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Synthesis and Application of a Triazene−Ferrocene Modifier for Immobilization and Characterization of Oligonucleotides at Electrodes

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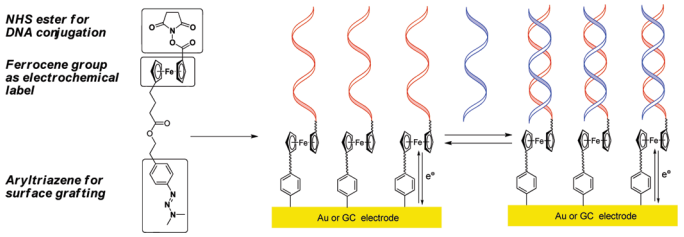

A new DNA modifier containing triazene, ferrocene, and activated ester functionalities was synthesized and applied for electrochemical grafting and characterization of DNA at glassy carbon (GC) and gold electrodes. The modifier was synthesized from ferrocenecarboxylic acid by attaching a phenyltriazene derivative to one of the ferrocene Cp rings, while the other Cp ring containing the carboxylic acid was converted to an activated ester. The modifier was conjugated to an amine-modified DNA sequence. For immobilization of the conjugate at Au or GC electrodes, the triazene was activated by dimethyl sulfate for release of the diazonium salt. The salt was reductively converted to the aryl radical which was readily immobilized at the surface. DNA grafted onto electrodes exhibited remarkable hybridization properties, as detected through a reversible shift in the redox potential of the Fc redox label upon repeated hybridization/denaturation procedures with a complementary target DNA sequence. By using a methylene blue (MB) labeled target DNA sequence the hybridization could also be followed through the MB redox potential. Electrochemical studies demonstrated that grafting through the triazene modifier can successfully compete with existing protocols for DNA immobilization through the commonly used alkanethiol linkers and diazonium salts. Furthermore, the triazene modifier provides a practical one-step immobilization procedure.

Introduction

Immobilization of DNA on surfaces is important for most DNA-based sensor technologies.[1,2] In recent years, DNA immobilization on electrode surfaces for the development of simple, cost-effective, and sensitive electrochemical biosensors has attracted much attention.[3−5] Covalent immobilization of DNA on electrode surfaces is most often required for obtaining stable DNA layers during hybridization and/or denaturing analysis. Commonly used covalent DNA attachment protocols include, e.g., anodized carbon using carbodiimide chemistry, where the carboxylic groups in the electrode surface are coupled to amine-modified DNA.[6,7] Another approach, most frequently used for gold surfaces, is DNA chemisorption onto electrodes through an alkanethiol or disulfide linker, producing functional DNA self-assembled monolayers (SAMs).[8,9] However, limited thermal[10] and

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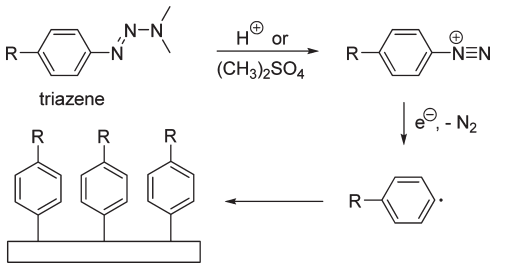
electrochemical stability[11−13] of the Au−S bond restricts application of the DNA-modified gold electrodes to moderate temperatures and potentials.

Recently, multistep procedures using diazonium salt derivatives for the immobilization of DNA have been demonstrated. The surfaces, e.g., carbon nanotube-,[14] silicon-,[15] or boron-doped, single-crystalline diamond films,[16] are functionalized with nitrophenyl groups, which are reduced either chemically or electrochemically to the amine. The DNA coupling is then performed by a glutaraldehyde linker[15] or by using maleimide chemistry.[14,16] A more direct approach utilizing a phenylmaleimide diazonium salt conjugated to Fc-labeled DNA was also reported,[17] but hybridization properties of the DNA-functionalized electrodes were not shown, which was ascribed to reaction of the diazonium species with the nucleic acid bases.[18] Another problem associated with conjugation of diazonium salts derivatives to DNA is that they are only stable in aqueous solutions at low pH of 2−3,[19] at which depurination of DNA may occur and cause degradation of the DNA.[20]

Here we introduce a new one-step electrochemical modification procedure of electrode surfaces by DNA through a DNA-conjugated triazene modifier. Triazenes can be used as masked diazonium salts, and they have been used for grafting of molecules to surfaces.[19,21] When masked as the triazene, the diazonium salt is not liberated until it is activated and grafting occurs, minimizing the chance of undesired byproducts. A general scheme for the immobilization of aryltriazenes via diazonium salts on surfaces is shown in Scheme 1.

Tour et al. have demonstrated grafting of small molecules via triazenes onto silicon[22,23] and single-walled carbon nanotubes.[24] In their studies, conversion of the triazene to the diazonium salt was done at pH 2, whereby the triazene is protonated and cleaved. As an alternative we have used Me_2SO_4, which was added directly to the DNA−triazene conjugate solution already placed on the electrode. It is assumed that Me_2SO_4 methylates the terminal nitrogen of the triazene, triggering the spontaneous cleavage to the aryl diazonium salt.[25] A one-electron transfer from the electrode reduces the diazonium group, whereby it decomposes to nitrogen gas and the aryl radical, which immediately reacts with the electrode surface and forms a covalent bond.

SCHEME 1. General Scheme for Immobilization of Aryltriazenes on Surfaces



A common approach to provide unambiguous evidence for successful immobilization of DNA onto the electrode surfaces is to use a redox-labeled DNA species for grafting.[4,17,26] Successful DNA grafting can also be revealed through specific hybridization of the immobilized DNA with a redox-labeled complementary probe.[1−5] This enables electrochemical characterization of the DNA grafting efficiency and properties of the produced DNA monolayers. The latter approach is, however, highly dependent on the hybridization efficiency of the immobilized DNA. To incorporate these features in one molecular unit we have synthesized the triazene ferrocene *N*-hydroxysuccinimide ester (TzFcNHS) modifier **1** (Figure 1). This modifier integrates (i) an activated ester for conjugation to amine-modified DNA, (ii) the triazene moiety for grafting to a surface, and (iii) a ferrocene redox reporter group for monitoring grafting efficiency and hybridization events. Application of this modifier provides a unique quality control of DNA monolayers, since it enables the direct and quantitative electrochemical characterization of the grafted DNA at the electrode.

Results and Discussion

For the synthesis of the TzFcNHS modifier **1**, the commercially available ferrocenecarboxylic acid was applied as the starting material (Scheme 2). After conversion into the methyl ester **2**,[27] a handle for the triazene moiety had to be attached to the unsubstituted ferrocene Cp ring. By a Friedel−Crafts acylation with succinic anhydride, **2** was converted into **3** in moderate yield of 40%, followed by removal of the excessive ketone in a Clemmensen reduction to give **4**.

The triazene moiety was prepared from 4-(2-aminoethyl)-aniline by first performing a selective Cbz protection of the amine to yield **5**. The aniline was then converted into a diazonium salt by treatment with sodium nitrite under acidic conditions and subsequently in situ trapped by dimethylamine to give the triazene **6**. The reaction had to be made in a solvent mixture containing THF, MeCN, H_2O, and HCl to avoid precipitation of starting material or intermediates. After deprotection of Cbz with H_2 and 10% Pd/C, the amine

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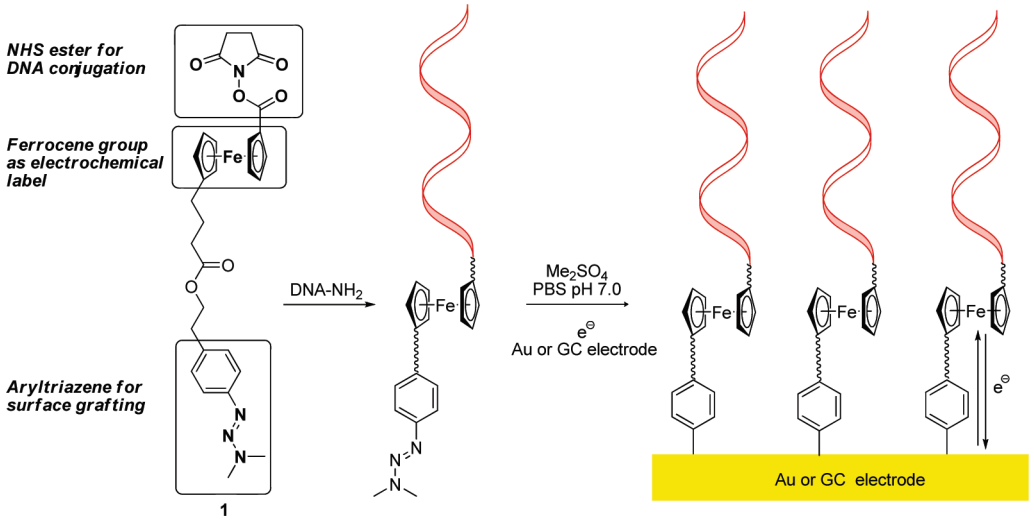
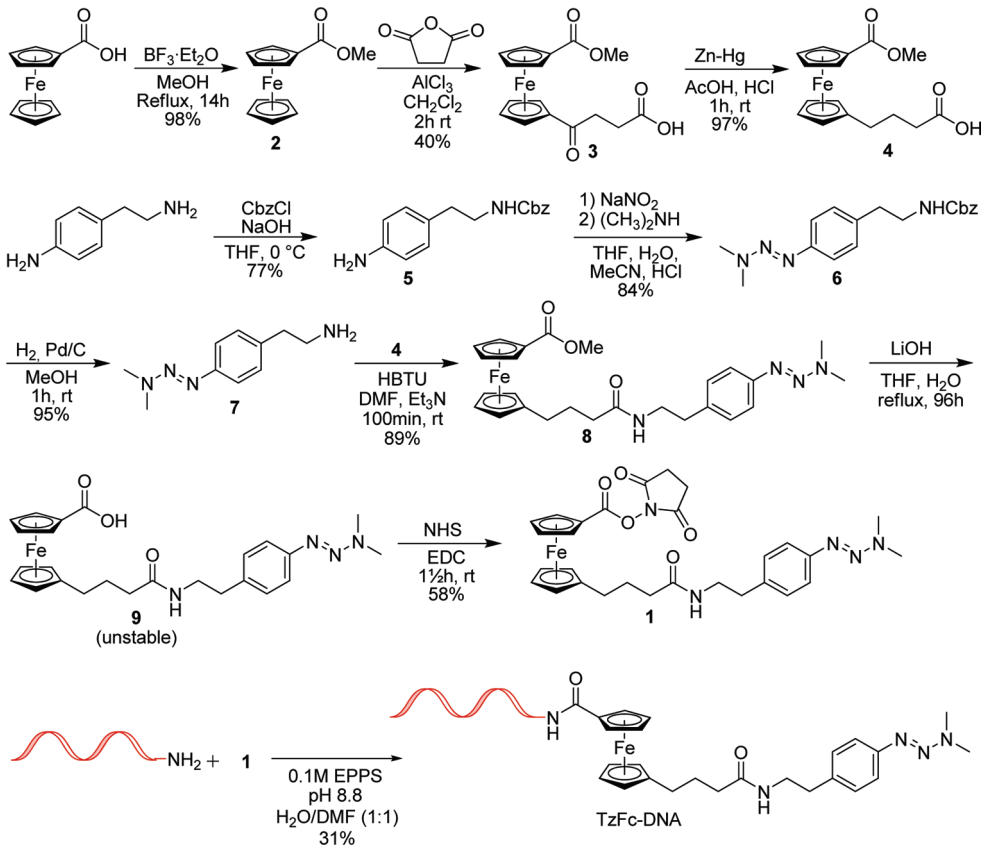


FIGURE 1. Formation, grafting, and characterization of the triazene−ferrocene−DNA conjugate. The surface coverage can then be determined by the redox activity of ferrocene.

SCHEME 2. Synthesis of TzFcNHS Modifier 1 and Conjugation to 3′-Amino-Modified ssDNA



in **7** was coupled to **4** using HBTU as the coupling reagent to give the assembled triazene ferrocene ester **8** in a satisfying yield of 89%. The use of HBTU or TBTU is preferred compared to acid chloride formation or EDC or DCC coupling, since it is a one-pot procedure which requires short reaction times and can be performed in commercial grade solvents. Furthermore, side products from the coupling reagent can easily be removed by extraction.[28]

The methyl ester in **8** was hydrolyzed with LiOH to give the unstable acid **9** of which pure ^{1}H NMR spectrum could not be obtained. The instability is caused by the acid lability of the triazene moiety, and it is important to continue the synthesis right after purification of the acid. In this last step, an activated ester was formed in a reaction with NHS and EDC giving the TzFcNHS modifier **1** in an overall yield

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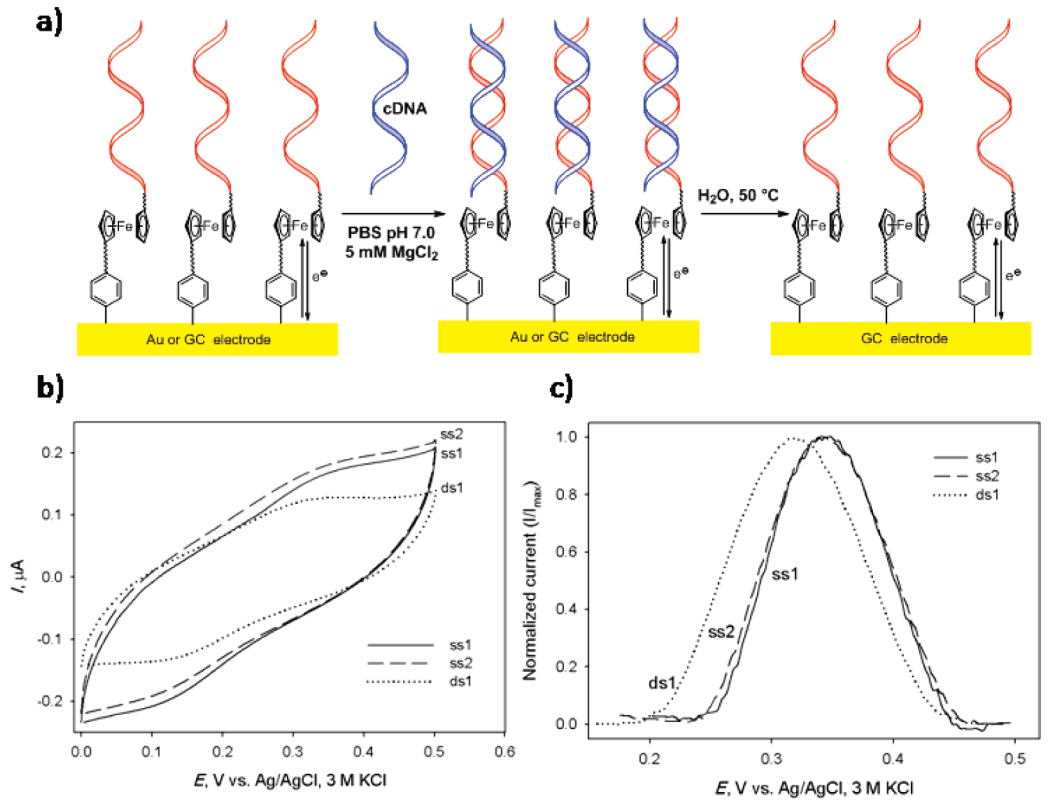

FIGURE 2. (a) Schematic illustration of the hybridization and denaturing events. (b) Cyclic voltammogram of the Fc-labeled DNA-modified GC electrode in PBS, pH 7, before (ss1) and after (ds1) hybridization, and after denaturation at 50 °C in water (ss2). Scan rate is 100 mV s^{-1}, and CVs recorded after the fifth scan are shown. (c) Normalized and background-subtracted anodic scans currents derived from the corresponding CVs.

of 26%. The NHS ester is relatively stable, and after storage for 1 year at 4 °C it was still sufficiently preserved to conjugate to amino-modified DNA.

The TzFcNHS modifier **1** was coupled to a 10-mer oligonucleotide modified with a C6 amino linker at the 3′-end (Scheme 2). The reaction, which proceeded in 31% yield, was performed in an aqueous 100 mM EPPS buffer containing 25 mM modifier and 50% DMF. After incubation at 45 °C for 16 h, the conjugate was HPLC-purified and redissolved in phosphate buffer at pH 8. UV−vis of the conjugate showed the expected absorbance at 260 nm from the DNA bases and a smaller peak at 320 nm from the triazene moiety (Supporting Information, Figure S1). As shown by HPLC, the conjugate was stable after three months at −18 °C.

The produced DNA−triazene conjugate was in one step activated by addition of Me_2SO_4 to release the diazonium salt, which was then immediately in situ electrochemically reduced to the aryl radical for immobilization on the electrode surface at a fixed potential of −200 mV vs Ag/AgCl (3 M KCl) for 20 min. In a separate experiment, HPLC analysis showed that the Me_2SO_4 treatment did not cause methylation of the DNA bases under the conditions applied for grafting.

The representative cyclic voltammograms (CVs) of the DNA-modified GC electrodes are shown in Figure 2. The CVs display the redox peaks with a mean potential of 231 ± 5 mV, which correlates well with the Fc redox label conjugated to the grafted DNA (Figure 2b,c). There is a small oxidative peak at around −200 mV in the first scan (not shown), and in the following scans the CV remains as shown in Figure 2b for ss1. The observed quasi-reversibility of this electron transfer (ET) process can have several origins such as the relatively large distance between the surface and the ferrocene redox probe, a relatively poor electrolyte, permeability of the electrografted layer and neighboring charge effects caused by the formation of ferrocenium ions. The peak current's linear dependency of the potential scan rate is characteristic for a surface-confined redox process,[29] and the amount of immobilized DNA, estimated by integration of the Fc redox peaks, was 29 ± 4 pmol cm^{-2}, which approaches the best values shown for the compact DNA monolayer surface coverage.[30] The ET rate constant k_s, determined from the cathodic and anodic peak separation,[31] was 0.28 ± 0.05 s^{-1}. Similar data were obtained for the DNA-modified gold electrodes via the TzFc-modifier (Supporting Information, Figure S2a), where the representative CVs demonstrate a pair of the Fc redox peaks from the Fc-labeled DNA grafted to the gold surface centered at +218 ± 4 mV. A close to compact DNA monolayer coverage of 31 ± 9 pmol cm^{-2} was obtained from the peak area integration, while the ET rate constant k_s between DNA ferrocenes and gold was close to 1 s^{-1}, which, as expected, is higher than what was observed with the glassy carbon electrodes.[32] The grafting was also attempted without the

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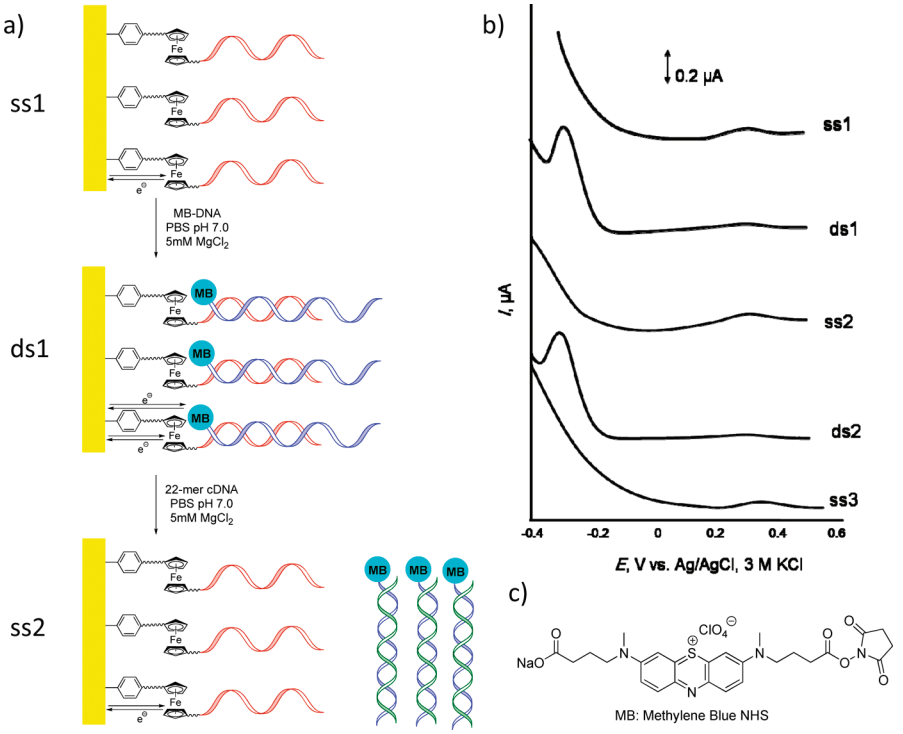

FIGURE 3. (a) Schematic illustration of the hybridization and dehybridization events of the 15-mer TzFc-modifier grafted DNA (red) with MB-labeled 22-mer DNA (blue) and competitive removal of the surface hybrid by addition of a competitive 22-mer DNA strand (green) that forms a duplex with the full MB-labeled 22-mer DNA. (b) Differential pulse voltammogram (DPV) of DNA-modified GC electrode (ss1) after grafting with 15-mer TzFc−DNA conjugate; (ds1) after hybridization with a complementary 22-mer MB-labeled DNA, (ss2) after dehybridization using the competitive hybridization with excess amount of 22-mer ss-DNA complementary to the MB-labeled DNA; (ds2) and (ss3) repetition of hybridization/dehybridization process, potential step 2 mV, modulation amplitude 50 mV and apparent scan rate 10 mV s^{-1}. (c) Structure of the modifier (MB−NHS ester) used to label DNA with the MB probe.

presence of Me_2SO_4 and without applying a reductive potential to the electrode. In either case, no grafting occurred as deduced from the absence of redox signals from Fc in the CVs performed after washing the electrode.

Hybridization properties of the electrode-grafted DNA were probed with a label-free cDNA (cDNA) sequence. After hybridization the voltammetric responses from the DNA-modified electrodes changed. For dsDNA-modified GC electrodes mean potential of Fc shifted −15 mV (Figure 2b,c), and the k_s increased slightly to 0.34 ± 0.04 s^{-1}. In addition, the amount of electrochemically active Fc groups, and thereby the amount of the covalently bound DNA, remained the same. After hybridization to the DNA-grafted gold electrodes, the redox potential for the Fc label shifted −14 mV, consistent with the data obtained for DNA grafted onto the GC electrodes (Supporting Information, Figure S2b), while k_s remained essentially the same. The peak width increased after hybridization, and we assume that this is caused by a contribution to the overall electrochemical signal from a minor population of single-stranded TzFc-grafted DNA at the electrode.

For both electrodes, denaturation was performed to regenerate the original ssDNA-modified electrode state. After the electrode was heated to 50 °C in pure water, the signal from the Fc label returned to the initial signal observed in CVs before hybridization with the cDNA (Figure 2 and Supporting Information, Figure S2b). For the GC electrode the magnitude of the electrochemical signal from the Fc redox label also appeared to be identical to the original ss1 signal. Thus, the stability of the produced C−C bond at the surface (346 kJ mol^{-1})[33] was sufficient to enable denaturation of the DNA duplex at elevated temperatures. However, the weaker bond between gold and carbon resulted in higher susceptibility of this bond to breakage: in the course of repeated cycles hybridization and thermal denaturation, the signal from the Fc redox label constantly decreased consistent with a lower amount of gold surface-tethered DNA.

To further investigate the hybridization/denaturation at the GC electrode surfaces we employed a cDNA sequence conjugated to methylene blue (MB) (Figure 3a). Application of MB as an additional redox probe enables us to simultaneously monitor both the grafted TzFc−DNA and the MB−cDNA electrochemically. The signals from the two redox probes are well resolved since MB has a redox potential 0.6 V more cathodic than the Fc redox probe.[34] A 15-mer DNA sequence was grafted onto the GC electrode via the TzFcNHS modifier as described above. A complementary 3′-amino-modified 22-mer DNA sequence was conjugated to MB via an activated ester (Figure 3c). In solution, the melting temperature (T_m) for the 15-mer hybrid between the two strands was measured to be 60.7 °C.

First the Fc signal from the TzFc−DNA grafted electrode was detected by CV (Supporting Information, Figure S3)

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and differential pulse voltammetry (DPV) verifying a successful grafting of the DNA (Figure 3b, ss1). Then MB−cDNA in a PBS buffer containing 5 mM $MgCl_2$ was added to the modified electrode and allowed to hybridize for 1 h at room temperature. After hybridization, two redox peaks, correlating both with the grafted Fc-conjugated DNA and with the hybridized MB-labeled cDNA, were observed (Figure 3b, ds1). A significant difference in the magnitude of the two signals is observed. In connection to this it should be kept in mind that, in contrast to cyclic voltammetry, the amount of surface-confined redox active molecules in DPV cannot be directly estimated by integration of the peak areas. In first approximation[35] this amount is directly proportional to the peak currents and, inversely, to the square of the number of electrons n involved in the ET reaction. For the MB label n is two and for Fc it is one, and therefore, more pronounced peaks from the MB-labeled cDNA are consistent with smaller peaks from the Fc-labeled DNA and correspond to the same amount of DNA when divided by n^2.

For regeneration of the ssDNA electrode, we applied another strategy than the water denaturation described above. The MB−cDNA sequence is designed with a single-stranded toehold extending from the duplex in ds1, and this toehold hybridizes instantly with a 22′mer DNA sequence which was added to the solution. This sequence is fully complementary to the MB−cDNA conjugate (T_m 71.9 °C). The 15-mer hybrid of TzFc−DNA and cDNA was therefore outcompeted, and the 22-mer duplex formed in solution was washed away from the electrode. After this procedure, only the Fc signal from the DNA grafted onto the electrode was observed in DPVs recorded with the DNA-modified electrodes (Figure 3b, ss2). This hybridization and subsequent competitive removal of MB−cDNA was repeated on the same electrode, giving the expected reappearance and disappearance of the MB signal (Figure 2b, ds2 and ss3). As observed for hybridization of the immobilized DNA to a nonlabeled DNA sequence (Figure 2), the hybridization to the MB-labeled cDNA also induced cathodic shift in the Fc-redox potential. In addition, a decrease in the magnitude of the Fc signal is also observed, and we assume that this is caused by shielding of the Fc moiety by the MB group.

Conclusion

We have synthesized a new triazene−ferrocene−NHS ester **1** which is conjugated to amino-modified DNA and applied for one-step immobilization of DNA on electrodes. Furthermore, the ferrocene redox label is used to determine the density and stability of the immobilized layer by the total charge of the redox peaks and to detect hybridization to cDNA by a shift in the redox potential. Remarkably close to compact DNA monolayer surface coverages of 29 ± 4 and 31 ± 9 pmol cm^{-2} were observed for immobilization on GC and gold electrodes, respectively. The k_s determined from the cathodic and anodic peak separation was close to 1 s^{-1} for gold and 0.28 ± 0.05 s^{-1} for the electrochemically slower carbon electrodes. The grafted DNA retained its ability for hybridization, which could be detected by a cathodic shift in the Fc label redox potential for both electrodes in the presence of a cDNA sequences. The formed C−C bond linking DNA to the GC surface was more stable than the commonly utilized chemisorption approach using thiol-modified DNA on gold electrodes. Denaturation at 50 °C water did not diminish the Fc signal and thereby the electrode surface coverage. Further proof for the stability of and accessibility to the grafted DNA surfaces was made by hybridizing with a MB-labeled complementary probe whereupon signals from both redox probes could be observed. During two cycles of hybridization and dehybridization, the initial Fc signal was restored.

By the application of this modifier we have demonstrated that the aryltriazene is a useful functionality for introduction of an aryldiazonium precursor in DNA. By tuning the conditions for release of the free diazonium salt with the electrochemical grafting a dense monolayer of DNA, which is fully capable for hybridization, could be immobilized on GC and gold electrodes. The increased stability of the layers immobilized on GC renders this an attractive alternative to the thiol−gold immobilization protocol. The triazene modifier may also be used for grafting DNA and also other biomolecules to other surfaces and materials, such as graphite, carbon nanotubes, and graphene.

Experimental Section

General Procedures, Instrumentation, and Materials. All chemicals used for organic synthesis, buffer solutions, and Me_2SO_4 were purchased from commercial suppliers. Flash chromatography was performed using silica gel 60 (230−400 mesh). All solvents were HPLC grade; THF was distilled from Na and Et_3N from CaH_2. NMR spectra were recorded at 400 MHz (^{1}H NMR) or 100 MHz (^{13}C NMR) and calibrated to the residual solvent peak. RP-HPLC was performed on a system with a fraction collector and UV detection at 260 nm, fitted with a column (3 vm, 50 × 4.6 mm). Solvents were MeCN in 0.1 M triethylammonium acetate (TEAA). Concentration determinations of oligonucleotides were performed by UV−vis spectroscopy. MALDI spectra were recorded on a MALDI-TOF spectrometer.

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were done using a three-electrode potentiostat equipped with GPES 4.9.006 software. Analysis of the voltammetric data and polynomial background correction was performed using GPES software facilities. An Ag/AgCl, 3 M KCl electrode was used as reference and a Pt wire as the auxiliary electrode.

Gold disk working electrodes (diameter 2 mm) were mechanically polished to a mirror luster stepwise in 1 μm diamond and in 0.1 μm alumina slurries on microcloth, ultrasonicated in ethanol/water bath for 10 min, electrochemically polished by cycling in 1 M H_2SO_4, as described elsewhere,[35,36] washed with water, and further kept in absolute ethanol for 30 min. The disk glassy carbon (GC) working electrodes (diameter 0.3 mm) were pretreated similarly to gold electrodes: mechanically polished, ultrasonicated, washed with water, and further kept in absolute ethanol for 15 min.

The gold electrode surface area was determined from the surface oxide reduction peak in 1 M H_2SO_4.[37,38] Surface roughness was found to be around 2.5. Before modification, a Teflon cap was placed on the top part of the electrode, thus forming a 5-μL volume well-like microcell, with the bottom representing

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the electrode surface with a diameter of 2 mm and a well wall height of 1.6 mm.[35]

The electrolyte was an aqueous 20 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl (PBS). The reproducibility of the measurements was verified with at least three equivalently prepared electrodes. All nucleotides were synthesized by standard automated oligonucleotide synthesis by DNA Technology A/S (Risskov, Denmark). Millipore water (18.2 MΩ) was used throughout the work.

DNA Sequences. Grafting of DNA to gold and GC electrodes and hybridization: 5′-TGG TAC GTTA-$(CH_2)_6$-NH_2-3′ (TzFc modified) and 5′-TAA CGT ACC A-3′ (cDNA).

Grafting of DNA to GC, hybridization with MB-labeled DNA and strand exchange: 5′-H_2N-$(CH_2)_6$-TAC GTG AAC CTA CTG-3′ (TzFc modified), 5′-TCA GCA TCA GTA GGT TCA CGT A-$(CH_2)_6$-NH_2-3′ (MB modified), and 5′-TAC GTG AAC CTA CTG ATG CTG A-3′ (cDNA).

Methyl Ferrocenecarboxylate (2). To ferrocenecarboxylic acid (4.00 g, 17.4 mmol) in MeOH (120 mL) was added $BF_3 \cdot Et_2O$ (12.3 mL) and the mixture refluxed overnight. A 5% $NaHCO_3$ solution was added to adjust to pH 8, and the reaction mixture was extracted with CH_2Cl_2 (3 × 50 mL). The organic phases were washed with brine and dried over $MgSO_4$. The solvent was evaporated in vacuo to yield dark orange crystals (4.12 g, 16.96 mol, 98%). Analytical data were in accordance with literature data:[27] ^{13}C NMR ($CDCl_3$) δ (ppm) 188.9, 172.3, 71.4, 71.1, 70.2, 69.8, 51.7; ESI-TOF high-acc (m/z) calcd for $C_{12}H_{12}FeO_2$ MS ($[M + Na]^+$) 267.0084, found 267.0084.

Methoxycarbonyl-1-ferrocenesuccinic Acid (3). Methyl ferrocenecarboxylate **2** (4.12 g, 16.96 mmol) and succinic anhydride (3.70 g, 36.93 mmol) dissolved in CH_2Cl_2 (28 mL) were added dropwise to a solution of $AlCl_3$ (10.61 g, 79.58 mmol) in CH_2Cl_2 (28 mL). The reaction was stirred for 2 h at rt and poured onto ice (150 mL), and the organic phase was removed. The aqueous layer was acidified with concd HCl, and orange crystals were collected by filtration (2.28 g, 6.64 mmol, 40%). Analytical data were in accordance with literature data:[27] ^{1}H NMR (CH_3OD) δ (ppm) 4.91 (s, 4H), 4.87 (t, J = 1.8 Hz, 4H), 4.60 (t, J = 1.8 Hz, 2H), 4.57 (t, J = 1.8 Hz, 2H), 3.81 (s, 3H), 3.09 (t, J = 6.8 Hz, 2H), 2.65 (t, J = 6.8 Hz, 2H); ^{13}C NMR (CH_3OD) δ (ppm) 204.3, 176.5, 172.7, 80.9, 74.9, 74.3, 74.0, 72.8, 71.8, 52.3, 35.5, 28.5; ESI-TOF high-acc (m/z) calcd for $C_{16}H_{16}FeO_5$ ($[M + Na]^+$) 367.0245, found 367.0255.

1′-Methoxycarbonyl-1-ferrocenebutyric Acid (4). To a stirred mixture of zinc dust (3 g, 45.9 mmol) and $HgCl_2$ (0.25 g, 0.92 mmol) was added H_2O followed by concd HCl (0.2 mL) which was added dropwise. The solution was stirred for 10 min at rt before it was filtered and the precipitate (Zn−Hg) washed carefully with MeOH.

The Zn−Hg (2.4 g) was added in small portions to a stirred solution of **3** (0.5 g, 1.45 mmol) in AcOH (6.5 mL) and concd HCl (9.9 mL) over a period of 20 min. The slurry was stirred for 1 h at rt, H_2O (100 mL) was added, and the remaining Zn−Hg was removed by filtration. The solution was extracted with CH_2Cl_2 (3 × 50 mL) and washed, and the combined organic phases were washed with brine and evaporated in vacuo to yield an orange oil (466 mg, 1.41 mmol, 97%). Known compound:[27] ^{1}H NMR (CH_3OD) δ (ppm) 4.72 (t, J = 2.0 Hz, 2H), 4.43 (t, J = 2.0 Hz, 2H), 4.12 (s, 4H), 3.79 (s, 3H), 2.31 (t, J = 7.6 Hz, 2H), 2.29 (t, J = 7.6 Hz, 2H) 1.76 (k, J = 7.6 Hz, 2H); ^{13}C NMR (CH_3OD) δ (ppm) 177.2, 174.1, 91.2, 73.2, 72.4, 71.7, 70.9, 70.2, 52.1, 34.4, 28.8, 27.3; ESI-TOF high-acc (m/z) calcd for $C_{16}H_{18}FeO_4$ ($[M + Na]^+$) 353.0452, found 353.0463.

Benzyl 4-Aminophenethylcarbamate (5). To a solution of 4-(2-aminoethyl)aniline (1 g, 7.34 mmol) in THF (30 mL) and NaOH (1 M, 7.3 mL) was added benzyl chloroformate (1.03 mL, 7.35 mmol) in THF (10 mL) dropwise at 0 °C. The solution was stirred overnight at rt and the organic solvent evaporated in vacuo. The aqueous phase was extracted with EtOAc (3 × 15 mL) and washed with brine and the product purified by flash chromatography (CH_2Cl_2/EtOAc 2:1) as white crystals (1.44 g, 5.66 mmol, 77%): ^{1}H NMR (CH_3OD) δ (ppm) 7.31 (m, 5H), 6.94 (d, J = 8.4 Hz, 2H), 6.66 (d, J = 8.4 Hz, 2H), 5.04 (s, 2H), 3.25 (t, J = 7.4 Hz, 2H), 2.64 (t, J = 7.4 Hz, 2H); ^{13}C NMR (CH_3OD) δ (ppm) 158.7, 146.7, 138.4, 130.4, 130.1, 129.4, 128.9, 128.7, 116.9, 67.2, 43.8, 36.3; ESI-TOF high-acc (m/z) calcd for $C_{16}H_{18}N_2O_2$ ($[M + Na]^+$) 293.1266, found 293.1264; mp 81.1−81.5 °C.

Benzyl 4-(3,3-Dimethyltriaz-1-enyl)phenethylcarbamate (6). Benzyl 4-aminophenethylcarbamate (2.06 g, 8.09 mmol) was dissolved in THF (17 mL), H_2O (12 mL), MeCN (8.5 mL), and concd HCl (4.1 mL) and cooled to 0 °C. A solution of sodium nitrite (0.74 g, 10.76 mmol) in THF (2.5 mL) and H_2O (2.5 mL) was added slowly. The mixture was then added dropwise to a 40% aqueous solution of dimethylamine (22 mL) under vigorous stirring and immediately extracted with CH_2Cl_2 (3 × 15 mL). The combined organic phases were washed with H_2O and dried over Na_2SO_4, and the product was purified by flash chromatography (EtOAc/CH_2Cl_2 1:24) as light yellow crystals (2.22 g, 6.82 mmol, 84%): ^{1}H NMR ($CDCl_3$) δ (ppm) 7.35 (m, 7H), 7.14 (d, J = 8.4 Hz, 2H), 5.10 (s, 2H), 4.81 (bs, 1H), 3.45 (t, J = 6.8 Hz, 2H), 3.33 (bs, 6H), 2.80 (t, J = 6.8 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm) 156.4, 149.7, 136.7, 135.8, 129.6, 128.6, 128.1, 120.8, 66.7, 42.3, 35.7; ESI-TOF high-acc (m/z) calcd for $C_{18}H_{22}N_4O_2$ ($[M + Na]^+$) 349.1640, found 349.1639; mp 56.8−57.4 °C.

[4-(2-Aminoethyl)phenyl](3,3-dimethyl)triazene (7). Compound **6** (807 mg, 2.47 mmol) was dissolved in MeOH (10 mL) and 10% Pd/C (81 mg) was added. The suspension was stirred 1 h under hydrogen (1 atm) at rt and filtered through Celite and the solvent evaporated in vacuo to give the product as an orange oil (452 mg, 2.35 mmol, 95%): ^{1}H NMR ($CDCl_3$) δ (ppm) 7.35 (d, J = 8.4 Hz, 2H), 7.15 (d, J = 8.4 Hz, 2H), 3.31 (bs, 6H) 2.94 (t, J = 6.8 Hz, 2H), 2.64 (t, J = 6.8 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm) 150.7, 138.0, 130.2, 121.5, 44.2, 39.7; ESI-TOF high-acc (m/z) calcd for $C_{10}H_{16}N_4$ ($[M + H]^+$) 193.1453, found 193.1457.

Methyl 1′-[*N*-(4-(3,3-Dimethyltriaz-1-enyl)phenethyl)butylamide]ferrocene-1-carboxylate (8). 1′-Methoxycarbonyl-1-ferrocenesuccinate (236 mg, 0.71 mmol) and **7** (137 mg, 0.71 mmol) were suspended in dry DMF (7.5 mL) and Et_3N (0.8 mL). HBTU (271 mg, 0.71 mmol) was added and the mixture stirred for 100 min at rt. After addition of H_2O, the solution was extracted with EtOAc (3 × 20 mL). The product was purified by flash chromatography (EtOAc/CH_2Cl_2 1:2) as an orange oil (318 mg, 0.63 mmol, 89%): ^{1}H NMR ($CDCl_3$) δ (ppm) 7.37 (d, J = 8.4 Hz, 2H) 7.15 (d, J = 8.4 Hz, 2H), 5.49 (bs, 1H), 4.70 (s, 2H), 4.33 (s, 2H), 4.07 (s, 2H), 4.04 (s, 2H), 3.78 (s, 3H), 3.51 (q, J = 6.6 Hz. 2H), 3.33 (bs, 6H), 2.80 (t, J = 6.6 Hz, 2H), 2.25 (t, J = 7.6 Hz, 2H), 2.10 (t, J = 7.6 Hz, 2H), 1.77 (k, J = 7.6 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm) 172.7, 172.1, 149.7, 135.9, 129.3, 120.8, 89.9, 71.9, 70.7, 69.8, 69.1, 51.6, 40.6, 36.3, 35.2, 28.0, 26.8; ESI-TOF high-acc (m/z) calcd for $C_{26}H_{32}FeN_4O_3$ ($[M + Na]^+$) 527.1722, found 527.1722.

1′-[*N*-(4-(3,3-Dimethyltriaz-1-enyl)phenethyl)butylamide]-ferrocene-1-carboxylic Acid (9). LiOH (90 mg, 3.76 mmol) was added to a solution of **8** (77 mg, 0.15 mmol) in THF (6 mL) and water (0.5 mL). The mixture was refluxed for 96 h before the THF was evaporated in vacuo. AcOH (20%) was added dropwise until the product precipitated (pH 6−7), and the aqueous solution was extracted with EtOAc (3 × 10 mL) and washed with brine. The product was immediately purified by flash chromatography (EtOAc → EtOAc/MeOH 1:1) as an unstable orange oil (61 mg, 0.12 mmol, 82%). It was not possible to obtain pure spectroscopic data, and the compound should be used immediately in the proceeding reaction step due to degradation.

Succinimidyl 1′-[*N*-(4-(3,3-Dimethyltriaz-1-enyl)phenethyl)-butylamide]ferrocene-1-carboxylate (1, TzTcNHS). EDC (110 mg, 0.57 mmol) was added to a solution of **9** (134 mg, 0.27 mmol) and NHS (65 mg, 0.56 mmol) in CH_2Cl_2 (8 mL), and the reaction was stirred for 1.5 h at rt. The solution was then washed with water and brine. The product was purified by flash chromatography (EtOAc/CH_2Cl_2 1:1) as a red brown oil (93 mg, 0.16 mmol, 58%): ^{1}H NMR ($CDCl_3$) δ (ppm) 7.36 (d, J = 7.6 Hz, 2H), 7.15 (d, J = 7.6 Hz, 2H), 5.54 (bs, 1H), 4.82 (s, 2H), 4.51 (s, 2H), 4.26 (s, 2H), 4.24 (s, 2H), 3.50 (q, J = 6.2 Hz, 2H), 3.33 (bs, 6H), 2.81 (m, 6H), 2.33 (t, J = 7.2 Hz, 2H), 2.11 (t, J = 7.2 Hz, 2H), 1.79 (k, J = 7.2 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ (ppm) 172.8, 169.7, 167.5, 149.7, 136.0, 129.3, 120.8, 91.0, 73.6, 71.5, 71.0, 70.2, 64.6, 40.7, 36.2, 35.3, 28.1, 26.8, 25.8; ESI-TOF high-acc (m/z) calcd for $C_{29}H_{33}FeN_5O_5$ ([M + Na]$^+$) 610.1729, found 610.1724.

Triazene−Ferrocene−DNA Conjugate (10-mer). 3′-Amino-modified DNA (3 nmol) was evaporated to dryness and the pellet dissolved in 133 mM EPPS buffer pH 8.8 (7.5 μL) containing 33% DMF. A solution of 100 mM **9** in DMF (2.5 μL) was added and the reaction incubated overnight at 45 °C. The product was purified by RP HPLC (TEAA pH 8.5): (0−40%; 20 min) rt, 17.1 min; yield 995 pmol, 31%; ESI MS (m/z) calcd for ([M + Na]$^+$) 3708.6, found 3709.1.

Triazene−Ferrocene−DNA Conjugate (15-mer). 5′-Amino-modified DNA (6 nmol) was evaporated to dryness and the pellet dissolved in 133 mM EPPS buffer pH 8.8 (7.5 μL) containing 33% DMF. A solution of 100 mM **9** in DMF (2.5 μL) was added and the reaction incubated overnight at 45 °C. The product was purified by RP HPLC (TEAA pH 8.5): (0−40%; 20 min) rt, 16.8 min; yield 2578 pmol, 43%; ESI MS (m/z) calcd for ([M + Na]$^+$) 5203.4, found 5203.4.

Methylene Blue−DNA Conjugate. A solution containing 3′-amino-modified DNA (105 nmol) and methylene blue NHS ester (1.8 mg, 2.8 μmol) in 0.75 M sodium tetraborate buffer pH 8.5 with 14% DMSO was incubated at rt for 20 h.

After lyophilization the pellet was dissolved in H_2O and purified by RP HPLC (TEAA pH 7.0): (0−40%, 20 min) rt, 14.17 min; yield 49 nmol, 47%; MALDI MS TOF ([M + Na]$^+$) calcd 7333, found 7332.

Electrode Modification with the Triazene−Ferrocene−DNA Conjugate. In order to measure the combined redox signal from ferrocene and methylene blue, a two-step activation process for the GC surface was performed before the grafting step, according to methodology described elsewhere.[38] In brief, it was an electrochemical activation in 1 M KOH by a potential step to 1.2 V over 5 min followed by electrode cycling in a blank solution (1 M $HClO_4$, 100 mVs^{-1}) 10 times between 0 and 1.4 V.

Modification of the gold electrodes with the TzFc−DNA conjugate was performed by grafting the conjugate under potentiostatic control for 20 min at −200 mV vs Ag/AgCl (3 M KCl) from a 20 μL drop of 10 μM TzFc−DNA conjugate solution in PBS (20 mM pH 7.0, 0.15 M NaCl), placed on the top of the gold electrode. Modification of the GC electrodes with the TzFc−DNA conjugate was performed by grafting for 20 min at −400 mV vs Ag/AgCl (3 M KCl) from a 20 μL drop of 15 μM DNA conjugate solution in PBS (20 mM pH 7.0, 0.15 M NaCl). The triazene functional group was activated by the addition of 1 μL of a 100 mM solution of Me_2SO_4 in 100 mM PBS directly to the DNA drop (5 mM Me_2SO_4). After electrodeposition, the electrodes were rinsed with water to remove any nonspecifically adsorbed conjugate species.

On-Surface Hybridization/Denaturation Assay. Twelve microliters of a 42 μM solution of cDNA in PBS containing 4.2 mM $MgCl_2$ was placed on top of the DNA-modified electrode. Hybridization was performed for 1 h at rt, after which the electrodes were washed with PBS. Denaturation was performed by placing the electrodes in 50 °C water for 5 min.

For measuring the combined redox signal from immobilized TzFc−DNA conjugate on the GC electrode surface and methylene blue, the hybridization process was performed by placing 12 μL of a 15.8 μM solution of MB-labeled DNA in PBS containing 5 mM $MgCl_2$ on the electrode. After 1 h of hybridization at room temperature, the electrode was carefully washed with PBS buffer, and DPV was measured. For dehybridization excess amounts (200 pmol) of the 22′mer complementary to the MB-labeled DNA in PBS containing 5 mM $MgCl_2$ was added and allowed to hybridize for 2 h before DPV was performed.

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Supporting Information Available: UV spectrum of the TzFc−DNA conjugate, cyclic voltammograms of functionalized gold electrodes, and ^{1}H, ^{13}C NMR, and MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.