Ferrocenylnaphthalene Diimide-based Electrochemical Ribonuclease Assay

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Messenger RNA (mRNA) poly(A)+RNA (from mouse kidney) was immobilized on an N-hydroxysuccinimide(NHS)-activated carboxylic acid modified electrode prepared by the treatment of a gold electrode with 3,3′-dithiodipropionic acid, followed by NHS and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). An electrochemical measurement using this mRNA electrode was carried out in an electrolyte containing ferrocenylnaphthalene diimide (1), and showed an electrochemical signal based on 1 concentrated on immobilized mRNA. After treating this electrode with water containing varied amounts of ribonuclease A (RNase A), the current peak based on 1 decreased with increasing in the amount of RNase A with a linear correlation in the range of 0.2 – 10 pg of RNase A.

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Introduction

It is very important to maintain an RNase-free environment when working with RNA, for example, a recent RNA interference1 or mRNA expression analysis;2 also a simple, rapid and sensitive RNase detecting method in the environment is strongly required to ensure success in these RNA studies. So far, a hypersensitive fluorometric RNase detection method is known with the RNase-substrate oligonucleotide having fluorescein and rhodamine dyes at both termini.3–6 This oligonucleotide substrate shows fluorescence quenching of the fluorescein by the proximal rhodamine in an aqueous solution. However, in the presence of RNase, the fluorescence of the fluorescein was recovered by cleavage of the oligonucleotide due to RNase digestion. RNase assays by using this oligonucleotide are useful, but there still remains a difficulty for automatization, and a relative expensiveness of fluorogenic oligonucleotide.

Electrochemistry is generally expected to achieve high-sensitive analysis with an inexpensive, compact instrument; this technique has been applied to DNA detection.7–21 Electrochemical DNA analysis so far reported is briefly grouped into two methods based on the ways to be used for electrochemically active labeling of DNA10–15 and an electrochemically active DNA indicator.7,9,16–21 Highly sensitive DNA detection was achieved in both methods. An electrochemical DNA enzyme assay has also been reported in DNA polymerase,18 telomerase,6 DNase I,15 and S1 nuclease.13 However, an electrochemical RNase assay has not yet been reported to the best of our knowledge.

In the present work, we intended to construct a sensor electrode immobilizing mRNA to achieve an electrochemical RNase assay by using 1. Our idea of the 1-based electrochemical RNase assay is illustrated in Fig. 1. 1 developed for a double-stranded DNA binder6–8 could bind to RNA, depending on its amounts by an electrostatic interaction between cationic 1 and anionic RNA under selected conditions. Thus, after treating a test sample with RNase, we could estimate the amount of RNase due to the decreasing amount of 1 concentrated on the electrode. Here, we selected RNase A and poly(A)+RNA from mouse kidney as examples of RNase and mRNA in this experiment.

Fig. 1 Principle of ferrocenylnaphthalene diimide (1)-based electrochemical RNase A assay and the chemical structure of 1.
Experimental

Reagents and chemicals

mRNA (poly(A)^−RNA from mouse kidney) was purchased from Wako Chemicals Inc. (Osaka, Japan). Concentrated nitric acid was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), and 3,3′-dithiodipropionic acid were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Bovine pancreatic ribonuclease A (RNase A) was obtained from Ambion (Austin, TX). RNase A inhibitor from human placenta was obtained from Toyobo (Osaka, Japan). Water was purified by a Milli-Q system Gradient-A10 coupled with an Eliss3 kit (Millipore, Bellerica, MA). RNase-free Gengard water was obtained by filtering Milli-Q water with a Millipore cartridge (Millipore).

Poly(A)^−RNA from mouse kidney was diluted to a concentration of 10 or 5 ng/μl with Gengard water and stored at −30°C until assays. One hundred millimolar 3,3′-dithiodipropionic acid in ethanol was diluted to 1 mM with Gengard water. EDC and NHS were dissolved by Gengard water at a suitable concentration. Ferrocenylnaphthalene diimide (I) was synthesized according to a route described previously.9

Preparation of mRNA- or tris(hydroxymethyl)aminomethane(Tris)-immobilized electrode

Gold electrodes of 2 mm^2 in area (Bioanalytical Systems (BAS) Inc., Tokyo, Japan) or 0.79 mm^2 in area (ECA chip composed of 25 electrodes with a diameter of 1.0 mm, Toppan Printing Co., Ltd., Tokyo, Japan) were polished with 6 and 1 μm of diamond slurry, and 0.05 μm alumina slurry in this order and sonicated in Gengard water for 1 min (2 times). These electrodes were soaked in boiling 2 M NaOH for 30 min and washed with Gengard water. The electrodes were then soaked in concentrated nitric acid for 20 min and washed with Gengard water, and sonicated in Gengard water for 1 min (3 times).9 After the electrodes were dried by nitrogen gas, they were soaked in a 1 mM 3,3′-dithiodipropionic acid aqueous solution for 2 h at room temperature.22 After the electrodes were washed with Gengard water, 30 μl of a solution containing 5 mM EDC and 8 mM NHS in 0.02 M phosphate buffer (pH 7.0) was placed on a gold electrode held upside down and kept in a clean bench for 2 h at room temperature.22 After the electrodes were washed with Gengard water, 2.5 - 10 ng/μl of poly(A)^−RNA from mouse kidney was placed on the electrode for 20 min at room temperature. The mRNA-immobilized electrode thus obtained was washed with a solution containing 5 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, pH 7.4) and 20 mM NaCl to quench any unreacted activated carboxylic groups on the electrode for 30 min at room temperature according to references from a paper reported previously.20,21,23 A Tris-immobilized electrode as a masking electrode was also prepared by soaking the activated electrode described above in a solution containing 5 mM Tris-HCl (pH 7.4) and 20 mM NaCl for 30 min at room temperature.

Enzyme reaction

The mRNA-immobilized electrode was soaked in a solution containing varied amounts of RNase A or Gengard water as a reference for 1 h at room temperature.

Electrochemical measurement

All of the measurements were performed with an ALS Model 650 electrochemical analyzer (CH Instrument Inc., Austin, TX).

Cyclic voltammetry and differential pulse voltammetry measurements were carried out at room temperature with a normal three-electrode configuration consisting of an Ag/AgCl reference electrode, a Pt counter electrode, and a mRNA-immobilized electrode as the working electrode. A differential pulse voltammetric signal was recorded before and after an enzyme reaction in 0.1 M AcOK–AcOH (pH 5.5) buffer containing 0.1 M KCl and 0.05 mM I. The conditions for differential pulse voltammetric measurements were as follows: initial potential, 0 V; final potential, 0.6 V; scan rate, 100 mV s⁻¹; pulse amplitude, 0.05 V; sample width, 16.8 ms; pulse period, 0.15 s; pulse width, 0.05 s; quiet time, 2 s. An electrochemical measurement using the ECA chip was carried out with the ECA chip reader STR3000 (Toppan Printing Co., Ltd.).

Results and Discussion

Immobilization of mRNA on a gold electrode

mRNA was immobilized on a gold electrode following the scheme depicted in Fig. 2.

Firstly, the immobilization of 3,3′-dithiodipropionic acid and subsequent activation by EDC and NHS of the carboxylic acid group on the electrode surface was carried out according to a similar procedure reported previously.20-22 The immobilization of mRNA on the electrode thus obtained was then conducted based on the fact that deoxyguanosine or deoxycytidine of DNA could react with NHS-activated carboxylic acid on the electrode.20-21 Since the mRNA used in this experiment had a lot of guanosine and cytidine,23 the mRNA was expected to be immobilized through a similar reaction. Poly(A)^−RNA from mouse kidney as mRNA had an averaged length of 1 - 2 kb, confirmed by an Agilent 2100 bioanalyzer (data not shown).

Figure 3 shows cyclic voltammograms of a Tris- and mRNA-immobilized electrode in 0.1 M AcOK–AcOH buffer (pH 5.6), 0.1 M KCl, and 50 μM I containing 0.04 units/μl RNase inhibitor to obtain a stable current (compared with (a) and (b) in Fig. 6). The redox peaks, based on one-step electron transfer of the ferrocene unit, were observed at 407 and 457 mV vs Ag/AgCl (half-wave potential (E_1/2), 401 mV; peak potential separation (∆E_p), 50 mV) or 392 and 450 mV vs Ag/AgCl (E_1/2, 393 mV; ∆E_p, 58 mV) for mRNA- or Tris-immobilized electrode, respectively (scan rate, 250 mV/s). These ∆E_p value for the mRNA-immobilized electrode was smaller than that of 59 mV in theory,25 and a smaller ∆E_p for mRNA-immobilized electrode is expected for the effective concentration of I on an electrode through its RNA binding.

The intensity in the case of the mRNA-immobilized electrode was larger than that of the Tris-immobilized electrode. This
result showed that 1 concentrated on the mRNA-immobilized electrode, and is reasonable when considering the fact that 1 can bind to nucleic acid, although the binding affinity of 1 with single-stranded RNA was smaller than that with dsDNA. Finally, these results showed that mRNA could immobilize on the electrode by this procedure.

Treatment of mRNA-immobilized electrode with RNase A

In the next step, we measured cyclic voltammograms of the mRNA-immobilized electrode before and after a treatment with 3 ml of 1 μg/ml RNase A in 0.1 M AcOK–AcOH buffer (pH 5.6) and 0.1 M KCl containing 0.05 mM 1 and 0.04 units/μl RNase inhibitor. Figure 4 shows cyclic voltammograms of the mRNA-immobilized electrode before and after the RNase A treatment. The $E_{1/2}$ in a one-step redox reaction of ferrocene of 1 was also observed at 401 and 399 mV vs. Ag/AgCl before and after a RNase treatment, respectively. The $\Delta E_{p}$s were 46 and 55 mV before and after a RNase A treatment, respectively. This result suggested that the concentration process of 1 on the electrode contributes to the electron-transfer reaction in both cases. However, $\Delta E_{p}$ after the RNase A treatment was larger than that before it, and this might be come from the proportion of the increased diffusion process in the electron-transfer reaction of 1 by RNase A digestion.

When compared with both cyclic voltammograms at 250 mV/s of the scan rate, the intensity of the peak current ($i_{pa}$, 0.96 μA) was larger than that after the RNase treatment ($i_{pa}$, 0.76 μA).

Figure 5 showed log-log plots of the oxidative current peak versus the scan rate for the oxidation of 0.05 mM 1 before (a) and after (b) a treatment with 1 μg/ml RNase A. Data were corrected from those in Fig. 4.
the peak current gradually decreased along with an increase of the time scale. However, the peak current was almost stable in the presence of the RNase inhibitor. These results suggested that ordinary water (even if Gengard water was used) was contaminated with RNase during the treatment procedure in the laboratory. In the presence of 1 μg/ml RNase, the peak decrease was observed at time scale. It seemed to reach a plateau, as shown in Fig. 6.

Differential pulse voltammograms of the mRNA-immobilized 25-multi electrode chip before or after a treatment with 3 ml of 5 ng/ml RNase A were also measured as shown in Fig. 7. A current peak at 360 mV vs. Ag/AgCl based on 1 concentrated on the electrode was observed in both cases, and the intensity of this peak decreased after the RNase A treatment. To estimate the amount of RNase A in Gengard water, peak currents before and after RNase A treatment were measure as $i_o$ or $i$ value, respectively. The $i_o$ value was always larger than the $i$ value, but these values were scattered on the individual electrode. However, plots of $i_o$ against $i$ showed a relatively linear slope, where the slope decreased along with an increase of the amount of RNase concentration. Figure 8 shows $i_o$-$i$ plots in the case of Gengard water and 5 ng/ml RNase A. These slopes had a linear relation. The slope in the presence of 1 μg/ml RNase was 0.99, whereas that of 5 ng/ml RNase A was 0.62 in the $i_o$-$i$ plot. This showed that the existence of RNase in water could be determined by the difference of the slope.

The slope in different amounts of RNase A was measured in different electrodes and plotted against the logarithm scale of the RNase A concentration. The result thus obtained shows the good correlation in the range of 0.2 - 10 ng/ml of RNase A, as shown in Fig. 9. When treated with 0.1 ng/ml RNase A, the slope was about 1.0 which is similar to that for Gengard water. Thus, the detection limit in this system seems to be 0.2 ng/ml (0.2 pg). The RNase A detection method previously reported was 0.5 pg RNase A and the the detection limit of this method is in good agreement with that previously reported. These results showed that RNase could be detected by the mRNA-immobilized electrode coupled with 1.

Conclusions

A electrochemical RNase A assay was successfully achieved by the mRNA-immobilized electrode coupled with 1. The detection range was 0.2 - 10 ng/ml of RNase A and the detection limit was 0.2 pg, which is comparable to that by a fluorescence method previously reported. This electrochemical detection method, based on decreasing current still remains to be improved. However, we could succeed in a highly sensitive electrochemical detection of RNase A, even under the conditions employed here.

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