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Analytical Biochemistry
Volume 381
Number 2
Page Range 233-239
Year 2008-10-15
URL http://hdl.handle.net/10228/5587
doi: info:doi/10.1016/j.ab.2008.07.014
Electrochemical Assay for DNase I Activity

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ABSTRACT

A thiolated oligonucleotide having three ferrocenes was immobilized on a gold electrode through the sulfur – gold linkage. This electrode showed a current response based on the redox reaction of the ferrocene moieties and this response was decreased after treatment with DNase I, suggesting the disappearance of the ferrocene moieties on the electrode by the DNase I digestion. The linear correlation between $i_0$ and $i$, which are current peaks before and after DNase I treatment, respectively, was observed and this slope was decreased with increasing in the amount of DNase I. No current decrease was observed in the presence of EDTA or RNase A instead of DNase I. These results suggested that the current decrease responded specifically to the amount of DNase I and this electrode could be used for an electrochemical DNase I assay. Under the optimum conditions of DNase I digestion at 37°C for 30 min, a quantitative analysis could be achieved in the range of $10^{-4} – 10^{-2}$ units/μl of DNase I.

KEYWORDS

DNase I, electrochemical detection, ferrocenylcarbodiimide, ferrocenyloligonucleotide
INTRODUCTION.

Deoxyribonuclease I (DNase I, EC 3.1.21.1) is a non-specific endonuclease that cleaves phosphodiester linkages of single- or double-stranded DNA to give di- and oligonucleotides with a 5’-phosphate and a 3’-hydroxyl group [1-3]. But it has some preference for the cleavage site of purine and pyrimidine bonds such as adenine-cytosine sequences [4, 5]. DNase I plays an important role in biological events such as DNA metabolism [6] and has been suggested to be involved in autoimmune disease systemic lupus erythematosus [7, 8] or apoptosis [9, 10]. Recently, Pulmozyme recombinant human DNase I was cloned, expressed, and produced for the treatment of patients with cystic fibrosis [11]. Diagnostic use of serum DNase I activity as a novel early-phase marker in acute myocardial infarction has also been reported [12]. Due to the biological importance of DNase I and related DNases and their clinical and diagnostic uses, it is essential to measure DNase activity. A variety of methods have been developed to determine the DNase I or DNase activity, but few simple and sensitive detection methods are known including a single radial enzyme diffusion (SRED) method [13] or fluorometric DNase detection methods using PicoGreen dye [14] or DNaseAlert QC System [15] with an oligonucleotide substrate having fluorescein and rhodamine dyes at both termini. These methods are useful, but still have certain drawbacks such as the difficulty in automatic assay readout or the relatively expensive cost of the fluorogenic oligonucleotide required.

The aim of this work was to develop an electrochemical DNase assay method as a rapid, simple and sensitive one with an inexpensive and compact instrument using a ferrocenyloligonucleotide-immobilized electrode. Especially, the ferrocenyloligonucleotide-immobilized electrode has been developed in the viewpoint of electrochemical DNA detecting technology [16-20]. However, an electrochemical DNase assay by using the ferrocenyloligonucleotide-immobilized electrode has not been reported yet except for our preliminary report [21], although an electrochemical detection of DNA cleavage by DNase I pioneered by Palecek [22-26] or an electrochemical detection of DNA-related enzymes [27-30] has been reported over the years. Our idea of the electrochemical DNase I assay is illustrated in Fig. 1. A thiolated oligonucleotide carrying ferrocene (Fc) moieties (Fc-oligo-SH) was
prepared by the reaction of an oligonucleotide disulfide derivative with ferrocenylcarbodiimide (FCDI) followed by the treatment with dithiothreitol (DTT) which was developed as a simple ferrocenylation reagent for DNA research by our group [31-33]. Different means of DNA labeling especially with electrochemically active reagents have been described in detail in the papers [31, 34]. When the electrode is treated with an aqueous solution containing DNase I, the Fc parts would be removed from the electrode after cleavage by DNase I. This should cause a decreased electrochemical response depending on the amount of DNase I. This system will be first report, but photosignal change after DNase I cleavage was reported in the homogenous medium by using the DNA duplex formed from oligonucleotides having fluorescence and Au nanoparticle [35].

In this paper, we describe the construction of a sensor electrode carrying Fe-oligo-SH to achieve the electrochemical DNase I assay.
EXPERIMENTAL SECTION

**Materials.** Ferrocenylcarbodiimide (FCDI, Fig. 1) as a ferrocenylation reagent for DNA was synthesized according to the route described previously [31]. An oligonucleotide disulfide derivative, 5’-HO(CH₂)₆-SS-(CH₂)₆-dA₁₀ ACA AAT AAC AAA TAT-3’, was custom-synthesized by Genenet Co. (Fukuoka, Japan). BioPak water was purified by a Milli-Q system Gradient A10 coupled with Elixs3 kit (Millipore, Billerica, MA). DNase I (RNase free) was purchased from TaKaRa Bio Inc. (Shiga, Japan). 10xDNase I buffer (400 mM Tris-HCl (pH 7.5), 80 mM MgCl₂, and 50 mM DTT) from TaKaRa Bio Inc. was used to dilute DNase I.

**HPLC.** 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 Co., Tokyo, Japan). Reversed phase HPLC was run using a Mightysil RP-18 column (0.5 x 2.5 cm) (Kanto Chemicals Co. Inc., Tokyo, Japan) with the gradient conditions, where the acetonitrile (CH₃CN) content in 100 mM triethylammonium acetate (TEAA) buffer (pH 7.0) was linearly changed from 10 to 95% over 30 min at a flow rate of 1.0 ml/min with detection at 260 nm.

**MALDI-TOF MS.** Oligonucleotides modified with FCDI was characterized by MALDI-TOF MS (Voyager™ Linear-SA, PerSeptive Biosystems Inc., FosterCity, CA) measurement of the products separated by HPLC. They were dissolved in a solution of 50 mg/ml 3-hydroxypicolinic acid (3-HPA) in 0.1% trifluoroacetic acid (TFA)/50% CH₃CN and dried. Mass spectra were measured by the negative mode.

**Preparation of Fc-oligo-SH.** Thirty microliters of a solution of 5’-HO(CH₂)₆-SS-(CH₂)₆-dA₁₀ ACA AAT AAC AAA TAT-3’ (30 nmol) in 50 mM borate buffer (pH 9.0) was mixed with 30 µl of a solution
of 100 mM I in 50 mM borate buffer (pH 9.0) containing 60 % DMSO. After overnight shaking at 37 °C, the reaction mixture was justified to 1 ml by 1 ml with 0.1 M TEAA buffer (pH 7.0) and then this solution was seeped into the top of NAP-10 column (Pharmacia Sephadex G-25, Amersham Biosciences Co., Uppsala, Sweden) to remove unreacted FCDI. NAP-10 column was replaced by 0.1 M TEAA buffer (pH 7.0) by passage of 1.5 ml of its solution. One ml of the first fraction was collected, and freeze-dried. The lyophilized product was dissolved in 60 µl of autoclaved BioPak water and purified by reversed phase HPLC. The fraction eluted at 20 min was collected, dried and dissolved in 50 µl sterilized water. The oligonucleotide disulfide modified by three ferrocenes thus obtained, HO(CH2)6-SS-(CH2)6-dA10 ACA ATFc AAC AAA TATFc-3’ (Fc: FCDI modified part) was identified by MALDI-TOF MS, and reduced by the treatment with 0.04 M DTT in water to afford the desired thiolated ferrocenyloligonucleotide (Fc-oligo-SH).

**DNase I Digestion of Fc-oligo-SH in Homogenous Solution.** The varied amounts of DNase I was mixed with a solution of 10 µM Fc-oligo-SH in 1xDNase I buffer. The reaction mixture was incubated at room temperature for 30 min and the enzymatic hydrolysis was stopped by heating at 80°C for 10 min. Fc-oligo-SH did not show any decomposition under such conditions of heating in the absence of the enzyme. The reactivity of DNase I to Fc-oligo-SH was assessed by reversed phase HPLC and MALDI-TOF MS.

**Preparation of Fc-oligo-SH-immobilized Electrode.** A gold electrode of 2.0 mm² in area (commercially available electrode, Bioanalytical Systems (BAS) Inc., Tokyo, Japan) was polished with 6 and 1 µm of diamond slurry and with 0.05 µm of alumina slurry in this order, and sonicated in Milli-Q water for 15 min (3 times). This electrode was electrochemically polished by scanning 40 times from -0.2 to 1.5 V at a scan rate of 100 mV/s in a 0.5 M H2SO4 aqueous solution and sonicated in Milli-Q water for 15 min (3 times). One microliter of 1 M NaCl solution containing 0.5 µM Fc-oligo-SH was
placed on the gold electrode and kept in a closed container under high humidity for overnight at room temperature. After washing with Milli-Q water, 1 µl of 1 mM 6-mercapto-1-hexanol was placed on the electrode for 1.5 h at 45°C.

**DNase I Reaction on the Electrode and Electrochemical Measurement.** DNase I reaction was carried out by the treatment of the ferrocenyloligonucleotide-immobilized electrode with 1 µl of the water containing varied amounts of DNase I for 30 min at 25°C or 37°C. Electrochemical measurement was made on an ALS Electrochemical Analyzer Model 900 (CH Instrument Inc., Austin, TX) in 10 mM NaHPO4/NaH2PO4 buffer (pH 7.0) and 0.1 M NaClO4. The redox behavior of the electrode immobilized with the *Fc-oligo-SH* was monitored by the cyclic voltammetry (CV) measurement over a scan range of 0 - 0.5 V at a several scan rate of 10 - 50 mV/s. The Osteryoung square wave voltammetry (SWV) method was used in the experiments for *Fc-oligo-SH*-immobilized electrodes before and after DNase I treating with amplitude of 50 mV, applied potential of 10 mV, and frequency of 10 Hz. The measurement cell was furnished with three-electrodes consisting of an Ag/AgCl reference electrode, a Pt counter electrode, and a *Fc-oligo-SH*-immobilized electrode as the working electrode. The current peak i₀ or i in SWV measurements were carried out before or after treating with varied amounts of DNase I.

**Quartz Crystal Microbalance (QCM) Measurement.** QCM measurements were performed on AffinixQ (Initium Co., Tokyo, Japan) by using the gold surface (2.5 mm in diameter and 4.9 mm² in area) of 27 Hz and an AT-cut QCM sensor chip (Initium Co.). A frequency change of 1.0 Hz is equivalent to 30 pg mass change on the surface in aqueous solution [36]. The QCM chip was treated with a Piranha solution (7:3 = conc. H₂SO₄:30% H₂O₂, CAUTION. Because the Piranha solution reacts violently, even explosively, with organic materials, it should not be sorted or combined with significant quantities of organic material) for 10 min and washed with Milli-Q water. One hundred µl of 0.5 µM *Fc-oligo-SH* was put on this chip and kept for overnight at room temperature. After washing with Milli-Q water, 100 µl of 1 mM 6-mercapt-1-hexanol was put on this chip for 1.5 h at 45°C and washed with
Milli-Q water. QCM measurements were carried out on the chip in 1xDNase I buffer at 37°C with stirring rate of 600 rpm. DNase I was added to the chip in a final concentration of $5 \times 10^{-6}$ unit/$\mu$l, and the time course of the frequency changes was measured.

RESULT AND DISCUSSION

Synthesis of Fc-oligo-SH. Twenty-five-meric oligonucleotide disulfide derivative having three thymines, 5'-HO(CH$_2$)$_6$-SS-(CH$_2$)$_6$-dA$_{10}$ ACA AAT AAC AAA TAT-3', was allowed to react with FCDI in 50 mM borate buffer (pH 9.0) containing 30% DMSO at 37°C overnight and the product was purified by reversed phase HPLC. Fig. 2(A) showed the HPLC traces before (a in Fig. 2(A)) and after (b in Fig. 2(A)) reaction of the oligonucleotide disulfide derivative with FCDI. The peak at 15 min of the oligonucleotide disulfide derivative was changed to that at 20 min. This peak product was assigned as 3:1 adduct of FCDI with the oligonucleotide disulfide derivative by MALDI-TOF MS as shown in Fig. 2B (The parent peak of 9377 was in good agreement with the theoretical value of 9380 within the error).

DNase I Reaction in Homogenous Solution.

A solution of 10 $\mu$M Fc-oligo-SH was treated with 2.5 units/$\mu$l DNase I in 1xDNase I buffer at room temperature for 30 min. Reversed phase HPLC analysis of the reaction mixture showed many peaks as shown in Fig. 3(A). The HPLC fractions of the retention times of 5 – 10 min, 20 – 25 min, 33 – 35 min, and 30 min were collected and assigned by the MALDI-TOF MS analysis (Fig. 3(B)-(E)). The fraction of 5 –10 min showed many periodical peaks started from $m/z = 313.2$. These peaks could be assigned as mono-, di-, tri-, and tetra-adenine fragments with or without HS-(CH$_2$)$_6$- moiety (Fig. 3(B)). The peak at the retention time of 20 – 25 min contained the fragment with $m/z$ of 2627.7 and 2938.9, which are assigned as CAA AT$^{Fc}$ A A and A CAA AT$^{Fc}$ A A, respectively, and supported that DNase I cleaved AC site (Fig. 3(C)). The main peak at the retention time of 33 – 35 min contained the fragment with $m/z$ of 3077.6 assigned as CAA AT$^{Fc}$ A T$^{Fc}$ (Fig. 3(D)) and the peak at the retention time of 30 min has $m/z$ of
473.1 assigned as FCDI (Fig. 3 (E)). DNase I clearly cleaved the phosphate bond of AC site of purine–pyrimidine sequence, which is in agreement with the paper previously reported [1-3].

Then the solution of 10 μM Fc-oligo-SH was treated with the varied amounts of DNase I. After treatment for 30 min at room temperature, DNase I was deactivated by heating and the digestion ratio was estimated from the fragment peak areas in HPLC. Fig. 4 shows the plot of the digestion percentage of Fc-oligo-SH by DNase I in the homogenous solution. The digestion percentage was increased with an increase in the amount of DNase I. This also suggested that the Fc-oligo-SH acted as a DNase I substrate and its activity could be monitored.

**Estimation of the Amount of the Immobilized Fc-oligo-SH on the Electrode.**

Fig. 5(A) shows the cyclic voltammograms (CVs) of the Fc-oligo-SH-immobilized electrode at the varied scan rates. Oxidation and reduction current peaks were observed at 186 and 180 mV, respectively. The peak potential separation, ΔE_p, was 6 mV, which was smaller than that of the theoretical value of 57 mV based on the diffusion of the ferrocene in a homogenous medium and this value is in agreement with the electron transfer reaction of the immobilized ferrocene on the electrode [37, 38]. Fig. 5(B) shows the plots of the oxidation current response at its oxidation peak against the scan rate, and a good linear correlation was observed. This result also shows that Fc-oligo-SH could be immobilized on the gold electrode and could contribute to an electron transfer process from the ferrocene moiety to the electrode. The amount of the immobilized Fc-oligo-SH was estimated to be 1.02 ± 0.09 pmol/cm² (0.6 x 10¹² molecules/cm²) from the area of the oxidative current in Fig. 5(A) where 1 μl of 0.5 μM Fc-oligo-SH was used. Twenty five-meric Fc-oligo-SH was selected in this experiment because it seems to be standing with highly extended configuration on the electrode surface as discussed by Steel et al.[39]. They also suggested that the surface coverage begins to decrease with probe length for the thiolated oligonucleotides longer than 24 bases because of their increasingly polymeric nature [39]. Average distance between the Fc-oligo-SH immobilized on the electrode surface was expected to be 127 Å.
calculated by the equation of \(1/\sqrt{(N_A \Gamma)}\), whereas \(N_A\) and \(\Gamma\) refer to Avogadro's constant and immobilization density (0.6 x 10^{12} molecules/cm^2), respectively. This value was larger than the size of DNase I (45 Å x 40 Å x 35 Å)[40]. Furthermore, the efficiency of DNase I reaction on the electrode was 48%, 72%, or 73% when treated with 1 μl of 2 μM, 1 μM, or 0.5 μM Fc-oligo-SH, respectively (data not shown). These results also suggested that the enough space between the immobilized oligonucleotide or the density and the conformation of the probe on the electrode are important for the effective reaction of DNase I as shown in the papers [41, 42].

**Monitoring of DNase I Activity by QCM.** DNase I activity could be monitored by QCM measurement. Thus Fc-oligo-SH immobilized on QCM chip was treated with DNase I in 1xDNase I buffer at 37°C. In the presence of DNase I, frequency was observed and reached to plateau as shown in Fig. 6(a). The frequency change at the plateau was 24 Hz or the mass change was 16 ng/cm^2, equivalent to 21 pmol/cm^2 or 1.5 x 10^{12} molecules of Fc-oligo-SH/cm^2. This value was larger than that in the case of the electrode prepared above. However, this is reasonable because 10-times largest amount of the oligonucleotide Fc-oligo-SH was used in this case. On the other hand, no frequency change was observed in the case of 6-mercaptop-1-hexanol immobilized on QCM chip (Fig. 6(b)). This result supported that DNase I could cleave the oligonucleotide on the gold surface and did not show a non-specific surface absorption.

**Electrochemical Detection of DNase I Activity.** Finally, we attempted to construct an electrochemical DNase I assay system by using the ferrocenyloligonucleotide-immobilized electrode. Thus, we intended to test the ability of this system according to the principle in Fig. 1. Firstly, the peak current \(i_o\) value was collected by the SWV measurement of the Fc-oligo-SH-immobilized electrode in 10 mM phosphate buffer (pH 7.0) and 0.1 M NaClO_4. One microliter of a solution containing varied amounts of DNase I was put on this electrode, kept at 25 or 37°C for 30 min, and the \(i\) value was observed by the SWV measurement. Typical SWV experiment to obtain \(i_o\) and \(i\) values using 2x10^{-3}
units/μl DNase was shown in Fig. 7(A). The peak current decreased after treatment with DNase I suggesting that the DNase I digested the **Fc-oligo-SH** on the electrode. However, the intensity of the peak current was changeable depending on the individual electrode used suggesting the variation of the immobilized amount of **Fc-oligo-SH** on the electrode. To solve this problem, a relationship between the peak currents $i_o$ and $i$ before and after treatment with DNase I was checked (Fig. 7(B)). The $i_o – i$ plot showed a good linear correlation and the slope decreased with an increase of the amount of DNase I, or its activity. Fig. 8 shows the correlation between DNase I activity and the slope in the $i_o – i$ plot at 25 and 37°C as for the digestion temperature. Both showed a good linear correlation. However, in the case of 37°C, a good linear correlation was observed in the range of $10^{-5} – 10^{-3}$ units DNase I/μl, and the detection limit of $10^{-4}$ units/μl of DNase I was expected. This value was 100-times smaller than that in the case of 25°C and a similar detection limit of DNase I using DNase Alert QC System [15] had been reported. This result in the case of 25°C is reasonable when considering the fact that the optimum temperature of DNase I is 37°C. To evaluate the specificity of this sensor electrode for DNase I activity, DNase I treatment in the presence of 5.4 mM ethylenediamine tetraacetic acid (EDTA) (Fig. 9(A)) and RNase A treatment instead of DNase I (Fig. 9(B)) were carried out considering the inhibitory activity of EDTA for DNase I and RNA specific activity of RNase A. The peak potential also varied slightly with the individual electrode used. This reason is not clear now, but it is known that the peak potential varies depending on the density of the oligonucleotide having electrochemically active molecules such as ferrocene or methylene blue immobilized on the electrode [43-45]. The current peaks of the **Fc-oligo-SH**-immobilized electrode were relatively stable within the error before and after treatment of DNase I solution with EDTA or RNaseA solution suggesting that this electrode could be used for the detection of DNase I activity specifically.

**CONCLUSION**

A thiolated ferrocenyloligonucleotide, **Fc-oligo-SH**, was prepared by the reaction of **FCDI** with an oligonucleotide disulfide derivative followed by the treatment with DTT and could be immobilized on
the gold electrode. This electrode was successfully used to realize the electrochemical DNase I activity assay. The current peak of this electrode decreased after treatment with DNase I, and the activity was well correlated with the slope in the $i_0 - i$ plot, where $i_0$ and $i$ refer to the peak current before and after treatment with DNase I. The detection limit of DNase I in this electrochemical assay system was $10^{-4}$ units/$\mu$l under 37°C digestion conditions. This method requires the labeling step of a thiolated oligonucleotide by FCDI. However, multi-labelings with the ferrocene could be achieved by a simple reaction with FCDI, enabling a simple detection of DNase I activity in vitro. The previous papers connected with the electrochemical detection of enzymatic cleavage of DNA duplex [22-26] or DNA base damage [46] might be useful to improve the sensitivity of this method. Improvement of the surface homogeneity of the individual electrode should lead this system to be the higher sensitive assay system. This system also can be extended to the electrochemical nuclease assay systems for other nucleases changing the oligonucleotide parts of $\text{Fc-oligo-SH}$ to an appropriate oligonucleotide structure as their substrates.

**ACKNOWLEDGEMENT**

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. S.S. is grateful for the financial support from the Japan Society for the Promotion of Science.
**Fig. 1.** Principle of electrochemical DNase I assay based on \textbf{Fc-oligo-SH}-immobilized electrode and chemical structure of \textbf{FCDI}. 
Fig. 2. (A) Reversed phase HPLC before (a) and after (b) reaction of 30 nmol HO(CH$_2$)$_6$-SS-(CH$_2$)$_6$-dA$_{10}$ACA AAT AAC AAA TAT-3’ with 50 mM FCDI in 50 mM borate buffer (pH 9.0) containing 30 % DMSO at 37°C for overnight. (B) MALDI-TOF MS of the HPLC fraction at 20 min in (A) (b). Matrix, 3-HPA; mode, negative. $m/z$ [M-H] = 9377 (theory for C$_{332}$H$_{447}$Fe$_3$N$_{124}$O$_{138}$P$_{25}$S$_2$, 9380).
Fig. 3. (A) Reversed phase HPLC of 10 µM Fc-oligo-SH after treatment with 2.5 units/µl DNase I in 1xDNase I buffer at room temperature for 30 min. MALDI-TOF MS of the HPLC fractions of the retention time of 5-10 min (B), 20-25 min (C), 33-35 min (D), and 30 min (E). The peak product at the retention time of 11.5 min was always derived from the DNase I buffer and did not change during the DNase I digestion reaction.
Fig. 4. Plot of the digestion percentage of **Fc-oligo-SH** by DNase I. The digestion percentage was determined from the area of the fragment peaks based on the total peak one after multiplied by 100 in HPLC measurement. n=3.
Fig. 5. (A) CVs of the Fe-oligo-SH-modified electrode after treatment with 1 µl of 0.5 µM 2 in 10 mM NaHPO₄/NaH₂PO₄ buffer (pH 7.0) and 0.1 M NaClO₄ at the varied scan rates of 10, 20, 30, 40, and 50 mV/s from center to outside. (B) Correlation between the oxidative current response at its peak and the scan rate.
**Fig. 6.** Frequency change of QCM chip immobilized with the **Fc-oligo-SH** (a) or 6-mercapto-1-hexanol (b) after the addition of $5 \times 10^{-6}$ units/µl DNase I.
Fig. 7. (A) SWV curves of **Fc-oligo-SH**-immobilized electrode before (solid line) and after (dotted line) treatment with $2 \times 10^{-3}$ units/μl DNase. (B) Plots of the peak current $i_0$ against $i$ for the same experiments in (A).
Fig. 8. Relationship between DNase I activity against the slope in the i₀-i plot at 25°C (○) or 37°C (●).
Fig. 9. SWV curves of Fe-oligo-SH-immobilized electrode before (solid line) and after (dotted line) of 1 unit/ml DNase I in the presence of 5.4 mM EDTA (A) and 1 unit/ml RNase (B).
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