An Electrochemically Controlled Microcantilever Biosensor

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ABSTRACT: An oligonucleotide-based electrochemically controlled gold-coated microcantilever biosensor that can transduce specific biomolecular interactions is reported. The derivatized microcantilever exhibits characteristic surface stress time course patterns in response to an externally applied periodic square wave potential. Experiments demonstrate that control of the surface charge density with an electrode potential is essential to producing a sensor that exhibits large, reproducible surface stress changes. The time course of surface stress changes are proposed to be linked to an electrochemically mediated competition between the adsorption of solution-based ions and the single- or double-stranded oligonucleotides tethered to the gold surface. A similar potential-actuated change in surface stress also results from the interaction between an oligonucleotide aptamer and its cognate ligand, demonstrating the broad applicability of this methodology.

INTRODUCTION

Nanoscale sensors, which can rapidly provide label-free biomarker detection with high sensitivity, have been explored for application to low-cost point-of-care diagnostics, biological warfare agent detection, drug-discovery platforms, and observations of single-molecule biochemical processes in real time. Most nanoscale sensor systems transduce surface stress changes using mechanical devices1–6 or conductance using field-effect transistor concepts.7,8 In both methods, the largest signals that result from a specific binding event are generated by changes in surface charge density, leading to changes in stress detected by microcantilever (cantilever) sensors9–11 or changes in the conductivity of nanowires.7,8 These transduction mechanisms point to the delicate balance between the dual role of the sensor surface which serves as both a support for the functionalization chemistry and as a transducer for biochemical recognition events. Covering the entire sensor surface with (bio)chemical receptors will, however, often reduce charge density changes close to the surface and thus will reduce the magnitude of an analytical signal. The magnitude of the analytical signal can also be compromised by surface contamination, and time-dependent contamination can lead to apparent aging of the sensor.

Although many cantilever systems that detect biological targets have been reported,1–6 applications have been limited by problems with both the reproducibility and magnitude of the sensor signal. Given that understanding the origin of signal (surface stress) generation is critical to solving these measurement problems, a number of factors contributing to stress changes have been identified or modeled in the case of gold-coated cantilevers. Factors intrinsic to the coating on the gold, including both attractive and repulsive intermolecular interaction5,12,13 and configurational entropy changes,14 have been described as contributors to cantilever-based surface stress measurements. The resulting experimental challenges, including poor signal-to-noise ratios, cantilever-to-cantilever variation, and sensitivity to conditions such as temperature and sample constituents, have led to elegant engineering approaches that increase the precision and/or accuracy of surface stress measurements. These engineering approaches include the simultaneous measurements of multicantilever arrays and differential measurements that reject common mode noise (reviewed in refs 15 and 16).

Although many factors contribute to surface stress changes in a cantilever biosensor experiment, understanding and controlling the relative importance of these factors is difficult and their concomitant deconvolution has generally proven to be intractable. However, the surface charge density has been identified as a dominant factor in determining the deflection of metal-coated cantilevers9–11,13 and can be orders of magnitude larger than the factors associated with the surface coating itself.13 Surface stress has been shown to be sensitive (up to 1 N m/C) and is directly proportional to the surface charge density.17 Changes in the applied potential near the potential of

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zero charge (PZC), in the presence of either adsorbing (Cl\(^-\), Br\(^-\)) or nonadsorbing electrolyte (ClO\(_4\)^{-}), lead to large changes in surface stress.\(^{17}\) We have thus sought to develop a robust methodology where the principal measurement is the change in the surface stress signal and its temporal features as a function of an applied potential, both in the absence and presence of an analyte of interest. By building on our prior experience\(^{13,18,19}\) and that of others,\(^{20,21}\) we have explored the use of the electrochemical control of gold-coated cantilevers. We demonstrate that programmed square wave potential changes applied to nucleic acid-derivatized cantilevers yield characteristic time-dependent surface stress signatures that are related to the quantity of surface-tethered nucleic acid both before and after hybridization with complementary oligonucleotide or after ligand capture by an oligonucleotide aptamer.

## EXPERIMENTAL SECTION

### Cantilever Preparation

Silicon cantilevers (type CSC12 tipless, MikroMasch, Bulgaria) are coated with titanium by thermal evaporation to a depth of 2 nm at a rate of 0.04 nm/s and with gold to a depth of 100 nm at a rate of 0.14 nm/s at a pressure <5.0 \times 10^{-6} Torr and 130 \pm 20 °C (Thermionics model VE90 vacuum evaporator, Thermionics Laboratories, USA). A layer of Apiezon wax (Apiezon Wax W, APWK, Apiezon, USA) dissolved in trichloroethylene (TCE) (Fisher Scientific, USA) is deposited on a portion of the gold surface to yield a gold electrode surface area of 1.0 mm\(^2\). Gold-coated cantilevers are then electrochemically cleaned via repetitive electrochemical cycling (−0.8 to 1.3 V vs saturated Ag/AgCl) in KClO\(_4\) solution (50 mM) at a scan rate of 20 mV/s until a repeatable voltammogram is obtained (BASI CV-50W voltammetric analyzer, Bioanalytical Systems, Inc., USA). Cleaning of the cantilever surface in this manner was performed immediately before each experiment.

### Electrochemical Methods

The cleaned gold-coated silicon cantilever (Figure 1A) was clamped with a copper clip to a Kel-F rod and partially immersed in a buffer containing TN buffer (10 mM Tris-HCl, 50 mM NaCl, pH 7.4) in an argon-saturated 6 mL evaporation to a depth of 2 nm at a rate of 0.04 nm/s and with gold to a depth of 100 nm at a rate of 0.14 nm/s at a pressure <5.0 \times 10^{-6} Torr and 130 \pm 20 °C (Thermionics model VE90 vacuum evaporator, Thermionics Laboratories, USA). A layer of Apiezon wax (Apiezon Wax W, APWK, Apiezon, USA) dissolved in trichloroethylene (TCE) (Fisher Scientific, USA) is deposited on a portion of the gold surface to yield a gold electrode surface area of 1.0 mm\(^2\). Gold-coated cantilevers are then electrochemically cleaned via repetitive electrochemical cycling (−0.8 to 1.3 V vs saturated Ag/AgCl) in KClO\(_4\) solution (50 mM) at a scan rate of 20 mV/s until a repeatable voltammogram is obtained (BASI CV-50W voltammetric analyzer, Bioanalytical Systems, Inc., USA). Cleaning of the cantilever surface in this manner was performed immediately before each experiment.

### Electrochemical Methods

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### Blocking of the Gold Surface with 6-Mercapto-1-hexanol (MCH)

After deflection measurements were performed on the clean gold-coated cantilever, it was incubated for 1 h in a 1.0 mM ethanolic solution of MCH (Fisher Scientific, USA). Before an additional deflection measurement was performed, the cantilever was rinsed three times alternately with ethanol and 50 mM NaCl. Deflection measurements were performed by initially holding the surface potential at \(V_{\text{appl}} = −200\) mV for 2 min in TN buffer and then switching to +200 mV.

### DNA Oligonucleotide Functionalization and Hybridization Assays

Experiments were performed using a 25-mer thiolated single-strand oligonucleotide (probe-oligo) with sequence 5′-HS-SC\(_6\)-TCCGATTCCTACAGAATGGGATCGC-3′ (IDT Technology, USA) diluted to a final concentration of 100 μM in 40 μL of TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8.0). The probe-oligo is desalted by incubating with 0.1 M dithiothreitol (DTT) (Sigma-Aldrich, USA) for 30 min and then purified using a NAP-5 column filter (GE Healthcare, U.K.) with TN buffer. The purity of the filtered probe-oligo solution was confirmed with ESI mass spectrometry. In all experiments, the concentration of the probe-oligo was adjusted to a final concentration of 3.0 μM. Two methods are used to functionalize the cantilever with thiolated oligonucleotides. In the single-step functionalization procedure, the gold-coated cantilever is incubated in the probe-oligo solution for 30 min and then rinsed three times with Milli-Q water (resistivity <18 M\(\Omega\) cm, Millipore, USA). In the multistep functionalization procedure, the clean gold-coated cantilever was repetitively incubated in the probe-oligo solution for 5 min periods. The cantilever is subjected to a ±200 mV square wave potential regime (\(t_{\text{cycle}} = 10\) min; three cycles) after each incubation. The surface-tethered probe-oligo is hybridized with a complementary 25-mer oligonucleotide (target-oligo) whose sequence is 5′-GGCCATTTCCACATTGAGATCGC-3′ (IDT Technology, USA) to a final concentration of 3.0 μM in 100 μL of TE buffer. Hybridization of the target-oligo to the probe-oligo is performed by incubating the functionalized gold-coated cantilever in 3.0 μM target-oligo in TE buffer solution at 48 °C (the calculated melting temperature of the oligonucleotide sequence) for 15 min and cooling to room temperature to avoid mismatch binding. Following hybridization measurements using a protocol involving a periodic square wave potential of ±50 mV and a standard switching interval (\(t_{\text{cycle}} = 10\) min). The potential window of ±50 mV was based on the potential range of Cl\(^-\) adsorption on Au(111).\(^{25,26}\)

### Figure 1

Schematic diagram of the electrochemical cell and electrochemically induced surface stress. (A) The silicon cantilever coated with evaporated titanium (2 nm) and gold (100 nm). Insulating Apiezon wax selectively coats areas of the cantilever to control the area of the gold-coated cantilever surface which is exposed to the electrolyte solution. The cantilever working electrode is held in the electrochemical cell using a copper plate spring. An Ag/AgCl (saturated) reference electrode and a platinum wire counter electrode are used. In each experiment, the gold surface is electrochemically cleaned in 50 mM KClO\(_4\) (see Experimental Section) immediately before the measurement. The cantilever deflection is measured optically. (B) Deflections are induced by changes in the surface charge density, mediated by adsorption and desorption of ions in response to changes in the applied potential. The first schematic shows the induction of compressive stress (expansion of the gold surface) by the adsorption of component(s) of the electrolyte. The second schematic shows the induction of tensile stress (contraction of the gold surface) when adsorbed ions are released from the gold surface.
ization, the gold-coated cantilever is thoroughly rinsed three times with Milli-Q water to remove any physisorbed target-oligo from the gold surface.

**Oligonucleotide Aptamer-Ligand Binding Assay.** Experiments are performed using a 24-mer thiolated single-strand oligonucleotide aptamer (probe-aptamer) with sequence 5'-HS-S2-GATCGAAACGATTGGCCTTTGCAGTC-3' (IDT Technology, USA) diluted to 100 μM in 40 μL of TE buffer. This sequence has been previously shown to bind to t-Arm (N-argininamide dihydrochloride, Sigma-Aldrich, USA) with a dissociation constant of 220 μM and a melting temperature of 42 °C. The gold-coated cantilever is functionalized with desalted probe-aptamer so that its stress pattern response matches that of the ca. 12% surface coverage of probe-oligo obtained by the multistep functionalization method. The aptamer binding ligand, t-Arm, is diluted to a final concentration of 1.0 mM with TN buffer and adjusted to pH 7.0. After the detection of the probe-aptamer-coated gold-coated cantilever is measured, the cantilever is incubated in an t-Arm solution for 30 min and thoroughly rinsed with Milli-Q water three times at room temperature.

**Measurement of Oligonucleotide Surface Coverage.** Following the completion of a cantilever deflection experiment, a gold-coated cantilever is incubated in a 1.0 mM ethanolic solution of 12-ferrocenyl-1-dodecanethiol (Fc(CH2)12SH; Fc = (η5-C5H5)Fe(η5-C5H5)) for 1 min. Fc(CH2)12SH occupies available gold sites on the cantilever surface. The signature redox peaks of surface-confined Fc(CH2)12SH are determined by cyclic voltammetry (0 to 700 mV vs saturated Ag/AgCl; scan rate 20 mV/s) in 0.1 M NaClO4. The coverage of Fc(CH2)12SH is determined from the charge associated with the reduction peak in the cyclic voltammogram. The gold electrode area is assumed to be equal to its geometric area.

## RESULTS AND DISCUSSION

An electrochemical cantilever cell (Figure 1A) was used to examine the relationship between applied potential (Vapp) and the cantilever deflection in the presence of ions (Cl−) that are known to adsorb to gold in a potential-dependent manner (Figure 1B). These studies use an applied potential range over which Cl− adsorption changes. However, the release of the potential from fixed to open circuit conditions and monitoring the ensuing cantilever deflection as a function of the changing open circuit potential (OCP) reveals a little- appreciated challenge in the use of cantilevers as sensors: very large surface stress changes (σt up to 100 mN/m) occur as the open circuit potential moves toward the equilibrium rest potential value of the electrode (Figure 2A). Because the surface charge density (and thus surface stress) of the gold-coated cantilever is controlled by the electrode potential and the nature of the adsorbates (whose affinity for the electrode surface is invariably potential dependent), only fixed potential conditions can ensure stable and reproducible values of surface stress with time. Operating at the OCP effectively reduces the cantilever to serving as a complex voltmeter. Many factors determine the OCP of an electrode near its potential of zero charge, including the Nernstian potential of the solution and the speciation and quantity of adsorbates, including those making up the buffer and electrolyte. Changes in the adsorbate species and/or their quantity lead to a change in the OCP and thus the extent (and direction) of cantilever deflection.

To evaluate the contribution of surface charge density changes to the cantilever deflection, we compared the changes in surface stress resulting from the application of a series of ±200 mV potential steps to a clean gold-coated cantilever and one coated with 6-mercaptop-1-hexanol (MCH) (Figure 2B). A defined Vapp has a significant effect on the surface stress value because Vapp = ±200 mV on clean gold under the experimental electrolyte conditions leads to a surface stress change of 160 mN/m. Tightly packed MCH blocks ion adsorption and reduces the capacitance of the electrode. Consistent with previous findings, the amplitude of the surface stress change for the MCH-coated gold was greatly reduced (to 12 mN/m) from that observed for clean gold. The cleanliness of the gold surface is thus critical to obtaining large, reproducible signals. To evaluate how controlling the surface charge density could be used in sensor applications, the effect of applying periodic square wave potentials to the functionalized gold-coated cantilever was studied. Cantilevers, clean gold-coated and...
gold-coated functionalized with 25-mer thiol-terminated oligonucleotides (probe-oligo) (Figure 3), were switched between ±200 mV with an interval time of 10 min. The clean gold-coated cantilever exhibited a surface stress time course that was in phase with the changes in the applied square wave potential, with a highly reproducible surface stress change of 160 mN/m being observed after the first potential cycle, persisting up to 20 cycles (Figure 3, dark-blue trace). The large magnitude of this signal makes it unnecessary to apply noise rejection methods such as the use of two cantilevers and differential deflection sensing. Functionalization of the cantilever with probe-oligo using a single-step functionalization procedure (Experimental Section) results in a stress pattern that is also in phase with the applied potential but undergoes a smaller stress change (145 mN/m) than does clean gold (Figure 3, red trace). Moreover, in contrast to the clean gold-coated cantilever, the surface stress of the probe-oligo cantilever has complex time-course features. For example, the initial compressive stress observed at $V_{\text{appl}} = +200$ mV undergoes a small but reproducible tensile change (Figure 3, red trace, d–a’). Similarly, the significant tensile stress observed at $V_{\text{appl}} = −200$ mV undergoes a compressive stress change with time (Figure 3, red trace, b–c). Because the time course of the surface stress may reflect dynamic changes in the orientation of the surface-tethered probe-oligo, we sought to determine whether the characteristic temporal features could be used to discriminate between single- and double-stranded oligonucleotides on the cantilever surface. The gold-coated cantilever functionalized with probe-oligo was thus incubated in a hybridization buffer containing the non-thiolated complementary oligonucleotide (target-oligo) to form thiolated double-stranded oligonucleotide (hybridized-oligo). Compared to the surface stress-time pattern observed for probe-oligo, hybridized-oligo shows greater tensile stress when $V_{\text{appl}} = +200$ mV (Figure 3, green trace, d–a’ and a larger compressive stress when $V_{\text{appl}} = −200$ mV (Figure 3, green trace, b–c). These results suggest that the stress patterns are influenced by increasing the charge density as well as by changing the orientation of the nucleic acid tethered to the surface cantilever surface.

To better understand how the quantity of probe-oligo at the gold surface affects the temporal features of the potential-dependent stress signal patterns, probe-oligo was tethered to the gold surface using an alternative multistep functionalization method (Experimental Section). The resulting cantilever deflection associated with each functionalization cycle was recorded (Figure 4A). As the quantity of probe-oligo bound to the surface increases with each successive functionalization procedure, the net stress change decreases. Both the compressive stress when $V_{\text{appl}} = −200$ mV and the tensile stress when $V_{\text{appl}} = +200$ mV increase. The stress changes observed on switching $V_{\text{appl}}$ between +200 and −200 mV most likely derive from a competition between different adsorbates (Cl−, surface-bound nucleotides, and buffer components) and their respective influences on surface stress. The principal ionic species present at the gold–solution interface have a potential-dependent affinity for the gold surface in the ±200 mV range.25,26 Given that signal-generating adsorption events require unoccupied gold sites (Figures 2B and 3, light-blue trace), the decrease in the stress change is due to a lessening of the quantity of available gold caused by the functionalization of the probe-oligo (Figure 3, red trace). The observed compressive stress change when $V_{\text{appl}} = −200$ mV, for example, would arise from an increase in Cl− adsorption at unoccupied gold in the presence of the probe-oligo. However, when $V_{\text{appl}} = +200$ mV, the probe-oligo is expected to have significant affinity for the gold surface and will be able to compete to some extent with Cl− for gold adsorption sites, resulting in a tensile change in the surface stress.

The compressive stress changes observed when probe-oligo is tethered to the gold surface (Figure 3, red trace) and then subjected to periodic potential changes have been attributed to the reorientation of probe-oligo on the gold surface from a quasi-prone to a vertical state.31,32 We postulate that the single-step functionalization process as described creates a random orientation of probe-oligo on the gold surface. The resulting probe-oligo is not effectively repelled from the gold surface at −200 mV. In contrast, the multistep functionalization process allows for electrochemical conditioning in some fashion, leading to an increased loading of probe-oligo as the tethered 25-mers are repetitively switched between prone and vertical orientations by the alternating periodic square wave potential. The ratio of the compressive stress change ($\sigma$) to the stress amplitude ($\sigma$) in the case of the multistep functionalization process is dependent on the surface coverage of the probe-oligo (Figure 4B). The relatively small variation in stress changes compared to that of the cleaned gold-coated cantilevers in Figures 3 and 4A is due to the variation in the polycrystalline evaporated gold surface because the binding isotherm...
associated with each common crystal orientation is different to some extent. The stress pattern changes following oligonucleotide hybridization (Figure 3, green trace) show a significant increase in compressive stress when $V_\text{appl} = -200 \text{ mV}$. To use this stress pattern change as the basis for a biosensor, one needs to determine the optimum surface coverage of probe-oligo. To do so, clean gold-coated cantilevers were functionalized to different extents with probe-oligo, and the cantilever deflection was measured as a function of the periodic square wave potential (Figure 5A, red traces). Following incubation with target-oligo, the cantilever deflection was measured as a function of the periodic square wave potential (Figure 5A, green traces). Following the deflection measurements, each gold-coated cantilever was labeled with Fc(CH$_2$)$_{12}$SH (Experimental Section), an electrochemically active reporter that can be used to determine the quantity of the gold surface that remains unfunctionalized.

Increasing the surface coverage of probe-oligo increases the magnitude of the compressive stress and significantly changes the shape of the surface stress–time curve (Figure 5A, green trace). The difference between the probe-oligo and hybridized-oligo signals is most apparent at intermediate rather than high coverage levels (e.g. Figure 5A, c and c’). The surface-tethered probe-oligo and hybridized-oligo differ in their respective total charge, their stiffness, and their orientation, each of which may be a factor in the observed stress profiles. For example, potential changes to more negative values have been shown to drive the hybridized-oligo away from the gold surface. This would, in principle, allow for more Cl$^-$ adsorption and a concomitant rise in compressive stress when $V_\text{appl} = -200 \text{ mV}$. The hybridized-oligo, through its greater density of phosphate groups, would have more affinity for the gold surface at $+200 \text{ mV}$ than the single-strand oligonucleotide. Simulations by Rant and colleagues of equivalent systems also suggest that the lift-off rate of the hybridized oligo is greater...
than that of the single-strand oligonucleotide because of the greater chain stiffness in the former case.\textsuperscript{22}

Whereas the effects of oligonucleotide charge, stiffness, and orientation on the observed stress change profiles are challenging to separate, our results suggest that other interactions involving a single-stranded oligonucleotide, such as the interaction between an oligonucleotide aptamer and its cognate ligand,\textsuperscript{33,34} might also exhibit characteristic potential-dependent time courses. As a proof of principle, a 24-mer oligonucleotide aptamer (probe-aptamer) whose conformation changes following binding to L-argininamide (l-Arm) was studied. This binding results in increased stiffness and a looped structure of the probe-aptamer.\textsuperscript{34} The gold-coated cantilever was thus functionalized with probe-aptamer to the extent that its surface stress response profile parallels that of the ca. 12% surface probe-oligo coverage situation (Figure 5B, red trace). Following incubation in an l-Arm solution (1.0 mM for 30 min), the cantilever deflection shows a clear change in its stress time course as a function of switching between +200 and −200 mV. The more rapid initial increase in compressive stress when trace) is attributed to the increased stiffness and a looped structure of the probe-aptamer.\textsuperscript{34} The temporal evolution of the surface stress signal of an oligonucleotide-derivated gold surface, as a function of the applied potential, is dependent upon the surface loading of probe-oligo. This significantly changes the shape of the resultant periodic signal as well as its magnitude, providing a robust, information-rich read-out. Optimal coverage is not a densely packed monolayer because a large difference in the surface charge density associated with a specific binding event is sought. The result is a sensor that exhibits reproducible, characteristic signals upon (bio)chemical recognition events. This concept is demonstrated with a generic oligonucleotide aptamer sensor system, opening the possibility of microcantilever detection of DNA, proteins, and other small molecules.

**CONCLUSIONS**

The experiments reported here utilize oligonucleotides in solution as a prototypical molecular recognition system involving a number of charged species, each of which competes for surface sites. Electrochemical control offers the possibility to control the balance of ions close to and at the surface, generating large reproducible changes in the surface stress signal as a function of the applied potential. The temporal evolution of the surface stress signal of an oligonucleotide-oligonucleotide wrapping around the l-Arm, thus making available an additional area of the gold surface for Cl\textsuperscript{−} adsorption.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

TN buffer, Tris-HCl NaCl buffer; TE buffer, Tris-HCl EDTA buffer; PSD, position-sensitive detector; MCH, 6-mercaptopenta-1, hexanol; probe-oligo, thiolated single-strand oligonucleotide; hybridized-oligo, thiolated double-strand oligonucleotide; target-oligo, non-thiolated complementary oligonucleotide for probe-oligo; DTT, dithiothreitol; ferrocenyl C12-thiol, 12-ferrocenyl-1-dodecanethiol; l-Arm, l-argininamide dibydrochloride; OCP, open circuit potential; PZC, potential of zero charge

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