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An ultrasensitive electrochemical biosensor for detection of DNA species related to oral cancer based on nuclease-assisted target recycling and amplification of DNAzyme†

JingHua Chen,[ab] Jing Zhang,[a] Yang Guo,[a] Juan Li,[a] FengFu Fu,[a] Huang-Hao Yang*[a] and Guonan Chen*[a]

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A simple, label-free, ultra-highly sensitive and selective electrochemical sensor based on nuclease-assisted target recycling and DNAzyme for the detection of DNA species related to oral cancer in saliva is developed.

Currently, oral cancer is diagnosed either in cancer cell lines or in biopsy specimens from late invasive and metastatic cancers. However, non-invasive early diagnosis of oral cancer is the most effective means to reduce death from this disease. Recently, oral fluid has become a perfect diagnostic fluid with more and more cancer-related nucleic acids found in it. Compared with the blood, oral fluid has many obvious advantages.[1] However, a number of notable challenges associated with diagnostic technique in saliva still exist, such as, low sensitivity and poor specificity. So, it is very significant to develop highly sensitive and selective methods for oral cancer detection with saliva.

DNA electrochemical biosensors have shown great promise for rapid, sensitive, reliable and cost-effective DNA detection in clinical diagnostics.[2–5] To date, various sensing strategies have been developed to improve the sensitivity and selectivity of electrochemical DNA biosensors.[6–8] However, due to the low concentration (~fM) of salivary biomarkers and the complex background of saliva, conventional electrochemical biosensors do not meet the clinical diagnostic requirement of high signal-to-background ratio (SBR) for direct RNA/DNA detection in saliva.

Target recycling, wherein signal amplification is achieved by allowing a single RNA/DNA target molecule to interact with multiple nucleic acid-based signalling probes, represents an interesting alternative to conventional methods,[9–11] which is expected to become an alternative for the ultra-sensitive detection of RNA/DNA.

Catalytic nucleic acid (DNAzyme)-based methods have attracted increasing attention since they can catalyze various chemical reactions such as metal ion-dependent DNA/RNA cleavage, DNA self-modification *etc.*[12,13] As one of the most interesting DNAzymes, G-quadruplex-hemin DNAzyme, which is composed of hemin and a G-rich nucleic acid, has horseradish peroxidase-like activity and can effectively catalyze the H_2O_2-mediated oxidation. Therefore, it has shown considerable potential as a novel kind of catalytic label for the development of various DNA biosensors.

Herein, we developed an ultra-high sensitive and selective electrochemical sensor for the detection of DNA species related to oral cancer overexpressed 1 (ORAOV1) in saliva.[14] Our strategy contains two successive steps of isothermal enzymatic amplification. As shown in Fig. 1, a signalling probe (Sp), which is modified with a thiol group at its 3′ terminus, consists of target recognition sequence (black) and a G-rich sequence (blue). At the same time, the target recognition sequence of the Sp contains a nicking endonuclease recognition sequence. DNA nicking endonucleases is a special family of restriction endonucleases and occurs either naturally or *via* gene engineering. Nicking endonucleases can cut one strand of a double-stranded DNA at a specific recognition nucleotide sequences known as a restriction site and produce DNA molecules that are ''nicked'' rather than cleaved.[15] The nicking endonuclease used here is N.BstNB I (New England Biolabs), which recognizes a simple asymmetric sequence, namely 5′-GAGTC-3′, and cleaves only one DNA strand, 4 bases away from the 3′ end of its recognition site (see Table S1† in Supporting Information, the highlighted letters are the

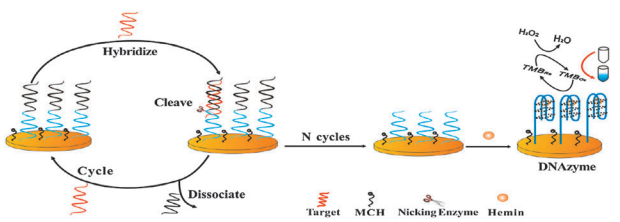

Fig. 1 Sketching of concept and experimental principle for the electrochemical DNA sensor.

[a] *Ministry of Education Key Laboratory of Analysis and Detection for Food Safety, Fujian Provincial Key Laboratory of Analysis and Detection for Food Safety, Fuzhou University, Fuzhou, Fujian, 350002, China. E-mail: gnchen@fzu.edu.cn, hhyang@fzu.edu.cn; Fax: (+86)-591-22866135*
[b] *Department of Pharmaceutical Analysis, Faculty of Pharmacy, Fujian Medical University, 350004, China*

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recognition sequence of N.BstNB I, and the arrow indicates the nicking position).

The Sp is covalently attached to a gold electrode (GE) through S–Au bonding. Then the Sp modified GE is blocked with 2-mercaptoethanol (MCH) to form a mixed monolayer (Sp/MCH/GE). In the presence of a perfectly matched target, the target DNA hybridizes with the Sp to form a double-stranded structure with a full recognition site of N.BstNB I. The nicking endonuclease in turn preferentially binds to the recognition site and selectively nicks the Sp, resulting in only the G-rich sequence part of the Sp remaining on the electrode surface. At the same time, the affinity of target DNA and the Sp is reduced and target DNA dissociates from the Sp. The released target DNA is able to hybridize with a new un-nicked Sp and catalyze a new cycle of probe transformation. In this way, a single target DNA is able to trigger the permanent transformation of multiple Sps to the G-rich nucleic acid sequences. Upon completion of the strand-scission cycle, the target recognized fragment of Sp can be easily removed by washing. Thus, only G-rich fragments remain on the sensor surface. Therefore, upon addition of hemin, the G-rich sequences can bind hemin to form the G-quadruplex-hemin DNAzyme, which can catalyze the H_2O_2-mediated oxidation of 3,3′,5,5′ tetramethylbenzidine (TMB), accompanied by a changed color of the reaction solution and an increased electrochemical current signal. However, in the absence of the target DNA, the nicking endonuclease cannot cut the Sp. Therefore Sp could not bind hemin to form the G-quadruplex-hemin DNAzyme due to the steric-hindrance effect.

This strategy will have ultra-high sensitivity due to the target recycling mechanism, in which a single target DNA is able to trigger the permanent transformation of multiple Sps from long linear structures to flexible G-rich sequences, yielding a large increase in signal amplitude. Furthermore, the nicking reaction is highly specific since it requires complete complementarity between Sp and the target at the restriction site and sufficient complementarity to allow hybridization outside of the enzyme recognition site.[16] By employing this strategy, we demonstrate that the proposed DNA biosensor has good discrimination for the detection of single-base mismatch. Moreover, this electrochemical sensor should be comparatively simple, portable, and sensitive, offer fast response times and be inexpensive. Most importantly, because the result of our assay will be clearly observable either with a simple hand-held electrochemical instrument or with the naked eye, this biosensor will facilitate the construction of inexpensive electrochemical or optical biosensors.

Previous studies have demonstrated that RuHex can bind to DNA by electrostatic interactions because the DNA backbone has a negative charge. In order to test whether indeed our biosensor works as expected, the CV behaviour of RuHex and the electrochemical impedance spectra is investigated at different stages of the biosensor preparation (see the Supporting Information†), the results of the CVs are consistent with that of the EIS, indicating that our biosensor indeed works as expected.

In order to confirm the catalytic nature of DNAzyme, we compared with the CV signals of TMB substrate at different stages of the biosensor preparation. From Fig. 2, we can see there are two pairs of redox peaks at the surface of the bare

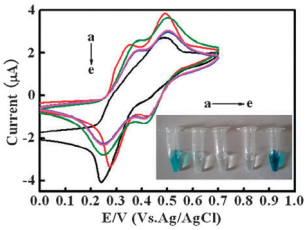

Fig. 2 CVs of bare electrode in TMB substrate (b), bare electrode in TMB substrate containing DNAzyme (e), DNAzyme/MCH/GE in TMB substrate (a) Sp/MCH/GE (c), and Sp-T1/MCH/GE (d) in TMB substrate. Inset: the photograph of reaction system after different electrodes immersed into TMB substrate.

electrode (Fig. 2b). When DNAzyme was added into TMB, a prominent catalytic reduction peak was found in CV of the bare electrode (Fig. 2e), reflecting that the DNAzyme efficiently catalyzed the reduction of hydrogen peroxide with the help of TMB (an electroactive co-substrate), leading to significantly amplified electrochemical current signals. At the same time, the solution colour observed with the naked eye gradually changed from colourless (inset b) to blue (inset e).

The different DNA modified biosensors were compared in the same TMB substrate (Fig. 2). We found that a prominent catalytic reduction peak appeared in CV of DNAzyme/MCH/GE (Fig. 2a) and the solution colour changed to blue too (see inset a). This is because only G-rich nucleic acid sequences remain on the surface GE after many strand-scission cycles. Therefore, upon addition of hemin, the G-rich sequences bind hemin to form the G-quadruplex-hemin DNAzyme, which catalyzed the H_2O_2-mediated oxidation of TMB, giving rise to enzymatically amplified electro-chemical current signal.

The selectivity of the sensor is investigated by using Sp/MCH/ GE to hybridize with various DNA sequences (complementary DNA T1, single-base-mismatch DNA T2–T5 and non-complementary DNA T6 mentioned in reagents section, see Table S1† in the supporting information). To quantitatively evaluate the discrimination ability of our sensor, we defined the discrimination factor (DF) as the ratio of net amperometric signal (the amperometric current of target minus that of the background) obtained with complementary

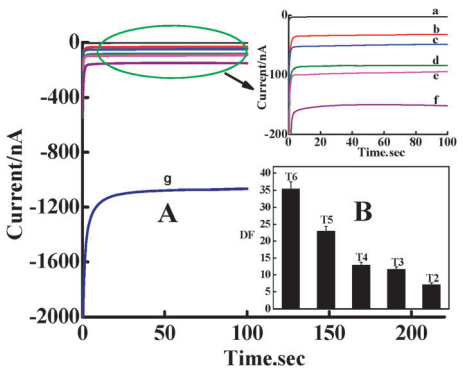

Fig. 3 A) DNA probe modified electrode to different target's specificity research, Sp/MCH/GCE(a), Sp-T6/MCH/GCE(b), Sp-T5/MCH/GCE(c), Sp-T4/MCH/GCE(d), Sp-T3/MCH/GCE(e), Sp-T2/MCH/GCE(f), Sp-T1/MCH/ GCE(g). B) The discrimination factors of the electrochemical biosensor for a single-base mismatch.

target DNA T1 to that obtained with T2–T6. It can be seen that DF is highly dependent on the position of mutation. Although T2, T3, T4 and T5 all have only a single mismatched base, they have a dramatically different effect on DF. From the data shown in Fig. 3B, we calculated that the DF of T2, T3, T4 and T5 was 7.1, 11.6, 12.9 and 22.9, respectively. We conclude that the difference of DF is due to one or both of the following effects of mutation:[17] (i) directly disturbing the enzyme binding and/or nicking sites, (ii) lowering the stability of the target-Sp hybrid. Mutation of T5 occurs at the centre of the enzyme binding site, resulting in the largest DF. Mutation of T4 is at the edge of the binding site, leading to the smaller drop in DF. Surprisingly, although mutation of T3 is at neither binding site nor nicking site, its effect is almost as strong as that of T4, probably because it can influence both binding and nicking of the enzyme. It was proposed previously that N.BstNBI has two structural domains: the N-terminal domain for DNA binding and the C-terminal domain for DNA cleavage. Mutation of T3 is at the linker region between the binding site and nicking site, and the mismatch "bubble" causes the linker region to be floppy. We guess the floppy nature of the mismatched linker region weakens the coupling between binding and cleavage, and as a result, slows down the cleavage rate. Mutation of T2 is the furthest from the binding site, and is also out of the linker region. So it has the least influence on DF among the four single-base-mismatch DNA. Yet it still causes a significant drop in the amperometric current, probably due to its influence on the stability of the duplex. In addition, as expected, no significant difference of peak current can be observed for the probe modified GE and its hybridization with non-complementary sequences (see Fig. 3), since no successful hybridization occurs due to the sequence mismatch between the probe and the non-complementary sequence. To sum up, compared with some sensing strategies recently reported such as "triple stem" and "gap ligation",[18,19] our biosensor has an excellent discrimination ability for single-base mismatch targets. In addition, our biosensor has more advantage because the above sensors need complicated labelling and fabrication procedures, and enzyme-aided reactions.

The sensitivity of the electrochemical DNA biosensor for detection of the target DNA is investigated. The current signals after the sensor hybridized with different concentrations of target DNA and incubated in the nicking endonuclease buffer solution with the same time is measured with amperometric techniques (see Fig. S3† in Supporting Information for detail). The average current shows a good linear correlation with the concentration of the complementary target DNA in the range of 0.08–8.0 fM.

We also found that the nicking reaction slowed down with the target concentration decreasing. This observation is consistent with the results obtained in the previous report.[16] The targets act as 'mobile catalytic sites' for the nicking reaction to occur: the fewer catalytic sites, the slower the nicking speed.[16] The detection limit ($3\sigma/S$, where σ is the standard deviation of the blank solution, $n = 8$) is calculated to be 0.02 f M for complementary target DNA. The relative standard deviation (RSD) of 8 different sensors, which were fabricated on the same electrode for the detection of 1.0 f M was 6.24%, and that of 3 different sensors fabricated on three different electrodes was 6.87%.

Compared to other methods previously reported,[20–22] the proposed method has a lower detection limit and better selectivity, meeting the clinical diagnostic requirement in saliva.

In order to evaluate the applicability of the electrochemical DNA sensor in saliva, we compared its performance in pure buffer and in saliva, and results show that our proposed electrochemical DNA biosensor may represent a promising path toward direct oral cancer detection in saliva (see Supporting Information† for detail).

In summary, a simple, label-free, ultra-high sensitive and selective electrochemical sensor based on nuclease-assisted target recycling and DNAzyme for DNA species related to oral cancer detection in saliva is developed here. The sensor can detect as low as 0.02 fM target DNA and exhibits ultra-high discrimination ability even for the detection of single-base mismatch. These features, as well as its other advantages, such as fabrication easiness, operation convenience and low cost, make it a promising candidate for a non-invasive early diagnosis for oral cancer detection in saliva.

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Notes and references

1 H. P. Lawrence, *J. Can. Dent. Assoc.*, 2002, **68**, 170–174.
2 E. C. Jorge, T. Moshe and K. Hagop, *Am. J. Med.*, 1996, **100**, 555–570.
3 C. H. Fan, K. W. Plaxco and A. Heeger, *Trends Biotechnol.*, 2005, **23**, 186–192.
4 F. Lucarelli, G. Marrazza, A. P. F. Turner and M. Mascini, *Biosens. Bioelectron.*, 2004, **19**, 515–530.
5 R. Gasparac, B. T. Taft, M. A. Lapicrre-Devlin, A. D. Lazareck, J. M. Xu and S. O. Kelley, *J. Am. Chem. Soc.*, 2004, **126**, 12270–12271.
6 D. Li, Y. Yan, A. Wieckowska and I. Willner, *Chem. Commun.*, 2007, 3544–3546.
7 W. Q. Yang, X. Zhu, Q. D. Liu, Z. Y. Lin, B. Qiu and G. N. Chen, *Chem. Commun.*, 2011, **47**, 3129–3131.
8 E. G. Hvastkovs and D. A. Buttry, *Analyst*, 2010, **135**, 1817–1829.
9 K. Hsieh, Y. Xiao and H. T. Soh, *Langmuir*, 2010, **26**, 10392–1039.
10 X. L. Zuo, F. Xia, Y. Xiao and K. W. Plaxco, *J. Am. Chem. Soc.*, 2010, **132**, 1816–1818.
11 S. Bi, J. L. Zhang and S. S. Zhang, *Chem. Commun.*, 2010, **46**, 5509–5511.
12 Y. Lu and J. W. Liu, *Acc. Chem. Res.*, 2007, **40**, 315.
13 I. Willner, B. Shlyahovsky, M. Zayats and B. Willner, *Chem. Soc. Rev.*, 2008, **37**, 1153.
14 L. Jiang, X. Zeng, H. Yang, Z. Wang, J. Shen, J. Bai, Y. Zhang, F. Gao, M. Zhou and Q. Chen, *Int. J. Cancer*, 2008, **123**, 1779–1786.
15 L. S. Higgins, C. Besnier and H. Kong, *Nucleic Acids Res.*, 2001, **29**, 2492–2501.
16 J. W. J. Li, Y. Z. Chu, B. Y.-H. Lee and X. L. S. Xie, *Nucleic Acids Res.*, 2008, **36**, e36.
17 F. Wei, J. H. Wang, W. Liao, B. G. Zimmermann, D. T. Wong and C. M. Ho, *Nucleic Acids Res.*, 2008, **36**, e65.
18 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.
19 Y. Xiao, X. H. Lou, T. Uzawa, K. J. I. Plakos, K. W. Plaxco and H. Tom Soh, *J. Am. Chem. Soc.*, 2009, **131**, 15311–15316.
20 W. Xu, X. J. Xue, T. H. Li, H. Q. Zeng and X. G. Liu, *Angew. Chem., Int. Ed.*, 2009, **48**, 6849–6852.
21 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.
22 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.

