

Accounts

Highly Sensitive Probe for Gene Analysis by Electrochemical Approach

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Electrochemically active DNA ligands, including ferrocenyl DNA oligonucleotides and ferrocenyl naphthalene bis(carboximide), are reviewed in connection with development of an electrochemical DNA sensor. Ferrocenyl oligonucleotides are used as an electrochemical probe DNA detectable by high performance liquid chromatography with the electrochemical detector (HPLC-ECD) system and they enable the target DNA analysis at femto mole level. Ferrocenyl oligonucelotides are useful also as a polymerase chain reaction (PCR) primer to realize quantitative PCR. Ferrocenyl naphthalene bis(carboximide) is a general DNA detecting ligand. When coupled with a probe DNA immobilized on an electrode, the target DNA from plasmid, chromosomal DNA, and total RNA extracted from several sources can be analyzed with very high sensitivity. Ferrocenyl naphthalene bis(carboximide) bound to the DNA duplex serves as a pseudo-polyferrocene array and this unique architecture leads to enhanced electron transfer between reduced glucose oxidase and the electrode. Unpaired base(s) present on the mismatched base(s) of the DNA duplex can be detected by observation of a decrease in the amount of ferrocenyl naphthalene bis(carboximide) bound to such a mismatched DNA duplex. Hybridized DNA on the DNA microarray was visualized electrochemically in the aqueous solution by the scanning electrochemical microscope (SECM) coupled with the ferrocenyl naphthalene bis(carboximide).

Thanks to the completion of the human genome sequencing, we are ready to apply the genetic information to human welfare. Analysis and use of any given gene begin with its detection on the basis of the fact that DNA exists as a duplex of complementary sequences. Thus, when a single stranded DNA of natural or synthetic origin is allowed to react with a mixture of genes, it will pick up its complementary target specifically. If a probe DNA is labeled appropriately, the duplex formed is detected easily and even more with high sensitivity. In DNA sensors or genosensors, this duplex formation or hybridization occurs on the sensing surface carrying a (single-stranded) probe DNA.[1–3] Such a type of sensor is important for gene diagnosis and related purposes. Information of the duplex formation can be monitored by the mass change on the quartz surface carrying a probe DNA (quartz crystal microbalance)[4,5] or by the dielectric character on the gold surface carrying the probe DNA (surface plasmon resonance).[6] To enhance sensitivity of detection, a large molecule such as a liposome[7] or antibody[8] is attached to the sample DNA in some cases. Fluorometric detection was performed in the optic fiber carrying a probe DNA coupled with a fluorescently labeled DNA sample. These DNA sensor techniques helped rapid detection of target DNA.[9,10]

Electrochemical DNA sensor is another means of DNA detection and it has a potential to surpass the existing sensors with respect to sensitivity, quickness and cost.[11,12] Initial research in this area aimed at obtaining an electrochemical signal of redox reaction in nucleic acid bases of DNA, which was enhanced by the addition of osmium(III) oxide-pyridine.[2] In the double-stranded DNA, nucleic acid bases are located inside the DNA duplex, shielded from the redox reaction which occurs outside the duplex including bulk solution and therefore the electrochemical signal based on nucleic acid bases of double-stranded DNA was smaller than that of single-stranded DNA.[13] Discrimination between single- and double-stranded DNA exploited this difference in electrochemical behavior. Stripping analysis in electrochemistry, which can pre-concentrate the DNA sample on the electrode, was also employed to develop a DNA sensor.[14,15] However, these techniques described above impair sample and probe DNA for analysis. Therefore, it is important to develop a non-destructive method and, more preferably, to use the DNA sensor repeatedly, as long as a labeling method for a probe DNA with a stable and electrochemically active molecule is aveilable. In addition, the development of an electrochemically active ligand with high specificity for double-stranded DNA is also important to detect the double-stranded DNA formed on the probe DNA immobilized on the electrode.

In this paper, we will discuss two types of DNA sensors developed by us and others. Some of the electrochemical DNA ligands give rise to electro-generated chemiluminescence,

which can be applied for DNA sensing.[16] In the meantime, it is known that the electric field can promote the rate of hybridization of DNA. Nanogene Inc. has developed a DNA sensor based on this principle, though the detection itself does not rely on electrochemistry.[17,18]

Electrochemically Active Probe DNAs

Ferrocene undergoes a stable redox reaction reversibly. Therefore incorporation of a ferrocene molecule in the DNA may be an effective means to construct an electrochemically active probe DNA. We first synthesized the ferrocenyl oligonucleotide **1** (Fig. 1) by the reaction of an oligonucleotide carrying amino alkyl chain at the 5′-terminus with an activated ester of ferrocenecarboxylic acid.[19,20] This probe DNA was allowed to hybridize with the sample DNA, the hybridized DNA was separated from probe DNA by HPLC and was detected from the electrochemical signal of the ferrocene with the ECD. This system can be run in the flow system of HPLC-ECD in a short period of time. Using this system, we succeeded in the detection of a cancer gene and yeast choline transport gene with a detection limit of 1 femto mole.[19] We can also detect and quantify picogram levels of mRNA from the total RNA extracted from rat brain.[19] We also used the ferrocenyl oligonucleotide as a PCR primer and obtained the ferrocenyl DNA double-stranded fragment from the PCR product, which is detectable in the HPLC-ECD system.[21] Since the PCR amplification proceeds exponentially (2^n) with the PCR cycle number n, extrapolation of a plot of the DNA amount against the PCR cycle should give the initial amount of DNA in the sample. This technique is called quantitative PCR. We applied ferrocenyl oligonucleotides to the quantitative PCR and quantified the amount of the muscular dystrophin gene from a human chromosome sample. Moreover, we were able to discriminate the two-fold difference in the initial DNA amount from male and female individuals.[21]

Ihara et al. developed a target DNA detection system using a sandwich method in which the target DNA can bind to two different probe DNAs attached to the electrode or ferrocene.[22] The target DNA is allowed to hybridize first with the ferrocenyl probe DNA (1) and then with another probe DNA (2) immobilized on the electrode, as shown in Fig. 2. Clinical micro sensors (CMS) Inc. exploits a DNA diagnosis chip based on a similar sandwich method.[23] These observations prove the importance of ferrocenyl oligonucleotides as an electrochemical probe DNA to detect the target gene. Letsinger's[24] and Yu's[25] groups separately reported a method to incorporate a ferrocene moiety in an oligonucleotide in a different manner (**2** and **3** of Fig. 1). An oligonucleotide carrying another redox active molecule of anthraquinone (**4**) was also reported together with its electrochemical behavior in an aqueous solution.[26]

Detection of Double Stranded DNA by an Electrochemically Active DNA Ligand

Some of the DNA ligands have a redox active character, which serves as an indicator of DNA information transduceable to an electrochemical signal. Table 1 summarizes typical DNA ligands, **5**–**12**, having an electrochemically active moiety. Figure 3 shows the principle of the DNA sensing method using an electrochemically active DNA ligand. Double-stranded DNA is formed between target DNA in the sample solution and the probe DNA on the electrode and therefore the ligand can be concentrated on the electrode as long as it has a preference for double-stranded DNA. This argument suggests that a ligand specific for double-stranded DNA is useful for DNA sensing with a DNA probe immobilized on the electrode. The DNA binding mode of the ligands is categorized into intercalation or groove binding for double-stranded DNA and electrostatic interaction. In principle, the former two interactions are associated with specificity for double-stranded DNA. However, when nucleic acid bases of single-stranded DNA are exposed to water, a stacking interaction of aromatic planes is also expected to work between nucleic acid bases and the ligand. Since the ligand has a cationic character so as to be soluble in aqueous medium, the ligand can also bind to single stranded DNA by the electrostatic interaction between the cationic ligand and polyanionic DNA. Therefore, these ligands in

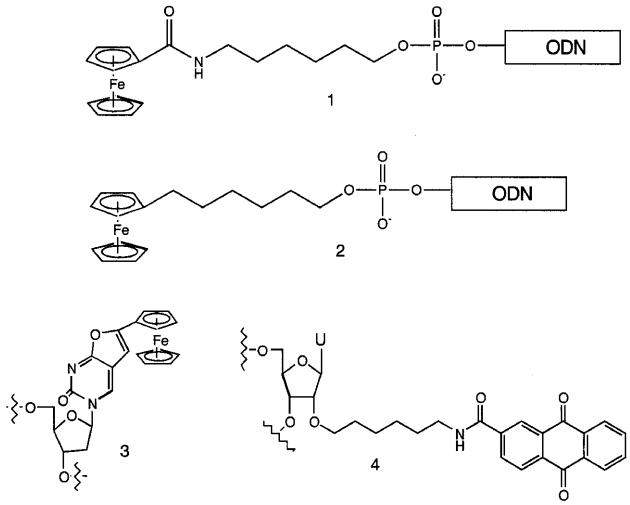


Fig. 1. Chemical structure of electrochemically active probe DNAs **1**–**4**. ODN refers to an oligodeoxynucleotide.

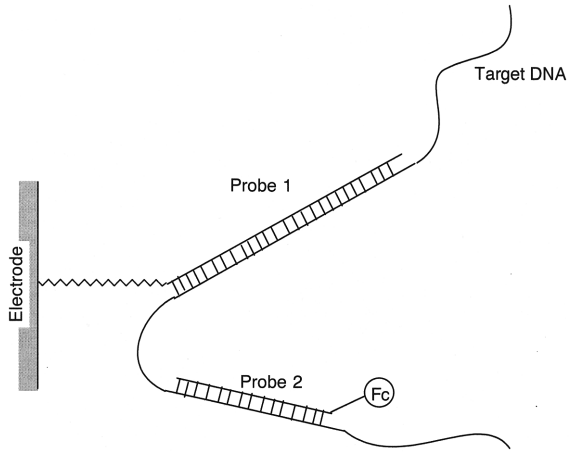
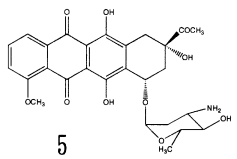
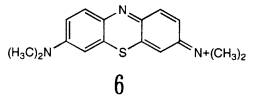
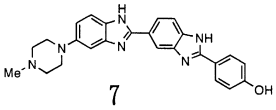
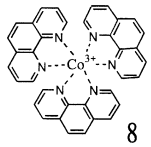
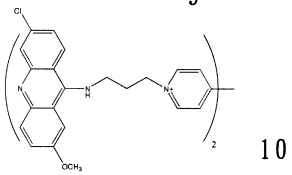
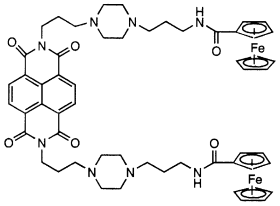
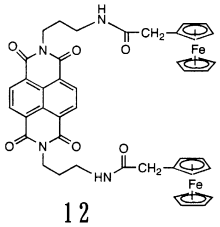


Fig. 2. Sandwich method using a ferrocenyl oligonucleotide **1** and a DNA-immobilized electrode.

Table 1. Example of the Redox Active DNA Ligands Reported Previously

Chemical structure	Binding mode	Base pair preference	Binding affinity M^{-1}	Redox potential V
 5	Intercalation	GC	2.35×10^5 [27]	−0.68[a] [28] (vs. SEC)
 6	Intercalation	GC	3.8×10^6	−0.26[b] [29] (vs. SEC)
 7	Groove binding	strong AT	7×10^6 [c] [30]	−0.55[d] [31] (vs. Ag/AgCl)
 8	Partial stacking, electrostatic interaction	None	1.74×10^3	−0.06[e] [32,33] (vs. Ag/AgCl)
 9	Partial stacking, electrostatic interaction	None	1.6×10^4 1.0×10^5	+0.14[f] [34] (vs. SCE) +1.06[g] [35] (vs. Ag/AgCl)
 10	Bisintercalation & groove binding	slightly AT	1.7×10^5	−0.34[h] [36] (vs. Ag/AgCl)
 11	Threading intercalation	None	1.3×10^5	+0.5[i] [37,38] (vs. Ag/AgCl)
 12	Threading intercalation	not determined	not determined	+0.36[j] [39] (vs. Ag/AgCl)

a) 5 mM sodium phosphate buffer–50 mM NaCl (pH 7.0). b) 25 mM sodium phosphate buffer–75 mM NaCl (pH 7.0). c) 50 mM Tris–100 mM NaCl (pH7.5). d) 1/15 M phosphate buffer (pH 7.0). e) 5 mM Tris–20 mM NaCl (pH 7.1). f) 5 mM Tris•HCl buffer–50mM NaCl (pH 7.1). g) 50 mM phosphate buffer–80 mM NaCl (pH 6.8). h) 0.1 M Tris–0.1 M NaCl (pH 8.0). i) 10 mM MES buffer–1 mM EDTA (pH 6.24). j) 0.1 M AcOK–AcOH buffer–0.1 M KCl containing 40% DMSO (pH 5.6).

common could not show so high discrimination ability for double-stranded DNA over single-stranded. In addition to specificity for double-stranded DNA, the ligand should undergo a stable and reversible redox reaction, though Hoechst 33258 (**7**) is not a reversible redox-active compound. Metal chelate derivatives (**8**, **9**) undergo a reversible redox reaction in

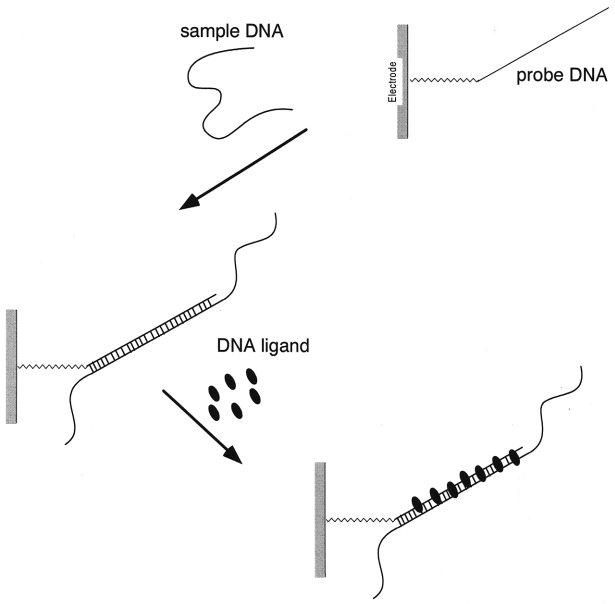


Fig. 3. Principle of a DNA sensor using a ligand specific for double stranded DNA coupled with a DNA-immobilized electrode.

an aqueous medium. As for the DNA binding manner, some of the chelates do intercalate partially into double-stranded DNA, but the main force in the DNA binding is the electrostatic interaction. Bard and co-workers studied the interaction of metal chelates in detail by the electrochemical method and showed that the potential shift of a metal chelate depended on the electrostatic or hydrophobic interaction with the DNA duplex.[40,41]

A DNA sensor system using metal chelating ligands was reported by Mikkelsen's[32,33] and Wang's groups.[14,15] After hybridization, the peak current is nearly doubled, suggesting the interaction between the ligand cationic site and anionic phosphate sites of DNA. The DNA phosphate sites are nearly doubled upon hybridization with DNA of the same length. When hybridized with a DNA fragment longer than the probe DNA, the current increased with an increase in the length of target DNA because of an increase in the amount of the phosphate sites of DNA on the electrode. Many ligands including **5**–**9** depicted in Table 1 do not show high specificity for double-stranded DNA.[42–46] We synthesized the mixed ligand, bisacridinyl viologen **10**, carrying intercalating and groove binding sites in the same molecule.[36] This ligand could bind to double-stranded DNA with increased affinity. We also succeeded in synthesizing mixed ligands carrying repeated viologen units[47–49] and the acridinyl viologen connected through a different length of alkyl chain.[50] The ligands are required to possess high discrimination ability between single- and double-stranded DNA, but not to show DNA sequence selectivity such as simple GC or AT preference, because if they showed high DNA sequence selectivity, the current response could differ among sample DNAs with a different GC content. Ordinary intercalators show GC preference, whereas groove binders show high AT preference, as shown in Table 1. Mixed ligand **10** synthesized by us prefers AT slightly.[48]

We synthesized a threading type intercalator **11** to achieve high specificity for double-stranded DNA.[37,38] The threading intercalator formed a DNA complex where one of the substituents is lying in the major groove and the other in the minor groove. These substituents act as an anchor to prevent dissociation of the ligand from double-stranded DNA. The threading type ligand does not have such stability for single-stranded DNA because it does not exert any special effect in the interaction of threading ligand and single-stranded DNA. Dissociation rate constants of the ligand **11** are 4.2 s^{-1} for single-stranded DNA and 0.056 s^{-1} or 80 times smaller for double-stranded one. This ligand binds to double-stranded DNA every one base pair, following the nearest-neighbor exclusion model. Incidentally, the ligand did not show base pair preference, as inferred from a binding study with $[poly(dA–dT)]_2$ and $[poly(dG–dC)]_2$. All of the data on this ligand show that it is a good candidate for DNA sensing using a probe DNA immobilized on the electrode. When this ligand was applied to the electrochemical DNA sensor with an oligonucleotide probe immobilized on the electrode, complementary oligonucleotide or natural DNA fragment from plasmid DNA could be detected with very high sensitivity.[38] Under the best experimental conditions so far attained, as small as several ten zepto moles of target DNA can be detected. The reason for such high sensitivity in this system is not clear now, but we have evidence which suggests that the catalytic current through the ferrocene-coated DNA duplex is responsible. When applying such a DNA sensor for clinical use, accumulation of data of the PCR product and chromosome DNA is needed, but the examples of electrochemical DNA sensors reported to date are limited to those on oligonucleotides and plasmid DNA. We applied this system to the PCR-amplified DNA, chromosome DNA and mRNA and were able to detect the target sequence using an oligonucleotide probe immobilized on the electrode.[51] Another advantage of this ligand **11** is that it gives the current response at the positive potential (+0.5 V relative to Ag/AgCl, Table 1), where there is no intervention from the redox reaction of oxygen dissolved in the electrolyte solution.

Catalytic Current in DNA Detection

The thiol–gold linkage is usually used in the immobilization of a probe DNA on the electrode. Thus, a gold electrode is treated with a solution containing a thiolated oligonucleotide.[37,38] Barton and co-workers studied the properties of DNA immobilized gold electrodes in detail.[29,52] They showed that the DNA duplex is arranged on the electrode surface as a monolayer with 60 pmol cm^{-2} with a tilt of the strand by about 40 degrees from the surface. This is like a DNA film covering the gold electrode. Barton and co-workers prepared a DNA film in which the distance between distamycin (**5**) and the electrode is varied by changing the site of attachment of **5** on the oligonucleotide and studied the electron transfer reaction between **5** and the electrode.[28] From these experiments, they proposed the electron transfer mechanism through the π-stack of the base pairs of double stranded DNA. Throp and co-workers showed the mediation reaction of the unpaired guanine bases

on double stranded DNA through ruthenium complex **9** bound to DNA.[53] They used an indium tin oxide electrode in these experiments, because this mediation could not be observed on the DNA-immobilized gold electrode because of cleavage of the sulfur-gold linkage under the voltage required in this measurement. Xanthon constructed a DNA sensor based on this mechanism.[54]

Ferrocenylnaphthalene bis(carboximide) **11** developed by us bound to DNA every other base pair on the double-stranded DNA where the ferrocene moieties are arranged along the DNA groove.[38] This complex is regarded as a pseudo-polyferrocene coating the DNA duplex and therefore the electron transfer through this array was expected (Fig. 4). In fact, we observed an electron transfer between the electrode and the reduced glucose oxidase generated from the reaction of glucose oxidase and glucose (Fig. 4).[55] Experimental results showed that ferrocenyl naphthalene bis(carboximide) **11** bound to the double-stranded DNA-immobilized electrode enhanced the catalytic current arising from the electron transfer between the reduced glucose oxidase and the electrode. This result suggested that the electrochemical signal is enhanced further upon duplex DNA formation in this system. In summary, the electron transfer occurs through the ferrocene array covering the groove of DNA duplex in our system, whereas it occurs through the π-stack base pairs of DNA duplex in Barton's system.

Mismatch Detection

DNA mismatch detection has become increasingly important from a viewpoint of single nucleotide polymorphisms (SNPs). It is now well perceived that a small difference in DNA sequences can be used as a marker of individual genetic traits. Moreover, analysis of SNPs will be a great help to predict the possible side effect of drugs and the risk diagnosis of such syndromes as hypertension and cancer. Ordinarily, this detection is performed as follows. Sample DNAs carrying a certain allele are allowed to hybridize with a proper DNA probe sequence to form a mismatched duplex, containing an un-paired region on the DNA duplex. Since the mismatched DNA duplexes are unstable compared with the fully matched DNA duplex, one can discriminate mismatched DNA duplexes from fully matched ones from a difference in their melting temperatures (T_m). Therefore, proper setting of the hybridization temperature and/or the washing temperature after hybridization is of utmost importance for successful detection of the mismatch by a DNA sensor. Heller and co-workers developed a catalytic mismatch detection system. Soybean peroxidase (SBP) was attached to an oligonucleotide (model of the sample DNA), which was then allowed to hybridize with the complementary oligonucleotide (model of the DNA probe) immobilized on the electrode with an osmium-containing polymer cover.[55] In the presence of hydrogen peroxide, a catalytic current was observed upon hybrid formation.

Barton and co-workers reported a novel mismatch detection system using the electron transfer reaction through the π-stack of nucleic acid bases of duplex DNA (Fig. 5A).[57] They observed an effective electron transfer reaction between the intercalator and the electrode in the system where the intercalator is bound to the surface of the double-stranded DNA-immobilized electrode. The signal was enhanced by the electrocatalytic reduction of $Fe(CN)_6^{3-}$ by Methylene Blue **6**.[58] When mismatched base(s) exist in the DNA duplex, the electric charge or the efficiency of electron transfer through the base π-stack decreased.[58]

We established another mismatch detection system based on the difference in the amount of **11** bound to matched or mismatched DNA duplex (Fig. 5B).[59] In this experiment, it is important to normalize the amount of probe DNA immobilized on

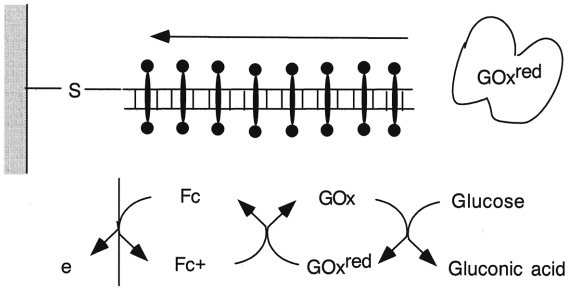


Fig. 4. Electron transfer between reduced glucose oxidase (GOx^{red}) and the electrode through a pseudo-polyferrocene wire with **11** bound to the DNA duplex.

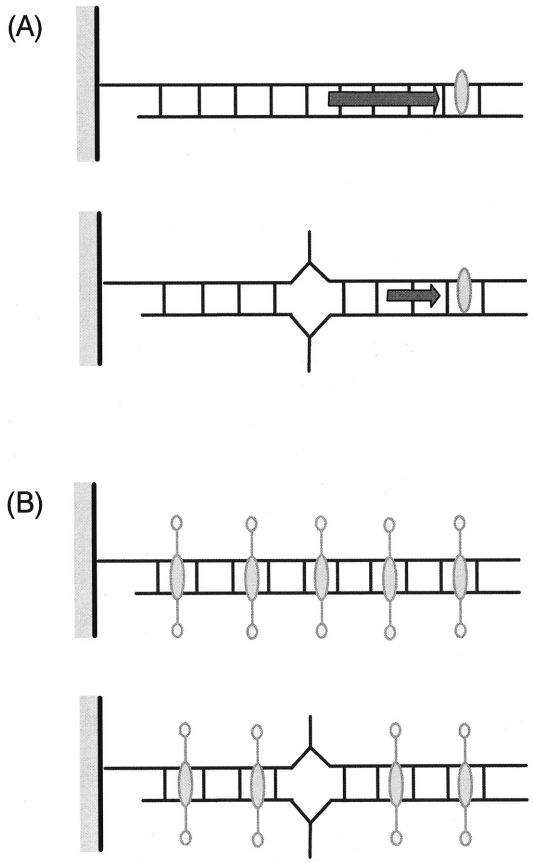


Fig. 5. Electrochemical detection of a single base mismatch based on charge transduction through DNA (A) and by reducing the amount of **11** bound to mismatched DNA duplex (B).

the electrode, as the immobilization efficiency of a thiolated probe DNA on a commercial gold electrode was variable by 20%. Therefore, we introduced the Δi value to correct for this variability. The Δi value, defined as $i/i_0 - 1$, where i_0 and i refer to the current before and after hybridization, respectively, represents a net increase in the current of the DNA duplex formed per single stranded DNA-immobilized electrode. We succeeded in the detection of single mismatched bases on the 20-mer DNA duplex using this system.[59]

The data were supported by quartz crystal microbalance (QCM) experiments, in which a frequency shift occurred in proportion to the amount of **11** concentrated per double stranded DNA on the electrode. It followed that nine molecules of **11** are bound to the duplex on average. In a similar experiment for a duplex with one mismatch, seven molecules of **11** were found to bind to it. In other words, the amount of bound **11** decreased with an increase in the amount of mismatched bases on the DNA duplex. Furthermore, the observation that nine molecules of **11** are bound to the fully matched DNA 20-mer is reasonable in light of the nearest-neighbor exclusion model. Additionally, we studied a temperature dependence of the current response of DNA-immobilized electrode after hybridization to obtain a T_m curve of the current response against temperature. Since the binding of **11** to duplex DNA enhanced the stability of the duplex, the difference in the stability between matched and mismatched DNA duplex was expanded in this system.

Application to a DNA Microarray

Recently, DNA microarray is receiving attention as a means of high-throughput analysis of DNA. This technique consists of the following procedures. Many different probe DNAs are immobilized on the surface of a glass plate (preparation of a DNA microarray). A labeled DNA sample is allowed to hybridize on the microarray plate. After washing the plate, the sample DNA bound on the plate is detected by the label's signal. However, the current DNA microarray technology cannot control the amount of probe DNA immobilized on the plate and therefore the quantity of the DNA sample on the plate is estimated only from the relative intensity of two different labels attached to the sample and reference DNA after hybridization with their equal mixture (competitive hybridization).

The electrochemical method described above has a potential to solve this problem, but there are several obstacles to overcome, such as the procedure to immobilize different DNA on different electrodes and the method to collect data from multiple electrodes rapidly. Calasso et al. reported a method to immobilize different DNA probes on different electrodes using electropolymerization (Fig. 6).[60] Thus, the DNA probe **13** carrying a pyrrole moiety was immobilized on a specific electrode by applying the voltage between this electrode and counter electrode. Nanogene Inc. reported another specific DNA immobilization method using electrophoresis.[17,18] When the voltage is applied to a specific avidin-coated electrode, the biotinilated DNA probe was electrophoresed or concentrated on this specific electrode where the DNA probe was immobilized

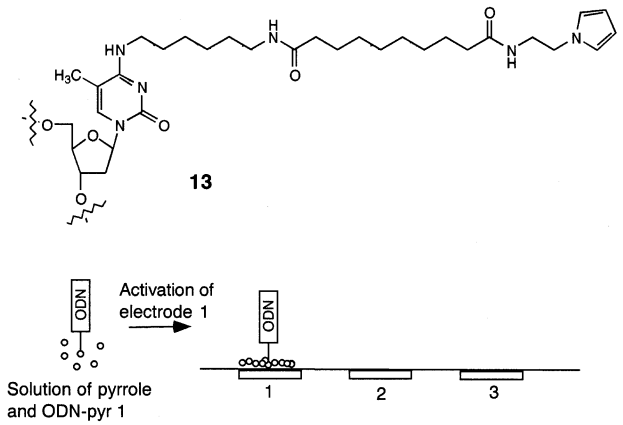


Fig. 6. Polypyrrole DNA chip on a silicone device. Electrochemical polymerization of oligonucleotide **13** attached to pyrrole (ODN-pyr, where ODN and pyr refer to oligodeoxynucleotide and pyrrole, respectively) occurrs on electrode 1 only to which the voltage is applied.

by the avidin-biotin interaction. However, both methods are time-consuming and unsuitable for the preparation of more than 100 microarray electrodes at a time.

We are developing the electrochemical visualization of an ordinary glass plate microarray using a scanning electrochemical microscope (SECM).[61] Thus, a DNA plate after hybridization was dipped in an electrolyte containing **11** and measured by SECM. Since **11** is bound to the double-stranded DNA region, the current was increased with an increase in the amount of DNA duplex region on the DNA microarray (Fig. 7). We succeeded in the detection of p53 polymorphism from the PCR product of patients. In SECM, which can deal with aqueous solutions, it is easy to set the temperature and salt concentration of the hybridization at will and this method is versatile and universal for gene analysis. Multi-electrode array should be also important for the quick diagnosis if the number of the electrodes is smaller than 100. When using **11** as an electrochemically active ligand for double stranded DNA, it is important how quickly the electrochemical signal of **11** can be acquired. In addition, it is desirable that the redox reaction occurs at a more negative side.

We designed and synthesized a new ferrocenyl naphthalene bis(carboximide) **12** which will meet these requirements. Note that the linker chain of **12** is shorter than that of **11**. Ligand **12** is present in non-ionic form in aqueous media, whereas **11** carrying two piperazine parts is present in dicationic form. The linker between the ferrocene and the amino moieties of **12** is electron-donating methylenes, while that of **11** is an electron-withdrawing carbonyl. Therefore, the redox potential of **12** is shifted to the negative side from that of **11** (+0.36 V, Table 1) because the ferrocene of **12** is more electron-rich than that of **11**, thereby shortening the time required for analysis.

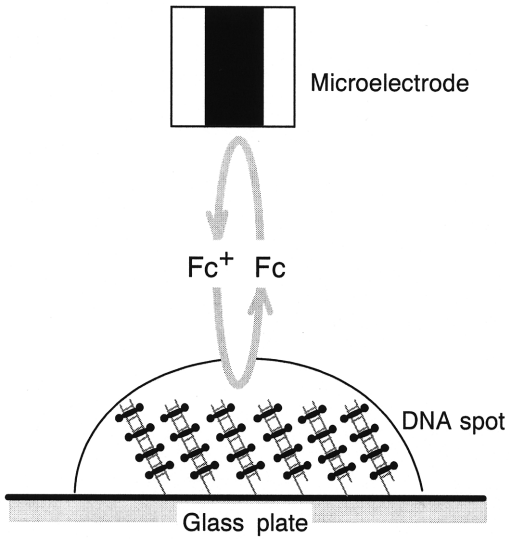


Fig. 7. Cartoon illustrating the mechanism of the increase in the current in the double stranded DNA region by the concentrated ferrocene parts of **11** in the SECM measurement. Fc and Fc^+ refer to ferrocene and oxidized ferrocene, respectively.

Conclusion

In the electrochemical DNA sensing, an electrochemically active ligand having high selectivity for double-stranded DNA is important not only as a universal DNA probe but also for highly sensitive DNA detection. One of the breakthroughs was presented in our electrochemical threading intercalators, **11** and **12**, consisting of ferrocene and naphthalene bis(carboximide). However, the demand for simpler, quicker and less expensive DNA sensing system never ceases and therefore a search and development of a ligand with even better properties have to be continued. In relation to the recent development of DNA microarray, the multi-electrode array system or electrochemical visualization by SECM will become more and more important in the near future

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