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Supported synthesis of ferrocene modified oligonucleotides as new electroactive DNA probes

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Abstract—The use of 1-[3-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphor amidityl)propyl]ferrocene and 1-[3-*O*-dimethoxytrityl propyl]-1′-[3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidityl) propyl] ferrocene as reactive synthons for DNA/RNA synthesizer allows to generate ferrocene-labelled oligonucleotides with remarkable DNA detection properties.

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 this context electrochemical methods received particular attention due to their high sensitivity, easy instrumentation, low production cost and compatibility to make small devices.[5] Many of electrochemical strategies are based on the change in the electrochemical response of external label grafted onto oligonucleotide probes. In that conjugation, ferrocene as label has been the subject of intense investigation due to its good stability, which affords convenient synthetic chemistry. Generally, the electroactive marker is bound on sugar or nucleic base of nucleotides, in using different coupling strategies that is, prior[6] to, during[7] or after[8] the ODN synthesis on DNA/RNA synthesizer.

To simplify the chemical approach of ferrocene-labelled oligonucleotide we developed a strategy based on a replacement of a nucleotide directly by a ferrocene unit during automated solid-phase DNA synthesis.[9] Furthermore, an increase of detection sensitivity can be considered by an easy incorporation of several electroactive markers at any position of the sequence. In order to achieve this strategy, a new type of ferrocene derivatives containing phosphoramidite and dimethoxytrityl (Dmt) groups were studied (Fig. 1).

Ferrocene phosphoramidites **1** and **2** were incorporated into ODN using an automated DNA/RNA synthesizer.[8] The monofunctional ferrocene **1** was introduced at the 5′-position and the bisfunctional ferrocene **2** was coupled at the first cycle of ODN synthesis. The coupling time was increased to 500 s for **1** and **2**, compared to 15 s for the other nucleotide synthons. The coupling yield of **1** was estimated to 80% from HPLC analyses of ODN crude syntheses. The obtained coupling yield for the bisfunctional ferrocene **2** was 95% from dimethoxytrityl

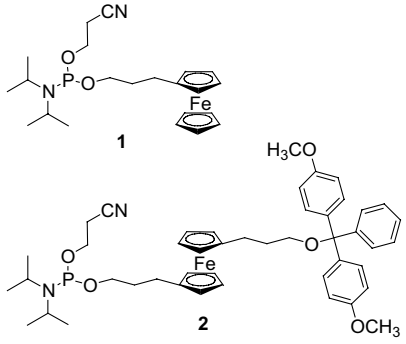

Figure 1. Structure of ferrocene phosphoramidites.

Keywords: Ferrocene; Oligonucleotides; Electrochemistry; DNA.
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Table 1. DNA oligonucleotide sequences

DNA entries	Sequence	MALDI-TOF mass spectrometry analyses	
		m/z Calculated	m/z Found[a]
Natural	5′ GTA TTC CTT GGA CTC ATA AGG T-C7-NH_2 3′		
ODN-3′	5′ GTA TTC CTT GGA CTC ATA AGG T-**Fe**-C7-NH_2 3′	7326.70	7326.40
ODN-3′,5′	5′ **Fe**-GTA TTC CTT GGA CTC ATA AGG T-**Fe**-C7-NH_2 3′	7632.80	7627.10
Target	5′ ACC TTA TGA GTC CAA GGA ATA C 3′		

[a] Accurate mass values were found to be with ±5 Da.

(Dmt) quantification. The A, T, C, G synthons classically reacted with an average coupling yield of 98% (measured via dimethoxytrityl quantification). After purification on preparative C18 reverse phase HPLC (Chromolith Performance column, Merck), the modified ODN were characterized by MALDI-TOF MS analyses (Table 1). The difference between calculated and found masses is attributed to difficulties encountered during calibration of the instrument. In fact, it is necessary to use standards for calibration with a behaviour similar to ferrocenyl-ODN, which are difficult to find. The same problem was observed in literature.[6] The thermal denaturation analyze of DNA duplex between the natural probe without ferrocenyl group and the target gave a melting temperature (T_m) at 69.1 °C. With the incorporation of ferrocenyl group, the resulting DNA duplexes had a T_m value at 68.6 °C for ODN-3′ and 68.3 °C for ODN-3′,5′, respectively (lower than the perfect match), which indicates a slight destabilization of DNA duplexes due to an alteration of the hybridization geometry.

We have investigated the electrochemical properties of ferrocene modified ODN using cyclic voltammetry (CV) (Fig. 2).† As expected a reversible one electron wave associated with oxidation and subsequent reduction of the ferrocenyl moieties is observed, confirming the good integration of ferrocene in ODN.

The differences between anodic and cathodic peaks are near 60 mV. On the one hand, the voltamogram reveals a reversible Nernstian behaviour for ferrocene/ferricinium redox system (Table 2). But on the other hand, the difference observed can be either explained by a response towards reduction limited by diffusion or by a slow electron transfer of Fc-ODN confined at the electrode surface. We observed ferrocene redox reaction only with scan rates from 10 to 100 mV s^{-1}. In this context, currents of the ferrocene redox system are directly proportional to scan rates (characteristic of an electrochemical reaction confined on surface) and to the square root of the scan rates (characteristic of a diffusion-controlled electrochemical reactions) because we are at the beginning of the curves.[10] So, to clarify this point the electrode was removed from the solution containing the oligonucleotide and placed in free phosphate buffer pH 6.8, NaCl 0.75 M. No electrochemical response was observed in buffer solution, which indicates a diffusion-controlled system. This does not represent an obstacle in practice to compare electrochemical properties of labelled ODN because the viscosity of the solution increases in the presence of ODN and small diffusion coefficients are expected for ODN.[8,11]

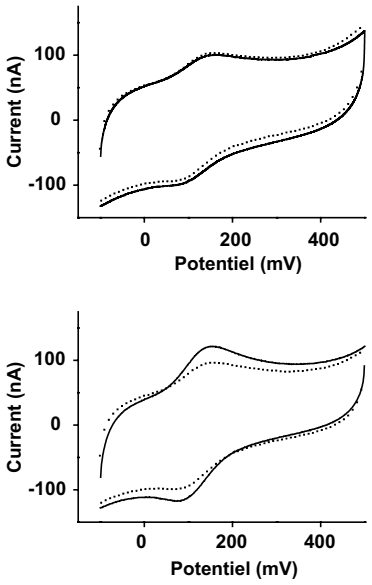

Figure 2. Cyclic voltammetry of labelled ODN-3′ (top) and ODN-3′,5′ (down); 25 μM in phosphate buffer pH 6.8, NaCl 0.75 M; before (dashed line) and after hybridization (solid line), $v = 10$ mV s^{-1}, 37 °C, ref. Ag/AgCl.

Table 2. Electrochemical data at 37 °C[a]

DNA entries	Before hybridization			After hybridization		
	E_{pa} (mV)	E_{pc} (mV)	Q (pmoles)	E_{pa} (mV)	E_{pc} (mV)	Q (pmoles)
ODN-3′	149	91	3.91	144	96	3.70
ODN-3′,5′	151	97	4.49	146	86	8.50

[a] ODN were at 25 μM in phosphate buffer pH 6.8, NaCl 0.75 M (1 mL of solution per assay), $v = 10$ mV s^{-1}, ref. Ag/AgCl.†

† Cyclic voltammetry data were acquired using a computer-based Bioanalytical instrument (BAS 100) electrochemical workstation with a three-electrode setup. The working electrode was a 3 mm diameter glassy carbon working electrode polished before each set of voltammograms with 1 μm diamond paste and ultrasonically rinsed in absolute ethanol. Counter electrode in platinum and Ag/AgCl reference electrode were used.

Therefore the area under the ferrocene anodic peak corrected from background current measured in the solution without ODN represents the Faradaic charge Q required for the full oxidation. The total amount of Fc-ODN on the electrode can be calculated quantitatively from $Q = nFN$ with n being the number of electrons transferred ($n = 1$), F the Faraday constant (C equiv^{-1}), and N the oxidized fc-ODN.

The estimation of the amount of labelled ODN oxidized at the electrode surface was 3.91 pmoles for ODN-3′ and 4.49 pmoles for ODN-3′,5′. After the addition of 1 equiv of target ODN directly in buffered aqueous solution containing the modified ODN followed by 10 min incubation always at 37 °C, the hybridizations were monitored by CV. After binding, the integration of the anodic peak indicates the oxidation of 3.70 and 8.5 pmoles of ferrocenyl groups for respectively ODN-3′ and ODN-3′,5′. These results show an increase of 89% of the area for ODN-3′,5′, which indicates that the 5′-ferrocenyl group become much more accessible for electrochemical reaction after hybridization.

In conclusion, the results presented here clearly show the potentiality of ferrocene phosphoramidites to allow generating electrochemically detectable DNA probes. The 5′-position appears very strategic to increase the sensitivity of binding detection. The results reveal that such a new type of modified ODN, bearing a Fc at each extremity of the chain can be used as probe for electronic detection of nucleic acids. The sensitivity should be further improved by incorporation several ferrocenyl groups at the 5′ extremity. In the meanwhile, we successfully used this strategy to prepare other labelled ODN with electroactive markers at different positions of the chain.

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