Synthesis and characterization of DNA with ferrocenyl groups attached to their 5′-termini: electrochemical characterization of a redox-active nucleotide monolayer

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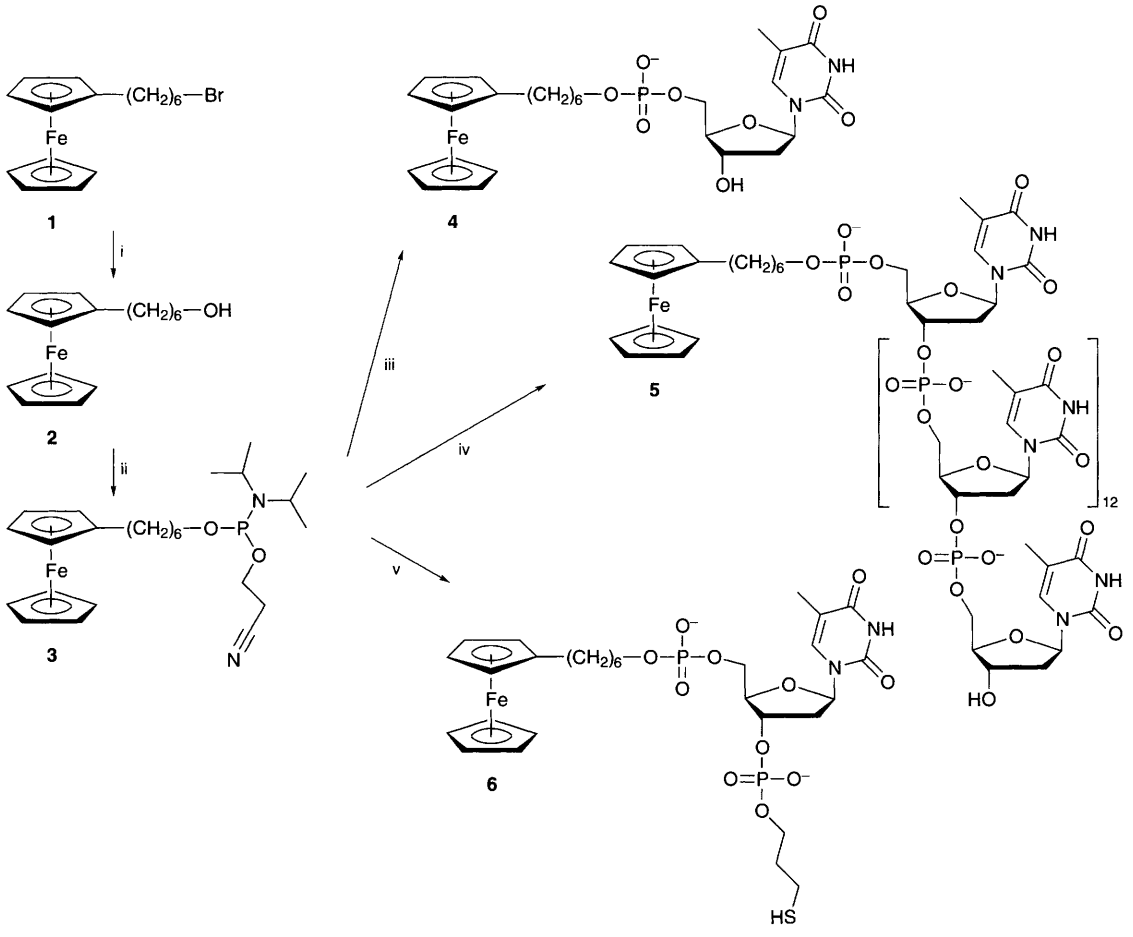
A novel 5′-ferrocenyl and 3′-thiol modified nucleotide is used to prepare and characterize, for the first time, a redox-active nucleotide monolayer on Au.

Here we report the synthesis and characterization of oligonucleotides with ferrocenyl moieties attached to their 5′ termini. Our aim is to use these molecules in a molecular architecture scheme that relies on the unique molecular recognition properties of complementary DNA strands to build multilayer structures on surfaces in a step-by-step fashion. Such structures could find utility in the preparation of novel diagnostic devices, light harvesting materials and NLO frequency doubling materials. Our goal is to develop such chemistry for surfaces with well-defined structures and with adsorbate chemistry that leads to stable, and characterizable monolayer films. In this regard, Au is an excellent choice for the following reasons. (i) Au(111) with large atomically flat terraces may be prepared by thermal evaporation of Au onto a variety of substrates including SiO_2 and mica.[1] (ii) Thiol adsorption onto Au is well documented and often leads to densely packed, highly oriented monolayer structures.[2] (iii) Au prepared by thermal evaporation methods is SERS-active with enhancement factors $> 10^3$, making characterization of monolayer structures by Raman spectroscopy straightforward.[3] (iv) Au can be used as an electrode to characterize the surface-adsorbed, redox-active ferrocenyl capped DNA.

Traditionally, oligonucleotides or DNA have been radiolabelled and used for diagnostic reasons. Recent advances have shown that high sensitivity detection can be achieved by alternative markers based on fluorescent, chromophoric or chemiluminescent detection.[4a] However, because oligonucleotides exhibit irreversible electrochemistry in standard



Scheme 1 *Reagents and conditions*: i, H_2O/HMPA, reflux, 6 h; ii, β-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite: *N*,*N*-diisopropylethylamine: THF, room temp., 3 h; iii, thymidine modified CPG: DNA synthesizer; iv, thymidine modified CPG: DNA synthesizer; v, 3′-thiol modified CPG: DNA synthesizer

electrolytic media, there are no reliable methods known which enable electrochemical detection of oligonucleotides in a non-destructive manner.[4b] Being able to form robust, densely packed oligonucleotide monolayer films is of paramount importance to our oligonucleotide molecular architecture goals. Equally important are methods to characterize such structures and to determine adsorbate surface coverages. In this regard, the use of redox-active ferrocenyl-capped oligonucleotides and conventional CV is a very appealing strategy. Here we present a method for the chemical introduction of a ferrocenyl redox group to the 5′-end of oligonucleotides, and we show how this moiety may be used to detect a monolayer of an oligonucleotide. Although others have used thiol adsorption chemistry to form monolayer films of oligonucleotides on Au surfaces[5a–c] and some have prepared chemically modified DNA with electrochemically reversible redox couples,[5d] there are no reports of the preparation and characterization of monolayers formed from nucleotides functionalized with a thiol in the 3′-end and a redox-group, such as ferrocene, in the 5′-end. A key advantage of attaching the ferrocenyl group to the 5′-terminus is that it is based on the widely adopted method of solid phase synthesis[6] and can be achieved routinely on a variety of commercially available DNA synthesizers. Our strategy to synthesize 5′-ferrocene-dT **4**† was based on the coupling of a ferrocenylphosphoramidite **3** with thymidine bound controlled pore glass (CPG) by standard automated DNA solid phase chemistry, Scheme 1.[6]

Compound **3** was synthesized from 6-bromohexylferrocene[7] **1**. In a typical preparation, **1** (7 mmol, 2.44 g) was stirred in 42 ml of a 15% (*v*/*v*) aqueous HMPA solution at 120 °C for 6 h to form 6-hydroxyhexylferrocene **2**;‡ this is a modification of a literature method.[8] Compound **2** (400 mg, 1.4 mmol) was then treated in THF (16 ml, with 7 mmol of *N*,*N*-diisopropylethylamine) at room temperature for 3 h with β-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (340 μl, 2.1 mmol) to form the amidite compound **3**.[9] Evaporation of the solvent, aqueous workup, and preparative TLC gave **3** as a yellow-orange oil.‡

The ferrocenylphosphoramidite **3** was then coupled to thymidine bound CPG. After the coupling reaction a yellow–orange colour was apparent on the solid support which remained after multiple rinses with acetonitrile. A green colour was momentarily seen during the last oxidation step of the DNA synthesis but quickly returned to yellow–orange; these colour changes are consistent with those seen for the oxidation and subsequent reduction of ferrocene. The ferrocene modified nucleotide was then removed from the solid support by standard treatment with concentrated ammonia at 55 °C for 16 h to give **4**.[4]‡

The new ferrocenyl-modified nucleotide **4** was purified by Reverse Phase-HPLC and characterized by ^{1}H and ^{31}P NMR, mass spectrometry and CV, which were fully consistent with its proposed structure. For example, the Fast Ion Bombardment (FAB^-) mass spectrum of **4** shows a molecular ion peak at *m*/*z* 589, M_{calc} = 589, and the CV of **4** in a buffered aqueous solution[10] (pH = 7, 0.1 mol dm^{-3} $NaClO_4$) exhibits one electrochemically reversible wave associated with oxidation and subsequent reduction of the pendant ferrocenyl moiety ($E_{1/2}$ = 0.110 V vs. Ag/AgCl), Fig. 1(*a*). To show the usefulness of this method, we also successfully applied it to the synthesis of a longer dA_{14} oligonucleotide strand to form **5**‡ and a nucleotide terminated in the 3′-end with a thiol **6**‡ by the use of 3′-thiol-modified CPG.

Compound **6** spontaneously adsorbed from aqueous solution onto the surfaces of Au substrates to form densely packed monolayer films. CV of an Au substrate modified in this manner exhibited a reversible wave with an $E_{1/2}$ = 220 mV *vs.* Ag/AgCl, Fig. 1(*b*), which is consistent with a surface-confined redox active adsorbate ($i_p \propto$ scan rate), Fig. 1(*b*). The shift to more positive potential for the surface-confined DNA species **6** as compared with the solution species **5** may be due to dielectric differences for the monolayer as compared with the adsorbate-molecule/electrolyte solution. We assigned this wave to oxidation/reduction of the surface bound ferrocenyl capped DNA molecules. Assuming that the ferrocenyl groups are fully electrochemically accessible (a reasonable assumption based on literature precedent),[1] integration of the current associated with one electron oxidation and subsequent reduction of the DNA adsorbate molecules allowed us to calculate a surface coverage for **4** of 3.0 × 10^{-10} moles cm^{-2}. This surface coverage value in conjunction with the surface roughness value for the Au substrate (roughness factor = 1.3) allowed us to estimate a molecular footprint for the surface-confined redox-active nucleotide (9.8 Å diameter cylinder), which was somewhat larger than that estimated for ferrocenylalkyl thiols adsorbed on Au(111) substrate, suggesting that the nucleotide portion of the molecule is size determining rather than the ferrocenyl group. Molecular models of **6** are consistent with this conclusion. Surface-enhanced Raman spectroscopy of the **6**-modified Au substrate also showed a characteristic band at 1489 cm^{-1} associated with the nucleotide framework.[11] These spectroscopic and electrochemical signatures will be useful for diagnostic purposes in studying hybridization and multilayer growth on such surfaces. Furthermore, it is worth noting that from molecular models, it is difficult to estimate the effective

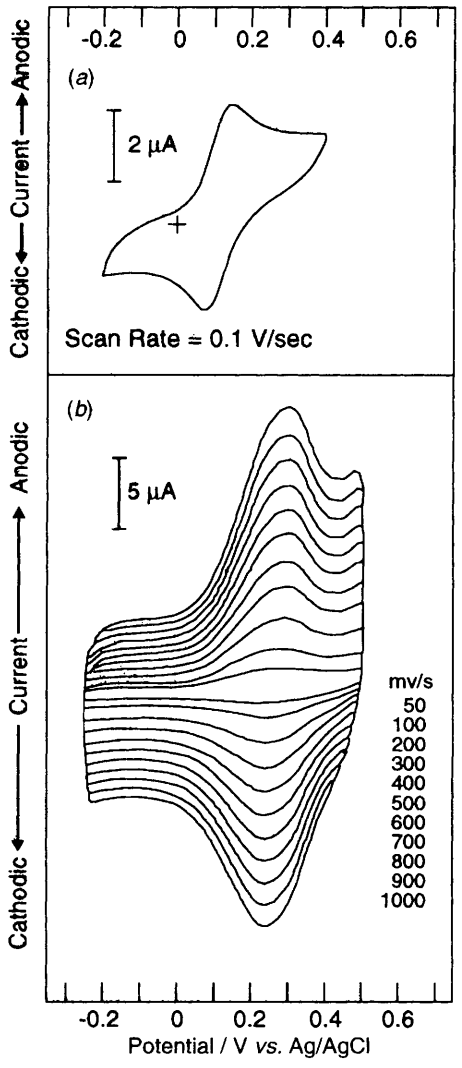


Fig. 1 (*a*) Cyclic voltammetry of **4** at an Au disk electrode (7 mm^2) in aqueous solution buffered at pH 7 with 0.1 mol dm^{-3} $NaClO_4$ supporting electrolyte, Pt mesh counter electrode, and Ag/AgCl reference electrode. (*b*) Cyclic voltammetry of a monolayer of **6** adsorbed on an Au thin film electrode [2000 Å on an oxidized Si(100) wafer with a 50 Å Ti adhesion layer]; electrolyte conditions and counter and reference electrodes are the same as above.

molecular footprints of DNA-based adsorbate molecules because of their degree of hydration and ionic character; indeed, the use of redox-active groups in conjunction with surface-confinable oligonucleotides may become a general strategy for assessing the molecular footprints of single and multistrand DNA-based adsorbate molecules.

We have demonstrated a method for covalently attaching ferrocenyl redox groups to oligonucleotides to prepare DNA that exhibits reversible electrochemistry. This methodology works for single nucleotides and oligonucleotides. Moreover we have used this strategy to prepare novel nucleotide adsorbate molecules for Au surfaces which form robust redox-active monolayer structures. This is an important step towards rationally constructing characterizable oligonucleotide multilayer structures on well-defined surfaces.

C. A. M. acknowledges NSF (CHE-9121859) for seed support for this work. C. A. M. also acknowledges use of the crystal growth facility which was purchased by the MRSEC Program of the NSF at the MRC of NU, under Award No. DMR-9120521. C. A. M. also acknowledges an A. P. Sloan Foundation Fellowship, a Dreyfus Foundation New Faculty Award, a National Science Foundation Young Investigator Award (URI3136YIP), a Dupont Young Professor Award, and a Naval Young Investigator Award for financial support. R. L. L. acknowledges support for this work from the National Institutes of General Medical Sciences (grant GM 10265) and the National Institute of Allergy and Infectious Diseases (grant UOI AI24846).

Footnotes

† Oligonucleotides were synthesized on a Milligene Expedite DNA synthesizer using phosphoramidite chemistry in dual column modes (1 μmol scales). All solutions were purchased from milligene (DNA synthesis grade). The 3′-thiol modified-CPG that was used for the synthesis of **6** was purchased from Glen Research, Sterling, VA. Cleavage of **6**-modified CPG was accomplished by treatment with dithiothreitol (0.05 mol dm^{-3}) in concentrated NH_3. For DNA syntheses, average coupling efficiency varied from 98–99.8%. When DNA-synthesis was completed the CPG material was dried under vacuum before removal from the column. Subsequently the CPG material was transfered to a screw-cap vial and heated at 55 °C in 1.5 ml concentrated NH_3 for 16 h. DNA was purified by RP HPLC. Reverse-phase HPLC was carried out on a Hewlett Packard ODS Hypersil column (4.6 × 200 mm, 5 μm particle size) with a 1% min^{-1} gradient of MeCN + 5% 0.03 mol dm^{-3} $Et_3NH^+OAc^-$ buffer in 0.03 mol dm^{-3} $Et_3NH^+OAc^-$ buffer (TEAA), pH 7.0. Flow: 1.0 ml min^{-1}. Melting curves were recorded on a Perkin-Elmer Lamba 2 UV–VIS spectrophotometer using solutions 100 mmol dm^{-3} in NaCl, 15 mmol dm^{-3} in phosphate buffer, 0.4 mmol dm^{-3} DTT and 5 μmmol dm^{-3} in each oligonucleotide. Changes in absorbance at 260 nm were followed while ramping the temperature at the rate of 1 °C min^{-1}. All NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer at 20 °C unless otherwise noted. The ^{31}P spectra were referenced to external 85% H_3PO_3. Cyclic voltammetry was performed using a Pine RDE4 bipotentiostat coupled with a Linseis LY1400 4000 X-Y recorder.

‡ *Spectroscopic data* for **2**: ^1H NMR ($CDCl_3$): 1.3–1.6 (m, 8 H, $C_5H_5CH_2CH_2CH_2CH_2CH_2CH_2OH$), 2.30 (t, J = 7.7 Hz, 2 H, $C_5H_5CH_2$), 3.62 (t, J = 6.3 Hz, 2 H, CH_2O), 4.03 (broad, 4 H, C_5H_4) and 4.08 (s, 5 H, C_5H_5); EI-MS: m/z 286 (M^+). For **3**: ^1H NMR ($CDCl_3$): 1.12–1.13 (m, 12 H, CH_3), 1.40–1.54 (m, 9 H, CH_2), 2.31 (t, 2 H, CH_2Fc), 2.62 (t, 2 H, 2 × CH), 3.67 (m, 4 H, $POCH_2CH_2CN$), 3.80 (m, 2 H, CH_2OP) and 4.10 (m, 9 H, C_5H_4 and C_5H_5); ^{31}P NMR ($CDCl_3$): 148.7. For **4**: ^1H NMR (400 MHz, D_2O): 1.10–1.48 [m, 8 H, $(CH_2)_4$], 1.87 (s, 3 H, CH_3), 2.12 (m, 2 H, 2′-H), 2.72 (t, 2 H, CH_2Fc), 3.68 (m, 2 H, CH_2O), 3.85 (m, 2 H, 5′-H), 3.95 (s, 1 H, 3′-H), 4.21 (br, 9 H, C_5H_4 and C_5H), 4.40 (br, 1 H, 4′-H), 6.18 (t, 1 H, 1′-H) and 7.61 (s, 1 H, thymine (CH). ^{31}P NMR (D_2O): 1.0 ppm; FAB-MS: m/z = 589 (M^-). For **5**: Characterized on the basis of its Reverse Phase HPLC retention time (27.4 min) as compared to that for an unmodified dA_{14} strand (18.5 min), and also, on its ability to form a hybrid pair with a complimentary T_{14} strand (T_m = 42 °C, 0.1 mol dm^{-3} NaCl), as compared with 41 °C for the same duplex lacking the ferrocene substituent. For **6**: FAB-MS: m/z 760 ($M + H_2O$). Fc = ferrocenyl.

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Received, 21st September 1995; Com. 5/06244F