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Two-Potential Electrochemical Probe for Study of DNA Immobilization

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A two-potential electrochemical method is applied to study DNA immobilization, by the simultaneous characterization of capture probe DNA self-assembled monolayers and hybridized target DNA molecules on a given gold electrode surface. Capture probe and target DNA strands are labeled with ferrocenes composed of differing chemical environments, to permit their simultaneous, yet independent signaling at different formal potentials, so that their respective signals may be de-convoluted and assessed for relative surface concentration. Some special attributes of the nondestructive two-potential electrochemical probe described herein include surface sensitivity, chemical and orientation specificity, and the ability to provide a real-time, in situ probe that does not need any wash steps for stringency. This electrochemical probe is applied to study the kinetics, surface architecture, coverage, and orientation of DNA during its immobilization on gold. On the basis of our results primarily from this electrochemical probe, and validated by N(1s) core-level X-ray photoelectron spectra, we judge significant DNA deposition within 5 min of incubation in the deposition solutions, with the capture probe DNA anchored predominantly via the thiol end, even at low coverages. Surface coverage for DNA immobilization plateaus within 30 min of incubation time to $\sim 2 \times 10^{13}$ molecules/cm^2 and the immobilization kinetics as determined from this electrochemical method are consistent with surface re-organization as the rate-determining step.

I. Introduction

The formation of continuous, well-organized, and close-packed self-assembled monolayers (SAMs) over varying surface conditions (area, surface structure, roughness, and contaminants) is a great challenge for applications spanning from molecular electronics[1] to sensors[2−5] and actuators[6,7] based on SAMs. While this process is well-studied for small molecules such as alkanethiols,[8−11] the mechanism is very much under discussion for larger biomolecules such as 15−25-mer thiolated DNA.[12−17] The details of molecular-scale surface architecture are not well understood, and despite an array of available surface analysis techniques and data, there is still a dire need for probes capable of providing structural information in real time and in situ.[17] We report here on one such probe; a unique two-potential electrochemical method to simultaneously characterize the immobilized DNA capture probe (thiol-modified 15−25-mer DNA strand deposited on the gold surface to function as a sensor) and hybridized DNA target (DNA strand in solution that is sensed by hybridization to a section of the surface-immobilized capture probe) on a given gold electrode, by labeling the capture probe and the target DNA strands with ferrocenes that signal at different formal potentials by methods detailed in earlier publications.[3,18,19] As a result of the simultaneous, yet independent signaling at different formal potentials in a standard voltammetric scan, the respective signals may be de-convoluted and assessed for relative surface concentration of the capture probe and target DNA. This two-potential electrochemical probe is applied here to study DNA immobilization kinetics and study the anchoring of the capture probe DNA via the terminal thiol to gold, at different coverages. The results demonstrate the formation of DNA SAMs anchored predominantly via the terminal thiol even at low coverages,

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and the monolayers are close-packed to the limit of $\sim 2 \times 10^{13}$ molecules/cm^2, where hybridization signals are maximum. The surface coverage peaks to this packing density within 30 min, and immobilization kinetics, as determined from this electrochemical method, are consistent with prior studies[15] where surface re-organization is the rate-determining step. These attest to the chemical and orientation specificity of the two-potential electrochemical probe to study DNA immobilization, which is vital to the study of molecular-scale surface architecture of SAMs.[15] X-ray photoelectron spectroscopy (XPS) is used to verify surface cleanliness and provide an alternative confirmation to the DNA adsorption results from the electrochemical method.

II. Experimental Methods

Vapor-deposited gold on silicon wafer substrates was used as the surface to immobilize DNA monolayers. Electron-beam-induced gold films were deposited at 10^{-7} Torr vacuum on a silicon dioxide substrate with a photoresist patterned by standard microelectronic lift-off techniques, and a top-layer plasma enhanced chemical vapor deposition silicon nitride was used to insulate the contact lines, with an etch step to expose 5−500-μm-wide gold electrodes for DNA deposition and electrochemical analysis. The substrates were used within a day of preparation. Each electrode was separated by about 1 mm from neighboring electrodes so that immobilization experiments could be carried out independently on each pad. A chip consisted of 36 working electrodes, a Ag/AgCl reference electrode, and a large Au auxiliary electrode (chip designs as described in prior work[3−5] on circuit board substrates).

The syntheses of DNA capture probes, signaling probes, target mimics, and ferrocene attachment to DNA for signaling at different formal potentials have been described in detail in prior publications.[2−4] The deposition procedure for DNA monolayers involves the incubation of 30-nL drops of the deposition solution consisting of DNA capture probe and other components of the SAM in a high-salt buffer for 1−30 min, as described in prior publications.[2−4] Plasma-based vapor phase surface treatment procedures were used prior to DNA deposition to adjust the contact angle on the insulating silicon nitride layer to 35°, adjacent to a clean and hydrophilic gold surface, thereby permitting each gold pad on the chip to be spotted with a particular capture probe DNA, for a particular immobilization time. Where necessary, contact angle measurements were conducted before DNA deposition using a standard VCA Optima Series goniometer. After capture probe DNA spotting, the substrates were placed in a high-humidity chamber for the respective time interval of the experiment, and no significant evaporation or accumulation was observed. The substrates were then rinsed in deionized water (DI), dried under a stream of nitrogen, and stored in nitrogen-filled foil-lined polythene bags until use. Hybridization and electrochemical measurements were carried out after equilibrating the chips in a solution of 100 nM target DNA in a 500 mM sodium chloride and 500 mM sodium perchlorate solution, with added components of 1 mM mercaptohexanol, 10% (v/v) fetal calf serum, and 10 mM trizma buffer to adjust the pH to 6.5. This target DNA concentration was chosen because the hybridization kinetics are rapid, and kinetics measurements show that the signals from hybridized DNA saturate within a few minutes. By spotting ferrocene-labeled and non-ferrocene-labeled capture probe DNA on different pads within the same chip, electrochemical peaks from the signaling of target DNA hybridized to the capture probe in either case were confirmed to be the same, thereby demonstrating that the attachment of ferrocenes to capture probe DNA did not greatly affect their immobilization and packing density on the gold surface.

XPS spectra were obtained on a Surface Science Instruments SSX-100 spectrometer using monochromatic Al Kα X-rays. Binding energies were referenced to Au($4f_{7/2}$) at 84.0 eV, and an analyzer pass energy of 50 eV (energy resolution $\sim$0.5 eV) was used. While the data reported here are using 1 mm^2 spot sizes to give the best signal-to-noise ratio, smaller spot sizes (300−500 μm^2) were used to correlate the spectra directly to electrochemical data (obtained on 200−500-um arrays). Scanning force microscopy (SFM) imaging was performed at room temperature in air using a Nanoscope IIIa microscope (Digital Instruments, Inc.) in contact mode using commercial Si_3N_4 tips. Electrochemical measurements using alternating current voltammetry (ACV) were performed with a custom potentiostat using the standard three-electrode configuration as described previously,[3,4,19,20] and the results from 200-μm gold electrodes are described here. Scans in the first harmonic were performed using a standard computer-based CH Instruments 660 electrochemical workstation. For fourth harmonic scans, the instrument consisted of a Pentium personal computer with a 16-bit, 100-kHz PCI_MIO_16XE-10 data acquisition (DAQ) card with an onboard digital-to-analog converter from National Instruments (Austin, TX) and a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA). A direct current (dc) voltage bias is applied to the cell and ramped from −100 to +500 mV (versus the Ag/AgCl reference) at 50 mV/s. An ac voltage of 200 mV (peak-to-peak) is applied at a typical frequency of 100 Hz in conjunction to the dc bias, and the potentiometer was used to determine the current. In general, all electrochemical data reported here were measured at the fourth harmonic, unless indicated otherwise. The electrochemical data had pad-to-pad variations of 10−12% within a chip, whereas the chip-to-chip variation was 15−18%; hence, the results reported here were performed on a single chip. However, because the use of multiple chips for the study of individual immobilization times was a far more convenient experiment, this procedure was performed also to cross-check the values with those obtained from a single chip. In this case two signal reference pads were set aside for spotting each chip with capture probe DNA immobilized at signal saturation conditions of 30 min of immobilization time, while other pads were spotted for the immobilization time of the experiment. The peak values from the signal reference pads were normalized between the chips to adjust the peak values and reduce chip-to-chip variations.

III. Results

Probing the Surface by Electrochemistry. The synthetic chemistry for ferrocene-containing molecules that signal at two distinctly different potentials and the labeling procedure to attach them to DNA molecules has been described in detail previously.[3,18,19] The expected surface architecture of the resulting SAM has also been described previously,[3,4] and the current work will aim to verify and quantify this architecture through immobilization kinetics measurements using the two-potential electrochemical probe. Alkyl ferrocynyl derivatives signal at $\sim$180 mV (used to label the target DNA) and electron-withdrawing carbamyl ferrocynyl derivatives signal at $\sim$350 mV (used to label the capture probe DNA). Representative two-potential electrochemical ACV scans of a gold pad with immobilized capture probe DNA signaling at 350 mV and hybridized DNA signaling at 180 mV are shown in Figure 1, for the first and fourth harmonic conditions. This figure shows the clear separation of the signals; however, the peaks were fitted by Gaussian curves for the respective harmonic values to accurately determine peak amplitude and redox potentials for each ACV scan. The current amplitude and phase data were used in the fitting algorithm. Due to greater signal sensitivity in the fourth harmonic as a result of improved signal over noise, it was used for all electrochemical data reported here, unless indicated otherwise.

DNA Immobilization Kinetics. The two-potential labeling system,[3] which has previously been successfully applied to detect single base mismatches,[4] was applied here to study the DNA immobilization kinetics as shown in Figure 2. Each row (six pads) on the chip was spotted

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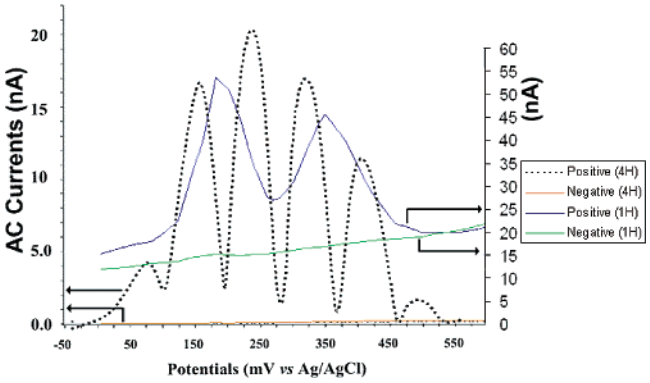

Figure 1. Representative two-potential scans of surfaces with ferrocene signaling at different formal potentials of ~180 and ~350 mV in the first and fourth harmonics to show the peak separation and deconvolution. The signals for surfaces without capture probes and hybridized DNA target molecules are also shown to distinguish between the positive and negative signals. The scale for the fourth harmonic signals is on the left axis, and that for the first harmonic signals is on the right axis.

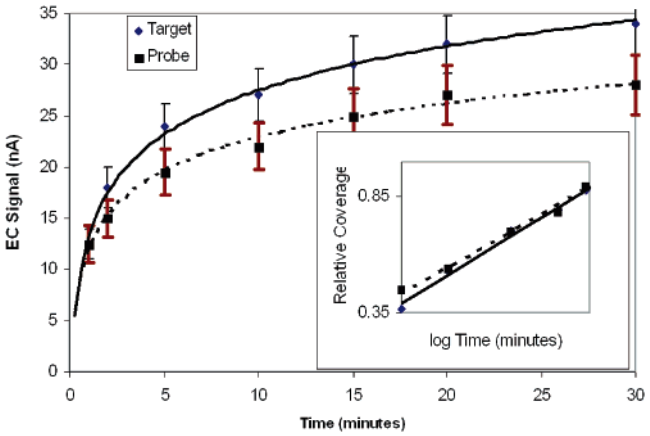

Figure 2. Electrochemical signals from immobilized capture probe DNA (squares) and hybridized target DNA (diamonds) as a function of deposition time. A logarithmic trend line (solid for the target and broken for the probe signal) is used to fit the data. The inset shows a linear fit for the *X* axis of log time versus relative surface coverages (determined as the ratio of the signal at a particular time to the signal upon saturation). The data points are plotted as average $\pm\sigma$ (standard deviation) determined for electrochemical analysis on six pads (68% confidence level for a Gaussian).

with capture probe DNA for the different immobilization times of 1, 2, 5, 10, 15, and 30 min and washed off in DI water while the other pads were still incubated for the longer immobilization times. The entire chip was then washed in DI water, flooded with target DNA for hybridization, and measured for electrochemical signals. Alternatively each chip was also made for a particular immobilization time, but due to larger chip-to-chip versus pad-to-pad (within a chip) signal variations, a signal reference pad was needed to compare the values. In such cases, the pads with 30 min of DNA immobilization time were used as signal reference pads to normalize against this value. Upon normalizing with the signal reference pad, both methods resulted in similar values for signals, and data from the former method are reported here. Assuming 100% signaling efficiencies for the ferrocene (confirmed by correlating signal strengths at saturation with expected molecular surface concentrations of $\sim 2 \times 10^{13}$ molecules/cm^2), the electrochemical signals are directly proportional to relative surface concentrations of capture probe and hybridized DNA. The difference in

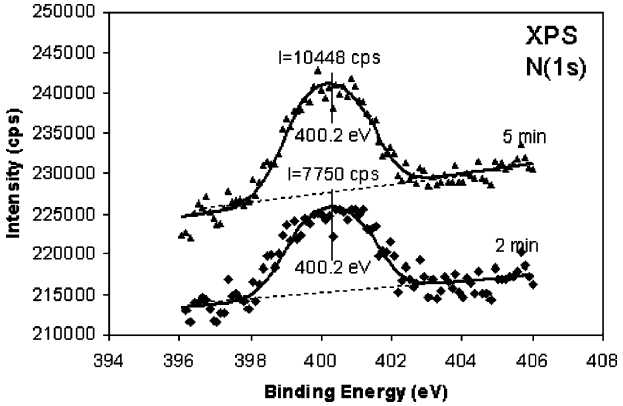

Figure 3. XPS N(1s) spectra of surfaces incubated for 2 and 5 min in deposition solution.

absolute intensity between the probe and target signals arises from the difference in the placement of ferrocenes on the DNA relative to the surface.[18−21] Electrochemical signals from alkyl ferrocynyl derivatives signaling at ~180 mV (used to label the target DNA) are more strongly coupled to the gold surface than those from electron-withdrawing carbamyl ferrocynyl derivatives signaling at ~350 mV (used to label the capture probe DNA), and these trends are consistent with prior studies.[20,21] Hence, due to these differing signaling efficiencies, target signals in Figure 2 seem higher than probe signals, and, hence, the relative trends of the signal versus time are shown in Figure 2 as a semi-logarithmic fit to show the similarities between the two signals. However, to make more definitive judgments about the trends, the signals at any given time were normalized to the signal at saturation (30 min of immobilization time is assumed to give complete coverage because probe and target signals do not increase any more with immobilization time) to give surface coverages as determined from probe and target signals. These normalized surface coverage values are indeed identical for the capture probe and target (see inset of Figure 2), except for immobilization times of 2 min or less, and the reasons for this are examined more closely in the discussion section. The semi-logarithmic fit of the data (linear plot in log time in inset), consistent with recently reported work at high salt conditions in deposition solution,[15] testifies to the role of molecular re-organization on the surface, rather than diffusion to the surface, as the rate-determining step.[22]

Monolayer Characterization by XPS and SFM. The electrochemical data took into consideration the necessary negative controls (nonspecific ferrocene-labeled target DNA labeled in solution and nonspecific DNA capture probe on other electrodes of the array), carried out with the same degree of stringency as described previously;[3,4] yet because it involves labeling to measure the surface condition, we present here XPS and SFM measurements for the surfaces after 2 and 5 min of deposition time. The best indication of DNA immobilization from the thiol end group is the N(1s) XPS peak at approximately 400.4 eV,[23,24] rather than −1.8 and −3.1 eV shifted bulk thymine-like N(1s) peaks that are seen for DNA laying flat on the surface.[15] The spectra in Figure 3 show the data that is

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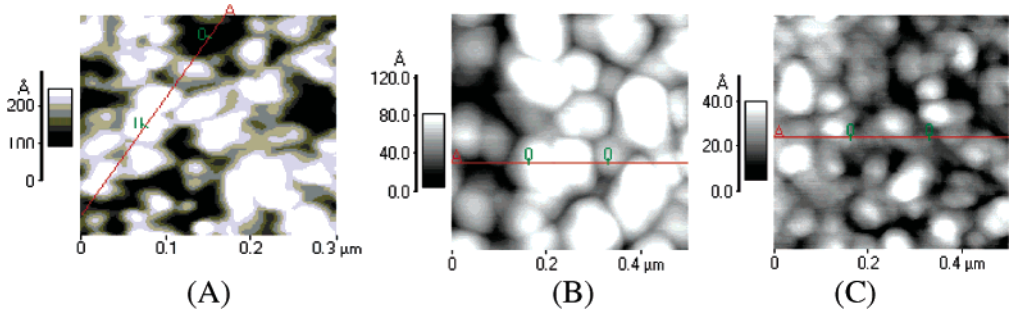

Figure 4. SFM scans of (A) a bare Au surface (averaged surface roughness = 5 nm); (B) after 2 min in DNA deposition solution (averaged surface roughness = 2.4 nm); and (C) after 5 min in DNA deposition solution (averaged surface roughness = 1.1 nm).

fit well with a single peak at 400.2 eV (90% Gaussian, 10% Lorentzian). While the higher signal-to-noise ratio at 2-min incubation times cannot completely rule out the shifted peaks due to flat DNA on the surface, clearly it is not significant in comparison to the main N(1s) peak. These spectra also support the prior observations with electrochemical data, on significant DNA immobilization at 2- and 5-min incubation times in the deposition solution. SFM results are presented here purely as a qualitative method for confirming significant DNA immobilization within 5 min. Comparisons of the scans of Figure 4a−c show a reduction in surface roughness due to smoothing of the grain boundaries, possibly due to DNA immobilization as mixed monolayers.

IV. Discussion

Surface Structure Determination through Electrochemistry. The study of DNA capture probe immobilization kinetics and its anchoring to the surface through the thiol end group is judged in prior research[15] using XPS and IR, rather than surface plasmon resonance because the former set of techniques is specifically sensitive to DNA versus other monolayer species. In our study we use a two-potential electrochemical labeling system[3,4] for the DNA capture probe and target to study DNA immobilization, and XPS is used merely to validate the electrochemical data. From prior work using this electrochemical system for study of charge transfer through monolayers,[20,21,25−27] we have determined that ferrocene signals are far larger for surface electrochemistry (adsorbed and possibly thiol-anchored capture probe) versus solution phase electrochemistry (un-adsorbed capture probe); hence, the probe signal is predominantly from adsorbed DNA on the gold surface. Furthermore, access to capture probe DNA nucleotides for purposes of hybridization with a target DNA is better for the thiol-anchored DNA versus that lying flat on the surface. Hence, the electrochemical signal from the hybridized DNA in proportion to the trends from the probe signal is another sign of a capture probe anchored via the thiol end group. Our results in Figure 2 do show such trends. Finally, the hybridization signal from binding of target DNA to non-surface-bound capture probe DNA is a great deal smaller than that from anchored DNA, as a result of the same reasons outlined previously: higher signals for surface electrochemistry versus solution phase electrochemistry. On the basis of all this we believe that the two-potential electrochemical probe described herein has some special attributes to study DNA immobilization, such as surface sensitivity, chemical and orientation specificity, and the ability to provide a real-time, in situ surface probe that does not need any wash steps for stringency (as needed with two-color fluorescent assays, for example). A close examination of relative coverages (inset of Figure 2) shows that surface coverage of the target is lower than that of the capture probe by 10% at 1 min and by about 2.5% at 2 min. This suggests that, for immobilization times less than 2 min, 10% or less of the capture probe DNA that contributes to the capture probe signal is not hybridized and, hence, does not contribute to the target signal. A possible explanation is that at these very early immobilization times, while most DNA capture probes are anchored via the terminal thiol, 10% or less of the capture probes is in a flat geometry to the surface bonded through thymine adsorption[15,23,24] where the orientation is not well-suited to DNA hybridization. Hence, these adsorbed DNA molecules contribute to the capture probe signal (as a result of being adsorbed on the surface) but not to the target signal (because the flat adsorption orientation is not well-suited to hybridization). However, given that this occurs only to a small percentage for immobilization times of 2 min or less, we judge that, for our deposition system, the capture probe DNA is predominantly anchored from the thiol end group even at low coverages. XPS data validate this as a result of absence of shifted N(1s) peaks. Of particular interest is the sensitivity of this electrochemical probe to the detection of nonthiol adsorbed capture probe DNA, down to 2% surface coverages, in comparison to those reported in the literature by XPS, IR, and surface plasmon resonance methods. The absolute quantification of capture probe surface concentration is addressed in a following section.

Surface Re-Organization. While the formation of alkanethiol-type monolayers is limited by transport of thiol to the surface,[9] recently reported data[15] as well as our data suggest that the deposition of DNA SAMs in high salt solutions is limited by surface re-organization. This includes the rearrangement of capture probe DNA from lying flat on the surface to that anchored to the surface through the thiol end group, which is likely affected a great deal by minor variations in surface area available for adsorption due to contaminants, micro- and nanoscale roughness,[25] electrostatics, and hydrophilicity[8,9] of the surface on which the monolayer forms. Because our XPS and electrochemical data show minimal DNA adsorption in the flat position, even at deposition times less than 5 min, we judge that the surface chemistry allows for rapid surface re-organization so that all capture probe DNA are anchored through the thiol end group. This surface chemistry stems from the use of plasma-based vapor phase surface treatments to obtain a clean and hydrophilic gold surface, which has been demonstrated in prior studies as

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a vital step in enhancing the formation and reproducibility of monolayers.[28−31] Furthermore, the monolayer components for the SAMs described herein include molecules with polar and hydrophilic tail ends (~10° water contact angles in air), based on the motivation that such molecules work better to resist nonspecific binding of target DNA and aid electron transport from ferrocenylated target DNA to the surface.[20] This may also work in favor of enhancing capture probe DNA immobilization, because hydrophilic end groups on the monolayer possibly enhance the spreading of the molecules initially over the hydrophilic gold surface,[9] with re-organization occurring over time as a result of increasing thiol surface coverage. This permits significant DNA immobilization via the thiol end, within 5 min of deposition time.

Quantification of Surface Concentration. The electrochemical data were quantified to demonstrate that DNA SAMs in this work are tightly packed at surface concentrations of $\sim 2 \times 10^{13}$ molecules/cm^2 when the electrochemical signal reaches the saturation condition. For this purpose, the electrochemical signal from the redox label was correlated to surface concentration using a similar approach to prior work,[14] modified for surface immobilization,[21,32] using the equation in ref 33. For example, a peak current of 34 nA at saturation for the target was correlated to a signal from 1.9×10^{13} molecules/cm^2. P32 labeling for the hybridized target was also used to confirm this surface concentration.

V. Conclusions

The major conclusions of this work are as follows:

1. The application of a nondestructive two-potential electrochemical method is demonstrated to probe DNA immobilization with high surface sensitivity and chemical and orientation specificity, as well as for providing a real-time, in situ surface probe that does not need any wash steps for stringency.

2. Significant DNA immobilization ($\sim 10^{13}$ molecules/cm^2) occurs within 5 min of incubation in the deposition solution, and the DNA surface coverage eventually plateaus within 30 min to a surface concentration of $\sim 2 \times 10^{13}$ molecules/cm^2. For this deposition system we judge that the capture probe DNA is predominantly anchored via the thiol end, even at low coverages.

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(33) Quantification of surface concentration from the electrochemical signal: $I_{avg}(E_0)$ = peak current in the first harmonic ACV averaged for a particular E_0; n = number of electrons transferred per redox event; N_{surf} = number of molecules on the surface; f = frequency of ACV scan; F = Faraday constant; E_0 = redox potential; E_{AC} = amplitude of ACV. The following equation is solved for N_{surf} in the high-amplitude limit: $I_{avg}(E_0) = 2nfFN_{surf}\{\sinh(nFE_{AC}/RT)/[1 + \cosh(nFE_{AC}/RT)]\}$. The following equation is solved for N_{surf} in the low-amplitude limit: $I_{avg}(E_0) = N_{surf} E_{AC} f F^2/(RT)$.