Electrochemical SNP Detection Using Glucose Dehydrogenase

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ABSTRACT

Single nucleotide polymorphisms (SNPs) are important biomarkers for evaluating sensitivity to drugs and for predicting whether people might have a disease in the future. In this study, we constructed an electrochemical detection system of an SNP of peroxisome proliferator-activated receptor γ_2 (PPAR γ_2) (C34G) using glucose dehydrogenase (GDH) based on a single base extension method. Target DNA was hybridized with capture DNA immobilized on a gold electrode. Biotinylated dCTP was inserted to probe DNA if target DNA had the SNP. Avidin conjugated GDH can bind to one base extended DNA resulting in increased response current after washing of redundant avidin conjugated GDH and addition of glucose. In this system, PPAR γ_2 (C34G) was detected specifically with 10^{-8} mol dm $^{-3}$ detection limit based on amperometric sensing system.

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1. Introduction

Single nucleotide polymorphisms (SNPs) are attractive biomarkers for predicting whether people will have a disease in the future.¹ Additionally, SNPs provides important clues for matching the right medicine to the right person, with the right dosage at the right time. Some SNPs are approved as biomarkers for diagnostics and selection of a suitable drug for each person by the U. S. Food and Drug Administration.

Many studies have developed SNPs genotyping systems as reviewed by Sobrino et al.² Although hybridization based DNA sensors are attractive for discriminating DNA specifically, they require a specific design.³ On the other hand, an enzyme based assay can discriminate SNPs specifically based on enzyme substrate specificity and Patolsky et al. reported an attractive approach based on a single base extension method.⁴

Figure 1 shows a scheme of SNP detection based on a single base extension method. DNA probes that are complementary DNA with downstream sequence of a mutation of target DNA are immobilized on a gold electrode. Target DNA hybridizes with immobilized probe DNA. Biotinylated base that is complementary with a mutant is extended on the probe DNA in the presence of DNA polymerase if the target DNA has a mutation. Then avidin conjugated with the enzyme (avidin-enzyme) binds to the DNA probe in which a biotin was inserted resulting in increase of current after washing of excessive avidin-enzyme. Whereas the DNA probe does not react with biotinylated base if the target DNA does not have an SNP resulting in no increase of current.

We previously reported a DNA sensor using avidin conjugated glucose dehydrogenase (GDH).⁵ Since the activity of GDH can be measured by a commercially available glucose sensing system that is miniaturized and an optimized biosensing system, GDH is an attractive enzyme for labeling an enzyme to detect DNA, protein and so forth.^{5–7} In this study, we tried to combine GDH with an SNP detection system based on single base extension method and detect SNP of PPAR γ_2 related to type 2 diabetes as the model target.⁸ Since PPAR γ_2 (C34G) would be a factor in reducing the risk of acquiring type 2 diabetes, it would be a biomarker for health control.

Figure 1. The scheme of the electrochemical SNP detection system using glucose dehydrogenase based on the single base extension method. (a) In the presence of SNP, avidin-conjugated glucose dehydrogenase binds to inserted biotinylated dNTP resulting in increase of current after addition of glucose and electron mediators. (b) In the absence of SNP, avidin-conjugated glucose dehydrogenase is washed out resulting in no increase of current after addition of glucose and electron mediators.

2. Experimental

2.1 Materials

Flavin adenine dinucleotide dependent GDH (FADGDH) was obtained by recombinant expression in *Escherichia coli*.⁹ 1-methoxyphenazine methosulphate (m-PMS) and glutaraldehyde solution was purchased from Wako Chemicals. Bio-11-dCTP (Biotin-11-2'-deoxycytidine-5'-triphosphate) was purchased from Enzo Diagnostics, Inc. Gene Taq polymerase was purchased from Nippon gene.

2.2 Preparation of avidin conjugated glucose dehydrogenase

Avidin-FADGDH conjugate was prepared using glutaraldehyde crosslinking, as has been described elsewhere as well as avidin-

PQQGDH.⁵ The activity of the PQQGDH-avidin conjugate was measured using 1 mM 2,6-dichlorophenolindophenol (DCIP), m-PMS, 20 mM glucose, and we defined 1 unit of GDH activity as the amount of enzyme required to reduce 1.0 μ mol DCIP per min in the presence of glucose.

2.3 Amperometric SNP genotyping

A gold electrode was immersed into the phosphate buffered saline (50 μ L) containing 1-nmol thiol-modified DNA probe (5'-SH-AGTGTATCAGTGAAGGAATCGCTTTCTG-3') for 12 h or more and the DNA probe was immobilized onto the gold electrode. After washing of the electrode by water, the electrode was incubated with 1% blocking reagent for hybridization (Roche applied science) to reduce nonspecific binding of DNA. PPAR-SNP-DNA (5'-TCA-CATAGTCACTTCCTTAGGCAAAGACGCAGTTATCC-3') or PPAR-DNA (5'-TCACATAGTCACTTCCTTAGGCAAAGACC-CAGTTATCC-3') (100 pmol), biotinylated dCTP (b-dCTP) (100 pmol) and Gene *Taq* DNA polymerase (2 U) were incubated with the DNA probe immobilized on the electrode for 10 min at 60°C. Then avidin conjugated FADGDH (1 U) was added to the solution and incubated for 20 min at 60°C. Unbound avidin conjugated FADGDH was removed by washing the electrode with the buffer [10 mM MOPS, 150 mM NaCl, 1 mM EDTA buffer (pH 7.0)] including 0.2% Tween20 for three times. Various concentrations of glucose solutions were added to the electrode and the response current was measured at 40°C at a potential of 0.1 V vs. Ag/AgCl in the measurement buffer [10 mM MOPS buffer (pH 7.0) containing 6 mM m-PMS].

3. Results and Discussion

3.1 Glucose dehydrogenase for labeling enzyme

To develop a highly sensitive detection system, the background signal should be decreased to reduce noise signals. Although we have already reported a DNA sensing system-using Pyrroloquinoline quinone dependent GDH (PQQGDH) that has high catalytic activity (ca. 5000 U mg⁻¹ protein), it binds to DNA nonspecifically via cationic amino acids on its surface resulting in high background signals (data not shown). Then we selected bacterial FADGDH, which has a lower pI value (pI = 6.0) than PQQGDH (pI = 10.5).

We reported the cloning and characterization of thermostable bacterial FADGDH derived from *Burkholderia cepacia*.⁹ Although FADGDH has a lower catalytic activity (ca. 95 U mg⁻¹ protein) than PQQGDH, FADGDH maintained a sufficient catalytic activity for labeling enzyme after conjugation with avidin (maintained 78% activity compared with intact FADGDH).

3.2 Amperometric SNP sensing

The SNP in PPAR-DNA was detected by the single base extension method using avidin conjugated FADGDH. First of all, target DNA was incubated with the electrode followed by reaction of single base extension and incubation with avidin conjugated FADGDH. However, it required several hours to measure SNPs. To shorten the sensing time, we tried to perform all steps simultaneously. High temperature would enhance the specificity of extension and decrease DNA mishybridization and nonspecific binding of avidin-FADGDH. Since FADGDH that derived from *B. cepacia* has thermo stability, FADGDH activity is not lost during incubation at 60°C. When all reagents were incubated simultaneously, we observed a small difference of response current between PPAR-SNP-DNA and PPAR-DNA (data not shown). This indicates that b-dCTP would bind to avidin conjugated FADGDH before extension of b-dCTP on the DNA probe.

To avoid competing of extension of b-dCTP against binding of b-dCTP to avidin-FADGDH, avidin-FADGDH was added after 10 min extension reaction by *Taq* DNA polymerase. Figure 2 shows

Figure 2. Amperometric detection of PPAR-SNP-DNA. 10⁻⁶ M PPAR-SNP-DNA or PPAR-DNA was added to hybridize with immobilized probe DNA. 1 U avidin-FADGDH was added and incubated with the electrode for 20 min after extension of b-dCTP by *Taq* DNA polymerase for 10 min. The operational potential: 100 mV vs. Ag/AgCl. Temperature for measurement: 40°C. Each bar represents Δ current (nA) in the presence of 30 mM glucose.

Figure 3. Calibration curve corresponding to changes of the electronic current upon detection of different concentrations of PPAR-SNP-DNA and PPAR-DNA. Black circle represents response current of PPAR-SNP-DNA. Open circle represents response current of PPAR-DNA. Glucose concentration is 30 mM. Error bars indicate s.d. (n = 4).

the results of SNP sensing. We observed a higher current in the presence of PPAR-SNP-DNA than in the presence of PPAR-DNA, control (40-mer randomized DNA) or without DNA. Since there was little difference between in the presence of PPAR-DNA and in the absence of DNA, little b-dCTP was reacted with PPAR-DNA.

Then we investigated the dose dependency of this system. Figure 3 shows the results of measurement of PPAR-SNP-DNA and PPAR-DNA [10⁻¹⁰–10⁻⁶ M (mol dm⁻³) DNA]. We observed the difference between PPAR-SNP-DNA and PPAR-DNA at 10⁻⁸ M concentration. This detection limit is sufficient to detect SNP combined with DNA amplification by polymerase chain reaction (PCR).

Although there are many reports on detecting SNPs, a single base extension method using DNA polymerase would discriminate SNPs more clearly than hybridization. Additionally, the activity of glucose dehydrogenase can be easily measured using a commercially available electrochemical glucose sensing system. It would be an attractive enzyme for developing a commercially available SNP biosensor. However, in this study, we observed a higher background signal in the absence of DNA although we used glucose dehydrogenase with a low pI value to avoid nonspecific interaction. In order to develop a more highly sensitive SNP

detection system without PCR amplification, optimization of the amount of immobilized probe DNA, blocking of electrode, selection of different GDH or improvement of GDH with protein engineering are required.

4. Conclusion

Since SNPs affect drug susceptibility and side effects, the development of a biosensor that can identify SNPs rapidly is required for selection of the right drug for the right person. We developed an amperometric SNPs detection system based on a single base extension method. We succeeded in discriminating SNP of PPAR γ_2 with 10^{-8} M DNA as the detection limit. Amperometric sensing systems are attractive for simple measurement with a miniaturized biosensing system. Although we need to optimize the detection system since the detection limit of this study was insufficient without PCR amplification, we were able to construct an electrochemical SNP detection system.

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