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Washing-free electrochemical DNA detection using double-stranded probes and competitive hybridization reaction†

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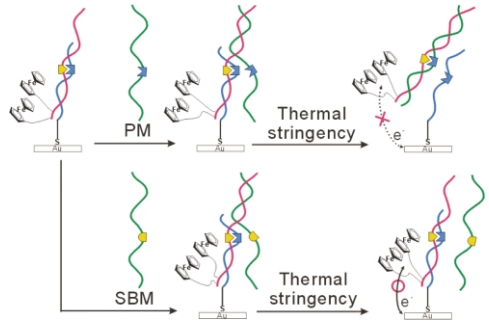
A new electrochemical DNA detection method using double-stranded probes and competitive hybridization reaction offers highly selective discrimination of single base mismatch without post-hybridization washing.

The development of microdevices for point-of-care testing (POCT) has been a critical issue in *in vitro* diagnostics technology.[1] To date, the most impressive advancement of such devices is DNA lab-on-a-chip for nucleic acid testing. Due to its compatibility with microfabrication technology, electrochemical[2–9] and electrical[10] DNA detection methods are potential candidates for POCT. However, previous electrochemical methods using direct hybridization,[2] amplification,[4–8,10] and sandwich-type[7–10] have inherent drawbacks in the convenience for end-users of lab-on-a-chip, because most of these methods require target labeling with a redox-active compound, or complex fluid control for signaling reagent addition and solution mixing. In addition, washing of residual target DNA or signaling reagents is required after target hybridization, in order to enhance the signal-to-noise ratio. However, when the detection is performed in a microchamber of the chip, efficient washing out of the chamber is very difficult due to insufficient mixing associated with viscous flow. One strategy to overcome these disadvantages is to preimmobilize a signaling DNA labeled with a redox-active compound on the sensor surfaces during the chip fabrication, and then remove it from the surface by hybridization with target DNA. Very recently, several groups have attempted to exploit this concept in DNA detection to realize a reagentless method using molecular beacon (MB) probes.[11] However, the instability resulting from the short stem of conventional MB on a solid support is problematic to the commercialization of DNA lab-on-a-chip, for which long-term stability is essential. Moreover, washing-free detection has not been attempted.

In this paper, we report a new electrochemical DNA detection method using double-stranded (DS) probes having a longer stem (11 base pairs) and an unprecedented detection scheme using a competitive hybridization reaction. To the best of our knowledge, this is the first report of the use of DS probes with electrochemical detection. The DNA sequence used in this study is a part of codon 72 in the *p*53 tumor suppressor gene. As italicized, the single mismatch position was a mutation site in this part of the gene. A DS probe prepared by the hybridization of capture DNA (5′-CCCCGCGTGGCC–$(CH_2)_6$–O–$(CH_2)_3$–SH-3′) and signaling DNA (5′-FcFcGCCACGCGGGGAGCAG-3′) was immobilized on a gold film electrode through a thiol linkage, which was used as the sensor surface. As a label for electrochemical detection, ferrocene-conjugated DNA monomer (Fc) of phosphoramidite form was synthesized,[12] and two Fc groups were serially coupled to the terminal of the signaling DNA strand using a DNA synthesizer. Perfect-matched (PM) target DNA (5′-CTGCTCCCC*G*CGTGG-3′) and single-base-mismatched (SBM) target DNA (5′-CTGCTCCCCCCGTGG-3′) were designed and synthesized to be competitors of capture DNA toward signaling DNA. The melting temperatures (T_m) of plausible duplexes for signaling DNA were obtained by measuring temperature-dependent UV absorption spectra of 1 μM duplex-containing 2 × saline-sodium citrate (SSC) buffer solutions. The T_m of the initial DS probe was 59 °C, and for the SBM and PM duplex the values were 61 °C and 70 °C, respectively. Scheme 1 shows competitive hybridization under the thermal stringency, by which PM target DNA is expected to displace capture DNA and simultaneously hybridize with signaling DNA to make duplex DNA, because the resulting duplex DNA exhibits much higher stability than the initial DS DNA probe. As a result, signaling DNA is removed from the sensor surface. In the case of SBM target DNA, the rate of displacement by the competition reaction is much slower than that of the PM target. Therefore, we expect that even SBM can be discriminated based on the different melting temperatures. As well as the MB method,[13] DS probes have been shown to exhibit a greater degree of specificity in mismatched-base discrimination than single-stranded probes due to the intrinsic nature of competitive hybridization.[14]

The washing step could be eliminated if our detection protocol could satisfy the following additional constraints. The buffer solution used for competitive hybridization should contain a sufficiently high concentration of supporting electrolytes for an electrochemical DNA detection. More importantly, the signaling DNA detached from the electrode surface should not influence on the electrochemical reaction. We also demonstrate a proof of the concept of washing-free DNA detection.

The surface density of DS probes on the electrode surfaces, as measured from the charge needed for the electrochemical oxidation of ferrocene on a cyclic voltammetric scan, has been controlled to near one-tenth of full monolayer coverage throughout this research. The sensor surface with a ferrocene-free DS probe did not exhibit any oxidation peak, and the modification of DS probe without thiol linker produced less than 10% of the peak intensity. The surface has been also investigated by grazing-angle reflectance FT-IR. A strong absorption band near 1090 cm^{-1} associated with symmetric P=O stretching vibration of the DNA backbone was initially observed.[8b] After thermal treatment of the DS probe on a surface at 46 °C of 2 × SSC buffer solutions for 3 hours, the oxidation peak was not observed and the intensity of the IR band was decreased by about 50%. These results support that the probe at the sensor



Scheme 1 Schematic diagrams of the electrochemical SBM detection by competitive hybridization reaction. Capture DNA (blue), signaling DNA (red), and target DNA (green).

† Electronic supplementary information (ESI) available: HPLC and MALDI-TOF MS data of the synthetic ferrocene-conjugated signaling DNA. Grazing angle FT-IR spectra. Sequences of PCR mimics. See http://www.rsc.org/suppdata/cc/b4/b402914c/

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surface was significantly denatured by heating at 46 °C although T_m of the probe at a solution is 59 °C. This is consistent with the report of Yu *et al.*[9b] where the T_m value at their sensor surface is lowered by 14 °C compared to that in the solution. Our control experiments demonstrated the specificity of the immobilization of DS probe.

For competitive hybridization, the sensor surfaces were dipped in three different target-containing 2 × SSC buffer solutions. To test SBM discrimination of the proposed protocol, the electrochemical responses for PM, SBM, and noncomplementary (NC) target DNA (5′-CCGATGGACGCACCGG-3′) were obtained. At 20 °C, the initial current intensity did not show any significant changes for all targets for 1 hour in the solutions. To enhance competition-reaction kinetics and hybridization specificity, thermal condition was optimized. As a result, heat cycles for temperature ranges between 30 °C and 46 °C were applied to the hybridizing cell for 1 hour. In this heating protocol, the sequence selectivity was maximized while thermal denaturation at blank solution was minimized.

Fig. 1A shows differential pulse voltammetry (DPV) results for various target DNA. After target hybridization, the resulting surfaces were thoroughly washed using 2 × SSC buffer solution. The results demonstrate that our method exhibits high selectivity in SBM discrimination. The selectivity might be regarded as a ratio of decrease by PM and by SBM from the current for the blank 2 × SSC buffer solution. This current was nearly same as the value for NC target DNA. The selectivity for SBM discrimination is at least fivefold. The current decreases by the blank and NC solutions might be caused by thermal dehybridization of the surface bound duplex. We also performed DPV for target-reacted electrodes without washing (*i.e.*, immediately after the target hybridization in a conventional three-electrode cell). Fig. 1B displays washing-free DPV scans that are very similar to the results with washing, which suggests a significant fact in that we have successfully achieved highly selective sequence discrimination without the trouble of surface washing. The results also indicate that the constraints for washing-free detection as mentioned above might be satisfied with our system.

It should be pointed out that real samples including long DNA strands, such as PCR products and enzymes such as polymerase, needed to be tested as part of our ongoing investigation. Such samples may be more adsorptive to the sensing surfaces than the short oligomers tested above, and hence interference with the electron transfer at the sensor surfaces must be considered in order to obtain reliable washing-free results. To study the case of more adsorptive target DNA, we synthesized PCR product mimics with 99 base pairs for use with our washing-free detection method. Immediately prior to the hybridization, the PCR-mimic-containing 2 × SSC buffer solution was heated to 95 °C for 2 minutes and then quickly quenched on ice. Fig. 2 shows washing-free detection results from DPV scans for wild-type PM sequences and mutations including SBM. The results demonstrate that the washing-free method is also able to discriminate a single-base mutation even in the more-adsorptive target DNA with high selectivity.

The current decrease by the competitive hybridization also depended on the concentration of target DNA, as demonstrated in Fig. 3. This phenomenon was also investigated without washing.

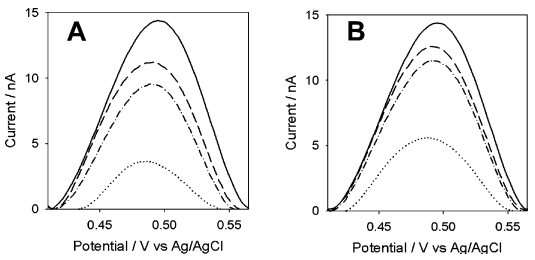

Fig. 1 The baseline-corrected DPV responses (A) with washing and (B) without washing electrode surfaces after hybridization with various target DNA (1 μM each). NC (dash), SBM (dash-dot), and PM (dot) reacted surfaces. Note that initial DS probe-modified surface (solid line) is common to (A) and (B).

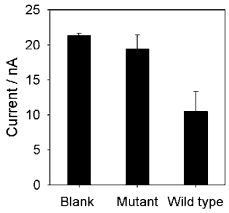

Fig. 2 The washing-free electrochemical responses for synthetic PCR product mimics of wild type and mutant (50 nM each).

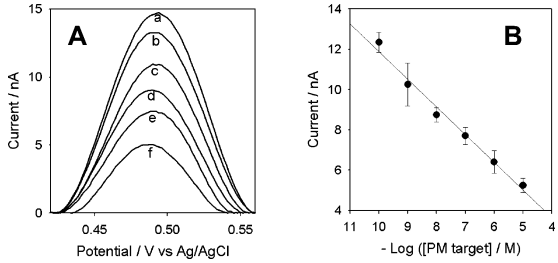

Fig. 3 (A) The baseline-corrected DPV of (a) an initial DS probe on electrode surface and washing-free surface upon the analysis of PM at (b) 0, (c) 1 × 10^{-9}, (d) 1 × 10^{-8}, (e) 1 × 10^{-7}, and (f) 1 × 10^{-5} M. (B) A calibration curve corresponding to changes in DPV peak currents.

The calibration curve was linear over five orders of magnitude, with a subnanomolar detection limit.

In summary, our electrochemical DNA detection method using the DS probe offers highly selective discrimination of SBM DNA as a result of a competitive hybridization reaction. Due to its reagent-free and washing-free advantages, the method might relieve end-users′ labors in detection steps. In practice, the method would be suitable for POCT using low-cost disposable microarrays. When the method is applied to multiplex DNA detection on microarrays, another advantage of DS probes might be the minimization of signal interference that is often caused by chemical cross-talk between arrays; we are currently investigating this in our laboratory.

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