Direct Detection of Single Nucleotide Polymorphism (SNP) with Genomic DNA by the Ferrocenylnaphthalene Diimide-based Electrochemical Hybridization Assay (FND-EHA)

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A ferrocenylnaphthalene diimide-based electrochemical hybridization assay (FND-EHA) was applied to the direct detection of a C-to-G transition in a codon (TCA) for Ser-447 of the human lipoprotein lipase (LPL) gene, which resulted in the termination of the LPL protein there. Either one of two 13-mer oligonucleotide probes, S447 WT and S447X MT, representing sequences complementary to those of the wild type (WT) and mutated (MT) forms, was immobilized on a gold electrode, followed by hybridization with chromosomal DNA extracted from human leukocytes under the condition in which both WT- and MT-type sequences can form a duplex. These two electrodes were soaked in an electrolyte containing FND under a condition [0.1 M HOAc/KOAc (pH 5.6) containing 0.1 KCl and 0.05 mM FND at 40˚C], in which only the MT duplex could undergo dissociation. FND was concentrated in proportion to the amount of the duplex formed on the electrode to give rise to a current signal. The electrochemical signal ratios obtained for WT/WT, WT/MT and MT/MT were close to the theoretical 2:1:0 with the S447 WT-modified electrode, and was again close to 0:1:2 with the S447X MT-modified one.

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Introduction

Most of the common diseases are induced by a combination of hereditary and environmental factors. It has also been realized that heterozygotes, rather than homozygotes, pose a serious problem in most hereditary diseases.1 Thus, a high-throughput method is desired for the detection of heterozygotes. DNA hybridization has been widely used for this purpose: a mutated region is amplified by PCR 2 and the product is allowed to hybridize with a fluorophore-labeled oligonucleotide containing the region in question.3 This method has, however, a few difficulties: several time-consuming steps are required, and it is difficult to choose the condition to remove mismatched oligonucleotides from the fully matched one. In order to circumvent these problems, the Molecular Beacon,4,5 Taqman,6 and Invador methods7,8 have been developed. However, the Taqman is not cost-effective because of a requirement for several kinds of fluorophore-labeled oligonucleotides, and the Invador method needs a special enzyme.9 On the other hand, although mass spectrometry has been developed for genotyping,10 it also requires PCR, and the result of an analysis is severely affected by the purity of the sample. In order to realize a quick and simple method for the routine detection of a genome-level mutation, a highly sensitive DNA detection method should be developed. An electrochemical method with ferrocenylnaphthalene diimide (FND) as a hybridization indicator, which we developed, is suitable for this purpose.11–15 FND is a multi-functional molecule based on a threading intercalator, naphthalene diimide, carrying two ferrocene moieties via two linkers: the naphthalene diimide moiety intercalates between the base pairs of double-stranded DNA, whereas the two ferrocene moieties are coordinated to the minor and major grooves of DNA.11,12 The ferrocene moieties also work as electrochemical sensor as well as an anchor. We succeeded in the atto mol-level detection of DNA fragment coding for the target sequence14 as well as in one-base mismatch detection in double-stranded DNA with this system.13,14 Especially, one-base mismatch or a single nucleotide polymorphism of the human lipoprotein lipase (LPL) gene was successfully detected with its PCR products of 221 bp.16 In the present work, we attempted to analyze a one-base mismatch directly with chromosomal DNA. The basic idea described here is non-specific hybridization of mismatched and fully matched DNAs with DNA probe immobilized on an electrode and a specific dissociation of a mismatched DNA sample from the electrode. This method can detect a heterozygous DNA sample in spite of the concentration of the DNA sample. Here, an example of a successful analysis is presented for a one-base mismatch of a C-to-G transition present on the LPL gene.
Experimental

Materials

All of the oligonucleotides used in this study were custom synthesized by Hokkaido System Science (Sapporo, Japan). The nucleotide sequences are illustrated in Fig. 1. Probe DNAs S447 WT and S447X MT have nucleotide sequences complementary to 13 bases containing S447 and S447X sites of the LPL gene, respectively. A thiol group was introduced on the 5'-terminus of the probes. Chromosomal DNAs from human leukocytes were prepared by using QIAamp (Qiagen) according to the manufacturer’s protocol. Typically, 3 – 12 µg of genomic DNA was obtained from 200 µl of blood. A 221-bp fragment of DNA containing the site of mutation in question was amplified by PCR with human chromosomal DNA as a template and the following primers: 5'-TGT AAA ACG ACG GCC AGT TTG TTC TAC ATG GCA TAT TCA CAT CCA-3' and 5'-CAG GAA ACA GCT ATG ACC AGC TCA GGA TGC CCA GTC AGC TTTA-3'. The amplified DNA was purified with Qiaquick a PCR Purification Kit (Qiagen) according to the manufacturer’s protocol. The concentration of DNA was determined with molar absorptivity: 6500 M⁻¹ cm⁻¹ for one base pair at 260 nm. All of the chemicals used were of the highest grade available.

Immobilization of probe DNA on the gold electrode

Probe DNA was immobilized on the surface of a gold electrode by means of a 5'-thiol group. Before immobilization of the probe DNA on the gold electrode, the gold surface was pretreated as follows. A piece of gold (BAS No. 2014, 2 mm²) was boiled in 2 M NaOH for 1 h, washed with water, and dried. It was swirled in concentrated nitric acid for 30 min at room temperature, washed with water, and dried. After a pretreatment, 1 µL of a probe DNA solution was placed on the gold surface, and the electrode was capped and incubated for 2 h at room temperature. The concentration of DNA was 2 pmol µL⁻¹ for synthetic oligonucleotides and PCR products and 1 fmol µL⁻¹ for chromosomal DNA. The electrode was then washed with water, dried, and 1 µL of 2-mercaptoethanol was added. After incubation for 2 h at room temperature, the electrode was washed with water and kept in water until use. The probe-immobilized electrode can be used several times by removing the target DNA by a formamide treatment. The electrode, itself, can be recycled several hundred times by a pretreatment.

Temperature-dependent duplex dissociation on the electrode

One microliter of oligonucleotides of 13 bases (10 pmol µL⁻¹ in 2 × SSC) complementary to the probe sequence with (MT) and without (WT) one base mismatch were allowed to hybridize at room temperature for 1 h with the S447 WT probe-immobilized electrode prepared above. The same experiment was carried out with the S447X MT probe. The electrode was then soaked in 2 mL of a measurement solution (0.1 M HOAc/KOAc, pH 5.6, 0.1 M KCl, and 0.05 mM FND). Differential pulse voltammetry (DPV) was determined at different temperatures. The conditions for the DPV measurements were as follows: initial potential, 0 V; final potential, 0.6 V; scan rate, 100 mV s⁻¹; pulse amplitude, 0.05 V; sample width, 16.7 ms; pulse period, 0.2 s; pulse width, 0.05 s; quiet time, 2 s; sensitivity, 10⁻⁸ A V⁻¹. The counter electrode and reference electrode were Pt and Ag/AgCl, respectively. All of the measurements were repeated 10 times.

Optimization of the experimental temperature with oligonucleotides

The experimental temperature was optimized with oligonucleotides of 13 bases. DPV was determined with a gold electrode modified with probe DNA (2 pmol). After the electrode was washed with water, 1 µL of sample DNA (10 pmol µL⁻¹, 2 × SSC) was dropped on it and hybridization was allowed to proceed at room temperature for 1 h. DPV was then determined again. The response currents at various temperatures were measured, and the largest difference in the response currents between WT and MT appeared at 40°C.

Optimization of the hybridization condition for PCR products

DPV was measured with a gold electrode modified with a DNA probe (2 pmol), and the electrode was washed with water. One milliliter of a PCR product (5 pmol µL⁻¹) was heated at 98°C for 10 min and cooled quickly with liquid nitrogen. After the addition of various amounts of 1 µL of SSC, hybridization was carried out by standing the electrode at room temperature for 1 h. The response current was obtained by a DPV measurement at the several temperatures. The response current of WT could be separated at 40°C from that of MT. All of the DPV measurements were performed at 40°C, according to the above results.

Detection with a genome sample

This time, a gold electrode was modified to lower density with the probe DNA (1 fmol). Preliminary experiments suggested that immobilization of the probe DNA to high density impairs the efficiency of hybridization, presumably because of a steric hindrance. It was found that 1-fmol was suitable for the detection of chromosomal DNA. After the electrode was washed with water, 40 µL of 20 mM HEPES (pH 6.5) containing 58% formamide and 0.4 M NaCl was placed on it. DNA prepared from human chromosome was heated at 98°C for 5 min and quickly cooled with liquid nitrogen; a 20 µL aliquot was placed on the electrode. After 10 min at 50°C, the temperature was lowered to 20°C over a period of 5 h. DPV was taken again to observe an increase in the current. The observed current increase was several hundred nA in those experiments.
Results

Duplex dissociation with a rise in temperature

Following hybridization with synthetic 13-meric oligonucleotides representing the WT or MT sequence of the LPL gene (Fig. 1), the DPV of a gold electrodes modified with 13-meric oligonucleotides, whose sequence is complementary to the sample DNA, was determined in the presence of 50 μM FND at several temperatures where a duplex is formed on the electrode. The correlation of the peak current with the temperature is shown in Fig. 2. In each case, the current decreased with the temperature, as the duplex tended to dissociate as the temperature was raised concomitant with the loss of FND from DNA to the bulk solution. With S447 WT probe, the mismatched duplex started to dissociate at a lower temperature than the fully matched case, simply because the former was less stable. On the other hand, with the S447X MT probe, the temperature dependence of the dissociation was barely different between the two types of duplexes, presumably because of a difference in the instabilities of the C-C mismatch and the G-G mismatch; the latter is less stable than the former.18 In both cases, the response current decreased with the temperature at a lower temperature, because the response current depends on the diffusion rate of FND. However, as the temperature is raised, the duplex on the electrode starts to dissociate, resulting in a decrease in the response current. These results showed that 40°C is good to discriminate between mismatched and fully matched DNA duplexes in this combination. It has also been shown that the S447 WT probe is more suitable for this purpose because of the larger difference in the response between mismatched and fully matched duplexes.

Optimization of the temperature for the detection of oligonucleotide DNAs

In order to find out the optimal condition for hybridization, various temperatures were tested with the same DNA-modified electrodes. With the S447 probe, the ratio of the response current increase was estimated near the temperature at which WT oligonucleotide DNA and MT hybridize. The difference in the peak current Δ%/ was defined as (i – i0)/i0 × 100, where i0 and i represent the peak current before and after hybridization, respectively; Δ represents the net increase in the current of FND bound to the duplex DNA formed per DNA probe immobilized on the electrode. As shown in Fig. 2, the maximum response current was observed at 40°C, which was regarded as being the optimum temperature, and was used in subsequent experiments. The average current increase and mean standard error with the WT probe were 17.6% and 7.4, respectively, under these conditions, whereas with the MT oligonucleotide DNA, the corresponding values were –0.66% and 4.5 (Fig. 3).

Hybridization of the PCR product with immobilized probe DNA

Part of exon 9 of the LPL gene containing the site of mutation was amplified by PCR. The resulting PCR product of 221 bp was allowed to hybridize with the probe DNA immobilized on a gold electrode at room temperature for 1 h in 2 × SSC. DPV was then determined at 40°C and the ratio of the current increase was estimated. Because the amount of the duplex DNA formed on the electrode reflects the population of the WT sequence in the PCR products, the ratio of WT/WT:WT/MT:MT/MT should be 2:1:0, which should be equivalent to the amount of FND concentrated on the electrode, as reflected in the magnitude of the electrochemical response. As shown in Fig. 4, the current increase and mean standard error were 122.7% and 42.7, respectively, for WT homozygotes, whereas the corresponding values for the WT/MT were 60.0% and 14.7, and for the MT/MT 17.8% and 4.2. Their ratio of 2.05:1:0.30 is close to the theoretical value of 2:1:0. Therefore, it seems certain that mutation can be detected with a PCR product under the optimal conditions.

Genotyping of genomic DNA

The results obtained above suggested that the detection of a mutation may be feasible even with a genomic DNA sample. Thus, genomic DNA purified from human white blood cells was tested with and without ribonuclease A (RNase A) treatment. Hybridization was carried out in 20 mM HEPES (pH 6.5) containing 58% formamide and 0.4 M NaCl with the temperature slowly being lowered from 50°C to room temperature. As shown in Fig. 5, the current increase and mean standard error for the WT were 116.9% and 23.1, whereas the corresponding values were 53.7% and 16.9 for WT/MT, and 8.0% and 11.8 for MT/MT, or 2.18:1:0.15, a ratio not far from...
the theoretical 2:1:0. Therefore, a mutation in genome DNA can be directly detected without PCR under the optimal conditions. The results without an RNase A treatment were essentially the same as those shown in Fig. 5: the average current increase and mean standard error were 135.1% and 54.6 for WT, whereas they were 48.3% and 10.5 for WT/MT, and 15.7% and 6.2 for MT/MT, or 2.80:1.0:3.3. In other words, the RNase A treatment may be omitted when the speed of analysis rather than the quality of the data is of primary concern.

**Discussion**

A quick and simple method has been desired for genotyping in order to realize a tailor-made diagnosis based on a result obtained from human genome analysis. The electrochemical method with FND has an advantage in that no chemical modification of the probe and the sample DNA is required. In order to demonstrate its usefulness in DNA analysis, we studied the detection limit in a model system with oligonucleotides and PCR products. We were able to demonstrate that the atto mol-level detection of target DNA sequences could be achieved with them. In addition to this high sensitivity, we succeeded to analyze a one-base mismatch under proper conditions. Although this achievement possesses an enormous potential to apply this method to the practical analysis of SNPs, there remained a few difficulties to overcome. For one thing, the margin to discriminate WT/WT from WT/MT was so small that meticulous care needed to be taken in all manipulations. Second, genomic DNA was not amenable to this analysis, and hence at least one PCR step was necessary. As described above, we were able to expand the gap between WT/WT and WT/MT significantly by a small alteration of the original technique. The point is that both the WT and MT sequences are dissociate by shifting the temperature. Even an RNase treatment can be omitted when the speed of analysis is of primary importance. Furthermore, a variation in the quantity of the sample applied does not affect the result very much, as in the case of heterozygotes, carrying WT and MT in equal quantities; either of them forms a duplex with an identical probability. Then, the MT is selectively removed from the electrode, and hence only the WT gives rise to a signal, whose magnitude is half that of the homozygote WT/WT. Although only a specific example of S447 mutation of the LPL gene was tested, this technique should be a general one and applicable to any kind of mutations and SNPs. Moreover, an electrochemical DNA microarray system based on this technique may be even more useful. This study is a first step toward such a microsystem where genome DNA prepared from cells can be analyzed directly without any labeling or amplification process.

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**References**


