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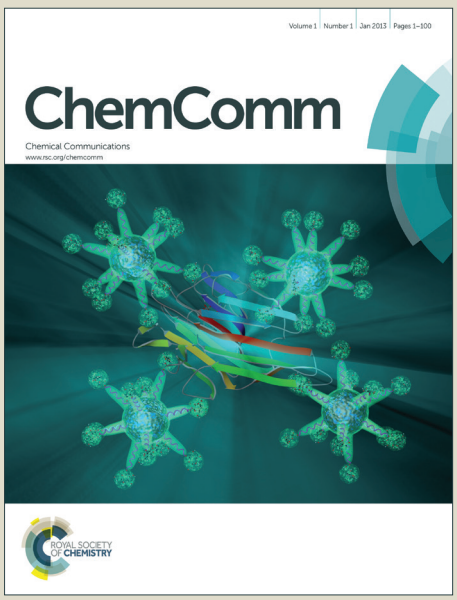

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Rapid and sensitive detection of HBV DNA with nanopore sensor

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Nanopore analysis has emerged as the simplest single-molecule technique. We combined DNA probes with nanopore electrochemical sensor for rapid and sensitive detection of pathogenic DNA. The novel nanopore biosensor allows the single-base discrimination and detection of picomolar DNA in serum samples.

Rapid and sensitive detection of sequence-specific DNA associated with human diseases is of great importance in a variety of applications, such as biomedical research, pathogen identification and early clinical diagnosis.[1, 2] For example, Hepatitis B virus (HBV) is one of the causative agents of viral hepatitis. The infection with HBV is a major health problem worldwide and can cause both acute and chronic disease.[3] According to the World Health Organization (WHO), more than 240 million people have chronic liver infections and about 600 000 people die every year due to the consequences of HBV. One way to control the spread of diseases is to perform fast and effective diagnosis of the virus as early as possible and to carry out efficacious treatment or quarantine. Therefore, a variety of assay methods, including polymerase chain reaction (PCR),[4, 5] DNA microarrays,[6] electrochemical biosensor,[7-9] surface-enhanced Raman Spectrometry,[10, 11] surface plasmon resonance imaging,[12] molecular beacon,[13, 14] colorimetry,[15] and quantum dots[16, 17] have been developed to achieve sensitive detection of DNA. Among the techniques, PCR is the most widely used technique to achieve the ultrasensitive detection of DNA due to its target amplification strategy. However, the use of multiple primers and special DNA polymerases in PCR[18] increase the experimental complexity and cost, which limits its practical applications. In addition, PCR requires the precise control of temperature cycling for successful amplification. Other methods such as eletrochemcial biosensor and molecular beacons, though very powerful and sensitive, but still shared the drawbacks of tedious procedures, false positives, or low sensitivity. Therefore, it is greatly desired to develop new approach for simple and rapid DNA detection without the use of time-consuming labeling or error-prone amplification methods.

The α-hemolysin (α-HL)-based protein nanopore has been devised as sensor elements for stochastic detection of various analytes.[19-21] A single heptameric α-HL protein inserts into a planar lipid bilayer and forms a transmembrane nanopore consisting of a vestibule and β barrel. Under an applied potential, when an analyte drives through the pore, the registering information of analyte within it is recorded by characteristic electric signals containing the magnitude of current and dwell time, by which the analyte can be identified and quantified. In this way, α-HL pores have been used to detect metal ions,[22, 23] small organic molecules,[19, 24-27] peptides,[28] proteins.[29] In the DNA researches, distinct nanopore sensors have been explored to examine unzipping kinetics of duplex DNA[30, 31] including oxidized lesions[32], DNA abasic site[33] and G-quadruplexes hybrid folds[34] except for nanopore DNA sequencing applications.[35-37] It is still a novel target in the emerged field to develop nanopore detectors that are capable of discriminating and quantifying specific pathogenic markers. Recently, two groups of Drndic[38] and Gu[39, 40] reported the use of solid and protein pores to detect cancer-related microRNAs respectively. However, so far few studies deals with the detection of pathogenic DNA using nanopore sensors.

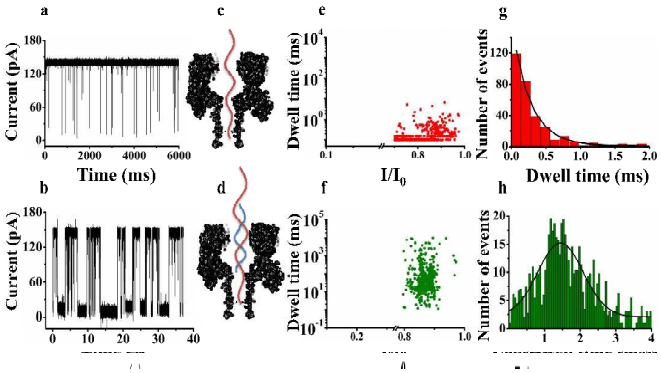

Figure 1. Nanopore detection of target HBV DNA. (a) Representative single-channel current traces of the probe DNA (250 nM); (b) Typical single-channel current traces of probe and target mixture (each DNA concentration: 250 nM); (c) and (d) Schematic illustration of the probe DNA and the probe/target hybrid DNA passing through the α-HL nanopore; (e) and (f) The scatter plot of the events generated by the probe and probe/target mixture (event dwell time versus normalized current amplitude I/I_0); (g) The histogram of dwell time for probe, which was fitted by the exponential function; (h) The dwell time histogram of probe/target translocation through the pore. The histogram is fitted into a Gaussian function. I in Figure 1c and 1d is current blockage of translocation events, and I_0 is the open pore current. I/I_0 is normalized current blockage. All the experiments were performed in solution containing 1M KCl and 10 mM Tris, pH 8.5. The transmembrane potential was +150 mV.

Single-stranded DNA can slide through α-HL nanopore at a bias voltage, but the signal is undistinguishable for DNA with similar length. To address the challenge, DNA probe technique was combined with nanopore detection in the present work. The novel nanopore DNA biosensor relies on the hybridization reaction between the short HBV target strand and deliberately designed

DNA probes. We demonstrated that the target HBV DNA could be detected with high sensitivity and selectivity. Furthermore, we showed that the potential of this approach by discriminating DNA at single-base resolution and quantitating picomolar levels of HBV-specific DNA in serum samples.

The sequence of the probe molecule specially designed for HBV DNA is shown in table S-1. The middle of the probe is a hybridization domain for target HBV DNA. The 3' and 5' end of the probe is flanked by two 25-mer poly (dA) overhangs (poly $(dA)_{25}$). The purpose of the overhang is to induce DNA into the vestibule of the pore for subsequent translocation and increase the frequency of translocation events. Our experiments demonstrated that compared with Poly (dC), Poly (dT) and Poly (dG), Poly (dA) yielded the largest blockage and longest translocation time, which will improve the signal to noise ratio and temporal resolution. As shown in Figure 1a and 1c, the addition of the probe molecules on the cis side (the vestibule side) produced transit events with an average current blockage of (89.7 ± 0.6) % (Figure 1e) and the mean translocation time of 266.32 ± 2.01 μs (Figure 1g). For the detection of target HBV DNA, the probe and target were mixed and prehybridized before adding to the cis solution to ensure maximum duplex formation and to minimize single-stranded events. Figure 1b, d, f, h display the results of probe/target mixture on the cis side of the nanopore. We observed that the events with short dwell time decreased significantly. Instead, a large number of prolonged events could be observed in the current trace (Figure 1b). The mean dwell time of the probe/target hybrid was about 27.95 ± 1.09 ms, 100 fold longer than that of probe translocation time (Figure 1h). The average current blockage dropped to (92.8 ± 0.4) % of the open pore current. The Scatter plot showed current blockage and dwell time (Figure 1f). It should be noted that in order to avoid the intervention from normal ssDNA translocation such as probe ssDNA and target ssDNA, we selected the events with dwell time longer than 1 ms for plotting. The discrimination between the probe and the probe/target hybrid was readily achieved based on translocation time. These long blockade duration translocation events were only observed in the presence of both probe and target, which is ascribed to the formation of probe/target hybrids that unzipped in the nanopore under a transmembrane voltage (1d). The conclusion is further supported by voltage dependence experiments. In the presence of the probe/target hybrid on the cis side of the pore, the mean dwell time decreased as the transmembrane potential increased (Fig. S1). Therefore, the dwell time is a good characteristic signature for target DNA identification.

The probe/target hybrid translocation through the pore could result in events having longer dwell time that is different from those events generated by probe or target alone (Fig. S2). The α-HL nanopore thus offers the potential to develop a sensitive sensor for specific sequence DNA detection. To testify this concept, HBV DNA at various concentrations were examined. As shown in Figure 2a, the frequency of long-lived events increased with an increase in the concentration of HBV DNA. Linear regression analysis showed a good linearity between the long-lived event frequency and target DNA concentration ranging from 100 pM to 50 nM with correlation coefficient of 0.98 (Figure 2b, inset). The detection limit can be as low as 10 pM target HBV DNA in the solution (S/N = 3). Such low detection limit is mainly due to the high sensitivity of the designed probe and the application of high voltage (Fig.S3, ESI). Above HBV concentration 100 nM, the change in long event frequency with smaller slope could be ascribed to the weak interaction between HBV DNA molecules (Figure 2b).

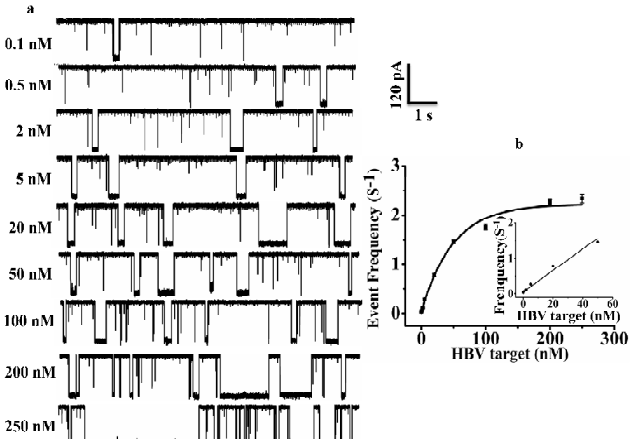

Figure 2. Detection of HBV DNA at various concentrations using single-channel recording with α-HL nanopore. (a) Representative single-channel current traces showing various concentrations of target DNA added to the *cis* chamber in the presence of 250 nM probe. The concentrations of target DNA were ranging from 0.1 nM to 250 nM, respectively (from up to down). (b) Plot of event frequency corresponding to target DNA at various concentration. The calculation of event frequency was based on the long-lived events. Inset: The calibration plot of event frenquency as a function of target DNA in the range of 0.1 nM to 50 nM. Traces were recorded at +150 mV in 10 mM Tris solutions containing 1M KCl (pH 8.5). Three separate experiments were performed, and the mean value is plotted.

To test the selectivity and specificity of the nanopore sensor for HBV DNA detection, a series of comparative studies using the single-base and two-base mismatch sequences were investigated as control experiments. From the results shown in Figure 3a and Figure 3b, we can see that the presence of one-base and two-base mismatch in the probe/target hybrid causes significant reduction of translocation time. The durations of signature events (τ_{sig}) were 4.67 ± 1.04 ms and 4.01 ± 1.03 ms, respectively, which was about 7 times shorter compared with complementary probe/target hybrid (Figure 3c). The results suggest that the translocation of the perfect complementary duplexes take much longer than that of duplexes containing mismatches. This is easily explained by duplex stability. Stable duplexes need more time and energy to unzip by the nanopore. The interpretation is consistent with the predicted theoretical hybridization free energy. We calculated hybridization free energy by the DINAMelt Web Server.[41] The values were -24.27, -20.72 and -17.19 kcal/mol for fully matched, single-base and two-base mismatched duplexes, respectively. Therefore, the fully matched probe/target hybrids can be distinguished from those probe/target hybrids that contain mismatches based on dehybridization time. The method developed in the present study has high selectivity of HBV DNA sequence with single-base resolution. In order to investigate the feasibility of the

nanopore biosensor to HBV DNA analysis in real samples, DNA in serum was detected with method as it provides a protein-containing background, relevant in a diagnostic setting. The results were shown Figure 3d and Figure S4. From Figure 3d (top panel), it can be seen that, although the serum samples were complex with many different species, the signal to noise of the nanopore current is still at a high level. In the presence of probe alone, only short-lived events were observed as

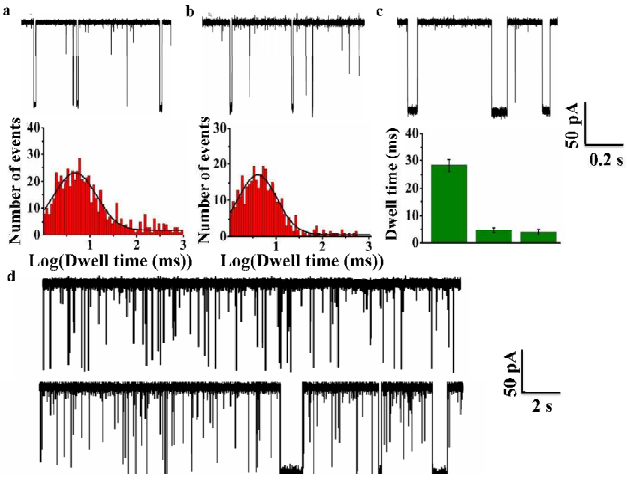

Figure 3. Discrimination of HBV DNA against the control DNA sequences with single-base and two-base mismatch. (a) Detection of control DNA sequence with single-base mismatch (top panel: current trace, bottom panel: the histogram of dwell time for signature events). (b) Detection of control DNA sequence with two-base mismatch (top panel: current trace, bottom panel: dwell time of signature events). (c) Top panel: current trace of probe/target hybrid with fully matched translocation through the pore; Bottom panel: dwell time of fully matched probe/target hybrids and probe/target hybrids containing mismatches. (d) Representative current traces for detection of HBV DNA in human serum samples before (top panel) and after (bottom panel) the addition of 100 pM HBV DNA to the *cis* solution. The concentration of probe in the serum sample was 250 nM. The experiments were carried out at +150 mV in solution containing 1 M KCl buffered with 10 mM Tris (pH 8.5). Three separate experiments were performed, and the mean value is plotted.

single-stranded probe DNA translocation through the pore. After the addition of HBV DNA to the serum containing the probe, HBV DNA with characteristic long-lived blocks can be identified and quantified (Figure 3d, bottom panel). The results demonstared that this nanopore sensor had strong anti-interference ability and could be applied to the detection in complex samples.

In conclusion, we demonstrated a highly sensitive and selective nanopore biosensor for label-free detection of HBV DNA. We achieved a low detection limit of 10 pM for target DNA. In addition, the nanopore sensor also exhibits excellent selectivity for target DNA containing a single and a double mismatch. More importantly, the proposed method can be used to detect target DNA in serum sample. Combined with the portable nanopore sensor chip that can work outside the lab,[42, 43] the DNA sensor should have a great potential for practical application in early diagnosis of diseases.

This work was financially supported by the National Science Foundation of China (21175105, 21375104 and 21327806), the Specialized Research Fund for the Doctoral Program of Higher Education of China (20126101110015).

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1. F. S. Collins, E. D. Green, A. E. Guttmacher and M. S. Guyer, *Nature*, 2003, 422, 835-847.
2. L. M. Staudt, *Trends Immunol.*, 2001, 22, 35-40.
3. T. L. Wright and J. Y. Lau, *Lancet*, 1993, 342, 1340-1344.
4. C. A. Heid, J. Stevens, K. J. Livak and P. M. Williams, *Genome Res*, 1996, 6, 986-994.
5. P. Gill and A. Ghaemi, *Nucleosides Nucleotides Nucleic Acids*, 2008, 27, 224-243.
6. C. M. Niemeyer and D. Blohm, *Angew. Chem. Int. Ed.*, 1999, 38, 2865-2869.
7. Y. Xiao, X. Lou, T. Uzawa, K. J. Plakos, K. W. Plaxco and H. T. Soh, *J. Am. Chem. Soc.*, 2009, 131, 15311-15316.
8. F. Xia, X. Zuo, R. Yang, R. J. White, Y. Xiao, D. Kang, X. Gong, A. A. Lubin, A. Vallee-Belisle, J. D. Yuen, B. Y. Hsu and K. W. Plaxco, *J. Am. Chem. Soc.*, 2010, 132, 8557-8559.
9. Y. W. Hu, F. H. Li, X. X. Bai, D. Li, S. C. Hua, K. K. Wang and L. Niu, *Chem. Commun.*, 2011, 47, 1743-1745.
10. E. Papadopoulou and S. E. Bell, *Angew. Chem. Int. Ed.*, 2011, 50, 9058-9061.
11. A. Barhoumi and N. J. Halas, *J. Am. Chem. Soc.*, 2010, 132, 12792-12793.
12. W. J. Zhou, Y. Chen and R. M. Corn, *Anal. Chem.*, 2011, 83, 3897-3902.
13. J. H. Huang, X. F. Su and Z. G. Li, *Anal. Chem.*, 2012, 84, 5939-5943.
14. K. Wang, Z. Tang, C. J. Yang, Y. Kim, X. Fang, W. Li, Y. Wu, C. D. Medley, Z. Cao, J. Li, P. Colon, H. Lin and W. Tan, *Angew. Chem. Int. Ed.*, 2009, 48, 856-870.
15. X. Liu, F. Wang, R. Aizen, O. Yehezkeli and I. Willner, *J. Am. Chem. Soc.*, 2013, 135, 11832-11839.
16. C. Y. Zhang and J. Hu, *Anal. Chem.*, 2010, 82, 1921-1927.
17. J. Zhou, Q. X. Wang and C. Y. Zhang, *J. Am. Chem. Soc.*, 2013, 135, 2056-2059.
18. T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T. Hase, *Nucleic Acids Res.*, 2000, 28, E63.
19. L. Q. Gu, O. Braha, S. Conlan, S. Cheley and H. Bayley, *Nature*, 1999, 398, 686-690.
20. S. Howorka and Z. Siwy, *Chem. Soc. Rev.*, 2009, 38, 2360-2384.

21. J. E. Reiner, A. Balijepalli, J. W. Robertson, J. Campbell, J. Suehle and J. J. Kasianowicz, *Chem. Rev.*, 2012, 112, 6431-6451.
22. O. Braha, L. Q. Gu, L. Zhou, X. Lu, S. Cheley and H. Bayley, *Nat. Biotechnol.*, 2000, 18, 1005-1007.
23. S. Wen, T. Zeng, L. Liu, K. Zhao, Y. Zhao, X. Liu and H. C. Wu, *J. Am. Chem. Soc.*, 2011, 133, 18312-18317.
24. X. F. Kang, S. Cheley, X. Guan and H. Bayley, *J. Am. Chem. Soc.*, 2006, 128, 10684-10685.
25. R. Kawano, T. Osaki, H. Sasaki, M. Takinoue, S. Yoshizawa and S. Takeuchi, *J. Am. Chem. Soc.*, 2011, 133, 8474-8477.
26. A. J. Boersma, K. L. Brain and H. Bayley, *ACS Nano*, 2012, 6, 5304-5308.
27. X. Zhang, J. J. Zhang, Y. L. Ying, H. Tian and Y. T. Long, *Chem. Sci.*, 2014, 5, 2642-2646.
28. L. Movileanu, J. P. Schmittschmitt, J. M. Scholtz and H. Bayley, *Biophys J.*, 2005, 89, 1030-1045.
29. D. Rotem, L. Jayasinghe, M. Salichou and H. Bayley, *J. Am. Chem. Soc.*, 2012, 134, 2781-2787.
30. A. F. Sauer-Budge, J. A. Nyamwanda, D. K. Lubensky and D. Branton, *Phys. Rev. Lett.*, 2003, 90, 238101.
31. S. Howorka, S. Cheley and H. Bayley, *Nat. Biotechnol.*, 2001, 19, 636-639.
32. Q. Jin, A. M. Fleming, C. J. Burrows and H. S. White, *J. Am. Chem. Soc.*, 2012, 134, 11006-11011.
33. N. An, A. M. Fleming, H. S. White and C. J. Burrows, *Proc. Natl. Acad. Sci. U S A*, 2012, 109, 11504-11509.
34. N. An, A. M. Fleming and C. J. Burrows, *J. Am. Chem. Soc.*, 2013, 135, 8562-8570.
35. J. Clarke, H. C. Wu, L. Jayasinghe, A. Patel, S. Reid and H. Bayley, *Nat. Nanotechnol.*, 2009, 4, 265-270.
36. I. M. Derrington, T. Z. Butler, M. D. Collins, E. Manrao, M. Pavlenok, M. Niederweis and J. H. Gundlach, *Proc. Natl. Acad. Sci. U S A*, 2010, 107, 16060-16065.
37. Y. L. Ying, J. Zhang, R. Gao and Y. T. Long, *Angew. Chem. Int. Ed* ., 2013, 52, 13154-13161.
38. M. Wanunu, T. Dadosh, V. Ray, J. Jin, L. McReynolds and M. Drndic, *Nat. Nanotechnol.*, 2010, 5, 807-814.
39. Y. Wang, D. Zheng, Q. Tan, M. X. Wang and L. Q. Gu, *Nat. Nanotechnol.*, 2011, 6, 668-674.
40. X. Zhang, Y. Wang, B. L. Fricke and L. Q. Gu, *ACS Nano*, 2014, 8, 3444-3450.
41. N. R. Markham and M. Zuker, *Nucleic Acids Res.*, 2005, 33, W577-581.
42. L. Cassiday, *Anal. Chem.*, 2007, 79, 3979.
43. X. F. Kang, S. Cheley, A. C. Rice-Ficht and H. Bayley, *J. Am. Chem. Soc.*, 2007, 129, 4701-4705.

Manuscript ID: CC-COM-08-2014-006135

Title: Rapid and sensitive detection of HBV DNA with nanopore sensor

The simplest single-molecules nanopore sensor can be used for the rapid and sensitive detection of pathogenic DNA at single-base recognition level.