutation analysis was performed on a DNA sample containing both methylated and unmethylated strands. DNA methylation is a central epigenetic modification, playing crucial roles in cellular processes such as genome regulation, development, and disease.[14-16] The proposed detection methodology is compatible with and easily applied to other sensing platforms, including nanofluidic and nanopore devices,

and offers a novel avenue for future genetic diagnosis at the single-molecule level.

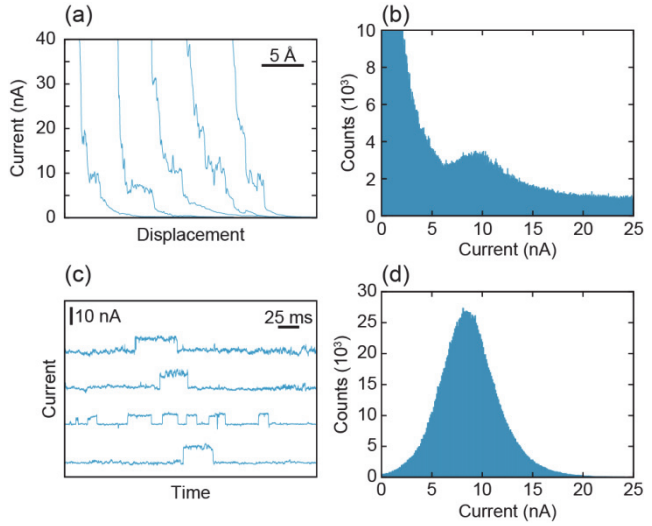

Fig. 2 (a), (c) Representative *I–z* and *I–t* plots, respectively, measured using the T_8 tip and S_8 sample. Each plot is offset either horizontally or vertically for clarity. Bias voltage: 0.2 V, initial set-point current: 75 nA (a) and 20 nA (c). (b), (d) current histograms obtained by *I–z* and *I–t* measurements, respectively. Bin size: 0.1 nA.

Gold STM tips functionalized with ssDNA served as a DNA tip to measure the tunneling current for Au(111) substrates on which target ssDNA was adsorbed. All the current measurements were conducted in a 0.1 M $NaClO_4$ aqueous solution at room temperature (see ESI† for details of the experimental procedures). Fig. 2a shows the results obtained using the current–distance (*I–z*) measurements, where the tunneling current was monitored as a function of the tip–substrate separation. The ssDNAs on the tip and substrate, referred to as T_8 and S_8, respectively (see Table S1 in ESI† for their sequences), were 8-mer long and complementary to each other. In these measurements, the DNA tip was brought close proximity to, but never contacted with, the substrate surface, and then current was recorded while the tip was retracted (Fig. 1a). The *I–z* traces were characterized by plateaus in which the tunneling current stayed constant despite the increased tip–sample separation. The appearance of the plateaus indicates the formation of a molecular junction. As demonstrated in our previous study,[11] the DNA tip undergoes hybridization with the target ssDNA adsorbed onto a substrate. The resulting dsDNA bridges the gap between the metal tip and substrate, and consequently induced electron transfer through this bridge. The *I–z* curves were statistically analyzed by constructing the current histogram created by using the current value of each data point of the *I–z* traces (Fig. 2b). Because of the plateaus in the *I–z* curves, a single pronounced peak appeared in the histogram. On the basis of the current value at the peak position, the conductance value of the dsDNA molecular junction created by the DNA tip and target ssDNA on the substrate was determined to be 48 nS, agreeing well with the previous study.[11] In addition, it was reported that the single-molecule conductance of 8-base-pair dsDNA having repeated GC sequence is 100 nS.[4] Given the fact that the G base has a relatively low highest occupied molecular orbital favorable for the charge transport,[5] the present results are in reasonable agreement with the literature. Thus, we conclude that the DNA tip enables single-molecule detection of the target sequence on the basis of electron transfer through the dsDNA formed by in-situ hybridization during the current measurement. In the previous study, it was discussed that the electron transfer as observed in the present work most probably takes place through stacked base pairs in the DNA duplex via a tunneling or super exchange process.[11] Although electron hopping is the dominant mechanism for the charge transport in dsDNA containing abundant G bases,[17] the tunneling process is the most probable mechanism in the present experiments because of the random DNA sequences.

In the present work, the DNA tip was further applied to the single-molecule detection in the time domain (Fig. 1b). For the current–time (*I–t*) measurements, the DNA tip was first brought close proximity to the sample surface. Then, the tip was held stationary by disabling the feedback control of the STM and probed the tunneling current with the sample surface. This measurement protocol eliminates the requirement for piezo actuators to drive the tip and, therefore, greatly facilitates the applicability to future single-molecule genetic analysis technologies. Fig. 2c presents the *I–t* measurements performed using the T_8 DNA tip and a substrate modified with the complementary S_8 DNA. The *I–t* plots exhibited sudden current jumps followed by plateaus over the stable background current, which is equal to the initial set-point current. These jump–plateau signatures indicate the formation of molecular junctions that connect the tip and substrate and induce electron transfer.[18] The increased amount of the current relative to the background value was evaluated for each data point in the plateaus, and current histogram was constructed based on the increased values (Fig. 2d). The histogram exhibits one distinct peak at 8.5 nA, which allows for determination of the conductance of the molecular junction to be 43 nS. Importantly, these values are nicely consistent with those obtained in the *I–z* measurements (Fig. 2b). Thus, the jump–plateau signals are ascribed to the electron transfer through the dsDNA between the tip and substrate. Control experiments were performed using a non-complementary sample DNA (S_8-nc, Table S1 in ESI†). The resulting *I–t* traces showed no obvious jump–plateau signatures, which corroborates the assignment that the formation of the dsDNA causes the current jump and plateau. In the present experiments, the substrate was modified with the target DNA at a low coverage by employing a low concentration of the DNA solution and short modification time (see ESI†), and this condition is important to form a single-molecule junction.[12] In addition, the low surface density of the immobilized DNA can increase the efficiency for the hybridization.[19] When the DNA tip was kept still in proximity to the sample surface, the target ssDNA, which freely diffuses on the gold substrate under this experimental condition, spontaneously hybridizes with the tip DNA. Since the melting temperature of the T_8–S_8 duplex is approximately room temperature (22.3°C),[11] the DNA on the tip and substrate can reversibly hybridize and dehybridize between the tip and substrate, generating the bistable current signals (Fig. 2c). Therefore, we conclude that single-molecule DNA detection is achieved based on the static current measurements. The measurement scheme is very simple and requires only current sensing within a nanogap that has a size comparable with the length

of a target DNA. Such measurements can be easily implemented with other devices such as nanofluidic and nanopore devices.

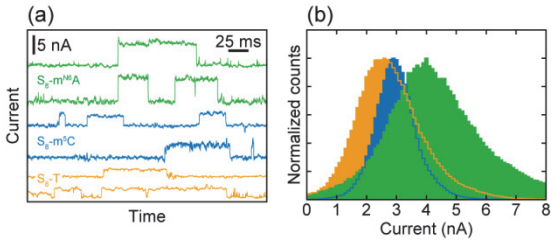

Fig. 3. (a) Representative *I–t* plots and (b) current histogram measured using the T_8 tip and S_8-T (yellow), S_8-m^5C (blue), and S_8-$m^{N6}A$ (green). Each *I–t* plot is shifted vertically for clarity. Bias voltage: 0.2 V, initial set-point current: 20 nA, bin size: 0.1 nA. The counts at the peak maxima are 44406, 67562, and 53701 for the S_8-T, S_8-m^5C, and S_8-$m^{N6}A$, respectively. The histograms in (b) were normalized by these maximum values.

We next investigated single-molecule detection of DNA mutations, which is essential for genetic analyses. *I–t* measurements were performed using the T_8 DNA tip and mutated sample DNAs. The samples include S_8-T DNA, which contain a mismatched T base, and DNAs containing methylated bases, 5-methylcytosine and N6-methyladenine (S_8-m^5C and S_8-$m^{N6}A$, respectively; see Table S1 in ESI†). Under all conditions, the *I–t* plots exhibited clear jump–plateau characteristics (Fig. 3a), indicating the formation of DNA duplexes between the T_8 probe and the mutated sample strands. Single distinct peaks characterized the resulting current histograms (Fig. 3b), and the single-molecule conductance of the mutation-containing dsDNA was successfully quantified. Both the mismatched and methylated bases were found to cause significant decreases in the conductance values (13 nS, 15 nS, and 21 nS for S_8-T, S_8-m^5C, and S_8-$m^{N6}A$, respectively) compared with the fully matched sample (43 nS). The effect of the DNA mutations is summarized in Fig. 4a. The results of the mismatched S_8-T DNA are in line with previous *I–z* measurements using metal tips.[5] The hybridization in the presence of the methylated bases and the subsequent formation of the dsDNA single-molecule junction is reasonable given that methylated bases stabilize dsDNAs[20] due to stronger base stacking that results from a methylation-induced increase in polarizability.[21] This increased stability renders conventional assays that rely on probe-sample hybridization inapplicable to methylation analyses. In contrast, the present method easily detects methylated bases because of the sensitivity of tunneling currents to local chemical environments. The observed decrease in conductance in the presence of methylated bases agrees with a previous measurement of the single-molecule conductance of dsDNA with an alternating G:C sequence.[8] One may expect the conductance increase, rather than decrease, upon methylation because of the increased stability of the duplex containing the methylated bases (see above). However, the higher thermal stability of the dsDNA does not necessarily imply better electronic coupling between the stacked base pairs for charge transfer. The conductance decrease can be explained by the alteration of the energy gap between the highest occupied and lowest unoccupied molecular orbitals by the methylated bases. The alternative scenario where DNA methylation cause worse electronic coupling in the base stacking has been discussed in the literature.[8]

The *I–t* measurements as in Fig. 3 demonstrate that the DNA tip provides a direct means to electrically identify the base mismatch and the epigenetic methylation of DNA at the single-molecule level.

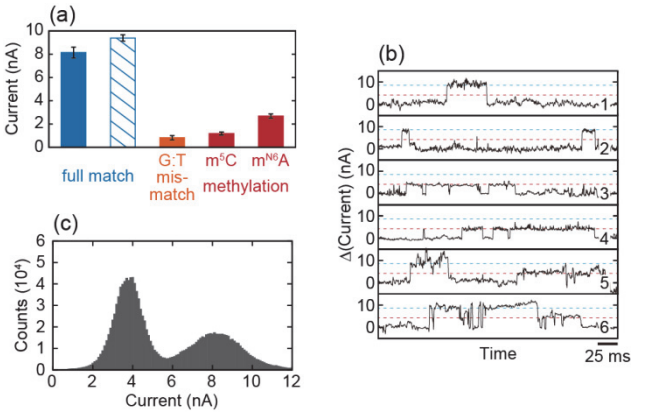

Fig. 4 (a) The peak current values in the histograms for the DNA tip and fully matched and mutated DNA samples. Solid and hatched bars indicate *I–t* and *I–z* measurements, respectively. (b) Representative *I–t* plots and (c) current histogram measured using the T_8 tip and a mixture of S_8 and S_8-$m^{N6}A$ sample DNAs. Bias voltage: 0.2 V, initial set-point current: 20 nA, bin size: 0.1 nA. In (b), the difference in current from the background is plotted, and the blue and red dashed lines indicate the peak current values for the pure S_8 and S_8-$m^{N6}A$, respectively.

As demonstrated above, the recognition achieved using the DNA tip is in marked contrast to that of conventional methods. Specifically, the measurements were performed on a single-molecule basis without averaging out the signals in ensemble measurements. Thus, the DNA tip is expected to achieve detection of the mutations even in mixed samples containing both unmutated and mutated DNAs, a condition that is mandatory for practical high-throughput applications. To demonstrate this, current measurements using the T_8 DNA tip were performed for a mixture of S_8 and S_8-$m^{N6}A$ (1:1) DNA as the sample. The jump–plateau signals were observed in the *I–t* traces, as shown in Fig. 4b. Careful examination of these traces revealed the existence of two types of signals, those showing larger and smaller current increases (traces 1 & 2 and 3 & 4 in Fig. 4b, respectively). Some *I–t* plots exhibited two such signals in succession (trace 5 & 6). The current histogram complied from the *I–t* traces was consequently characterized by two peaks (Fig. 4c). Two Gaussian functions fit the histogram very well and the current values at the peak positions were determined to be 3.8 nA and 8.1 nA. The peaks at the higher and lower current nicely agree with those obtained in the measurements using the pure complementary S_8 and methylated S_8-$m^{N6}A$ samples, respectively (Fig. 4a). A single hybridization process produces each jump–plateau signal in Fig. 4b. The presence of a mutation in a sample strand is read out from its current value, enabling one-by-one mutation analysis of a single DNA molecule.

In summary, we achieved single-molecule DNA detection by conducting *I–t* measurements using DNA molecular tips. The introduction of the mutated bases in target DNAs substantially affects the extent of electron transfer, allowing the identification of mismatched and methylated bases in single DNA molecules. Consequently, we successfully performed a mutation analysis of DNA samples containing both mutated and unmutated strands by using one-by-one single-molecule detection. The present methodology is

very simple; it can be easily realized by current sensing in a nanogap electrode and implemented with, e.g., nanofluidic or nanopore devices. Therefore, the DNA tip is a promising method that can be used for future single-molecule genetic diagnosis.

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Notes and references

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† Electronic Supplementary Information (ESI) available: sequences of oligonucleotide used as sample and tip molecules (Table S1), and experimental procedures. See DOI: 10.1039/c000000x/

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