Anal. Chem. **2002,** *74,* 5057−5062

Sequence-Specific Electrochemical Detection of Asymmetric PCR Amplicons of Traditional Chinese Medicinal Plant DNA

Thomas M. H. Lee and I-Ming Hsing*

Department of Chemical Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong

In this study, an electrochemistry-based approach to detect nucleic acid amplification products of Chinese herbal genes is reported. Using asymmetric polymerase chain reaction and electrochemical techniques, single-stranded target amplicons are produced from trace amounts of DNA sample and sequence-specific electrochemical detection based on the direct hybridization of the crude amplicon mix and immobilized DNA probe can be achieved. Electrochemically active intercalator Hoechst 33258 is bound to the double-stranded duplex formed by the target amplicon hybridized with the 5′-thiol-derivated DNA probe (16-mer) on the gold electrode surface. The electrochemical current signal of the hybridization event is measured by linear sweep voltammetry, the response of which can be used to differentiate the sequence complementarities of the target amplicons. To improve the reproducibility and sensitivity of the current signal, issues such as electrode surface cleaning, probe immobilization, and target hybridization are addressed. Factors affecting hybridization efficiency including the length and binding region of the target amplicon are discussed. Using our approach, differentiation of Chinese herbal species *Fritillaria* (*F. thunbergii* and *F. cirrhosa*) based on the 16-mer unique sequences in the spacer region of the 5S-rRNA is demonstrated. The ability to detect PCR products using a nonoptical electrochemical detection technique is an important step toward the realization of portable biomicrodevices for on-spot bacterial and viral detections.

In recent years, DNA polymorphism-based assays have been developed for the identification of Chinese herbal medicines.[1,2] These assays are well suited to miniaturization for rapid, cost-effective, and automated genotyping. The isolation of various cell types,[3,4] the separation of genomic DNA[5] from full blood samples, and the amplification of target genes by the polymerase chain reaction[6−10] (PCR) in miniaturized devices have been demonstrated. One major technical obstacle in the realization of a portable DNA analyzer is the ability to perform on-chip sequence-specific detection of PCR products. The fluorescence-based detection method,[11,12] although very sensitive and widely used in microarray applications, is not easy to implement on-chip because of its requirement in the bulky optical system. On the other hand, the electrochemistry-based detection method, which has an inherent advantage for miniaturization and is capable of producing portable electrochemical signals, is a good alternative for the portable device application.

Sequence-specificity in the electrochemistry-based detection comes from the evaluation of the hybridization reaction between unknown sample/target sequence and immobilized DNA probe on the electrode surface. Efficiency and reproducibility of immobilization of a single-stranded (ss) DNA probe onto an electrode surface is a prerequisite for the electrochemistry-based sequence-specific detection of DNA. Many researchers have reported their studies previously to optimize the immobilization of a DNA probe onto various electrode surfaces. For example, Wang et al.[13,14] demonstrated the adsorptive accumulation on anodically activated carbon paste electrodes. Mikkelsen et al.[15,16] reported the covalent attachment of deoxyguanosine residues of ss-DNA to chemically oxidized glassy carbon or to steric acid-modified carbon paste electrodes using water-soluble carbodiimide. The tin-doped indium

* Corresponding author. Tel: (852) 23587131. Fax: (852) 23580054. E-mail: kehsing@ust.hk.

(1) Cai, Z. H.; Li, P.; Dong, T. T. X.; Tsim, K. W. K. *Planta Med.* **1999**, *65*, 360−364.
(2) Carles, M.; Lee, T.; Moganti, S.; Lenigk, R.; Tsim, K. W. K.; Ip, N. Y.; Hsing, I. M.; Sucher, N. J. *Fresenius J. Anal. Chem.* **2001**, *371*, 190−194.
(3) Cheng, J.; Sheldon, L. W.; Heller, M. J.; O'Connell, J. P. *Anal. Chem.* **1998**, *70*, 2321−2326.
(4) Wang, X. B.; Yang, J.; Huang, Y.; Vykoukal, J.; Becker, F. F.; Gascoyne, P. R. C. *Anal. Chem.* **2000**, *72*, 832−839.
(5) Manz, A.; Becker, H. *Microsystem technology in chemistry and life science*; Springer-Verlag: Berlin, 1998; pp 215−231.
(6) Mullis, K. B.; Ferre, F.; Gibbs, R. A. *The polymerase chain reaction*; Birkhauser: Boston, 1994.
(7) Lee, T. M. H.; Hsing, I. M.; Lao, I. K.; Carles, M. C. *Anal. Chem.* **2000**, *72*, 4242−4247.
(8) Northrup, M. A. *Anal. Chem.* **1998**, *70*, 918−922.
(9) Kopp, M. U.; de Mello, A. J.; Manz, A. *Science* **1998**, *280*, 1046−1048.
(10) Wilding, P.; Kricka, L. J.; Cheng, J.; Hvichia, G.; Shoffner, M. A.; Fortina, P. *Anal. Biochem.* **1998**, *257*, 95−100.
(11) Dong, D.; Wang, E.; Hsie, L.; Cao, Y.; Chen, X.; Gingeras, T. R. *Genome Res.* **2001**, *11*, 1418−1424.
(12) Schena, M. *DNA microarrays: a practical approach*; Oxford University Press: Oxford, U.K., 1999; Chapter 2.
(13) Wang, J.; Cai, X.; Rivas, G.; Shiraishi, H. *Anal. Chim. Acta* **1996**, *326*, 141−147.
(14) Wang, J.; Cai, X.; Rivas, G.; Shiraishi, H.; Farias, P. A. M.; Dontha, N. *Anal. Chem.* **1996**, *68*, 2629−2634.
(15) Millan, K. M.; Mikkelsen, S. R. *Anal. Chem.* **1993**, *65*, 2317−2323.
(16) Millan, K. M.; Saraullo, A.; Mikkelsen, S. R. *Anal. Chem.* **1994**, *66*, 2943−2948.

10.1021/ac020068s CCC: $22.00
Published on Web 09/05/2002

oxide (ITO) electrode investigated by Thorp's group has been effectively used to attach the ss-DNA through adsorption[17] or via the chemical interaction between the dicarboxylate self-assembled monolayer and endogenous amines of the nucleobases.[18] Other immobilization techniques utilizing the chemisorption of a 5′-thiol-derivatized DNA probe on gold electrodes[19−21] were also reported.

In the electrochemical detection technique, instead of an optical signal, an electrochemical current signal is obtained. The hybridization event, resulting in double-stranded (ds) hybrid formation on the electrode, is transduced into an electrical signal via oxidization/reduction of a redox-active DNA intercalator (metal coordination complexes,[13−16] anthracycline antibiotics,[19] bisbenzimide dyes,[21] and redox species-modified DNA ligand[22]).

To our knowledge, most of the previous publications on the electrochemical transduction of the hybridization event employed short oligonucleotide targets (20−40-mer). However, in real applications, sequence-specific detection of long PCR amplicons (several hundred to thousand base pairs) is needed. Although there have been reports on the detection of PCR products, the samples are either purified[17] or labeled with redox-active species.[23,24] In this work, we report an electrochemistry-based approach to detect asymmetric PCR amplification products of Chinese herbal genes. In particular, the two closely resembled *Fritillaria* species, which have a different 16-mer sequence in the spacer region (∼ 600 bp),[1] are used as exemplary DNAs. Our strategy uses a 5′-thiol-derivatized ss-DNA probe chemisorbed on a gold electrode surface for the hybridization-based detection of unpurified PCR amplicons (comprising unreacted nucleotides, primers, enzymes, and so on). Subsequent signal transduction of the hybridization event is achieved by the linear sweep voltammetric scan of a commercially available redox-active DNA intercalator (Hoechst 33258) bound to the hybrid between the DNA probe and PCR amplicon. The current signal obtained can be used to identify the sequence of the PCR amplicon and to quantify the amount of DNA template present in the sample. To improve the reproducibility and sensitivity of the electrochemistry-based detection approach, issues such as electrode surface cleaning, probe immobilization, target amplification, and hybridization are addressed. Here, target amplification by the asymmetric PCR technique[25] is employed to generate an excess of one strand containing the recognition sequence complementary to the probe sequence for the direct hybridization. This approach offers a simpler and more reproducible hybridization condition than normal PCR, of which the ds-PCR amplicon has to be denatured (e.g., heated to 95 °C for a few minutes), and the reannealing of the two strands during the course of hybridization reduces the binding between the probe and target strand. Factors affecting hybridization efficiency including the length and binding region of the target amplicon are discussed.

EXPERIMENTAL SECTION

Reagents. Seven oligonucleotides were purchased from Synthetic Genetics (San Diego, CA) with the following base sequences: 5′-HS−$(CH_2)_6$−CAC AAA ACG GGG GCG G-3′ (probe, 16-mer), 5′-CCG CCC CCG TTT TGT G-3′ (target, with and without a Texas Red fluorescent label at the 3′ end, 16-mer, complementary to the probe), 5′-CAC AAA ACG GGG GCG G-3′ (negative control, with and without a Texas Red fluorescent label at the 3′ end, 16-mer, noncomplementary to the probe), 5′-GGA TTC GTG CTT GGG CGA GAG TAG TA-3′ (forward primer for PCR), and 5′-GGA TTC TTA GTG CTG GTA TGA TCG CA-3′ (reverse primer for PCR). Note that the negative control has the same base composition as the probe, except that the latter has a mercaptohexyl group at the 5′-phosphate end for immobilization onto the gold electrode surface by chemisorption.

Sodium chloride, potassium chloride, trisodium citrate dihydrate, sodium phosphate monobasic, sodium phosphate dibasic, tris(hydroxymethyl)aminomethane (Tris), boric acid, ethylenediaminetetracetic acid (EDTA) disodium salt dihydrate, hydrogen peroxide (30%), absolute ethanol, and sodium acetate were purchased from Sigma-Aldrich and were all the RdH brand. Sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO). Sulfuric acid (98%) and Hoechst 33258 were purchased from Arcos Organics. Potassium ferricyanide(III) was from Aldrich (Milwaukee, WI). All chemicals were of analytical reagent grade and used as received. Stock solutions were prepared with deionized water from a Millipore Milli-Q system.

Instrumentation. Cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were performed using an Autolab PGSTAT 30 potentiostat/galvanostat (Eco Chemie) controlled by General Purpose Electrochemical System (GPES) software (Eco Chemie). Electrochemical experiments were carried out in a 50-mL two-compartment cell using a gold working electrode (area of 1.37 cm^2, Maxtek, Torrance, CA), a Pt counter electrode, and a Ag/AgCl reference electrode (immersed in a 3 M NaCl filling solution saturated with AgCl, EG&G, Princeton Applied Research, Oak Ridge, TN). The DNA concentration was determined with a GeneQuant RNA/DNA calculator (Amersham Biosciences, Piscataway, NJ), and PCR was performed in a PTC-200 Peltier thermal cycler (MJ Research, Incline Village, NV). Fluorescence imaging was performed on a ScanArray 5000 (Packard Biosciences, CT), and subsequent intensity measurements were computed with QuantArray (Packard Biosciences) evaluation software.

Asymmetric PCR Protocol. PCR reagents were purchased from GIBCO BRL (Life Technologies, Rockville, MD), unless otherwise stated. For each PCR experiment, 100 μL of mastermix was prepared. It contained 10 μL of 10 × PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 4 μL of 50 mM $MgCl_2$, 2 μL of 10 mM dNTPs, 5 μL of 0.1 μM forward primer or negative control without the Texas Red label, 5 μL of 10 μM reverse primer, 10 μL of 5 μg/μL bovine serum albumin (BSA), 10 μL of 1 ng/μL DNA template, 0.5 μL of 5 units/μL Taq DNA polymerase, and 53.5 μL autoclaved double-deionized water. The DNA template consisted of the 5S-rRNA spacer gene of either *Fritillaria thun-*

(17) Armistead, P. M.; Thorp, H. H. *Anal. Chem.* **2000**, *72*, 3764−3770.
(18) Napier, M. E.; Thorp, H. H. *Langmuir* **1997**, *13*, 6342−6344.
(19) Kelley, S. O.; Boon, E. M.; Barton, J. K.; Jackson, N. M.; Hill, M. G. *Nucleic Acids Res.* **1999**, *27*, 4830−4837.
(20) Steel, A. B.; Herne, T. M.; Tarlov, M. J. *Anal. Chem.* **1998**, *70*, 4670−4677.
(21) Hashimoto, K.; Ito, K.; Ishimori, Y. *Anal. Chem.* **1994**, *66*, 3830−3833.
(22) Takenaka, S.; Yamashita, K.; Takagi, M.; Uto, Y.; Kondo, H. *Anal. Chem.* **2000**, *72*, 1334−1341.
(23) Uto, Y.; Kondo, H.; Abe, M.; Suzuki, T.; Takenaka, S. *Anal. Biochem.* **1997**, *250*, 122−124.
(24) De Lumley-Woodyear, T.; Campbell, C. N.; Freeman, E.; Freeman, A.; Georgiou, G.; Heller, A. *Anal. Chem.* **1999**, *71*, 535−538.
(25) Gyllensten, U. B.; Erlich, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7652−7656.

bergii or *Fritillaria cirrhosa* (~600 bp) cloned into the pCR2.1-TOPO TA vector (3.9 kb, Invitrogen, Carlsbad, CA).

The mastermix was subjected to the following thermal cycling profile: initial denaturation at 95 °C for 4 min, 45 cycles at 95 °C for 30 s, at 53 °C for 30 s, at 72 °C for 45 s, and a final extension at 72 °C for 10 min. To confirm the fidelity of the reaction, PCR products were loaded in a 1% agarose gel prepared with TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA) containing 0.5 μg/mL ethidium bromide and electrophoresed at 100 V for 1 h. Then, the sample was visualized by UV transillumination and its size was compared with a known DNA ladder.

Procedures. *Preparation of the Probe Immobilization Solution*. Thiol-modified DNA tends toward oxidative dimerization to form disulfide, resulting in reduced probe immobilization efficiency and variable surface probe density for different batches of experiments. To reduce dimerization, the DNA probe was treated with DTT prior to its immobilization. The probe/DTT solution (the final concentration of DTT was 0.1 M) was incubated at room temperature for 30 min, followed by the removal of DTT using a standard ethanol precipitation procedure.[26] One volume of the probe/DTT solution was mixed thoroughly with 0.1 volume of 3 M sodium acetate (pH 5.6) and 3 volumes of ice-cold absolute ethanol, kept at −80 °C for 30 min and centrifuged at 14000*g* for 15 min. The DNA pellet obtained was washed with 1 mL of 95% ethanol, centrifuged again, dried, and resuspended in water. The DNA concentration of this solution was determined and adjusted to 25 μM (the final salt composition was 500 mM NaCl/25 mM sodium phosphate, pH 7.0) as the probe immobilization solution.

Probe Immobilization. The gold electrodes (1.37 cm^2) and cover glasses (20 mm in diameter and 0.1 mm thick; Superior) were cleaned with H_2SO_4/H_2O_2 solution (7:3 volume ratio) in a sonic bath at room temperature for 10 min, followed by thorough rinsing with water. Immediately after that, 5 μL of the immobilization solution was pipetted onto each electrode and the cover glass was laid on top to spread it uniformly over the gold electrode surface. The electrode was incubated in a humidity chamber for 3 h at room temperature and then flushed with copious amounts of water to remove the nonimmobilized DNA probe.

Hybridization Experiments. Oligonucleotide (the target or negative control) solutions of 5 μM were prepared in a 2× SSC buffer (300 mM NaCl/30 mM Na citrate, pH 7.0). Asymmetric PCR samples were mixed with an equal volume of a 4× SSC buffer to achieve a final salt composition close to that of the oligonucleotide solutions (the hybridization rate is a strong function of the sample's ionic strength). For each hybridization experiment, 200 μL of the sample was applied to the probe-modified electrode surface. After 60 min of incubation at room temperature, the electrode was rinsed sequentially with 2× SSC/0.1% SDS and 2× SSC buffers to remove nonspecifically bound species.

Intercalator Binding and Electrochemical Measurements. The binding of the DNA intercalator with the probe or probe−target hybrid on the gold surface took place in the 100 μM Hoechst 33258 solution (in 100 mM NaCl/10 mM sodium phosphate, pH 7.0, PBS) for 5 min at room temperature. Excess intercalator was washed off with a large amount of PBS solution. The anodic peak current of the bound intercalator was measured with the LSV technique in PBS solution at a scan rate of 100 mV/s. Moreover, CV measurements with ferricyanide solution (1 mM ferricyanide in 100 mM KCl) were conducted at a scan rate of 50 mV/s. Note that all measurements were taken at room temperature.

(26) Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K. *Short protocols in molecular biology*; John Wiley & Sons: New York, 1992; Unit 2.1.

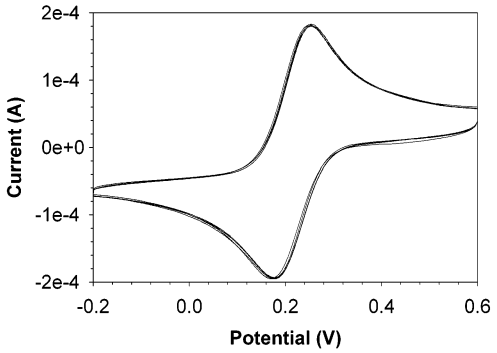

Figure 1. Cyclic voltammograms of 1 mM ferricyanide at the five bare gold electrodes, with 100 mM KCl supporting electrolyte and a scan rate of 50 mV/s.

Fluorescence Imaging and Intensity Measurements. The gold electrodes modified with a Texas Red-labeled DNA probe or with a hybridized Texas Red-labeled oligonucleotide target (in this case, the DNA probe was not fluorescently labeled) were scanned at a resolution of 30 μm (excitation wavelength of 594 nm; emission wavelength of 614 nm). To determine the surface densities of the immobilized probe and the hybridized target, the fluorescence intensities were compared to the intensity of a calibration plot generated by spotting a series of known amounts of fluorescently labeled oligonucleotide on a microscope slide.

RESULTS AND DISCUSSION

In the following sections, we first describe the voltammetric investigation in the ferricyanide system to ensure the cleanliness and reproducibility of the electrode surface, followed by a discussion of the electrochemical sequence-specific detection of the short oligonucleotides. Finally, we demonstrate the combination of the asymmetric PCR technique and the established detection strategy for the differentiation of the two *Fritillaria* species.

Characterization of the Bare and Probe-Modified Electrode with the Ferricyanide System. Throughout this study, a total of five gold electrodes were used. To start with, it is necessary to ensure that the active surface areas of all the electrodes are identical. Otherwise, the anodic current signal of the intercalator in subsequent experiments has to be corrected accordingly. This is significant from a practical point of view in that microfabricated electrodes with identical surface areas must have reproducible DNA probe coverage, leading to well-defined intercalator peak currents of the probe-modified electrodes. This, in turn, eliminates the tedious need to calibrate every electrode or batch of electrodes. Figure 1 shows the simultaneous plot of cyclic voltammograms of the ferricyanide system with the five bare electrodes. The peak currents of the five curves are nearly indistinguishable (with a relative standard deviation of 0.7%), indicating that the active surface areas of all the electrodes are the same.

Another use of the ferricyanide system is to qualitatively characterize the immobilization of the DNA probe onto the gold surface. When the DNA probe is immobilized onto the gold

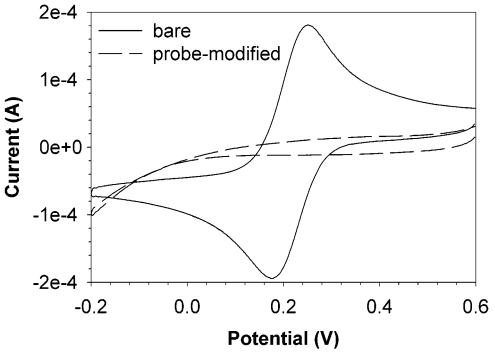

Figure 2. Cyclic voltammograms of the bare and probe-modified gold electrodes in a 1 mM ferricyanide/100 mM KCl solution, with a scan rate of 50 mV/s.

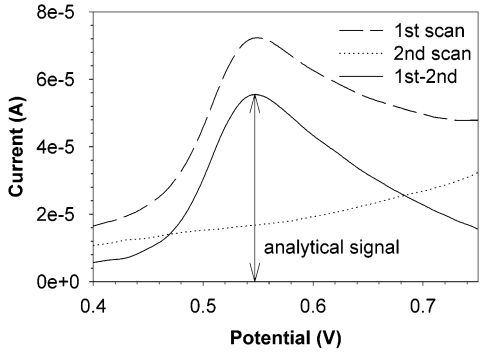

Figure 3. Two consecutive LSV scans of 100 μM Hoechst 33258 absorbed onto the bare gold electrode. The second scan is subtracted from the first scan to obtain the background-corrected analytical signal, as marked in the figure. LSV was performed in PBS solution at a scan rate of 100 mV/s.

surface by chemisorption, the surface becomes so negatively charged (due to phosphate groups along the DNA backbone) that it retards the anionic ferricyanide from approaching the electrode surface, resulting in a significant reduction in the peak currents and a greater separation of the cathodic/anodic peak potentials. With the immobilization procedure as described in the Experimental Section, a probe monolayer of high surface density is formed that suppresses the reduction of ferricyanide in the potential range studied, as illustrated in Figure 2. Quantitation of the amount of immobilized probe by the fluorescence method gives a surface coverage of $\sim 3 \times 10^{12}$ molecules/cm^2. If nonthiolated DNA (i.e., the negative control) is used instead, the immobilization efficiency is much lower, as indicated by a cyclic voltammogram close to that of the bare electrode (with slightly lower peak currents and a larger peak potential difference).

Typical cleaning procedures for gold electrodes involve either mechanical polishing with a diamond/alumina slurry or chemical treatment. The polishing is applicable to glass/plastic rod-sealed disk-type electrodes, but it suffers from the drawback of a changing background current from run to run. For the thin-film sputtered gold electrodes used in this work, a simple H_2SO_4/H_2O_2 treatment can completely restore the probe-modified surface to its original bare condition, as evidenced by the CV from the ferricyanide system (data not shown).

Characterization of the Intercalator. Hoechst 33258, a bisbenzimide dye used as a hybridization indicator, features irreversible oxidation with a peak potential of 550 mV at the bare gold electrode. Therefore, when two consecutive LSV scans are conducted, the second scan contains nothing but the contribution from the background current, which is subtracted from the first scan to extract the analytical signal (Figure 3). Figure 4 presents evidence for the binding of Hoechst 33258 with the DNA probe immobilized onto the gold electrode. The enrichment of the intercalator onto the probe-modified electrode surface leads to a 3-fold increase in the peak current compared to that of the bare electrode.

Performance Evaluation of the Probe Immobilization and Hybridization with Oligonucleotides. Before commencing the hybridization experiments, a reproducible probe immobilization procedure must be established. This is achieved by immobilizing the DNA probe onto the five electrodes simultaneously and then observing the current responses of Hoechst 33258 intercalated onto the probe-modified electrodes. Figure 5 shows that the peak

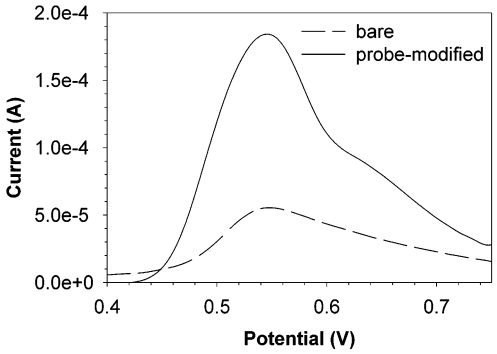

Figure 4. Background-corrected linear sweep voltammograms of 100 μM Hoechst 33258 absorbed onto the bare and probe-modified gold electrodes at a scan rate of 100 mV/s.

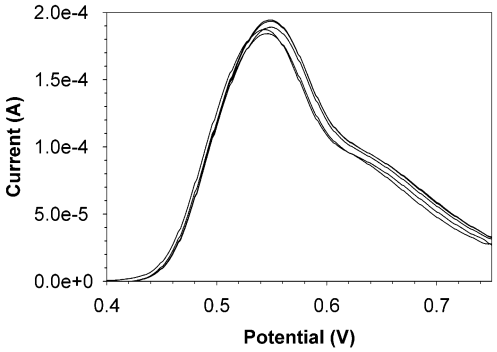

Figure 5. Background-corrected linear sweep voltammograms of 100 μM Hoechst 33258 intercalated onto the five probe-modified gold electrodes at a scan rate of 100 mV/s.

currents of the five probe-modified electrodes in a single batch of immobilization experiments are consistent (with a relative standard deviation of $\sim$2%). Nevertheless, there exists slight variation in the immobilization efficiency from batch to batch. Hence, a probe-only electrode surface is included in each set of experiments as the control.

The hybridization performance of the immobilized DNA probe is first evaluated with the short oligonucleotides (the target and negative control). A double-stranded DNA hybrid is formed on the electrode surface when the 16-mer target is allowed to interact with the probe. As a result of the higher binding activity of the intercalator with ds-DNA than with ss-DNA[21] (binding selectivity of $\sim$2), more Hoechst 33258 molecules are attached to the hybrid-

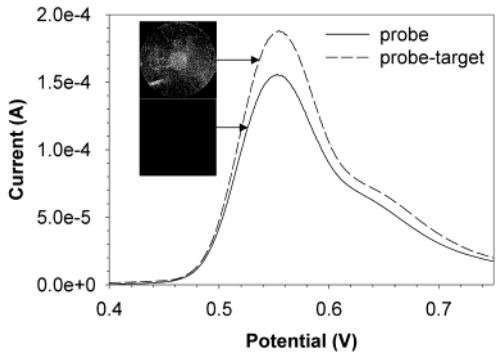

Figure 6. Background-corrected linear sweep voltammograms of 100 μM Hoechst 33258 intercalated onto the probe and probe−target hybrid-modified gold electrodes. The probe−target hybrid was formed by incubating the probe-modified electrode with a 5 μM concentration of the 16-mer target in 2× SSC buffer for 1 h. The inset shows fluorescent images of the gold electrode surface (1.37 cm^2) after hybridizing with the Texas Red-labeled target or negative control.

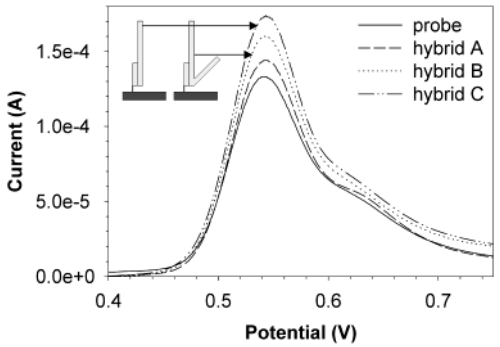

Figure 7. Background-corrected linear sweep voltammograms of 100 μM Hoechst 33258 intercalated onto four electrodes with different target hybridization species: (1) no target (probe); (2) 5 μM concentration of the 16-mer target sequence (hybrid A); (3) asymmetric PCR product (~600 bp) with binding region at ~200 bp from the 3′ end (hybrid B); (4) asymmetric PCR product (~400 bp) with binding region at the 3′ end (hybrid C). Hybridization was performed in 2× SSC buffer for 1 h.

modified electrode surface than to the probe-only surface. In our case, the hybridization of the 16-mer target with the probe gives rise to a 20% increase in the peak current of the intercalator during the LSV scan, as shown in Figure 6. On the other hand, if the negative control is incubated with the probe-modified electrode, the peak current of the intercalator is virtually the same as that of the probe-only electrode, demonstrating the capability of the present electrochemical system for sequence-selective detection of the DNA analyte. This is also confirmed by the fluorescent images of the electrode surface after hybridization with the Texas Red-labeled target and negative control (insets of Figure 6). The total dark image of the negative control indicates that the washing protocol is good enough to remove nonspecifically bound DNA.

The fluorescence intensity of the hybrid-formed electrode (only the oligonucleotide target was fluorescently labeled) is very close to that of the probe-modified electrode, suggesting that nearly all DNA probes are accessible to binding with the targets. An attempt is made to investigate the influence of the washing protocol on the stability of the hybrid on the electrode surface. Fluorescence imaging reveals that a gentle water rinse following the 2× SSC/0.1% SDS and 2× SSC buffer washes removes most of the bound targets as a result of the great electrostatic repulsion between the negatively charged phosphate backbones of the probe and target in an extremely low ionic strength environment.

Detection of Asymmetric PCR Amplicons. To realize DNA analysis in a lab-on-a-chip that comprises sample preparation, target DNA amplification, and post-PCR detection functionalities, one technical challenge is to develop an on-chip detection scheme capable of directly analyzing PCR products without the need for cumbersome purification/separation steps. One such candidate would be the electrochemical methodology discussed here that separates the target PCR amplicon/probe hybrid from other interfering substances (nonspecific PCR amplicons and PCR reagents) by simple buffer washes. Up until now, research efforts in the field of electrochemistry-based DNA detection have mainly focused on short oligonucleotides. Here, a DNA probe immobilized on the gold electrode is used for sequence-specific detection of unpurified PCR products.

To distinguish the two close species of *Fritillaria* (*F. thunbergii* and *F. cirrhosa*), two primers (forward and reverse) complementary to the coding region of the highly conserved 5S-rRNA gene can be used to amplify the variable spacer regions (~600 bp) that are different for the two species. After asymmetric PCR, both samples contain sufficient amounts of ss-DNA amplicons for species identification. As the probe sequence used in this work is unique to *F. thunbergii*, only amplicons from this species have the complementary sequence for hybridization with the probe. Electrochemical measurement with this hybrid (hybrid B in Figure 7) produces a peak current higher than that of the probe/16-mer target hybrid (hybrid A in Figure 7). However, considering that there are a lot more intercalators bound to the probe−PCR target hybrid (the PCR amplicon is ~40 times longer than the oligonucleotide target), the hybridization efficiency for the asymmetric PCR amplicon is much lower than for the short oligonucleotide. This may be accounted for by a lower PCR target concentration and higher steric effect (bulky end groups contiguous to the binding region, ~200 bp at the 3′ end and ~400 bp at the 5′ end) of the PCR amplicon. One way to minimize the steric effect is to change the limiting primer in the asymmetric PCR to the nonthiolated probe sequence (i.e., the negative control) so that the binding region of the amplicon is exactly at its 3′ end. The hybridization efficiency of this shorter amplicon (~400 bp) is slightly higher than that of its longer counterpart (~600 bp), as suggested by a higher intercalator peak current during the LSV scan (hybrid C in Figure 7). A correlation curve between the concentration of the asymmetric PCR amplicon (*F. thunbergii*, ~400 bp) and the intercalator peak current is shown in Figure 8. It can be seen that the current response is proportional to the concentration of the asymmetric PCR target amplicon.

CONCLUSIONS

A 5′-thiol-derivatized DNA probe chemisorbed onto a gold electrode surface has been utilized for hybridization-based detection of target DNA molecules. Signal transduction of the hybridization event was achieved by the linear sweep voltammetric scan of the electrochemically active DNA intercalator Hoechst 33258 bound to the formed hybrid on the electrode surface. Combining the asymmetric PCR technique and the established electrochemical sequence-specific detection scheme, differentiation of the two

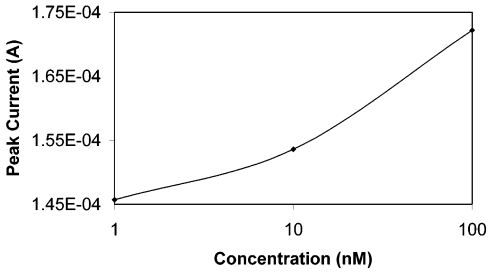

Figure 8. Calibration plot of the intercalator peak current against the concentration of the asymmetric PCR amplicon (*F. thunbergii*, ~400 bp).

closely related *Fritillaria* species based on a 16-mer unique sequence in single-stranded amplicons of the spacer region of the 5S-rRNA gene has been demonstrated. Even though the present work is solely targeted on a particular herbal gene, it can be extended not only to other herbal species differentiation but also to genetic disease diagnostics and viral/bacterial identification. In future work, we will implement the established detection approach in an integrated PCR-electrochemical cell microdevice for portable DNA analysis applications.

ACKNOWLEDGMENT

The authors are thankful for the funding support from the Institute of Integrated Microsystem (I^2MS) of HKUST and the Innovation and Technology Commission of the Hong Kong Special Administrative Region (Industry Support Fund Project No.: AF/150/99). The authors also thank Dr. Maria Carles for helpful discussions and Prof. Karl Tsim of the Biology Department at HKUST for providing the raw materials of the *F. thunbergii* and *F. cirrhosa* clones.

Received for review January 30, 2002. Accepted July 30, 2002.

AC020068S