Electrochemical Molecular Beacon for Nucleic Acid Sensing in a Homogeneous Solution

Yusuke KITAMURA,† Kotaro MISHIO, Pelin ARSLAN, Boui IKEDA, Chiharu IMOTO, Yousuke KATSUDA, and Toshihiro IHARA†

Division of Materials Science, Faculty of Advanced Science and Technology, Kumamoto University, 2-39-1 Kurokami, Chuo-ku, Kumamoto 860-8555, Japan

† To whom correspondence should be addressed.

E-mail: ykita@kumamoto-u.ac.jp (Y. K.)
E-mail: toshi@chem.kumamoto-u.ac.jp (T. I.)
Abstract

A ferrocene (Fc) and β-cyclodextrin (βCyD) were modified at each end of stem-loop structured DNA as an electrochemical signal generator and its quencher, respectively, to give an electrochemical molecular beacon (eMB). Relatively high efficiency of the signal quenching was achieved by the inclusion complex (βCyD ⇌ Fc) formation that was induced on the stem structure of the closed form (= stem-loop structure) of eMB. With the addition of the target DNA, the structure of eMB opened to form a linear duplex, where the Fc dissociated from the βCyD to restore its intrinsic electrochemical signal. The signal contrast of the electric current for this off/on-type sensor was high, ca. 95. This technique did not require any modification of the electrode surface and it realized the detection of the target nucleic acids in a homogeneous solution with a high sensitivity using high-performance liquid chromatography (HPLC)
equipped with electrochemical detector.

**Keywords:** Nucleic Acids, Electrochemical biosensor, Molecular beacon, Inclusion complex, Ferrocene, Cyclodextrin, Homogeneous assay
Introduction

Various fluorescent molecules or nano-materials have been developed as probes for monitoring targeted biomolecules using various forms of spectroscopy. In most cases, these probes are required to provide a signal in a spatiotemporal manner. That is, they are designed to generate a signal by specific bio-chemical or -physical stimuli that are relevant to the biological events of interest. Molecular beacon (MB) is a typical stimulus-responsive fluorescent molecular probe consisting of an oligodeoxyribonucleotide (ODN) and a FRET (Förster resonance energy transfer) pair. The fluorescence from a dye attached to one end of the ODN is quenched with the acceptor attached on another end by FRET in the hairpin-form (stem-loop) structure of the MB. The interaction with the target DNAs or RNAs changes the conformation of the MB to a rigid linear duplex to break up the FRET pairing, then intrinsic fluorescence of
the dye is restored.² MBs have been widely used as general signal transducers for various biomolecules, even those other than DNA or RNA, such as small molecules, proteins with combined use of aptamers for their targets.³

Electrochemical techniques are cost-effective and potentially sensitive and versatile. Although various analytical platforms based on electrochemical responses have been proposed for studying specific interactions involving nucleic acids and/or proteins, the number of applications used in practical assays is quite limited compared with fluorescent techniques. This is mostly due to the lack of the means for modulation of an electrochemical signal in a homogeneous solution. Most electrochemical methods, therefore, require one of the participants in the interactions to be immobilized onto the electrode because signal contrasts arise as a result of changes in the distance between the electrochemically active probe and the electrode surface on the targeted events.⁴ The preparation of biomolecule-modified electrodes is time-consuming
and, generally, it causes difficulty in obtaining reproducible results using two electrodes
prepared even with the same protocol. If molecular quenchers of electrochemical signals are
available, electrochemistry-based MBs (eMBs) could be designed and electrochemical
monitoring for biological events could be performed in a homogeneous solution.

Previously, we proposed electrochemical DNA sensing using a ferrocene (Fc)-modified
DNA probe.\textsuperscript{4a, 4b, 4c, 5} In that series of studies, we found that a β-cyclodextrin (βCyD) modified
on the end of an ODN almost completely suppressed the electrochemical signal from the Fc.
The βCyD and Fc tethered to the ends of different ODNs form a tight inclusion complex on the
DNA scaffolding (single-stranded target DNA). The Fc in the cavity of the βCyD is shielded
from the bulk solution, so its electron transfer with the electrode is suppressed. In this case, the
βCyD is therefore a good quencher for electrochemical signals, similar to an azo-quencher used
in FRET-based conventional MBs.\textsuperscript{5} This means that the eMB can be constructed based on an
appropriate hairpin form of an ODN by tethering the Fc and βCyD on each end, respectively (Fig. 1). eMB works in a homogeneous solution and frees the electrochemical sensing of the targets from the need to anchor the sensor molecules on the electrodes.

**Experimental**

**General**

The Fc carboxylic acid and \( O -(\text{Benzotriazol-1-yl})-N,N,N',N'\)-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 1-Hydroxybenzotriazole monohydrate (HOBt) was purchased from Watanabe Chemical Industries (Hiroshima, Japan). The βCyD and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and Dojindo Laboratories (Kumamoto, Japan), respectively. All ODNs were synthesized by an automated DNA
synthesizer (8900, Expedite or NTS M-2-MX, Nihon Techno Service) using conventional phosphoramidite methods. The phosphoramidite monomers were purchased from Proligo (Hamburg, Germany) and Glen Research (Sterling, VA, USA). All ODNs and ODN conjugates were purified by reversed phase HPLC (Column: InertSustain Swift C18, 4.6 mmφ × 150 mm, GL Science, Tokyo) using an appropriate linear gradient with 0.1 M triethylamine-acetic acid (TEAA)-acetonitrile (pH 7.0), and identified with MALDI-TOF mass spectrometry on a Bruker Daltonics Autoflex-III (Billerica, MA, USA) using 3-hydroxypicolinic acid as a matrix. All other reagents were obtained as the highest grade and used without further purification.

**Preparation of the eMB**

The outline of total syntheses of the eMB is shown in Scheme 1. The eMB was synthesized by two successive post-synthetic modifications of the Fc and βCyD with the alkyl amino groups modified on each end of an ODN. The first coupling of the Fc carboxylic acid
was performed with the 5´-aminated ODN tethered to the support resin (CPG, controlled pore glass). Afterward, βCyD modification at the 3´-end of an ODN was carried out in a solution after cleavage from the CPG.

**Fc-ODN**: Prior to coupling, CPG (0.4 mg, carrying 15 nmol ODN as expected) was treated with 500 μL of 10 % triethylamine in acetonitrile for 10 min, and the solution was removed from the CPG after settling. The Fc carboxylic acid (3.5 mg, 15 μmol), HOBt (2.0 mg, 15 μmol) and TBTU (4.8 mg, 15 μmol) were dissolved in a 100 μL mixed solution of acetonitrile and N,N-dimethylformamide (DMF) (7:3) to prepare the Fc solution. To the 20 μL of the Fc solution, CPG was added and incubated for 1 h at room temperature with gentle agitation. Another 20 μL of the fresh Fc solution was added to the CPG suspension and mixed overnight at room temperature. The CPG suspension was allowed to settle and the solution was removed. Then, the CPG was washed with acetonitrile. The ODNs were cleaved from the CPG
by methylamine and the \textbf{Fc-ODN} was purified with reversed phase HPLC and identified with MALDI-TOF MS according to the standard protocols.\textsuperscript{6} MALDI-TOF MS: m/z calcd for [M-H]\textsuperscript{–}: 9667.1; found: 9666.6.

\textbf{Fc-ODN-ssPy}: The \textbf{Fc-ODN} (20 nmol) was dissolved in 40 μL of 10 mM phosphate buffer (pH 9.0, containing 10 mM EDTA). To this solution, SPDP (0.6 mg, 2.0 μmol) dissolved in 20 μL dry DMSO was added. The resulting suspension was shaken for 20 min at room temperature and subjected to a NAP-10 column (Sephadex G-25) to remove excess SPDP. Finally, the \textbf{Fc-ODN-ssPy} was isolated with reversed phase HPLC and identified with MALDI-TOF MS according to the standard protocols. MALDI-TOF MS: m/z calcd for [M-H]\textsuperscript{–}: 9864.4; found: 9868.1.

\textbf{eMB}: Thiolated βCyD was prepared via tosylated βCyD according to a procedure previously reported.\textsuperscript{5} The \textbf{Fc–DNA-ssPy} (11 nmol) was dissolved in 20 μL of 10 mM phosphate buffer.
buffer (pH 7.0, containing 10 mM EDTA). To this solution was added thiolated βCyD (100 nmol) dissolved in 10 μL dry DMSO. The resulting suspension was stirred at room temperature overnight. The solution was subjected to reversed phase HPLC to purify the eMB using a standard protocol. The eMB was identified with MALDI-TOF MS. MALDI-TOF MS: m/z calcd for [M-H]⁻: 10904.3; found: 10964.4.

UV melting experiments

Thermal denaturation experiments were carried out in Tris-HCl buffer solution (20 mM, pH 8.5) containing 1.0 M NaCl. The concentration of each DNA component was 1 μM. All experiments were performed on a UV/Vis spectrophotometer equipped with a Peltier thermal controller (1650pc, Shimadzu). Prior to beginning each denaturation experiment, the samples were degassed at 85 °C for 5 min, then annealed with slow cooling to 0 °C. After equilibration
for 30 min at 0 °C, the solutions were heated slowly at a rate of 0.5 °C min\(^{-1}\) while blow drying the cuvette with N\(_2\) gas (below room temperature) to prevent condensation. The melting profiles were monitored by absorption change at 260 nm. The temperature that gave the maximum first derivative of the melting curve was used as the melting temperature (\(T_m\)), an index of the thermal stability of the structures. Thermal denaturation experiments were repeated two to three times for each sample and the reported \(T_m\) values were the average of all duplicates or triplicates for a given sample.

**Electrochemical measurements**

Differential pulse voltammetry: Electrochemical measurements were performed using an Electrochemical Analyzer (ALS 842B, BAS) with a conventional three-electrode system at room temperature in 10 mM phosphate buffer (pH 7.0) containing 500 mM KCl. A glassy
carbon disk (1.0 mm φ), platinum wire, and Ag/AgCl (with 3.0 M NaCl) were used as the working, auxiliary, and reference electrodes, respectively. Differential pulse voltammetry (DPV) was performed with scanning potentials from +0.2 to +0.6 V with a 200 ms pulse period, 25 mV pulse amplitude, 50 ms pulse width, and 4 mV potential increment.

Electrochemistry in the flow system: Quantitative electrochemical measurements using the \textit{eMB} were performed with the HPLC equipped with an electrochemical detector (ECD) (ECD-700, Eicom). After addition of the targets to \textit{eMB}, sample solutions were incubated for 15 min at room temperature prior to the measurements. Ion exchange chromatography (TSKgel DNA-STAT, 4.6 mm φ × 100 mm, Tosoh) was adopted for determining DNAs with a linear gradient using 0.02 M Tris-HCl (pH 8.5) (Eluent A) and 0.02 M Tris-HCl with 2.0 M NaCl (pH 8.5) (Eluent B) as the eluents (gradient: 10%–50% B in 30 min; flow rate: 0.6 mL/min). The chromatograms were recorded at several applied potentials using the ECD with a glassy carbon
electrode. Hydrodynamic voltammogram was obtained from plots of the current peaks at several potentials for the same amount of eMB (200 pmol).

Results and Discussion

The sequences and the structure of the eMB and other ODNs used in this study are shown in Fig. 2. The 5´- and 3´-ends of the ODN were modified with the Fc and βCyD, respectively, to prepare the eMB. The length of the linker chains tethering both ends of the ODN were designed to be almost the same so a stable inclusion complex, βCyD ⊘ Fc, would form at the end of its hairpin conformation. The eMB was obtained by two-step post modifications of the ODN with amino alkyl linkers on both ends. To conduct each successive modification selectively, the Fc carboxylic acid was attached to the 5´-end amino group of the ODN on the CPG by coupling using TBTU/HOBt. Then, monothiolated βCyD was modified to the 3´-end of the Fc-ODN.
after liberation from the CPG through a bifunctional linker, SPDP. The yield of the Fc coupling to the ODN on the CPG was very low, typically around 20\%–30\% (Fig. S1). Although we tried to improve the yield by modifying various conditions, such as the CPG pretreatment method, the kinds of coupling reagents, the quantity of chemical ingredients, the reaction times and the temperatures, we could not find remarkable solutions. We finally adopted the amide bond formation on the support resin using TBTU/HOBt, which is one of the most common reactions in peptide synthesis. Surface structures of the CPG used in DNA synthesis might not be ideal for such coupling reactions to form an amide bond. Other reactions were conducted as described in the literature,\textsuperscript{7} in some cases, with minor modifications. Their yields were reasonable (Figs. S2 and S3).

Melting experiments were carried out for the eMB in the absence and presence of two ODNs, T\textsubscript{L} and T\textsubscript{S}, each of which consisted of a 20 mer sequence complementary to the central
loop and a partial loop plus a stem at the βCyD side of the eMB, respectively. The eMB itself gave a gentle transition around 38 °C, which was derived from the conformational change from the hairpin to the coil (Fig. S4a). However, relatively sharp transitions were observed for the duplexes formed between the eMB and T₇ or T₈ (eMB/T₇ and eMB/T₈) at ca. 65 °C and 71 °C, respectively (Fig. S4b). Generally, the steepness of the melting curve reflects the degree of entropy change; the steeper the melting is, the more the -ΔS it shows. The gentle and sharp transitions observed for eMB and its duplexes (eMB/T₇ and eMB/T₈) agree with their intramolecular and intermolecular transitions, respectively. Considering the Tₘ values, the open form (duplex) of the eMBs (eMB/T₇ and eMB/T₈) were thermodynamically more stable than the closed form (hairpin) of the eMB.

The fundamental electrochemical property was studied by DPV measurements. As shown in Fig. 3, an obvious peak derived from the oxidation of the Fc was observed at +0.40 V (vs.
Ag/AgCl) for the Fc-ODN. However, the signal of the eMB was ca. 90% smaller than that of the Fc-ODN. This implied that an inclusion complex of βCyD ⇔ Fc formed in the closed hairpin form of the eMB to shield the Fc from outside, resulting in the effective suppression of its electron transfer.

The electrochemical signal from the eMB was also monitored on the HPLC equipped with an ECD. At the beginning, optimization of voltage applied to the electrochemical cell was carried out by increasing it from +0.2 V to +0.7 V (vs. Ag/AgCl) using the Fc-ODN. The oxidation peak of the Fc was found around 7.2 min on the chromatogram (Fig. 4 inset). The peak currents were increased by raising the voltage, and reached a plateau at +0.5 V, which was a little higher than the oxidation potential of the Fc. Thus, the voltage applied on the ECD in the following experiments was set at +0.5 V (Fig. 4).

In general, the detection limit of MBs based on the fluorescent signal depends on a $T_m$
value of the duplex formed between the MB and the target, which determines the amount of
open form of the MB and, accordingly, the amount of signal. Although the $T_m$ value is essential
for the detection limit of this system, it can be controlled by the length of the complementary
sequence of the MB with the target and salt concentration. Therefore, the detection limit of this
system is also expected to change according to the length of target and given experimental
conditions, and can be controlled by the probe design and buffer conditions, like that of a
conventional MB. Therefore, we tried to find the potential (theoretical) detection limit of this
system, which is simply affected by the amount of the Fc rather than the amount of the open
form of the eMB. For these reasons, the peak currents were measured by changing the amount
of the Fc-ODN to assess the potential detection limit of this system. As shown in Fig. 5, a linear
relationship was confirmed between the amount of Fc-ODNs and peak currents in the range
from 1 pmol to 100 pmol. Even only 500 fmol of the Fc-ODN was clearly detected as an
obvious peak. This implies some amount of the target DNA that opens 500 fmol of the eMB can be detected in this system.

The chromatograms obtained by HPLC-ECD are shown in Fig. 6. Contrary to our expectation, the electrochemical response of the eMB was hardly restored by the addition of TL. Scarce signals observed at 7.4 and 8.4 min would be attributed to the closed forms of the eMB and eMB/TL, respectively. Although it is inconsistent with the result of the UV melting experiment with TL, we thought that some unknown factors under the conditions of the ion-exchange mode in HPLC destabilized the eMB/TL and/or promoted the formation of more complicated DNA complexes. Complementarity of 5-nucleotides-long stem sequences on both ends and flanking the Fc and βCyD of the eMB or eMB/TL might be responsible for the discrepancy. Thus, similar experiments of chromatography were conducted using TF, which has a fully complementary sequence of eMB, instead of TL. Remarkable restoration of the
An electrochemical signal was observed for T_F (Fig. S5), probably due to the masking of the 5-nucleotide sequences in the stem region of the eMB. However, the generality of the probe design is lost if it is necessary for the targets to be complementary to not only the loop but also both stem sequences of the eMB. To ensure the generality, we used T_S as a model target, which is complementary to the loop and one stem region of the eMB. This design of the probe did not restrict the target sequence. By the addition of T_S, a large recovery of the current signal was observed, as shown in Fig. 6; the signal ratio in the absence and presence of T_S (on/off ratio) was ca. 95. Previously, Aoki et al. reported an electrochemical DNA probe modified with a Fc and βCyD. Although it showed a ca. 60 mV shift in its oxidation potential when binding with the target sequence, the on/off ratio of the electric current was ca. 5 at an arbitrary potential.\textsuperscript{11} The binding constant between the Fc and βCyD was not high (K = 1.65 × 10^4 M\(^{-1}\)). Therefore, it seems that the signal suppression in the absence of the targets was sacrificed, resulting in low
signal contrast. However, that probe design, which did not involve a stem region, was simple and versatile. The results of present study indicate that the short sequence of stem moiety is essential when designing an eMB with a high on/off ratio in its electric current. Stem structure is important not only for the complete quenching of the electrochemical signal by the placing auxiliary units in close proximity using stem hybridization as a guide, but also for avoiding a false opening of the MB. The stem-induced hairpin form of the MB is a metastable structure that competes with the duplex formed with the target strand, making the sequence recognition of the probe very strict. To the best of our knowledge, this eMB is the first molecular beacon consisting of a conventional stem-loop structure based on electrochemical signaling.

**Conclusions**

We proposed a molecular beacon based on an electrochemical signal induced by a
single-stranded target DNA or RNA. The eMB consists of a stem-loop DNA, an electrochemical
signal generator (Fc), and its quencher (βCyD), which is similar to the fluorescence-based
conventional MB. This technique did not require any modification of the electrode surface
essential for a traditional electrochemical biosensor and it realized the detection of target nucleic
acids in a homogeneous solution. In addition to the intrinsic high sensitivity of the
electrochemical detection system, a relatively high quenching efficiency of the signal was
achieved by the DNA-templated formation of an inclusion complex (βCyD ⊙ Fc) at the
terminus of the stem moiety to give a high on/off ratio between the absence and presence of the
target DNA. Owing to the simplicity and generality of the probe design, it can become a
universal electrochemical sensor for several biomolecules other than the nucleic acids combined
with DNA/RNA aptamers. In addition, as with a fluorescence-based multi-color assay, it would
be possible to achieve a multi-target assay by simultaneous use of several electrochemical active
molecules that have different redox potentials from each other and can form inclusion complexes with CyD.

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Supporting Information

RP-HPLC chromatograms for the isolation of the Fc-ODN, Fc-ODN-ssPy2, and eMB after each synthesis. UV melting curves for the eMB, eMB/T_L, and eMB/T_S. Chromatograms of the eMB in the absence and presence of T_F. This material is available free of charge on the Web at
http://www.jsac.or.jp/analsci/

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Figure Captions

Fig. 1 Schematic illustration of electrochemical molecular beacon (eMB).

Fig. 2 (a) Structure of eMB and (b) the sequence of DNAs used in this study.

Scheme 1 Synthesis of eMB.

Fig. 3 Differential pulse voltammograms of Fe-ODN (black dashed curve) and eMB (red solid curve). Measurements were carried out in 10 mM phosphate buffer (pH 7.0) containing 500 mM KCl at 25 °C with the scanning potential from +0.2 to +0.6 V with 200 ms pulse period, 25 mV pulse amplitude and 50 ms pulse width, 4 mV potential increment. Concentrations of Fe-ODN...
or eMB were 40 μM.

Fig. 4 Hydrodynamic voltammogram of **Fc-ODN** measured by HPLC equipped with EDC.

Inset shows chromatograms of **Fc-ODNs** at several applied potentials. Column: TSKgel DNA-STAT, 4.6 mm × 100 mm, Tosoh; eluent A: 0.02M Tris-HCl (pH 8.5); eluent B: 0.02 M Tris-HCl with 2.0 M NaCl (pH 8.5); gradient: 10-50 %B in 30 min; flow rate: 0.6 mL/min.

Amount of **Fc-ODNs** injected were all 200 pmol.

Fig. 5 (a) Amount of **Fc-ODN** vs current plot measured by HPLC equipped with EDC in the range from 1 to 100 pmol. (b) HPLC chromatogram for the detection of 500 fmol **Fc-ODN**.

Fig. 6 Chromatograms of **eMB** in the absence (red dashed curve) and presence of **Tl** (black
dashed curve) or $T_S$ (red solid curve). Column: TSKgel DNA-STAT, 4.6 mm $\phi \times 100$ mm, Tosoh; eluent A: 0.02M Tris-HCl (pH 8.5); eluent B: 0.02 M Tris-HCl with 2.0 M NaCl (pH 8.5); gradient: 10-50 %B in 30 min; flow rate: 0.6 mL/min. Amount of eMBs and $T_L$ or $T_S$ injected were all 100 pmol.
Fig. 1 Schematic illustration of electrochemical molecular beacon (eMB).
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**(a)**

**(b)**

- **eMB**: 5’ Fe-CCGCGTTTGGCCAAATGTTCTACGCGG-CyD 3’
- **T_L**: 3’ CAAACGGTTTACAGAAGATA 5’
- **T_S**: 3’ GTTTPACGAGATAGCGGC 5’
- **T_F**: 3’ GCCGCAAACGGTTTACAGAAGATAGCGGC 5’
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Graphical Index

Electrochemical molecular beacon