# The first automated synthesis of ferrocene-labelled phosphorothioate DNA probe: A new potential tool for the fabrication of DNA microarrays

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Short Communication

# The first automated synthesis of ferrocene-labelled phosphorothioate DNA probe: A new potential tool for the fabrication of DNA microarrays

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We report the synthesis and the characterisation of the first electroactive ferrocene-labelled oligonucleotide phosphorothioate (ODN-Fc-Ps) probe obtained by automated synthesis. The grafting of ODN-Fc-Ps probe on gold electrode resulted in the appearance of the ferrocene redox couple in cyclic voltammetry confirming the effectiveness of the ODN grafting. The electrochemical response of the modified electrode was analysed in aqueous media before and after hybridisation with ODN target. The hybridisation with ODN target induces a large conformational change in the surface-confined DNA structure monitored by cyclic voltammetry of the ferrocene marker which confirms the potential of ferrocene-labelled oligonucleotide phosphorothioate to develop electrochemical DNA chips.

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During the last years extensive research has been directed towards the detection of specific DNA sequences using real-time methods without radioactive isotopes, for applications in clinical diagnostics [1], environmental protection [2], food quality control [3] and forensic science [4]. In contrast to existing optical approaches, electrochemical methods have received particular attention due to their high sensitivity, easy instrumentation, low production cost and compatibility to make small devices [5–7]. In previous works we have described a new strategy to prepare ferrocene labelled oligonucleotides (ODN-Fc) probes based on a replacement of a nucleotide directly by a ferrocene unit during automated solid-phase DNA synthesis [8–10]. ODN-Fc were characterised by cyclic voltammetry in solution and have shown different electrochemical behaviour before and after hybridisation reaction with complementary strand. In this context a fully automated synthesis of a ODN probe with ferrocene unit and successive internucleosidic phosphorothioate groups at 3' position using Beaucage's reagent was developed [11]. The choice of phosphorothioate group was determined for its compatibility with automated synthesis with the multi-point anchoring of the DNA which gives stable immobilisation of DNA on the gold electrode [12]:

ODN 3.S
5'GTA TTC CTT GGA CTC ATA AGG T-**Fc**-TpsTps TpsTpsTpsT 3'
Ps = phosphorothioate linkage

This new electrochemical ODN 3.S probe combines the significant advantages of electrochemical detection (sensitivity and rapidity) to monitor the effective layer formation and the hybridisation reaction.

ODN 3.S was synthesized on ABI 394 DNA/RNA synthesiser according to classical protocols. 1-[3-O-dimethoxytritylpropyl]-1'-[3'-O-(2-cyanoethyl-N,N-diisopropyl phosphoramidityl) propyl]ferrocene was prepared as described previously (Fig. 1) [8–10].

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**Abbreviations: ODN-Fc**, ferrocene labelled oligonucleotides; **Ps**, phosphorothioate linkage

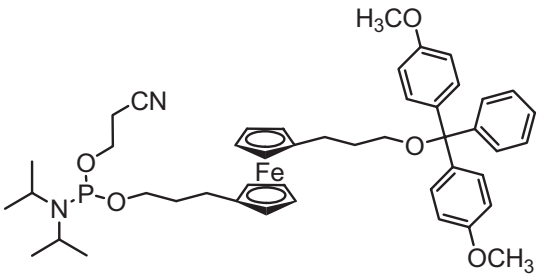


**Figure 1.** Structure of 1-[3-O-dimethoxytritylpropyl]-1'-[3'-O-(2-cyanoethyl-N,N-diisopropyl phosphoramidityl) propyl]ferrocene.

Classical nucleoside phosphoramidites and reagents were obtained from Glen Research (Sterling, Virginia). Phosphodiester and phosphorothioate linkages were obtained by using either 0.02 M $I_2$ in THF/Pyridine/$H_2O$ or 0.05 M 3-H-1,2-Benzodithiole-3-one-1,1-dioxide in anhydrous acetonitrile [11]. DMTr ON oligonucleotides were purified after ammonium hydroxide deprotection (28%, 6 h at 60°C) on MOP cartridge (CTGEN, San Jose, CA). DMTr was removed using 80% acetic acid solution (30 min) and oligonucleotide was precipitated in ethanol. HPLC characterisation was carried out on a Kontron HPLC using a chromolith RP18e column (Merck) with a gradient (5% ‡ 80% CH3CN in TEAAc 0.05 M during 35 min). ODN 3.S was purified on preparative reverse phase column. ODN purities were controlled by reverse phase HPLC. The ODN was eluted with a retention time (25.79 min) superior than the one of natural ODN probe (13 min) confirming the modification of the fragment. The width of the peak observed was due to diastereoisomeric forms of phosphorothioate linkages. ODN 3.S was characterised by MALDI-TOF mass spectral analyses at negative mode in a saturated solution of hydroxypiccolinic acid (HPA) in water. The result for modified oligonucleotide ODN 3.S was: m/z calcd 9008.7, found 9014.2 that illustrate the successful incorporation of ferrocenyl moiety into the oligonucleotide. The difference (0.06%) between calculated and found masses was attributed to difficulties encountered during calibration of the instrument. In fact, the two well known oligonucleotides used for calibration had a mass inferior to the one of ODN 3.S and therefore, the range of calibration was not strictly accurate for the sample analysis. The same problem was observed in literature [13]. The concentration of ODN 3.S was determined by UV absorption at 260 nm, assuming a molar extinction coefficient (ε260) of 264214 L/(mole·cm$^{-1}$).

The ODN 3.S was anchored on the gold electrode via chemisorption as the protocol described by Ihara et al. [12]. A 5 µl droplet of a 50 µM aqueous solution of ODN 3.S (containing 100 µM KCl) was casted on a freshly-polished gold disk electrode. The electrode was washed with 100 µM KCl solution and then ultrasonicated for 10 min in 0.1 M $LiClO_4$ at room temperature and stored at 5°C before use.

In the absence of target DNA, a ferrocene redox couple was observed in cyclic voltammetry using the ODN 3.S modified gold electrode (Fig. 2).

A BAS 100 from Bioanalytical Instrument was used for electrochemical measurement. The working electrode was a 1.6 mm diameter (ca. 0.02 cm$^2$ geometrical area) gold disk working electrode polished before each set of modification with 1 µm diamond paste, rinsed with water and then ultrasonicated for 10 min in degassed absolute ethanol. All cyclic voltammetry experiments were carried out at room temperature (20°C) using a cell equipped with a jacket allowing circulation of water from the thermostat. 0.10 M KCl/$H_2O$ was used as the supporting electrolyte solution.

The apparent formal potential (E°′) of the electroactive label is 0.212 V *vs* Ag/AgCl, as estimated from $E_{1/2} = (E_{red} + E_{ox})/2$ with 239 and 185 mV for respectively $E_{ox}$ and $E_{red}$. The difference of 54 mV bewteen $E_{ox}$ and $E_{red}$ can be interpreted as reversible Nernstian behavior for ferrocene/ferrocinium in a diffusion process for ODN 3.S in solution or a slow transfer electron in ODN 3.S adsorbed at the electrode surface. The linear relationship between peak currents and scan rates confirms that the redox species are confined to the electrode surface with a slow transfer electron [14]. The value of E°′ is in the typical redox potential range of ferrocene [13, 15]. We thus ascribe this redox couple to the redox conversion of ferrocene labels in close proximity to the gold electrode. The Faradaic charge *Q* required for the oxidation was determined by integration of the area under the ferrocene anodic peak corrected from background current. From *Q*

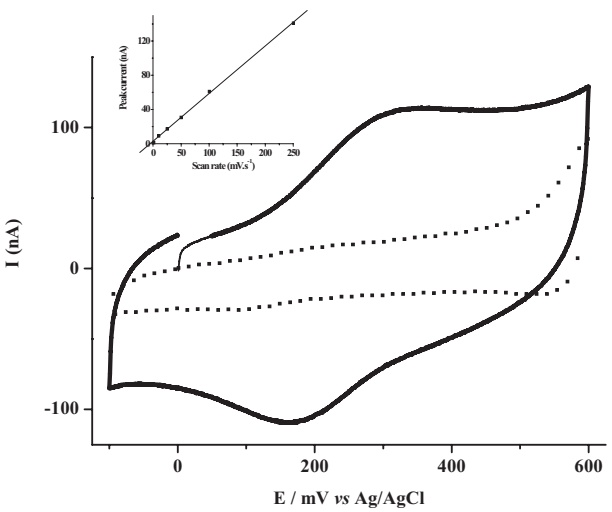


**Figure 2.** Cyclic voltammograms for a gold electrode before (dotted line) and after (solid line) modification with the **ODN 3.S** in the absence of target DNA (0.1 M KCl/$H_2O$, v=25 mV.s$^{-1}$). (*Inset*) The linear relationship between peak currents and scan rates.

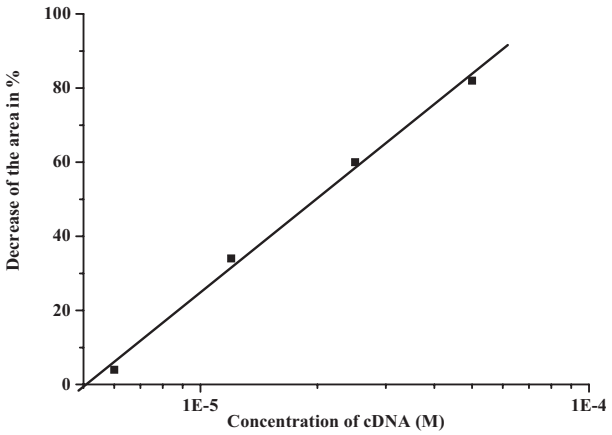


**Figure 3.** Calibration curve shows peak area *versus* target concentration (0M, 12 µM, 25 µM, 50 µM).

= n*FN* the oxidised Fc-ODN *N* was calculated (n=1, *F* = 96485 cb). Q was found to correspond to 60–75 pmoles.cm$^{-2}$ of ferrocene confined at the electrode surface in good agreement with values reported by Ihara et al. [12].

Modified electrode was incubated with a complementary target sequence at different concentrations (6 to 50 µM) in phosphate (25 mM) buffer aqueous solution (pH = 6.8) at 37°C for 3 h. No significant signal change was observed for electrodes incubated in DNA-free hybridization buffer. At lower complementary target concentrations, only partial loss of the signal was observed. For example, after incubation at 6 µM target, the electrochemical signal decrease to 4% of its initial value. Contrary, after incubation at 50 µM target, the electrochemical signal decrease to 82% of its initial value (Fig. 3).

Under these conditions, we easily observe measurable decreases in the areas in relation with target DNA concentrations. The sensitivity limit of the DNA detection estimated by extrapolation of the calibration curve is around 3 µM. The modified electrode exhibits a dynamic range where the peak area is logarithmically related to target concentration. Whereas similar logarithmic signal-versus-concentration or current relationships have been reported for other solid-state DNA sensors but the mechanism underlying the relationship has not been clarified [16–18]. However, Yang et al. indicate that phosphorothioate-ODN is most likely to be loaded in an approximate parallel fashion onto the gold surface due to coordination of the sulfur atom that dominates the interaction between the DNA and the gold surface [19]. In this context, we could imagine that hybridisation moves away the redox system from the gold surface which leads to a decrease of the electrochemical signal. As observed in solution for ODN-Fc no significant variations were observed with non complementary DNA target [9, 10].

The electrochemical DNA sensor is readily reusable. We have successfully recovered up to 90% of the original signal by washing the electrode with 0.1 M $LiClO_4$ at 55°C for 30 min in phosphate buffer 2.5 mM (pH = 6.8) and rechallenging with the target sequence. We believe that the minor signal loss during recovery arises from a low stability of ferrocene in aqueous solution at high temperature.

In conclusion, we have demonstrated that DNA probe with internal redox marker and anchoring site can be entirely synthesised by automated solid-phase DNA synthesiser. The anchoring of ODN-Fc-Ps probe on the gold electrode *via* chemisorption could be monitored by the appearance of the ferrocene redox couple in the electrochemical response which directly confirmed the effectiveness of the ODN layer formation. The electrochemical response of the modified electrode was analysed in aqueous media before and after hybridisation with ODN target and showed a change in current intensity. Moreover we have successfully recovered the DNA sensor after denaturation of the system. Thus the ODN-Fc-Ps probe proposed here presents a promising basis for simple and highly selective sensing system.

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