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Author(s)	Sato, Shinobu; Takenaka, Shigeori
Issue Date	2013-06-14
URL	http://hdl.handle.net/10228/5811
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Electrochemical RNase A detection using an electrode with immobilized ferrocenyl deoxyribooligonucleotide containing cytidine residue

Shinobu Sato, Shigeori Takenaka*

Department of Applied Chemistry, Kyushu Institute of Technology, Kitakyushu, Fukuoka 804-8550, Japan

* e-mail: shige@che.kyutech.ac.jp

Received: ((will be filled in by the editorial staff))

Accepted: ((will be filled in by the editorial staff))

Abstract

A ferrocenyl deoxyribooligonucleotide (FcODN(rC)) with contiguous cytosine bases and a single ribonucleotide, cytidine, was immobilized on a gold electrode, and this electrode was used to detect RNase A. RNase A activity in a solution was assessed using cyclic voltammetry, and it was found that the current response of the sensor electrode decreased with increasing enzyme concentration. An extremely low detection limit of 1.0×10^{-11} g mL⁻¹ RNase A was observed, with 15–90% changes in the current signal. RNase activity can be an indicator of a number of diseases; therefore, this probe has great potential for applications in medical diagnostics.

Keywords: RNase A, electrochemical detection, ferrocenyl deoxyribooligonucleotide

DOI: 10.1002/elan.((will be filled in by the editorial staff))

1. Introduction

Living organisms are known to produce their own specific ribonucleases (RNases) that have many important functions in cellular metabolic activity. The most important of these is the hydrolysis of phosphodiester linkages in RNA [1]. Different organisms secrete RNase in different ways (e.g., it is present in human sweat) to prevent invasion by foreign RNA such as viral RNA. RNase A is known to be an extremely stable enzyme since its activity is not lost even after boiling in water for an extended period, while DNase (a DNA digesting enzyme) becomes denatured and deactivated after the same procedure. However, even though RNase A is stable at high temperatures, it is not resistant to contamination; thus, considerable attention needs to be paid to the experimental environment when conducting studies involving this enzyme. The detection of RNase A in a solution would be highly useful in diagnostic medicine as its level in the blood serum can be a diagnostic marker for many diseases, including myocardial infarction and pancreatic cancer [2]. Detection of RNase A has previously been achieved by monitoring RNA cleavage by using an absorption technique [3] or gel electrophoresis [4]. Recently, a method based on an RNA probe containing a fluorescence resonance energy transfer (FRET) molecule at each of its termini was developed. In this system, fluorescence was quenched on cleavage of the probe by RNase A [5]. The use of electrochemical techniques is a highly promising approach to detecting RNase A, as it is user-friendly, cost-effective, and can be miniaturized. However, such a strategy has not been extensively investigated, and only our group [6] and Wang's group [7] have reported on it. The electrochemical method developed by

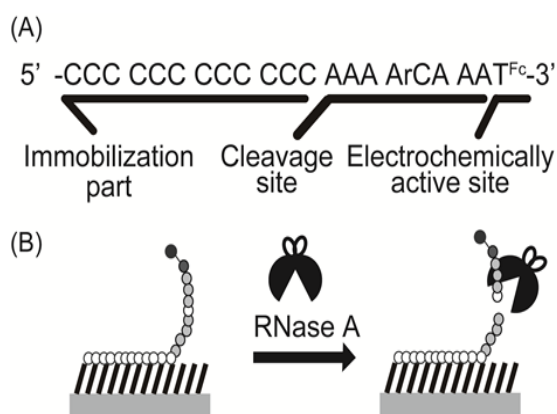


Figure 1. (A) Sequence of ferrocenyl deoxyribooligonucleotide (FcODN(rC)) with deoxycytidine dodecamer as the electrode immobilization section, one ribonucleotide cytidine (rC) as the target of RNase A, and ferrocenyl thymine as the electrochemical signalling part. (B) Principle of electrochemical RNase A assay based on the electrode with immobilized FcODN(rC).

our group was based on an electrode on which RNA is immobilized [6], with measurements performed in an electrolyte containing ferrocenylnaphthalene diimide (FND). The current signal from the FND decreased as a result of RNA cleavage by RNase A, and it was possible to detect the enzyme over the range of 0.2–10 ng mL⁻¹.

Moreover, we constructed an electrode modified by a thiolated oligonucleotide carrying a ferrocene moiety at the terminal position through the Au–S linkage and successfully employed it in the detection of DNase I [8]. A section of DNA with immobilized

ferrocene was cleaved on the electrode by DNase I, thereby decreasing the current and enabling quantification of DNase activity within the range of 10^{-4} to 10^{-2} unit μL^{-1} . Furthermore, we developed an electrode with a ferrocene-modified oligonucleotide, which was synthesized by the reaction of a ferrocenyl oligonucleotide (FcODN) carrying a continuous deoxycytidine sequence (C_{12}) using an activated carboxylic acid-modified electrode [9]. In order to produce an electrode for RNase A detection by using this method, the design of the probe was altered to include ribonucleotide cytidine (rC) as a target for the enzyme. The ferrocenyl deoxyribooligonucleotide probe containing a cytidine cleaving site (FcODN(rC)), as shown in Fig. 1A, was prepared, and the working principles of this probe immobilized on an electrode are illustrated in Fig. 1B.

2. Experimental

2.1. Materials

Ferrocenylcarbodiimide (FCDI) was synthesized as a ferrocenylation reagent for thymine or guanine in single-stranded DNA, according to a previously described protocol [10]. The deoxyribooligonucleotide containing a cytidine ribonucleotide, 5'-TAA ArCA AAA CCC CCCCCCCC-3' [ODN(rC)], was custom-synthesized by Hokkaido System Science (Sapporo, Japan). BioPak water was purified by a Milli-Q system Gradient A10 coupled with an Elix 3 kit (EMD Millipore, Billerica, MA). RNase A and RNase-free water were obtained from Life Technologies (Carlsbad, CA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), 3,3'-dithiodipropionic acid, and guanidine thiocyanate were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan).

2.2. Apparatus

The HPLC system used in this experiment was composed of the following components: Hitachi C-7300 column oven, L-7450H diode array detector, L-7100 pump, and D-7000 interface chromatograph (Hitachi High-Technologies, Tokyo, Japan).

The mass spectrometer (MALDI-TOF MS) used for characterization of FcODN(rC) and the products of the enzymatic reaction was a VoyagerTM Linear-SA from PerSeptive Biosystems (Foster City, CA). FcODN(rC) purified using HPLC was dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid and 50 mg mL^{-1} 3-hydroxypicolinic acid and then dried. Mass spectra were measured in negative mode using 3-HPA as the matrix.

All electrochemical measurements were performed with an ALS Model 650 analyzer (CH Instrument, Austin, TX). Cyclic voltammetry (CV), and Osteryoung square wave voltammetry (SWV) measurements were performed at 25 °C with a normal three-electrode configuration consisting of an Ag/AgCl reference electrode, a Pt counter electrode, and an electrode with immobilized FcODN(rC), which is used as a working electrode.

2.3. Synthesis of FcODN(rC)

Forty microliters of a solution of 500 μM ODN(rC) in 50 mM

borate buffer (pH 8.5) was mixed with 40 μL of a solution of 150 mM FCDI in 50 mM borate containing 60% DMSO. The mixture was then vortexed for 15 h at 37 °C, and the resulting reaction product was purified by ethanol precipitation. FcODN(rC) was further purified using reversed-phase HPLC, run on an X Terra MS C18 column (4.6×100 mm, 5 μm , Nihon Waters, Tokyo, Japan) under gradient conditions. The acetonitrile concentration in 0.1 M triethylammonium acetate (pH 7.0) was changed linearly from 10 to 40% over 40 min at a flow rate of 1.0 mL min^{-1} with detection at 260 nm.

The mass of the product was determined using MALDI-TOF MS. The parent peak was found at m/z $[M-H]^-$ 6661.5 (Calcd. for $C_{221}H_{289}N_{80}O_{120}P_{20}Fe-H$: 6661.4)

2.4. RNase A digestion of FcODN(rC) in homogenous solution

The reaction between the obtained FcODN(rC) and RNase A was performed by the incubation of a mixture of 100 μL of 1.0×10^{-5} g mL^{-1} RNase A and 3.0 μM FcODN(rC) at 37 °C for 30 min. The resulting solution was purified using a NAP-10 column after equilibration with BioPak water, and 1 mL of eluent was collected and freeze-dried. Next, 100 μL of BioPak water was added and the products were analyzed using HPLC monitored at 260 nm.

2.5. Preparation of the electrode on which FcODN(rC) is immobilized

A Au electrode (ϕ 1.6 mm, theoretical area 0.02 cm^2) (Bioanalytical Systems (BAS), Tokyo, Japan) was polished with a 6 μm and 1 μm diamond slurry and then a 0.05 μm alumina slurry, followed by sonication in BioPak water for 5 min (3 times). The electrode was subsequently electropolished and ultrasonicated according to a previously reported procedure [11]. CV traces using the resulting electrode were recorded in 0.05 M H_2SO_4 . A large reduction peak at 0.9 V vs. Ag/AgCl with a normalized area of 390 ± 10 $\mu\text{C cm}^{-2}$ was observed for the polycrystalline Au electrode. After the electrode was dried with air blower, it was soaked in 300 μL of an ethanolic solution of 1 mM 3,3'-dithiodipropionic acid overnight at 25 °C. It was then washed with BioPak water and soaked in 7 mL of an aqueous solution of 5 mM EDC and 8 mM NHS, and incubated for 20 min at 25 °C. The resulting activated electrode was soaked in 50 μL of a 10 nM FcODN(rC) solution containing 0.05 M NaCl at 25 °C for 30 min. After washing with Milli-Q water, the electrode was soaked in 300 μL of 5 mM Tris-HCl, pH 7.4, containing 20 mM NaCl for 30 min at 4 °C to quench any remaining activated carboxyl groups on the surface.

Oligonucleotides carrying non-continuous C or continuous A could not be immobilized on the activated electrode, as described by Mikkelsen [12].

2.6. RNase A reaction on the electrode on which FcODN(rC) is immobilized

An RNase A assay was run with an electrode on which FcODN(rC) is immobilized and which is dipped in 100 μL of a set concentration of RNase A (1.0×10^{-12} to 1.0×10^{-5} g mL^{-1}). After incubating the mixture at 37 °C for 30 min, the electrode was

washed with BioPak water.

2.7. Electrochemical measurements

CV or SWV measurements were made in 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.0) containing 0.10 M NaClO_4 . The scan rates were 152 V s^{-1} or lower for Laviron's plots [15].

The Osteryoung SWV signal was recorded before (i_0) and after (i) the enzymatic reaction in 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.0) containing 0.10 M NaClO_4 . The current change was evaluated using Δi , which was defined as $[(i_0 - i)/i_0 \times 100]$. SWV measurements were made with an amplitude of 30 mV, applied potential of 5 mV, and frequency of 150 Hz.

3. Results and discussion

3.1. Synthesis of ferrocenyl deoxyriboligonucleotide containing cytidine ribonucleotide, FcODN(rC) and its testing as RNase A target

It is known that RNase A can recognize ACAAA and AUAAA motifs and cleave the ribonucleotide that has a cytosine (C) or uracil (U) at the 5'-terminus, respectively, in any sequence of RNA [13]. — For this reason, in the present study, the probe was designed to contain the rC as an RNase A target. To enable monitoring of current signal changes on RNA cleavage, a ferrocenyl moiety (FCDI) was incorporated into the probe. The prepared ferrocenyl deoxyriboligonucleotide containing cytidine ribonucleotide (rC), FcODN(rC), was analyzed using HPLC according to a previously reported protocol [10]. The results show a single peak that was shifted from 9.9 min to 11.1 min after reaction of ODN(rC) with FCDI (Figure S1A & B).

The product was collected at 11.1 min and the MALDI-TOF MS spectrum was recorded in negative mode. The obtained parent peak observed at -6661.5 was comparable to the calculated mass of FcODN(rC) (-6662.50) (Figure S1C). The presence of a single peak demonstrates that the reaction progressed quantitatively and that the FcODN(rC) did not decompose during the analysis. Additionally, FcODN(rC) was found to be stable for at least three

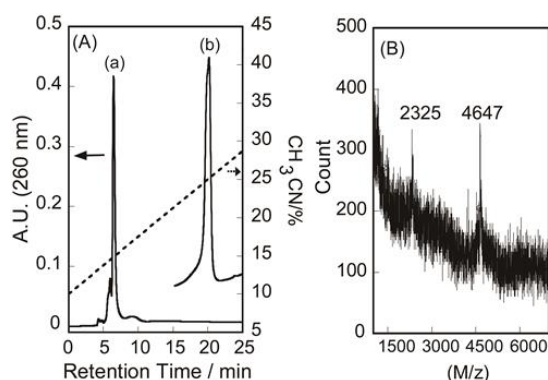


Figure 2. (A) Reversed-phase HPLC chromatograms of 100 μL of 3.0 μM FcODN(rC) before (b) and after treatment with $1.0 \times 10^5 \text{ g mL}^{-1}$ RNase A at 37 $^\circ\text{C}$ for 30 min (a). (B) MALDI-TOF MS of the solution after treatment with RNase A. m/z 2328.17 and 4660.05 were assigned as $5'\text{-T}^{\text{Fc}}\text{AA ArC}\cdot 9\text{Na}$ ($[\text{C}_{73}\text{H}_{90}\text{N}_{24}\text{O}_{30}\text{P}_5\text{FeI}\cdot 9\text{Na}]^-$) and A AAA CCC CCCCC CCC-3' ($[\text{C}_{148}\text{H}_{192}\text{N}_{56}\text{O}_{90}\text{P}_{15}]^-$), respectively.

months when kept at $-80 \text{ }^\circ\text{C}$.

After RNase A digestion of FcODN(rC) in a homogenous solution, the HPLC peak was shifted from 21 min [Figure 2A (b)] to 6.42 min [Figure 2A (a)]. The masses of the resulting products observed in the MALDI-TOF MS spectra were 2325 and 4647, which are in agreement with theoretical masses of $5'\text{-T}^{\text{Fc}}\text{AA ArC}\cdot 9\text{Na}$ ($[\text{C}_{73}\text{H}_{90}\text{N}_{24}\text{O}_{30}\text{P}_5\cdot 9\text{Na}]^- = 2328.17$) and dA AAA CCC CCCCC CCC-3' ($[\text{C}_{148}\text{H}_{192}\text{N}_{56}\text{O}_{90}\text{P}_{15}]^- = 4660.05$) (Fig. 2B). These results show that FcODN(rC) can act as a substrate for RNase A and that it can be cleaved with a preference for the rC site.

3.2. Immobilization of the FcODN(rC) probe on the electrode and analysis of its behavior

CV analysis of the electrode with immobilized FcODN(rC) after overnight incubation did not reveal any redox peaks. It is likely that this was due to decomposition owing to RNase contamination of the incubation solution, even though all reagents used in the experiment were prepared with RNase-free water. Therefore, the CV analysis was carried out after a shorter incubation time. Figure 3 shows the CV trace from the electrode with immobilized FcODN(rC), after 30 min incubation in 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer (pH 7.0) containing 0.10 M NaClO_4 .

The redox peaks can be seen at 0.18 V (anodic peak potential, E_{pa}) and 0.16 V (cathodic peak potential, E_{pc}) vs. Ag/AgCl (half-wave potential $[E_{1/2} = (E_{\text{pa}} + E_{\text{pc}})/2]$, 0.17 V; peak potential separation (ΔE_p), 0.02 V) with a recording scan rate of 0.025 V s^{-1} . Even though the immobilization method was different from that used for our previously reported FcODN probe [9], the density of immobilization was similar, reaching 3.8 pmol cm^{-2} and 6.0 pmol cm^{-2} , for FcODN(rC) and FcODN, respectively. However, ΔE_p for FcODN was 0 V, whereas that for FcODN(rC) was 0.02 V. The scan rate dependence of the electrode was measured next, and a linear relationship between oxidation peak and scan rate was observed up to 0.4 V s^{-1} . This demonstrates that the electron transport reaction from the ferrocene section of the FcODN(rC) to the electrode surface follows the scan rate up to 0.4 V s^{-1} , but not above it. These results show that FcODN(rC) was immobilized on the electrode with certain degree of flexibility, and that the immobilization reaction of FcODN(rC) onto the activated electrode did not involve any adenine base(s).

In order to analyze the behavior of FcODN(rC) on the electrode, Laviron's plot was constructed as described by Anne *et al.* [14].

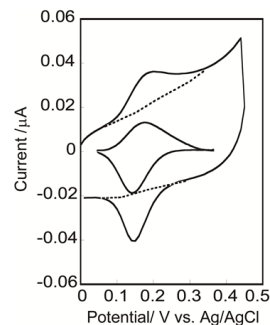


Figure 3. Cyclic voltammograms of an electrode with immobilized FcODN(rC) in 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer (pH 7.0) and 0.10 M NaClO_4 with a scan rate of 0.025 V s^{-1} . The dashed curves correspond to background signals. Background subtracted signal is also shown.

The CV trace of the electrode having the immobilization density of 3.8 pmol cm^{-2} of FcODN(rC) was measured over scan rates of $0.05\text{--}152 \text{ V s}^{-1}$ in an electrolyte of $10 \text{ mM NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH7.0) and 0.10 M NaClO_4 containing $0.04 \text{ units } \mu\text{L}^{-1}$ of RNase inhibitor (porcine liver) to prevent degradation with RNase A.

Figures 4a and 4b show the plots of $i_{pa}/(N_0\nu)$ and $i_{pa}/N_0\nu^{1/2}$ against $\log \nu$, respectively, where N_0 represents immobilized amount of FcODN(rC). Figure 4a shows the deviation from Nernstian behavior for the ferrocene redox over $\log \nu = -0.5$ ($\nu = 0.9 \text{ V s}^{-1}$). Anne *et al.* [14], showed that a 20-meric single stranded ferrocenyl oligonucleotide immobilized on the electrode retained Nernstian behavior up to $\log \nu = 4$, whereas a double stranded analog only retained it up to $\log \nu = 2$, demonstrating that the more rigid ferrocenyl oligonucleotide had smaller $\log \nu$ values. According to these observations, the FcODN(rC) investigated here appears to be immobilized on the electrode with a more restricted conformation than in the case of the double stranded oligonucleotide mentioned above. Figure 4c shows the Laviron's plot for $\Delta E_{pa} = E_{pa} - E_{1/2}$ and $\Delta E_{pc} = E_{pc} - E_{1/2}$ against $\log \nu$. The symmetric shapes of the plots for ΔE_{pa} and ΔE_{pc} gave $\alpha = 0.5$. Using this value, the electron transfer rate constant was estimated to be 245 s^{-1} by using the equation $k_0 = anF\nu c/RT$ where n represents number of electrons per molecule for reduction ($n = 1$), F Faraday constant, ν_c critical scan rate where obtained by plotting (ΔE_{pa} and ΔE_{pc}) vs. $\log \nu$ and by extrapolating to $\Delta E_{pa} = 0$ and $\Delta E_{pc} = 0$ in Figure 4c [15]. The values of k_0 for the ferrocenyl 15-meric single- and double-stranded oligonucleotides were reported to be 270 s^{-1} and 40 s^{-1} , respectively, by Inouye's group [16]. By comparing these reported rates, it can be concluded that FcODN(rC) was immobilized on the electrode with a rigid conformation and that the ferrocene part was located close enough to the electrode surface to take part in electron transfer. Assuming that FcODN(rC) occupied the

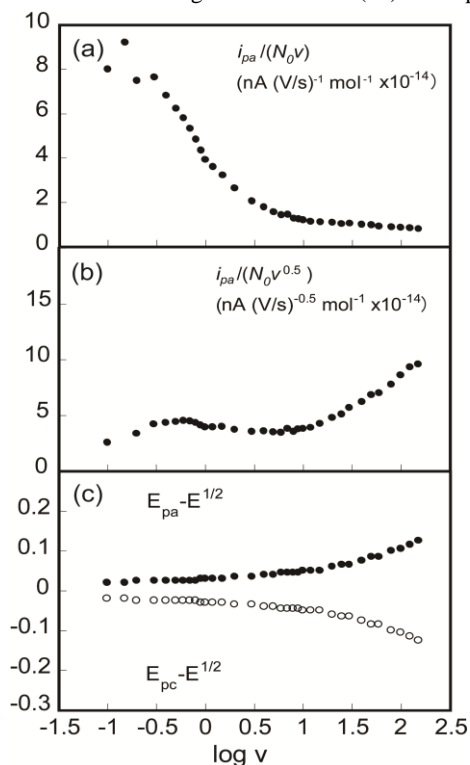


Figure 4. Scan rate ν and $\sqrt{\nu}$ anodic peak current i_{pa} (a, b), and the anodic and cathodic peak potentials, E_{pa} and E_{pc} , from the CV (c) recorded with the electrode with immobilized FcODN(rC). In (a) and (b) the peak current is normalized versus ν and $\sqrt{\nu}$, respectively, and versus the total number of Fc heads ($N_0 = 3.1 \times 10^{13} \text{ mol}$).

molecular area of $(3.4 \times 21 \times 10^{-10} \text{ m}) \times (1 \times 10^{-9} \text{ m}) = 7.14 \times 10^{-18} \text{ m}^2 = 7.14 \times 10^{-14} \text{ cm}^2$, total immobilized area of FcODN(rC) on the electrode was $7.14 \times 10^{-14} \text{ cm}^2 \times 3.80 \times 10^{-12} \text{ mol cm}^{-2} \times 0.02 \text{ cm}^2 \times 6.02 \times 10^{23} \text{ molecules} = 0.0032 \text{ cm}^2$ and the area occupied (in percentage) by FcODN(rC) on the electrode was estimated to be 16%. This result shows that FcODN(rC) occupied a significant amount of space on the electrode. Since the immobilization density of $3.80 \times 10^{-12} \text{ mol cm}^{-2}$ of FcODN(rC) gives a distance of 5.12 nm between FcODN(rC) molecules, it is expected that RNase A (3 nm [17]) and DNase I (4.5 nm [18]) would be able to freely access the FcODN(rC) on the electrode.

3.3. Electrochemical behavior of FcODN(rC) on the electrode after RNase A treatment

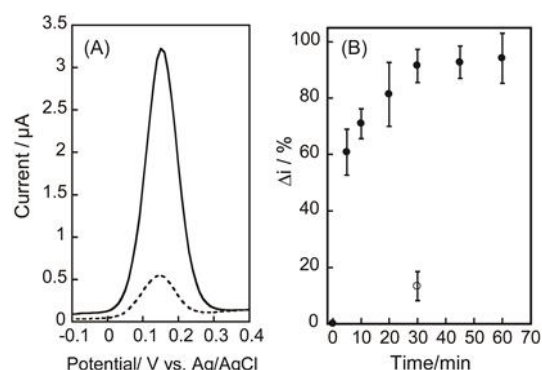


Figure 5. (A) SWV curves from the electrode with immobilized FcODN(rC) before (solid) and after (broken) treatment with $1.0 \times 10^{-6} \text{ g mL}^{-1}$ RNase A (30 min, 37°C) (B) Plot of the digestion percentage of FcODN(rC) on the electrode after treatment with (filled circle) or without RNase A (open circle). $n = 3$.

SWV measurements of the electrode with immobilized FcODN(rC) were carried out after treatment with $100 \mu\text{L}$ of $1.0 \times 10^{-6} \text{ g mL}^{-1}$ RNase A (Figure 5A). A current decrease was observed after the enzyme treatment, demonstrating that the electrode was capable of detecting RNase A. Figure 5B shows the percentage decrease in current (Δi) after treatment with RNase A for different durations. The Δi values can be seen to increase with reaction time, reaching a plateau at approximately 90% in 30 min. As a control, the experiment was performed in the absence of RNase A, and after 30 min, a much lower value of Δi was observed ($15 \pm 1\%$). To confirm whether the cleavage site was in agreement with that expected for RNase A, MALDI-TOF MS was carried out to determine the products of enzymatic reactions on the electrode. Twelve electrodes with 0.18 pmol FcODN(rC) (total: 2.16 pmol) were treated with $100 \mu\text{L}$ of $1.0 \times 10^{-6} \text{ g mL}^{-1}$ RNase A at 37°C for 30 min, and the resulting solution was then freeze-dried and dissolved in $20 \mu\text{L}$ of RNase-free water (theoretically $0.1 \mu\text{M}$ of FcODN(rC) was obtained). The obtained MS peak with an m/z of 2174.04 was in agreement with the mass calculated for the $\text{T}^{\text{Fc}}\text{AAArC}$ fragment (2131.2, $\text{C}_{74}\text{H}_{100}\text{N}_{24}\text{O}_{30}\text{P}_3\text{FeI}$) with two sodium ions (Figure S2). This result shows that the electrode works well and that RNase A cleaved the phosphodiester bonds between the expected bases.

3.4. Evaluation of the performance of the electrode with immobilized FcODN(rC)

Excellent correlation was observed between RNase activity and Δi values in the range of 1.0×10^{-13} g mL⁻¹ to 1.0×10^{-5} g mL⁻¹ (Figure 6A). The Δi values of 15 and 90% were obtained at concentrations of 1.0×10^{-12} g mL⁻¹ and 1.0×10^{-6} g mL⁻¹, respectively. Based on the value of Δi for the experiment with RNase-free water ($15 \pm 1\%$), statistical analysis was carried out (*t*-test), and detection limit value of 1.0×10^{-11} g mL⁻¹ RNase A ($22 \pm 3\%$, $p = 0.01$) was obtained. This result shows that this system has a detection limit of 1.0×10^{-11} g mL⁻¹ RNase A, which is higher than that for the previously reported FRET-based probe (1.0×10^{-9} g mL⁻¹ RNase A) [5].

RNase A contamination was then investigated in a series of different water samples. In addition, experiments were performed using a commercially available RNase A detection system (RNase Alert) for comparison (Table 1).

Elix, Milli-Q, BioPak, and RNase-free water samples were used, and the first three were tested immediately after filtration. Commercially available RNase-free water was used immediately after opening the bottle. The electrode with immobilized FcODN(rC) gave Δi values of 48 ± 17 , 18 ± 5 , 22 ± 4 , and $15 \pm 1\%$ for the Elix, Milli-Q, BioPak, and RNase-free water samples, respectively. Using the calibration curve shown in Figure 6A, the contamination of RNase A in each water sample was estimated to be 1.0×10^{-8} , 1.0×10^{-11} , 1.0×10^{-11} , and 1.0×10^{-12} g mL⁻¹, respectively. It was found that the RNase Alert assay detection limit was quite high, with fluorescence intensities in the range of 9–10 observed for all samples. This suggests a similar RNase A content in all samples. However, the stated detection limit of this assay is 1.0×10^{-9} g mL⁻¹; therefore, it is difficult to detect enzyme below this concentration. These results show that the electrode with immobilized FcODN(rC), described in the study, had a lower detection limit than the commercially available RNase Alert system. Furthermore, while the commercial system required 30–60 min of incubation with the sample solution, the electrochemical method only needed 30 min.

The SWV of the electrode with immobilized FcODN(rC) was measured after treatment with 1.0×10^{-9} g mL⁻¹ of RNase A, in the presence of 10 mM guanidine thiocyanate as an RNase inhibitor. A Δi value of $-2 \pm 2\%$ was achieved, which was comparable to background signal and much lower than the $31 \pm 7\%$ observed in the absence of guanidine thiocyanate (Table 1). This result shows

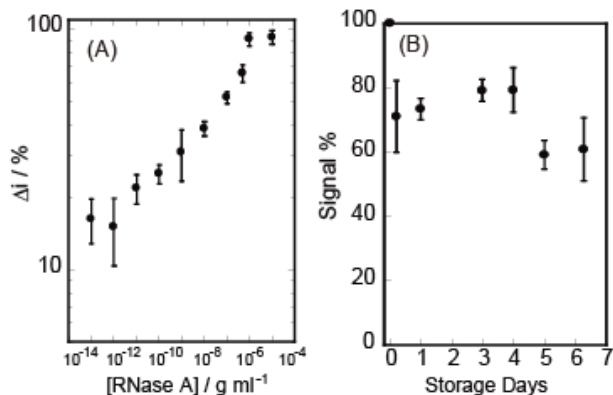


Figure 6. (A) Relationship between Δi value and RNase A activity (37 °C, 30 min). (B) Time-dependence of the oxidative peak current of an electrode with immobilized FcODN(rC) kept in 100 μ L RNase-free water at 4 °C. All CVs were measured in 100 μ L of 10 mM Na₂HPO₄/NaH₂PO₄ buffer (pH 7.0) and 0.10 M NaClO₄. The bar represents a standard error for four determinations each.

that this electrode can detect RNase A in a specific manner, causing changes in the current signal that correlate with RNA cleavage by the enzyme. Furthermore, we tested the effect of DNase I activity on the probe. The SWV of the electrode with immobilized FcODN(rC) was tested with 100 μ L of 0.005 units μ L⁻¹ DNase I at 37 °C for 30 min, and a value of $71 \pm 5\%$ was observed. However, the Δi values were seen to decrease dramatically to $22 \pm 10\%$ in the presence of 50 mM EDTA as a DNase I inhibitor, and subsequent addition of 1.0×10^{-7} g mL⁻¹ RNase A gave $\Delta i = 95 \pm 4\%$ (Table 1). These results demonstrate the capability for highly specific detection of RNase A or DNase I by employment of inhibitors.

The stability of the electrode with immobilized FcODN(rC) was also evaluated (Figure 6B). After preparation of the electrode, it was kept in water containing guanidine thiocyanate [19] at 4 °C. SWV measurements were performed after different storage times after warming back to r.t. The Δi values were found to be approximately 20% lower than those achieved immediately after electrode preparation, and then, the values gradually decreased, suggesting that contamination with RNase A from the atmosphere occurred. Nevertheless, the sensing electrode was shown to be usable after up to 6 days of storage, as long as it was kept in water

Table 1. $\Delta i/\%$ values for the detection of RNase A in several kinds of water using the system proposed in this paper and results for the commercially available RNase Alert assay.

Water samples	$\Delta i/\%$	F.I. obtained from RNase Alert system
RNase Free Water	15 ± 1	10 ± 1.0
BioPak Water	22 ± 4	9.3 ± 1.7
Milli-Q Water	18 ± 5	12 ± 1.7
Elix Water	48 ± 17	10 ± 2.1
1.0×10^{-9} g mL ⁻¹ RNase A	31 ± 7	
1.0×10^{-9} g mL ⁻¹ RNase A + 10 mM Guanidine thiocyanate	-2 ± 2	
1.0×10^{-12} g mL ⁻¹ RNase A	17 ± 4	12 ± 1.9
0.005 unit μ L ⁻¹ DNase I	71 ± 5	
0.005 unit μ L ⁻¹ DNase I+50 mM EDTA	22 ± 10	
0.005 unit μ L ⁻¹ DNase I+50 mM EDTA + 1.0×10^{-7} g mL ⁻¹ RNase A	95 ± 4	
1.0×10^{-7} g mL ⁻¹ RNase A	52 ± 3	

containing guanidine thiocyanate.

4. Conclusions

An electrode with RNase A-sensing immobilized ferrocenyl deoxyribooligonucleotide (FcODN(rC)) was successfully prepared using a chimeric oligonucleotide. Detection of RNase A was possible within an RNase A concentration range of 1.0×10^{-11} to 1.0×10^{-6} g mL⁻¹, with a decreasing current range of 15–90%. The detection limit of the electrode was found to be 1.0×10^{-11} g mL⁻¹ RNase A, which is superior to that of the commercially available RNase Alert system [20]. The investigated probe also responded to DNase I because it is a DNA–RNA chimeric probe. Further, RNase A and DNase I could be detected simultaneously or separately by employing specific inhibitors. Although the electrode with immobilized FcODN(rC) deteriorated gradually, it could be used efficiently within one week of preparation, as long as it was stored properly. With some further improvements in performance, this sensor electrode should be effective for detecting RNase or DNase

activity in blood serum. This electrode has huge potential in the field of medical diagnostics because abnormal levels of RNase and DNase in the blood are indicators of many diseases.

5. Acknowledgements

This work was supported in part by Grants-in-Aid for Feasibility Study of Kurume City Area Program (Development Stage) and for Knowledge Cluster Initiative implemented by Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

4. References

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