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Automated synthesis of new ferrocenyl-modified oligonucleotides: study of their properties in solution

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ABSTRACT

We have developed new ferrocenyl-modified oligonucleotide (ODN) probes for electrochemical DNA sensors. A monofunctional ferrocene containing phosphoramidite group has been prepared, and a new bisfunctional ferrocene containing phosphoramidite and dimethoxytrityl (DMT) groups has been developed. These ferrocenyl-phosphoramidites have been directly employed in an automated solid-phase DNA synthesizer using phosphoramidite chemistry. The advantages of this method are that it allows a non-specialist in nucleotide chemistry to access labeled ODNs and that it has demonstrated good results. ODNs modified at the 3' and/or 5' extremities have been prepared, with the incorporation of the ferrocenyl group into the chain. The 5' position appears to be more important due to its particular behavior. The thermal stability and electrochemical properties of these new ODN ferrocenes were analyzed before and after hybridization with different ODNs. The feasibility of using these new ferrocenyl-labeled ODNs in DNA sensors has been demonstrated.

INTRODUCTION

The detection of specific DNA sequences using real-time methods has received increasing attention for applications in clinical diagnostics (1), environmental protection (2,3), food quality control (4) and forensic science (5). Detection methods based on radioactive isotopes are sensitive but are also hazardous and not well suited for *in situ* measurements. The development of alternative detection systems, which are convenient and efficient, is necessary for real-time applications. Several non-radioactive detection techniques such as fluorescence or luminescence detection (6–9) or quartz crystal microbalance measurements (10) have been described. In this context, electrochemical methods have received particular attention due to their high sensitivity (11–15). Furthermore,

the simplicity of the detection apparatus results in the development of inexpensive and compact devices.

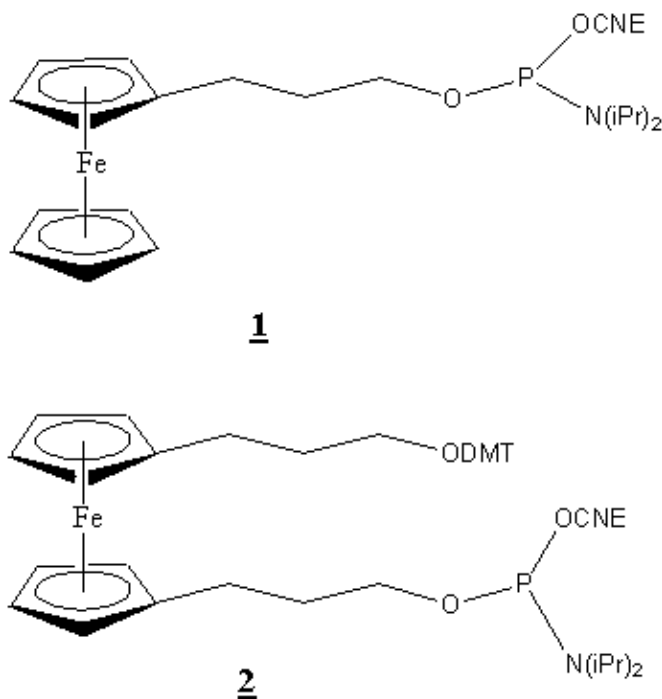
One of the strategies of monitoring the DNA hybridization with a complementary strand is based on the change of the electrochemical response of labeling DNA with metal complexes (16–22) or electroactive compounds (23–36). In this context, ferrocene has received particular attention due to its good stability, enabling convenient synthetic chemistry. For example, ferrocenyl-oligonucleotides (ODNs) obtained by the coupling of amino-terminated ODNs with a ferrocene derivative bearing an activated ester have been developed. After the formation of a duplex between a probe ODN anchored on a gold electrode with its DNA target, the redox-active ferrocenyl-modified ODN was used to obtain a sandwich-type ternary complex which gives a redox current (28–30). In a similar approach developed by Yu *et al.* (31), the redox-active ferrocenyl-modified ODN was directly prepared using a DNA/RNA synthesizer and a nucleoside phosphoramidite bearing a ferrocenyl moiety at the 2' position. This latter strategy is chemically more difficult but has the advantage of giving access to ODNs containing ferrocene at various positions (31,32). In this way, the electroactive marker is bound on a sugar or nucleic base of nucleotides, using different coupling strategies, i.e. prior (31,32) to, during (35,36) or after (37) the ODN synthesis on DNA/RNA synthesizer.

In order to simplify the chemical synthesis and to increase the selectivity and sensitivity of such electronic microsensors, we developed a new strategy to elaborate ferrocenyl-modified ODN based on a replacement of a nucleotide by an electroactive marker. This is directly inserted during the DNA synthesis.

In this paper, we report on a new type of ferrocene derivatives containing phosphoramidite and DMT groups (Scheme 1).

These phosphoramidites **1** or/and **2** have been directly employed with the four classical commercial nucleotide phosphoramidites in an automated solid-phase DNA synthesizer using phosphoramidite chemistry. The advantage of this method is the possibility for a non-specialist in nucleotide chemistry to have access to labeled ODN. To validate this strategy, modified ODN sequences have been synthesized with one or several ferrocenyl derivatives selectively incorporated

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Scheme 1. Phosphoramidites 1 and 2.

into any position of the chain (38,39). The thermal stability and electrochemical properties of these new ODN ferrocenes are discussed below.

MATERIALS AND METHODS

Chemical materials

Lithium aluminium hydride (AlLiH_4), *N,N*-dimethylamino-pyridine (DMAP), 4,4'-dimethoxytrityl chloride (DMTCl), *N,N*-ethyl-diisopropylamine, triethylamine (TEA), 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, ferrocene and triethyl phosphonoacetate were purchased from Aldrich. Diethyl ether (anhydrous), tetrahydrofuran (THF) (anhydrous) and pyridine (anhydrous) were purchased from Acros. Dichloromethane, absolute ethanol, ethyl acetate and cyclohexane were purchased from CarloErba, silica gel (240–400 mesh) from Merck and MgSO_4 from SDS. All aqueous solutions were made with MilliQ purified water (Maxima ELGA). Phosphate buffers (pH = 6.8) were made with 0.25 M KH_2PO_4 , 0.25 M Na_2HPO_4 and 0.75 M NaCl.

Instrumentation

Uncorrected melting points were obtained from Electrothermal 9100 apparatus. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker AC-250 at 250 MHz and 62.5 MHz, respectively. ^{31}P NMR spectra were recorded on a Bruker 200 MHz spectrometer, and ultraviolet (UV) spectra were obtained on Varian Cary 1E. Elemental analyses were made at the Service Central d'Analyses of CNRS (Vernaison, France). FAB mass spectra for organic compounds were obtained on a JEOL FX 102 Mass spectrometer (Laboratoire de Mesures Physiques, USTL, Montpellier, France).

High-performance liquid chromatography (HPLC) analyses were run on a Kontron instrument equipped with a HPLC pump 422, an autosampler 465 and a diode array detector 440. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra of ODNs were recorded at the Laboratoire de Chimie Organique Biomoléculaire de Synthèse (Université Montpellier II, France) on a Voyager DE (PerSeptive Biosystems, Framingham, MA) mass spectrometer equipped with an N_2 Laser. The matrix used for ODN mass analyses was the hydroxy-picolinic acid. Calibration was carried out using internal data base references. Melting temperature measurements were performed on a UV spectrophotometer Uvikon 943 (Kontron). The temperature of the cell was controlled by a Huber temperature programmer (Ministat) connected to a refrigerated ethylene glycol/water bath. The absorbance of ODN solutions was recorded at 260 nm versus temperature increased at a rate of $0.33^\circ\text{C min}^{-1}$.

Synthesis of ferrocene phosphoramidites

1-[(2-Ethoxyethyl)ethenyl]ferrocene 5. An aliquot of 0.93 g (4.67 mmol) of triethyl phosphonoacetate and a solution of 1 g (4.67 mmol) of ferrocene monocarboxaldehyde prepared as described previously (40) in 20 ml of absolute ethanol was added drop by drop at 0°C to a solution of 0.11 g (6.67 mmol) of sodium in 46 ml of absolute ethanol. The mixture was stirred at room temperature for 1 h and concentrated under reduced pressure. The crude product was purified on a silica gel column and eluted with ethyl acetate–cyclohexane mixture (5/95). The desired fractions were pooled and concentrated to give quantitatively (1.32 g) of a red powder corresponding to **5**: mp $74\text{--}75^\circ\text{C}$; ^1H NMR (250 MHz, CDCl_3) δ : 1.25 (t, $J = 7.16$ Hz and $J = 7.12$ Hz, 3H, $\text{CH}_2\text{-CH}_3$), 4.07 (s, 5H, H_{Fc}), 4.14 (q, $J = 7.12$ Hz, 2H, $\text{CH}_2\text{-CH}_3$), 4.32–4.41 (m, 4H, H_{Fc}), 5.95 (d, $J = 15.74$ Hz, 1H, H_{eth}), 7.49 (d, $J = 15.74$ Hz, 1H, H_{eth}). ^{13}C NMR (62.5 MHz, CDCl_3) δ : 14.5, 68.7, 69.8, 70.9, 78.9, 115.1, 145.7, 167.4 (C=O); MS (EI): 284 (M^+ , 100), 185 (59); UV-Vis (CH_2Cl_2) λ_{max} nm (log ϵ) 252 (4.22), 296 (4.27), 366 (3.34), 464 (3.14). Elemental analysis calculated for $\text{C}_{15}\text{H}_{16}\text{O}_2\text{Fe}$: C, 63.41; H, 5.68; O, 11.26; Fe, 19.65. Found: C, 63.33; H, 5.74; O, 12.04; Fe, 18.89.

1-[(2-Ethoxyethyl)ethyl]ferrocene 4. A solution of 1 g (3.52 mmol) of **5** and 0.10 g (1 mmol) of palladium-on-charcoal in 60 ml of ethylacetate was degassed under argon for 30 min before saturated with hydrogen. The mixture was vigorously stirred for 24 h. The filtration and the concentration under reduced pressure gave 1 g (98%) of yellow crystals corresponding to title compound: mp $30\text{--}31^\circ\text{C}$; ^1H NMR (270 MHz, CDCl_3) δ : 1.20 (t, $J = 7.10$ Hz and $J = 7.17$ Hz, 3H, $\text{CH}_3\text{-CH}_2\text{-O}$), 2.46 (m, 2H, $\text{CH}_2\text{-CO}$), 2.59 (m, 2H, Fc-CH_2), 4.09 (m, 11H, $\text{CH}_3\text{-CH}_2\text{-O}$ and H_{Fc}); ^{13}C NMR (62.5 MHz, CDCl_3) δ : 14.7, 25.3, 36.1, 60.7, 67.8, 68.3, 68.9, 87.9, 173.5 (C=O); MS (EI): 286 (M^+ , 100), 55 (15), 22 (31); UV-Vis (CH_2Cl_2) λ_{max} nm (log ϵ) 226 (3.99), 427 (2.04). Elemental analysis calculated for $\text{C}_{15}\text{H}_{18}\text{O}_2\text{Fe}$: C, 62.96; H, 6.34; O, 11.18; Fe, 19.52. Found: C, 63.08; H, 6.47; O, 11.62; Fe, 18.83.

Synthesis of 1-(3-hydroxypropyl)ferrocene 3. A solution of 0.40 g (10.48 mmol) of AlLiH_4 in 20 ml of diethyl ether was prepared and stirred for 10 min. A solution of 3 g

(10.48 mmol) of **4** in 20 ml of anhydrous diethyl ether was added drop by drop to this solution to maintain the reflux. After 1 h, the mixture was cooled at room temperature before adding 15 ml of water. Then, the mixture was filtered and extracted with diethyl ether (2 × 50 ml). The combined organic phases were washed with water, dried (MgSO₄) and evaporated *in vacuo* to give 2.46 g (96%) of yellow oil corresponding to **3**. ¹H NMR (250 MHz, CDCl₃) δ: 1.42 (sl, 1H, OH), 1.71 (m, 2H, CH₂), 2.35 (t, *J* = 8.01 Hz and *J* = 7.51 Hz, Fc-CH₂), 3.60 (t, *J* = 6.44 Hz, 2H, CH₂-OH), 3.99 (m, 4H, H_{Fc}), 4.03 (s, 5H, H_{Fc}); ¹³C NMR (62.5 MHz, CDCl₃) δ: 25.9 (C₆), 34.0 (C₇), 62.4 (C₈), 67.5 (C₂C₅), 68.4 (C₃C₄), 68.9 (C₁-C₅'), 89.1 (C₁); MS (EI): 244 (M⁺, 100), 55 (8), 161 (16), 179 (30). Elemental analysis calculated for C₁₃H₁₆OFe: C, 63.96; H, 6.61; O, 6.55; Fe, 22.88. Found: C, 63.55; H, 6.85; O, 8.75; Fe, 20.85.

1-[3-O-(2-Cyanoethyl-N,N-diisopropylphosphoramidyl)propyl]ferrocene 1. A mixture of 0.20 g (0.823 mmol) of 1-(3-hydroxypropyl)ferrocene **3** and 0.014 g (0.12 mmol) of DMAP was co-evaporated with dry pyridine (2 × 2 ml) and dry THF (2 × 2 ml) followed by the addition of 5 ml of dry THF. The mixture was stirred at room temperature under nitrogen, and 0.287 ml (1.65 mmol) of diisopropylethylamine (DIPEA) was added. An aliquot of 0.202 ml (0.905 mmol) of 2-cyanoethyl-diisopropylchlorophosphoramidite was then introduced drop by drop, during which a precipitate appeared. After 4.5 h at ambient temperature, the solvent was removed by evaporating *in vacuo*. The product was purified on a silica gel column using ethyl acetate-TEA-cyclohexane (15-0.5-84.5). The removal of solvents *in vacuo* gave 0.28 g (77% yield) of an yellow-orange oil corresponding to the title compound. ¹H NMR (250 MHz, CD₃CN) δ: 0.99 (d, *J* = 6.8 Hz, 12H, 4CH-CH₃), 1.60 (m, 2H, CH₂), 2.22 (t, *J* = 8.16 Hz and *J* = 7.34 Hz, 2H, Fc-CH₂), 2.44 (t, *J* = 5.92 Hz and *J* = 5.74 Hz, 2H, CH₂-O), 3.33-3.61 (m, 6H, 2CH, CH₂P, CH₂CN), 3.82-3.94 (m, 9H, H_{Fc}); ³¹P NMR (62.5 MHz, CD₃CN): 148.23 (P). ¹³C NMR (62.5 MHz, CDCl₃) δ: 20.9, 24.8, 25.9, 32.6, 43.1, 58.3, 63.3, 67.3, 68.2, 68.6, 88.6, 117.8 (CN). MS (EI): 444 (M⁺, 10), 102 (57), 43 (26). FAB high resolution mass spectrometry (HRMS): C₂₂H₃₃O₂N₂PFe: calculated 444.3357; found 444.1629.

1,1'-bis[(2-ethylxycarbonyl)ethenyl]ferrocene 9. An aliquot of 0.81 ml (4.08 mmol) of triethyl phosphonoacetate and a solution of 0.47 g (1.94 mmol) of ferrocene dicarboxaldehyde prepared as described previously (40) in 10 ml of absolute ethanol was added drop by drop at 0°C to a solution of 0.09 g (4.08 mmol) of sodium in 25 ml of absolute ethanol. The mixture was stirred at room temperature for 1 h and concentrated under reduced pressure. The product was purified on a silica gel column with ethylacetate-cyclohexane mixture (5/95). The desired fractions were pooled and concentrated to give 0.56 g (75%) of red crystals corresponding to **9**: mp 92-93°C; ¹H NMR (250 MHz, CDCl₃) δ: 1.26 (t, *J* = 7.15 Hz and *J* = 7.12 Hz, 6H, 2CH₂-CH₃), 4.15 (q, *J* = 7.14 Hz and *J* = 7.11 Hz, 4H, 2CH₂-CH₃), 4.31-4.38 (m, 8H, H_{Fc}), 5.91 (d, *J* = 15.80 Hz, 2H, H_{eth}), 7.33 (d, *J* = 15.79 Hz, 2H, H_{eth}); ¹³C NMR (62.5 MHz, CDCl₃) δ: 14.2, 60.1, 69.7, 72.2, 79.9, 116.2, 143.7, 166.9 (C=O); MS (EI): 382 (M⁺, 100); UV-Vis (CH₂Cl₂) λ_{max} nm (log ε) 226 (4.43), 255 (4.46), 307 (4.35),

386 (3.53), 472 (3.33). Elemental analysis calculated for C₂₀H₂₂O₄Fe: C, 62.85; H, 5.80; O, 16.74; Fe, 14.61. Found: C, 63.15; H, 5.92; O, 16.92; Fe, 14.01.

1,1'-bis-[(2-Ethylxycarbonyl)ethyl]ferrocene 8. A solution of 0.40 g (1.05 mmol) of **9** and 0.1 g (0.94 mmol) of 10% palladium-on-charcoal in 40 ml of ethyl acetate was degassed under argon for 30 min prior to being saturated with hydrogen. The mixture was vigorously stirred for 72 h. Then, the solution was filtrated and concentrated under reduced pressure to give 0.40 g (99%) of yellow oil corresponding to the expected compound: ¹H NMR (250 MHz, CDCl₃) δ: 1.25 (t, *J* = 7.15 Hz and *J* = 7.12 Hz, 6H, 2CH₂-CH₃), 2.49 (m, 4H, CH₂-CO), 2.57 (m, 4H, Fc-CH₂), 4.00 (s, 8H, H_{Fc}), 4.06 (q, *J* = 7.13 Hz and *J* = 7.15 Hz, 4H, 2O-CH₂-CH₃). ¹³C NMR (62.5 MHz, CDCl₃) δ: 14.2, 24.6, 35.7, 60.1, 68.1, 68.4, 87.4, 172.7 (C=O); MS (EI): 386 (M⁺, 100), 221 (35), 136 (5); UV-Vis (CH₂Cl₂) λ_{max} nm (log ε) 227 (4.11), 259 (3.90), 376 (3.08), 443 (3.03). Elemental analysis calculated for C₂₀H₂₆O₄Fe: C, 62.19; H, 6.78; O, 16.57; Fe, 14.46. Found: C, 62.45; H, 6.96; O, 16.32; Fe, 14.27.

1,1'-bis-(3-Hydroxypropyl)ferrocene 7. A solution of 0.14 g (3.70 mmol) of AlLiH₄ in 7 ml of anhydrous diethyl ether was prepared and stirred for 10 min. A solution of 1.43 g (3.70 mmol) of **8** in 9.5 ml of anhydrous diethyl ether was added drop by drop to this in order to maintain the reflux, and then 15 ml of anhydrous THF was added. After 1 h, the mixture was cooled at room temperature prior to adding 15 ml of water. Then, the mixture was filtered and extracted with diethyl ether (2 × 25 ml). The combined organic phases were washed with water, dried with MgSO₄ and evaporated *in vacuo* to give 0.81 g (75%) of yellow oil corresponding to **7**. ¹H NMR (250 MHz, CDCl₃) δ: 1.80 (m, 4H, 2CH₂), 2.04 (sl, 2H, 2OH), 2.12 (t, *J* = 8.20 Hz and *J* = 7.40 Hz, 4H, 2Fc-CH₂), 3.69 (t, *J* = 6.12 Hz, 2CH₂-OH), 4.04 (m, 8H, H_{Fc}); ¹³C NMR (62.5 MHz, CDCl₃) δ: 26.0, 34.5, 62.9, 68.3, 69.1, 89.0; MS (EI): 302 (M⁺, 100), 179 (40), 161 (21). UV-Vis (CH₂Cl₂) λ_{max} nm (log ε) 227 (4.00), 259 (2.73), 426 (2.58). Elemental analysis calculated for C₁₆H₂₂O₂Fe: C, 63.59; H, 7.34; O, 10.59; Fe, 18.48. Found: C, 62.73; H, 7.66; O, 12.64; Fe, 16.97.

1-[3-O-Dimethoxytritylpropyl]-1'-[3'-hydroxypropyl]ferrocene 6. A mixture of 0.20 g (0.662 mmol) of 1,1'-dihydroxypropylferrocene **7** and 0.016 g (0.132 mmol) of DMAP were co-evaporated with dry THF (2 × 5 ml). Then, 5 ml of dry THF was added to the residue followed by 0.25 g (0.728 mmol) of 4,4'-dimethoxytritylchloride and 0.115 ml (0.662 mmol) of DIPEA. The mixture was stirred at room temperature under nitrogen. After 4 h at ambient temperature, MeOH (2 ml) was introduced to stop the reaction and the solvents were evaporated under reduced pressure. The resulting crude product was dissolved in dichloromethane (25 ml). The organic phase was washed with saturated solution of NaHCO₃ in water (5 × 25 ml) and dried with magnesium sulfate. After removing solvents *in vacuo*, the product was purified by silica gel chromatography (neutralized by TEA) with gradient of MeOH in CH₂Cl₂ to obtain an orange oil (0.30 g, 76% yield). ¹H NMR (250 MHz, CDCl₃) δ: 1.64-1.86 (m, 4H, CH₂), 2.38-2.53 (m, 4H, 2CH₂, Fc-CH₂), 3.11 (t, ³*J* = 6.3 Hz, 2H, CH₂O-C), 3.55 (t, ³*J* = 6.4 Hz, 2H, CH₂OH), 3.78 (s, 6H, 2CH₃O), 3.97 (s, 8H, CH, H_{Fc}), 6.82-6.96 (m, 4H, CH, H_{ph}), 7.17-7.68 (m, 9H, CH, H_{ph}).

MS (EI); 604 (M^{*+} , 40), 303 (100), 57 (52). FAB HRMS: m/z $C_{37}H_{40}O_4Fe$ calculated $[M+H^+]$ 604.5676; found 604.2277.

1-[3-O-Dimethoxytritylpropyl]-1'-[3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)propyl]ferrocene 2. A mixture of 0.17 g (0.280 mmol) of 1-[3-O-dimethoxytritylpropyl]-1'-[3'-hydroxypropyl]ferrocene **6** was co-evaporated twice with dry acetonitrile (3 × 2 ml). Then, 1.4 ml of dry acetonitrile was added to the residue. The mixture was stirred at room temperature under argon. Then, 98 μ l (0.560 mmol) of DIPEA and 77 μ l (0.308 mmol) of chlorophosphine were added. After 5 min at ambient temperature, the mixture was concentrated *in vacuo* without heating to obtain 1 ml. The product was purified by silica gel chromatography with elution in ethyl acetate–TEA–cyclohexane solution (10–0.5–89.5). An yellow–orange oil was obtained (0.19 g, 83% yield). 1H NMR (200 MHz, CD_3CN) δ : 1.17 (m, 12H, 2NCH(CH₃)₂), 1.75 (m, 4H, 2CH₂), 2.36 (m, 4H, 2Fc-CH₂), 2.61 (m, 2H, OCH₂CH₂CN), 3.02 (m, 2H, CH₂OC), 3.61 [m, 6H, OCH₂CH₂CN, 2NCH(CH₃)₂, CH₂OP], 3.75 (s, 6H, 2CH₃), 3.90 (m, 8H, H_{Fc}), 6.62–7.26 (m, 13H, DMT); ^{31}P NMR (200 MHz, CD_3CN) δ : 148.25 (P); MS (EI): 802 (M^{*+} , 20), 303 (100), 102 (19), 57 (45), 43 (33). FAB HRMS: m/z $C_{46}H_{57}O_5N_2PFe$ calculated $[M+H^+]$ 804.3355; found 804.3356.

Preparation of labeled ODNs

All DNA ODNs were synthesized using an Applied Biosystems 394 RNA/DNA synthesizer. Ferrocene phosphoramidite compounds **1** and **2** were dissolved in anhydrous acetonitrile ($C = 0.09$ M) and the solutions were dried on 3 Å molecular sieves for 24 h. The solutions were filtered on 0.22 μ m polyvinylidene fluoride filter before loading on DNA synthesizer. The standard 1 μ mol coupling cycle was used for ODN elongations. The coupling reaction time was increased from 15–500 s for ferrocene synthons.

After ODN synthesis (DMTr ON), controlled pore glass (CPG) support was treated in NH_4OH (30% aqueous) for 16 h at 55°C, then supernatant was recovered and evaporated to dryness under vacuum. The pellet was dissolved in 500 μ l of MilliQ water plus 500 μ l of MOP buffer. The ODN was purified on MOP column (CTGen, San Jose, CA) as follows: column was first washed with 2 ml of CH_3CN/H_2O (1:1) and 2 ml of TEAAc buffer 0.1 M (pH = 7) successively before loading the ODN solution on column. The truncated sequences were eluted with 4 ml of TEAAc buffer 0.1 M (pH = 7). The ODN was eluted with 1 ml of CH_3CN/H_2O (1:1) and the solvents were evaporated to dryness. Then, ODN was detritylated with 300 μ l of 80% acetic acid/water for 30 min. Acetic acid was eliminated by evaporation, and ODN was precipitated twice with 0.3 M AcONa (300 μ l) and ethanol (900 μ l) before lyophilization in water. The oligonucleotides **ODN 5** and **ODN 5.3** were purified by HPLC on a RP-18e Lichrospher 100 (300 × 7.5, 10 μ l; Merck) with linear gradient of acetonitrile (5–40%) in 0.05 M aqueous triethylammonium acetate (pH = 7). The HPLC analyses of ODN purities were run on a reverse-phase RP-18e Chromolith Performance column (100 × 4.6 mm; Merck) with the same eluents than the ones described above. The concentrations of ODNs were determined by the UV absorption at 260 nm, assuming molar extinction coefficients (ϵ_{260}) indicated in Table 1.

Table 1. Molar extinction coefficients of ferrocenyl-modified ODN

ODN	Extinction coefficient (ϵ_{260}) L mol ⁻¹ cm ⁻¹
Natural	212100
ODN 5	215512
ODN 3	215614
ODN 5.3	219026
ODN 3.3	219129

Molar extinction coefficients of ferrocenyl compounds **3** and **7** were found to be 3412 l mol⁻¹ cm⁻¹ and 3514 l mol⁻¹ cm⁻¹ respectively, in methylenechloride. The molar extinction coefficients of modified ODN were calculated by adding the epsilons of **3** and **7** to the ϵ value of the unmodified strand (1 mol⁻¹ cm⁻¹). The DNA melting curves were obtained by monitoring the absorbance of DNA solutions at 260 nm depending on the temperature. The melting temperatures (T_m) were measured in 0.75 M NaCl, 250 mM phosphate buffer (pH 6.8), containing 3 μ M ODNs. Each T_m data resulted from duplicate measures and were estimated to be accurate within $\pm 0.5^\circ C$.

Electrochemical experiments

Cyclic voltammetry (CV) 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 μ m diamond paste and ultrasonically rinsed in absolute ethanol. A counter electrode in platinum and an Ag/AgCl reference electrode were used. Cyclic voltammograms were recorded without ohmic drop compensation and at a scan rate of 10 mV s⁻¹. All CV experiments were carried out at 37°C using a cell equipped with a jacket allowing circulation of water from the thermostat. Phosphate buffer at pH 6.8 containing 0.75 M NaCl was used as the supporting electrolyte solution.

RESULTS AND DISCUSSION

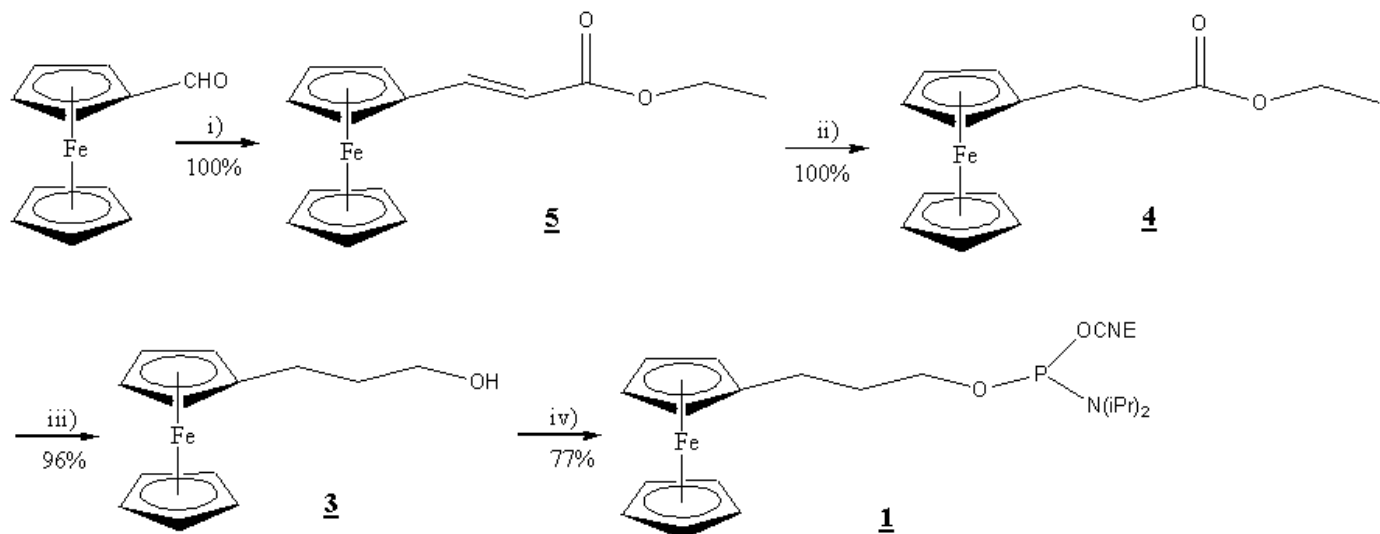
Monomers

Synthesis of phosphoramidite 1. Compound **5** was obtained quantitatively by Wittig–Horner reaction of 1-formylferrocene prepared as described previously (40) using substituted phosphonate anion derived from the triethyl phosphonoacetate (Scheme 2).

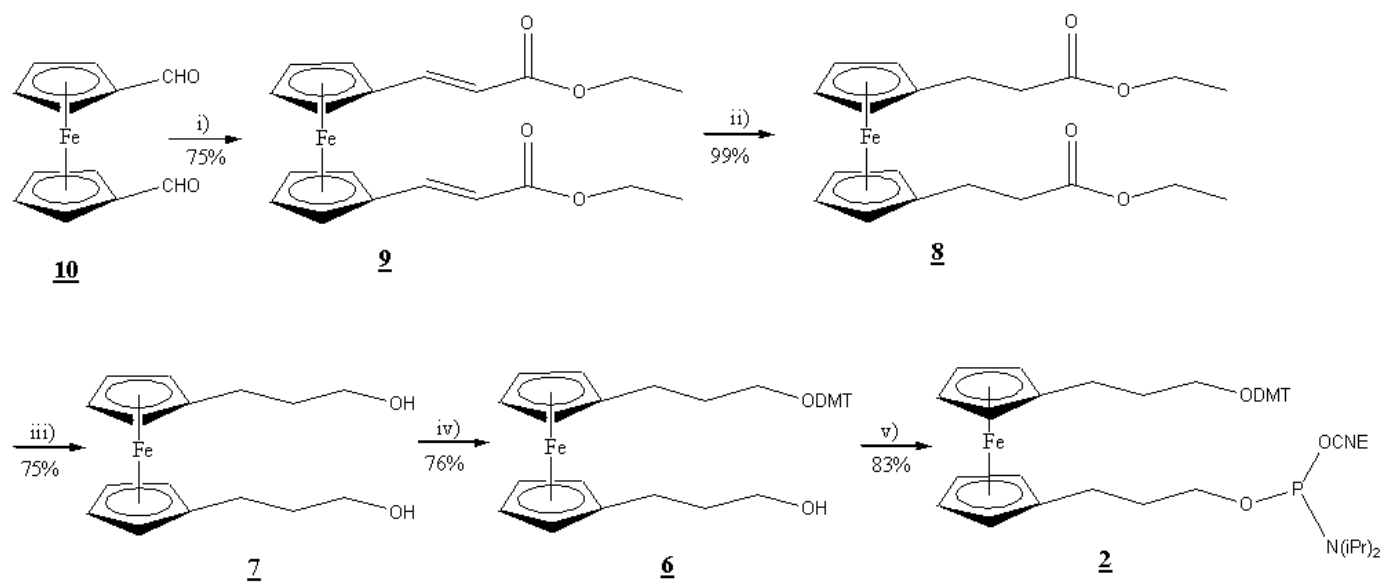
The compound **4** was obtained in quantitative yield by catalytically hydrogenation of double bond with palladium-on-charcoal. The hydroxypropyl ferrocene **3** was classically prepared by the reduction of carboxylic esters with $AlLiH_4$ in 96% yield. Phosphitylation from compound **3** with standard reagents, i.e. with 2-(cyanoethoxy)-bis diisopropylchlorophosphoramidite yield the corresponding compound **1** (41).

Synthesis of phosphoramidite 2. The same synthetic scheme was used to obtain compound **2** (Scheme 3).

Compound **9** was obtained in 75% yield by Wittig–Horner reaction with phosphonate anion derived from the triethyl phosphonoacetate and 1,1'-bisformylferrocene prepared as



Scheme 2. (i) Triethyl phosphonoacetate, Na, EtOH, 0°C, 1 h. (ii) Pd/C, ethylacetate, H₂, room temperature, 24 h. (iii) AlLiH₄, diethyl ether, 1 h. (iv) 2-cyanoethyl-diisopropylchlorophosphoramidite, THF, room temperature, 4.5 h.



Scheme 3. (i) Triethyl phosphonoacetate, Na, EtOH, 0°C, 1 h. (ii) Pd/C, ethylacetate, H₂, room temperature, 24 h. (iii) AlLiH₄, diethyl ether, 1 h. (iv) 4,4'-Dimethoxytritylchloride, THF, room temperature, 4 h. (v) 2-cyanoethyl-diisopropylchlorophosphoramidite, THF, room temperature, 4.5 h.

described previously (40). The near-quantitative reduction of the double bond with 10% palladium-on-charcoal followed by the reduction of carboxylic esters with AlLiH₄ led to compound **7**. One of the hydroxy groups of compound **7** was protected by DMAP and DMT in a one-pot reaction to give compound **6** in 76% yield. The phosphitylation of the free hydroxy group from compound **6** was carried out under the same conditions for compound **1** and gave the phosphoramidite **2** (41).

Labeled ODNs

Synthesis and characterization. Ferrocene phosphoramidites **1** and **2** were incorporated into oligodeoxyribonucleotides

using an automated DNA/RNA synthesizer. Table 2 lists the sequences of the synthesized oligodeoxyribonucleotides.

A 3' amino modified CPG was used for natural and labeled ODN syntheses. The introduction of an amino arm at the 3' position of all ODN probes was achieved to further be able to immobilize them on a reactive support, e.g. polypyrrole film bearing activated ester (42). The monofunctional ferrocene **1** was introduced at the 5' position of ODNs **5**, **5.3**, and the bifunctional ferrocene **2** was coupled at the first cycle of ODN synthesis for ODNs **3**, **5.3** and **3.3**. The reagents and reaction times of the coupling cycle are indicated on Table 3. Both ferrocenyl synthons were used at 0.09 M in dry acetonitrile. The coupling time was increased to 500 s for compounds **1** and **2**, compared with 15 s for the other nucleotide

Table 2. Sequence of ferrocene labeled ODNs

DNA entry	DNA ODNs sequences	T_m values with natural target ($^{\circ}\text{C}$)
Natural probe	5'-GTA TTC CTT GGA CTC ATA AGG T-C7-NH ₂ -3'	69.1
ODN 5	5'-Fe-GTA TTC CTT GGA CTC ATA AGG T-C7-NH ₂ -3'	69.0
ODN 3	5'-GTA TTC CTT GGA CTC ATA AGG T-Fe-C7-NH ₂ -3'	68.6
ODN 3.3	5'-GTA TTC CTT GGA CTC ATA AGG T-Fe-Fe-C7-NH ₂ -3'	68.2
ODN 5.3	5'-Fe-GTA TTC CTT GGA CTC ATA AGG T-Fe-C7-NH ₂ -3'	68.3
SM3 target	5'-ACC TTA TGA GTC-3'	
SM5 target	5'-TCC AAG GAA TAC-3'	
Natural target	5'-ACC TTA TGA GTC CAA GGA ATA C-3'	

Table 3. Electrochemical data for ferrocene-labeled ODN at 37 $^{\circ}\text{C}$ ^a

ODN	$E_{1/2} \pm 6$ (mV) versus Ag/AgCl		N (pmol)	
	Before hybridization	After hybridization	Before hybridization	After hybridization
5	135	139	3.06	4.00
3	113	118	3.91	3.70
5.3	114	115	4.49	8.50
3.3	126	128	9.66	8.15

^aTo determine $E_{1/2}$, the measurements were repeated four times.

synthons. The coupling yield of compound **1** was estimated to 80% from HPLC analyses of ODNs **5** and **5.3** crude syntheses, by comparing peak areas of full length ODN and truncated sequence (minus **1** at the 5' extremity). The obtained coupling yield for the bisfunctional ferrocene **2** was 95% from DMT quantification. The A, T, C and G synthons classically reacted with an average coupling yield of 98% (measured via DMT quantification) (Table S1). Except for ODNs **5** and **3**, all ODNs were synthesized in keeping the last DMT on the sequence, allowing a rapid and efficient purification on MOP column, as described in Materials and Methods. The ODNs **5** and **3** were purified on preparative reverse phase column. The ODN purities were controlled by reverse-phase HPLC. The chromatographic profiles of ferrocenyl-modified ODNs **5**, **5.3** and **3** are overlaid on the graph of Figure 1. In all the cases, the ODNs are eluted with a retention time superior than the one of natural ODN probe (13 min) confirming the modification of the fragments. The ferrocene moiety introduced at the 5' extremity seems to bring more hydrophobic character to the sequence than on the 3' position. The ODN sequence modified at both extremities appears to be the most hydrophobic as it is retained the longest on the column.

All labeled ODNs were characterized by MALDI-TOF mass spectral analyses. The results for modified oligonucleotides ODN **5** (containing one ferrocenyl group at the 5' position, m/z calculated 7268.70, found 7263.70), ODN **3** (containing one ferrocenyl group in 3' position, m/z calculated 7326.70, found 7326.40), ODN **5.3** (containing one ferrocenyl group at 3' and 5' positions, m/z calculated 7632.80, found 7627.10) and ODN **3.3** (containing two ferrocenyl groups at 3', m/z calculated 7674.90, found 7677.22) illustrate the successful incorporation of ferrocenyl moieties into the ODNs. The difference between calculated and found masses is attributed to difficulties encountered during the calibration of the instrument. In fact, it is necessary to use standards for calibration with a behavior similar to ferrocenyl-ODN that are difficult to find. The same problem has been observed previously (31).

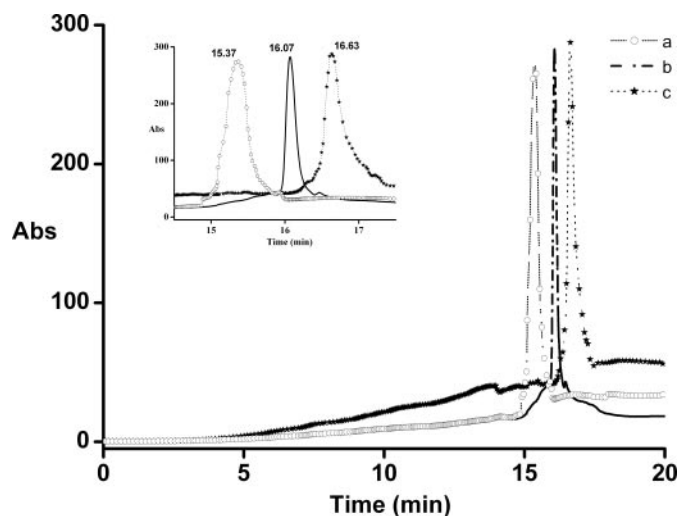


Figure 1. HPLC profile of labeled-ODN. A reverse-phase RP-18e Chromolith Performance column (100 \times 4.6 mm; Merck) was used with linear gradient of acetonitrile (5–30%) in 0.05 M aqueous triethylammonium acetate (pH = 7); a: ODN **3**; b: ODN **5**; c: ODN **5.3**.

Thermal denaturation studies of ferrocene-labeled oligodeoxynucleotides. To study the ability of these new modified oligodeoxynucleotides to form DNA duplexes, UV thermal melting studies were performed for each sequence in the presence of a complementary target. The denaturation of the duplexes under increasing temperature was followed at 260 nm. The T_m was calculated from experimental curves. The obtained T_m values are indicated on Table 2.

The thermal denaturation curves of DNA duplex formed between natural ODN probe and its target gave a T_m value of 69.1 $^{\circ}\text{C}$ corresponding to the dissociation of natural duplex formed with two complementary strands of length 22 base. With respect to this reference value, the addition of the ferrocenyl derivative **1** at the 5' extremity of ODN probe had no

impact on duplex stability. However, the presence of ferrocenyl monomer **2** at the 3' extremity slightly destabilized the hybridization, as the observed T_m was $\sim 1^\circ\text{C}$ lower than the reference. These lower values could be explained by a slight alteration of the hybridization geometry of the DNA duplexes. Nevertheless, the results clearly show that these new redox probes are specific of DNA targets.

Electrochemistry of ferrocene-modified oligodeoxyribonucleotides in solution

Before hybridization. The electrochemical responses of the ferrocene modified ODN were investigated by CV experiments in buffered aqueous solution [25 μM in phosphate buffer (pH 6.8), NaCl 0.75 M] at 37°C . Only one concentration was used because each labeled ODN was synthesized in low quantities with the final goal of grafting onto organic conducting polymer to develop DNA chips.

In all the cases, a redox system associated to the electrochemical response of the ferrocenyl moiety is observed with a

difference of ~ 60 mV between anodic and cathodic peaks (Figure 2).

This difference could indicate reversible Nernstian behavior for ferrocene/ferrocinium in a diffusion process or a slow transfer electron in Fc-ODN adsorbed at the surface electrode. The current was measured at different scan rates; with data proportional to the fast scan rate, an electrochemical reaction confined on surface is expected. However, the current measured directly proportional to the square root of the scan rate at slower scan rates indicate a diffusion-controlled electrochemical reaction (43). Unfortunately, with our labeled ODNs, the ferrocene redox system was only observed with scan rates varying from 10 to 100 mV s^{-1} and the current measurements were proportional to scan rate and square root of the scan rate. Linear fit of the plot for ODNs **3.3** and **5.3** gives R values between 0.990 and 0.999 (Figure S1). To discriminate the process, the electrodes were removed from ODN solutions and placed in free phosphate buffer (pH 6.8, NaCl 0.75 M). No electrochemical responses were observed that could indicate a diffusion process. Previous works have shown that ODN

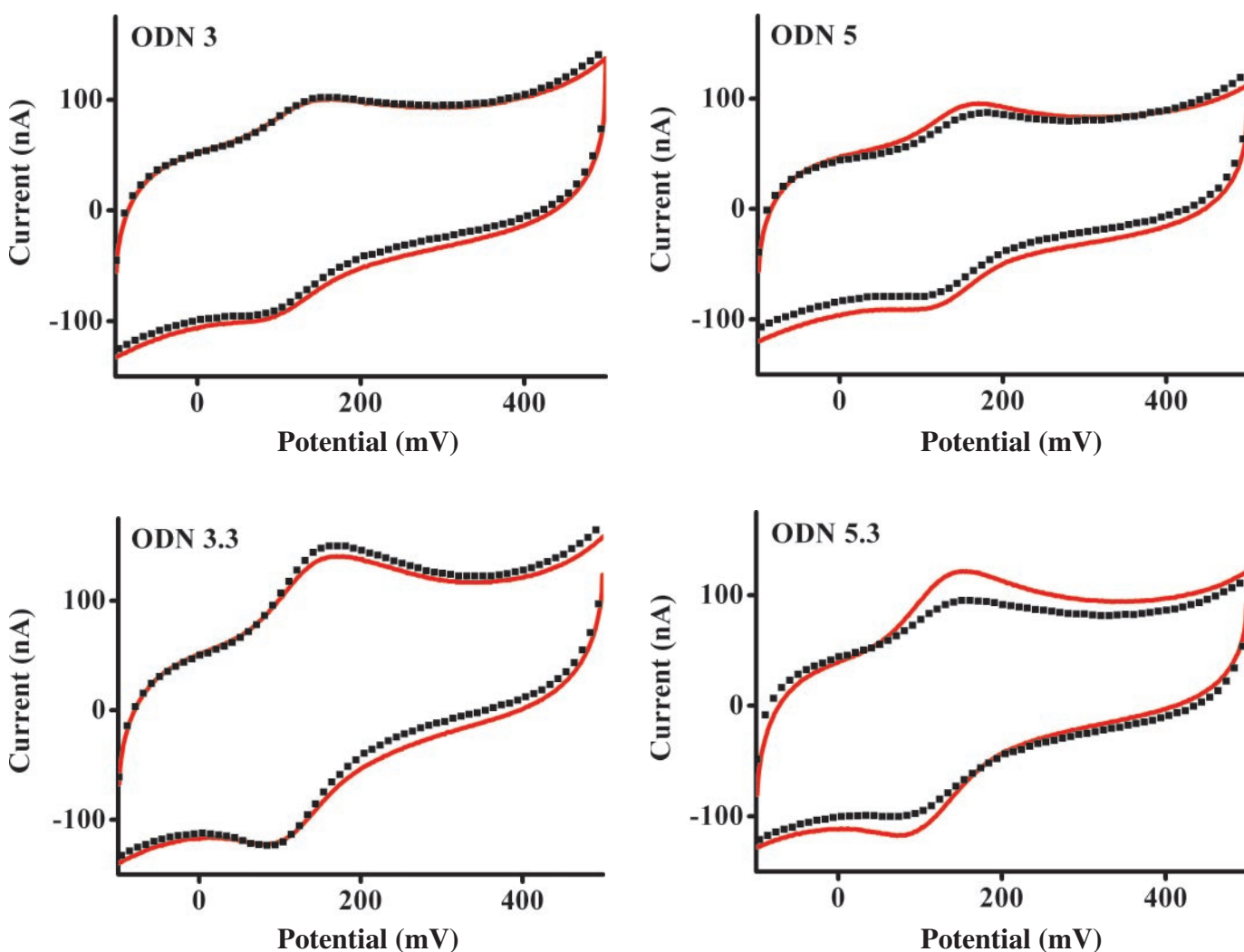


Figure 2. CV data of labeled ODN [25 μM in phosphate buffer (pH 6.8), NaCl 0.75 M] before hybridization (dashed black line) and after hybridization (red solid line), $v = 10\text{ mV s}^{-1}$, 37°C , reference Ag/AgCl, glassy carbon working electrode (diameter 3 mm).

in aqueous solution have small diffusion coefficients therefore allowing a study of the electrochemical properties of differently labeled ODN (28). The calculated coefficients of diffusion for the four labeled ODNs give a low value of $\sim 8.50 \cdot 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. However, on the basis of the shape of the CV curves which exhibits peak symmetry and the stability of the film (Figure S2), an electrochemical process principally governed by adsorption phenomena could be suggested. The small interactions can be attributed to the vitreous carbon electrode that is made from sp³ carbon compared with pyrrolytic carbon electrode made from sp² carbon. This phenomenon was reported in the case of proteins especially with cytochrome *c* (44). Due to the small interaction adsorption on vitreous carbon, it is difficult to have more information about the DNA films. In this case, as not to disturb the films, the starting potential of the CV curves recorded must be at the rest potential (80 mV). In practice, the CV experiments starting from rest potential or from -50 mV were identical, which indicates that the films are not drastically disturbed by the starting potential (Figure S3).

The Faradaic charge Q required for the oxidation in each case was determined by the integration of the areas under the ferrocene anodic peak corrected from background current. From $Q = nFN$, the oxidized Fc-ODN N was calculated with n being the number of electrons transferred ($n = 1$), F the Faraday constant (C/equiv.). The electrochemical data including $E_{1/2}$ and N are listed in Table 3.

In comparison to **ODN 5**, the electrochemical data of the other labeled ODN show a negative shift of the $E_{1/2}$ with the introduction of ferrocenyl group at the 3' position attributed to the electron donating influence of their second alkyl linker. The lowest negative shift of $E_{1/2}$ was observed for **ODN 3.3** with two ferrocenyl moieties in 3' position. This result suggests that the introduction of several consecutive redox systems at the 3' extremity leads to a loss in electroactivity in aqueous media probably due to the increase of local hydrophobicity around ferrocenyl group modifications. In contrast, the introduction of one ferrocene at both 3' and 5' positions did not lead to the loss of electroactivity. The comparison of N for **ODNs 3** and **3.3** reveals a strong electrochemical response of ferrocenyl groups with 3.90 and 9.66 pmol, respectively. These results indicate the presence of 3.90 and 4.83 pmol of **ODNs 3** and **3.3** at the surface electrode and demonstrate the advantage of adding several ferrocenyl groups in labeled ODN. In contrast, the N values of **ODNs 5** and **5.3** show a difference in behavior with an area of anodic wave corresponding respectively to 3.06 and 4.49 pmol of oxidized ferrocenyl groups. While the value obtained for **ODN 5** is in accordance with **ODN 3**, the value for **ODN 5.3** indicates either a lesser quantity of ODN at the surface electrode or that one ferrocenyl group is not electroactive. The comparison of $E_{1/2}$ for **ODNs 3** and **5.3** (respectively 113 and 114 mV versus Ag/AgCl) suggests that the electrical response of **ODN 5.3** is mainly due to the ferrocenyl group at the 3' position, while the ferrocenyl moiety in 5' position seems to be masked.

After hybridization with exact target. The hybridization reactions were monitored by CV after the addition of 1 equivalent of target ODN directly into the buffered aqueous solution containing the modified ODN. Increasing the hybridization

incubation time by 10 min at 37°C did not improve the results. In all the cases, the hybridization reactions lead to a negative shift of $E_{1/2}$ except for **ODN 3.3**. For **ODNs 3** and **3.3**, a small decrease in area under the CV peak was observed after incubation and therefore we cannot definitively attribute this to the hybridization reaction. Using **ODN 5**, an increase in area of 31% was observed, which indicates a more favorable position of 5' for electrochemical analysis. The strongest effect of hybridization was obtained for **ODN 5.3**. In fact, after hybridization the integration of the CV peak indicates the oxidation on 8.5 pmol of the labeled ODN corresponding to an increase of 89% in area. This reveals that the same quantity of **ODN 5.3** was at the electrode surface before and after the hybridization reaction. Kraatz *et al.* (45) have shown that electrochemical response in Fc-labeled double-stranded DNA grafted on Au electrode is more effective when the redox probe is in 5' position at the terminus of the DNA duplex. The orientation or/and geometric effects predispose the 5'-labeled Fc to be more accessible to the base pairs (45). In our case, the single-strand Fc-labeled ODNs were not grafted onto electrode surface, but an orientation of Fc-labeled ODN can be presumed due to an interaction between the terminal amino group and glassy carbon surface. Thus, the 3' ferrocenyl groups are probably located at the electrode surface. In fact, amine compounds can be grafted on glassy carbon electrode surface under appropriate conditions (46). Therefore, the electrochemical responses would be more efficient with ferrocenyl groups in 3' position. In this context, the electrochemistry of the 3' ferrocenyl group is unchanged by hybridization, and ferrocenyl groups in 5' position appears more electroactive due to the base pairs proximity (45).

After hybridization with smallest targets. The **ODN 5.3** was hybridized with two 12mer complementary targets. The smallest target **SM3** and **SM5** are complementary to the 3' and 5' side of **ODN 5.3** respectively. An equivalent of these new target ODNs has been added directly in buffered aqueous solution containing the modified **ODN 5.3**, followed by 10 min incubation at 37°C. The CV was recorded before and after hybridization (Figure 3). The charging current observed shows a difference due to the quality of the initial surface of the electrode after cleaning. In practice, it is not a problem because the Faradaic peak currents are quasi-similar with background-subtracted current.

With **SM5** target, the CV is identical before and after the hybridization reaction, which indicates that the electrochemical response of **ODN 5.3** is not affected by the target. With **SM3**, the CV increases by 45% of the area oxidation wave of ferrocenyl group after hybridization. The comparison at the 89% increasing of the area obtained with an exact target can be explained by two hypotheses. First, the hybridization with **SM3** leads to an ordering of the DNA into a more favourable conformation for increasing the electroactivity of the 3' ferrocene unit. Second, the electroactivity of the ferrocenyl group in position 3' is the same and the ferrocenyl group in position 5' is more accessible to electrochemical reaction. This hypothesis is based on an orientation of the labeled ODN at the surface electrode and a possible role of the double-stranded DNA in the electron transport effect. A more detailed investigation is underway on these new results. However, the first results of grafted Fc-ODN on conducting polymers correspond

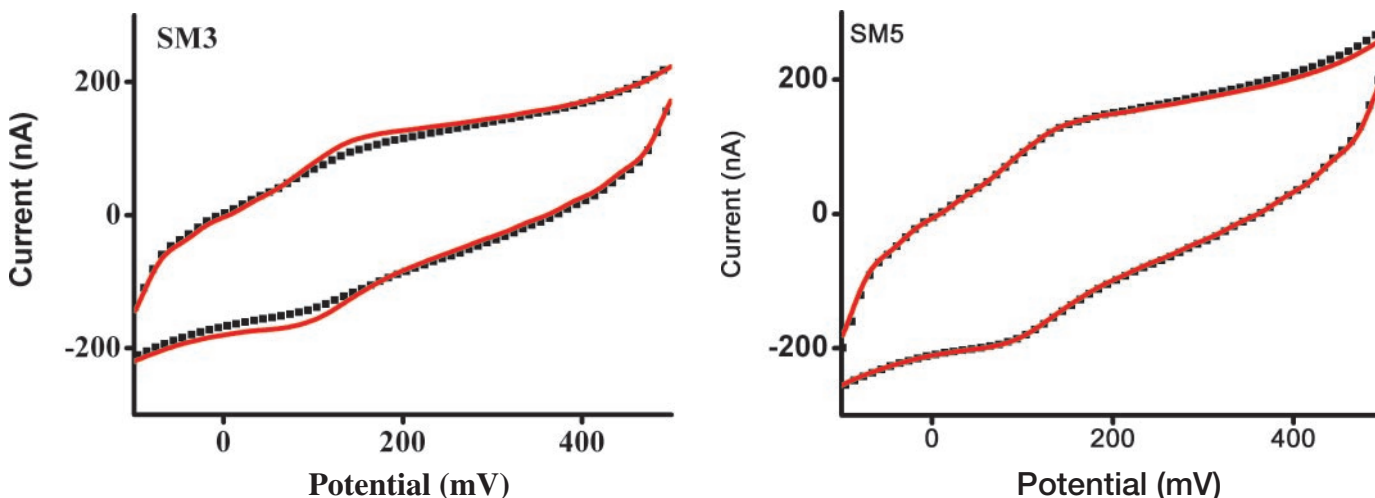


Figure 3. CV data of labeled ODN 5.3 [25 μ M in phosphate buffer (pH 6.8), NaCl 0.75 M] before hybridization (dashed black line) and after hybridization (red solid line), $v = 10 \text{ mV s}^{-1}$, 37°C , reference Ag/AgCl.

with results obtained in aqueous solution and seem to confirm the second hypothesis.

These results demonstrate that all ferrocene-modified ODNs are electrochemically detectable and an increase in the sensibility of detection can be reached by incorporation of several ferrocenyl groups at 3' and 5' positions. The results reveal that this new type of modified ODN can be used as signaling probes in the electronic detection of nucleic acids.

CONCLUSION

We have described a new strategy toward ferrocene-labeled ODNs as potent markers of DNA hybridization without the need for nucleotide chemistry. Two new phosphoramidite ferrocenes have been synthesized, characterized and incorporated during automated DNA synthesis at various positions along the ODN sequence. Melting temperature measurements demonstrated that the introduction of a ferrocenyl group at the 3' position and/or at 5' position has no drastic effect on the thermal stability of DNA duplex. Electrochemical data of the ferrocene-labeled ODN show that these complexes have an important electrochemical response. For all compounds, different electrochemical behaviors were observed before and after hybridization reaction with complementary strand. The best electronic detection sensitivity of hybridization was performed with labeled ferrocene ODN at both 5' and 3' extremities. The particular behavior of ODN 5.3 appears to be extremely important for DNA chip applications due to the increase in the electrochemical ferrocene response only with the exact target. Meanwhile, we successfully used this strategy to prepare other labeled ODNs with electroactive markers at different positions of the chain and develop DNA chips.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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