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DNA sensor: A novel electrochemical gene detection method using carbon electrode immobilized DNA probes

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The authors have developed a novel, rapid, convenient, and specific gene detection method, named the 'DNA sensor,' using a graphite electrode loaded with DNA probes. Synthesized oligonucleotide (5'-TGCAGTTCCGGTGGCTGATC-3') complementary to oncogene v-*myc* was employed for a model probe. The oligonucleotide was chemically adsorbed on a basal plane pyrolytic graphite (BPPG) electrode. The sensor was able to be applied to a hybridization reaction (40°C) in a linearized pVM623 solution carrying the *Pst* I fragment of v-*myc* (1.5 kbp).

After the hybridization reaction, the sensor was immersed into an acridine orange solution (1 μM) and washed with a phosphate buffer (pH 7.0). Acridine orange intercalated between base pairs of the formed double stranded DNAs on the electrode. The anodic peak potential of acridine orange that interacted with the DNAs on the electrode was measured. The positive shift of the peak potential increased in proportional to the pVM623 concentration in the hybridization reaction. 10^{-11} g/ml of pVM623 was able to be detected in the buffer solution using the sensor. This gene detection was completed within an hour.

INTRODUCTION

Recently, much attention to gene diagnoses based on nucleic acid hybridization using DNA probes have been paid for early and precise diagnoses of infectional diseases and so on. Various novel techniques have been developed to replace traditional methods using radioactive labelled probes.[1,2] Biotin,[3,4] digoxigenin,[5] and fluorescent dyes[6] became popular reagents for the non-radioactive labelling of nucleic acid probes in research uses. Furthermore, the polymerase chain reaction (PCR), which is a major advancement in molecular biology, has been widely used to detect gene deletions and point mutations.[7,8]

However, all these conventional methods need labelled probes and such a long time for detection as 10 h and complicated operations accompanying skilled techniques. In these methods, gene detection is performed at different places from the hybridization reaction. The authors considered that they could simplify the gene detection method if both the hybridization reaction and the gene detection could proceed at the same place. Intercalators and antitumor agents were intercalated between DNA base pairs in an approximately perpendicular position to the double-helix axis. Some of these molecules were electrochemically active. Bard and coworkers have recently reported voltammetric studies on the interaction of intercalators with DNAs in solution.[9–11] They used electrochemically active substances as probes for the determination of DNA structures and DNA sequences. Generally, an electrochemical reaction is one of the easiest ways to analyze various molecules. Both enzyme sensors[12] and immunosensors[13,14] have been investigated for the determination of biological substances. These sensors performed both the reaction and detection on the surface of the electrode.

The authors have devised a rapid, easy, and non-radioactive gene detection method based on the electrochemical reaction of acridine orange intercalated into specific hybrids formed on oligonucleotide probes immobilized on an electrode. A graphite electrode has a good adsorptive nature that a large quantity of molecules is immobilized on the electrode with easy handling.[15] So, the authors selected a basal plane pyrolytic graphite (BPPG) electrode for the immobilization of oligonucleotide probes. The electrochemical signals of acridine orange intercalated did not depend on the targeted gene sequences because the sequence was independent of the intercalation of acridine orange to DNAs. The authors were able to detect targeted genes using acridine orange for an electrochemical probe without troublesome labelling of the oligonucleotide probes. This paper reports the

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results of confirming the principle of the DNA sensor by detecting an oncogene as a model gene.

EXPERIMENTAL SECTION

Materials

Acridine orange was obtained from Molecular Probe Inc., and reagents for oligonucleotide syntheses were all purchased from Applied Biosystems. Other reagents were commercially available or laboratory grade ones. The solutions were prepared with high-purity water (ρ = 18 MΩcm).

Procedure

The authors employed an electrochemical analyzer manufactured by Bioanalytical Systems (Model BAS-100B) with a data storage and a TOSHIBA J-3100 computer system for electrochemical analyses. All voltammetric experiments were performed in double-compartment cells of 500 ml at 25°C [supporting electrolyte: 1/15 M sodium phosphate buffer (pH 7.0), reference electrode: Ag/AgCl (Toa Electronics, Ltd.), counter electrode: Pt flag (4.9 cm^2)].

Differential pulse voltammetry (DPV), linear sweep voltammetry (LSV), and cyclic voltammetry (CV) were examined under the following parameters; (DPV): pulse amplitude = 200 mV, pulse width = 80 mV, sampling width = 100 mV, sweep rate = 5 mV/s, pulse period = 2s, (LSV): sweep rate = 25 mV/s, and (CV): sweep rate = 100 mV/s.

DNA preparation

A model DNA for the detection (plasmid pVM623) was prepared by inserting a 1.5-kilobase pair (kb) *Pst* I fragment of oncogene v-*myc* (Takara) into the vector pUC119. The plasmid DNA was prepared by a conventional method[16] and purified with a Biogel A-25 (Bio-Rad Labs) column (ϕ5 × 200 mm). The plasmid pVM623 was linearized with endonuclease *Hin* dIII. The oligonucleotide (5'-TGCAGTTCCGG-TGGCTGATC-3') complementary to the 3' end of v-*myc*[17] was synthesized with a DNA synthesizer (Applied Biosystems; Model 391 PCR-MATEEP), and purified with NAP columns (Pharmacia). The concentrated stock solutions were stored at 4°C.

Immobilization of oligonucleotide probes on the electrode

A basal plane pyrolytic graphite (BPPG, Tomoe Kogyo) electrode with a geometric area of 0.20 cm^2 was employed for the immobilization of oligonucleotide probes. The BPPG electrode was sequentially polished before experiments with 6 μm and 1 μm diamond pastes, and then with 0.05 μm alumina (Buehler) on a polishing pad. The electrode was subjected to ultrasonic cleaning in 95% ethanol and then in distilled water for ca. 1 min.

The synthesized oligonucleotide probes were adsorbed on polished BPPG electrodes in a solution [10 μg/ml in 1 mM Tris-HCl (pH 8.5)] containing 100 mM NaCl for 30 min at 100°C. Then, the electrode was washed with distilled water at 100°C to remove not-adsorbed probes. The quantity of adsorbed probes was estimated by measuring the irreversible anodic peak current derived from guanine residues configurating the DNAs. The electrode was dried in an oven at 100°C and dipped into a stearylamine solution (1 mM in ethanol) for 30 min at 50°C to prevent non-specific adsorption of acridine orange and sample DNAs on the electrode. The DNA sensor was stored in a TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] at 4°C before use.

Sensing procedure of the DNA sensor

The DNA sensor was immersed into a 2 × SSC [SSC: 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0)] solution containing heat denatured pVM623. The hybridization reaction was carried out for an hour at 40°C with shaking. After the reaction, the electrode was washed with distilled water to remove non-specifically bound DNAs on the electrode.

After the electrochemical oxidation of the hybrid DNAs on the electrode, the sensor was immersed into an acridine orange solution [1 μM in 1 mM Tris-HCl (pH 8.5)] containing 1 M sodium chloride in a plastic vessel for 5 min at room temperature under a dark condition, and then washed with a 1/15 M phosphate buffer (pH 7.0) for 5 min. The electrochemical signals of acridine orange intercalated into the hybrids formed on the sensor were measured. DPV was performed to determine the concentration of pVM623.

RESULTS

Immobilization of oligonucleotides on the electrode

Purine residues of DNAs both guanine and adenine were electrochemically oxidized at the graphite electrode.[18-22] So, the authors confirmed the immobilization of oligonucleotides on the electrode with a voltammetric method. The adsorbed oligonucleotides showed two clear and irreversible anodic waves by only one scan at ca. 1.0 V and 1.4 V (vs. Ag/AgCl) (Fig 1). These anodic waves were derived from guanine and adenine residues, respectively.

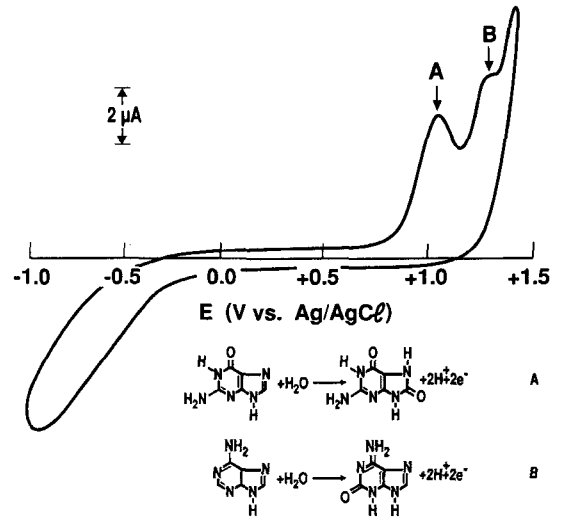

Figure 1 Cyclic voltammograms of oligonucleotide probes adsorbed on the BPPG electrode. Sweep rate; 100 mV/s. Inset: Electrochemical oxidation of (A) guanine and (B) adenine on the graphite electrode.[18,19]

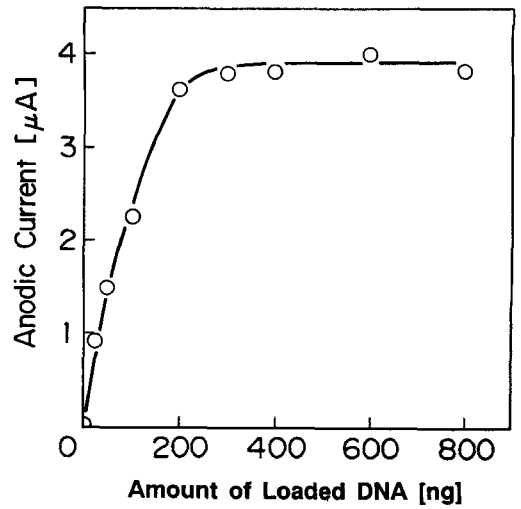

Figure 2 Correspondence between anodic current and loaded oligonucleotide probes on BPPG electrode. Geometric area of the electrode; 0.20 cm^2.

Then, a given quantity of oligonucleotides (0 to 800 ng) was loaded on the surface of the electrode, and dried at 100°C in an oven. Voltammetric experiments were examined after washing the electrode with distilled water at 100°C to remove excess loaded probes. The anodic peak current derived from guanine residues of the immobilized oligonucleotides was proportional to the loaded quantity of oligonucleotides on the electrode ranging from 0 to 200 ng (Fig 2), and the peak current reached a plateau over 200 ng of loaded oligonucleotides. The authors suggest that the quantity of oligonucleotides adsorbed on the electrode can be estimated by measuring the anodic peak currents, and about 200 ng of oligonucleotides were adsorbed on the BPPG electrode (ϕ0.5 mm) in saturation.

The authors determined the conditions so that a larger quantity of oligonucleotides will be adsorbed on the BPPG electrode. The quantity of oligonucleotides on the electrode would influence the efficiency in the hybridization reaction. The adsorbed oligonucleotides increased by increasing the concentration of sodium chloride in the range from 0 to 100 mM (Fig. 3). Generally, a polymer DNA is condensed under high ionic strength conditions (ion condensation theory of polyelectrolytes).[23] The 20-mer oligonucleotides were considered to behave like a polymer, and the occupied area of one molecule on the surface of the electrode decreased in a high ionic strength condition. Furthermore, the adsorbed oligonucleotides increased with temperature (Fig 4). These results suggest that the oligonucleotides would be strongly adsorbed on the electrode through chemisorption. The adsorption state of the oligo-nucleotides on the

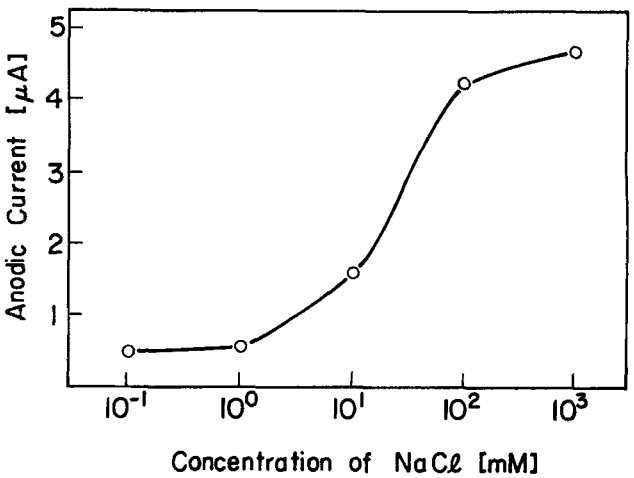

Figure 3 Effect of salt concentration on oligonucleotide adsorption.

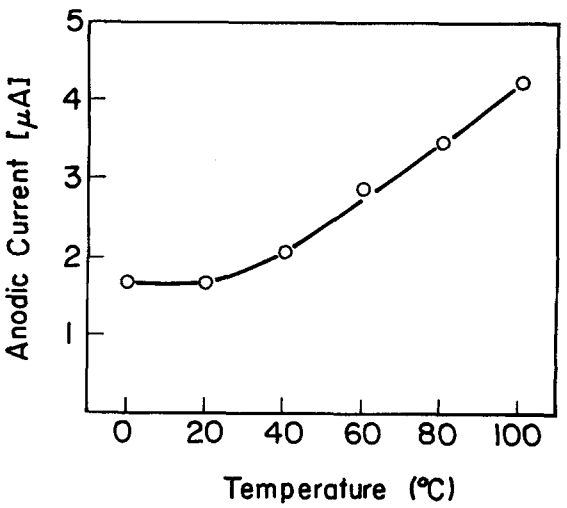

Figure 4 Effect of temperature on oligonucleotide adsorption.

electrode was so stable that desorption was not observed neither when heating up to 100°C nor applying a negative potential (−500 mV) to the electrode were performed. The authors employed this DNA sensor for specific gene detection.

Gene detection by the DNA sensor

When the DNA sensor reacted with pVM623 (1 μg/ml), a newly obtained and clear anodic wave of guanine residues derived from the hybridized DNAs was observed at ca. 1.0 V (Fig 5). In contrast, a smaller anodic wave was observed when the DNA sensor reacted with pUC118. Plasmid pUC118 did not have a complementary region to the oligonucleotide probe, so it was considered that the small wave was derived from pUC118 bound non-specifically to the DNA sensor. The selectivity of the DNA sensor was depended on the oligonucleotide probe sequence to targeted genes. The optimization of the hybridization conditions (temperature, ion strength and some other parameters) will result in the high selectivity of the DNA sensor.

Acridine orange revealed a clear and irreversible anodic wave [peak potential; 724 mV (DPV)] in the phosphate buffer (pH 7.0). The sensor reacting with pVM623 was immersed into the acridine orange solution, and washed with the phosphate buffer. Voltammetric experiments (DPV) showed that the electric capacity derived from acridine orange increased and the anodic peak potential shifted to more positive values (ca. 40 mV) when the DNA sensor was reacted with pVM623 (1 μg/ml), compared with the data in a solution without the targeted DNA pVM623 (Fig 6). The average anodic peak potentials were 727 mV (with pVM623) and 695 mV (without pVM623), respectively. The positive values of the anodic peak potential shift was related to the concentration of the targeted DNA pVM623 in hybridization reactions ranging from 10^{-11} to 10^{-5} g/ml (Fig 7).

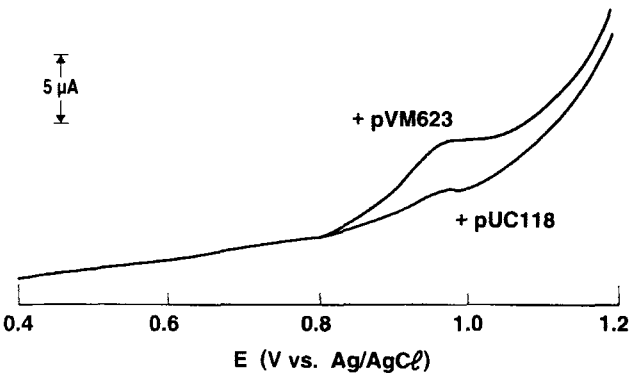

Figure 5 Linear sweep voltammograms of oligonucleotide probe adsorbed BPPG electrode when reacted with 1 μg/ml of pVM623 and 1 μg/ml of pUC118. Sweep rate; 25 mV/s. Hybridization condition; in 2 × SSC buffer, 1 h, 40°C.

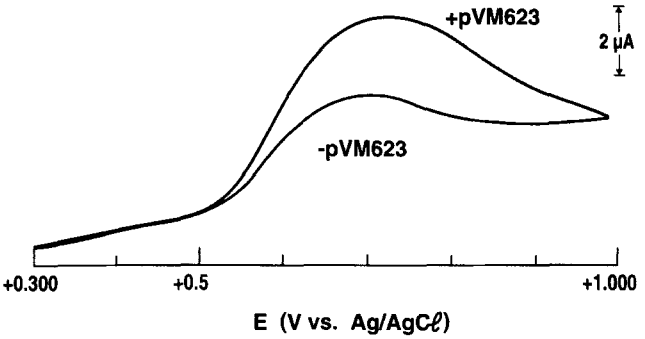

Figure 6 Differential pulse voltammograms of acridine orange on oligonucleotide probe-adsorbed BPPG electrode reacted with 1 μg/ml of pVM623 and without pVM623. Sweep rate; 5 mV/s. Acridine orange concentration; 1 μM.

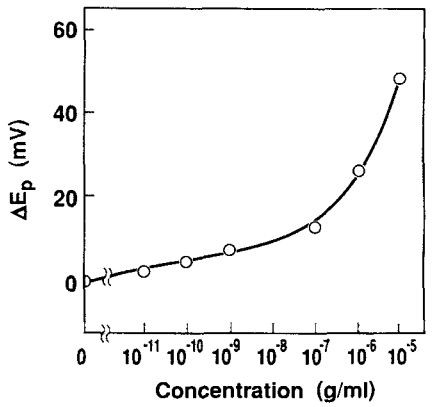

Figure 7 Calibration curve for targeted DNA pVM623. Hybridization condition; in 2 × SSC buffer, 1 h, 40°C.

DISCUSSION

The adsorption state of oligonucleotides on the electrode will dominate the efficiency in the hybridization reaction. The authors demonstrated by a voltammetric method that the oligonucleotide probes adsorbed on the electrode could be hybridized with complementary DNAs under normal hybridization conditions (previous data). The authors hardly know the details of the oligonucleotide adsorption on the electrode surface yet. Berg reported that the adsorption depended on the conformation of nucleic acids in solution, which was a function of electrode potential, ion strength, pH, temperature and some other unknown parameters.[22] Bravec presented the different adsorption states of double stranded DNA (dsDNA) and single stranded DNA (ssDNA) on the graphite electrode.[21] However, this phenomenon has not been analyzed in details. We suggest that the DNA sensor can be prepared if only the adsorption conditions will be controlled. These are most important problems in the DNA sensor for

Table 1 Effect of lipids for preventing adsorption of acridine orange on BPPG electrode

Lipid[a]	*Response (%)*[b]
PEDP[c]	59
n-decylamine	65
1,10-diaminodecane	41
Cetylamine	16
Stearylamine	12
Aminononadecane	12
n-hexadecane	90
Stearic acid	95

[a] The electrode was dipped into the lipid solution (1 mM in ethanol), dried, and immersed into the acridine orange solution (1 μM). Voltammetric experiments were examined after washing with a phosphate buffer.
[b] The ratios indicate the percentages of the anodic current of acridine orange using a coated electrode compared with that of a non-treated electrode.
[c] PEDP; Phosphatidylethanolamine, dipalmitoyl.

detecting genes specifically. The authors will continue to make these problems clear.

The authors realized the direct adsorption of acridine orange on the electrode surface and the interactions of acridine orange with single stranded ssDNAs on the DNA sensor in the gene detection experiments of the DNA sensor using acridine orange. Acridine orange is considered to be adsorbed on the graphite electrode through physical adsorption. The authors tried to prevent the former by coating the oligonucleotides-adsorbed electrode with lipid. The screening of several lipids showed that stearylamine was effective for coating the electrodes (Table 1). Stearylamine has a cationic functional group ($-NH_2$) and a long hydrocarbon chain. The electrode coated with stearylamine prevents the adsorption of acridine orange by the effect of repulsive force. The long hydrocarbon chain is considered to contribute to the stability of the molecule on the electrode because of hydrophobic interactions between the lipid and the electrode.

The binding of acridine orange to nucleic acids is related not only to the intercalation into the hydrophobic state of but also to the electrostatic interaction with phosphate groups of the DNA backbone. So this dye is bound to ssDNAs as well as dsDNAs. The electrostatic interactions can be suppressed by changing the conditions of the ion concentration and pH. A high salt condition showed a decrease in the binding constant for a non-intercalated bound dye.[24] The isoelectric points of DNA and acridine orange were about 2 and 10, respectively. For conditions under pH 3 or over pH 10, dsDNAs dissociate into ssDNAs in a solution. The authors examined what specific interaction occurred between acridine orange and dsDNAs, and chose one molar of sodium chloride and pH 8.5 for the experiments (data not shown).

Bard and coworkers reported a voltammetric study on the interaction of an intercalator with DNAs.[9] Tris(1,10-phenanthroline)cobalt complex[$Co(phen)_3^{3+}$] was interacted with DNA through hydrophobic interactions with the interior of the DNA molecules (intercalation). The redox potentials of $Co(phen)_3^{3+}$ mixed with DNAs shifted to more positive values compared with those of the $Co(phen)_3^{3+}$ solution. On the other hand, those of tris(2,2'-bipyridine)cobalt complex [$Co(bpy)_3^{3+}$] shifted to more negative potentials for a solution without DNAs. $Co(bpy)_3^{3+}$ interacted with DNAs through electrostatic interactions which involved an outer anionic coat along the DNA backbone. They showed change in the volammetric behavior of the intercalators, and positive shifts of the peak potential were observed in the case of the binding form via hydrophobic interactions. In the authors' cases, the peak potential of acridine orange bound to oligonucleotide probes on the DNA sensor shifted to more negative values compared with the data on the acridine orange solution (724 → 695 mV). Acridine orange was bound to ssDNAs through electrostatic interactions. On the other hand, the peak potential of acridine orange using the DNA sensor reacted with pVM623 shifted to more positive values compared with that in a solution (724 → 727 mV). The authors' results suggest that specific hybrids are formed on the DNA sensor and acridine orange was intercalated between these base pairs. The quantity of the formed dsDNAs on the electrode is considered to affect the positive shift values of the peak potential of acridine orange. The authors were able to determine specifically the concentration of targeted genes by measuring the positive values of the anodic potential shift derived from the acridine orange intercalated into the formed dsDNAs on the DNA sensor. The peak potential shift caused by the intercalation of acridine orange was not much in the low concentrations of the targeted DNA pVM623 on the DNA sensor, because fewer hybrids formed were less acridine orange bound to DNAs. We considered that a specific intercalater, which would bind to dsDNA more selective than acridine orange, would make electrochemical signals much clearer. The authors have been screening better intercalators, and have not optimized the experimental conditions in the DPV measurements. These improvements will lead the DNA sensor to the more sensitive detection and the practical applications.

These results imply that the DNA sensor based on the electrochemical reaction of the intercalator with an oligonucleotide probe adsorbed electrode can be an additional quick and convenient method for determining genes specifically. A successful development of this method for detecting genes may markedly facilitate the understandings of human pathological conditions.

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