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Immobilization, hybridization, and oxidation of synthetic DNA on gold surface: electron transfer investigated by electrochemistry and scanning tunneling microscopy

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Abstract

Fundamental understanding of interfacial electron transfer (ET) among electrolyte/DNA/solid-surface will facilitate the design for electrical detection of DNA molecules. In this report, the electron transfer characteristics of synthetic DNA (sequence from pathogenic *Cryptosporidium parvum*) self-assembled on a gold surface was electrochemically studied. The effects of immobilization order on the interface ET related parameters such as diffusion coefficient (D_0), surface coverage (θ_R), and monolayer thickness (d_l) were determined by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). DNA surface density (Γ_{DNA}) was determined by the integration of the charge of the electro-oxidation current peaks during the initial cyclic voltammetry scans. It was found that the DNA surface density at different modifications followed the order: Γ_{DNA} (dsS-DNA/Au) > Γ_{DNA} (MCH/dsS-DNA/Au) > Γ_{DNA} (dsS-DNA/MCH/Au). It was also revealed that the electro-oxidation of the DNA modified gold surface would involve the oxidation of nucleotides (guanine and adenine) with a 5.51 electron transfer mechanism and the oxidative desorption of DNA and MCH molecules by a 3 electron transfer mechanism. STM topography and current image analysis indicated that the surface conductivity after each surface modification followed the order: dsS-DNA/Au < MCH/dsS-DNA/Au < oxidized MCH/dsS-DNA/Au < Hoechst/oxidized MCH/dsS-DNA/Au. The results from this study suggested a combination of variations in immobilization order may provide an alternative approach for the optimization of DNA hybridization and the further development for electrical detection of DNA.

Keywords

DNA Immobilization; Scanning tunneling microscopy (STM); Gold electrode; Hoechst 33258; Electron transfer; DNA oxidative damage

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1. Introduction

Electrochemical biosensors play an important role in biological, pharmaceutical, clinical, and agricultural areas of study because of their association with viruses, microorganisms, and genetic and biological materials. The introduction of the electrochemical DNA hybridization biosensor initiated an area of research, which has continually grown in popularity over the past decade [1]. Compared with other biosensing techniques, such as fluorescence-based DNA analysis which has some limitations (e.g., expensive instrumentation and sophisticated numerical algorithms to interpret the data), electrochemical biosensors have gained great attention in molecular diagnostics due to their high sensitivity, small dimension, low cost, ease-of-use and compatibility with micro-fabrication, and integrated array technology [2,3]. A basic DNA hybridization biosensor consists of single stranded DNA (ss-DNA) immobilized on a transducer surface which can then hybridize a complementary target DNA (t-DNA) sequence. The double strand DNA duplex, which is formed on the transducer surface, is known as a hybrid [4] and can be electrochemically studied by measuring the interfacial ET behaviors.

The DNA hybridization event can be converted into a measurable analytical signal via interaction with redox active molecules. Different redox active molecules, coupled with electrochemical techniques, can be used to specify and amplify detection signals. Metal complexes like ruthenium(III) hexamine (RuHex) and cobalt(III) tris(2,2'-bipyridine) (CoBPY) have shown that electrostatic binding to DNA is not affected with nucleic immobilization to an electrode surface [5]. Electroactive tags such as ferrocene (Fc) yield higher electron transfer rates than $\text{Ru}(\text{NH}_3)_6^{3+}$ [6]. Cationic phenoxazine dyes such as methyl blue (MB) [7] and nile blue (NB) have been utilized because of π -stacking intercalation [8]. External labels such as bisbenzimidazole dyes (Hoechst 33258) bind to the minor groove and are more selective than intercalators [9]. More recently, these external small electroactive molecules have been applied to probe the electron transfer characteristic of DNA lesion [10] and single base mutations [11] on a modified gold electrode surface.

Cryptosporidium parvum, a deadly waterborne pathogenic parasite, has been recognized by the World Health Organization as a significant global threat [12,13]. There is an increasing need for fast, reliable, and cost-effective methods of detection. Electrochemical hybridization biosensors for the detection of *Cryptosporidium parvum* DNA have been reported. One approach utilized a metal complex, $\text{Co}(\text{phen})_3^{3+}$ to improve the signal of adsorptive immobilized probe DNA on a carbon paste transducer for the capture and detection of a rRNA oligonucleotide specific to *C. parvum* [14]. Another approach was a DNA-hybridization assay with electrochemical detection through covalent attachment on a gold location that was separate from the working electrode. An alkaline phosphatase conjugate was used to produce an electrochemically active species (*p*-aminophenol; PAP_R , *p*-aminophenyl phosphate; PAPP) for detection at the working electrode, which was amplified with time [15]. These reported electrochemical approaches to detect *C. parvum* DNA focused on the improvement of detection limits; however, they did not reveal specific interfacial electrochemical (or ET related) characteristics of *C. parvum* DNA.

Scanning tunneling microscopy (STM) has also been utilized to investigate modified and unmodified DNA with a variety of substrates [16–18]. This surface imaging technique offers the unique capability of high resolution imaging DNA at the atomic or nano scale, as well as detects electronic properties of DNA [16]. STM has also been used to observe the binding of Hoechst 333258 with DNA [19].

In this study, we used synthetic DNA (40 bases) from the heat shocked protein (*hsp* 70) of the human genotype *C. parvum* (Genebank Access No. AF221535). Our present paper is divided into two sections. In the first section, we investigated the interfacial electron transfer of DNA

molecules at a gold modified electrode surface by electrochemical techniques. Then, in the second section, we investigated oxidation events that occurred at the DNA modified gold surface by electrochemical techniques and scanning tunneling microscopy. The ultimate goal of this work is to develop a highly specific and sensitive biosensor array to detect different genotypes of *C. parvum*. An understanding of the interfacial ET behaviors will assist to achieve this goal.

2. Experimental

2.1. Chemicals and Reagents

All chemicals were of analytical reagent grade and were supplied by Sigma-Aldrich unless otherwise stated. Molecular grade water was used in all solutions. Probe DNA (ss-DNA, 5'-HS-(CH₂)₆-AAATCGAAGATCAATACATTTCTCTCGCCAGTTCCTTTCT-3'), target DNA (t-DNA, 5'-AGAAAGGAACTGGCGAGAGAAATGTATTGATCGATTT-3'), and synthetic double strand DNA (dsS-DNA), a complementary hybrid of the probe and target DNA, were synthesized and purified from SynGen Inc. (San Carlos, CA) and were diluted to a 100 mM concentration stock solution. The DNA stock solution was then diluted in 0.01 M PBS buffer to desired concentrations for further experiments. 1 mM 6-mercapto-1-hexanol (MCH) was prepared in Molecular grade water. 100 μ M Hoechst 33258 solution was also prepared by dissolving it directly in 0.01 M PBS buffer, sealed and stored at 4 °C, and then used for up to 1 week.

2.2. Surface treatment of gold electrode

A gold electrode (diameter 2 mm; 0.0314 cm²) were cleaned with 25% H₂O₂/75% H₂SO₄ (piranha solution) for 10 minutes to remove organic impurities and then rinsed with double distilled water (Milli-Q). **CAUTION: Piranha solution reacts violently with organic solvents and is a skin irritant. Extreme caution should be exercised when handling piranha solution.** The gold surface was then 'renewed' by sequential hand polishing, with a polishing kit (CHI120) (CH Instruments, Austin, TX, USA), with 1.0 micron alpha alumina powder on a nylon polishing pad for ~60 seconds then with 0.3 micron alpha alumina powder on a nylon polishing pad for ~90 seconds, and finally with 0.05 micron gamma alumina powder on a micro-cloth polishing pad for ~120 seconds. The gold surface was rinsed with double distilled water between each polishing step.

It has been cited in literature that effectiveness in the monolayer is correlated to the method of substrate preparation [20]. In order to reduce the surface roughness and improve reproducibility [21] the hand polished gold electrode was then electrochemically cycled (~15 cycles at 60 mV s⁻¹) from a potential of 0.0 to +1.57 V in 1 M H₂SO₄ solution until stable gold oxidation peaks at ~1.06 V vs. Ag/AgCl were observed. The reactivity of the gold electrode is distinctly affected by the presence of absorbed layers of organic contaminants used in the common polishing step [21–23]. In each cycle, a monolayer of chemisorbed oxygen was formed and reduced. The reduction charge per microscopic unit area has been experimentally determined as 390±10 μ C cm⁻² [24]. The microscopic surface area was obtained by integrating the reduction current peak to obtain the reduction charge, and dividing this by 390 μ C cm⁻². By this method, our polished gold electrode indicated a roughness of *ca.* 1.

2.3. DNA modification of gold electrode surface

Schematic 1 is an illustration of the gold substrate modification that we used in this study. The freshly prepared gold electrode (step 1) was immersed in 1 μ M ss-DNA in 0.01 M PBS buffer at 4 °C for 24 hours. The ss-DNA modified electrode was then rinsed with 0.01 M PBS, pH 7.4, to remove any probes that were non-specifically absorbed. The electrode was mounted in an electrode holder for CV and EIS measurements. Next, ss-DNA/Au electrode (step 2) was

immersed in 1 mM MCH solution for a period of 1 to 12 hours, and then rinsed with PBS buffer to form MCH/ss-DNA/Au (step 3). After that, the resulting probe DNA modified gold electrode was then incubated with 2×SSC (saline sodium citrate) buffer containing t-DNA (1 μ M) for 90 min at 37 °C with interval agitation. After the reaction, the substrate was washed with 2×SSC buffer solution to remove non-specifically bound DNA, to form a t-DNA/MCH/ss-DNA/Au (step 4) modification. After being rinsed with PBS buffer, the modified gold electrode was then immersed in a 100 μ M Hoechst 33258 in 0.1 M KCl solution for 5 minutes at 25 °C to form the Hoechst/t-DNA/MCH/ss-DNA/Au (step 5) modification. The electrode was then rinsed with Tris-HCl (10 mM) buffer to remove non-specifically adsorbed Hoechst 33258. Finally, the Oxidation/Hoechst/t-DNA/MCH/ss-DNA/Au (step 6) modification was achieved by running cyclic voltammetry oxidation of the gold electrode surface (step 5) in 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4 between 0 – 1.3 V at 30 mV s^{-1} to irreversibly oxidize Hoechst 33258 (bound to hybridized ds-DNA) [9] and/or oxidize immobilized DNA during the substrate gold oxidation [25]. All experiments were carried out at ambient laboratory temperature (25 °C). Finally, if necessary, the oxidized electrode surface (step 6) was re-immersed in 100 μ M Hoechst 33258 solution for 5 min to see if the CV behavior changed after the DNA or bound Hoechst was oxidatively damaged.

2.4. Electrochemical measurements

Cyclic voltammetry (CV) was performed with a CHI 1220 Electrochemical Analyzer via the CHI software Version 4.30 (CH Instruments, Austin, TX, USA). Initial experiments were undertaken using a three-electrode setup; consisting of a platinum wire counter electrode (CHI115), a Ag/AgCl reference electrode (CHI111), and a gold working electrode (CHI101) (CH Instruments, Austin, TX, USA). All potentials refer to the Ag/AgCl reference electrode. The CV curves were measured between 0.6 V and –0.1 V in a solution of 0.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ + 0.01 M PBS over a range of scan rates; 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.08, 0.1, 0.15, 0.2, 0.3, and 0.4 V s^{-1} . Electrochemical impedance spectroscopy (EIS) was performed using a VMP2/Z multi-channel potentiostat (Princeton Applied Research, TN, USA) via the EC-Lab software Version 9.32 (Bio-Logic, France). These EIS measurements were conducted in a solution of 0.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ + 0.5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ + 0.01 M PBS under an AC amplitude of 0.01 V and a frequency range from 100 kHz to 0.01 Hz. The EIS parameters (R_Ω , C_d , R_{ct} , Z_W , K_a^0 , θ_R , and d_i) were obtained by modeling the EC-Lab data using ZSimpWin 3.21 (EChem Software, Ann Arbor, Michigan, USA).

2.5. STM imaging

STM imaging for DNA/MCH coated Au (111) substrate in air was conducted with a PicoPlus AFM system (Agilent Technologies, AZ) with Picoscan 5.3 version data acquisition software. STM tips ($\text{Pt}_{0.8}\text{Ir}_{0.2}$ wire) were used. The Au (111) substrate used for STM imaging was a fresh mica substrate coated with a ~200 nm thickness of gold layer (Agilent Technologies, AZ). Immediately before use, the gold substrates were annealed in a hydrogen flame for 90 seconds. A gas chamber was used to provide an argon environment, and an STM scanner with a maximum scan range of 1.5 μm was used. The annealed gold substrate was immersed in 1.0 μM of oligonucleotide solution (HS-ssDNA or HS-dsDNA) of 1.0 M phosphate buffer at 4 °C for different coating times. Then, the gold substrate was removed from the solution, rinsed thoroughly with distilled water, and dried in an argon gas stream. Unless otherwise stated, the images were acquired using the same parameters with the tip scanning from left to right. The scan rate was kept at 2.7~3.1 lines/second. The modifications of the Au (111) followed the same aforementioned procedures as the polycrystalline gold (Au) working electrode.

2.6. Electron transfer characterization by CV and EIS

The association of electroactive species (Hoechst 33258) with DNA structure facilitates the electron transfer of immobilized DNA at the electrode surface. The electrode reaction mechanisms of the electroactive species at the electrode surface can be determined by either CV or EIS measurement.

The Randles–Sevcik equation [26], Eq. 1, depicts a linear relationship between the peak current i_p and scan rate $v^{1/2}$ (where T is $\sim 25^\circ\text{C}$).

$$i_p = (2.69 \times 10^5) n^{3/2} A D_0^{1/2} C v^{1/2} \quad (1)$$

In Eq. 1, n is the number of electrons transferred, A the area of the working electrode, and C the concentration of the redox species in solution. The slope can then be used to determine the rate of species diffusion, or the diffusion coefficient (D_0), of the electroactive species to or from the electrode surface. The magnitude of D_0 reflects the mass transport characteristic of electroactive species diffusing to or from the electrode surface.

The electron transfer through a DNA self-assembled monolayer on metal substrates has been intensively studied using EIS [27–29], and is generally described as an equivalent circuit model [30]. The basic equivalent circuit model (Randles model) consists of four components; the electrolyte resistance (R_Ω), charge-transfer resistance (R_{ct}), double-layer capacitance (C_d), and Warburg impedance (Z_W) due to mass transport at the electrode surface. A Nyquist plot, imaginary part (Z_{im}) versus real part (Z_{re}), was used for the impedance analysis. The Nyquist plot for an electrode is generally, a semicircular region lying on the Z_{re} axis followed by a straight line. The semicircle portion, measured at higher frequencies, corresponds to a direct electron transfer limited process, whereas the straight linear portion, observed at lower frequencies, represents a diffusion-controlled electron transfer process. Modification of the working electrode surface with an organic layer tends to decrease the double layer capacitance and delays the interfacial electron transfer rate as compared to a bare working electrode [31–33].

The general features of the Nyquist plot are understood intuitively [34]. The imaginary component of the impedance comes solely from C_d . The C_d contributions fall to zero at high frequencies because it offers no impedance. All the current is the charging current, and the only impedance seen is the ohmic resistance. As the frequency continues to drop, the finite impedance of C_d is manifest as a significant Z_{im} . At the very low frequencies, the capacitance (C_d) offers high impedance; hence the flow passes mostly through R_{ct} and R_Ω . Therefore, the imaginary impedance component falls off again. Generally, a departure from the low frequency regime is expected and the Warburg impedance (Z_W) will become important [30]. When the Nyquist data is modeled, the above mentioned parameters are determined and can be further explored.

Kinetic parameters related to the electron transfer at the DNA modified electrode surface can also be obtained by EIS analysis. The heterogeneous standard charge-transfer rate constant (K_a^0) was obtained by using Eq. 2 [26],

$$R_{ct} = \frac{RT}{n^2 F^2 A K_a^0 C_0^{*(1-\alpha)} C_R^\alpha} \quad (2)$$

where the charge-transfer coefficient α is assumed to be 0.5, $C_0^{*(1-\alpha)} = C_R^\alpha = C_0$, C_0 being the concentration of the bulk solution. The other constants in Eq. 2 are as follows; $T = 298\text{ K}$, $A =$

0.0314 cm^2 , $n = 1$ (for the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox probe used here), F Faraday's constant (96485 C mol^{-1}), and R universal gas constant ($8.31 \text{ J mol}^{-1} \text{ K}^{-1}$)

Furthermore, the surface coverage (θ_R) for each addition to the monolayer was calculated by using Eq. 3 [35], where $R_{\text{ct}}^{\text{Au}}$ and $R_{\text{ct}}^{\text{SAM}}$ are the charge-transfer resistance values for the bare and DNA-modified Au electrode, respectively. An increase in θ_R would yield a decrease in D_0 .

$$\theta_R = 1 - \left(\frac{R_{\text{ct}}^{\text{Au}}}{R_{\text{ct}}^{\text{SAM}}} \right) \quad (3)$$

On the other hand, Eq. 4 was used [36] to estimate the monolayer thickness from the capacitance values:

$$d_i = \frac{\epsilon_0 \epsilon_i A}{C_d} \quad (4)$$

where d_i is the thickness of the i^{th} layer, C_d is the differential capacitance of the double layer, ϵ_0 is the permittivity of free space ($8.854 \times 10^{-12} \text{ s}^4 \text{ A}^2 \text{ kg}^{-1} \text{ m}^{-3}$), ϵ_i is the dielectric constant of the layer, and A is the electrode area (0.0314 cm^2). For our calculations, we use the dielectric constant of water (80) as the ϵ_i value [37].

3. Results and Discussion

3.1. STM image of DNA immobilized on Au (111)

Figure 1 gives the STM topography images of Au (111) surface with different modifications under constant current mode: (A) bare Au (111), (B) ss-DNA modified 13 hours, (C) MCH modified 6 hours, (D) ss-DNA 24 hours/MCH 6 hours, (E) dsS-DNA 24 hours, (F) dsS-DNA 24 hours/MCH 6 hours, (G) MCH 6 hours/dsS-DNA 24 hours. Figure 1 (A) illustrates the atomically flat Au (111) surface with a depth of $\sim 2.5 \text{ \AA}$ between two adjacent terraces. The topography of Au (111) surface modified with 40 base ss-DNA is shown in Figure 1 (B). The gold atom layers are still observed and wave-like features appear on the flat gold surface which is related to the immobilized ss-DNA. It was also observed the zig-zag-like structure on the Au (111) surface modified with shorter DNA oligonucleotide (not shown), similar to literature [38]. Compared to ss-DNA modification, MCH modification presents smoother surface features than the ss-DNA/Au (111) but rougher than bare Au (111). It is expected that MCH, like other alkanethiol molecules, form self-assembly monolayer structures on the gold surface after 6 hours immobilization. However, the immobilizations of ss-DNA for 24 hours and then subsequent MCH for 6 hours result in atomically flat, non-distinctive, gold layers shown in Figure 1 (D). This observation is because the MCH molecules have filled in the boundaries of the gold atom layers. The role of MCH immobilization is to replace non-specifically bound ss-DNA from the gold surface [5].

Different surface topography features are observed for the synthetic double strand DNA (denoted as dsS-DNA) modified Au (111) surface. Immobilization of dsS-DNA for 24 hours forms DNA cluster structures which aggregate on the Au (111) surface, as indicated in Figure 1 (E). The cross section analysis from one of these clusters (not shown here) indicates a size of $20 \times 10 \text{ nm}$ (length $\times$ height), which is similar in size for ds-DNA imaged by STM in air [39], but much larger than the heights for ds-DNA imaged by AFM [40]. For AFM investigation in air, the heights for ds-DNA immobilized on a solid substrate vary from 0.5 to 1.9 nm [40], which is smaller than the helix diameter, probably due to elastic deformations of the ds-DNA

caused by the AFM tip [40]. The dsS-DNA “forest”, as shown in Figure 1 (F), is observed for the coating of dsS-DNA for 24 hours, followed by MCH for 6 hours. Figure 1 (F) and Figure 1 (D) give different surface topography features, respectively, for the dsS-DNA and ss-DNA confined gold surfaces. In both cases, the DNA molecules are perpendicularly oriented to the electrode surface due to the replacement by MCH. The former produces more densely packed DNA molecules and no observable gold atom layer, as compared with the latter where the terrace of the gold atom layer is vague yet distinguishable. If the coating order is reversed, i.e., MCH for 6 hours first and then dsS-DNA for 24 hours, then such rod-like structures are observed, as in Figure 1 (G). Similar rod-like structures have been observed in literature for ds-DNA with mercapto-hexyls on 5' end of each chain [39]. It is apparent that by reversing the immobilization order between MCH and dsS-DNA yields visible differences seen in STM topography imaging.

3.2 Electron transfer behaviors of the Hoechst/t-DNA/MCH/ss-DNA modified electrode surface

Figure 2 illustrates cyclic voltammograms in 0.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ + 0.01 M PBS of the sequentially modified gold electrode. The electrochemical behaviors of the electroactive $\text{Fe}(\text{CN})_6^{3-/4-}$ redox couple is used to reflect the ET characteristic of the gold surface at each modification. As for bare Au (curve 1), a pair of redox peaks for $\text{Fe}(\text{CN})_6^{3-/4-}$ appeared at 0.176 V and 0.263 V at a scan rate (v) of 60 mV s^{-1} giving a formal potential E^0 of 0.22 V and the peak potential difference ΔE_p of 0.087 V. The ΔE_p increased with increasing the scan rate and the peak current was directly proportional to $v^{1/2}$ (Figure 2 insert), which indicates a quasi-reversible surface electrode process [8]. After the immersion in 1 μM ss-DNA for 24 hours, ss-DNA/Au (curve 2), there was a decrease in the redox peak current and an increase in the ΔE_p . This could be attributed to a modification of the gold surface with the 5'-end thiolated 40-mer ss-DNA (via gold-thiol bond) that blocks the interfacial electron transfer between the gold surface and bulk solution [41]. Next, with the addition of 1 mM MCH for 1 hour to form MCH/ss-DNA/Au, the redox peaks are completely suppressed (curve 3), which indicate further coverage by MCH on the gold surface. As mentioned previously, it is believed that MCH plays a two-fold role in the treatment of ss-DNA modified electrodes. First, it can take part in the replacement of thiolated ss-DNA (via thiol-thiol exchange) to remove or decrease non-specifically bound ss-DNA; and second, it can form a barrier (via the gold-thiol bond) to inhibit electron transfer [41]. Next, the probe DNA modified gold electrode is then immersed in 1 μM t-DNA in 2 $\times$ SSC hybridization buffer for 1.5 hours to form t-DNA/MCH/ss-DNA/Au (curve 4). At this stage a ds-DNA duplex is formed, yet the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox peaks remained negligible. The DNA prevents a redox response of the bulk solution (potassium ferricyanide ions) after the immobilization or hybridization of DNA on the gold electrode surface [23]. Finally, the interaction of 100 μM Hoechst 33258 with the t-DNA hybridized gold surface for 5 minutes (curve 5, Hoechst/t-DNA/MCH/ss-DNA/Au) restores some of the modified surface conductivity, as compared with curve 4. This means that Hoechst bound ds-DNA duplexes facilitate the interfacial electron transfer reaction [9].

Table 1 lists the calculated diffusion coefficient (D_0) of the $\text{Fe}(\text{CN})_6^{3-/4-}$ ions obtained by the Randles-Sevcik equation (Eq. 1). For simplicity, the cathodic peak currents were used in this calculation. The bare gold electrode gives rise to D_0 of $6.59 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, which is close to the literature reported value [42]. After ss-DNA immobilization, D_0 decreases to $1.54 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ because the DNA layer acts as a barrier to block the diffusion of electroactive $\text{Fe}(\text{CN})_6^{3-/4-}$ ions at the ss-DNA modified gold surface. This role is further evidenced by the subsequent immobilization of MCH and the t-DNA hybridization where no D_0 values are obtained. However, D_0 returns to a very low level of $9.3 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ after Hoechst binds with the hybrid ds-DNA duplex.

Figure 3 shows the EIS Nyquist plots after each electrode modification as described in Figure 2. Bare Au (curve 1) possesses a straight line in the low frequency region with unit slope, but has no semicircular portion in the higher frequency region. The low frequency signal indicates that the redox couple electron transfer process is diffusion-controlled. On the other hand, for the ss-DNA/Au (curve 2) modified electrode immersed in 1 μ M ss-DNA for 24 hours, there is a semicircular portion in the higher frequency region, which corresponds to a direct electron transfer limited process, and a straight line in the low frequency region which represents a diffusion-controlled electron transfer process. After immersion in 1 mM MCH for 1 hour, MCH/ss-DNA/Au (curve 3), the semicircular portion in the higher frequency region increased and the straight line in the low frequency region has a decreasing slope. For the hybridization with 1 μ M t-DNA for 1.5 hours, t-DNA/MCH/ss-DNA/Au (curve 4), the semicircular portion in the higher frequency region increased substantially revealing increased barrier properties of the resultant hybridized ds-DNA duplex on the modified gold surface. The effect of immersion in 100 μ M Hoechst for 5 minutes on the hybridized ds-DNA is evidenced in curve 5 (Hoechst/t-DNA/MCH/ss-DNA/Au) where there is a greater decrease in the semicircular portion. The insert in Figure 3 is the modified Randles circuit used to model the EIS data.

The values for circuit components R_{Ω} , C_d , R_{ct} , Z_W , K_a^0 , θ_R , and d_i are listed in Table 2. For this set of experiments, the solution resistance (R_{Ω}) had an average value of 155.2 Ω , which is expected for measurements under identical experimental conditions. The interfacial double-layer capacitance (C_d) for the bare gold electrode is estimated at 1.12 μ F, which is in the range of 20–50 μ F cm⁻² [43] (considering our electrode geometric area 0.0314 cm²). C_d increases to 9.64 μ F with the ss-DNA modification due to the fact that ss-DNA is a charged macromolecule, then this value decreases to 2.92 μ F with the subsequent MCH modification due to the replacement of ss-DNA by MCH, and increases to 3.76 μ F after the formation of the hybrid ds-DNA duplex, and remains constant (3.75 μ F) with the final binding of Hoechst. It is not surprising that Hoechst interaction does not affect the C_d due to Hoechst attachment with the inner duplex of the helical ds-DNA via a minor groove binding mechanism [9], and this binding may not significantly change the surface charge of the ds-DNA modified electrode surface. The electron transfer characteristics of the step-by-step modification could be reflected by the changes in the charge transfer (R_{ct}) and heterogeneous charge-transfer rate constant (K_a^0), which are associated with each other by Eq. 2. In each step, from bare gold to t-DNA, the modification results in the rapid increase of R_{ct} and an inversely significant decrease in K_a^0 . For example, the immobilization of ss-DNA on the bare gold surface leads to a 37-fold increase of R_{ct} , from 1 k Ω to 37 k Ω ; correspondingly, K_a^0 decreases from 1.51×10^{-2} cm s⁻¹ to 4.54×10^{-4} cm s⁻¹ (approximately a two order of magnitude decrease). The subsequent increase of R_{ct} (or decrease of K_a^0) is due to the formation of the thiolated ss-DNA layer on the gold surface after this modification, which prevents the electron transfer of the redox couple $\text{Fe}(\text{CN})_6^{3-/4-}$ at the modified Au/solution interface. It is interesting to note that the binding of Hoechst to the hybridized ds-DNA duplex yields an approximate 10-fold decrease of R_{ct} (conversely a ~ 10 -fold increase of K_a^0), compared to that obtained from the t-DNA/MCH/ss-DNA/Au modified surface. This is evidence that the binding of Hoechst to the ds-DNA on the modified gold surface increases electrode surface conductivity, and thus facilitates the ET of $\text{Fe}(\text{CN})_6^{3-/4-}$ at this modified electrode surface. This effect is seen in the restored D_0 value (Table 1) after the Hoechst binding. There is not much change in the Warburg impedance (Z_W) and the electrolyte resistance (R_{Ω}) for each modification, except the variations in R_{Ω} and Z_W after the t-DNA hybridization step.

It is also found that there are changes in surface coverage (θ_R) and thickness (d_i) for each modification of the gold electrode surface (Table 2). For each modification step, the values for both θ_R and d_i are calculated from Eq. 3 and Eq. 4. The initial immobilization of ss-DNA results in θ_R of 0.97 and d_i of 2.31 nm. The subsequent modification with MCH may replace some immobilized ss-DNA and/or non-specifically bound ss-DNA to allow more ss-DNA

molecules to stand-up vertically due to charge repulsion (step 3 in Schematic 1). Therefore, it is not surprising to observe a slight increase in θ_R (to 0.988) and a significant increase in d_i (to 7.63 nm), the latter resulting in a thickness increase (Δd_i) of 5.32 nm due to the MCH modification. Considering that the estimated (or theoretical) thickness of MCH is closer to a height of 7~8 Å (with a 30° tilt angle toward the substrate), this increased thickness would imply the formation of a multi-layer MCH structure as seen in step 3 of Schematic 1, similar to other thiol multi-layers formed on gold surfaces [44–46]. This multi-layer MCH structure could be due to hydrogen bonding between the –O~H and –S~H that exist between the inter-layer MCH molecules. This hypothesis of multi-layer MCH structures will need to be further verified by other surface analysis tools such as X-ray Photospectroscopy (XPS), and/or ellipsometry. The further hybridization of t-DNA with MCH/ss-DNA/Au causes a slight increase of θ_R from 0.988 to 0.995 despite a decrease in d_i from 7.63 nm to 5.92 nm. It is understandable that the formed ds-DNA duplex might have a decreased thickness compared to the MCH/ss-DNA/Au, where MCH helps the ss-DNA to orient vertically. After the hybridization with t-DNA, the resulting ds-DNA would have an increased probability to orient more parallel to the electrode substrate surface, which could account for the decreased thickness at the ds-DNA/MCH/ss-DNA/Au modification. On the other hand, during hybridization, the t-DNA could replace the multi-layer MCH structures previously formed on the modified gold surface shown in step 3 of Schematic 1. This replacement would also result in the decrease of the layer thickness. The K_a^0 value increases by about one order of magnitude with the binding of Hoechst to the ds-DNA (due to the increase of the conductivity of the bound ds-DNA duplex), at this modification step, the interfacial double layer capacitance C_d (3.75 μ F) and layer thickness d_i (5.93 nm), however, remain unchanged. This reaffirms the idea that Hoechst binding within the inner minor groove of the helical structure of the hybrid ds-DNA duplex does not significantly change C_d and d_i but facilitates the interfacial electron transfer indicated by an increase in K_a^0 .

3.3 Electron transfer behaviors of the Hoechst/CV oxidation/Hoechst/MCH/dsS-DNA modified electrode surface

To compare the ET characteristic difference between ss- and ds-DNA, the electrochemical behaviors of synthetic ds-DNA (“dsS-DNA”) were also investigated. This ds-DNA duplex was formed by the hybridization of ss-DNA (probe DNA) with t-DNA (each sequence is 40 bases). Figure 4 depicts cyclic voltammograms of the sequentially modified gold electrode beginning with bare Au, followed by dsS-DNA, MCH, Hoechst binding, CV oxidation, and Hoechst re-binding. In this comparison study, the same bare gold electrode CV curve in Figure 2 (curve 1) is used. After the bare electrode is immersed in 1 μ M dsS-DNA for 24 hours, dsS-DNA/Au (curve 2), there is a pair of compressed redox peaks for $\text{Fe}(\text{CN})_6^{3-/4-}$ and an increase in the peak potential difference, similar to the ss-DNA/Au modification seen in curve 2, Figure 2. However the diffusion coefficient D_0 for dsS-DNA, $3.84 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (Table 3), is more than double that of ss-DNA, $1.54 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (Table 1). This implies that the ss-DNA would form a more densely compact or uniform DNA layer, than dsS-DNA, on the bare gold electrode for the same 24 hours immobilization time. This is also verified by the d_i value difference shown in Table 2 and Table s1. Similarly, the subsequent modification of the DNA immobilized gold in 1 mM MCH for 6 hours exhibits no $\text{Fe}(\text{CN})_6^{3-/4-}$ redox peaks. This also suggests that the replacement of dsS-DNA by MCH could have achieved a better surface coverage for the MCH/dsS-DNA/Au modification (curve 3). Next, the immersion of in 100 μ M Hoechst 33258 for 5 minutes to form the Hoechst/MCH/dsS-DNA/Au modification (curve 4), shows a little more conductivity of the modified gold electrode, seen by a slight increase in the current responses for both the reduction and oxidation branches of the CV curve (compared to curve 3). The binding of Hoechst with the immobilized dsS-DNA yields the same D_0 of $9.3 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ (Table 3) as that of the hybrid ds-DNA duplex as shown in Table 1. After three cycles of voltammetric oxidation (Figure 4, inset) in 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4 between 0

– 1.3 V at 30 mV s⁻¹, the CV oxidation/Hoechst/MCH/dsS-DNA/Au is formed and the well-defined Fe(CN)₆^{3-/4-} redox peaks return (curve 5), giving peak potential and peak current heights that are close to those of the bare gold electrode (curve 1). We propose that a combined effect of the oxidative DNA damage and oxidative desorption of the immobilized dsS-DNA and MCH due to this CV oxidation increases surface conductivity and contributes to the return of the Fe(CN)₆^{3-/4-} redox peaks in curve 5. This cyclic oxidation of the Hoechst bound dsS-DNA modified gold surface results in an increase of D_0 by three orders of magnitude, from $9.3 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ to $7.26 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (Table 3). A second immersion in 100 μM Hoechst 33258 (5 minutes) of the oxidized Hoechst bound dsS-DNA would form the Hoechst/Oxidation/Hoechst/MCH/dsS-DNA/Au modification (curve 6). Unfortunately, the D_0 value is not obtained as the CV data are missing (Table 3).

The EIS Nyquist plots for the steps described above are displayed in (Supplementary Materials) Figure s1. The same EIS equivalent circuit model from Figure 3 was used and the simulated values for R_{Ω} , C_d , R_{ct} , Z_W , K_a^0 , θ_R , and d_i are listed in Table s1.

Compared to the ss-DNA (Table 2), with the same immobilization time (24 hours) and probe concentration (1 μM), the first step in the immobilization of the synthetic dsS-DNA gives smaller values: double layer capacitance ($C_d = 7.64 \text{ }\mu\text{F}$), charge transfer resistance ($R_{ct} = 4.8 \text{ K}\Omega$), and surface coverage ($\theta_R = 0.766$) (Table s1). The larger values of these three parameters for the ss-DNA immobilization imply the ss-DNA layer is more densely spaced than the ds-DNA layer on the gold electrode surface under current experimental conditions. A larger K_a^0 ($3.54 \times 10^{-3} \text{ cm s}^{-1}$) is observed due to the dsS-DNA immobilization. This K_a^0 value is approximately one order of magnitude greater than the ss-DNA immobilization of $4.54 \times 10^{-4} \text{ cm s}^{-1}$ (Table 2). The measured thickness for dsS-DNA is about 2.91 nm, which is a little larger than the ss-DNA layer of 2.31 nm. After further modification with MCH, the decreases in both C_d (3.1 μF) and K_a^0 ($1.86 \times 10^{-4} \text{ cm s}^{-1}$) are observed, which are 40% and 5.3% of the respective values obtained for the ss-DNA immobilization. As expected, the MCH modification shows an increase in R_{ct} (91 K Ω), θ_R (0.988), and d_i (7.19 nm), respectively. At step 4, the self-assembled dsS-DNA (with MCH) molecules are then bound with Hoechst (CV curve shown in Figure 4 and EIS plot not shown in Figure s1). In step 5 a very conductive gold surface is produced, due to the oxidative damage of the dsS-DNA and oxidative desorption of the dsS-DNA and MCH, as evidenced by the increase in K_a^0 ($4.04 \times 10^{-3} \text{ cm s}^{-1}$). In spite of the similarity between the values for R_{ct} and θ_R in step 2 versus those of R_{ct} (4.2 K Ω) and θ_R (0.733) in step 5, the values for C_d (2.63 μF) and d_i (0.85 nm) in step 5 (CV oxidation) are significantly less than those at step 2 (7.64 μF and 2.91 nm), respectively. The significant decrease in both θ_R and d_i , (compared to step 3), are a result of the desorption of dsS-DNA and MCH from the gold surface, after the electrochemical oxidation at a high positive potential (up to 1.3 V). The resulting surface conductivity would be comparable to the immobilized dsS-DNA only in terms of R_{ct} (or K_a^0). Especially, the thickness d_i (0.85 nm) after the CV oxidation, which is similar to a single layer of MCH (or the length of the linking spacer, $-\text{S}(\text{CH}_2)_6-$, of the synthetic dsS-DNA or pure MCH). After the re-binding with Hoechst for the oxidized gold surface, the increase of R_{ct} (5.5 K Ω), θ_R (0.799), and d_i (2.13 nm) are accounted for by non-specifically bound Hoechst. Most dsS-DNA molecules have been oxidized and removed away from the modified gold surface at step 5, thus there is a less likely chance for Hoechst to bind with dsS-DNA.

For comparison purposes, the electron transfer behaviors of the Hoechst/CV oxidation/dsS-DNA/MCH modified electrode surface were also investigated by CV and EIS. The results are shown in supplementary materials Figures s2 – s3 and Tables s2 – s3.

3.4. Hoechst interactions with ss-DNA, hybridized ds-DNA, dsS-DNA, and oxidatively damaged dsS-DNA

Figure 5 shows cyclic voltammograms of Hoechst 33258 bound to DNA modified gold electrodes in 0.01 M PBS buffer. Curve 1 represents the CV response of the bare gold electrode between 0 to 0.9 V in a 100 μ M Hoechst 33258 + 0.01 M PBS solution. It exhibits an irreversible oxidation peak for Hoechst at the peak potential of 0.647 V and peak current of -0.634×10^{-6} A, which is consistent with reported literature values [47]. After modification with ss-DNA for 1 hour, the ss-DNA modified electrode is run by CV with the same conditions, as shown in curve 2. Hoechst bound ss-DNA in the presence of the Hoechst solution shows an oxidation peak shifted to the potential 0.580 V with a larger current response of -0.682×10^{-6} A (I_{ss}), indicating the oxidation potential of Hoechst is more negative than that measured on the bare gold surface. Then the ss-DNA modified gold electrode is hybridized with t-DNA to form a ds-DNA(hybrid)/Au. This ds-DNA duplex interaction with Hoechst results in a slightly positively shifted peak current at 0.586 V and a further increase in current to -0.74×10^{-6} A (curve 3, $I_{ds,hybrid}$). As a control, synthetic ds-DNA (dsS-DNA) is immobilized on the bare gold surface for 12 hours and then moved to the same Hoechst PBS buffer solution for CV analysis, as shown in curve 4. This voltammogram gives a similar shape, peak potential (0.588 V), and peak current (-0.722×10^{-6} A, I_{ds-S}) as those in curve 3 (dsS-DNA hybrid). This means the Hoechst prefers to bind with ds-DNA other than ss-DNA, which yields a larger current response ($I_{ds,hybrid} \sim I_{ds-S} > I_{ss}$). Finally, the (12 hours) dsS-DNA modified gold electrode is first oxidized in 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4 by cycling at 100 mV s^{-1} (as illustrated in Figure 6A), and then run with CV in the 100 μ M Hoechst 33258 PBS buffer. As shown in curve 5, the Hoechst binding to the oxidatively damaged dsS-DNA leads to a significant negatively shifted oxidation peak of Hoechst at 0.539 V and a decreased peak current of -0.40×10^{-6} A ($I_{ds,damaged}$). The binding of Hoechst 33258 with ds-DNA is attributed to a minor groove binding mechanism [48–50], where Hoechst selectively binds to A-T base pairs [51,52]. The first scan in Figure 6B is the same as curve 5 in Figure 5. The continuous scans (scan 2, 3, etc) gives rise to almost no further oxidation of Hoechst due to irreversible oxidation on the electrode surface (Figure 5, curve 5). It is interesting to observe that the oxidation currents of Hoechst on the DNA modified electrode follow the order: $I_{ds,hybrid} \sim I_{ds-S} > I_{ss} > I_{ds,damaged}$.

From the chemical structure of Hoechst 33258, the most probable oxidation sites are in the N-3 and N'-3 of the two benzimidazole rings [50]. Since the imidazole ring shows a partial electrostatic charge, when binding with ss-DNA, it would bind to the phosphate groups of DNA by electrostatic binding in the ss-DNA state [23]. The Hoechst 33258 binding mode changes to a minor groove binding when Hoechst 33258 binds to the ds-DNA duplex.

3.5 Comparison of Current vs. Time curves of DNA oxidations on electrode surfaces with different modifications

The current versus time curves of three cases: (A) Hoechst 33258 (5 min)/MCH (6 hours)/dsS-DNA (24 hours)/Au (corresponding to the inset in Figure 4), (B) dsS-DNA (24 hours)/MCH (6 hours)/Au (corresponding to the inset in Figure s2), and (C) dsS-DNA (12 hours)/Au (corresponding to Figure 6A) are shown in Figure s4 (see Supplementary Materials). Each CV illustrates the first three cyclic voltammetry scans and are numbered 1, 2, and 3. The surface densities of ds-DNA in these cases can also be obtained from the electro-oxidation charges of A or G nucleotides [25,53] on a ds-DNA modified gold electrode. From the CV scans in each case, it can be seen that the oxidation of the gold substrate is not prevented but only retarded. The first current peaks for these cases (A)-(C) appear at 1.11 V, 1.11 V, and 1.00 V, respectively, whereas the reduction peaks for all cases appear close to, *ca.* 0.46~0.47 V. The small reduction peaks observed in each of the cases (A)-(C) may be attributed to the electro-reduction of electro-oxidation products produced on the electrode surface at each oxidation

process. It is not clear what the small reduction peaks actually represent in Figure s4(D). These small reduction peaks (are absent in A-C) are from the last three CV scans of the electro-polishing processes by repetitive CV cycles of the bare gold electrode in a solution of 1.0 M H₂SO₄ between 0 – 1.3 V at 60 mV s⁻¹. Table 4 lists all the charges for the first three electro-oxidation peaks and corresponding electro-reduction peaks obtained from the individual CV curves.

The surface density (Γ_0) of the DNA immobilized on the gold surface can be obtained from Eq. 5, where Q_{DNA} is the oxidation charge difference between the first scan (Q_{01}) and the second scan (Q_{02}):

$$Q_{\text{DNA}} = Q_{01} - Q_{02} \quad (5)$$

$$\Gamma_0 = \frac{Q_{\text{DNA}}}{nFAR_s} \quad (6)$$

In Eq. 6, R_s is the roughness factor (R_s is approximately 1 for the gold electrodes used here). The surface density (Γ_{DNA} , mol cm⁻²) of DNA immobilized on the gold surface can be calculated from Eq. 7 below:

$$\Gamma_{\text{DNA}} = \Gamma_0 \frac{z}{m} \quad (7)$$

Where m is the number of base pairs in the DNA strand and z is the charge of the redox molecules.

Therefore, the surface DNA densities (Γ_{DNA}) on the different modified gold electrodes are determined as: 3.33×10^{-11} mol cm⁻² for Hoechst/MCH/ds-DNA/Au, 2.94×10^{-11} mol cm⁻² for ds-DNA/MCH/Au, and 3.92×10^{-11} mol cm⁻² for ds-DNA/Au, by the DNA electro-oxidation charge ($Q_{\text{DNA, ox}}$) given in Table s5, assuming an average electron-transfer of 5.51 electrons per base pair. The calculation for the number of electrons transferred through DNA is carried out as follows: if we assume 6 and 4.7 electrons for the electrochemical oxidation of the adenine and guanine moieties [25], the average number of electrons needed for the oxidation of each base pair is given by the relationship $(0.625 \times 6) + (0.375 \times 4.7) = 5.51$ electrons, because the current 40 bp oligonucleotide contains A-T and G-C in a ratio of 0.625/0.375 (25 A-T pairs/ 15 G-C pairs). The immobilization of ds-DNA directly on the bare electrode for 12 hours gives a DNA density of 3.92×10^{-11} mol cm⁻², which is close to the literature value [6,54]. Our results show that the treatment of MCH on the DNA-modified gold surface leads to a slightly lower DNA surface density than that of the gold surface without the MCH treatment. A similar observation has been reported [41]. It is found that the DNA densities at these modifications follows this order: Γ_{DNA} (dsS-DNA/Au) > Γ_{DNA} (MCH/dsS-DNA/Au) > Γ_{DNA} (dsS-DNA/MCH/Au).

On the other hand, assuming that ds-DNA molecules are adsorbed flat on the gold substrate surface in a saturated monolayer, a surface density of 6.1×10^{-12} mol cm⁻² can be estimated from the area of a single ds-DNA molecule (length $\times$ width) of 27.2 nm² for a 40 bp duplex. It is obvious that the actual surface density obtained from the CV is larger than the theoretical calculation. This may be due to the fact that ds-DNA is not actually laying flat on the substrate, but more likely it is in a 'coiled' format, rather than in a compact (flat) format, on the gold surface. The former format would yield a smaller single molecule area, and thus lead to a larger

surface density. This coiled effect becomes more plausible for the long 40 bp oligonucleotide. From the EIS measurement, ss-DNA/Au and ds-DNA/Au give the thickness values of 2.31 nm for ss-DNA (Table 2) and 2.91 nm for ds-DNA (Table s1), both are greater than the theoretical 2 nm thickness (using a DNA width of 2 nm).

Meanwhile, according to the oxidative desorption charge ($Q_{\text{ox-des}}$) for each individual modified electrode at voltammetric scans, one could calculate the surface density of the oxidatively desorbed thiolated molecules immobilized on the gold surface by assuming a three-electron transfer of desorption [55].

It is seen that the $Q_{\text{ox-des}}$ decreases with an increase in the number of scans for all three modified electrodes (Table s5). Applying the individual $Q_{\text{ox-des}}$ to Eq. 6 and 7, one can obtain the DNA oxidative desorption density. For instance, if the gold electrode is immobilized with dsS-DNA (i.e., dsS-DNA/Au, case C), the surface density due to DNA oxidative desorption is $1.26 \times 10^{-10} \text{ mol cm}^{-2}$ for the 1st scan, $1.15 \times 10^{-10} \text{ mol cm}^{-2}$ for the 2nd scan, and $1.02 \times 10^{-10} \text{ mol cm}^{-2}$ for the 3rd scan, respectively, which are about three times larger than the surface density due to DNA oxidation, $3.92 \times 10^{-11} \text{ mol cm}^{-2}$. These calculation details can be found in the Supplementary Materials. It is understandable that the surface density, due to DNA oxidative desorption, decreases over the cyclic scanning time, because more and more of the adsorbed thiol-group DNA molecules (and MCH in the cases of the other two modifications) are oxidized to a more soluble RSO_2^- species that results in the gradual removal of the DNA from the gold surface.

3.6. STM imaging of electro-oxidation of DNA modified gold surface

In order to observe more surface details of DNA modified gold electrode before and after electro-oxidation, we used the same immobilization procedures mentioned above to modify a single crystalline gold surface (111). This Au (111) electrode surface is atomically flat and suitable to image DNA. In this study, we utilized the nanoscale resolution of STM imaging to assess the surface topography and surface conductivity of the gold surface for different modifications. Figure 7 illustrates typical STM topography (A) and current (B) images that were obtained using a constant current mode on the dsS-DNA modified Au (111) surface at different oxidation stages. The procedures to process these STM images are described in detail in the Supplementary Materials. A bright spot was chosen at random, as indicated by the black arrows in images (A) and (B), and then, for further analysis, magnified images ($5.3 \times 5.3 \text{ nm}$) were created, as shown in topography (C) and current (D) images, respectively. One simple way to analyze both images can easily be done by cross section analysis for both the topography and current images (see Supplementary Materials). The cross section profiles (c1) and (c2) were obtained by drawing the green line and red line in the topography image (C), respectively. Similarly, (d1) and (d2) correspond to the green line and the red line drawn in the current image (D), respectively.

It is interesting to note that the area where the green line crosses in the topography image (C) yields a different cross section profile as does the red line. The red line crosses a dark area in the topography image (C) and yields a decrease in height (c2), whereas the green line shows an increase in the height of the area it crosses (c1). This implies that the bright spots on the Au (111) surface are probably accounted for the molecules modified on the gold surface and the dark areas (or pinholes) are the gold substrate. These pinholes are often observed in the reductive desorption of alkanethiols due to the breaking of the Au-S bond formed on the Au (111) surface [38,56,57]. It is also noteworthy to observe that the corresponding bright spot in the current image (D) is at the same location for the topography image (C). The bright spot

crossed by the green line (d1) gives a much higher conductance than the spot crossed by the red line (d2). Considering that our sample is a ds-DNA modified flat gold (111) surface, it is plausible to tentatively assign the bright spot in both the topography (height information) and current images (conductance information) to at least three possible species: i) the oxidized thiol linker ($-\text{HS}(\text{CH}_2)_6-$) of the adsorbed ds-DNA molecules, ii) the electroactive Hoechst molecules that could be bound with oxidized DNA or native DNA molecules on gold surface, and iii) oxidized and/or broken DNA fragments.

For i), the charged species is most likely in the format of $^-\text{O}_2\text{S}(\text{CH}_2)_6-$, as shown in Eq. 8. When the STM tip approaches the conductive species, like $-\text{O}_2\text{S}(\text{CH}_2)_6-$, the topography image will give an increase in the height and the current image will exhibit an increase in conductivity. From the cross section of the green line (c1) in the topography image (C), the height of the bright spot is estimated to be $\sim 6\sim 7$ Å (depth relevant to the substrate). Considering the length of MCH to be 1.2 nm [58], this bright spot corresponds to a tilt angle of about 30° toward the substrate for mercaptohexane groups. For ii), the dimension of the bright spot in image (C) is about 1.3×0.48 nm², which is comparable to the theoretical size of single Hoechst 33258 [9], 2.12×0.48 nm² (length $\times$ width). Finally, for iii), since it does not resemble the observed island structure as shown in Figure 1 (E) after the electro-oxidation on the dsS-DNA modified gold electrode, it is speculated that the thiolated 40 bp dsS-DNA previously immobilized on the gold surface could be broken down to DNA fragments or pieces of nucleotides (by opening the duplex helix), after voltammetric oxidation. On the other hand, the $6\sim 7$ Å height of the bright spot is close to the thickness of ss-DNA [58]. Thus, after the oxidization of bases at a very positive potential, it could be likely that the helical duplex structure of ds-DNA would be opened to become either single strand DNA or DNA fragments.

Nevertheless, it is difficult to definitively distinguish which species or combination of species could be assigned to the numerous bright spots shown on the gold surface after the electro-oxidization (it is known that these bright spots represent a conductive species on the electrode surface).

By analysis of the STM current images, we were able to obtain the current (conductance) distribution for the successive modifications of dsS-DNA (24 hours), MCH (6 hours), voltammetric oxidation, and binding with Hoechst 33258. Triplicate images are analyzed at each modification and the results are shown in Figure 8. It was observed that the surface conductivity (based on the current of the bright spots in Figure 7) after each modification follows the order: dsS-DNA/Au < MCH/dsS-DNA/Au < oxidized MCH/dsS-DNA/Au < Hoechst/oxidized MCH/dsS-DNA/Au. This trend is consistent with the results shown in the CV curves of Figure 4 and the EIS plots of Figure s1 which were obtained for the polycrystalline gold electrode using the same surface modification order. STM was previously applied to observe the Hoechst 33258 bound ds-DNA [19]; however, no quantitative information was obtained in that application. From the results obtained in this present work, conductivity changes of the gold electrode surface could be estimated by at least three methods: cyclic voltammetry (cyclic shape changes), electrochemical impedance spectroscopy (EIS parameter changes, e.g., R_{ct}), and STM current image (changes in current). Furthermore, it will be of interest to utilize the combined *in situ* electrochemical STM (*in situ* EC-STM) to quantitatively correlate the results obtained by these different methods.

4. Conclusion

In this study we have demonstrated the formation of DNA SAM by a variety of step-by-step immobilization procedures to understand the DNA-mediated electron transfer behaviors on modified gold electrode surfaces by electrochemical methods (cyclic voltammetry and electrochemical impedance spectroscopy) and STM. As expected, in these immobilization

procedures MCH plays an essential role in the replacement of thiolated DNA molecules (via thiol-thiol group exchange) and removal of non-specifically bound DNA from the modified electrode surface, and therefore further adjusting the interfacial electron transfers on electrode/DNA/electrolyte. These roles of MCH are elucidated by investigating the changes in CV curves and Randles equivalent circuit model parameters for each step of surface modification. The change of layer thickness (d_l), as shown in Eq. 4, obtained from the change in the interfacial double layer capacitance (C_d) before and after MCH immobilization, implies that there may be a multilayer structure of MCH that could be attached on the modified electrode by the hydrogen bond between MCH molecules (H-S...HO). However, this hypothesis needs to be confirmed by other surface analysis techniques.

It is proposed that the electro-oxidation of the DNA-confined electrode in PBS buffer solution involves oxidative DNA damage (via 5.51 electron transfer mechanism) as well as the oxidative desorption (via 3 electron transfer mechanism) of DNA and MCH (if immobilized), according to the electrical charges obtained from the voltammetric oxidation. Hoechst 33258, as an electroactive non-intercalator, has been introduced to probe the conductivity changes before and after the electro-oxidation of the ds-DNA modified electrode surface. The constant layer thickness and remarkable increase in electron transfer rate before and after Hoechst binding indicate that Hoechst 33258 prefers to bind to ds-DNA, rather than ss-DNA, by a minor groove binding mechanism. STM topography and current image analysis are, for the first time, introduced to study the conductivity of the modified electrode surfaces. The results suggest that there is a possibility of the co-existence for oxidative DNA damage and oxidative desorption on the modified electrode surface at a positive potential (e.g., > 1.1 V vs. Ag/AgCl). Our results highlight that the combined techniques, such as EC-STM, may provide a new tool to qualitatively and quantitatively measure the DNA-mediated electron transfer passing through the DNA nucleotides/bases, as well as the visualization of the DNA oxidative damage on modified electrode surface at nanoscale resolutions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

STM topography images of (A) bare Au (111) electrode (-0.5 V, 0.01 nA), (B) ss-DNA modified 13 hr (-0.5 V, 0.03 nA), (C) MCH modified 6 hr (-0.5 V, 0.01 nA), (D) ss-DNA 24 hr/MCH 6 hr (-0.5 V, 0.03 nA), (E) dsS-DNA 24 hr (-0.5 V, 0.05 nA), (F) dsS-DNA 24 hr/MCH 6 hr (-0.5 V, 0.05 nA), (G) MCH 6 hr/dsS-DNA 24 hr (-0.5 V, 0.05 nA). The numbers in parenthesis are the bias and tunneling current set points under which the images are collected. All coatings of DNA and MCH are conducted in 5 mM phosphate buffer. dsS-DNA is designated as the synthetic double strand DNA. Arrows in (E) indicate the DNA cluster structures and aggregates. The arrows in (G) show the rod-like structure.

Figure 2.

Voltammetric behaviors of $\text{Fe(CN)}_6^{3-/4-}$ redox couple (0.5 mM $\text{K}_3\text{Fe(CN)}_6$ + 0.01 PBS) on step-by-step ss-DNA modified electrodes: The data corresponds to electrode modifications: bare Au (1), ss-DNA/Au (2), MCH/ss-DNA/Au (3), t-DNA/MCH/ss-DNA/Au (4), and Hoechst/t-DNA/MCH/ss-DNA/Au (5) (all scans performed in 0.5 mM $\text{K}_3\text{Fe(CN)}_6$ + 0.01 M PBS, pH 7.4; at scan rate 60 mV s^{-1}). Inset: Dependence of i_{pa} and i_{pc} on $v^{1/2}$, for scan rates: 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.08, 0.1, 0.15, 0.2, 0.3, and 0.4 Vs^{-1} .

Figure 3.

Electrochemical impedance spectroscopy (EIS) of step-by-step ss-DNA modified electrodes: bare Au (1), ss-DNA/Au (2), MCH/ss-DNA/Au (3), t-DNA/MCH/ss-DNA/Au (4), and Hoechst/t-DNA/MCH/ss-DNA/Au (5). Inset: modified equivalent Randles circuit used to model impedance data; R_{Ω} , electrolyte resistance; Z_W , Warburg impedance; R_{ct} , charge-transfer resistance; and C_d , double-layer capacitance. The coating conditions are the same as Figure 2. EIS was conducted in 0.5 mM $K_3Fe(CN)_6$ + 0.5 mM $K_4Fe(CN)_6$ in 0.01 M PBS, pH 7.4 with 10 mV AC amplitude.

Figure 4. Voltammetric behaviors of $\text{Fe(CN)}_6^{3-/4-}$ redox couple ($0.5 \text{ mM K}_3\text{Fe(CN)}_6 + 0.01 \text{ PBS}$) on step-by-step synthetic ds-DNA modified electrodes: bare Au (1), dsS-DNA/Au (2), MCH/dsS-DNA/Au (3), Hoechst/MCH/dsS-DNA/Au (4), Oxidation/Hoechst/MCH/dsS-DNA/Au (5), and Hoechst/Oxidation/Hoechst/MCH/dsS-DNA/Au (no data) (6). Inset: $5 \text{ mM Na}_2\text{HPO}_4 + 5 \text{ mM NaH}_2\text{PO}_4$ between $0 - 1.3 \text{ V}$ at 30 mV s^{-1} .

Figure 5.

Voltammetric behaviors of modified electrodes between 0 V to 0.9 V in 100 μM Hoechst 33258 + 0.01 M PBS buffer: bare Au (1), ss-DNA for 1 hr (2), t-DNA and ss-DNA hybrid (3), dsS-DNA for 12 hrs (4), and oxidized dsS-DNA (5). Scan rate: 100 mV s^{-1} . Oxidation was conducted in 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4 between 0 – 1.3 V at 30 mV s^{-1} for three cycles. All DNA concentrations are 1 μM .

Figure 6.

Cyclic voltammograms (A) oxidation of dsS-DNA (pre-immobilized for 12 hrs) in 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4 solution between 0 – 1.3 V at 30 mV s^{-1} for three cycles; (B) continuous scanning of Hoechst bound dsS-DNA-modified gold electrode in 100 μM Hoechst 33258 + 0.01 M PBS, pH 7.4; scan rate 100 mV s^{-1} . The first scan in (B) is the same as curve 5 in Figure 5.

Figure 7.

STM topography (A) and current (B) images of Au (111) surface after being modified by dsS-DNA for 12 hrs, subsequently by voltammetric oxidation, and then binding with Hoechst 33258 for 5 min. scan area 100×100 nm. The bright spot (black arrow) of the inset in topography image (A) corresponds to that spot (black arrow) of the inset in current image (B). Magnified images are shown in topography image (C) and current image (D). Scan area 5.3×5.3 nm. Cross section profiles (c1) and (c2) correspond to the green line and red line in (C), respectively. Similarly, (d1) and (d2) correspond to the green line and red line in (D), respectively. Voltammetric oxidation was conducted in 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4 between 0 – 1.3 V at 30 mV s^{-1} for three cycles.

Figure 8.

Current distributions obtained from STM current images for the modified gold surface at the following modification order: dsS-DNA modification (24 hrs), MCH modification (6 hrs), voltammetric oxidation, and binding with Hoechst 33258. Triplicate images are analyzed at each modification.

Schematic 1.

The sequential modification of the gold working electrode beginning with the bare Au substrate (1), addition of single strand-DNA (ss-DNA) (2), addition of 6-mercapto-1-hexanol (MCH) (3), hybridization with target-DNA (t-DNA) (4), binding Hoechst 33258 (5), and the cyclic voltammetric oxidation of the DNA modified on electrode surface(6).

Table 1

Diffusion coefficients (D_0) for successive modifications on bare gold by ss-DNA, MCH, t-DNA, and Hoechst binding.

Step	Modifications	$D_0^\theta / \text{cm}^2 \text{s}^{-1}$
1	Bare gold	6.59E-06
2	ss-DNA	1.54E-06
3	MCH	--
4	t-DNA	--
5	Hoechst	9.30E-09

^{θ} Calculated from Equation 1 by using cathodic peak currents.

Table 2
Measured EIS parameters for successive modifications on bare gold by ss-DNA, MCH, t-DNA, and Hoechst binding

Step	Modifications	$\bar{R}_O/\Omega$	$\bar{C}_d/\mu F$	$\bar{R}_{ct}/\Omega$	$\bar{Z}_W/\mu\Omega s^{-1/2}$	$f_{K_a}^0 / cm\ s^{-1}$	ϵ_{θ_R}	ψ_{d_f}/nm
1	Bare gold	147.9	1.12	1120	80.4	1.51E-02	-	-
2	ss-DNA	150.2	9.64	37360	68.7	4.54E-04	0.970	2.31
3	MCH	145.7	2.92	95930	63.9	1.77E-04	0.988	7.63
4	t-DNA	184.5	3.76	255400	32.3	6.63E-05	0.995	5.92
5	Hoechst	147.9	3.75	32360	72.7	5.24E-04	0.965	5.93

$\bar{Y}$ Calculated based on the modified Randles equivalent circuit

f Calculated using Equation 2

ϵ Calculated using Equation 3

ψ Calculated using Equation 4.

Table 3

Diffusion coefficients (D_0) for successive modifications on bare gold by dsS-DNA, MCH, Hoechst, CV oxidation, and Hoechst binding.

Step	Modifications	$D_0^\phi/\text{cm}^2 \text{ s}^{-1}$
1	Bare gold	6.59E-06
2	dsS-DNA	3.84 E-06
3	MCH	--
4	Hoechst (1)	9.30 E-09
5	CV Oxidation	7.26 E-06
6	Hoechst (2)	--

^{ϕ} Calculated from Equation 1 by using cathodic peak currents.

Table 4
Oxidation/Reduction charges for cyclic voltammetric oxidations of different modified gold electrodes.

	Modifications	Q _{O1}	Q _{R1}	Q _{O2}	Q _{R2}	Q _{O3}	Q _{R3}
A	Hoechst 33258 (5 min)/MCH (6 hrs)/dsS-DNA (24 hrs)/Au	102.04	47.38	77.18	48.33	74.47	47.94
B	dsS-DNA (24 hrs)/MCH (6 hrs)/Au	120.33	47.41	96.77	48.34	92.80	47.96
C	dsS-DNA (12 hrs)/Au	119.08	47.49	89.42	47.48	85.72	48.50

(A)-(C) were conducted in 5 mM Na₂HPO₄ + 5 mM NaH₂PO₄ between 0 – 1.3 V at 30 mV s⁻¹; QO1 (QR1), QO2 (QR2M), and QO3 (QR3) are the charges for the 1st, 2nd, 3rd oxidation (reduction) peaks, respectively. The columns QO1, QO2, and QO3 can be broken down to three columns shown in Table s5. Unit of charge: μC .