Electrochemical Analysis of Nucleic Acids with a Ferrocenylated Oligonucleotide or Intercalator

Yoshihiro Uto

Department of Biochemical Engineering and Science
Faculty of Computer Science and Systems Engineering
Kyushu Institute of Technology

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CONTENTS

Abbreviations

CV	cyclic voltammetry
DMD	Duchenne muscular dystrophy
DMSO	dimethylsulfoxide
DPV	differential pulse voltammetry
DTT	dithiothreitol
ECD	electrochemical detection
EDTA	ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography
IR	infrared
MES	morpholinoethanesulfonate
NMR	nuclear magnetic resonance
OD	optical density
PCR	polymerase chain reaction
POD	horse radish peroxidase
Rf	ratio of flow
RFLP	restriction fragment length polymorphism
SDS	sodium dodecyl sulfate
SHE	standard hydrogen electrode
S/N	signal to noise ratio
dNTPs	deoxyribonucleoside triphosphates
TEAA	triethylammonium acetate

TLC	thin layer chromatography
T_m	melting temperature
UV	ultraviolet

Publication List

Takenaka, S., Uto, Y., Kondo, H., Ihara, T. and Takagi, M. (1994) Electrochemically active DNA probes: Detection of target DNA sequences at femtomole level by high-performance liquid chromatography with electrochemical detection. *Anal. Biochem.*, **218**, 436-443.

Ihara, T., Maruo, Y., Uto, Y., Takenaka, S. and Takagi, M. (1995) DNA ligand - redox active molecule conjugates as an electrochemical DNA probe. *Anal. Sci. Tech.*, **8**, 887-894.

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Chapter 1. Introduction

The advent of gene manipulation technology brought about a drastic alteration in medical sciences in the following four major categories: (A) Research on gene structure and function, (B) production of useful materials such as biologically active peptides, (C) gene diagnosis and (D) gene therapy. (A) is the most basic one and research on genes for eukaryotes including humans and on their expression regulation is now progressing rapidly. The knowledge acquired is applied for understanding and analyzing a specific gene concerned with a disease. (B) is utilized for the production of various peptide hormones, lymphokines or cytokines, various bioactive peptides and vaccine antigens. (C) and (D) are medical applications of (A) and are expected to develop in the future.

Gene diagnosis or DNA (deoxyribonucleic acid) diagnosis refers to analysis of a gene or DNA. As soon as a given human gene is cloned, it becomes easy to detect a change, if any, in that gene with the former as a probe. This can be done roughly by comparing restriction enzyme fragments of the reference and sample DNA. But in order for this method to be successful, some restriction enzyme sites must be present in the gene in question. A fortunate situation like this does not happen frequently, however. Hence, one has to resort to a restriction fragment length

polymorphism (RFLP) for this purpose. The principle of RFLP lies in the use of a very small difference in individual genomes except sites of important function such as the protein coding regions and gene regulatory regions. This difference is called polymorphism and can trace transmission of polymorphic genes through generations in a family.

An alternative gene diagnosis method is the DNA probe method. This method is based on the fact that double stranded DNA with a mismatch in its base sequence dissociates at lower temperatures than those with no mismatch. When a probe DNA is designed properly, it cannot form a stable duplex with DNA with one base pair mismatch under certain conditions, so it can detect a point mutation directly. The DNA probe method described above consists of a nucleic acid hybridization technique that detects and quantitates a specific gene and its transcription product. This principle is based on the fact that complementary nucleic bases bind with each other to form a duplex. The nucleic acid used for detection of a target sequence is called a nucleic acid probe or a DNA probe.

The principle of nucleic acid hybridization is explained briefly below. DNA has a duplex structure with two polynucleotide chains intertwined with each other. Each polynucleotide chain consists of deoxyribose, phosphate backbone and one of four nucleotide bases

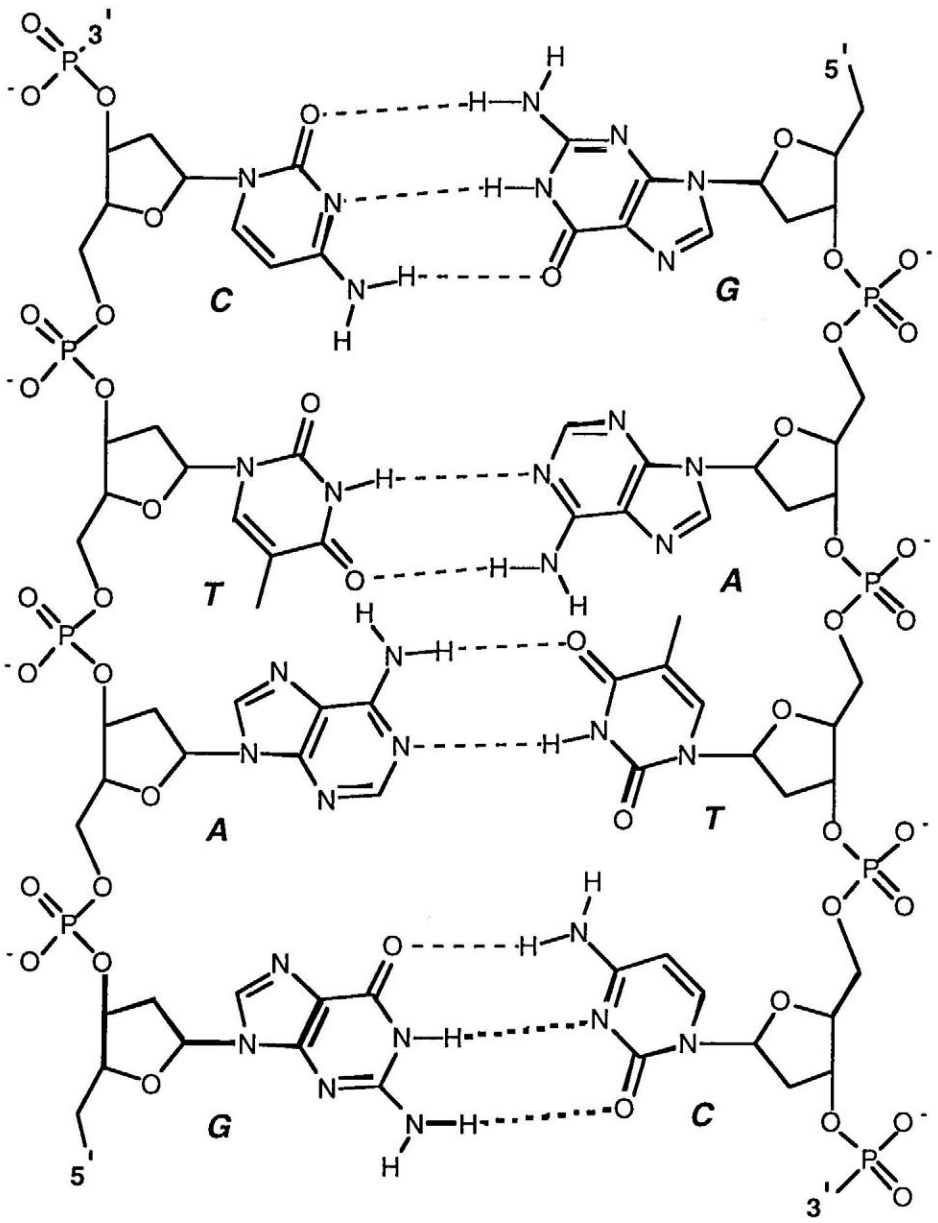


Fig. 1. The chemical structure of deoxyribonucleic acid (DNA). The symbols used in the figure represent the following nucleic bases: **A**, adenine; **G**, guanine; **T**, thymine; **C**, cytosine.

(adenine, guanine, thymine and cytosine) (Fig. 1). The two polynucleotide chains recognize each other to form pairs of adenine/thymine and guanine/cytosine through hydrogen bonds. This duplex structure is dissociated into two single stranded chains by heating (thermal denaturation), alkali (alkaline denaturation) or low ionic strength in the solution. But since DNA is most stable in the double stranded form, the two single stranded chains so generated tend to form spontaneously a duplex again if conditions such as temperature, ionic strength and pH are adjusted appropriately. This phenomenon is called renaturation or annealing. The renaturation is very specific and occurs only when the two polynucleotide chains are complementary with each other. Nucleic acid hybridization is based on this complementary nature of DNA. When exogenous polynucleotide chains (nucleic acid probe) are added to anneal with the denatured DNA they recognize their complementary sequence of denatured DNA and form a stable hybrid double stranded DNA (Fig. 2).

The advantage of this hybridization method with a nucleic acid probe is the excellence in sensitivity and specificity. In the Southern hybridization technique, oligonucleotide probes less than 20 bases in length can detect a single copy gene in the human genome (1). In the detection with nucleic acid hybridization, affinity is of utmost importance. In fact, of all the biological macromolecular interactions the hybridization of complementary

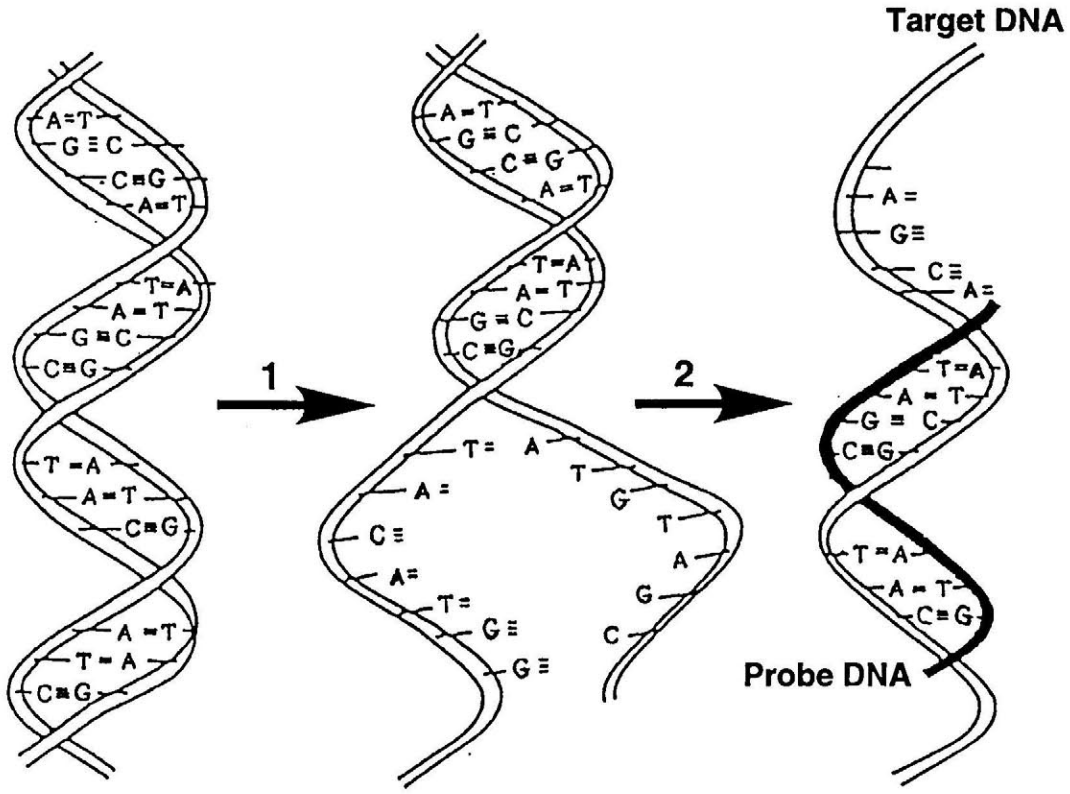


Fig. 2. The principle of DNA hybridization. The target double strand DNA is denatured by heating (1) and allowed to hybridize with the target-specific probe DNA (2).

polynucleotide chains is the strongest. Thus, the dissociation constant of a double stranded DNA, for instance, human β-globin gene fragment (272 base pairs) was estimated from a statistic physical theory to be nearly 10^{-23} M at the temperature 5 °C below the melting temperature (T_m), where double stranded and single stranded DNA are present in equal quantities. This dissociation constant is much smaller than those of typical protein-protein and protein-ligand non-covalent interactions: antigen-antibody reactions, 10^{-5} - 10^{-9} M; a repressor dimer and λO_R operator sequence, 3.0×10^{-9} M (2); tryptophan synthase subunits, 2.5×10^{-14} M, avidin and biotin, 1.0×10^{-15} M; trypsin and trypsin inhibitor, 1.0×10^{-9} M (3).

The specificity of a nucleic acid probe is also excellent. For example, theoretical calculation suggests that the dissociation constant of double stranded DNA of β-globin gene fragment (272 bp) is increased about 40-fold upon introduction of every one base mismatch. Accordingly in the presence of ten non-complementary bases, it is expected that the dissociation constant at the temperature 5 °C below the T_m increases from 10^{-23} to 10^{-7} M. If these ten mismatches occur at random in the 272 bp sequence, a genome can hybridize with very few probability, and hybridization between the sample DNA chain and the probe on a filter membrane would not be observed. This theoretical consideration is supported by experiments. In an extreme example, precise discrimination of

one base replacement was realized by such people as Wallace (4), Myers and Maniatis (5), and Lerman (6).

Virtually all of the practical hybridization reactions are carried out on a filter membrane on which denatured DNA is fixed and allowed to react with the probe DNA. The reason for this is the following. When a hybridization reaction is carried out actually, two antagonized reactions compete. The probe DNA binds with the sample DNA but it is also possible for the sample DNA to reanneal with each other. It is clear that in these circumstances the probe DNA hybridizes with the target DNA with more difficulty. Hence the denatured DNA needs to be fixed on a membrane filter and renaturation of sample DNA is prevented, whereas the probe DNA can gain free access to the sample DNA fixed on the filter membrane to react with it.

For analysis or diagnosis of genes the nucleic acid hybridization involves the following major steps. (a) The sample DNA is prepared, (b) a specific nucleic acid probe is prepared, (c) the probe is labeled, (d) the sample and probe DNA are allowed to hybridize and (e) the hybrid is detected. Of all these steps the labeling and detection dictate the sensitivity of the system and to attain maximum sensitivity radio-isotopes have often been used. However, there are some serious problems in the use of radio-isotopes in terms of safety, economy and necessity of a special facility. Recently, non radio-isotopic methods were developed

which rely on a bio-probe, photo-biotin, chemiprobe, blugene, labezyme-POD or enhanced chemiluminescence. Although these methods work well usually, there is a demand for a method which surpasses them in sensitivity.

That is why development of a novel non radio-isotopic gene detection system by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) (Fig. 3) was undertaken in this thesis (7). The ECD method is similar to polarography and voltammetry in that it detects an electric current change on the electrode and then quantitates the target material when electrochemically active material is oxidized or reduced on the electrode surface. This method was advocated by Kissinger for the sensitive analysis of catecholamines (8), and in fact is a very good method in terms of sensitivity and linearity of the calibration curve. It can detect as small as picograms or even less of samples and the ECD response is linear over a range of nearly five orders of magnitude. But this method has never been applied to DNA analysis except for DNA hydrolysates (9), because DNA has a very small electrochemical activity in an aqueous solution. Takenaka *et al.* reported previously that groove-binding viologen was useful as an electochemical probe of DNA (10), but they could not obtain high enough sensitivity because the reduction of viologen takes place at cathodic potentials, where reduction of dioxygen competes and

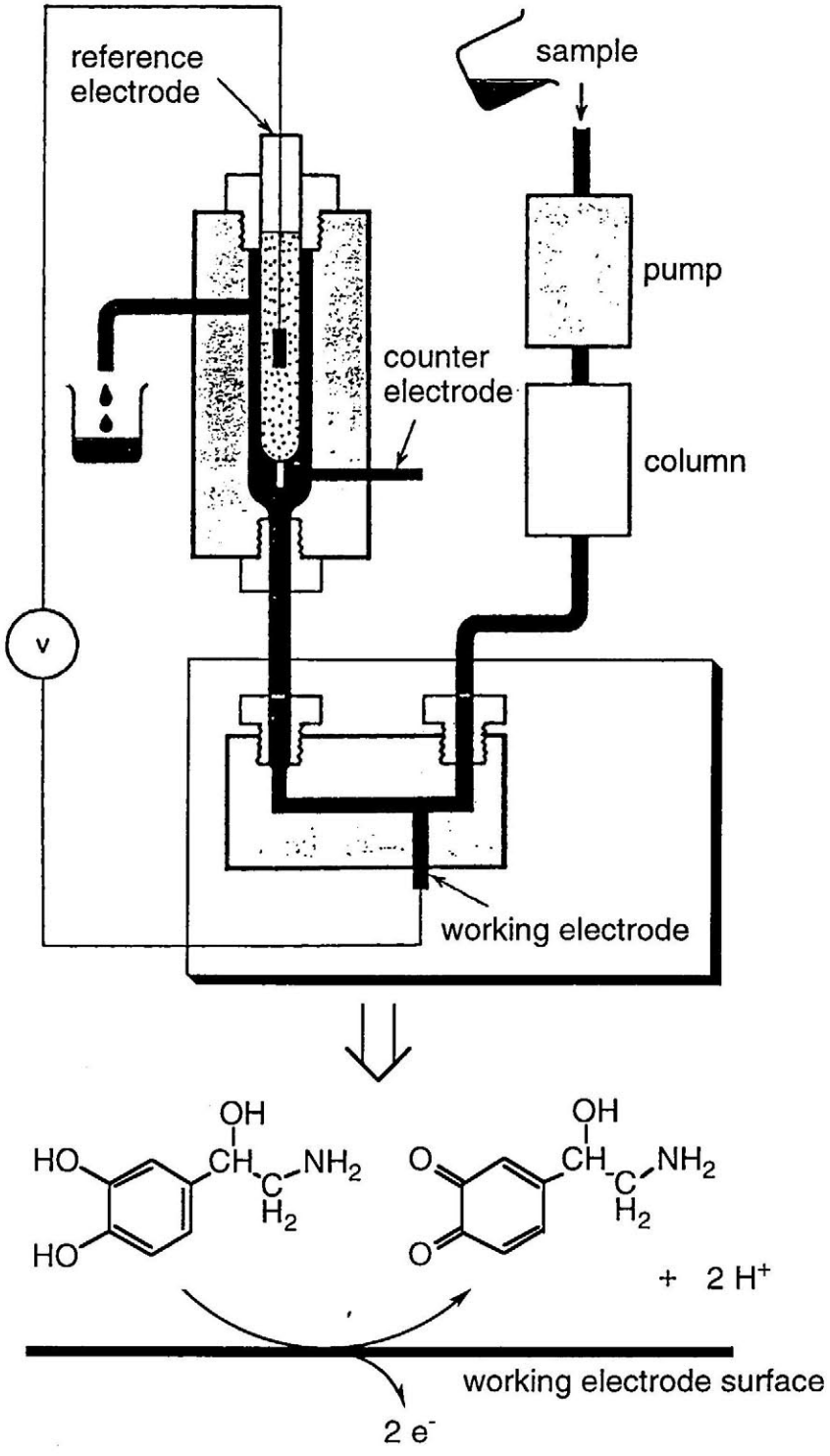


Fig. 3. The principle of an electrochemical detection (ECD). In this figure the electrochemical reaction of catecholamine (norepinephrine) on the electrode surface (+ 0.8 V vs. Ag/AgCl) is exemplified.

obscures the selective detection (Fig. 4). To circumvent this problem ferrocene is employed here instead of viologen as an electrochemical probe. Since ferrocene has an oxidation/reduction potential at 0.50 V vs. SHE (standard hydrogen electrode) (11), analysis can be carried out at low anodic potentials and the analytical sensitivity must be high (Fig. 4). If ferrocene is covalently bound to DNA, the resulting molecule may serve as an electrochemical probe suitable for analysis by the HPLC-ECD method. This notion is tested in Chapter 2.

To improve further the sensitivity of DNA analysis, polymerase chain reaction (PCR) was coupled with the HPLC-ECD method (Fig. 5) (12). PCR is a means of in vitro gene amplification which proceeds between a certain region of a DNA sequence specified by two oligonucleotide primers (13 - 14). The PCR technique has been currently used in many fields of molecular biology for direct DNA sequencing, genomic cloning, DNA typing, site-directed mutagenesis and so on (13, 15 - 17). Since each step of PCR can double the target DNA fragment, almost 10^6-fold DNA amplification can theoretically be achieved in 20 cycles of PCR. Using the correlation between the amount of the amplified DNA fragment and PCR cycle number, one can estimate the initial amount of the target gene in the sample which is equal to the amount of the target gene at zero cycle (quantitative PCR method) (18 - 21). In this method, the separation and the quantitation of

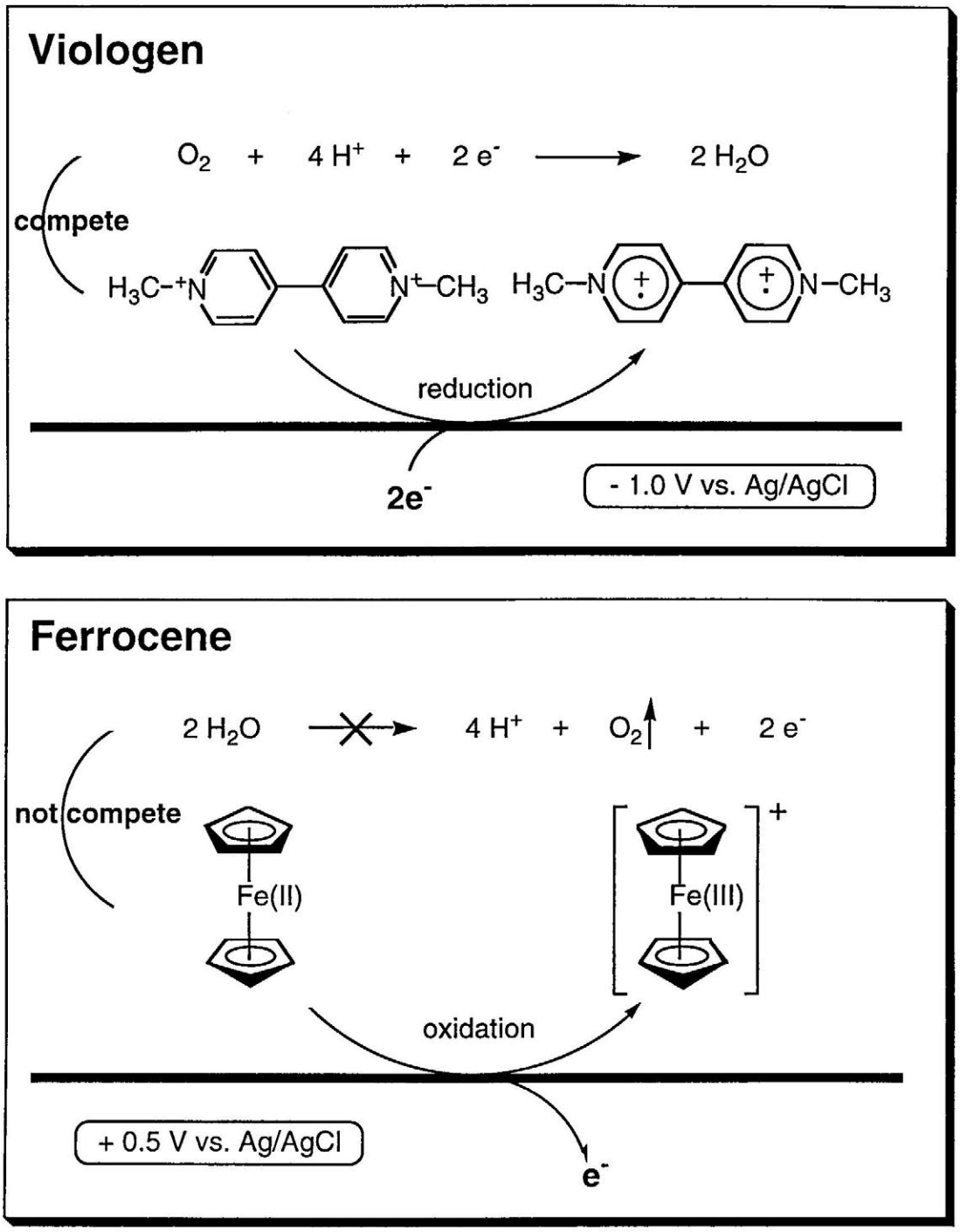


Fig. 4. Difference in the electrochemical properties of viologen (upper) and ferrocene (lower). The reduction of viologen takes place at cathodic potentials, where reduction of dioxygen competes to hamper selective detection. On the other hand, the oxidation of ferrocene takes place at anodic potentials so that it is free from any other electrochemical reactions.

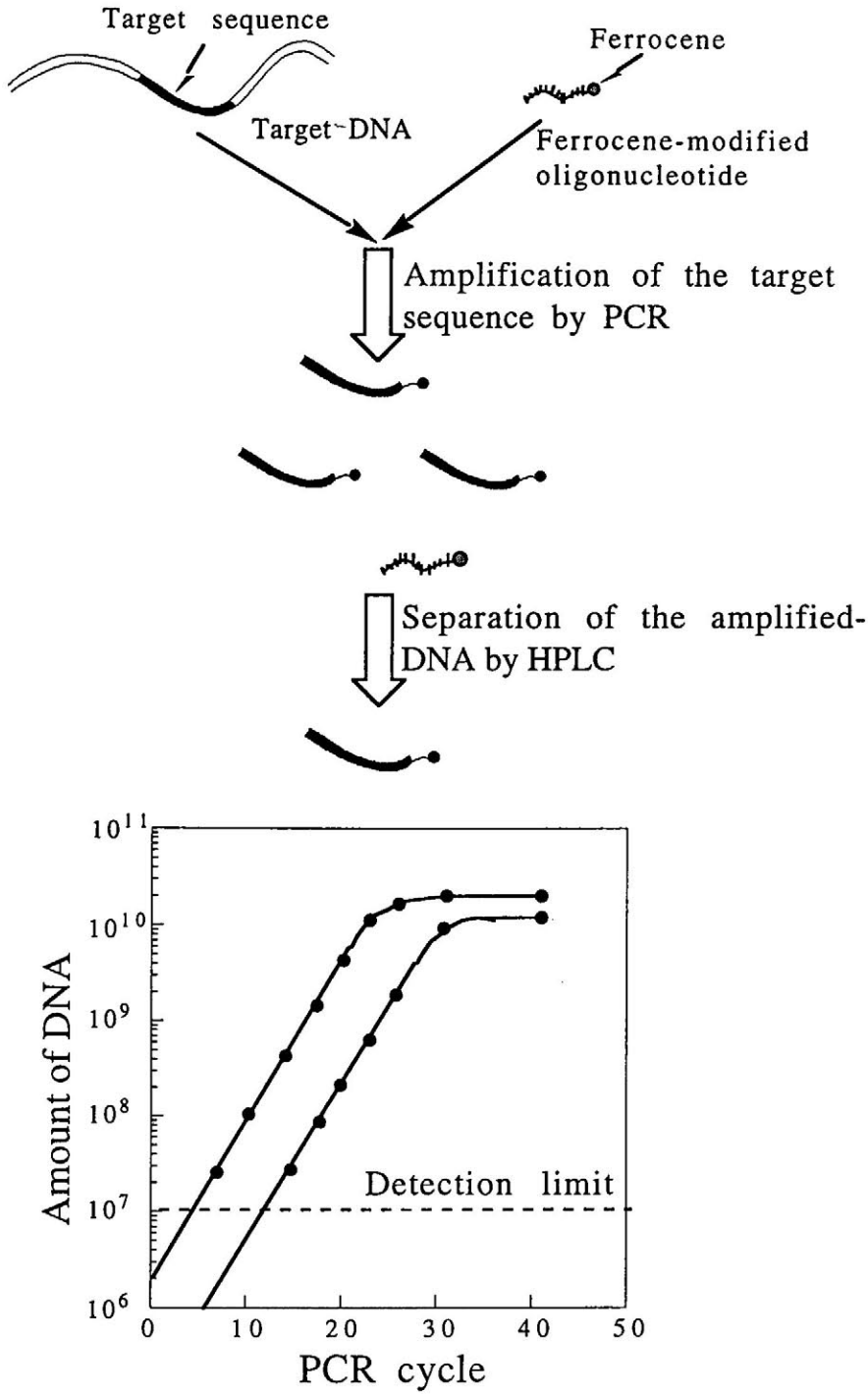
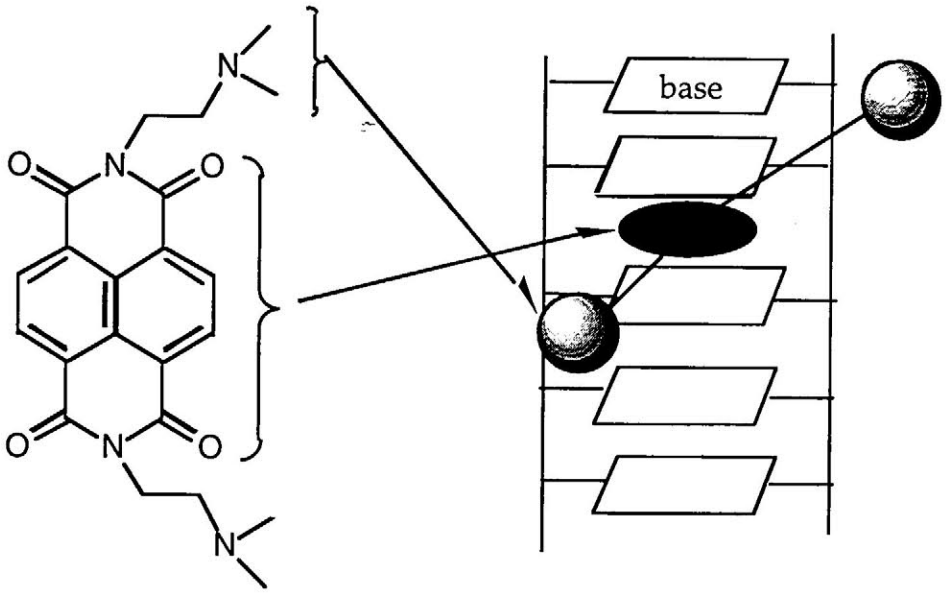


Fig. 5. The principle of a gene detection system relying on a combination of the polymerase chain reaction (PCR) and the HPLC-ECD method. The initial amount of DNA can be estimated at n = 0 (n is the number of PCR cycles) by extrapolation of the dependence of the amounts of the amplified DNA on PCR cycles.

PCR products are usually performed by gel electrophoresis (13 - 21), which is time-consuming and difficult to automate. To overcome these drawbacks of gel electrophoresis, a DNA probe method was developed by using ferrocenylated oligonucleotides. It is expected that the HPLC-ECD will facilitate separation and quantitation of the target gene hybridized with the ferrocenylated DNA probe with high sensitivity. Ferrocenylated oligonucleotides are used as a PCR primer, which should give PCR products incorporating the ferrocenyl substituent at the 5'-terminal position. This will allow rapid analysis and quantitation of the target gene by following the procedures described above by the HPLC-ECD system. The results of these experiments are described in Chapter 3.

Finally a novel DNA sensor was developed in Chapter 4 based on a ferrocenylated hybrid-specific ligand. Until now, DNA sensors suffer low sensitivity for hybrids because of the lack of a good double stranded DNA specific ligand (22, 23). To solve this problem, a threading intercalator was used as a hybrid-specific ligand (24, 25). One of the two substituents of this compound protrudes in the major groove of DNA, while the other in the minor groove when it is intercalated into DNA (Fig. 6). In order for the ligand to assume this conformation one of the substituents needs to thread the DNA double strand and upon dissociation of the ligand from DNA these substituents serve as a stopper. For this reason this compound undergoes dissociation extremely slowly and has a



Naphthalene Diimide

Complex of a threading intercalator with double strand DNA

Fig. 6. The binding complex of a threading intercalator (naphthalene diimide) with double stranded DNA.

specificity for double stranded DNA (24, 25). In this research naphthalene diimide was chosen as a threading intercalator (24-26) and the one carrying electrochemically active ligands was prepared. The detection procedure of this DNA sensor is shown in Fig. 7. When the target DNA exists in the sample solution, it can form a complex with the probe DNA fixed on an electrode. The ferrocenylated naphthalene diimide binds with the complex specifically, so it is concentrated near the electrode surface. This is why the target DNA is detected with high sensitivity.

Based on the results described in Chapters 2-4, the usefulness of this novel DNA analysis system is discussed in Chapter 5. In

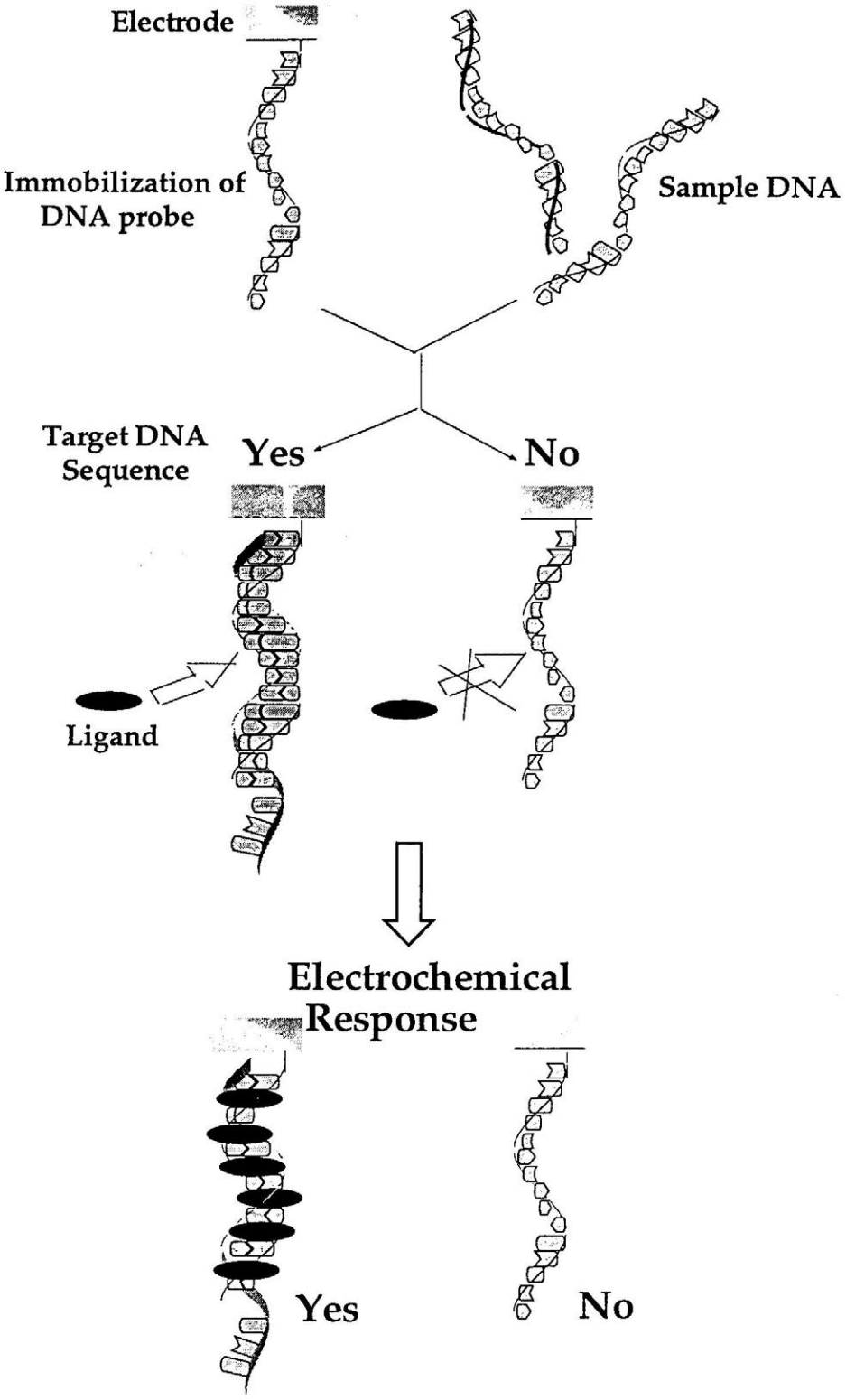


Fig. 7. The principle of a novel DNA sensor system based on a double stranded DNA-selective ligand.

addition, some subjects which remain to be overcome are discussed briefly.

Chapter 2. Detection of target sequences of DNA and RNA by an HPLC-ECD system

2-1. Introduction

Detection of specific DNA and RNA sequences is required in many aspects of nucleic acid chemistry and gene manipulation. The most commonly used assay takes advantage of hybridization of a probe DNA with a target DNA having a complementary sequence. The probe DNA is often radio-labeled to ease analysis (27). Although this method gives one of the highest analytical sensitivity, it suffers a serious drawback of using potentially hazardous radio-isotopes. Non-radioisotopic methods are also widely used that rely on fluorescence, bioluminescence and chemiluminescence for detection (28). In these methods sample DNAs are usually fixed on a membrane and allowed to hybridize with the probe (27, 28). As this hybridization is a solution-solid reaction, considerable time is required to complete the hybrid formation. Hence, an alternative method is needed which is both quick and does not rely on radio-isotopes.

Electrochemical methods appeared to be a promising alternative; they are not only quick to run but highly sensitive (29). In fact, they are routinely used for the analysis of biomaterials such as dopamines (30). They cannot be applied to DNA directly, however, because DNA is electrochemically inactive in aqueous

media at -1.0 to +1.0 V. One has to convert DNA into electrochemically active form. Several workers determined pg quantities of adenine and guanine, which are electrochemically active, indirectly by this method after acid hydrolysis of DNA (9, 31, 32). Recently, Takenaka *et al.* showed that viologen can be used as an electrochemical probe for DNA (10, 33). Since viologen is reduced at cathodic potentials where dissolved oxygen interferes with the analysis, high sensitivity was not obtained. In this chapter ferrocene is used in place of viologen as an electrochemical probe. It is shown that the ferrocene covalently bound to DNA can detect target DNA and RNA with high sensitivity in a short period of time.

2-2. Materials and methods

A. Materials.

(Dimethylaminomethyl)ferrocene (**1**) and ferrocenecarboxylic acid (**2**) were purchased from Aldrich Chemical Co. Polyadenylic acid, poly[dA] (MW > 10^5), and polydeoxyadenylic-thymidylic acid, poly[dA-dT]poly[dA-dT] (MW 7.7 x 10^6) were obtained from Sigma Chemical Co. Oncogene probe *v-myc* (1.52 kb) was purchased from Clontech Laboratories, Inc.

Recombinant plasmid pCT111 harboring the yeast choline transport gene was a gift of Dr. J. Nikawa. This DNA has one site of dA13 sequence on 3693 base pairs (34). An *Eco* RI and *Kpn* I digestion fragment of pCT111 was ligated with pBluescript II SK^+.

The resulting recombinant DNA was propagated in *Escherichia coli* strain JM 105 (Fig. 8). The linear DNA fragment (*CTR* gene fragment) was prepared by *Pst* I digestion of this recombinant.

Rat brain polyA$^+$ RNA (from 0.3 to > 9 kb in size, >98% in purity) was prepared by the selection with several rounds of oligo(dT)-cellulose columns from total RNA isolated by the modified guanidium thiocyanate method (Clontech Laboratories). Total cellular RNA from yeast strain D452-2 was isolated as described previously (35).

All DNA concentrations were determined by optical measurements. The following molar absorption coefficients (nucleotide phosphate unit) were used: poly[dA], 15400 $M^{-1}cm^{-1}$ at 260 nm (36); poly[dA-dT]poly[dA-dT], 6600 $M^{-1}cm^{-1}$ at 262 nm (37); plasmid DNAs, 6600 $M^{-1}cm^{-1}$ at 260 nm (38). RNA concentrations were determined by measuring the OD_{260}, where $OD_{260} = 1$ is equivalent to approximately 40 μg of RNA per ml (39).

Compounds **7**, **8** and **9** were synthesized by the route shown in Scheme 1.

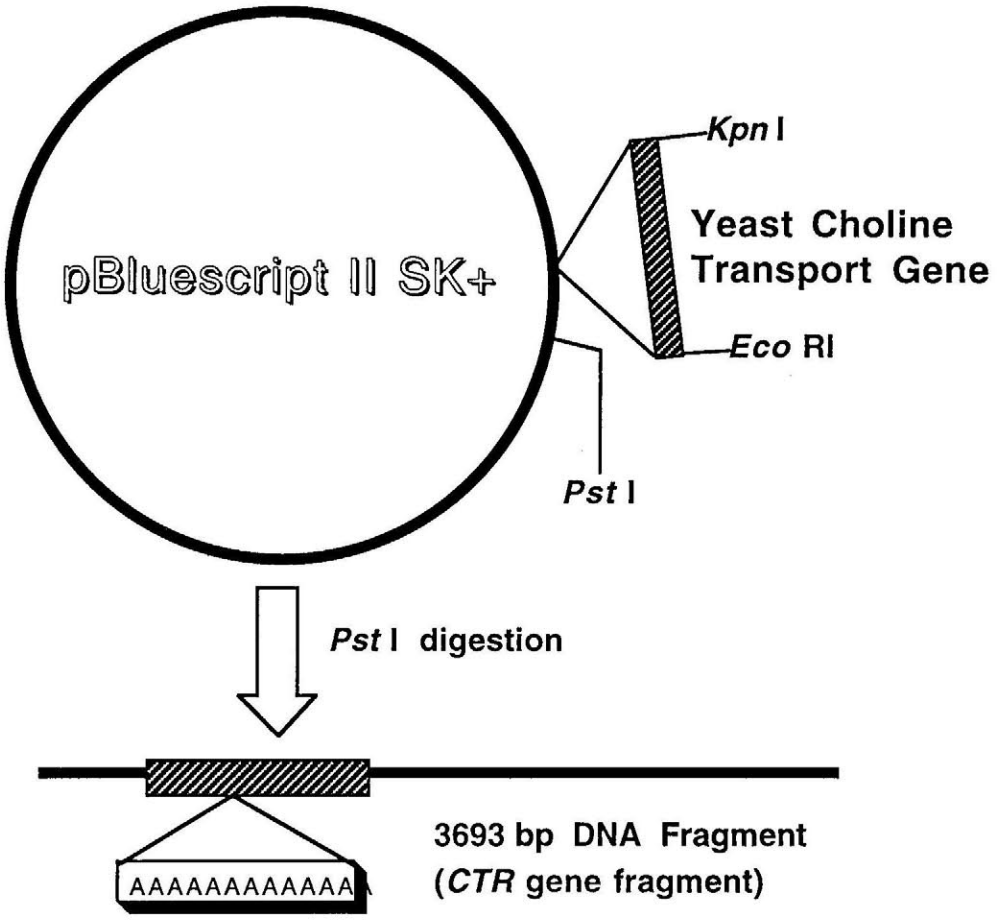
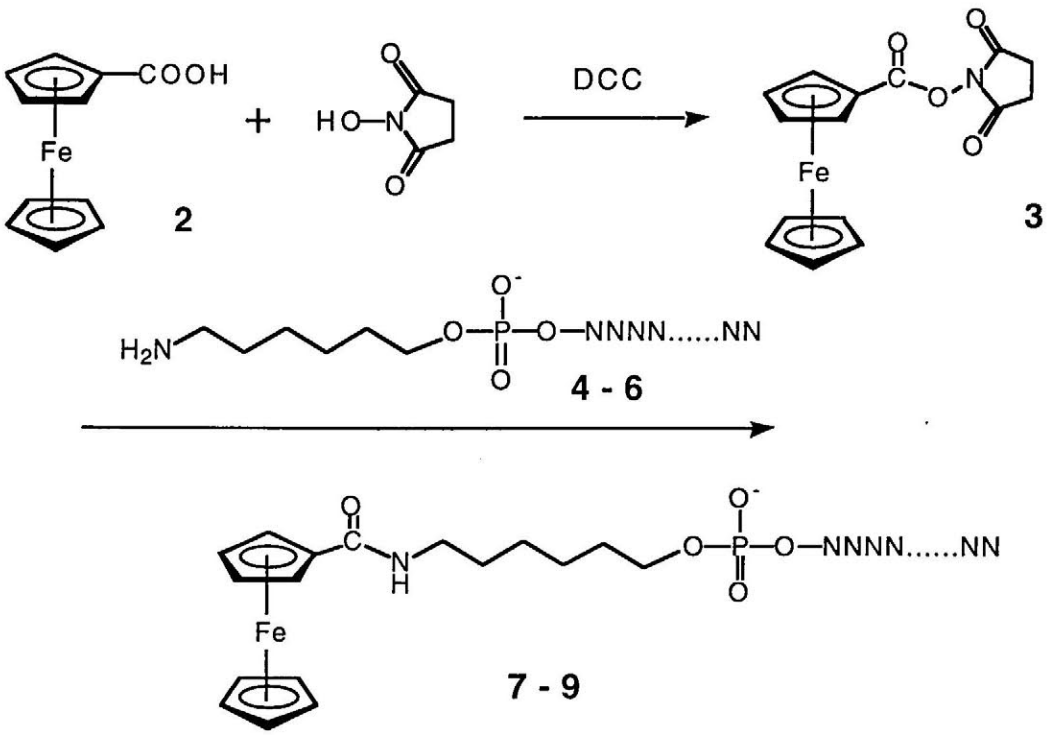


Fig. 8. Construction of pBluescript SK+ carrying a choline transporter (*CTR*) gene fragment. This DNA has one site of dA13 sequence on 3693 base pairs. This DNA was digested with *Pst* I for linearization.



Compound	NNNN.....NN
4,7	5'- TTTTT TTTTT TT -3'
5,8	5'- TGCAG TTCCG GTGGC TGATC -3'
6,9	5'- TTTTT TTTTT TTTTT TTTTT -3'

Scheme 1. Synthesis of ferrocenylated oligonucleotides 7 - 9.

B. Synthesis of *N*-hydroxysuccinimide ester of ferrocenecarboxylic acid

A solution of ferrocenecarboxylic acid (**2**, 0.50 g, 2.2 mmol) and *N*-hydroxysuccinimide (0.29 g, 2.5 mmol) in 20 ml of dioxane was added with stirring to a solution of dicyclohexylcarbodiimide (0.50 g, 2.5 mmol) in 5 ml of dioxane. The mixture was stirred at room temperature for 24 h and the precipitates formed were filtered off. The filtrate was concentrated to dryness and the solid obtained was chromatographed on a column of silica gel (Merck 60, chloroform eluent) to give 0.65 g of **3** as a yellow solid (yield 92%). ^{1}H NMR ($CDCl_3$) δ = 2.92 (4H, s), 4.44 (5H, s), 4.61 (2H, m), 4.99 (2H, m). IR (KBr) 1770, 1740, 1220, 1080 cm^{-1}. Found: C, 54.95; H, 4.11; N, 4.52%. Calculated for $C_{15}H_{13}FeNO_4$: C, 55.05; H, 3.99; N, 4.28%.

C. Synthesis of aminohexyl-linked oligonucleotides

Aminohexyl-linked oligonucleotides (**4 - 6**), whose sequences are shown in Scheme 1, were synthesized on a DNA synthesizer (Pharmacia, Gene Assembler) by using cyanoethyl phosphoramidite chemistry (40). Oligonucleotides were assembled by a standard procedure using standard amidite blocks. An aminohexyl linker (Aminolink 2, Applied Biosystems) was used in the final coupling step to facilitate attachment of a ferrocene onto the 5' end of the oligonucleotides. The concentrations of aminohexyl linked oligonucleotides were calculated by the method described

previously (41): **4** (ε_{260} = 97,800 $M^{-1}cm^{-1}$); **5** (ε_{260} = 162,600 $M^{-1}cm^{-1}$); **6** (ε_{260} = 173,600 $M^{-1}cm^{-1}$). **6** is a part of the complementary sequence to oncogene *v-myc* (42).

D. Synthesis of ferrocenylated oligonucleotides

Aminohexyl-linked oligonucleotide (26 nmol) was dissolved in 20 ml of 0.5 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.0). To this were added 6 ml of a DMSO solution of **3** (1.3 mmol). The mixture was stirred at room temperature overnight, then diluted to 1 ml with 0.1 M triethylammonium acetate (TEAA) buffer (pH 7.0) and chromatographed on an NAP-10 column (Pharmacia, Sephadex G-25). An effluent from 1.0 to 2.5 ml was collected and concentrated. This crude material was further purified by HPLC.

E. HPLC purification of oligonucleotide derivatives

The HPLC system consisted of an L-6200 dual plunger pump (Hitachi, throughout), a sample injector with a 20 μl sample loop, L-4200 variable-wavelength detector, and a D-2500 data processing apparatus. Aminohexyl-linked oligonucleotides (**4** - **6**) and ferrocenylated oligonucleotides (**7** - **9**) were purified to homogeneity by HPLC at 25 °C: flow rate, 1.0 ml/min; buffer A, TEAA buffer (pH 6.9); buffer B, acetonitrile; linear gradient, 10 - 40% B in 30 min. Lichrophore 100 RP-18 (e) (4.6 x 150 mm, hydrophobic column, Cica-MERCK) was used for **4** and **7**, and TSK

gel ODS-80TM (4.6 x 150 mm, hydrophobic column, GL Tosoh) for other samples. Their retention times were the following: **4**, 11.0 min; **5**, 20.6 min; **6**, 8.8 min; **7**, 16.0 min; **8**, 30.1 min; **9**, 18.2 min.

F. HPLC with electrochemical detection

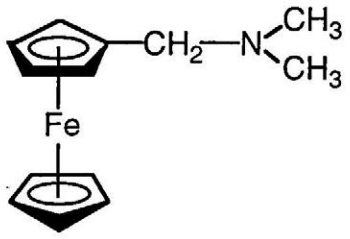
The HPLC system described above was used together with an Eicom ECD-100 electrochemical detector with a WE-3G graphite working electrode. The electrode potential was set at +0.8 V relative to the Ag/AgCl reference electrode. The analytical column and ECD system were maintained at 20 °C using an RTE-110A constant temperature circulator (M&S Instruments Trading Inc.). TSK gel HA-1000 (4.6 x 75 mm, hydroxyapatite column, Tosoh) was mainly used in this system. The hold-up time of these columns were estimated by injection of milli-Q water prepared by filtering deionized water through a 0.2 μm-pore size filter. The mobile phase was 0.5 M sodium phosphate buffer (pH 6.8) containing 0.1 M NaCl. The flow rate was 1.0 ml/min. In the flow injection mode, the column was omitted from the above system. Hydrodynamic voltammograms were obtained from plots of the peak current at several voltages for the same amount of electrochemically active compounds.

2-3. Results

A. Electrochemical behavior of (dimethylaminomethyl)ferrocene in the HPLC-ECD system

In order to assess whether ferrocene can be used as a highly sensitive electrochemical probe, (dimethylaminomethyl)ferrocene (**1**) was tested first in an electrochemical detection system with HPLC. Compound **1** could be eluted at 2.7 min from an HPLC column, TSK gel HA-1000 (flow rate, 1.0 ml/min) with 0.5 M sodium phosphate buffer (pH 6.8) containing 0.1 M NaCl. The hold-up time of this column was estimated to be 2.2 min. The ECD response (peak height) for 10 femtomole (fmol) (sample volume 10 μl) of **1** was obtained at various potentials and the results are plotted as a hydrodynamic voltammogram in Fig. 9 (a).

The optimum potential for detection of **1** was found to be +0.4 V. This agrees with the cyclic voltammetric observation for **1** that the oxidation potential was +0.44 V (43). A maximam peak current of 45 nA was obtained for 10 fmol of the ferrocene. Since the noise level in this system is less than 0.01 nA, the lower limit for electrochemical detection of the ferrocene with an S/N (signal to noise ratio) > 2 will be nearly 0.002 fmol. Fig. 10 (A) shows the chromatograms for various amounts (1.0 - 0.01 fmol) of **1** under these conditions. A good S/N ratio was obtained even at 0.01 fmol, as was expected from the arguments given above.



compound **1**

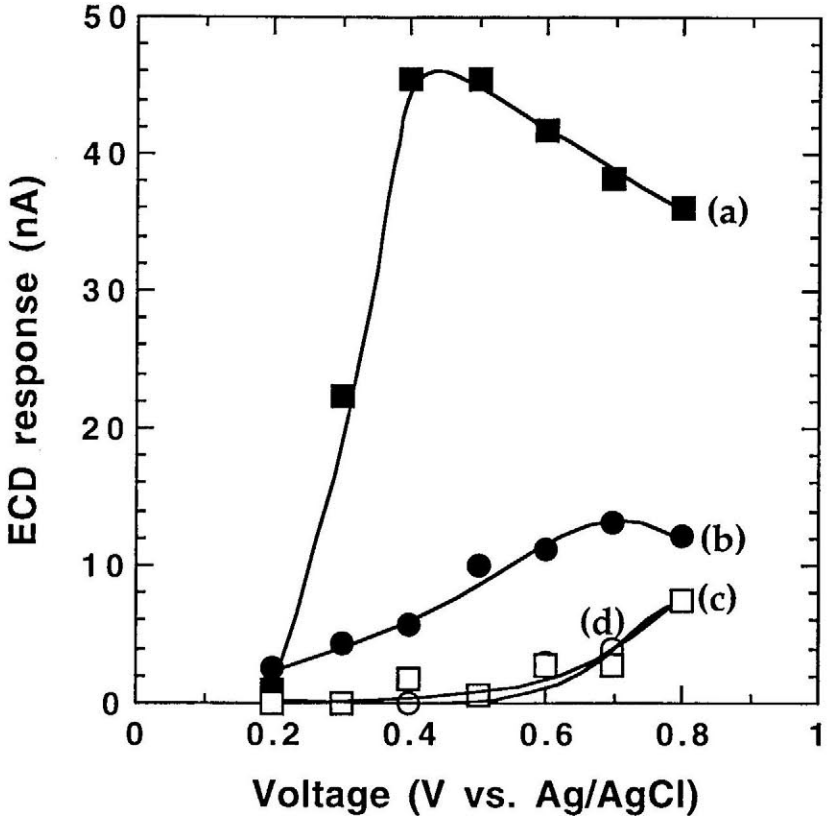


Fig. 9. Hydrodynamic voltammograms for 10 fmol of (dimethylaminomethyl)ferrocene **1** (a), 10 fmol of ferrocenyl thymidine dodecanucleotide **7** (b) and its hybrid complex with poly[dA] (c, d). The hybridization of 10 fmol of **7** with 1.0 pmol of poly[dA] was performed in 0.1 M sodium phosphate buffer (pH 6.4) and 1.0 M NaCl at 65 °C for 3 h. Samples (a, b, c) (10 µl) were injected directly into the ECD system. Sample (d) was injected into the ECD system through a SUPELCOSIL LC-ABZ column.

The ECD response was proportional to the amount of **1** in this range (Fig. 10 (A)). These results suggested that ferrocene can be utilized as a highly sensitive electrochemical probe, once it is connected covalently to DNA.

B. Electrochemical behavior of oligonucleotides in the HPLC-ECD system

Hydrodynamic voltammograms for 10 fmol of **7** are shown in Fig. 9 (b, c). These data were obtained by operating the detector at various potentials in the flow injection mode, and the response (peak height) shown is for a 10 fmol sample. The oxidation of **7** began at +0.2 V and the oxidation current reached a plateau at +0.5 V. The maximum oxidation current of 13.0 nA for **7** was about one third that for **1**. After hybridization with poly[dA], the start-up potential was shifted to +0.4 V. The ECD response of the hybrid with poly[dA] was decreased to one third that of free **7**. This phenomenon may be explained by the fact that the diffusion of ferrocene to the electrode becomes slow as its size increases. Alternatively, the ferrocene moiety of the hybrid interacts predominantly with the hydrophobic interior of DNA strand, so that oxidation of the ferrous ion of ferrocene is made more difficult than the unbound state. It was reported that an apparent oxidation potential of metal complexes is affected in the hydrophobic milieu of DNA (44).

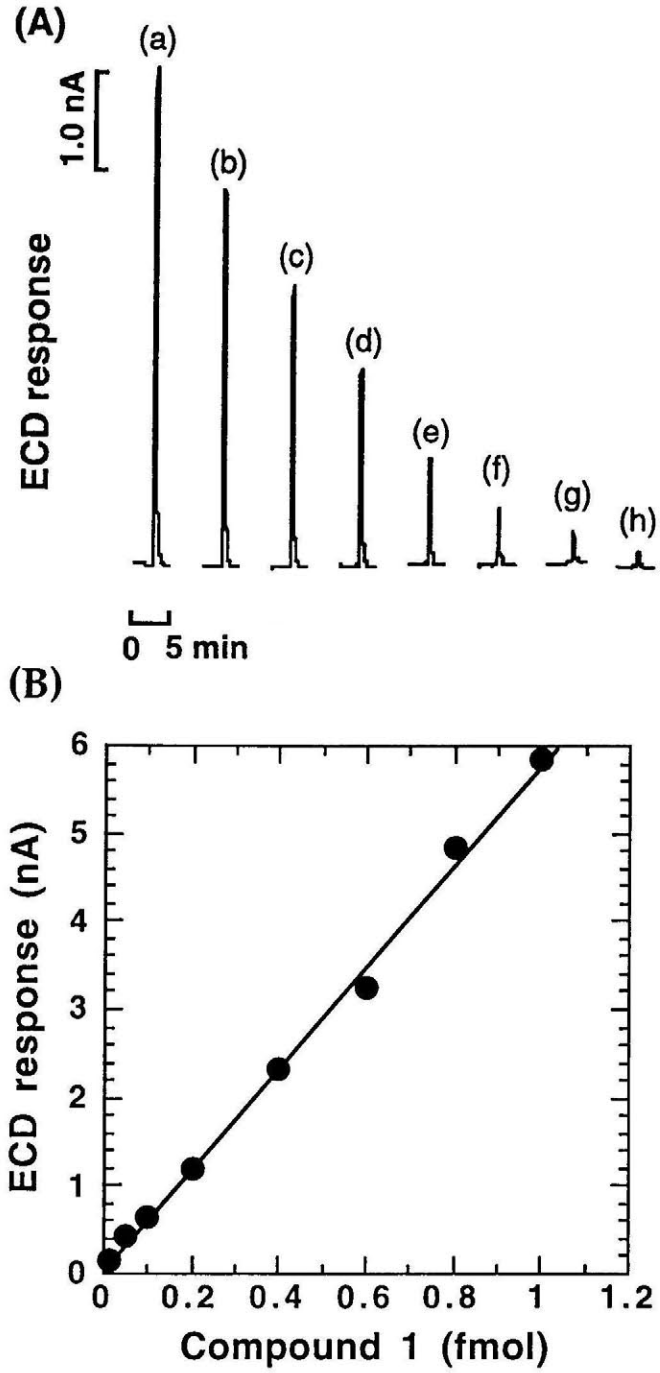


Fig. 10. (A) Chromatograms for **1**. The amounts of **1** were 1.00, 0.80, 0.60, 0.40, 0.10, 0.05, and 0.01 fmol for (a) - (h), respectively. Each sample was injected into the ECD system through a TSK gel HA-1000 column. Conditions were as follows: flow rate, 1.0 ml/min; applied potential, +0.7 V. (B) Linearity of the ECD response for **1**. This figure is a re-plot of the peak heights in (A).

To develop an HPLC-ECD system, the hydrodynamic voltammogram of **7** was examined with several types of columns, TSK gel G2000SW (gel permeation column, Tosoh), TSK gel DEAE2SW (ion exchange column, Tosoh), SUPELCOSIL LC-ABZ, and TSK gel HA-1000. It was found that up to 1 picomole (pmol) of **7** was captured completely by these columns at low organic solvent contents. In contrast to **7** alone, the hybrid of **7** with poly[dA] was eluted under the conditions by using SPELCOSIL LC-ABZ and TSK gel HA-1000. Fig. 9 (d) illustrates a hydrodynamic voltammogram for this hybrid with the SUPELCOSIL LC-ABZ column in 0.1 M phosphate buffer (pH 6.4) and 1.0 M NaCl at 25 °C. The shape of the hydrodynamic voltammogram of this hybrid is similar to that of the directly injected sample shown in Fig. 9 (c). It can be seen from the figure that the hybrid of **7** with poly[dA] (Fig. 9 (b)) is detected with the highest sensitivity at an applied potential of +0.7 V. The peak current of 5 nA was obtained for 10 fmol of the ferrocene and hence as small as 0.2 fmol of ferrocene was detectable even in this case. Fig. 11 (A) shows the chromatograms for various amounts (10 - 1 fmol) of the hybrid of probe **7** with poly[dA] under the above conditions. The ECD response was proportional to the amount of probe **7** in this range (Fig. 11 (B)). Sensitive as it was, this column did not retain the hybrid and the sample was eluted broadened (see Fig. 11 (A)).

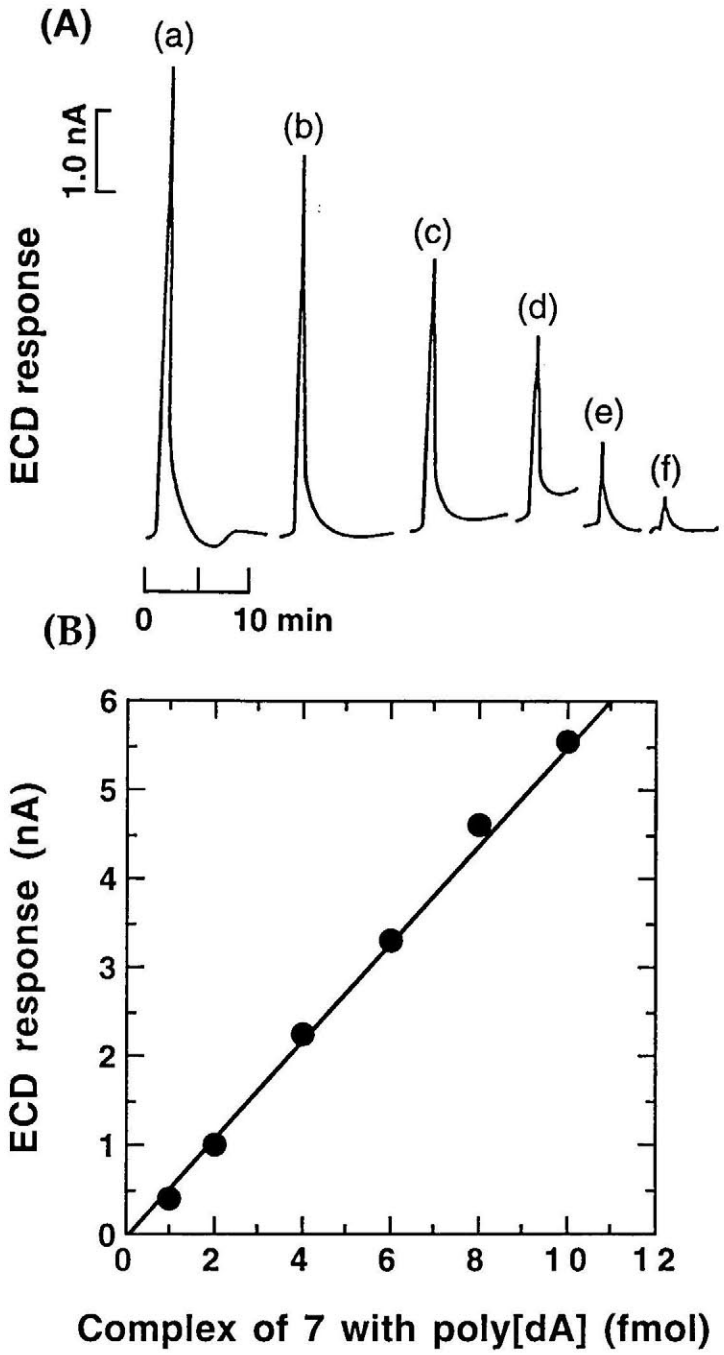


Fig. 11. (A) Chromatograms for 7 hybridized with poly[dA] on a reversed phase column. The amounts of 7 were 10.0, 8.0, 6.0, 4.0, 2.0 and 1.0 fmol for (a) - (f), respectively. The hybridization of 7 with a 100-fold molar excess of poly[dA] was performed in 0.1 M sodium phosphate buffer (pH 6.4) and 1.0 M NaCl at 65 °C for 3 h. The sample was injected into the ECD system through a SUPELCOSIL LC-ABZ column. Conditions were as follows: flow rate, 1.0 ml/min; applied potential, +0.7 V. (B) Linearity of the ECD response for 7 hybridized with poly[dA]. This figure is a re-plot of the peak heights in (A).

In contrast to the SUPELCOSIL LC-ABZ column, a TSK gel HA-1000 column was able to retain the hybrid of **7** with poly[dA], which was eluted with a retention time of 2.7 min by 0.1 M sodium phosphate buffer (pH 6.4) and 1.0 M NaCl, whereas the hold-up time was 2.2 min. Fig. 12 (A) shows the chromatograms for a various amount (100 - 20 fmol) of probe **7** hybridized with poly[dA] under these conditions. The ECD response was proportional to the amount of probe **7** in this range (Fig. 12 (B)). It should be noted that although a stable base line and sharp peaks were obtained with this column, the sensitivity of electrochemical detection was lower than that for SUPELCOSIL LC-ABZ column. This may be due to a difference in the salt concentration of the eluent. It is known that the stability of DNA double helix increases as the salt concentration is increased (45): T_m values of the hybrids of **7** were estimated by the equation previously described to be 31 °C in 0.5 M phosphate and 0.1 M NaCl and to be 40 °C in 0.1 M phosphate and 1.0 M NaCl.

These results confirmed that fmol level detection can be achieved by the ferrocenylated oligonucleotides using ECD with HPLC.

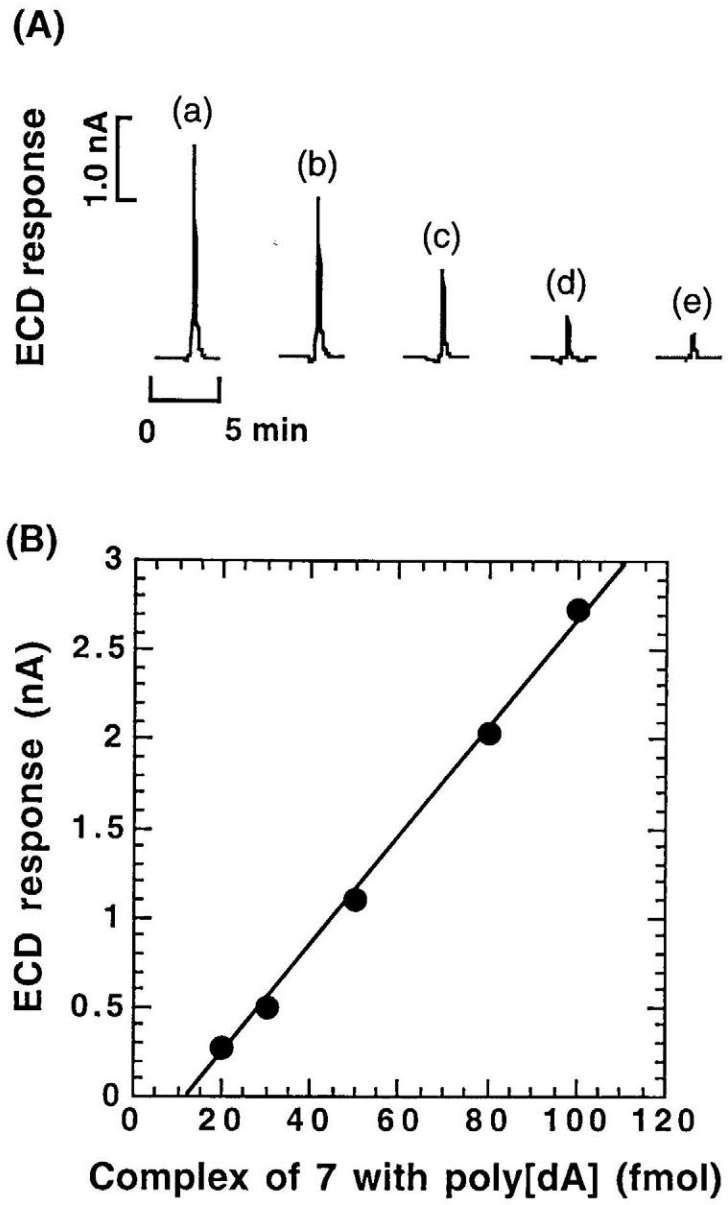


Fig. 12. (A) Chromatograms for 7 hybridized with poly[dA] on a hydroxyapatite column. The amounts of 7 were 100, 80, 50, 30 and 20 fmol for (a) - (f), respectively. The hybridization of 7 with a 100-fold molar excess of poly[dA] was performed in 0.1 M sodium phosphate buffer (pH 6.4) and 0.5 M NaCl at 65 °C for 3 h. The sample was injected into the ECD system through a TSK gel HA-1000 column. Conditions were as follows: flow rate, 1.0 ml/min; applied potential, +0.7 V. (B) Linearity of the ECD response for 7 hybridized with poly[dA]. This figure is a re-plot of the peak heights in (A).

C. Sequence recognition by ferrocenylated oligonucleotides

In order to examine whether ferrocenylated oligonucleotides can be used for the specific detection of the recognized sequence, sample 7 was analyzed by ECD and uv detector with HPLC after hybridization with poly[dA] or poly[dA-dT]poly[dA-dT]. 7 has a complementary sequence of poly[dA] and may hybridize with it, but may not form a hybrid with poly[dA-dT]poly[dA-dT]. In the case of poly[dA-dT]poly[dA-dT], two uv-absorbing peaks appeared, but neither was ECD positive (Fig. 13 (B)). Judging from the nature of the hydroxyapatite column (TSK gel HA-1000) (46), the first peak may be assigned to a single strand oligonucleotide, poly[dA-dT], and the second peak to a double strand oligonucleotide, poly[dA-dT]poly[dA-dT]. In the case of poly[dA], a single uv-absorbing and ECD positive peak appeared at a position of the putative single strand DNA in poly[dA-dT]poly[dA-dT] (Fig. 13 (A)). These results show that 7 can hybridize specifically with poly[dA] and that this hybrid was detected by ECD, whereas 7 cannot hybridize with poly[dA-dT]poly[dA-dT]. In other words, ferrocenylated oligonucleotides can recognize the target sequence properly and the resulting hybrid is detected by ECD with high sensitivity.

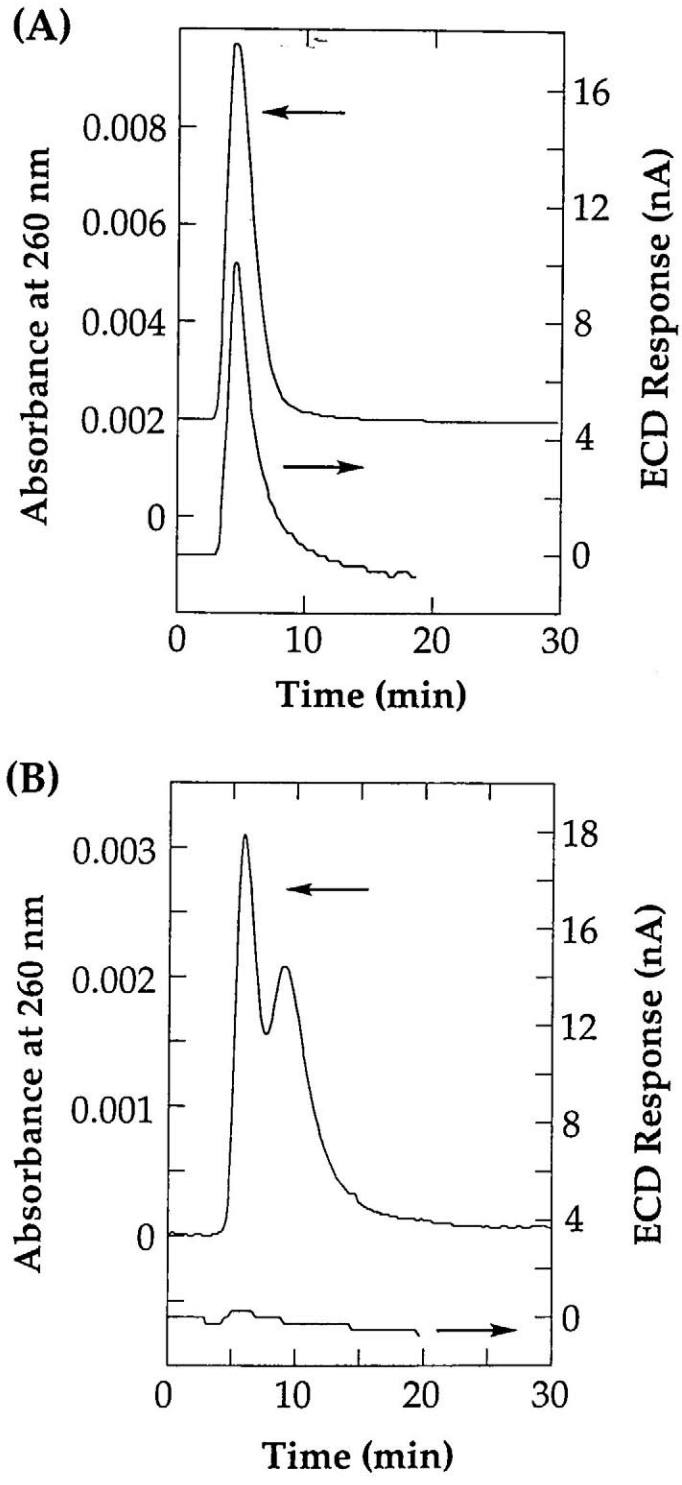


Fig. 13. Chromatograms for the hybridized mixture of **7** with poly[dA] (A) or poly[dA-dT]poly[dA-dT] (B). The hybridization of 100 nmol of **7** with 10 mmol of DNA were performed as in Fig. 12.

D. Electrochemical detection of natural genes

To assess the usefulness of this method, probe **7** was used for the detection of native DNA having one recognized site. The promoter region of the yeast choline transport gene (34) was selected which contains one dA13 sequence in the 79-base pair DNA. This DNA was introduced to pBluescript II SK$^+$ plasmid and the resulting recombinant was linearized by digestion with *Pst* I to yield a 3693 bp DNA. This DNA fragment (*CTR* gene fragment) was allowed to hybridize with a 10-fold molar excess of probe **7** in 0.5 M sodium phosphate buffer (pH 6.8) and 0.1 M NaCl at 82 °C for 3 h. The analysis of a various amount (100 - 20 fmol) of this DNA fragment hybridized with probe **7** was achieved under the above conditions. The ECD response was proportional to the amount of probe **7** bound to the *CTR* gene fragment in this range (Fig. 14). The lower limit of electrochemical detection in Fig. 14 was 20 fmol and was almost the same as that for poly[dA]. In contrast, a *Pst* I-digested DNA fragment of pBluescript II SK$^+$ plasmid which is devoid of the dA13 sequence was not detected under the same conditions.

Next, detection of oncogene *v-myc* was attempted with the selective probe **8** by ECD with HPLC. This oncogene *v-myc* DNA fragment was allowed to hybridize with a 10-fold molar excess of probe **8** in 0.5 M sodium phosphate buffer (pH 6.8) and 0.1 M NaCl at 80 °C for 3 h.

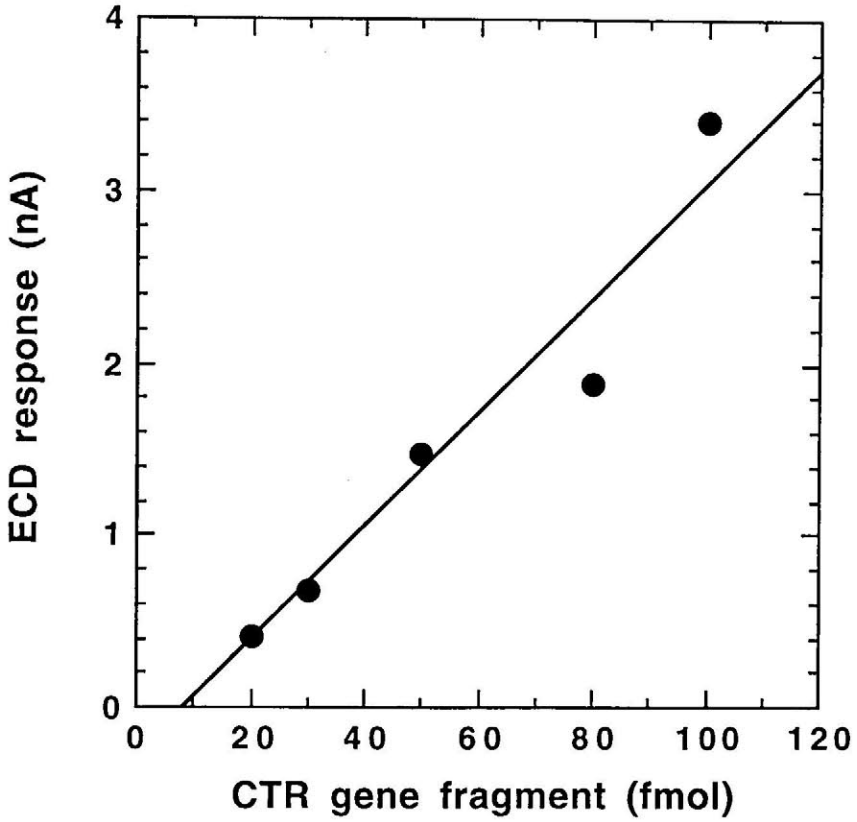


Fig. 14. Determination of the choline transport gene fragment (*CTR* gene fragment) through hybridization with 7. The hybridization of a 100-fold molar excess of 7 with the DNA fragment was performed in 0.1 M sodium phosphate buffer (pH 6.8) and 0.5 M NaCl at 82 °C for 3 h. The sample was injected into the ECD system through a TSK gel HA-1000 column and the ECD response was plotted against the amount of the *CTR* gene fragment.

Fig. 15 shows the analysis of a various amount (10 - 1 fmol) of this DNA fragment hybridized with probe **8**. The ECD response was proportional to the amount of probe **8** bound to oncogene probe *v-myc* in this range (Fig. 15). The lower limit in Fig. 15 was 2 fmol. This value is one twentieth that for **7**. This discrepancy arose presumably from a difference in the stability of the hybrids of **7** and **8**. The melting temperature of the hybrid of **7** with its target is estimated by the equation described previously to be 31 °C, whereas the corresponding value for **8** to be 53 °C (47). Hence a larger fraction of **7** exists in unhybridized form under the chromatographic conditions, leading to the lower sensitivity of detection.

E. Electrochemical detection of messenger RNA

Most eukaryotic messenger RNAs possess a stretch of polyadenylic acid at their 3'-end and thus will be detectable by **9** (48). mRNA from rat brain was mixed in a 100-fold molar excess of probe **9** in 0.5 M sodium phosphate buffer (pH 6.8) and 0.1 M NaCl at 20 °C and analyzed by ECD with HPLC. The standard curve for mRNA assay is shown in Fig. 16. It was found that picogram (pg) quantities of mRNAs could be detected.

Furthermore, the effectiveness of this method was tested in the detection of mRNAs from eukaryotic total RNA with the same

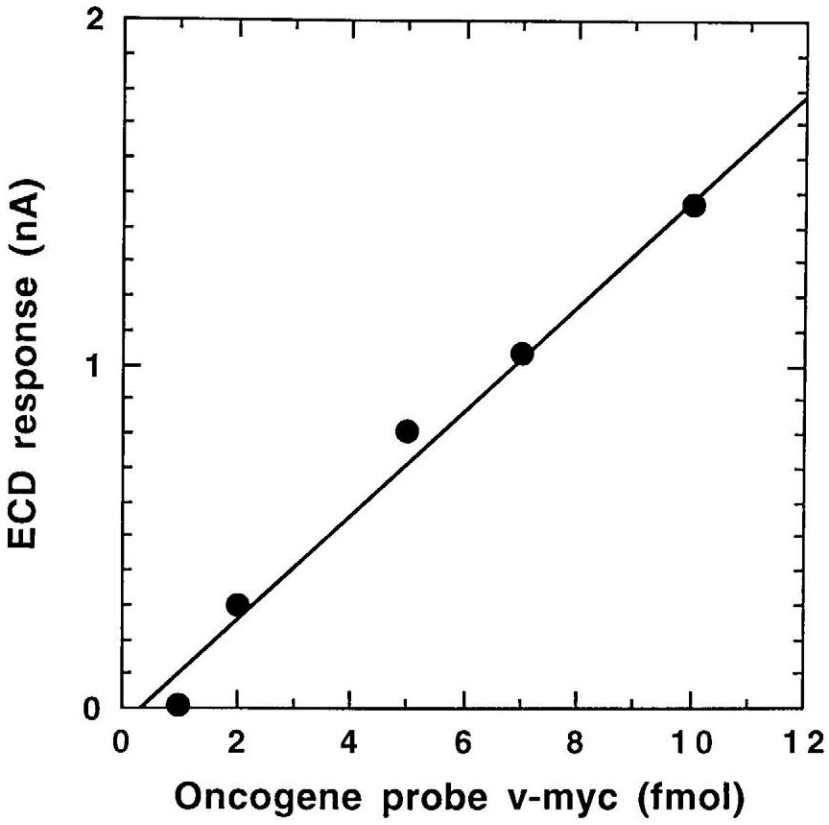


Fig. 15. Determination of the DNA fragment having oncogene *v-myc* through hybridization with 8. The hybridization of a 100-fold molar excess of 8 with the DNA fragment was performed in 0.1 M sodium phosphate buffer (pH 6.8) and 0.5 M NaCl at 80 °C for 3 h. The sample was injected into the ECD system through a TSK gel HA-1000 column and the ECD response was plotted against the amount of *v-myc* DNA.

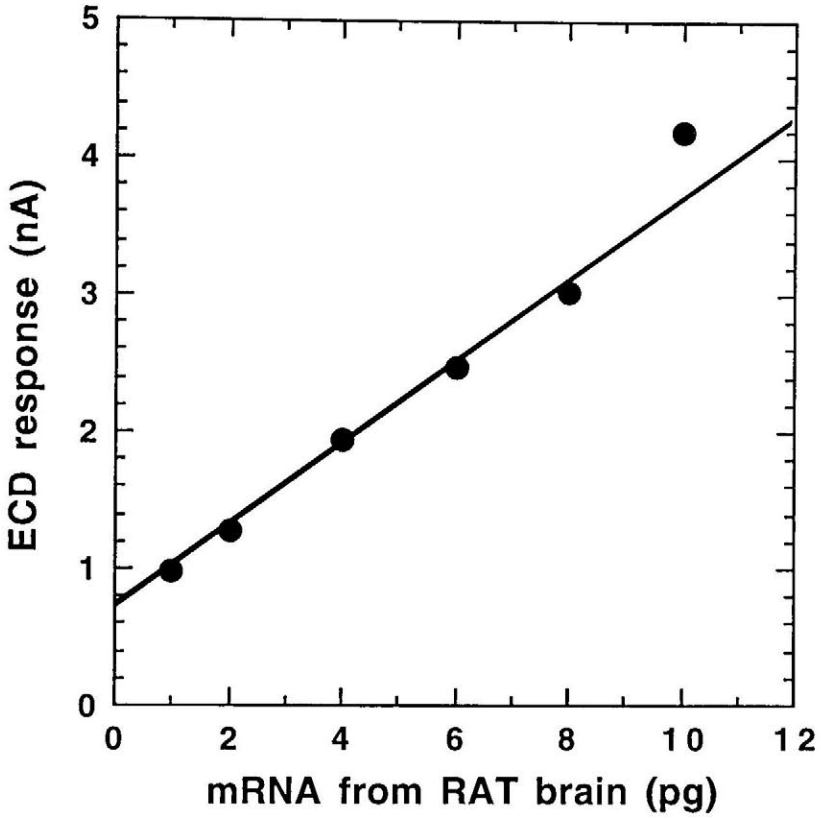


Fig. 16. Determination of mRNAs taken from rat brain. The hybridization of a 10-fold molar excess of 9 with RNA was performed in 0.5 M sodium phosphate buffer (pH 6.8) and 0.1 M NaCl at 25 °C. This sample was injected into the ECD system through a TSK gel HA-1000 column. Shown are the ECD responses or the peak heights against the amounts of mRNA.

probe 9. Total cellular RNA isolated from yeast strain D452-2 was allowed to hybridize with a 100-fold molar (phosphate unit) excess of probe 9 under the same experimental conditions as above. A response of 1.55 nA was obtained for 0.20 nanogram (ng) of total RNA. Using Fig. 16 as the working curve, the amount of mRNAs in total RNA was estimated to be 3 pg. In other words, 1.5% of the total cellular RNA was mRNAs. This value is in good agreement with the reported value of 1 - 5% (40).

2-4. Discussion

It was shown that an electrochemical method can be used for the analysis of polynucleotides with high sensitivity. This method relies on hybridization of target nucleotides with a complementary oligonucleotide carrying an electrochemically active ferrocene group. The resulting hybrids are analyzed by HPLC-ECD. It was found that as small as 1 fmol of DNA can be detected by this method with **9**. Even in less sensitive cases the detection limit was 20 fmol. These levels of sensitivity are nearly of the same order as that of the 5'-terminally ^{32}P-labeled oligonucleotides whereas this method is inferior to the most sensitive fluorescence and luminescence methods coupled with an enzyme amplification reaction (28). Compared with such methods which take more than 2 days, however, the electrochemical method is quick to run, as the key

hybridization step takes place in solution and the subsequent detection by HPLC-ECD is completed within minutes.

The analytical procedures of this method are summarized as follows: (a) A ferrocenylated oligonucleotide with a desired nucleotide sequence is synthesized for use as a hybridization probe. (b) A sample of DNA or RNA is allowed to hybridize with this probe. (c) The hybrid thus formed is injected into a column of HPLC with ECD. (d) The target DNA or RNA is quantitated from the ECD response or the peak height.

The sensitivity of this method appears to be dictated by the stability of the hybrid complex as measured by its T_m under the chromatographic conditions. The size of the probe DNA is one of the factors affecting the stability of hybrid. Thus, when probe 7 carrying a T_{12} sequence hybridized with its target, the hybrid formation appeared to be incomplete because of the low T_m of the hybrid. As a result, the detection limit was 20 fmol. By contrast, when probe 9 carrying a T_{20} sequence was used, the detection limit was 3 fmol (1 pg of mRNA corresponds nearly to 3 fmol). This figure probably represents the highest sensitivity attainable with this probe and the ECD system. As the hybridization of homopolymers of thymidine with homopolymers of adenosine is the weakest of all combinations of the nucleotide sequences, it is secure to conclude that the highest sensitivity will be obtained if one

uses an oligonucleotide greater than 20-mer, regardless of sequence.

Of the various commercial HPLC columns tested, LC-ABZ and HA-1000 were found to be suited for the electrochemical analysis of DNA hybrids. The former column consists of a reversed phase gel carrying extra polar groups near the silica surface for "electrostatic shielding" and the latter is a hydroxyapatite column. The ferrocenylated oligonucleotides **7 - 9** stuck to these and other tested columns, but their hybrids were eluted from the two columns by phosphate buffer. Although these columns clog with captured probes in a sense, as revealed by an increase in the pressure, they can be regenerated after repeated use by washing with 0.1 M sodium hydroxide, 60% methanol and water successively.

The LC-ABZ and HA-1000 columns have advantages and disadvantages. With the eluent used, the former column gave the higher sensitivity of detection, but the tailing of the chromatograms was significant. Even worse, the DNA hybrids were eluted in the void volume. Since excess probe, when present, is captured by the column, the only peak detected by ECD should be the hybrid in principle. Hence one has to take care that no other components are contained in this peak.

Although the sensitivity of detection was lower with the HA-1000 column than with the LC-ABZ column because of the lower salt buffer used, the base line and peak shapes were good. In this

study, the buffer composition of the sample injected to HPLC was the same as that of the eluent. However, in case that the buffer composition of the sample is different from that of the eluent, depending upon the hybridization condition, the artifact peak will appear at the unretained time. Above all, this column is able to retain the hybrid and hence overlap with artifact peaks is less likely to occur. In conclusion, HA-1000 column is the best to work with at present.

As for the eluent, any aqueous buffer is acceptable unless it contains electrochemically active material. Water-miscible organic solvents may also be acceptable as long as their content is kept lower than 20%.

Chapter 3. Electrochemical analysis of DNA amplified by the polymerase chain reaction with a ferrocenylated oligonucleotide as a primer

3-1. Introduction

It was described in the preceding chapter that oligonucleotides labeled with electrochemically active ferrocene can be used for the convenient analysis of DNA with high sensitivity (7). It was possible to detect DNA sequences at fmol level with a combination of HPLC and ECD. In the meantime, polymerase chain reaction (PCR) is currently in use in many fields of molecular biology (13, 16, 17). It then occurred to the author that if PCR is incorporated into the HPLC-ECD method, its sensitivity of DNA analysis would be enhanced drastically. Since DNA amplification proceeds exponentially under low cycles of PCR (49, 50), even quantitation of DNA may be realized by the ECD method under favorable circumstances. These two points will be addressed in this chapter. Two types of DNA were employed as template of PCR: 1.5 kb linear duplex and chromosomal DNA. Part of their sequences was amplified by PCR with ferrocenylated oligonucleotides as a primer and the resulting PCR products were analyzed by HPLC-ECD at sub-attomole level of template DNA.

3-2. Materials and methods

A. DNA samples

A DNA fragment of oncogene *v-myc* (1.52 kb) was purchased from Clontech Laboratories, Inc. Human chromosomal DNA was purified from leukocytes by the standard procedures (29). Oligonucleotides used as PCR primers (primers **10** - **13**) were custom-synthesized by Takara Shuzo, Kyoto: **10**, 5'-GTCAA CATCC ACCAA CACAA CTACG-3'; **11**, 5'-CTATG CACGA GAGTT CCTTA GCTGC-3'; **12**, 5'-TTGAA TACAT TGGTT AAATC CCAAC ATG-3'; **13**, 5'-CCTGA ATAAA GTCTT CCTTA CCACA C-3'. Two of them (**11** and **13**) were 5'-end labeled with a ferrocenecarboxylic acid (**2**) by a procedure described previously (7). The DNA concentration was determined by using the molar extinction coefficient of 6600 $M^{-1}cm^{-1}$ at 260 nm (51). Primers **10** and **11** were used to amplify a 0.97 kb DNA fragment of the *v-myc* gene. Primers **12** and **13** were to amplify a 0.51 kb fragment of exon 48 of the human dystrophin gene (52).

B. PCR amplification

PCR amplifications were performed by the following procedure. A sample solution containing a proper amount of template DNA was mixed with other reagents to give final concentrations as follows: 0.10 μM each of two primers, 250 μM each of four deoxyribonucleoside triphosphates (dNTPs), 2.5 units

of *Taq* DNA polymerase (Takara Shuzo), 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% (w/v) gelatin. The final volume of the solution was 20 μl. The solution thus prepared was overlaid with mineral oil and amplified by a required number of cycles using a DNA Thermal Cycler (Astec, Fukuoka). PCR amplification of the *v-myc* and dystrophin genes was carried out by repeating the following cycle: 94 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min. Samples containing chromosomal DNA was pre-heated at 94 °C for 2 min prior to PCR amplification.

C. HPLC-ECD system

The HPLC system consisted of an L-6200 dual plunger pump (Hitachi, throughout), a sample injector with a 20 μl sample loop, L-4200 variable-wavelength detector and D-2500 data processing apparatus. The ECD system consisted of an Eicom ECD-100 electrochemical detector with a WE-3G graphite working electrode. The electrode potential was set at a desired voltage relative to the Ag/AgCl reference electrode. The analytical column and ECD system were maintained at 25 °C using an RTE-110A constant temperature circulator (M&S Instruments Trading Inc.). Asahipak GF-510HQ (7.6 x 250 mm, gel permeation column, Asahi Kasei) and TSK gel HA-1000 columns were used as the stationary phase for the *v-myc* and dystrophin gene analysis, respectively. The mobile phase was 0.5 M sodium phosphate buffer (pH 6.8)

containing 0.1 M NaCl. The flow rate was set at 0.5 ml/min. Hydrodynamic voltammograms were obtained by determining the peak current at several voltages. A cDNA spun column packed with a gel permeation chromatographic resin (Pharmacia) was used for the purification of the PCR product for the *v-myc* gene.

3-3. Results

A. PCR with a ferrocenylated oligonucleotide as a primer

A 0.97 kb DNA fragment of the oncogene *v-myc* was amplified by PCR with a 1.52 kb fragment as template. Primer **10** and ferrocenylated primer **11** were used as primers. After 30 cycles of PCR, the reaction solution was electrophoresed on agarose gel and stained with ethidium bromide (Fig. 17). A DNA band was observed at a position of 0.97 kb in the electrophoretogram of this sample. Since a band was observed at the same position with the non-ferrocenylated primer **11**, the 0.97 kb DNA band should be the desired DNA fragment. Likewise, a 0.51 kb fragment of exon 48 of the dystrophin gene was amplified in the presence of primer **12** and ferrocenylated primer **13** with chromosomal DNA prepared from leukocytes of healthy men and women as template. Again, the same DNA band was obtained by PCR amplification with non-ferrocenylated primer **13**, proving that the ferrocenylated oligonucleotides can be used as a PCR primer to give correct PCR products.

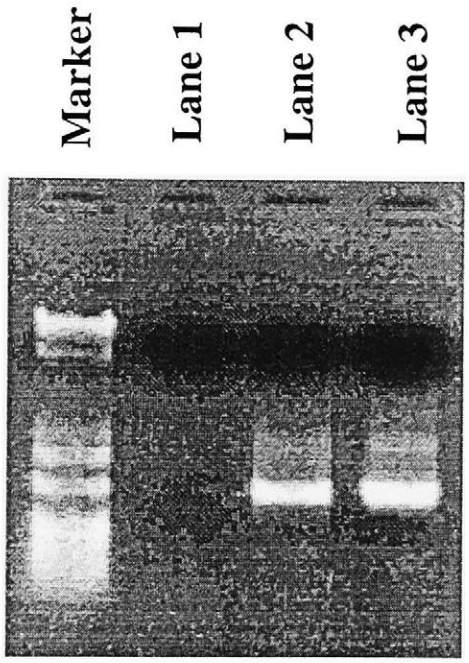


Fig. 17. Agarose gel electrophoresis of PCR products for *v-myc* DNA. Lanes 1 and 2 depict reaction mixtures before and after PCR, respectively. Lane 3 shows the PCR product after recovering the main band from twice the amount of lane 2 using a glass milk method.

B. Purification of PCR products

The 0.97 kb PCR product for *v-myc* was purified by the following procedures.

1) By a glass milk method (EASYTRAP^{TM} Ver. 2, Takara) (53). Lane 3 of Fig. 17 shows a gel electrophoretogram of recovered PCR product from twice the amount of lane 2. Comparison of the fluorescence intensity of DNA bands on Lanes 2 and 3 reveals the recovery efficiency of procedure being close to 50%.

2) By HPLC in the gel permeation mode on an Asahipak GF-510HQ column. Fig. 18 (a) shows a chromatogram of 10 μl of a solution after PCR under isocratic conditions. The retention time of the peak around 10 min is identical with that of the PCR product recovered by the glass milk method. Peaks from 16 to 21 min are due to the polymerase, primers and dNTPs.

3) The PCR product was separated from the reaction mixture by a cDNA spun column. Fig. 18 (b) shows the recovery efficiency of greater than 90%.

C. Electrochemical activity of PCR products

The PCR product for the *v-myc* gene using the ferrocenylated primer was analyzed by an HPLC-ECD system equipped with an Asahipak GF-510HQ column. The ECD response was observed at 10 min, as was with UV detection. By contrast, no ECD response

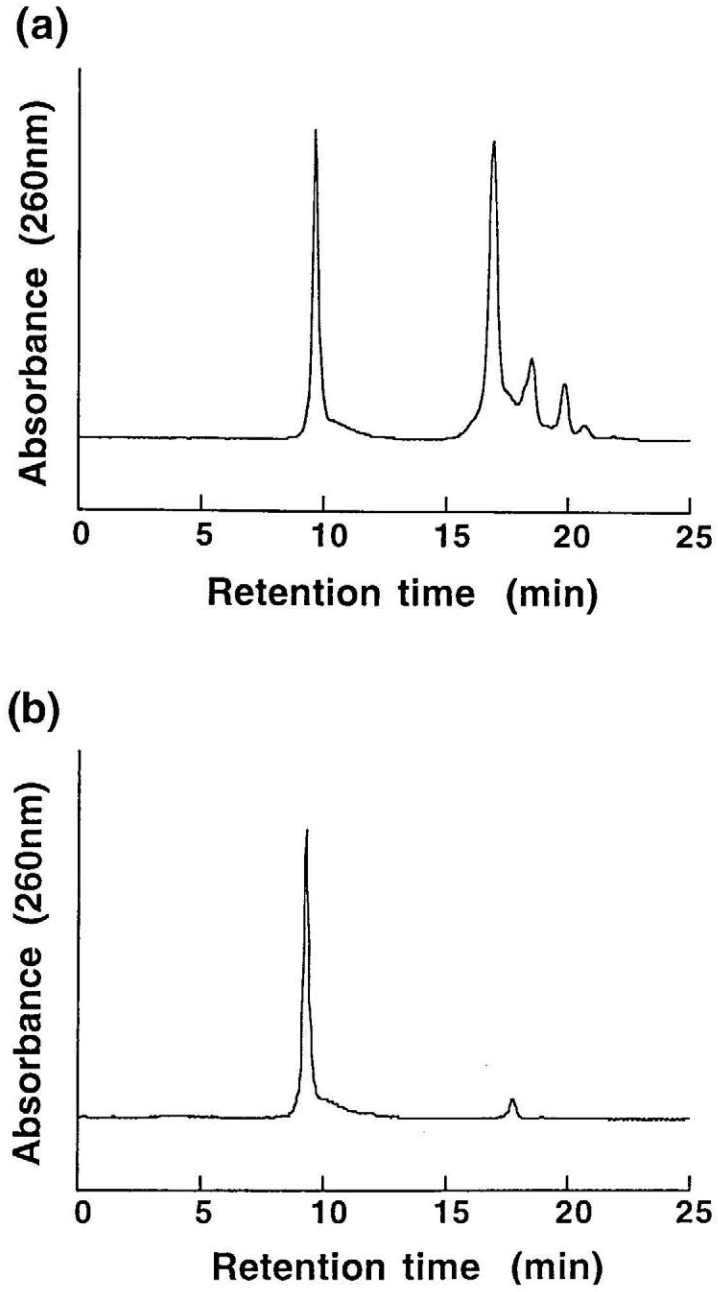


Fig. 18. Chromatograms of a PCR product for *v-myc* DNA. Twenty cycles of PCR were run for 0.10 fmol of *v-myc* DNA as template and other ingredients specified under Materials and methods on a 20 µl scale. After PCR, 10 µl of the sample solution were injected into the HPLC equipped with a UV/VIS detector (a). The PCR product purified by a cDNA spun column is also shown in (b). Conditions were as follows: column, Asahipak GF-510HQ; flow rate, 0.5 ml/min; detection wavelength, 260 nm.

was observed for the PCR product with the non-ferrocenylated primer.

This result reinforces the view that the ferrocenylated oligonucleotides serve as PCR primers to give correct ferrocenylated PCR products. To investigate the electrochemical behavior of the ferrocenylated PCR product, a hydrodynamic voltammogram was determined with the HPLC-ECD system. The ECD responses for 10 fmol of the ferrocenylated oligonucleotide **11** and its PCR product were measured at various potentials from 0 to +0.7 V at 0.1 V intervals (Fig. 19). In both cases, the oxidation current increased with an increase in the potential and reached a maximun at +0.7 V. This behavior is in agreement with the previous result (Chapter 2, Fig. 9) and suggests that these currents are based on the ferrocenyl part (7). The maximum oxidation current of 6.0 nA for the PCR product was about half that for the oligonucleotide primer **11**. This result is explicable in terms of a difference in their size (7).

D. Correlation of the ECD response with the concentration of PCR product

To assess the range suitable for quantitative analysis of the ferrocenyl PCR product, the ECD responses were determined at various concentrations of product by the HPLC-ECD system

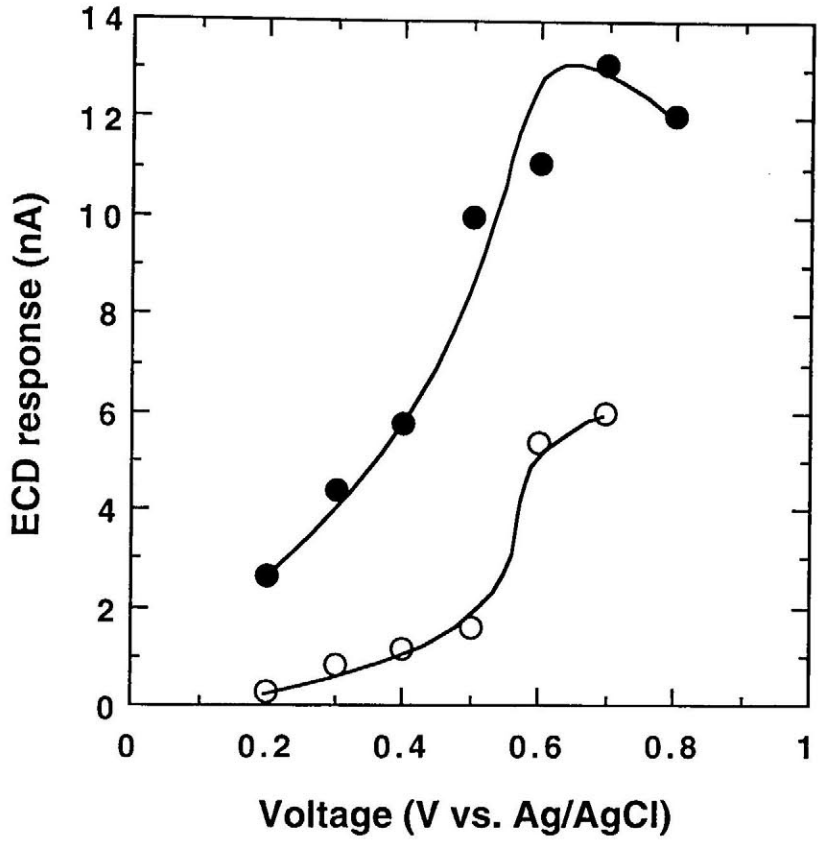


Fig. 19. Hydrodynamic voltammograms for 10 fmol of ferrocenylated PCR primer (closed circles) and PCR product from *v-myc* DNA (open circles). Ten microliter samples were injected into the HPLC-ECD equipped with an Asahipak GF-510HQ column.

utilizing the Asahipak GF-510HQ column. First, the ferrocenylated PCR product was obtained from the *v-myc* gene by 30 cycles of amplification and was purified with a cDNA spun column. The concentration of this product was determined by the absorbance at 260 nm.

Various amounts of the sample, prepared by diluting the PCR product sequentially, were injected into the HPLC-ECD system to give rise to a calibration curve shown in Fig. 20. The ECD response is linear over a broad concentration range of the PCR product (1 - 1000 fmol). The following equation was obtained from the plot in Fig. 20.

$$Y = 0.69\ X^{0.74}$$

where X and Y denote the concentration of the PCR product and its current response, respectively.

E. Quantitative PCR with a ferrocenylated primer

Because DNA is amplified exponentially by PCR, the initial amount of DNA can be estimated at n = 0 (n is the number of cycles) by extrapolation of the dependence of the amounts of the amplified DNA on PCR cycles (49, 50). Alternatively, the initial amount of DNA can be estimated by comparing it with an internal standard DNA which is co-amplified in the same solution (16, 20). Although some difficulties are involved, data under low cycle numbers of PCR can be used to quantitate or at least semi-quantitate a given

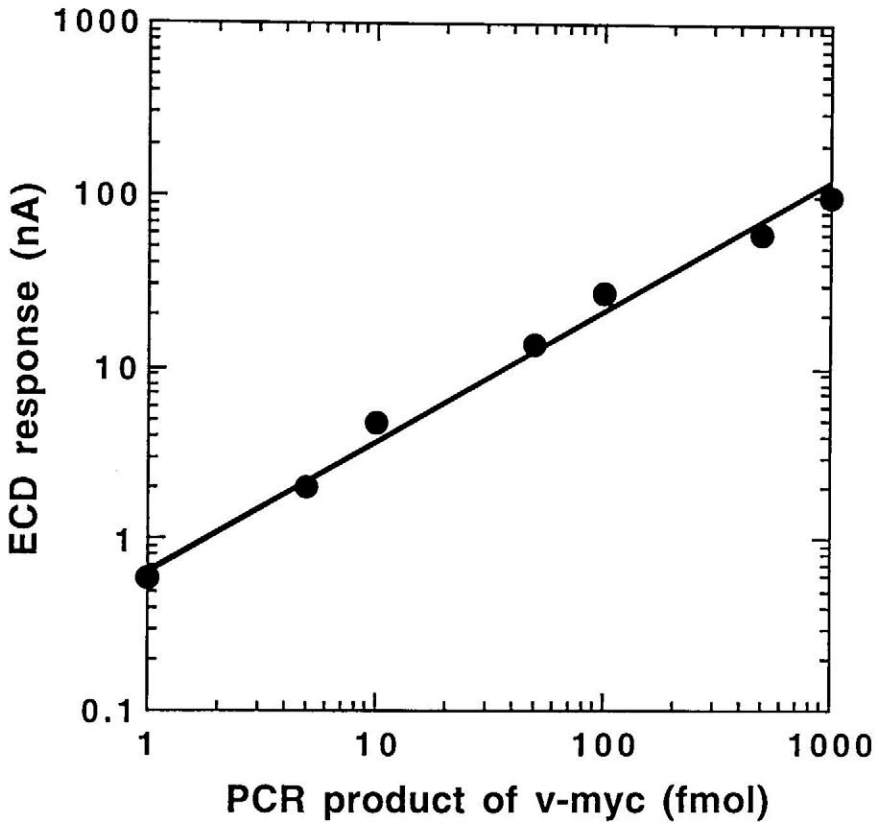


Fig. 20. Calibration curve for the quantitation of the *v-myc* gene fragment. The PCR product purified by a cDNA spun column was quantitated by absorbance measurement at 260 nm (ε=6600 $M^{-1}cm^{-1}$) and, after dilution, injected into the HPLC-ECD equipped with an Asahipak GF-510HQ column. The ECD responses obtained were plotted against the amount of the PCR product injected.

gene. In light of the fact that a radio-labeled oligonucleotide is used often for this purpose and that the ferrocenylated oligonucleotide has a sensitivity comparable to that of a radio-isotope, quantitative PCR may be possible with the ferrocenylated oligonucleotide as a PCR primer.

The initial amount of the *v-myc* gene in the solution was estimated by the extrapolation method first. A sample solution containing 0.10 fmol of template DNA was prepared and the DNA was amplified with different numbers of PCR cycles: 5, 10, 12, 14 and 16. The products obtained were analyzed by HPLC-ECD utilizing the Asahipak GF-510HQ column. ECD responses of each PCR product were converted into the DNA concentration using the above equation and plotted against the number of PCR cycles (Fig. 21). An intercept of 0.09 ± 0.04 (95% confidence interval) fmol was obtained using the least squares method. This value is in good agreement with the initial amount used in the sample solution (0.10 fmol). This model experiment suggests that the ferrocenylated primer can be utilized for the quantitative PCR analysis of DNA.

To explore further the scope of this analytical method for DNA, It was next attempted to estimate the muscular dystrophin gene in the chromosome prepared from leukocytes of healthy men and women. The muscular dystrophin gene is located in the X-chromosome and therefore healthy women have twice as many copies as that of healthy men (19). The ferrocenylated PCR product

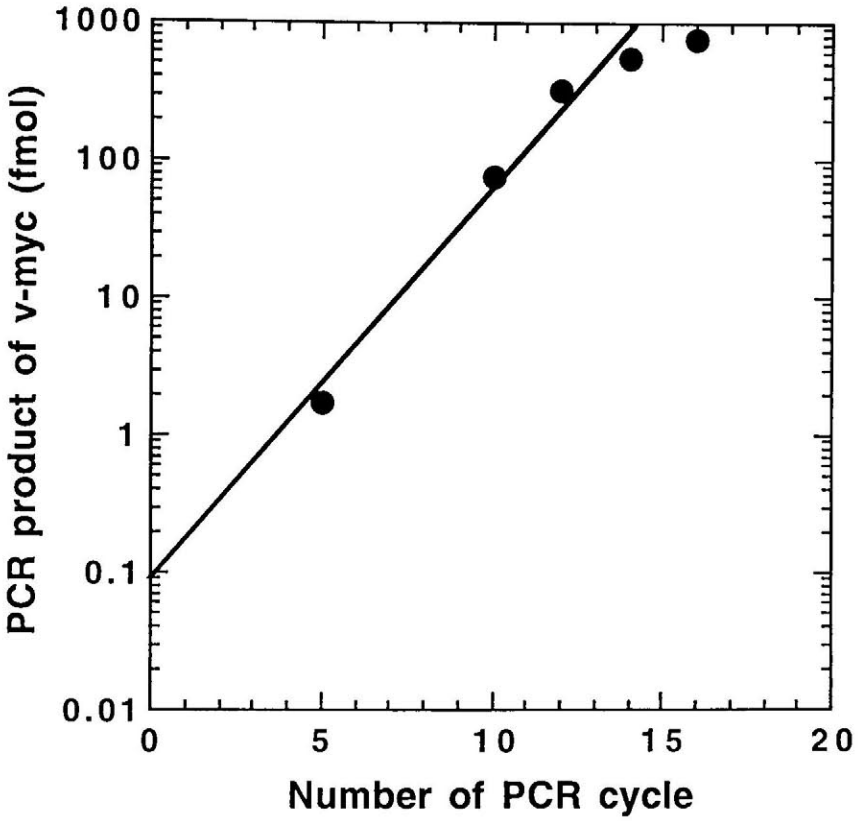


Fig. 21. Correlation between the concentration of PCR product for *v-myc* DNA and the number of PCR cycles. The PCR amplification was performed with 2 pmol each of primers and 0.10 fmol of *v-myc* DNA as template. Each reaction mixture was injected into the HPLC-ECD through an Asahipak GF-510HQ column.

of each cycle were obtained from 0.10 attomole (amol) of the template DNA. First, it was tried to separate the PCR product by HPLC utilizing the Asahipak GF-510HQ column, but the mixture could not be separated under isocratic conditions. Then a TSK gel HA-1000 hydroxyapatite column was tested in HPLC and succeeded in separating the PCR product from the other materials, as the latters were trapped by the column. Fig. 22 shows chromatograms of the PCR product (a) and 10 pmol of primer **13** (b) under isocratic conditions. Although a 5-fold molar excess of primer over the amount used for PCR amplification was injected, only a very small peak was detected in (b), whereas the peak was observed after PCR amplification (a). Thus, the purification of the PCR product was achieved with this procedure. The ECD response was plotted against the number of PCR cycles as shown in Fig. 23. No peak was obtained by HPLC-ECD with the non-ferrocenylated primer under the same experimental conditions. Both plots of Fig. 23 were almost linear, and the slopes of both lines were the same within error (0.027 ± 0.005). The ratio of the intercepts of both lines was 0.7 ± 0.2 for male/female. This value is in agreement with the theoretical value of 0.5, given the quality and complexity of the chromosomal DNA used. It should be noted that these PCR products could not be detected by fluorescence stain with ethidium bromide after gel electrophoresis. To confirm these results a sample of patients with Duchenne muscular dystrophy (DMD)

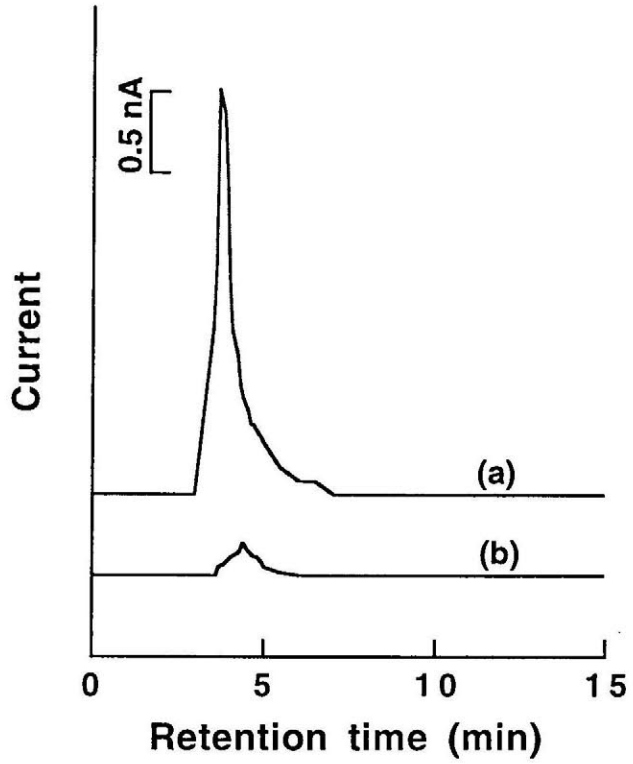


Fig. 22. Chromatograms of the PCR product after 20 cycles of amplification of the sample containing 0.10 amol of the muscular dystrophin gene (a) and 10 pmol of PCR primer (b). Conditions were as follows: column, TSK gel HA-1000; flow rate, 1.0 ml/min; applied potential, +0.7 V.

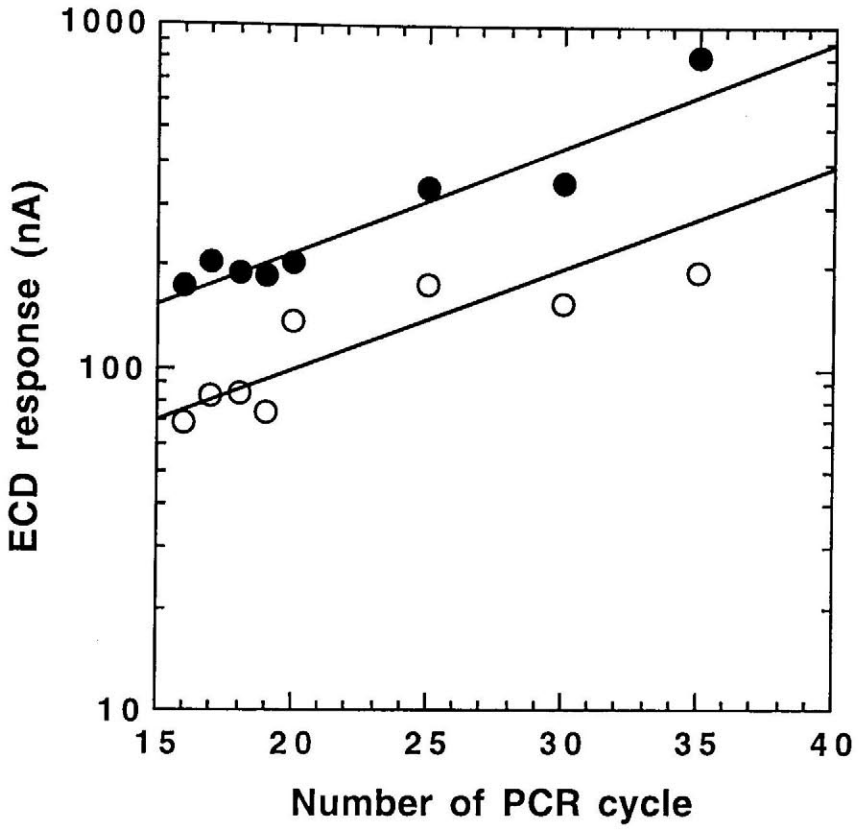


Fig. 23. Correlation between the ECD response of the PCR product from the muscular dystrophin gene and the number of PCR cycles. The PCR amplification was performed using 2 pmol each of primers and 0.10 amol of a male (open circles) or female (closed circles) chromosome sample. Each reaction mixture was injected into the HPLC-ECD equipped with a TSK gel HA-1000 column directly. Conditions were as follows: column, TSK gel HA-1000; flow rate, 1.0 ml/min; applied potential, +0.7 V.

lacking the DNA region in question was analyzed analogously but it gave no ECD peaks as expected.

3-4. Discussion

As described above, ferrocenylated oligonucleotides were employed for the first time as PCR primers (13). It was found that they serve as good primers to give correct ferrocenylated PCR products. The latters were in turn analyzed by HPLC-ECD at fmol level. Since PCR amplified the sample DNA at least 10^3 fold, the detection limit of the HPLC-ECD analysis was lowered to amol level as far as the starting DNA is concerned. In addition to qualitative analysis, quantitation of DNA was also attempted. PCR amplification of target DNA proceeded exponentially with the ferrocenylated primers under the conditions employed (Figs. 21 and 23). Extrapolation of the calibration curves to PCR cycle number zero allowed to estimate the initial amount of template DNA or gene with reasonable accuracy. Thus, this method solves some of the difficulties which conventional quantitative PCR encountered. Quantitation of DNA by means of PCR is usually carried out by co-amplifying the sample and reference DNA in one vial (21, 54). This technique is based on an assumption that two of them are amplified at the same rate but it is not certain whether this assumption holds in any case. In other words, competitive PCR is successful only when a reliable reference is available. On the other hand, one does

not worry about a reference, since this technique does not rely on it. The PCR/ECD technique relies only on the regularity of DNA amplification, which usually holds at low cycles of PCR and is verifiable experimentally (Figs. 21 and 23). The only problem to worry about is the small quantity of DNA amplified under such conditions and hence the analytical sensitivity is crucial. Electrochemical detection, fluorescence and several other methods meet this requirement but the linearity of the calibration curve holds in a range of three orders of magnitude in ECD (Fig. 19) vs two orders in fluorescence (55). This is obviously one of the advantages of the ECD method.

Chapter 4. Development of a double stranded DNA selective sensor based on a ferrocenylated threading intercalator

4-1. Introduction

DNA sensors are of crucial importance for the quick diagnosis of genetic diseases. Currently available DNA sensors utilize piezoelectric (56, 57) or electrochemical transducers (22, 58 - 61) and are based on the double helix formation of a probe DNA bound on an electrode with sample DNAs in solution. Electrochemically active ligands are then allowed to bind to the resulting duplex, thereby bringing about a current response. But because of the low selectivity of these ligands for single and double stranded DNA (23, 24), the sensitivity of this method is not high enough to be employed practically.

Recently, threading intercalators were developed in which their substituents can penetrate between the base pairs when bound to DNA (25, 28). This type of intercalators form a very stable complex with DNA, and they dissociate much more slowly from DNA than do classical intercalators (Fig. 24) (61). When electrochemically active groups are attached to a threading intercalator, the resulting molecule will serve as a double stranded DNA selective ligand. This idea was substantiated with a naphthalene diimide carrying two ferrocenylated moieties at its ends (**15**). Naphthalene diimides are typical threading intercalators

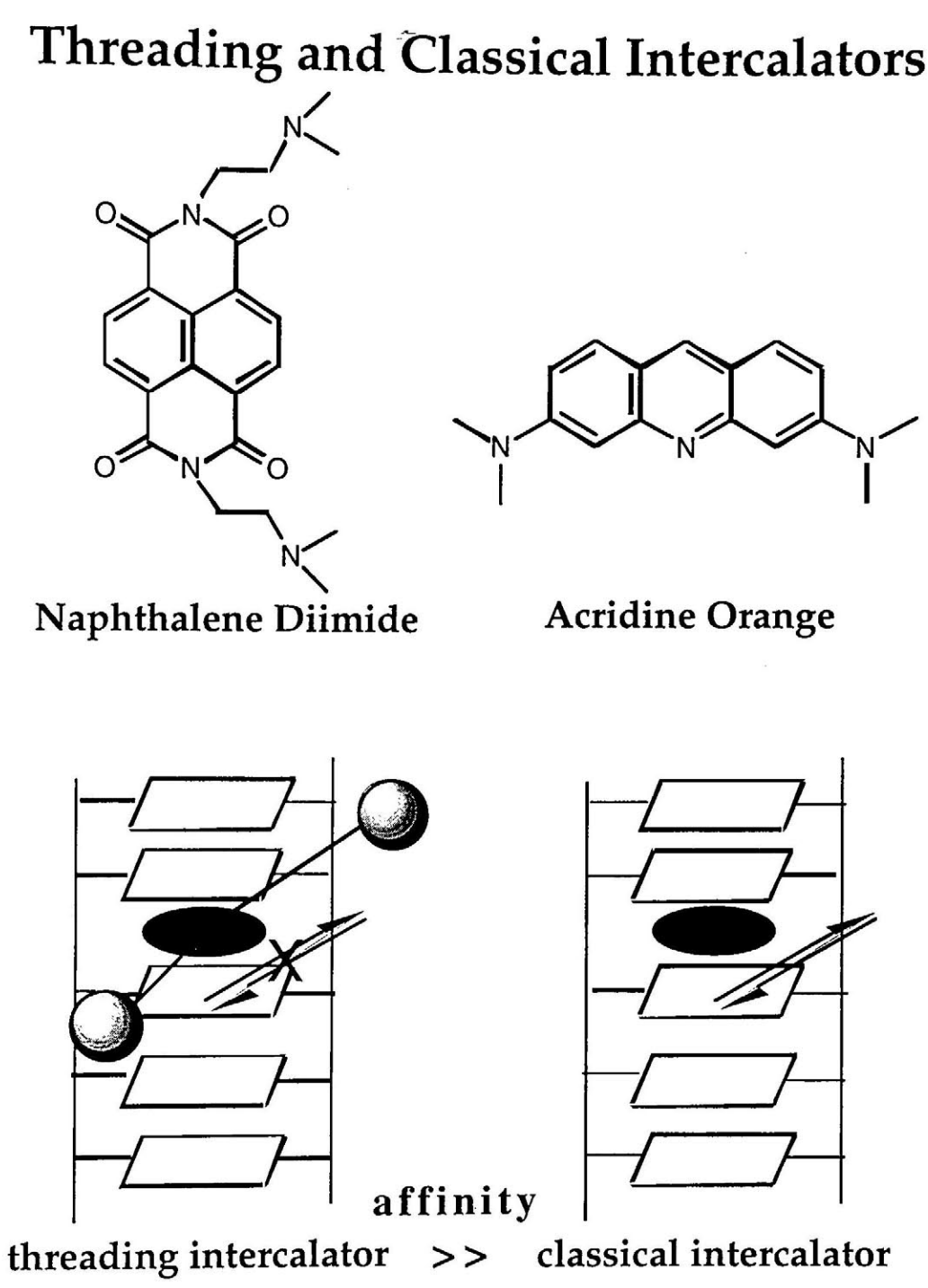


Fig. 24. The difference in the binding affinity of a threading or classical intercalator for double stranded DNA

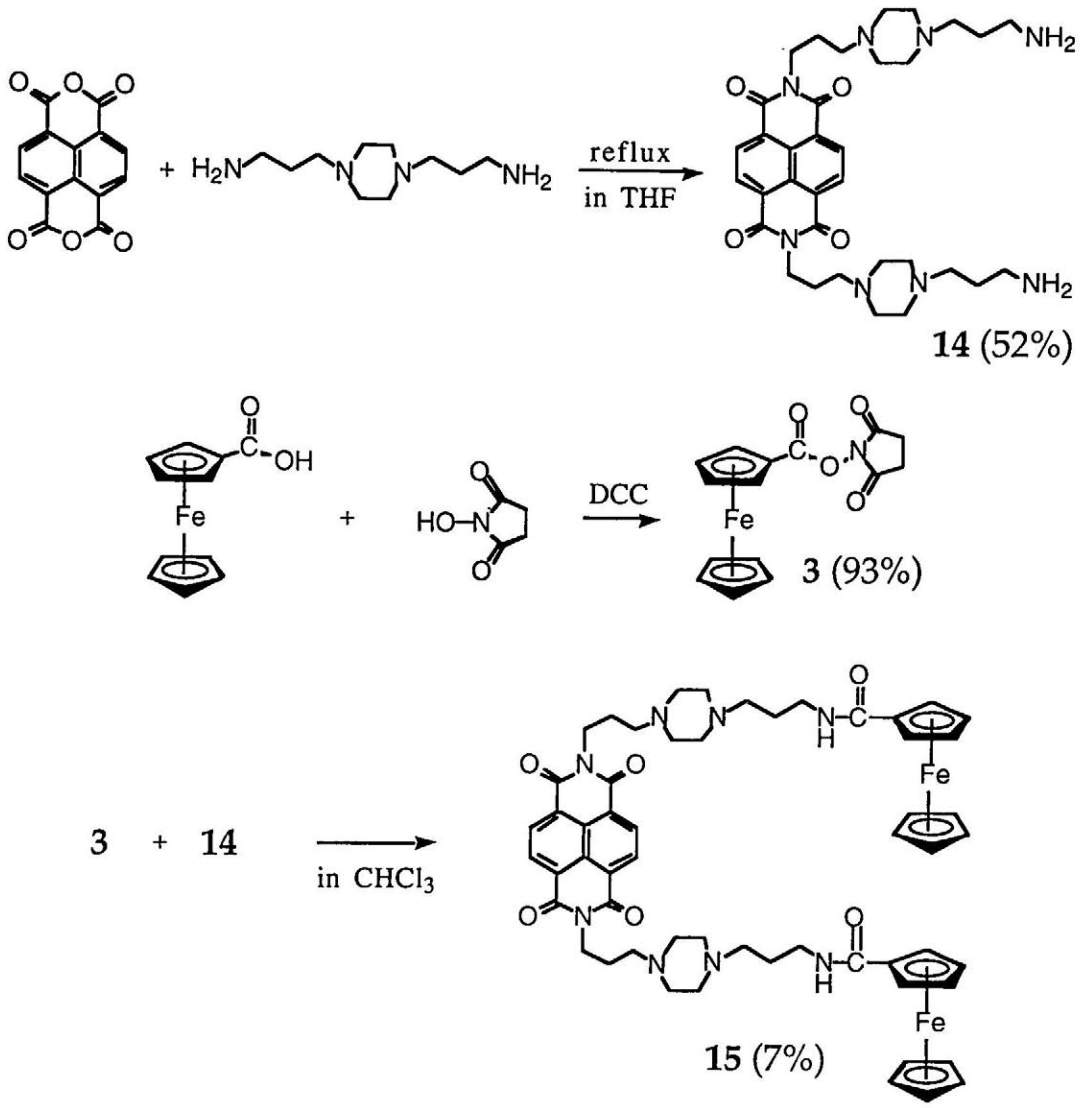
(22, 61) and ferrocene undergoes a stable reversible redox reaction (7). In addition, the bulkiness of ferrocene should serve as an anchor to protect the complex of **15** with the double stranded DNA from dissociation.

4-2. Materials and methods

Ferrocenylated naphthalene diimide (**15**) was synthesized according to the route shown in Scheme 2.

A. Synthesis of *N,N'*-bis[[4-(3-aminopropyl)piperazinyl]propyl] naphthalene diimide

Two grams of naphthalene-1,4,5,8-tetracarboxylic dianhydride (Aldrich) and 40 mL of *N,N'*-bis(3-aminopropyl)-piperazine were refluxed in 30 ml of tetrahydrofuran for 8 h. The solution was allowed to cool and poured into ether (1 L). The precipitate formed was dissolved in a small amount of chloroform-methanol (1:1) and poured into ether (1 L). The filtrate was concentrated and the residue was recrystallized from ether to yield 0.80 g (18%) of **14** as a solid: mp > 300 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.58 (4H, m), 1.95 (4H, m), 2.27 - 2.52 (20H, m), 2.71 (4H, t), 4.28 (4H, t) and 8.75 (4H, s). Found: C, 60.27; H, 7.51; N, 16.58%. Calcd for $C_{34}H_{48}N_8O_4 \cdot 2.5H_2O$: C, 60.27; H, 7.83; N, 16.54%.



Scheme 2. Synthesis of ferrocenylated naphthalene diimide **15**

B. Synthesis of *N,N'*-bis[[4-(3-ferrocenylaminopropyl)piperazinyl] propyl]naphthalene diimide

Compounds **14** (300 mg, 0.47 mmol) and **3** (600 mg, 1.8 mmol) were dissolved in chloroform (30 ml) and the solution was stirred at room temperature for 50 h. The solvent was removed and the residue was chromatographed on a column of silica gel (Merck 60, methanol). The fraction showing an Rf of 0.2 on TLC (methanol) was collected and the solvent was removed under reduced pressure. The residue was dissolved in a small amount of chloroform and the solution was poured into ether after filtration. The solid obtained was dissolved in a small amount of methanol and poured into water. The solid obtained was further purified by recrystallization from acetone. Yield 33 mg (7%) as a crimson solid: mp 237 - 240 °C; TLC Rf = 0.2 (methanol); IR (KBr) 1650, 1630 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.70 (4H, m), 2.0 (4H, m), 2.5 (20H, m), 3.4 (4H, q), 4.2 (10H, s), 4.3 (8H, m), 4.7 (4H, t), 8.8 (4H, s). Found: C, 62.72; H, 6.10; N, 10.37%. Calcd for $C_{56}H_{64}Fe_2N_8O_6$: C, 63.60; H, 6.10; N, 10.60%.

C. Absorbance measurements

Absorption spectra were measured on a Hitachi U-3210 UV-visible spectrophotometer with a 2-nm band pass in a 1-cm cell with temperature regulation.

The binding affinity of **15** for double stranded DNA was determined by Scatchard analysis using the condition probability method of McGhee and von Hippel (62) shown below:

$$r/L = K(1-nr)\{((2\omega-1)(1-nr)+r-R)/(2(\omega-1)(1-nr))\}^{n-1}$$
$$\{(1-(n-1)r+R)/2(1-nr)\}^2$$
$$R = \{(1-(n+1)r)^2+4\omega r(1-nr)\}^{1/2}$$

where r is the moles of ligand bound per base pair, L is the free ligand concentration, K is the observed binding constant, n is the maximum number of ligands bound per base pair, and ω is the cooperativity parameter.

The binding affinity of **15** for denatured DNA was determined by a Benesi-Hildebrand plot with the following equation:

$$1/\Delta A = 1/l\Delta\varepsilon CT + (1/K'l\Delta\varepsilon CT)(1/CN^0)$$
$$K' = \text{y-intercept/slope}$$

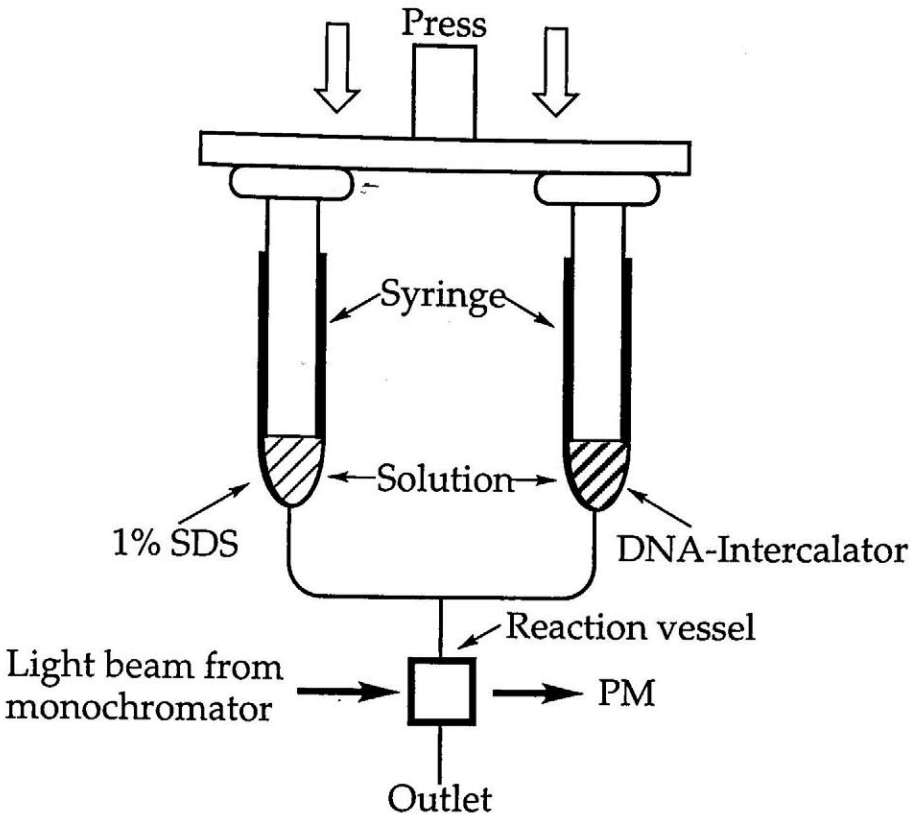
where ΔA denotes the difference in the observed absorption, l is the cell length, Δε is the extinction coefficient of the bound diimide minus that of the free diimide (εB-εF), K' (nK, K) is the affinity constant, n is the number of binding sites per base pair, CN^0 is the total concentration of polyU in base, and CT is the total diimide concentration.

The dissociation constant (k_d) of **15** from single or double stranded DNA was determined by the stopped flow method. Two kind of reaction solutions (1% sodium dodecyl sulfate (SDS) and DNA - **15** complex) were mixed instantaneously using a piston, and then the change in the absorption spectrum was measured soon after mixing. This absorbance change arose from dissociation of the DNA - **15** complex by SDS into an SDS - **15** complex (Fig. 25). In this reaction, the dissociation of the DNA - **15** complex was rate-limiting because of a very fast association of **15** and SDS. Accordingly the value determined can be approximated as a k_d.

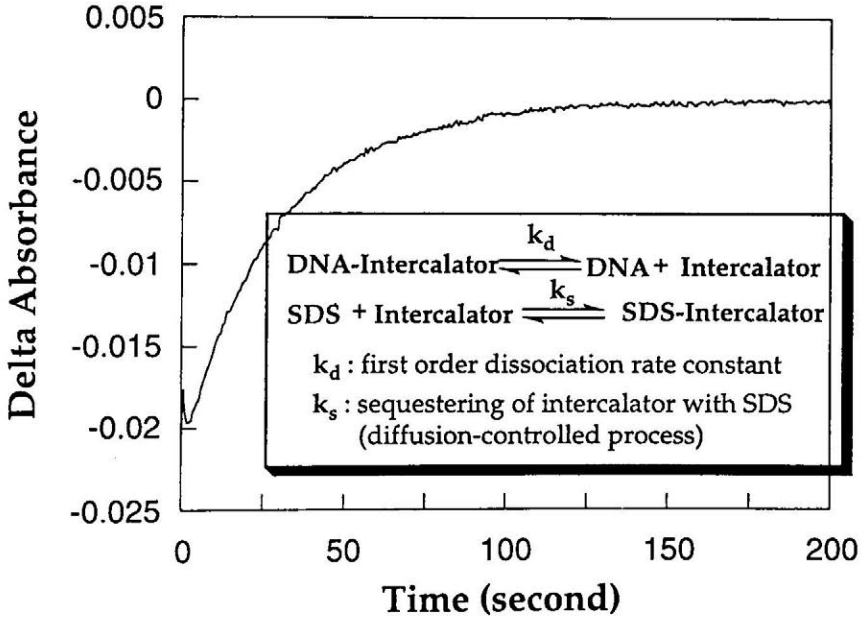
The value of k_d and the half life of the complex (τ) were obtained by fitting the data to the equation shown below, which is composed of two terms. Terms A_1 and A_2 would represent the dissociation processes from the major and minor grooves, respectively. Alternatively, they could represent the dissociation processes from the AT-rich and GC-rich sites, respectively.

$$A = A_1e^{-k_1t}+A_2e^{-k_2t}$$

$$k_d = 1/\tau = (A_1k_1+A_2k_2)/(A_1+A_2)$$



The stopped-flow technique



Stopped-flow kinetic trace for SDS-driven dissociation

Fig. 25. Calculation procedure of dissociation rate constants.

D. Preparation of a gold film electrode

The electrode and insulation pattern were drawn on a transparent sheet with 20-fold magnification. A high-resolution plate (Konica) was set on a photographing stand and exposed for 25 s. Next this plate was developed in a developer for 2 min. After the plate was washed with a 10% acetic acid solution, it was soaked in a fixation liquid for 1 min and washed with tap water. A photomask was obtained by drying this plate and it was used to prepare a film electrode by the lift-on method (Fig. 26). After a positive resist (OFPR II) was swabbed on a polycarbonate sheet by a spinner, it was exposed by the photomask with the electrode pattern. This sheet was soaked in a developer and it was vapor-deposited with gold using a vacuum deposition apparatus. The resist thus obtained was dissolved in ethanol and subjected to

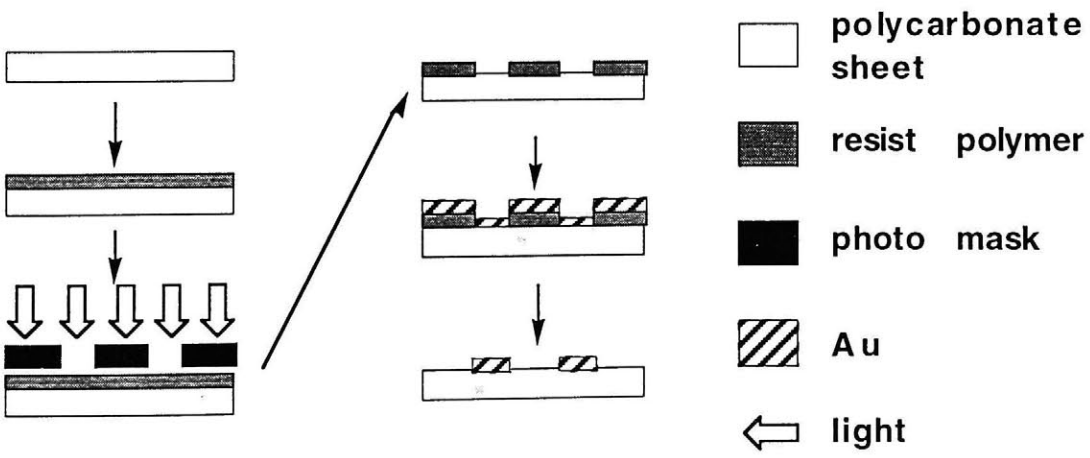
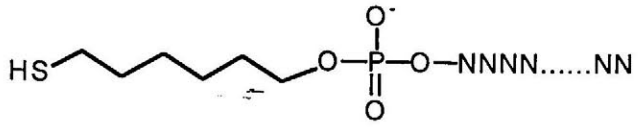


Fig. 26. Preparation of a gold film electrode by the lift-on method.



16, 17

Compound	NNNN.....NN
16	5'- TTTTT TTTTT TTTTT TTTTT -3'
17	5'- CCGCT TATCT TCAGT TTTCG -3'

Fig. 27. Structures of mercaptohexyl-oligonucleotides **16** and **17**.

ultrasonic washing. After drying of this sheet, a positive resist was swabbed with the same operation.

E. Modification of a gold film electrode with a mercaptohexyl-oligonucleotide

Oligonucleotides and mercaptohexyl-oligonucleotides (Fig. 27) were custom synthesized by Takara Shuzo, Kyoto: **18**, 5'-TTTTT TTTTT TTTTT TTTTT-3'; **19**, 5'-AAAAA AAAAA AAAAA AAAAA-3'. The target sequence of **17**, the *CTR* gene fragment was prepared as described in Chapter 2. The DNA concentration was determined by optical measurements. The following molar absorption coefficients (nucleotide phosphate unit) at 260 nm were used: **16**, 162,600 $M^{-1}cm^{-1}$ at 260 nm; **17**, 197,200 $M^{-1}cm^{-1}$; **18**, 162,600 $M^{-1}cm^{-1}$; **19**, 243,400 $M^{-1}cm^{-1}$ (41).

Modification of the gold electrode with a mercaptohexyl-oligonucleotide (oligos **16** and **17**) were performed in the following way. First, the custom synthesized mercaptohexyl-oligonucleotide was mixed with dithiothreitol (DTT) to prevent formation of a disulfide bond. Then 100 µl of ethyl acetate were poured into 100 µl of the oligonucleotide solution and after this mixture was stirred for 10 min, the aqueous phase was recovered and lyophilized. The mercaptohexyl-oligonucleotide was dissolved in 10 mM tris buffer (pH 7.5) and 1 µl aliquot was dropped on the gold electrode and the latter maintained at 4 °C for 2 h. The modified gold electrode was washed with 10 mM tris buffer (pH 7.5) to recover the unreacted mercaptohexyl-oligonucleotide.

F. Quantitation of a modified oligonucleotide on the electrode surface

The modification rate of mercaptohexyl-oligonucleotide was estimated by HPLC analysis. The HPLC system consisted of an L-6200 dual plunger pump (Hitachi, throughout), a sample injector with a 1 ml sample loop, L-4200 variable-wavelength detector and D-2500 data processing apparatus. Five hundred µl of the mercaptohexyl-oligonucleotide solution before or after reaction were injected into the HPLC system at 25 °C: flow rate, 1.0 ml/min; buffer A, 0.1 M TEAA buffer (pH 6.9); buffer B, acetonitrile; linear gradient, 10 - 40% B in 30 min. TSK gel ODS-80TS (4.6 x 150 mm,

Tosoh) was used for all the samples. Their reaction efficiencies are shown in their respective figure legends.

G. Electrochemical detection of target DNA

All cyclic (CV) and differential pulse (DPV) voltammetry experiments were performed in a single compartment cell with a three electrode configuration on an CV-50W potentiostat (BAS) equipped with a GATEWAY 2000 computer and a gold electrode, and the reference electrode was an Ag/AgCl. A platinum stick was used as the counter electrode. Solutions (50 mM potassium acetate (pH 5.2)) were deoxygenated by purging with nitrogen gas for 20 min prior to the measurements.

Measurement was carried out in the following way (Fig. 28): hybridization of target DNA with the immobilized probe DNA was performed that 2 μl of adequate concentrated target DNA solution was dropped onto an electrode surface at 4 °C for 20 min. In the case of plasmid DNA, the solution was heated at 80 °C for 30 min before hybridization. After hybridization, the electrode was washed with 50 mM potassium acetate (pH 5.2) for 5 s to eliminate the unhybridized target DNA. Next the electrode was immersed in a 1 mM solution of **15** for 5 min to allow intercalation. And then the electrode was washed with milli-Q water for 5 s with vortexing to eliminate the non-specifically bound **15**. Finally electrochemical analysis was performed for this electrode.

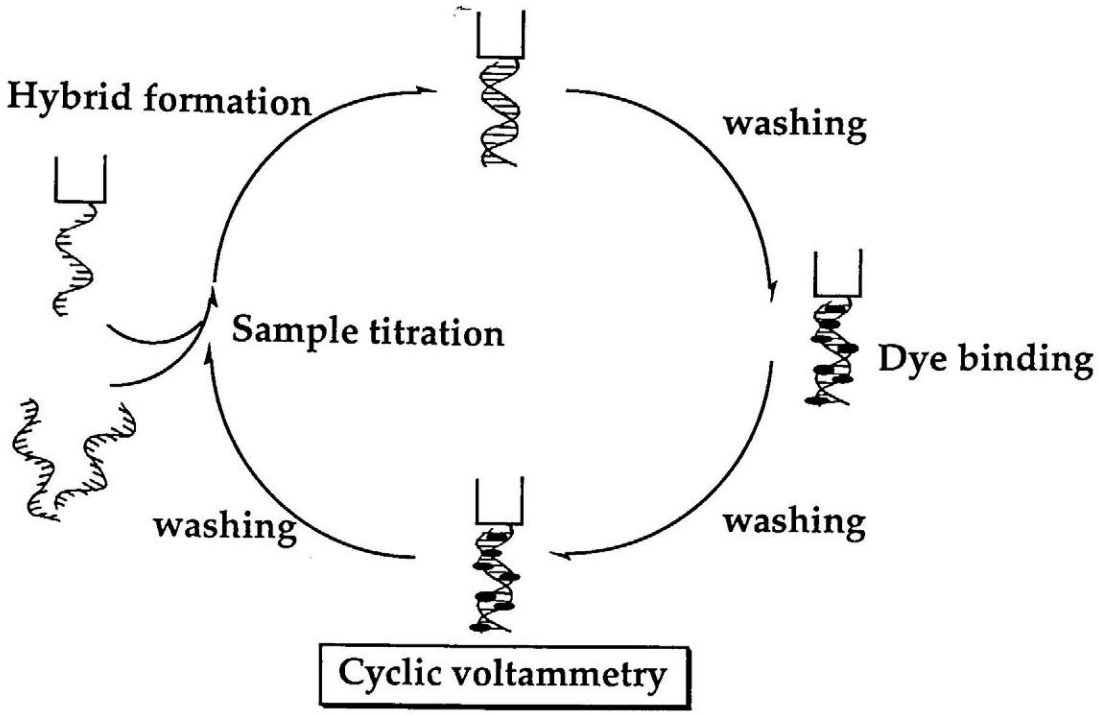


Fig. 28. Procedure of electrochemical detection with a DNA sensor.

4-3. Results

A. Absorption spectroscopy

Compound **15** was titrated spectroscopically with sonicated calf thymus DNA to examine whether it binds to DNA by intercalation (Fig. 29). Compound **15** undergoes hypochromic and bathochromic shifts of the absorption band of the naphthalene diimide chromophore upon binding to sonicated calf thymus DNA, which is indicative of DNA intercalation.

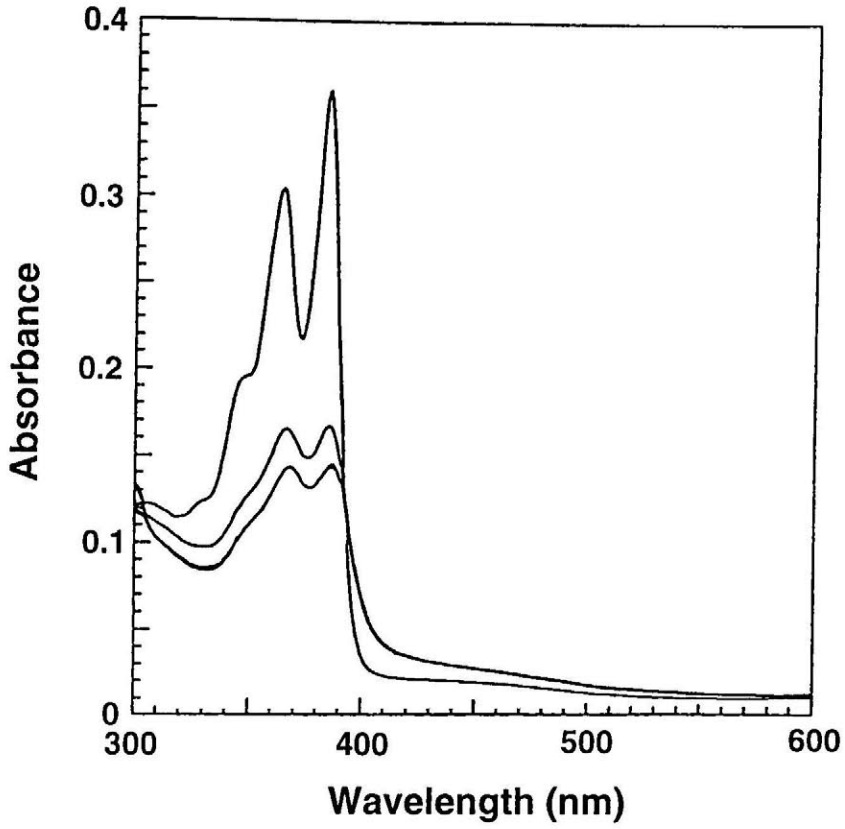


Fig. 29. The change of absorption spectrum for **15** with calf thymus DNA. The experiment was performed in 10 mM MES and 1 mM EDTA (pH 6.24) at 20 °C. The concentration of **15** was 12.3 μM and that of calf thymus DNA were 0, 35, 69, 104, 138 μM (from top to bottom). The last three traces overlapped.

Spectrophotometric titration data on **15** with calf thymus DNA analyzed by Scatchard plots gave a binding constant of 1.3 x 10^5 M^{-1} in 10 mM morpholinoethanesulfonate (MES) buffer and 1 mM EDTA (pH 6.24) at 20 °C (Fig. 30). Whereas, the binding constant of **15** for the denatured DNA was 3.0 x 10^4 M^{-1}, a value one fourth that for duplex DNA (Fig. 31).

Next, the SDS-driven dissociation experiments were carried out with the complex of **15** and intact or denatured calf thymus DNA. It was found that the half life of the complex of **15** with the intact DNA was 100 s at 20 °C (Fig. 32), whereas the dissociation of **15** from the denatured DNA was completed within 4 s (data not shown). These results suggest that **15** can be utilized as a double stranded DNA selective ligand for a DNA sensor.

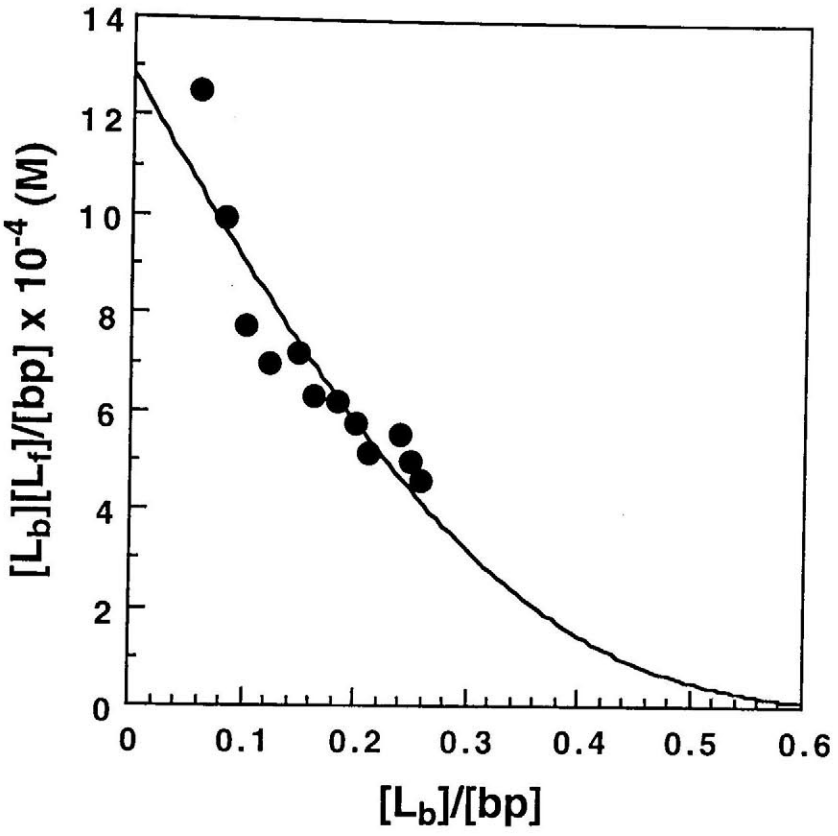


Fig. 30. Scatchard plots for the binding of **15** to calf thymus double stranded DNA. The experiment was performed in 10 mM MES and 1 mM EDTA (pH 6.24) at 20 °C. The solid lines indicate computer fits of the data with use of the McGhee-von Hippel model with the n and K values. $[L_b]$ and [Lf] represent the concentrations of ligand bound to DNA and free ligand, respectively, and [bp] is the concentration of DNA (per base pair).

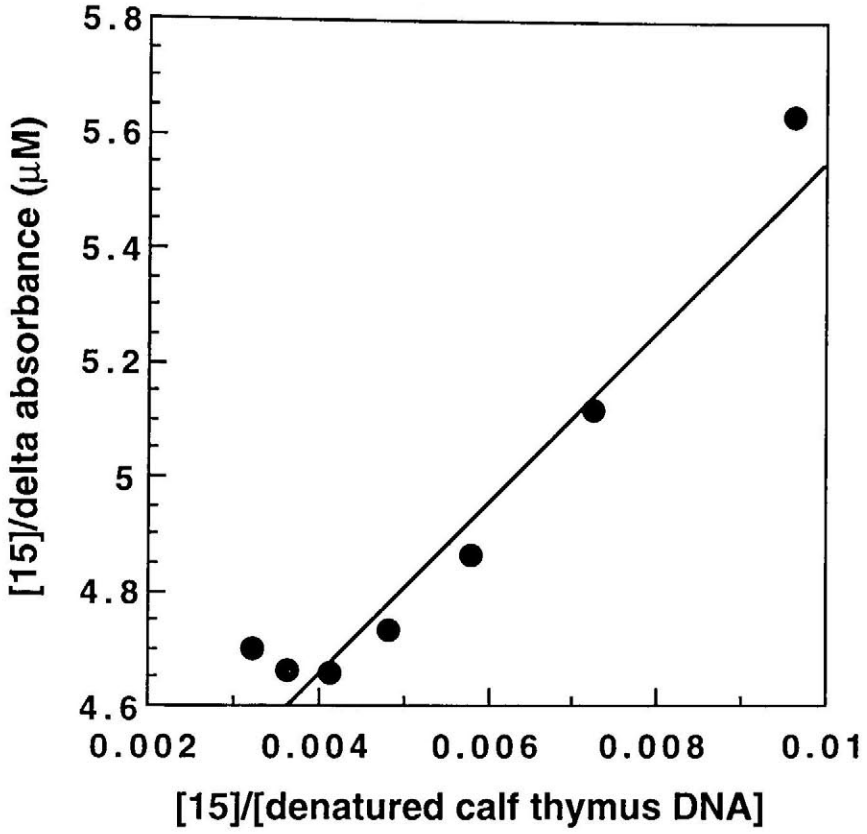


Fig. 31. Benesi-Hildebrand plot for equilibrium binding of **15** to denatured calf thymus DNA. The experiment was performed in 10 mM MES and 1 mM EDTA (pH 6.24) at 20 °C. Data were fitted by the linear least-squares regression and the correlation coefficient was 0.93.

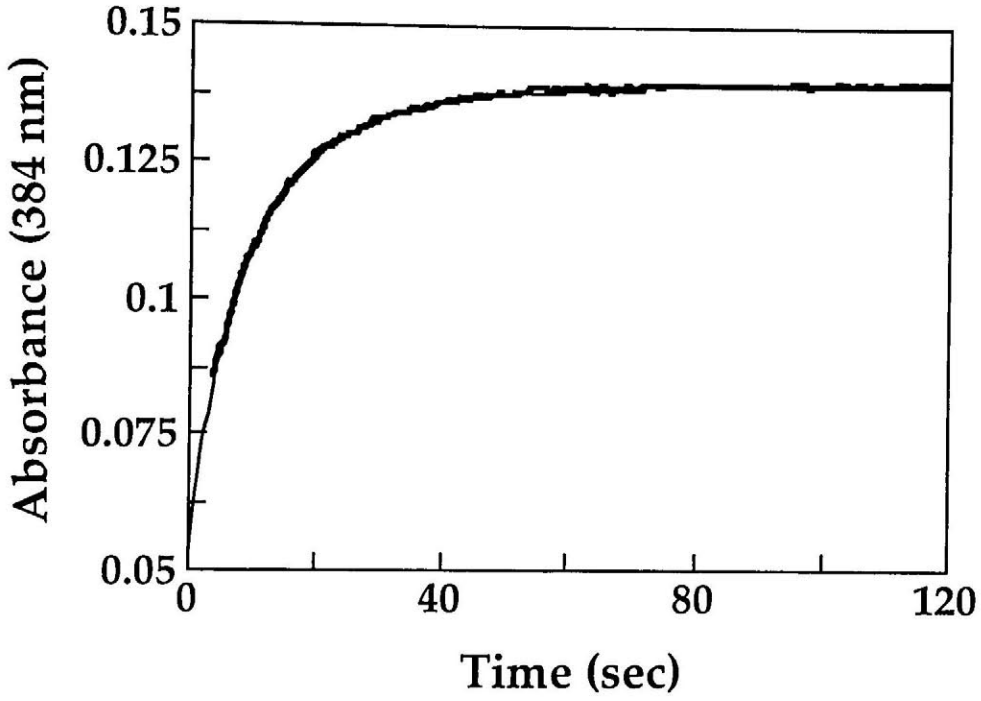


Fig. 32. The SDS-driven dissociation experiments carried out with the complex of **15** and calf thymus DNA. The experiment was performed in 10 mM MES and 1 mM EDTA (pH 6.24) at 20 °C. The concentration of compound **15** was 10 mM and that of calf thymus DNA 99 μM.

B. Electrochemical detection of 20-meric adenine

In order to assess whether an oligonucleotide-modified electrode can be used for electrochemical analysis, cyclic voltammetry (CV) was run first with dT_{20}-modified electrode and dA_{20} target oligonucleotide. Fig. 33 shows that the cyclic voltammogram with the dT_{20}-modified electrode gave an oxidation-reduction response for ferrocene in the range of +0.4 to +0.6 V. This result suggested that this electrode can be used for electrochemical analysis. CV was also determined with unmodified and dT_{20}/dA_{20} complex modified electrodes and the current response was plotted against the scan rate (Fig. 34). It was found that the current response for the modified electrode was larger than that for the unmodified one and that the response for the single stranded DNA-modified electrode was larger than that for the double stranded DNA-modified one. This phenomenon can be explained by the fact that the DNA density on the electrode surface was lower for the single strand-modified electrode than for the double stranded one, so that compound **15** was more accessible to the electrode surface.

Next the effect of washing of the electrode was investigated. Washing was supposed to eliminate non-specific binding to the double stranded site. Fig. 35 shows cyclic voltammograms of compound **15** for single or double stranded modified electrode. In the voltammogram of the single stranded modified electrode there

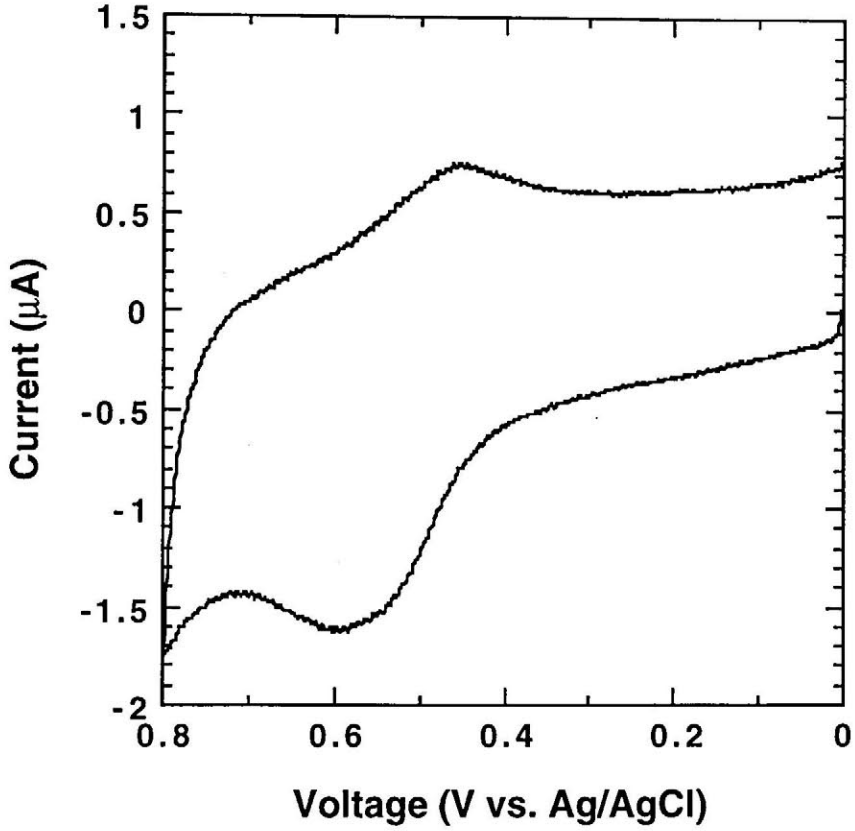


Fig. 33. Cyclic voltammogram for **15** with a dT_{20}-modified gold electrode. Conditions were as follows: solution, 0.1 mM **15** in 50 mM KCl; amount of modified **16**, 50 pmol/mm^2; scan rate, 500 mV/s.

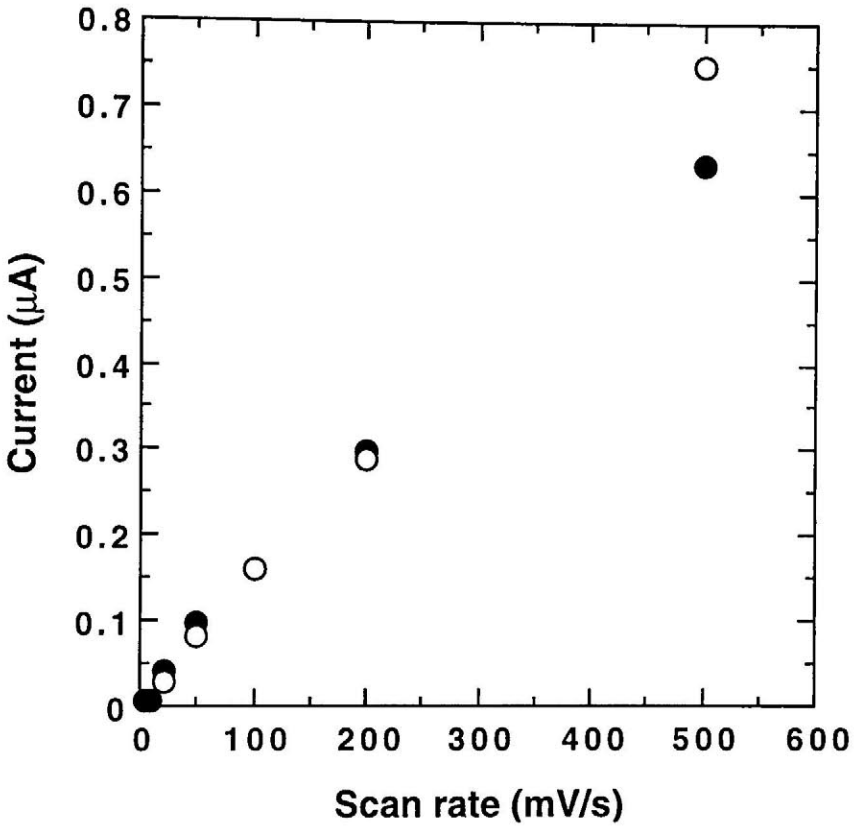


Fig. 34. Behavior of **15** on double strand- (closed circles) and single strand- (open circles) modified electrode surfaces. Conditions were as follows: solution, 0.1 mM **15** in 50 mM KCl; amount of single strand DNA-modified (**16**), 50 pmol/mm^2; amount of double strand DNA (**16**, **19**), 50 pmol/mm^2 each.

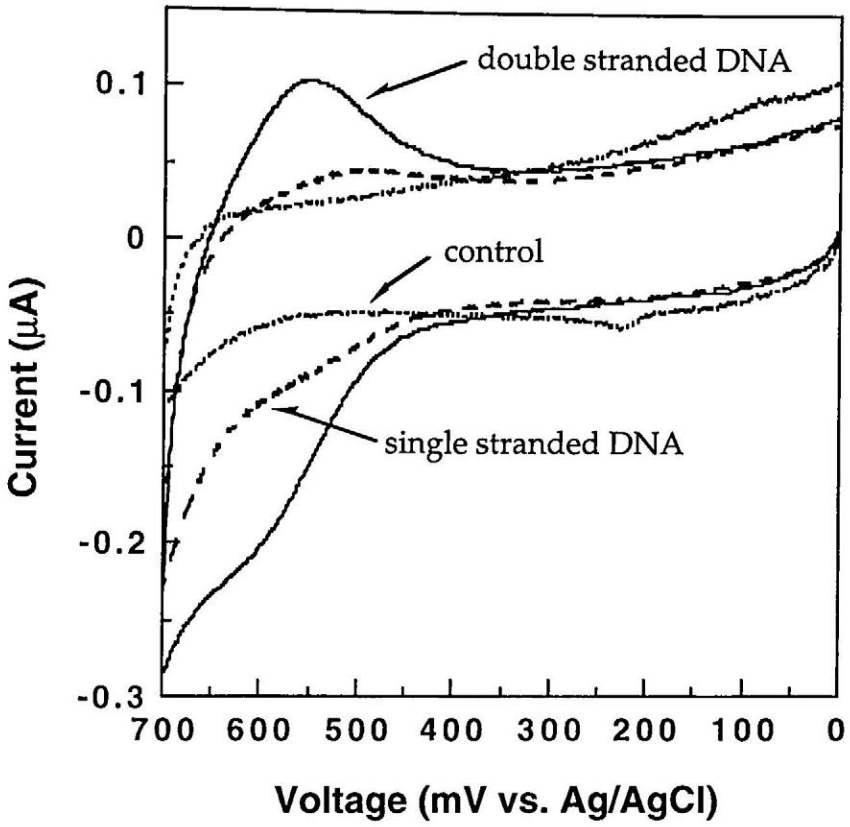


Fig. 35. Cyclic voltammograms for **15** after washing. Conditions were as follows: solution, 0.5 mM **15** in 41 mM AcOK/AcOH buffer (pH 5.2)/30% DMSO; scan rate, 100 mV/s.

is no current response of **15** observed under conditions where the double stranded modified electrode gave 0.1 μA at +550 mV. These results implied that only those intercalators bound to the double stranded site were resistant to washing of the electrode.

Finally quantitative analysis of dA_{20} target DNA was attempted with the dT_{20}-modified electrode (Fig. 36). A current response due to the ferrocenyl moieties of **15** increased linearly up to 100 pmol with an increase in the amount of dA_{20} (correlation coefficient 0.994) and then leveled off as shown in Fig. 36 (a). The amount of dA_{20} at the break point (109 pmol) was in agreement with the amount of the probe dT_{20} immobilized on the electrode (95 pmol). The detection limit was about 10 pmol of target oligonucleotide. DNA (dT_{20}) non-complementary to the sequence of dT_{20} was tested as a control, but the current response obtained was barely above background (Fig. 36, b). This procedure could be repeated several times and the current response per immobilized oligonucleotide was constant even with electrodes of different lot.

C. Electrochemical detection of a natural gene

Encouraged by this result, analysis of an actual sample was attempted by this method. The sample adopted was part of the yeast choline transport gene in a plasmid vector (3.0 kb) as a target sequence (34). Oligonucleotide **17** was immobilized on a gold electrode to 24 pmol/mm^2 and then the plasmid sample (10 fmol)

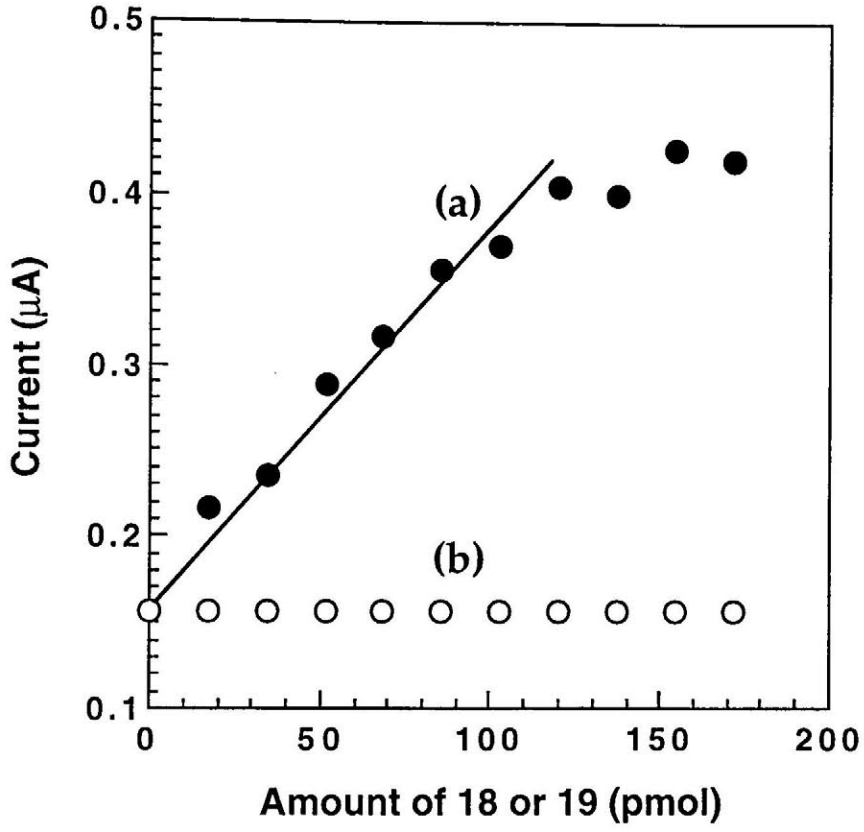


Fig. 36. Titration of **16**-modified Au electrode with **18** or **19**. Conditions were as follows: solution, 50 mM KCl; amount of modified **16**, 95 pmol/mm^2; scan rate: 20 mV/s; pulse amplitude, 50 mV. In this figure, (a) and (b) represent the titration of **19** and **18**, respectively.

was allowed to hybridize on the electrode.

Differential pulse voltammography (DPV) was run as shown in Fig. 37 (a). The current of 0.36 μA was obtained for 10 fmol of the plasmid DNA. This value is 10^3-times greater than that for dA_{20} (Fig. 36, a), demonstrating that the efficiency of detection is much greater for the sample with a larger size, presumably because the extra duplex region of the plasmid not involved in the complexation with the probe can bind **15**. No response was observed for plasmid DNA without the choline transport gene under the same conditions (Fig. 37, b).

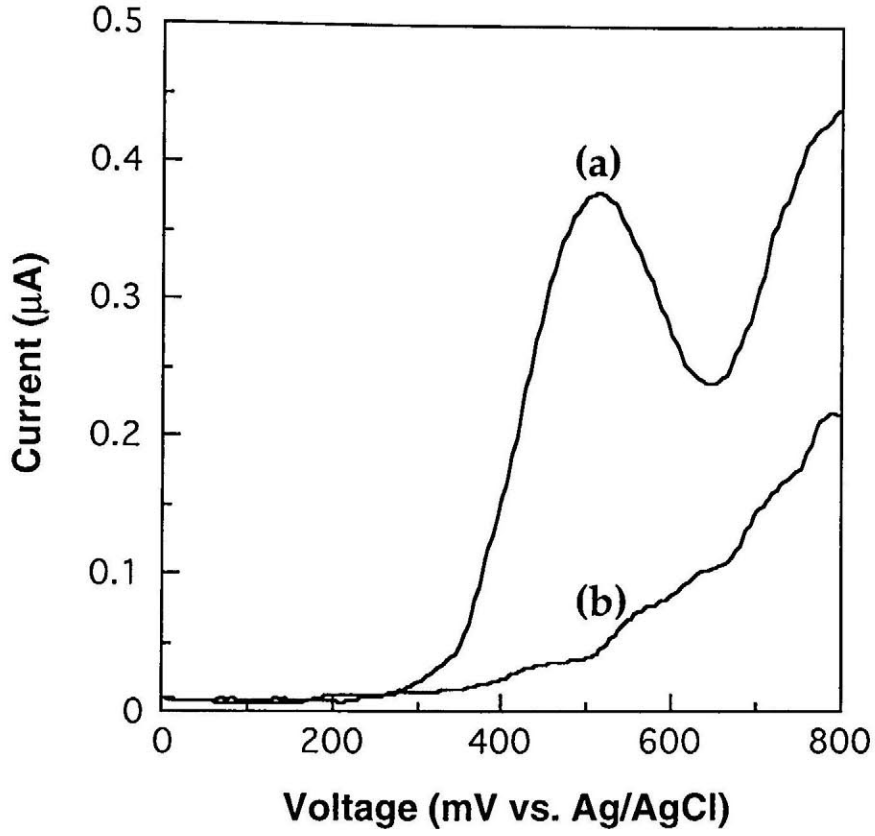


Fig. 37. Differential pulse voltammograms of **15** in the presence (a) or absence (b) of target sequence on plasmid DNA. Conditions were as follows: solutions, 50 mM AcOK/AcOH (pH 5.2); amount of modified **16**, 28 pmol/mm^2; scan rate, 20 mV/s; pulse amplitude, 50 mV.

4-4. Discussion

Although some pioneering work was reported on DNA sensors (22, 56 - 61), none are in practical use, solely because they are not sensitive enough. This happens because no ligands are known which can discriminate double stranded from single stranded DNA rigorously. As described above, compound **15** can do this by a fairly large margin. Owing to this excellent property of **15** the unhybridized portion of the probe does not participate in the redox reaction on the electrode. In conclusion, the electrochemically active threading intercalator **15**, coupled with a DNA-carrying electrode, enabled the sensitive detection of target DNA fragments. The whole manipulation was carried out in a short period of time as long as the sensor is ready and yet the sensitivity of detection is ultra-high, making this sensing system most feasible for practical use in DNA analysis and gene diagnosis.

Chapter 5. Conclusion

As described above, a novel analytical method for nucleic acids has been devised, which is based on the redox property of ferrocene and an electrochemical detection system.

By ferrocenylating an oligonucleotide it was made electrochemically active so that it can be used as a probe for detecting DNA and RNA at fmol level with the HPLC-ECD method. It was confirmed that the ferrocenylated DNA probe binds with the target DNA as strongly as do non-ferrocenylated counterparts (62). The resulting complex of the ferrocenylated DNA was as stable as the non-ferrocenylated counterparts and was feasible for analysis by HPLC. The detection limit of this HPLC-ECD method was 1 fmol in the case of 1.52 kb of *v-myc* DNA (7). This level of sensitivity is nearly of the same order as that of the end-radiolabeling method. As for the future prospect, the HPLC-ECD method may find use (a) in the rapid detection of a target gene, (b) in gene diagnosis, and (c) in the rapid monitoring of eukaryotic messenger RNAs in the cell.

Next, PCR was coupled with the HPLC-ECD method to improve analytical sensitivity. Although PCR is used in many fields of molecular biology, ferrocenylated oligonucleotides have never be used as a PCR primer. It was found for the first time that a ferrocenylated oligonucleotide can serve as a PCR primer to give

correct PCR products, which in turn were feasible for electochemical analysis. The detection limit of the PCR/ECD method was lowered to sub-amol level as far as the starting DNA is concerned. In other words, the analytical sensitivity was enhanced at least a thousand fold by coupling HPLC-ECD with PCR .

The key step in the ECD method lies in the separation of PCR products from an excess of primers. This is currently done by means of HPLC with a gel filtration or hydroxyapatite matrix under isocratic conditions. The former matrix is obviously better as long as the PCR product is long enough, about 1 kb, since the latter traps the primer and is deteriorated upon repeated use. The former has some drawbacks, however; it cannot separate PCR products of 0.5 kb from 20-25 base long primers under isocratic conditions. It seems certain, therefore, that availability of matrices capable of separating DNA of several hundred bases in length under isocratic conditions makes this method even more versatile in DNA analysis.

Finally, based on the information obtained above, a novel DNA sensor was devised. Thus, ferrocene was attached to a threading intercalator, naphthalene diimide. The resulting compound (**15**) showed high selectivity for double stranded DNA; it undergoes dissociation much more slowly from double stranded DNA than from single stranded one. This property is ideal as a DNA sensor, since the latter has to discriminate hybridized double

stranded DNA from unhybridized single stranded one. Several DNA sensors had been reported in the literature, but none is in practical use solely because they are not selective enough for double stranded DNA. In this sense, this DNA sensor is the first to have a potential to be used in practice. In the near future, it will be possible, by extending this DNA sensor system, to prepare a micro-electrode which enables one to handle many DNA samples at a time and/or automatically.

In conclusion, the electrochemical method for nucleic acid analysis developed in this work opened a new vista in analytical molecular biology. The author hopes that this novel method facilitates analysis of nucleic acids considerably and contributes to diagnosis of genetic disorders in humans.

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