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Enzyme-Modulated Cleavage of dsDNA for Studying Interfacial Biomolecular Interactions

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Abstract: This work describes the chemistry and methodology for constructing multilayers of bis-biotinylated dsDNA on metal substrates after enzyme cleavage and demonstrates its use for amplified microgravimetric and impedimetric analyses of anticancer drug, cisplatin. Specific chemical modification of dsDNA prior to immobilization was achieved via a bisulfite-catalyzed transamination of cytosine after endonuclease cleavage of plasmid DNA. The specificity of the reaction of cytosine residues at ss- versus dsDNA loci after endonuclease cleavage was characterized using circular dichroism, mass spectrometry, and absorption spectrophotometry. The biotinylated dsDNA consisting of 2961 base pairs was then used as a ligand at avidin-modified gold electrodes. Ac impedance spectroscopy and quartz crystal microbalance measurements clearly showed that the response to cisplatin increased linearly with target concentrations. The impedance spectroscopy resulted in a detection limit of 1 nM and a surface density of 4.8×10^{13} molecules/0.1 cm^2. The immobilization of dsDNA on surfaces is a significant improvement over existing approaches in that it enables the attachment of long pieces of unmodified double-stranded DNA via a simple biotinylation step. The immobilization technique provides a generic approach for dsDNA-based sensor development and for monitoring DNA−analyte interactions.

Introduction

Nucleic acids provide a powerful means of recognizing and monitoring many compounds of medical or environmental importance. Major research activities in these areas are directed toward the design of sequence-selective biosensors through hybridization effects. Nucleic acid biosensors use substrates (e.g. quartz crystal, electrodes, fused silica, gold nanoparticles, gold films and optical fibers substrates) to immobilize the recognition elements.[1] The instability of DNA recognition elements, when immobilized onto these surfaces, appears to be at a marked disadvantage with respect to sensitivity and the time taken for the reaction to proceed to equilibrium.[2]

A novel approach that may potentially be used to study biomolecular interactions including the determination of affinity constant and kinetic binding parameters is described. Although hybridization assays will continue to be important for routine applications such as gene identification and genetic screening, difficulties regarding sensitivities and reaction times could be overcome with alternative amplification steps requiring no DNA hybridization. With the growing quest to construct supramolecular architectures associating several chemical functionalities

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in a spatially ordered manner,[3−5] a stepwise design of multilayer DNA probes could be achieved by means of enzyme-modulated cleavage of dsDNA onto underpotentially deposited silver monolayers. A key component of developing nucleic acid biosensors is the immobilization of biomacromolecules of choice onto suitable substrates. The protocol for attaching short nucleic acid probes, proteins and oligonucleotides (usually single-stranded) ranges from the self-assembly of thiols or disulfide moieties on gold to the use of adjunct layers of polymers such as polyacrylamide.[6] It is also possible to take advantage of the irreversible adsorption of protein monolayers onto the quartz crystal microbalance (QCM) gold electrode surface.[7] In its various forms in particular, avidin has been used to immobilize biotinylated probes. It can bind up to four molecules of biotin with high affinity (dissociation constant $K_d = 10^{-15}$ M) and is involved with one of the strongest known protein−ligand interactions.[8] Despite the wide applications of this combination in analytical biochemistry, it has not been used extensively to study the bimolecular interactions at sensor surfaces.[9]

In this contribution, we report the chemistry and methodology for constructing bis-biotinylated dsDNA multilayers on metal substrates after enzyme cleavage and demonstrate its use for amplified microgravimetric and impedimetric analyses of the anticancer drug, cisplatin. Ac impedance spectroscopy and quartz crystal microbalance measurements clearly showed that the response to cisplatin increased linearly with target concentration. The impedance spectroscopy resulted in a detection limit of 1 nM and a surface density of 4.8×10^{13} molecules/0.1 cm^2.

Experimental Section

Materials. Avidin and biocytin hydrazide was obtained from Pierce (Rockford, IL). The 2961bp pBluescript II SK(−) phagemid dsDNA vector and T4 DNA ligase 300 units were purchased from Stratagene Inc. (La Jolla, CA). Two oligonucleotides (oligo 1: 5′-TATGCCCAAA-TTTCGCC, and oligo 2: 5′-biotin (12 atom linker)-CGGCGAAATTT-GGGCATAGGATCC-3′) were obtained from SYNTHEGEN (Houston, TX) and were resuspended in 100 μL of water; all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All chemicals were used as received from vendors without further purification.

Instruments. A Potentiostat/Galvanostat (EG&G 263A) and an EG&G lock-in amplifier (model 5210) were used for electrochemical measurements. A QCA 917 quartz crystal analyzer (Seiko EG&G) was used for the microgravimetric QCM experiments. Mass spectrometry assay was conducted at the Biotechnology Laboratory, Cornell University, Ithaca, NY, using Bruker Biflex III MALDI-TOF. Circular dichroism (CD) spectra were obtained from a J-810 (JASC) Spectropolarimeter. A UV160U UV−visible recording spectrophotometer (Shimadzu, Columbia, MD) was used for absorption measurements.

Methods. (a) Circular Dichroism Measurements. The far-UV CD spectra were recorded at 25 °C with a J-810 Spectropolarimeter (JASC) in the wavelength range between 190 and 350 nm using a 1 cm path length quartz cell. The spectra were background corrected.

(b) DNA Hybridization. The oligonucleotides were hybridized as previously described.[10] 10 μL of oligo 1 and 10 μL of oligo 2 were mixed in the hybridization buffer to a final volume of 1 mL. Different buffer systems used include: ammonium acetate buffer, pH 7.2; 2 X SSC buffer (pH 7.0), 10 mM Tris-Cl (pH 7.5, 0.5 M NaCl) and 2 X SSC buffer (300 mM NaCl, 30 mM sodium citrate (pH 7.0)). The mixture was heated at 90 °C for 10 min, allowed to cool to hybridization temperature (37 °C), and incubated at the hybridization temperature for 2 h without shaking. The samples were then dialyzed overnight against Nanopure water using MWCO 6000−8000 tubing and were freeze-dried for 24 h before analysis by mass spectrometry. Ligation step contained 70 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP (pH 7.0) buffer supplemented with 50 μg/mL BSA, $(1-2) \times 10^3$ cpm polynucleotide substrate (200 ng), and 0.2 units of T4 DNA ligase.

(c) Quartz-Crystal Microbalance Measurements. An open-circuit system was used for QCM measurements in which only the gold-coated quartz crystal working electrode was connected but in the absence of auxiliary and reference electrodes. The Au-coated quartz crystals (AT-cut, 9 MHz) of 0.2 cm^2 geometric area per face were obtained from EG&G instruments (Princeton Applied Research). The resonant frequency was determined using QCA 917 quartz crystal analyzer (Seiko EG&G).

(d) Electrochemical Measurements. A 2-mL, three-electrode electrochemical cell with an EG&G lock-in amplifier, model 5210, combined with an EG&G 263A potentiostat was used for the electrochemical measurements. Data acquisition was conducted using EG&G M270 and M398 software for cyclic voltammetry and impedance measurement, respectively. The impedance measurements were carried out in the presence of 0.1 M phosphate buffer (pH 7.0) or a 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox probe at the formal potential of the system ($E^o = 200$ mV) using alternating voltage of 10 mV. The frequency range was from 100 mHz to 10 kHz. The impedance spectra were plotted using complex plane diagrams (Nyquist plots). All measurements were carried out at ambient temperature (22 ± 2 °C).

Results and Discussion

Of all the bases in DNA, cytosine is the most susceptible to nucleophilic attack.[11] We have utilized a method involving the nucleophilic attack of cytosine, which was described by Shapiro et al. for generating specific chemical modification of nucleic acids.[12] The reaction consists of a bisulfite-catalyzed transamination of cytosine and cytidine, resulting in N4-substituted labels. The transamination reaction was reported to be single-strand specific[13] and was widely used in analytical biochemistry for the preparation of nonisotopic probes for nucleic acid

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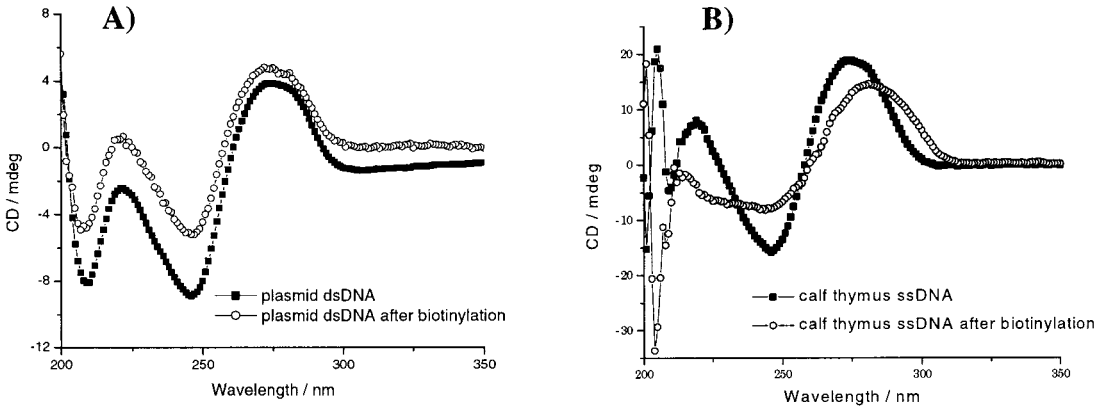
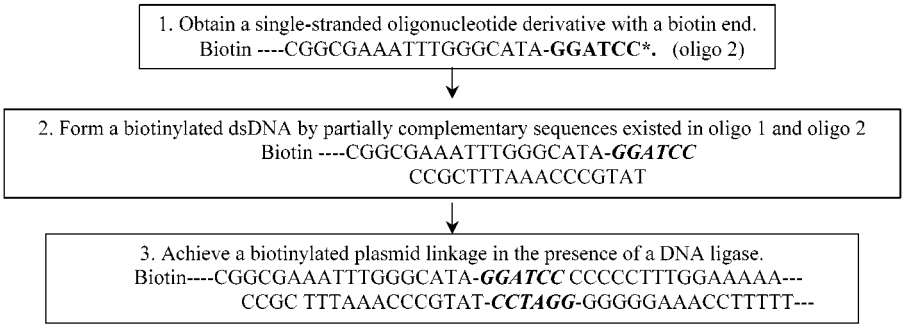


Figure 1. Circular dichroism spectrums for (A) plasmid dsDNA and (B) calf thymus ssDNA using a J-810 (JASC) spectropolarimeter.

Scheme 1. Schematic of the Biotinylation of Double-Stranded Plasmid



* CCCCCTTTGGAAAAA- - -*CCTAGG*-GGGGGAAACCTTTTT- - -obtained after enzyme cleavage of plasmid DNA by *Bam*HI digestion. (The recognition sequence for B*am*HI is 5′-G/GATCC-3′.)

hybridization assays. However, to our knowledge, this reaction has not been reported for DNA immobilization on solid substrates or for biosensor purposes. To obtain the biotinylated dsDNA, we linearized a 2961bp pBluescript SK(−) phagemid dsDNA vector (from Stratagene) after digestion with *Bam*HI, and carried out the transamination reaction for 24 h at 37 °C to have a high reaction rate. The linearization process yielded free cytosines in the double-stranded sequences that are identical to the original. The specific recognition sequence of 5′-G↓G-A-T-C-C-3′ was thus obtained.[14a] This process may be repeated to continue the enrichment of the sequence. Assuming that two biotin bound to each avidin in the array, products accumulate at a rate of 2^n, where the enrichment factors of the order of 10^7 from 30 cycles of amplification could be realized within a time span of hours.[14b]

It was possible to verify the restriction cleavage of dsDNA using HPLC and electrophoresis in 1.0% agarose gels.[15] These methods show that the digested plasmids resulted in the expected bands from known restriction sites on the plasmid map (Supporting Information). The cleavage of this type yielded DNA fragments having 3′ and 5′ overhangs or "sticky ends" that were then used for the single-strand specific transamination reaction.[15] Evidence that the reaction was single-strand specific was obtained from CD spectra obtained before and after the transamination reaction. Figure 1 shows the CDs of plasmid dsDNA and ssDNA before and after biotinylation. The CD spectra of plasmid dsDNA showed few differences, while those of ssDNA changed dramatically with a red-shift, suggesting a change in the secondary structure caused by biotinylation.

Characterizing the Specificity of the Reaction of Cytosine Residues at ss- versus dsDNA Loci. Although the transamination reaction of cytosine residues with biotin after endonuclease cleavage has been reported to be single-strand specific, the structural characterization has never been reported. To understand the nature or specificity of the reaction of cytosine residues at ss versus dsDNA loci, we examined the hybridization reaction following the description shown in Scheme 1. To achieve these steps, we obtained two oligonucleotides containing biotinylated cytosines that were derivatized at a small number of sites (oligo 1: 5′-TATGC-CCAAATTTCGCC-3′, FW 5105 and oligo 2: 5′-biotin (12-atom linker) −CGGCGAAATTTGGGCATAGGATCC-3′, FW 7925). These oligonucleotides were purified using reverse-phase HPLC and characterized by mass spectrometry. The oligomers were then annealed to a partial complement such that one end of the resulting duplex was blunt-ended, and the other was sticky-ended corresponding to the recognition sequence of a commercially available restriction enzyme, *Bam*HI. The sticky end was annealed to a corresponding sticky end in the linearized plasmid DNA in the presence of a T4 DNA ligase. The linearization was achieved using *Bam*HI such that only one sticky end complementary to the small duplex was present in the large duplex and was attached covalently with DNA ligase. Scheme 1 demonstrates the methodology of such a buildup.

Finally we characterized the reaction using matrix-assisted laser desorption ionization time-of flight (MALDI-TOF) spectrometry and time-course absorbance measurements using both

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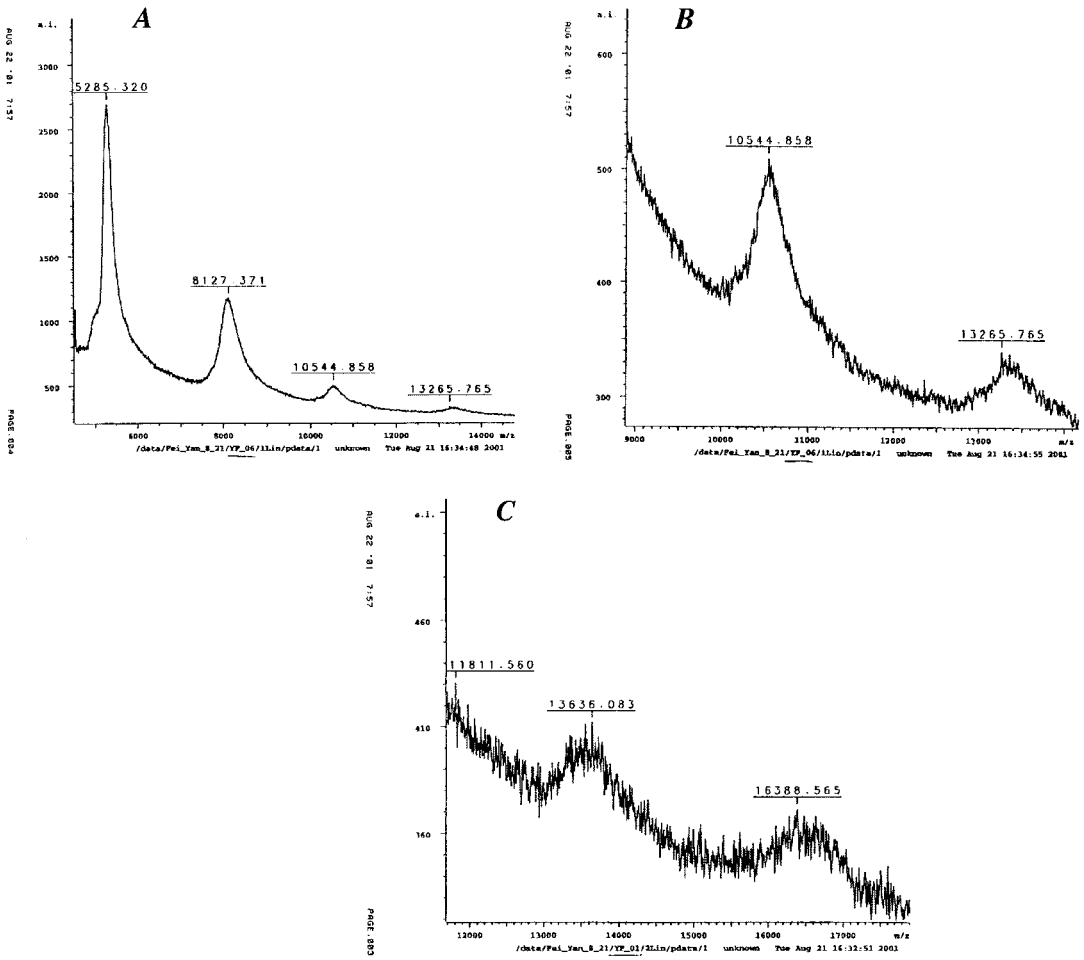

Figure 2. (A) MALDI-MS spectrum of the hybridization product resulting from the reaction of oligo1 and oligo2. (B) The magnified portion corresponding to the presence of dsDNA as shown in A. (C) Spectrum of the hybridized product in 0.1 M ammonium acetate buffer (pH 7.2).

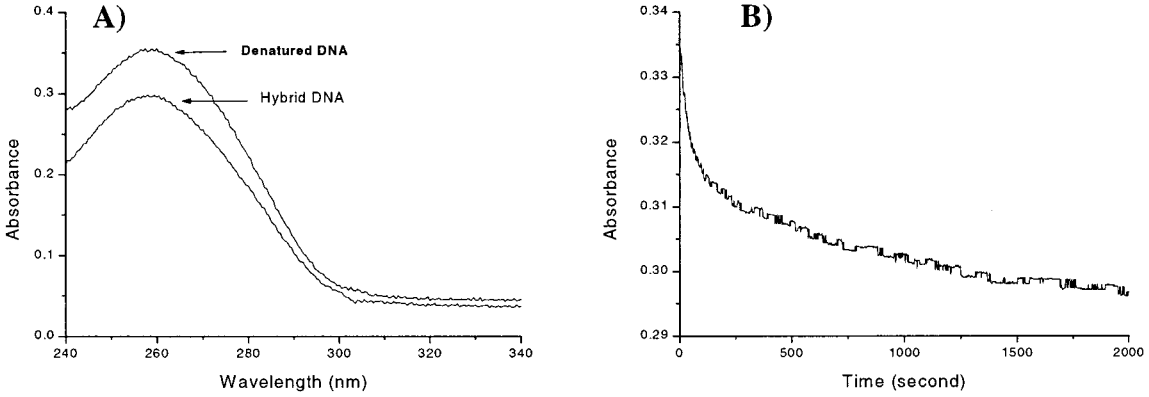

Figure 3. (A) Absorbance spectra of denatured DNA (at temperature 90 °C) and hybrid DNA (room temperature). (B) Time course absorbance monitoring as the temperature decreases drastically from 90 °C to room temperature. The absorbance results were recorded in an increment of 1 s for a total 2000 s.

denatured and hybrid DNA from 90 °C to room temperature. Considerable interference was noticed with the use of phosphate and Tris-Cl buffers due to the presence of sodium or possible denaturation during desalting process. We then considered using 0.1 M ammonium acetate buffer (pH 7.2). Figure 2A shows the MALDI-TOF data after desalination. The small, diffuse peaks at ca. 13 kDa was noticeable, corresponding to the presence of double-stranded DNA. The magnified figure is shown in Figure 2B. While ammonium acetate is ideal for MS analysis, it does not provide strong enough counterion to allow a high percentage of hybridization. The in situ absorbance monitoring at 260 nm wavelength was used to further confirm the complementary sequence of the two oligonucleotides when heated to 90 °C and then allowed to cool to room temperature. This was considered because with nucleic acids, getting rid of too much sodium would cause the strands to denature.

We followed the reaction by monitoring the absorbance at 260 nm. It is expected that the single-stranded pieces will generate very high absorbance, which will gradually decrease as the strands combine. As shown in Figure 3, both of the denatured oligonucleotides absorbed light strongly at the 260-nm wavelength region. However, the absorption of light was significantly reduced when the bases packed into a helical structure. This phenomenon, called *hypochromism*, results from

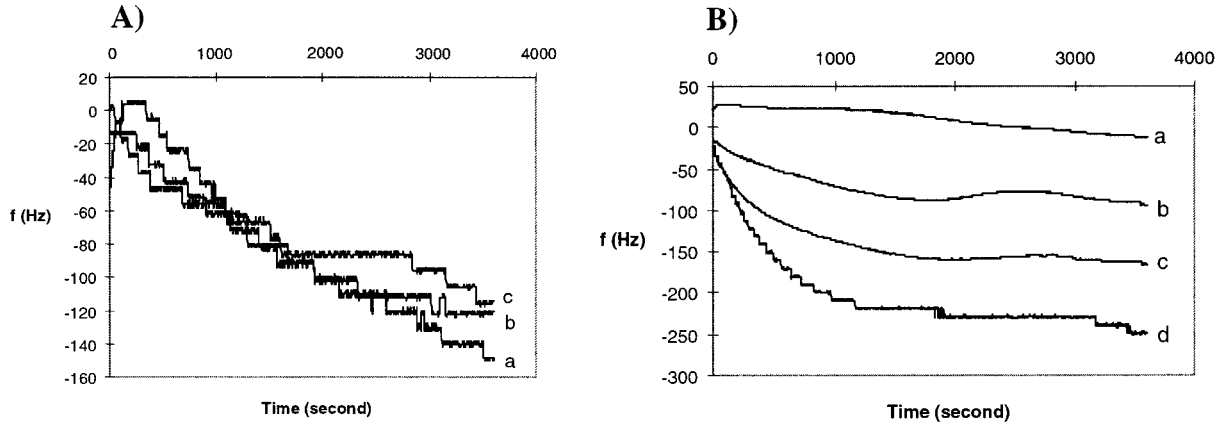

Figure 4. (A) QCM frequency change vs time for the serial docking of biotinylated pBluescript SK(−) phagemid dsDNA vector from PBS buffer onto an avidin-modified quartz crystal, (a), (b), and (c) for repeated trials; (B) In situ monitoring of frequency change when cisplatin (25 μl, 1 mM) was injected to (a) bare quartz crystal, (b) 1A1B, (c) 2A2B, (d) 3A3B functionalized substrate. A: Avidin; B: Biotinylated pBluescript SK(−) phagemid dsDNA vector.

close interaction of the light-absorbing groups, the purine and pyrimidine rings.[16] Furthermore, as evident from Figure 3, the two oligonucleotides showed complementary sequences as the absorbance decreased dramatically when the temperature changed from 90 °C to room temperature within a few minutes. These experiments thus confirmed that a hybrid was formed under the current conditions.

The biotinylated dsDNA was later used as a ligand at a QCM gold electrode containing avidin on a freshly electrodeposited silver submonolayer. It has been reported that underpotentially deposited films of copper and silver on polycrystalline gold substrates provide a densely packed and highly stable substrate for self-assembly of adsorbed alkanethiolate monolayers.[17] Furthermore, submonolayer amounts of underpotentially deposited silver provide an enhanced stability for self-assembled monolayers on polycrystalline gold substrates.[18] We utilized the potential-modulation method based on chronoamperometry to produce uniform coverage of silver.[19] The silver was electrochemically deposited onto polycrystalline gold QCM electrodes in the absence of additional supporting electrolyte to avoid anion-discharge problems. This approach thus generated a fresh surface while increasing the reproducibility of avidin monolayer formation, which is a key factor for an efficient biotin binding.[9a]

Experiments to form dsDNA multilayers were performed thereafter using avidin bis-biotinylated dsDNA. The average frequency change (±standard deviation) recorded for the self-assembly of avidin monolayer for triplicate experiments was −96 ± 12 Hz. The experimental frequency change was consistent with the formation of a monolayer of close-packed avidin in a flat orientation.[20] The docking of biotinylated dsDNA was confirmed by a decrease in frequency. The average frequency change (±standard deviation) corresponding to that of multilayer formation structures was −127 ± 9 Hz. (Figure 4A). The proposed multilayer formation was confirmed with in situ monitoring of cisplatin binding onto the surface-immobilized dsDNA and a frequency increase in the multilayer growth (see Figure 4B). A duplicate experiment showed an excellent reproducibility for the interaction between cisplatin and surface-bound dsDNA, and a standard deviation of less than 5% for 10 repetitive measurements.

Comparative analysis of cisplatin detection was performed using electrochemical impedance spectroscopy (EIS). EIS has been used to monitor the interfacial properties of layered electrodes upon formation of oligonucleotide complexes and to precipitate enzymatic product.[21] We have previously used EIS to study antibody−antigen interactions on conducting polymer-modified electrodes and other bioaffinity surfaces.[22] In EIS experiments, a sinusoidal voltage signal of small amplitude was applied to dsDNA-modified electrode, and the current response was measured under potentiostatic control. The impedance was calculated as the ratio of the system voltage phasor and the current phasor generated by the frequency response analyzer during the experiment. Cisplatin interaction was detected as a shift in impedance in the presence of an electroactive redox probe in solution (i.e., $Fe(CN)_6^{3-/4-}$). The interfacial electron-transfer resistance (R_{et}) of cisplatin upon binding to the biotinylated dsDNA was monitored.

The typical shape of ac impedance spectra (presented in the form of a Nyquist plot) includes a semicircle followed by a straight line. In general, two frequency regions can be distinguished in the presence of the electroactive species. The semicircle portion observed at higher frequencies corresponds to the electron-transfer-limited process, while the straight line represents the diffusion-limited, electron-transfer process. The intercepts of the semicircle with the Z_{re}-axis (real impedance) at high and low frequencies correspond to R_s (solution resistance) and $R_s + R_{et}$ respectively. Thus, the diameter of the semicircle is equal to R_{et} while increase in diameter can be used to correlate the serial assembly of the insulating layers to the electrodes.[21,23] For example, at the dsDNA-modified gold-quartz electrode, R_{et} recorded was 907.6 Ω , whereas R_{et} increased to 1125.8 Ω upon association of the complex between avidin and biotinylated plasmid dsDNA. These results are consistent with the fact that

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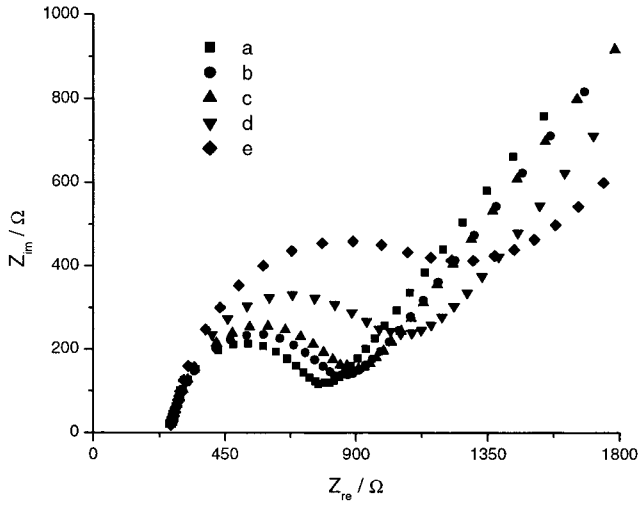

Figure 5. Nyquist diagram (Z_{im} vs Z_{re}) for the ac impedance measurements in the presence of 10 mM $Fe(CN)_6^{3-/4}$ at 1A1B modified gold electrode for cisplatin concentration range between 10 nM and 1 μM.

the negative charge associated with the phosphate groups of the plasmid increases after the multistep organization of the assembly. Figure 5 shows the interaction of cisplatin with surface-modified dsDNA. Cisplatin was found to inhibit the interfacial electron transfer with increasing concentrations. The electron-transfer resistances obtained in the presence of $Fe(CN)_6^{3-/4-}$ after cisplatin accumulation were 1003.2, 1018.1.9, 1222.6, 1582.1.5, and 2163.8 Ω for 10, 50, 250, and 500 nM and 1 μM cisplatin, respectively.

Conclusions

In summary, we have demonstrated a novel methodology for forming a multilayered assembly composed of avidin and biotinylated dsDNA. Specific chemical modification of the dsDNA prior to immobilization was achieved via a bisulfite-catalyzed transamination of cytosine after endonuclease cleavage of plasmid DNA. The specificity of the reaction of cytosine residues at ss versus dsDNA loci after endonuclease cleavage was characterized using mass spectrometry and absorption spectrophotometry. Using two oligonucleotides containing biotinylated cytosine, hybridization reactions were examined with MALDI-MS. The results showed that the two starting oligonucleotides were present in the final spectra, thus confirming the formation of nucleic acid hybrid formation. The resulting biotinylated dsDNA supramolecular multilayer system was formed through strong and specific avidin−biotin interaction. The assembly showed an enhanced sensitivity for small-molecule (e.g., cisplatin) binding. This indicated that the sensitivity might be tuned by controlling the number of layers, which could provide hybridization approach for some specialty applications. Such an oriented-dsDNA sensing element may be useful for the study of the kinetics of interactions of small molecules with dsDNA. Apart from QCM and EIS, similar strategies can be used for specific DNA immobilization utilizing other transduction mechanisms such as thermometric, optical, and PCR technologies.

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Supporting Information Available: Experimental selectivity details including agarose gel electrophoresis and HPLC identification of uncut and linearized pBluescript SK(−), surface characterization of each step involved in dsDNA immobilization using SEM with EDAX elemental analysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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