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Unmediated by DNA Electron Transfer in Redox-Labeled DNA Duplexes End-Tethered to Gold Electrodes

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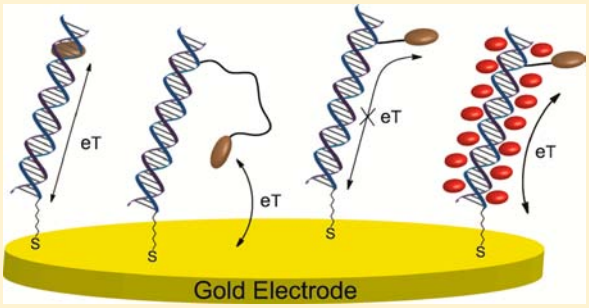
Supporting Information

ABSTRACT: Electron transfer (ET) between gold electrodes and redox-labeled DNA duplexes, immobilized onto the electrodes through the alkanethiol linker at the 3′-end and having internal either methylene blue (MB) or anthraquinone (AQ) redox labels, was shown to depend on the redox label charge and the way the redox label is linked to DNA. For loosely packed DNA monolayers, the conjugation of the positively charged MB to DNA through the long and flexible alkane linker provided ET whose kinetics was formally governed by the diffusion of the redox label to the negatively charged electrode surface. For the uncharged AQ label no ET signal was detected. The conjugation of AQ to DNA through the short and more conductive acetylene linker did not provide the anticipated DNA-mediated ET to the AQ-moiety: ET appeared to be low-efficient if any in the studied system, for which no intercalation of AQ within the DNA duplex occurred. The ET communication between the electrode and AQ, built in DNA through the acetylene linker, was achieved only when $Ru(NH_3)_6^{3+}$ molecules were electrostatically attached to the DNA duplex, thus forming the electronic wire. These results are of particular importance both for the fundamental understanding of the interfacial behavior of the redox labeled DNA on electrodes and for the design of biosensors exploiting a variation of ET properties of DNA in the course of hybridization.



INTRODUCTION

Nanotechnological applications of DNA as DNA-based molecular electronics[1−7] and electrochemical DNA biosensors[8−19] strongly depend on the electron transfer (ET) properties of the individual DNA molecules. At present, it is widely accepted that a π-stacked double-stranded (ds) DNA molecule is a one-dimensional electrical conductor that transports electrons with an efficiency comparable to that of conventional conducting polymers[20−24] and only 100 times less efficiently than a metal wire.[25] However, data on ET obtained along the dsDNA helix are often controversial, and debates on the exact mechanisms of DNA conductivity and particularly on the ET properties of dsDNA are ongoing.[26−31] It is still not completely clear how efficiently DNA can transport electrons over long distances, since large variations exist in the reported values for the ET distance-decay constant β, ranging between 1.4 and 0.64 $Å^{-1}$, characteristic of an ET medium comparable to proteins and saturated hydrocarbons,[27,28,32−34] and less than 0.2 $Å^{-1}$, which is more typical of the electronic wire.[26,35,36] On the basis of these data, it is difficult to unambiguously conclude what is the actual distance dependence of the ET in DNA and what are the precise conditions for the varying ET properties. It is clear that in many cases results dramatically depend on the techniques used for analysis, sample preparation, and on the whole structural design of the electron acceptor−DNA−electron donor systems.

That is particularly true for electrochemical studies of ET in redox-labeled DNA tethered to electrodes, when an electrochemical signal from the faradaic reaction between the electrode and the redox label conjugated to the essentially robust DNA duplex (the persistence length of around 50 nm[37]) is generally considered as the evidence of the electronic conductivity of the DNA double helix.[38−41] However, in contrast to the electrochemically unambigously demonstrated directional ET through the protein media[42−46] or through dsDNA with intercalated redox probes,[9,13,47−49] data on directional ET in redox-labeled DNA coming from different groups are inconsistent.[10,38−41,50−55]

In general, several cases of the redox probe−DNA interactions and the corresponding mechanisms of ET can be discussed (Figure 1): (a) intercalation of the redox probe in DNA enabling directional ET through the dsDNA strand;[9,13,47−49] (b) conjugation of the redox probe through the short linker, in some cases providing the ET response[40,41,56] and in other cases not;[54,56] (c) conjugation of the redox probe through the longer and more flexible alkanethiol linker, the results depending on the applied electrochemical conditions and demonstrating both the presence[38−40,55,57,58] and the absence of the faradaic signal from the redox probe;[10,50−53] and, finally, (d) intercalation of the redox probe, DNA-conjugated

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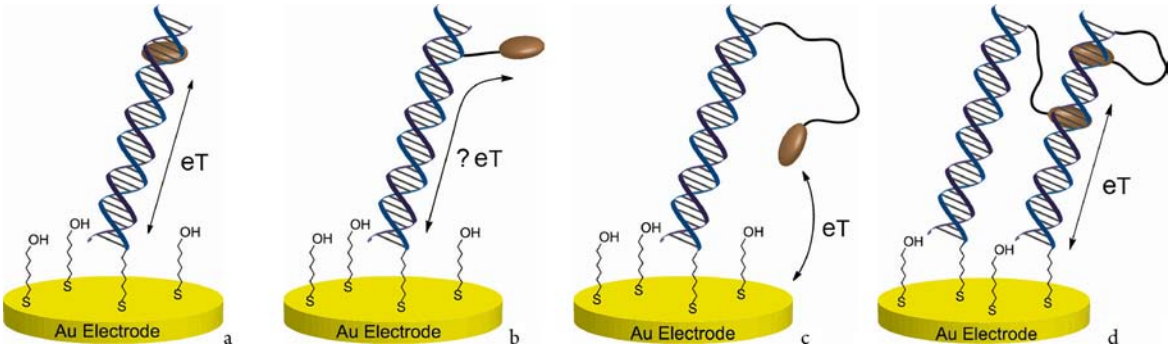

Figure 1. Schematic representation of the possible modes of the redox probe−DNA interaction and ET reactions proceeding in DNA tethered to the Au electrode through the alkanethiol linker at one end of the DNA sequence (see the text for details).

via the corresponding linker, into the DNA duplex, thus providing conditions for directional ET. In the latter case (d), intercalation is either experimentally proved[41,59] or its probability may be considered in some of the (b) and (c) cases. Namely, directional ET mediated by the DNA base-pair π-stack was observed in compact monolayers composed of dsDNA tethered to electrodes and modified by the Nile Blue- and pyrrolo-quinoline-quinone (PQQ)-redox probes,[38,40,41] which may also be discussed in terms of the redox probe−DNA π-stacking interactions.

In loosely packed DNA monolayers the situation appears to be quite ambiguous, and ET depends both on the electrochemical conditions and redox probes used. The loosely packed DNA monolayers are of particular importance for the development of electrochemical hybridization biosensors, since they provide favorable conditions with no steric hindrance for DNA on-surface hybridization.[60] Depending on the experimental conditions, for very similar DNA lengths, both the presence[39,51,53,55,56] and absence[10,50,52,54,61] of the faradaic signals from the DNA-conjugated redox probes were reported. Electrochemical analysis of the ET kinetics, performed by us[55] and others,[51,53,62] revealed that in the loosely packed DNA monolayers ET between the electrodes and redox probes conjugated to the surface-tethered dsDNA was governed by the motional movements of the probe versus the electrode surface. In other works, surface electrochemistry of the redox probes was shown.[39,56] These observed inconsistencies do not allow predictions of the system behavior, crucial to any biosensor design, and motivate us to scrutinize general ET concepts currently used in the interpretations of the electrochemical behavior of the redox-labeled dsDNA.

In the present work, we have studied the ET reactions proceeding in dsDNA tethered to the gold electrodes through the alkanethiol linker. DNAs were labeled with electrochemically active probes possessing redox potentials low enough to provide a "vertical" orientation of dsDNA on the negatively charged electrode surface. By these means the "horizontal" orientation of dsDNA, shown to orientate itself almost flat at positively polarized interfaces, was excluded.[63−65] We aimed at the analysis of the ET reactions proceeding in dsDNA−electrode systems similar to those presented in Figure 1b and c. Here, we studied ET in loosely packed DNA monolayers to avoid interactions between neighboring DNA molecules that may interfere with the diffusional movements of the redox probes or allow their possible intercalation in the neighboring DNA double strands. Along with that, the surface concentration of dsDNA was sufficient to generate electrochemical signals suitable for kinetic analysis. Two types of linkers, a short, conductive acetylene linker and a longer, less conductive alkane linker, and two types of redox probes, uncharged and positively charged, have been studied. Our hypothesis was that if the experimental conditions exclude the redox probe intercalation into dsDNA, then DNA-mediated ET should not be observed, and the faradaic signal from the redox probe is either absent or due to the movements of the redox probe toward the electrode surface. Kinetic studies should then enable an explanation and predication of the electrochemical response from the electrode-tethered DNA.

MATERIALS AND METHODS

Materials. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), hexaammine ruthenium(III) chloride, methylene blue (MB), anthraquinone (AQ), and all components of buffer solutions were purchased from Sigma-Aldrich. 6-Mercapto-1-hexanol (MC_6OH) was from Fluka. *N*-Hydroxysuccinimide ester (NHS) activated methylene blue (3,7-bis(*N*-(3-carboxypropyl)-*N*-methylamino)phenothiazin-5-ium perchlorate, succinimidyl ester, MB-NHS) and NHS activated anthraquinone (2-carboxylic acid succinimidyl ester, AQ-NHS) were from EMP Biotech GmbH, Berlin, Germany. DNAs were synthesized by conventional phosphoramidite chemistry. AQ-DNA, with AQ conjugated to the uracil base of the DNA strand at the X position through an acetylene linker[40] (Table 1, Figure 2a), was synthesized by Metabion International AG, Martinsried, Germany. Amino-modified and unmodified oligonucleotides for duplex formation were synthesized by DNA Technology A/S, Risskov, Denmark. The amino-modified oligonucleotides had an internal dC-conjugated C_6-amine modification (Link Technologies Ltd., Bellshill, Scotland, U.K.) at the X position (Table 1) and were used for the synthesis of AQ-C_6-DNA and MB-C_6-DNA (Figure 2b and c) and in the control experiments. All DNA probes had a C_6-disulfide ($-(CH_2)_6-S-S-(CH_2)_6-OH$) modification at the 3′-end. All solutions were prepared with Milli-Q water (18 MΩ, Millipore, Bedford, MA, U.S.A.).

Table 1. DNA Sequences Employed in the Present Work

DNA	DNA sequence
AQ-DNA with AQ conjugated to DNA through the acetylene linker in position X	5′-GTT GTG CAG XGC CTC ACA AC-3′
cDNA1 complementary to AQ-DNA	5′-GTT GTG AGG CAC TGC ACA AC-3′
AQ-C_6-DNA with AQ conjugated to DNA through the C6 alkane linker in position X	5′-GTT GTG CAG XGC CTC ACA AC-3′
MB-C_6-DNA with MB conjugated to DNA through the C6 alkane linker in position X	5′-GTT GTG CAG XGC CTC ACA AC-3′
cDNA2 complementary to AQ-C_6-DNA and MB-C_6-DNA	5′-GTT GTG AGG CGC TGC ACA AC-3′

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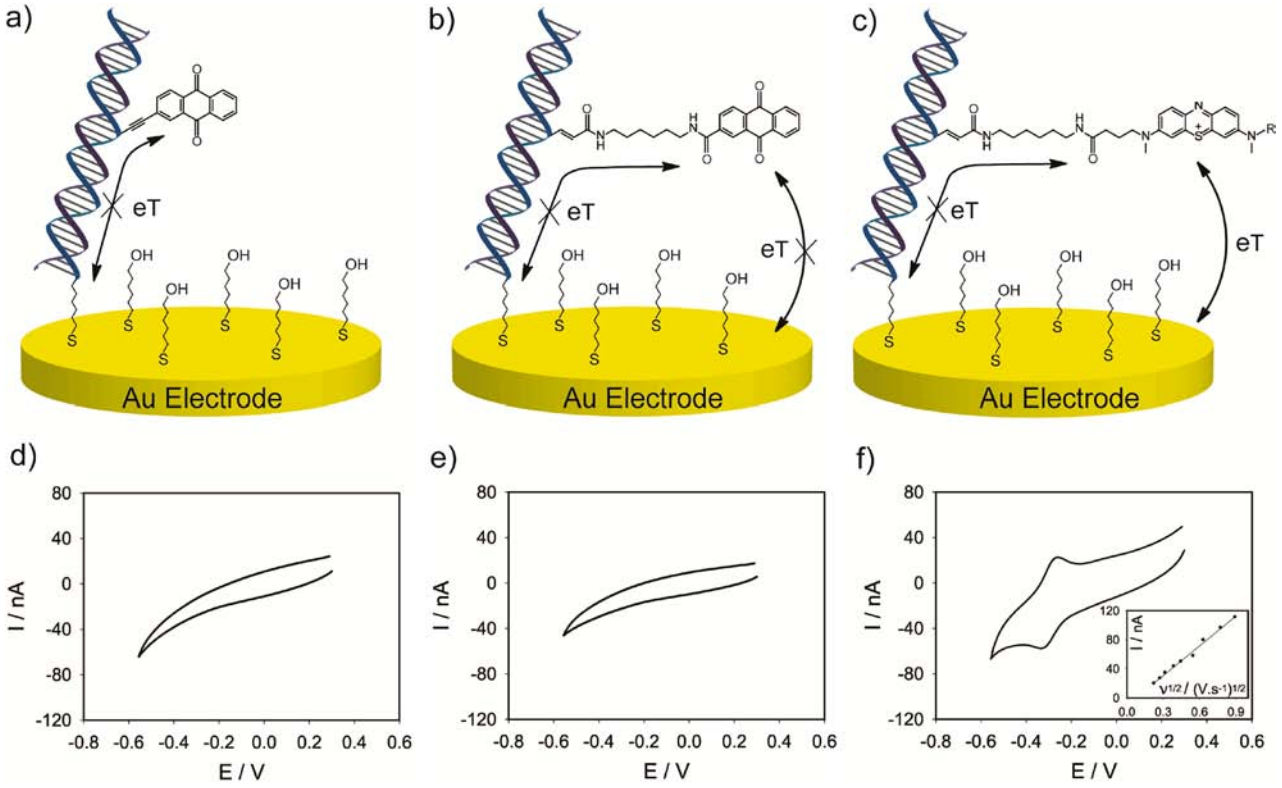

Figure 2. Schematic representation of ET reactions proceeding in (a) AQ-DNA, (b) AQ-C_6-DNA, and (c) MB-C_6-DNA tethered to the Au electrodes; and (d–f) representative CVs recorded with the corresponding electrode modifications in 10 mM PBS, potential scan rate 50 mV s^{-1}. (f) Inset: Dependence of the MB anodic peak current I on the square root of the potential scan rate $v^{1/2}$. The representative DNA surface coverage was 4.1, 4.3, and 4.1 pmol cm^{-2} for AQ-DNA-, AQ-C6-DNA-, and MB-C6-DNA-modified electrodes, respectively.

Melting temperatures T_m of the DNA duplexes, measured in 10 mM PBS, pH 7, using the Varian Cary 100 Bio spectrophotometer (Analytical Instruments AS, Værløse, Denmark), were 51.0 ± 0.8, 55.7 ± 1.3, and 55.5 ± 0.7 °C for AQ-DNA, MB-DNA, and unlabeled DNA, correspondingly (Figure 3S, SI).

AQ and MB Conjugation to Amino-Modified DNA. AQ and MB were conjugated to oligonucleotides with internal amine modifications by the reaction between the amine group of DNA and either AQ-NHS or MB-NHS, correspondingly, by a slightly modified protocol.[66] In brief, a solution containing amino-modified DNA (8 nmol) and AQ-NHS or MB-NHS (0.250 mg) in a 0.075 M MOPS buffer containing 14% dimethyl sulfoxide was incubated overnight at rt. After ethanol precipitation, the pellet was dissolved in 0.1 M triethylammonium acetate, filtered if necessary, and purified by reversed-phase HPLC.

Electrode Modification with DNA. Prior to modification, gold electrodes (CH Instruments, Austin, TX; diameter 2 mm) were mechanically polished on a microcloth pad using 1 μm diamond and 0.1 μm alumina slurries (both from Struers, Copenhagen, Denmark), washed with Milli-Q water and ultrasonicated in 1:1 ethanol–water solution for 10 min. The electrodes were then polished electrochemically in 1 M H_2SO_4 and1 M H_2SO_4/10 mM KCl.[67] Electrochemical surface area was estimated from the gold surface oxide reduction peaks in 0.1 M H_2SO_4.[68] Before immobilization of DNA, the electrodes were kept in absolute ethanol for 30 min. Prior to immobilization, disulfide bonds of the DNA probes were reduced by treatment with 10 mM TCEP for 1 h. Then 10 μL of a freshly prepared 10 μM DNA solution was incubated onto the surface of the gold electrode, under the lid, for 24–36 h, 4 °C, in the dark. After modification, the electrodes were carefully washed with a buffer solution (10 mM K_2HPO_4/KH_2PO_4 (PBS), 0.15 M NaCl, pH 7) and then immersed in 14 mM MC_6OH solution in the same buffer solution for 30 min, followed by thorough rinsing with 10 mM PBS, pH 7. DNA duplexes were prepared by 1 h hybridization of the electrode-tethered DNA with 15 μM cDNA in 10 mM PBS, pH 7.0, containing 0.15 M NaCl and 0.1 M $MgCl_2$. The DNA-modified electrodes were immediately used after their modification.

Instrumentation. All electrochemical measurements were performed in a conventional three-electrode cell using a μAutolab electrochemical system (Type III, Eco-Chemie B.V., Utrecht, The Netherlands) equipped with GPES (version 4.9.007) and NOVA (version 1.8.17) software. An Ag/AgCl (3 M KCl) and a Pt wire served as reference and auxiliary electrodes, respectively. Ten millimolar PBS was used as the supporting electrolyte if not stated otherwise. Working solutions were deaerated with N_2 for 30 min prior to data acquisition and kept under N_2 flow during the experiments. The DNA surface coverage (referred to the electrochemically determined electrode surface area) was estimated by cyclic voltammetry (CV) and by chronocoulometry in 10 mM PBS in the presence of 0.2 mM $Ru(NH_3)_6^{3+}$ (Figures 4S and 5S, SI), according to the protocol established by Steel et al.[69] In chronocoulometry, the potential was stepped from +0.1 to −0.4 V with a pulse period 0.5 s. In intercalation studies, dsDNA-modified electrodes (with no redox labels) were incubated in a 10 μM solution of MB in 10 mM PBS/0.15 M NaCl for 6–16 h, followed by washing in 10 mM PBS/0.15 M NaCl. Low solubility of AQ in water did not allow studies of its intercalation. To be consistent with the neutral charge on the AQ redox label used, we preferred not to use more soluble negatively charged AQ sulfonates[13,70,71] but restricted ourselves to MB studies. In square wave voltammetry (SWV) the square-wave amplitude was 25 mV, and the step potential was 1 mV. All experiments were carried out at 21 ± 1 °C and under conditions avoiding direct light (dark-cell experiments), with at least three equivalently prepared electrodes.

■ RESULTS AND DISCUSSION

Two DNA–electrode systems (b and c of Figure 1) were studied. The first system represented dsDNA with an AQ redox

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probe conjugated to DNA through the short and robust acetylene linker, originally designed to provide ET through dsDNA to the AQ moiety[40] (Figure 2a, AQ-DNA). The second system involved dsDNA with either AQ or MB conjugated to DNA through the commonly used flexible C_6-alkane spacer (b and c of Figure 2, AQ-C_6-DNA and MB-C_6-DNA, correspondingly). AQ and MB have essentially negative redox potentials $E^{o\prime}$, determined as −0.453 and −0.3 V, correspondingly. Thus, ET studies were performed under conditions where dsDNA molecules were orientated upright on the negatively charge electrode surface[63−65] and a direct ET contact between the redox probe and the electrode surface was unlikely. The charge of the used AQ probe was neutral, while MB was positively charged, which enabled us to study the effect of the probe charge on the ET reaction. Otherwise, the nucleotide (nt) composition of DNA was almost the same (Table 1).[72] The redox probes were placed in the internal 11th (from the electrode surface) nt position within the DNA duplex, to minimize the distance between the redox probe and electrode and, along with that, to ensure the dsDNA integrity in low ionic strength solutions (T_m > 50 °C). DNAs were immobilized at Au electrodes through the alkanethiol linker under conditions avoiding the redox probe intercalation into neighboring DNA duplexes (ssDNA immobilization). The DNA surface coverage determined by chronocoulometry[69] in the presence of $Ru(NH_3)_6^{3+}$ was 4.3 ± 0.5, 5.1 ± 0.8, 4.7 ± 0.6, and 4.6 ± 0.8 pmol cm^{-2} for the AQ-DNA, AQ-C_6-DNA, and MB-C_6-DNA and unlabeled DNA-modified electrodes, respectively (SI). These surface coverages are less than 40−50% of the limiting values[38,73,74] and are consistent with the loosely packed dsDNA monolayers with presumably noninteracting individual DNA molecules free-standing on the electrode surface.

Electrochemistry of AQ-DNA: AQ Conjugation through the Acetylene Linker. For AQ-DNA, a pronounced electrochemical signal resulting from directional ET through the DNA duplex and further to AQ via the conductive acetylene linker had been expected according to the literature.[40] CVs recorded with the AQ-DNA-modified electrodes showed no faradaic signal at potentials of the AQ redox probe (Figure 2a,d), consistent with ET between the electrode and AQ of a low efficiency (if any). Apparently similar results were obtained with unlabeled DNA (Figure 6S, SI).

In an earlier work,[40] immobilization of AQ-DNA was performed from dsDNA solutions and under conditions providing the compact monolayer formation. Such immobilization allows an AQ intercalation into the neighboring DNA duplexes (Figure 1d), and as a result, a corresponding ET signal from AQ-DNA was observed in that work.[40] Along with that, the dsDNA surface coverage, estimated from the AQ signal,[40] was at least 6 times lower than the surface coverage of dsDNA with covalently attached intercalated redox probes, estimated by the same group under similar conditions of DNA immobilization.[47] In this connection, the lower than expected AQ signal may be connected to partial AQ intercalation (not all DNA-conjugated AQ molecules may be intercalated in the neighboring duplexes). However, no independent estimate of the DNA surface coverage (e.g., with $Ru(NH_3)_6^{3+}$)[69] was reported. In the present work, special care was taken to avoid interactions between the neighboring dsDNA molecules. On the basis of our results, we can conclude that the electronic coupling between AQ, conjugated to DNA through the acetylene linker, and the DNA base-pair π-stack is insufficient to achieve ET between the electrode and AQ redox probe. A conjugation mode that allows AQ intercalation into the duplex[59] or simple intercalation of the AQ redox indicator[13,71] is required for the DNA-mediated ET to occur.

Electrochemistry of AQ-C_6-DNA and MB-C_6-DNA: Redox Probe Conjugation through the C_6-Alkane Linker. Pronounced faradaic signals both from ferrocene (Fc) and MB, conjugated to the electrode-tethered dsDNA through alkane linkers, have been reported numerous times.[39,50,51,53,55,58] These two redox probes provide essentially different conditions for ET reactions in redox-labeled DNA. An analysis of the ET kinetics in Fc-labeled dsDNA is complicated by the potential-induced attractive interactions between the positively charged electrode surface and negatively charged DNA[63−65] and depends to a large extent on such experimental conditions as potential scan rate (i.e., on the duration of the applied electric field) orientating dsDNA flat onto the electrode surface.[50,51,53]

For AQ- or MB-labeled DNA the situation is different, since DNA double strands stand upright on the negatively charged electrode surface, and thus the intimate interactions between dsDNA and the electrode may be avoided. The ET kinetics in MB-labeled dsDNA was then shown to be governed by the diffusion of the MB redox probe toward the electrode surface.[55] Our original hypothesis[55] was that those diffusional movements are induced by the applied electric field that pulls bulky and hydrophobic MB molecules bound to the alkanethiol linker at the electrode/solution interface closer to the electrode surface.[75,76] The charge of the organic redox label might also play a particular role in such movements. Here, we studied both the effect of the linker and the probe charge on the ET efficiency. No faradaic signal from AQ-C6-DNA was detected, similar to the experiments with AQ-DNA and unlabeled DNA (Figure 2b,e). In contrast to AQ-DNA (Figure 2a,d) and AQ-C_6-DNA (Figure 2b,e), a couple of redox peaks was seen in CVs recorded with MB-C_6-DNA-modified electrodes (Figure 2c,f, and Figure 7S [SI]). The ET process was limited by the diffusion of the MB redox probe to the electrode surface (a square root dependence of the peak currents on the potential scan rate,[77] Figure 2f, inset), consistent with our previous results.[55] In contrast to these data, the electrochemical signal from MB intercalated into unlabeled dsDNA showed a linear dependence on the potential scan rate (Figure 8S, SI), characteristic of a surface process,[77] with a heterogeneous ET rate constant k_s[78] of 1.9 ± 0.2 s^{-1} ($2e^-$ ET, SI).

Thus, in the absence of the redox probe intercalation into the DNA duplex and at negative electrode potentials, preventing the "flat" orientation of dsDNA on the electrode surface (i.e., excluding a direct contact between the redox probe and the electrode), no faradaic signals could be detected from the AQ redox probe, conjugated to dsDNA neither through the short and conductive acetylene linker nor through the more flexible and longer alkane linker. For the uncharged AQ label, the anticipated contribution of the redox probe diffusion to the overall ET reaction[55] appeared to be negligible, in contrast to the positively charged MB probe. The ET kinetics in the redox-labeled dsDNA thus depends significantly on the redox probe charge and its compatibility with the charge of the electrode.

Electrochemistry of Redox-Labeled DNA Mediated by the $Ru(NH_3)_6^{3+/2+}$ Wire. The electronic communication between the electrode and AQ attached to DNA through the acetylene linker was established via cationic $Ru(NH_3)_6^{3+}$ redox species that can electrostatically interact with the polyanionic sugar−phosphate backbone of DNA[69] (Figure 3a). $Ru(NH_3)_6^{3+}$

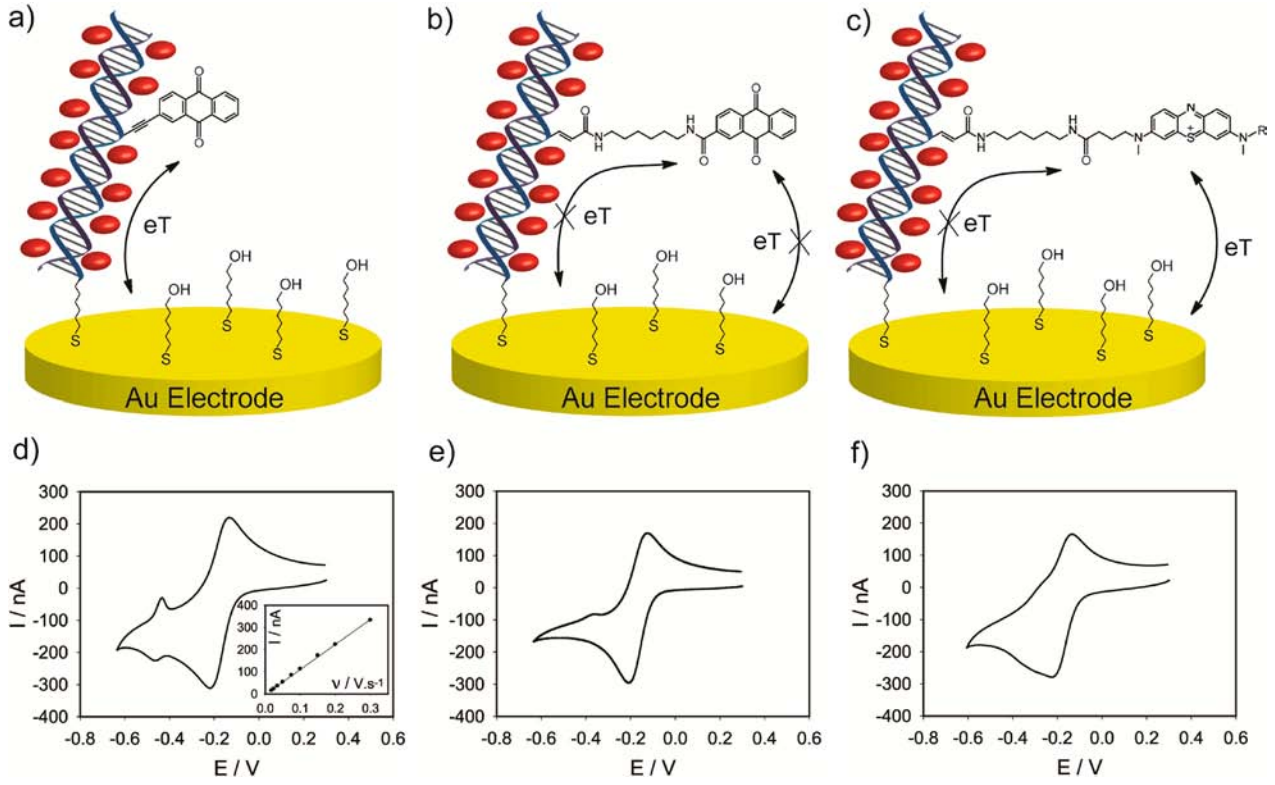

Figure 3. Schematic representation of ET reactions proceeding in (a) AQ-DNA (b) AQ-C_6-DNA, and (c) MB-C_6-DNA tethered to the Au electrodes, and (d–f) representative CVs recorded with the corresponding electrodes in 10 mM PBS, 50 μM $Ru(NH_3)_6^{3+}$. Deaerated solutions, potential scan rate 50 mV s^{-1}. (d) Inset: the dependence of the AQ anodic peak currents I on the potential scan rate v. Representative surface coverage estimated by chronocoulometry was 4.1, 4.3, and 4.1 pmol cm^{-2} for AQ-DNA, AQ-C_6-DNA, and MB-C_6-DNA, respectively.

produced an "electrographic" effect on the AQ redox process, similar to that observed in the silver-based photography in the presence of hydroquinone: A distinct couple of redox peaks (a mean potential of −0.455 V) consistent with the AQ redox chemistry was detected in low ionic strength solutions, when the $Ru(NH_3)_6^{3+}$ molecules were electrostatically attracted to the DNA duplex, thus forming the electronic wire mediating ET between the electrode and AQ (Figure 3d). The linear dependence of the AQ peak currents on the scan rate designated a surface-confined ET process[77] (Figure 3d, inset, Figure 9S, SI). The AQ-DNA surface coverage obtained by the integration of the AQ peak areas in CV (Figure 3d) was 4.1 ± 0.8 pmol cm^{-2}, consistently with the surface coverage estimated by chronocoulometry according to Steel et al.[69]

The ET rate constant k_s for heterogeneous ET between the electrode and AQ through the $Ru(NH_3)_6^{3+}$ bridge, estimated by the Laviron approach,[78] was 1.3 ± 0.3 s^{-1}. This k_s value is far below the ET rate constants of 10^5 - 10^{10} s^{-1} reported for the photoinduced ET between two DNA-intercalated compounds separated by similar or larger ET distances,[26,31,32,79,80] but in good agreement with 1.5 s^{-1} reported for the DNA-mediated ET in PQQ-labeled 12-base pair DNA duplexes.[38] These moderate ET rates may be limited by ET through the alkane linker tethering the DNA duplex to the electrode surface.[47] Along with that, more complex mechanisms (such as ECE) of the redox probe transformation (a $2e^-/2H^+$ electrochemical reaction versus a $1e^-$ photoinduced ET) may also contribute to the lower k_s values. The limiting step of the ET reaction was estimated by SWV enabling analysis of ET reactions of weakly bound species.[81] The SWV analysis of ET rates was performed within the Komorsky–Lovrić – Lovrić formal approach[82] by recording the AQ and $Ru(NH_3)_6^{3+/2+}$ peak currents at various frequencies (Figures 9S and 10S, SI). The resulting $I_p/f - \log f$ plots (here, I_p is the SWV peak current, measured at specific frequency f) were of a typical bell-shaped dependence (Figure 4), with a maximum at a frequency, which can be directly

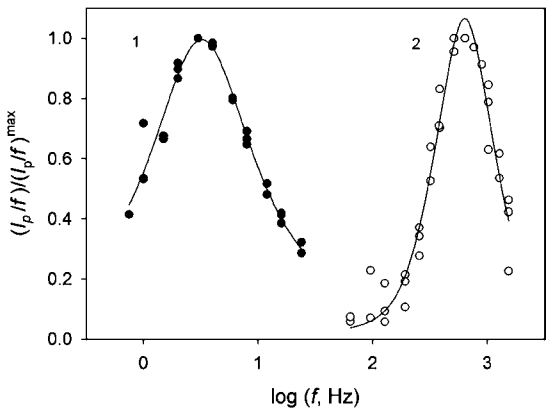

Figure 4. Dependence of the normalized relation between the SWV peak currents and the frequency at which the SWV has been recorded on the logarithmic frequency for (1) AQ peak recorded with the AQ-dsDNA-modified electrodes in 10 mM PBS, 50 μM $Ru(NH_3)_6^{3+}$; (2) Ru peak recorded with the dsDNA-modified electrodes, incubated for 5 min in 10 mM PBS containing 300 μM $Ru(NH_3)_6^{3+}$ and then after washing in 10 mM PBS transferred to blank 10 mM PBS. Amplitude E_{sw} is 25 mV. For a comparative analysis, the dependence of I_p/f on the log f is replaced by the normalized $(I_p/f)/(I_p/f)^{max} - \log f$ dependence, where $(I_p/f)^{max}$ is the parameter magnitude at the maximum of the dependence, i.e. at a critical frequency f^{max}.

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related to the value of the ET rate constant:[82] $k_s = \kappa_{max} \times f_{max}$, where κ_{max} is a critical kinetic parameter that depends on the transfer coefficient α and the product of nE_{sw} (E_{sw} is the square-wave amplitude). The k_s values estimated for the AQ ET reaction (2.4 ± 0.2 s^{-1}, $\alpha = 0.5$, $n = 2$) were similar to those obtained by CV and far below the k_s estimated for the ET reaction of electrostatically adsorbed $Ru(NH_3)_6^{3+}$ (659 ± 102 s^{-1}, $\alpha = 0.5$, $n = 1$). Thus, a redox transformation of AQ (neither ET through the alkanethiol tether or $Ru(NH_3)_6^{3+/2+}$ wire) can be suggested as a kinetically limiting step of the overall ET reaction.

To prove that electrons are eventually shuttled from the electrode to the AQ moiety along the ET bridge formed by $Ru(NH_3)_6^{3+}$ electrostatically attracted to the sugar−phosphate backbone of DNA, the AQ-DNA-modified electrode was incubated in the $Ru(NH_3)_6^{3+}$ solution and further transferred to the $Ru(NH_3)_6^{3+}$-free solution. The produced effect was similar to that observed in the presence of $Ru(NH_3)_6^{3+}$, though the AQ signal (as well as that from $Ru(NH_3)_6^{3+/2+}$) consequently degraded with scanning, due to the gradual desorption of $Ru(NH_3)_6^{3+}$ from the DNA surface (Figure 10S, SI). Along with that, the AQ signal significantly decreased in high ionic strength solutions, where electrostatically attached $Ru(NH_3)_6^{3+}$ molecules were replaced from the DNA surface by the electrochemically inactive Na^+ ions, present in a much higher concentration and incapable of mediating ET between the electrode and AQ (Figure 11S, SI). In the control experiments with unlabeled DNA and intercalated MB, no enhancement of the signal from intercalated MB in the presence of the $Ru(NH_3)_6^{3+}$ wire attached to the phosphate backbone of DNA was detected (Figure 13S, SI). In contrast to that, when ET mediating redox species are not forming wire along the sugar−phosphate backbone of DNA, the catalytic amplification of the redox signal from the intercalator mediated by the soluble redox indicators may be observed.[9,83,84]

When the AQ probe was conjugated to dsDNA through the longer and less conductive alkane linker (Figure 3b), the efficiency of $Ru(NH_3)_6^{3+}$ wiring was lower than in the case of the acetylene linker. For the DNA surface coverage lower than 3 pmol cm^{-2} the AQ signal was not seen, while at a higher DNA surface density a small AQ oxidation peak appeared. The latter correlated with surface electrochemistry of AQ (Figure 14S, SI) and was consistent with 1.1 pmol cm^{-2} of electrochemically active AQ molecules (representative data in Figure 3e and SI), representing 25% of the total DNA surface coverage. The observed AQ signal was ascribed to direct interactions between the AQ probe, conjugated to DNA through the sufficiently long and flexible linker, and neighboring $Ru(NH_3)_6^{3+}$-decorated DNA double strands. The AQ signal consistently disappeared at lower DNA surface coverages.

For MB-C_6-DNA in the $Ru(NH_3)_6^{3+}$ solutions, the signal from MB became essentially masked by the diffusion-limited $Ru(NH_3)_6^{3+}$ currents (Figures 3f and 12S, SI). After the deconvolution of the $Ru(NH_3)_6^{3+}$ and MB peak currents in the recorded CVs, the faradaic signal from MB appeared to be practically the same as that without $Ru(NH_3)_6^{3+}$ (Figure 15S, SI).

Therefore, it may be concluded that the conjugation of the redox probe to dsDNA through the alkane linker does not allow the $Ru(NH_3)_6^{3+}$-mediated ET between the electrode and the redox probe, since no $Ru(NH_3)_6^{3+}$ wire may be formed along the alkane linker, and the own conductivity of the linker is insufficient. In the case of the short and conductive acetylene linker the ET is established, with electrons passing through the $Ru(NH_3)_6^{3+}$ molecules bound to the sugar−phosphate backbone of DNA and further, through the conductive linker, to AQ. Such wiring is inefficient when the redox probe is intercalated inside the DNA duplex.

Plausible Mechanisms of ET in the Surface-Tethered dsDNA. Under experimental conditions used in the present work, an upright orientation of dsDNA molecules on the negatively charged electrode surface[63−65] prevented a possible direct ET contact between the electrode and the redox probe. On the other hand, redox probe-DNA intermolecular interactions (such as intercalation into the neighboring DNA duplex) were avoided by the formation of loosely packed dsDNA monolayers, with individual dsDNA molecules presumably free-standing and not contacting each other. These experimental conditions provided the results that allowed us to address certain inconsistencies existing in the hitherto published data on ET in the surface-tethered DNA duplexes.

Based on the analysis of the kinetics of ET proceeding in the AQ-conjugated dsDNA, we can conclude that the short and conductive acetylene linker does not provide electronic conjugation between the AQ redox probe and DNA base pair π-stack, essential for DNA-mediated ET (Figure 1b, no ET). In this context, the data reported for compact dsDNA monolayers[40] may be attributed to the intercalation of AQ into the neighboring DNA duplexes, thus enabling the DNA-mediated ET mechanism (Figure 1d). A scrutiny of the data on the DNA-mediated ET in the redox-labeled dsDNA[38,40,41] reveals that intercalation of the probe into DNA may be always expected when densely packed DNA monolayers are formed and/or electrode modifications through the immobilization of dsDNA (not ssDNA) are used. Then intercalation of the DNA-conjugated AQ,[40] PQQ,[38] or Nile Blue[41] into the adjacent dsDNA strands may occur in the course of either immobilization[40,41] or the following modification protocol.[38] Such intercalation ensures electrochemistry consistent with the directional ET through the DNA duplex.[85]

In the other works, the Fc probe conjugation to DNA through the conductive isoquinoline linker was reported as providing conditions for directional ET through the DNA duplex; along with that the electrochemical response from the single-mismatched dsDNA was inconsistent with the discussed ET mechanism.[56] Later, the same group reconsidered their results and concluded the diffusion-limited ET (at high scan potential rates) and direct ET between Fc and the electrode (at low scan rates), due to the elastic bending of dsDNA at positive charges of the electrode surface.[86] In this context, in these works the Fc signal from mismatched DNA duplexes seems to be consistent with incomplete hybridization of dsDNA,[87] resulting in the Fc-linked ssDNA overhanging tail, flexible enough to approach the electrode surface more readily than fully complementary dsDNA.

ET between the electrode and AQ conjugated to DNA via the conductive linker may be established through the $Ru(NH_3)_6^{3+}$ wire formed along the sugar−phosphate backbone of dsDNA. Such a wire works as an ET mediator only in the case of the conductive linker, providing further ET to the redox probe, but not in the case of the longer and less conductive alkane linker. ET wiring depends to a large extent on the electrolyte conditions and the wire location versus the redox probe. ET mediation is low efficient under conditions of weak

binding of $Ru(NH_3)_6^{3+}$ molecules to dsDNA (high ionic strength solutions) and when the redox probe is intercalated into the DNA duplex, which does not allow direct electronic contact between the intercalated probe and the $Ru(NH_3)_6^{3+}$ wire.

In the case of the redox probe conjugation to dsDNA through the commonly used alkanethiol linkers, the ET reaction and its mechanism depends both on the charge and redox potential of the used redox probe. Namely, the electrochemistry of the Fc-labeled dsDNA is always complicated by the attractive electrostatic interactions between the DNA and positively charged electrode, which is preconditioned by the redox potential of the Fc probe. Depending on such experimental conditions as the DNA surface coverage, electrode surface blocking by alkanethiols, DNA length, and potential scan rate, the hitherto published data fit two extreme cases: ET due to direct contact of the Fc group with the electrode surface, under conditions of the DNA "horizontal" orientation on the electrode surface,[39,56] and ET due to the diffusion of the Fc probe, under conditions of dsDNA electrostatic bending toward the positively charged electrode surface.[10,50−53,56,86] By varying experimental conditions both cases can be achieved. Several models of the diffusion-limited ET were proposed, including the rotational diffusion of a dsDNA rod around its anchoring linker or elastic bending of the dsDNA in the applied electric field,[50,51,53] and both may end up, at low potentials scan rates and for relatively short DNA sequences, in dsDNA lying almost flat onto the electrode.

Due to the essentially negative redox potentials, the AQ and MB redox probes do not provide the conditions for electrostatic attraction between dsDNA and the electrode, but vice versa, and that affects the overall mechanism of the ET reaction. No ET was observed for the uncharged AQ probe conjugated to DNA through the alkane linker, and a pronounced signal, consistent with the previously published results,[55,57,58] was detected in the case of the positively charged MB redox probe. In the latter case, the kinetics of ET is limited by the diffusion of MB to the electrode surface (Figure 2c,f) and depends both on the length of the dsDNA duplex[55] and the DNA surface crowding, the MB signal being suppressed with increasing surface coverage dsDNA.[88] The driving force behind the diffusional movements of the MB probe is expected to be different from the force bending negatively charged dsDNA at positive charges of the electrode surface. In our previous work[55] we suggested that the applied potential, interfacial forces and the charge of the organic redox label might play a particular role in the movement of the redox probe closer to the electrode surface.[75,89] As follows from our present data, the charge of the redox probe is particularly important and is primarily responsible for the observed diffusion-limited ET between the positively charged MB label and the negatively charged electrode. For such uncharged probes as AQ no ET reaction has been observed, which is connected with the absence of the electrostatic stimuli for the redox probe diffusion toward the electrode surface.

CONCLUSIONS

ET reactions proceeding in individual, presumably free-standing (noninteracting with each other) dsDNA molecules grafted on electrodes and having a redox probe conjugated to DNA through the linker do not obligatorily follow the DNA-mediated ET pathway, which requires strong π-stacking interactions between the redox indicator intercalated into the DNA duplex and DNA bases.[13,47,48,71,90,91]

In loosely packed DNA monolayers and with immobilization protocols, excluding redox probe intercalation into the neighboring dsDNA molecules, DNA-mediated ET between the electrodes and DNA-conjugated redox probes is hardly probable and, at negative charges of the electrode surface, depends primarily on the redox probe charge and the way it is conjugated to the DNA duplex. Conjugation of the redox probe to DNA through the short unsaturated linker does not provide electronic coupling between the redox probe and the dsDNA base pair π-stack required for the DNA-mediated ET, and for noninteracting dsDNA molecules the faradaic signal from the redox probe is not observed. In this case, ET between the redox probe and the electrode can be established through the electronic wire formed by $Ru(NH_3)_6^{3+}$ molecules electrostatically attracted to the sugar−phosphate backbone of the DNA duplex. When the redox probe is conjugated to DNA via the long alkanethiol linker, ET may not be observed if electrostatic compatibility between the probe and electrode is absent. Otherwise ET is due to the diffusion of the redox probe toward the electrode surface, which results from the electrostatic interactions between the positively charged redox probe (MB) and the negatively charged electrode surface, producing the effect somehow similar (but not identical) to dsDNA elastic bending at positive charges of the electrode surface.[51,53]

These results are of particular importance for the fundamental understanding of the interfacial behavior of DNA, modulation of the DNA ET-based reactivity, and the development of advanced biosensors and bioelectronic devices exploiting ET properties of the individual DNA molecules. They contribute to the general concept of ET reactions that might proceed in the redox-labeled dsDNA molecules tethered to electrodes, hitherto mostly restricted to the DNA-mediated ET, and may further guide nanotechnological applications of DNA in molecular electronics and electrochemical biosensor fields.

ASSOCIATED CONTENT

Supporting Information

Oligonucleotide characterization (including melting curves data) and electrochemical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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