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An electrochemical study of enzymatic oligonucleotide digestion

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Abstract

This paper describes the synthesis and application of a novel ferrocene (Fc) label that can be efficiently attached to oligonucleotides. We demonstrate how pulse electrochemical methods can be used to measure very low concentrations of ferrocene label and, importantly, show good electroanalytical discrimination between a labelled oligonucleotide and an enzyme digested labelled oligonucleotide, in which the ferrocene label nucleotide conjugate has been released. Real time in situ analysis gives a much greater understanding of the process. Potential applications include the detection of specific nucleic acid sequences and measurement of nuclease activity.

Keywords: Ferrocene; Oligonucleotide; Sensor; Enzymatic digestion

1. Introduction

The use of electrochemical probes to rival fluorescence in DNA and oligonucleotide interrogation has recently attracted attention [1,2]. Typically, ferrocene (Fc) or other electroactive moities such as ruthenium and osmium inorganic complexes [3] are used as the reporter molecule as they have well-understood electrochemistry, good stability and convenient synthetic chemistry [4]. In electrochemical studies of DNA, ferrocene-labelled intercalators and other transition metal complexes have been used as intercalators predominately for hybridisation detection [5]. A review of the use of electrochemistry to study both DNA hybridisation (for base sequence determination), as well as the presence of point mutations and damaged DNA is given by Palecek and Fojta [6] and complemented by Wang's biosensing review [7]. In an alternative approach, several workers [8–10] have ferrocene labelled a base phosphoramidite and then incorporated it into the oligonucleotide by synthesis. We report a ferrocene-based electrochemical label that allows the enzymatic digestion of oligonucleotides to be easily detected using Differential Pulse Voltammetry (DPV). Van Berkel et al. [11] have reported the detection of the ferrocene dimer (FcNHCONHFc) during derivatisation experiments to form ferrocene urethanes. Ferrocene carboxylic acid based labels have been used previously, but have relatively high oxidation potentials (ca. 0.4 V vs. Ag|AgCl) [1]. The advantage of ferrocene urea compounds is that the urea stabilises the oxidised ferrocene, thus giving a lower oxidation potential. Hence, potential problems from interferants such as peroxides can be avoided.

2. Experimental

4-(3-Ferrocene-ureido)-benzoic acid 5 was synthesised by a variation of the method of Van Berkel et al. [11]. Ferrocene acyl chloride 2 was reacted with sodium azide to form the ferrocenoyl azide 3. This was heated to form 4 which was coupled to 4-aminobenzoic acid to yield 5. The acid was converted to the *N*-hydroxysuccinimide ester 6 using standard coupling conditions (Scheme 1).

The oligonucleotide coupling followed the method of Takenaka [12]. Compound 6 (0.46 mg, 1 μmol) in 50 μl anhydrous DMSO was added to C6 amino modified oligonucleotide (20 nmol) dissolved in 50 μl of 500 mM potassium carbonate buffer (pH 9.0). The mixture was shaken at 25 °C for 15 h. The conjugate 7 was purified from unconjugated ferrocene and other low molecular weight species using a sephadex G-25 column (Sigma) (Scheme 2).

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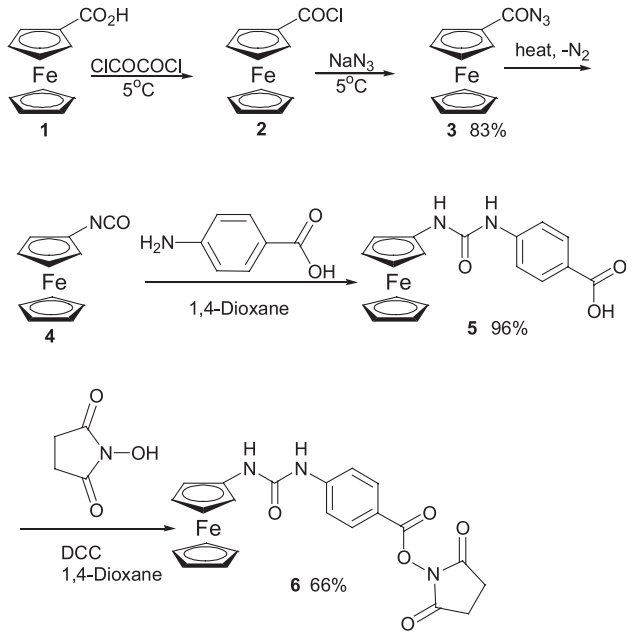


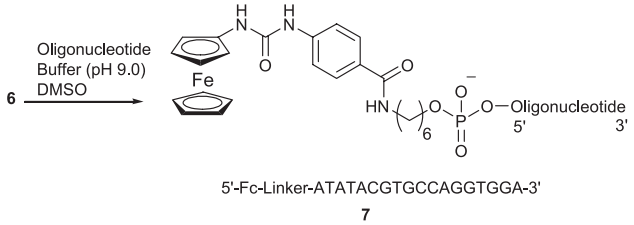
Scheme 1. Synthesis of 4-(3-ferrocene-ureido)-benzoic acid 5 and conversion to the *N*-hydroxysuccinimide ester 6.

The S1 nuclease digestion used 5 μM oligonucleotide, 0.25 U/μl S1 nuclease and a buffer consisting of 0.25 M ammonium acetate and 4.5 mM zinc acetate dihydrate (pH 6.5). The control sample omitted the enzyme, which gives the same result as using a heat-denatured enzyme (data not shown). The samples were incubated at 37 °C with electrochemical measurements of both a control system (without enzyme) and the digest being made in situ approximately every 8 min up to 90 min (Fig. 3).

The control and digest samples were electrochemically measured using DPV with an Ecochemie Autolab. The electrochemical cell consisted of a 200 μl low volume cell (BAS), glassy carbon working electrode, a silver/silver chloride (3M KCl) reference electrode and a platinum wire counter electrode.

3. Results and discussion

We report the change in electrochemical response of the labelled conjugate 7, before and after enzyme digestion. This single-stranded Fc-labelled oligonucleotide is digested by the



Scheme 2. Attachment of the oligonucleotide to 6 to form conjugate 7.

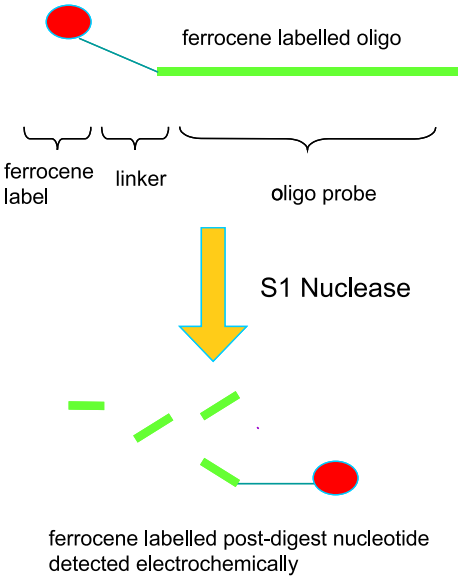


Fig. 1. Schematic of experimental procedure. Labelled oligonucleotide 7 has very low electrochemical oxidation current. Following endonuclease action, Fc probe cleaved and detected by differential pulse voltammetry.

S1 nuclease enzyme, as detailed above (Fig. 1). This enzyme is an endonuclease that degrades single-stranded nucleic acids by cleavage of the phosphodiester bond to yield 5′ phosphorylated products (mononucleotides or short oligonucleotides). As the linker is not affected by the enzyme, some of these fragments will be ferrocene labelled. Fig. 2 clearly shows the large increase in ferrocene oxidation current (measured by DPV) following endonuclease action compared to the labelled oligo before enzyme digestion.

DPV is a pulse technique which allows much higher sensitivity than conventional sweep techniques when detecting very low concentrations of a redox probe [13]. This is achieved by applying a small voltage pulse superimposed

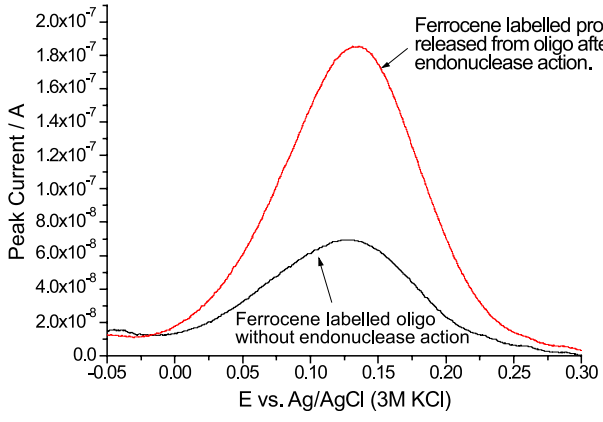


Fig. 2. Differential Pulse Voltammogram of the labelled oligonucleotide before enzyme digestion and following enzyme digestion following 90 min incubation at 37 °C. The area of the glassy carbon electrode is 0.07 cm^2. Voltammetric parameters are as follows. Scan rate, 3 mV s^{-1}; modulation time, 40 ms; interval time, 100 ms; step potential, 0.3 mV; modulation amplitude 50 mV.

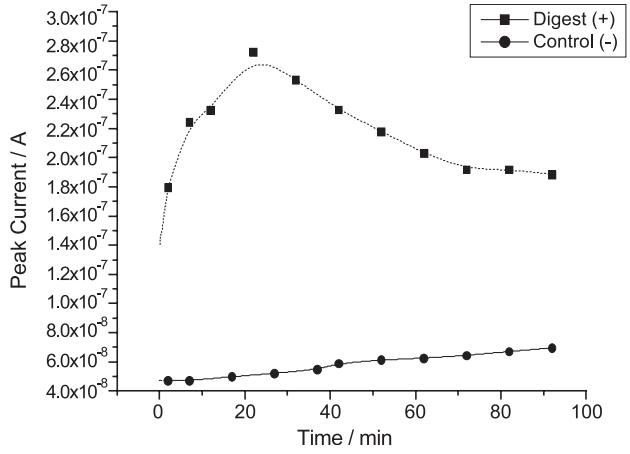


Fig. 3. Comparison of the Digest (+) and Control (−) samples, during S1 nuclease digestion.

on the linear voltage sweep, and sampling the differential current at a short time after the pulse. Hence, the measured current is only a product of the Faradaic process, with the capacitive charging current eliminated.

The difference in the electrochemical response of the labelled oligonucleotide before and after endonuclease action shown in Fig. 2, is attributed to both differences in the diffusion coefficient of the free probe compared to the labelled oligo and the possibility that the oligonucleotide assumes a secondary structure that may hinder access of the ferrocene label to the electrode.

A time course of the S1 digestion, using gel electrophoresis, showed the digestion to be virtually complete after 25 min. Real time analysis of the digestion at 37 °C, by measuring consecutive DPV traces (Fig. 3), gives a current maximum for the digest (+) shortly after 25 min, which then decays. We propose that the initial rapid increase of signal is due to the enzymatic digestion and liberation of small ferrocene-labelled fragments, which are able to readily diffuse to the electrode surface. The rate of increase slows to the maximum and then decays probably due to the effect of electrode fouling from the enzyme and oligo fragments. In contrast when the enzyme is omitted, the control (−), the much weaker response increases linearly with time. Although the origin of this behaviour is not entirely clear, one possibility is that this is indicative of nonspecific absorption of the ferrocene-labelled oligo probe, which creates an electroactive film that slowly thickens with time, giving a gradual increase of background signal. Further work is underway to determine the exact nature of this adlayer.

In conclusion, we have shown that using our ferrocene-labelled probe gives us good discrimination between digested and undigested oligonucleotide, using DPV and a standard electrochemical cell. The time resolved work gives a good understanding of the digestion and the behaviour of the electrode surface, particularly the effect of fouling. Moreover, the good correlation between the gel electrophoresis time course measurement and the time at which the maximum current was observed is of interest. Further applications of these (and related) probes are currently being investigated, a potential application being homogeneous, nuclease-based assays for the sequence specific detection of DNA.

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Appendix A. Notes and references

This work is the subject of patent applications.

Synthesis of 4-(3-ferrocene-ureido)-benzoic acid *N*-succinimide ester 6. Further synthetic detail is available form the authors on request.

DCC (194 mg, 0.939 mmol, 1.14 equiv.) was dissolved in anhydrous 1,4-dioxane (2 ml) and charged to a purged flask, under nitrogen. *N*-Hydroxysuccinimide (108 mg, 0.938 mmol, 1.14 equiv.) was charged. The 4-(3-ferrocene-ureido)-benzoic acid 5 (300 mg, 0.824 mmol 1.0 equiv.) was dissolved in anhydrous 1,4-dioxane (13 ml) and charged dropwise to the flask. The solution was stirred at room temperature for 23 h. A small amount of light brown solid was removed from the red/orange reaction mixture by Buchner filtration. Water (100 ml) and ethyl acetate (50 ml) were charged to the reaction mixture. The ethyl acetate phase was separated and the aqueous was extracted with ethyl acetate (100 ml). The ethyl acetate phases were combined, dried with sodium sulphate and concentrated in vacuo to afford the crude product as an orange oil, which was purified using silica flash chromatography with a gradient system from ethyl acetate 60/petroleum ether (bp 40–60 °C) 40 to ethyl acetate. Drying in a vacuum oven yielded 6 as fine orange coloured crystals (237 mg, 66%). ^{1}H-NMR δ (300 MHz, d6-DMSO) 2.88 (4H, s, CH_2CO), 3.98 (2H, t, J = 1.8 Hz, Cp*), 4.16 (5H, s, Cp), 4.55 (2H, t, J = 1.8 Hz, Cp*), 7.68 (2H, m, ArH), 8.00 (2H, m, ArH), 8.11 (1H, s, CpNH), 9.16 (1H, s, ArNH). ^{13}C-NMR δ (100 MHz, d6-DMSO) 25.9 (Ar), 60.7 (CH, Cp*), 63.8 (CH, Cp*), 68.7 (Cp), 95.8 (C, Cp*), 116.0 (CCO_2N), 117.2 (Ar), 131.4 (Ar), 146.4 (Ar), 152.0 (CONH), 161.0 ($COCH_2$), 170.2 (CO_2N). IR (KBr) ν (cm-1) 3379, 2928, 2851, 1762, 1734, 1718, 1600, 1560, 1540. MS (FAB + m/z) 462.0 [M + H]. HRMS (ES+): $C_{22}H_{20}FeN_3O_5$ [M + H] requires 462.0747 found 462.0748.

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