An ultrahighly sensitive and selective electrochemical DNA sensor *via* nicking endonuclease assisted current change amplification†

Jinghua Chen, Jing Zhang, Juan Li, Fengfu Fu, Huang-Hao Yang* and Guonan Chen*

Received 3rd April 2010, Accepted 1st June 2010
DOI: 10.1039/c0cc00748j

Based on the "nicking endonuclease assisted" detection strategy, a novel electrochemical biosensor for the sequence-specific detection of DNA with ultrahigh sensitivity and selectivity is developed. By employing the above strategy, this DNA biosensor can detect as low as 0.068 fM target DNA and exhibits high discrimination ability even against a single-base mismatch.

Detection of ultralow amounts of DNA is extremely important in clinical diagnostics, gene therapy, mutation detection and a variety of other biomedical applications.[1] To date, the detection of trace specific DNA sequences relies heavily upon various target amplification and signal amplification methods, such as polymerase chain reaction (PCR)[2] and rolling circle amplification.[3] However, these methods suffer from several drawbacks including complex handling procedures, vulnerability to contamination and high cost. In recent years, owing to the fact that electrochemical detectors are simple, portable, sensitive and have a fast response and low cost, electrochemical biosensors have been recognized as a promising tool for DNA detection.[4–10] Despite these attractions, a number of notable challenges associated with electrochemical DNA sensors still exist, such as, relatively low sensitivity and relatively poor specificity on the detection of a single-base mismatch and single-nucleotide polymorphisms (SNPs).

DNA nicking endonucleases are a special family of restriction endonucleases and occur either naturally or *via* gene engineering. Nicking endonucleases can cut one strand of a double-stranded DNA at a specific recognition nucleotide sequence known as a restriction site and produce DNA molecules that are "nicked" rather than cleaved.[11] Until now, over 200 nicking endonucleases have been studied and many of them are available commercially. Recently, a novel nucleic acid detection technique called nicking endonuclease enhanced signal amplification (NESA) has been reported by Kiesling *et al.*[12] As a new nucleic acid detection technique, NESA has been reported to have distinct specificity for the detection of a single-base mismatch. However, the sensitivity of previously reported NESA was not high enough (only in the picomolar level), even when molecular beacons,[13]a dual-labeled fluorescent DNA probe,[14] or nanoparticles[15] were used to increase the sensitivity. Furthermore, the NESA-based DNA detection methods previously reported have other limitations such as complexity of fabrication and are time consuming.

In order to overcome the above limitations, we herein firstly report a proof-of-concept nicking endonuclease assisted current change amplification (NEACA) electrochemical DNA biosensor, which is simple, ultrahighly sensitive and has excellent selectivity for a single-base mismatch. The detailed concept of NEACA is illustrated in Fig. 1A. The hairpin capture probe (HP) contains a thiol group at the 5′ end and a ferrocene (Fc) tag at the 3′ end. A nicking endonuclease recognition sequence is embedded into the loop portion of the HP. The nicking endonuclease used in this case is N.BstNB I (New England Biolabs), which recognizes a simple asymmetric sequence, 5′-GAGTC-3′, and cleaves only one DNA strand, 4 bases away from the 3′ end of its recognition site (Fig. 1B, the red highlighted letters are the recognition sequence of N.BstNB I, and the arrow indicates the nicking position). The HP is covalently attached to a gold electrode (GE) through S–Au bonding. Then the GE is blocked with 2-mercaptoethanol (MCH) to form a mixed monolayer (HP/MCH/GE). The coverage of MCH can effectively prevent the non-specific adsorption of endonuclease on the electrode surface and displace the weaker adsorption contacts between HP and the GE.

In the absence of target DNA, the nicking endonuclease cannot cut the HP. So the formation of a hairpin-like conformation brings the Fc group close to the surface of GE and results in a high redox signal being observed. However, when a perfectly matched target DNA hybridizes to the HP, a full recognition site will form for the nicking endonuclease. Then the nicking endonuclease can bind to and nick the recognition site. After nicking, the HP is cleaved into two

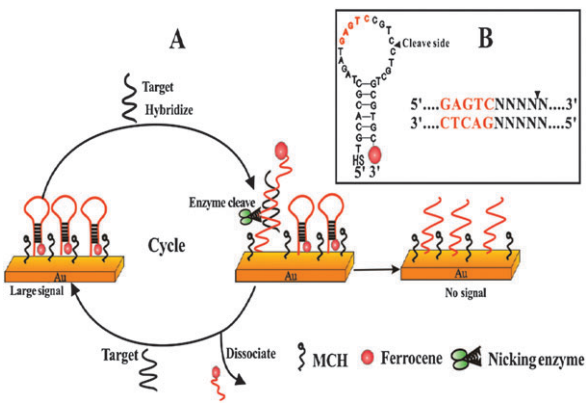

Fig. 1 (**A**) Sketch of the concept and the experimental principle of the NEACA electrochemical DNA sensor. (**B**) Recognition sequences of Nt.BstNBI. The recognition sequence, GAGTC, is highlighted in red and the cleavage site is indicated by small arrows.

Ministry of Education Key Laboratory of Analysis and Detection for Food Safety, Fujian Provincial Key Laboratory of Analysis and Detection Technology for Food Safety, Department of Chemistry, Fuzhou University, Fuzhou, Fujian 350002, China. E-mail: gnchen@fzu.edu.cn, hhyang@fzu.edu.cn; Fax: (+86)591-83713866

† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/c0cc00748j

pieces and the Fc labeled piece dissociates from the GE. At the same time, the affinity of the target DNA and the HP is reduced and the target DNA dissociates from the HP. The released target DNA can then hybridize to another HP and initiate the second cycle of cleavage. Eventually, each target strand can go through many cycles, resulting in the cleavage of many HPs. As a result, the peak current of Fc dramatically decreases to approximately zero. Therefore, this NEACA strategy will have ultrahigh sensitivity for the detection of target DNA. Furthermore, the nicking reaction is highly specific since it requires complete complementarity between the HP and the target at the restriction site and sufficient complementarity to allow hybridization outside of the enzyme recognition site.[13] By employing this strategy, we demonstrate that the NEACA based DNA biosensor has good discrimination for the detection of a single-base mismatch.

To prove the design, the electrochemical behavior of ferrocene at different sensing interfaces was investigated, and the results are shown in Fig. S3. In the absence of target DNA, the stem-loop structure of the hairpin probe held the ferrocene tag in close proximity with the electrode surface, thus ensuring rapid electron transfer and efficient redox of the ferrocene label. Consequentially, a significant signal was achieved. A relatively low peak separation (about 40 mV) between the anodic and cathodic potential demonstrated the successful immobilization of the ferrocenyl-labeled HP on the gold surface. The full width at half-height of either the cathodic or anodic wave was estimated to be 125 mV, somewhat larger than the theoretically expected value, (94.2 mV) for an ideal Nernstian process of a one electron redox system (Fc/Fc^+) at 37 °C, suggesting no significant inter-molecular interaction between the immobilized redox moieties.[16] The dependence of CVs on the scan rates was also investigated. The background-corrected current intensities were plotted against the scan rates, and the results showed that the peak current increased linearly with the increase of the potential scan rate, reflecting the existence of a surface-confined Faradaic reaction. This provided further evidence for the conclusion that ferrocene was confined at the electrode surface by the formation of the stem-loop structure.

On hybridization with the target sequence, a change in redox currents was observed. This was because the ferrocene label was separated from the electrode surface (Fig. S3, Inset curve b). When the target DNA concentration was high enough, hybridization between target DNA and the HP could produce enough signal difference for target DNA detection. This kind of electrochemical strategy has so far been employed for the detection of DNA,[17] however, it generally suffers from the problem of low sensitivity. We also detected the same target DNA by using the same strategy without nicking enzyme, and the results showed that with 1 pM target DNA, there was only a partial loss of signal after 60 min (Fig. S3, Inset curve c). This meant that this concentration was already below the detection limit of the hybridization assay without the nicking enzyme. On the other hand, in the nicking endonuclease assisted system, it was found that 1 pM of the target became detectable immediately after the nicking endonuclease was added. Furthermore, the intensity of the current decreased to nearly zero in about half an hour (Fig. S3,

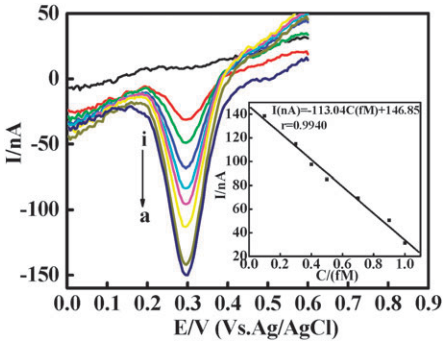

Fig. 2 Background-subtracted DPVs for the HP/MCH/GE in the presence of complementary DNA at 0, 0.1, 0.3, 0.4, 0.5, 0.7, 0.9, 1.0 and 10 fM (from a to i). The nicking reaction time was fixed at 30 min. The electrolyte is 1M $NaClO_4$. Inset shows the calibration curve demonstrating peak height *versus* target concentration.

Inset curve a). This indicated that the endonuclease could bind and cleave the HP, and the process continued until either all the HPs were cleaved or the enzyme was inactive. After cleavage, the ferrocene tag fragment of the cleaved HP can be easily removed by washing which results in electronic signals of nearly zero. By employing this nicking endonuclease assisted strategy, we demonstrated that this prototype sensor had ultrahigh sensitivity and can be used to detect an otherwise undetectable amount of target DNA.

As anticipated, the nicking reaction was extremely rapid. In the presence of 10 fM complementary target DNA and 10 U of enzyme, the nicking endonuclease cleaved approximately 100% of the HP in 30 min (Fig. 2, curve i). However, we didn't see any apparent changes in the current signal when the target concentration was lower than 0.05 fM until after 1 h, compared with 0 fM target (negative control, Fig. 2 curve a). We also found that the nicking reaction rate slowed down with decreasing target concentration (see Fig. S2 in the ESI†). This conclusion was consistent with the result obtained in the previous report.[13]

The sensitivity of the electrochemical DNA biosensor for the detection of the target DNA was investigated by varying the target DNA concentration. The sensor was hybridized with different concentrations of the target and incubated in the nicking endonuclease buffer solution for the same amount of time. Then, the response of the current to Fc was measured with DPV. The average currents showed a good linear correlation with the concentration of the complementary target DNA in the range of 0.1–1.0 fM (Fig. 2).

The detection limit ($3\sigma/S$, where σ is the standard deviation of the blank solution, $n = 8$) was calculated to be 0.068 fM for the complementary target. Compared to the existing electrochemical methods based on the conformational change of the redox-labeled probe strategies[7a,17,18a] and other strategies,[7b–10,18b–20] the proposed NEACA method can get a lower detection limit and has a higher sensitivity. The relative standard deviations (RSD) of the sensor for 8 replicate determinations of 0.5 fM is 5.8%.

Previous studies have demonstrated that nicking endonucleases have a high specificity in distinguishing the target from other sequences.[12,13] In this study, the selectivity of the sensor was also investigated by using HP/MCH/GE to

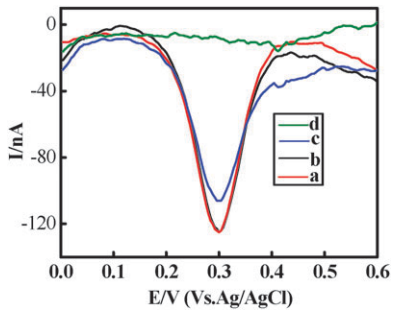

Fig. 3 Background-subtracted DPVs for the HP/MCH/GE (a) and hybridization with 1 pM non-complementary sequence (b), one-base mismatch sequence (c), and complementary sequence (d) after 30 min nicking reaction, respectively. The electrolyte is 1M $NaClO_4$.

hybridize with various DNA sequences (complementary DNA T1, one base mismatch DNA T2 and non-complementary DNA T3, see ESI† for the DNA sequences). As shown in Fig. 3, the highest peak current of Fc was observed on the HP/MCH/GE (Fig. 3a). After hybridization with T1, the peak current of Fc dramatically decreased to approximately zero (Fig. 3d). However, when HP/MCH/GE was hybridized with T2, the peak current of Fc only decreased slightly (Fig. 3c), indicating that a single-base mismatch was sufficient to inhibit the cleavage of the HP. It has been reported that N.BstNBI has two structural domains: the N-terminal domain for DNA binding and the C-terminal domain for DNA cleavage.[13] In the current study, the position of the mismatch is at the linker region between the binding site and nicking site. The mismatch "bubble" causes the linker region to become floppy, which weakens the coupling between enzyme binding and cleavage, and as a result, slows down the cleavage rate.[13] We also found that the DNAs with one base mismatch at the binding or nicking site lead to a slight decrease in the DPV signal compared with T1. In addition, no significant change of peak current was observed after hybridization with T3 (see Fig. 3a and b), since no successful hybridization occurs due to the sequence mismatch between the HP and the non-complementary sequence. These facts clearly indicated that our sensor had excellent discrimination ability for the detection of a single-base mismatch.

In summary, a novel electrochemical DNA biosensor for the sequence specific detection of DNA targets based on NEACA is reported in this study. The sensor exhibits ultrahigh sensitivity and discrimination ability even for the detection of a single-base mismatch. These features, as well as its other advantages, such as ease of fabrication and operation convenience, make it a promising alternative to conventional SNP analysis and genotyping. Since the sensor reported in this work is an electrochemical biosensor, one might expect the design of an integrated, portable and low cost device for DNA detection based on NEACA described in this study. It must be mentioned that the DNA biosensor based on NEACA can only be applied to the DNA sequence containing the recognition site of the nicking endonuclease. But with more and more nicking enzymes available commercially, this limitation will be no longer a problem.

This project was financially supported by National Basic Research Program of China (No.2010CB732403), NSFC (20735002, 40940026, 20905013), the Special Foundation for Young Scientists of Fujian Province, China (2008F3057).

Notes and references

1 (*a*) J. G. Hacia, L. C. Brody, M. S. Chee, S. P. A. Fodor and F. S. Collins, *Nat. Genet.*, 1996, **14**, 441; (*b*) F. Ricci, R. Y. Lai, A. J. Heeger, K. W. Plaxco and J. J. Sumner, *Langmuir*, 2007, **23**, 6827–6834.
2 (*a*) M. Kubista, J. M. Andrade, M. Bengtsson, A. Forootan, J. Jonak, K. Lind, R. Sindelka, R. Sjoback, B. Sjogreen and L. Strombom, *Mol. Aspects Med.*, 2006, **27**, 95–125; (*b*) S. J. Weissenborn, U. Wieland, M. Junk and H. Pfister, *Nat. Protoc.*, 2010, **5**, 1–13.
3 (*a*) P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas and D. C. Ward, *Nat. Genet.*, 1998, **19**, 225–232; (*b*) W. A. Zhao, M. M. Ali, M. A. Brook and Y. F. Li, *Angew. Chem., Int. Ed.*, 2008, **47**, 6330–6337.
4 A. A. Lubin, B. V. S. Hunt, R. J. White and K. W. Plaxco, *Anal. Chem.*, 2009, **81**, 2150–2158.
5 K. J. Cash, A. J. Heeger, K. W. Plaxco and Y. Xiao, *Anal. Chem.*, 2009, **81**(2), 656–661.
6 W. Miao and A. J. Bard, *Anal. Chem.*, 2004, **76**, 5379–5386.
7 (*a*) Y. Huang, Y. L. Zhang, X. M. Xu, J. H. Jiang, G. L. Shen and R. Q. Yu, *J. Am. Chem. Soc.*, 2009, **131**, 2478–2480; (*b*) D. Li, Y. Yan, A. Wieckowska and I. Willner, *Chem. Commun.*, 2007, 3544–3546; (*c*) J. Wang, O. Rincon, R. Polsky and E. Dominguez, *Electrochem. Commun.*, 2003, **5**, 83–86; (*d*) J. Zhang, B. P. Ting, N. R. Jana, Z. Q. Gao and J. Y. Ying, *Small*, 2009, **5**, 1414–1417.
8 (*a*) Y. Zhang, A. Pothukuchy, W. Shin, Y. Kim and A. Heller, *Anal. Chem.*, 2004, **76**, 4093–4097; (*b*) J. Zhang, S. P. Song, L. Y. Zhang, L. H. Wang, H. P. Wu, D. Pan and C. H. Fan, *J. Am. Chem. Soc.*, 2006, **128**, 8575–8580; (*c*) Y. Xiao, X. H. Lou, U. Takanori, K. J. I. Plakos, K. W. Plaxco and H. T. Soh, *J. Am. Chem. Soc.*, 2009, **131**, 15311–15316.
9 (*a*) S. Pinijsuwan, P. Rijiravanich, M. Somasundrum and W. Surareungchai, *Anal. Chem.*, 2008, **80**(17), 6779–6784; (*b*) J. H. Chen, J. Zhang, K. Wang, X. H. Lin, L. Y. Huang and G. N. Chen, *Anal. Chem.*, 2008, **80**, 8028–8034.
10 (*a*) S. D. Keighley, P. Estrela, P. Li and P. Migliorato, *Biosens. Bioelectron.*, 2008, **24**, 906–911; (*b*) A. Liu and J.-i. Anzai, *Anal. Chem.*, 2004, **76**, 2975–2980; (*c*) G. Liu, Y. Wan, G. Vincent, J. Zhang, L. H. Wang, S. P. Song and C. H. Fan, *J. Am. Chem. Soc.*, 2008, **130**, 6820–6825.
11 (*a*) R. D. Morgan, C. Calvet, M. Demeter, R. Agra and H. Kong, *Biol. Chem.*, 2000, **381**, 1123–1125; (*b*) L. S. Higgins, C. Besnier and H. Kong, *Nucleic Acids Res.*, 2001, **29**, 2492–2501; (*c*) H. Wang and J. B. Hays, *Mol. Biotechnol.*, 2000, **15**, 97–104.
12 T. Kiesling, K. Cox, E. A. Davidson, K. Dretchen, G. Grater, S. Hibbard, R. S. Lasken, J. Leshin, E. Skowronski and M. Danielsen, *Nucleic Acids Res.*, 2007, **35**, e117.
13 J. W. J. Li, Y. Z. Chu, B. Y.-H. Lee and X. L. S. Xie, *Nucleic Acids Res.*, 2008, **36**, e36.
14 K. Hosoda, T. Matsuura, H. Kita, N. Ichihashi, K. Tsukada, I. Urabe and T. Yomo, *RNA*, 2008, **14**, 584–592.
15 W. Xu, X. J. Xue, T. H. Li, H. Q. Zeng and X. G. Liu, *Angew. Chem., Int. Ed.*, 2009, **48**, 6849–6852.
16 T. Y. Dong, L. S. Chang, I. M. Tseng and S. J. Huang, *Langmuir*, 2004, **20**, 4471–4479.
17 C. H. Fan, K. W. Plaxco and A. J. Heeger, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 9134–9137.
18 (*a*) Y. Xiao, X. Qu, K. W. Plaxco and A. J. Heeger, *J. Am. Chem. Soc.*, 2007, **129**, 11896–11897; (*b*) C. E. Immoos, S. J. Lee and M. W. Grinstaff, *J. Am. Chem. Soc.*, 2004, **126**, 10814–10815.
19 Y. Xiao, A. A. Lubin, B. R. Baker, K. W. Plaxco and A. J. Heeger, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 16677–16680.
20 R. Miranda-Castro, P. de-los-Santos-Alvarez, M. J. Lobo-Castañón, A. J. Miranda-Ordieres and P. Tun-Blanco, *Anal. Chem.*, 2007, **79**, 4050–4055.