Label-free biosensing with functionalized nanopipette probes

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Nanopipettes, characterized by the submicron to nanoscale size of the pore at the tip, are of great interest because of their unique physicochemical properties and potential for various biomedical and biological applications. By pulling a single glass capillary, one can easily and cost-effectively create a pair of nanopipettes that can be used for molecular deposition onto a solid surface (1, 2), for delivery of a single cell (3) and its inner compartments (4, 5), or for biomolecular sensing as described hereafter. These applications can be optimized by an enhanced understanding of the physical and chemical interactions at the pore region, which has been a subject of theoretical studies (6). Advances in both technical and theoretical fronts will further demonstrate the utility of nanopipette-based devices for many purposes.

Biomolecule sensing with a nanopipette probe has been performed with and without the aid of optical methods. Fluorescence-based pH sensing (7) shows the submicron spatial resolution and millisecond time resolution of such sensors. Fully-electrical detection of DNA-conjugated gold nanoparticles (8) uses resistive pulses caused by the translocation of fairly large (10 nm) particles, the underlying principle identical to that of nanopore biosensors (9) and DNA sequencers (10). Unlike other nanostructure-based chemical sensors (11), which often require access to semiconductor facilities, nanopipette biosensors can be created and tailored at the bench, thereby reducing turnaround time. Nanopipettes also have enormous potential for detecting a small number of molecules from a tiny amount of clinical samples or live single cells, a feature useful for medical diagnostics and molecular and cellular biology research.

A key challenge for nanopipette biosensors is adapting to applications where specific molecules can be targeted. One approach would be to separate the sensing and actuating functions, an idea embodied by an engineered ion channel fused with a surface receptor protein responsible for target binding (12). However, incorporating a recognition element directly on the sensor (e.g., a natural ion channel with a selectivity filter) would be another, more straightforward approach. Immobilizing antibodies onto the sensing region, for example, has been found to work well in various nanosensors (13–17), including conical nanotubes, which resemble nanopipettes with respect to geometry (18). The second approach should work for nanopipette biosensors when the tip surface is successfully functionalized with antibodies or other recognition elements.

Here we report the development of nanopipette biosensors based on 2 main strategies. The first strategy uses electrostatic interaction (e.g., of charged polymers) with the nanopipette tip surface to modulate the rectification property of the glass nanopipette. The second strategy relies on specific binding of biotin-streptavidin or antigen-antibody molecules. Biotin-streptavidin interactions changed current amplitude when the nanopipette tip surface coated with biotinylated BSA molecules was exposed to streptavidin. Specific antigen-antibody interactions also affected the current amplitude. Both strategies showed a reasonable promise for future applications in molecular diagnostics and other applications that benefit from real-time label-free biosensing.

Study Design

Overview. Quartz nanopipettes with an opening of ~50 nm in diameter at the tip were fabricated as previously reported (8, 19), except that capillaries with filaments were used for smooth backfilling by capillary force. The nanopipettes were backfilled with 100 mM potassium chloride (KCl) aqueous solution supplemented with 2 mM phosphates (pH 7; called working buffer hereafter), and an Ag/AgCl wire electrode was inserted to comprise a nanopipette electrode module. Up to 2 independent modules were used for the biosensing experiments, typically with one as a probe and the other as a control (Fig. 1A).

Target molecules were added to the external working buffer solution in a bath in which the nanopipettes were immersed (Fig. 1A). Up to 2 nanopipettes were positioned in the same bath, while the reference electrode was located in another bath to prevent contamination from the added molecules. All bath solutions were electrically connected through a 1% (wt/wt) agar gel layer containing the same concentration of salts. The electrolyte solution and the gel registered a conductivity value of 13 mS/cm.

Sensing Strategies. Two strategies were used to sense proteins and other biomolecules (Fig. 1B). The first strategy was based on electrostatic attraction of highly charged target molecules to the oppositely charged surface. The second strategy was based on specific interactions between target molecules and the nanopipette tip surface functionalized with a recognition element. The

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biotin-streptavidin interaction, known as the strongest noncovalent form of molecular binding, was tested using the nanopipette tip surface on which biotinylated bovine serum albumin (biotin-BSA) was physisorbed. Functionalization with antibody molecules was also tested by chemically modifying the tip surface similar to an ELISA system, providing a wide variety of target molecules that could be captured and detected. In both strategies, we applied a voltage between the electrode inside the nanopipette and the reference electrode, and focused on changes in the measured current over time. The measured current represents the flow of mobile ions affected by molecular interactions in the nanopipette tip region.

Results

Sensing the Electrostatic Interaction of Polyelectrolytes with the Charged Glass Surface. Because the native quartz surface is negatively charged at neutral pH, it was expected that positively charged polymers should be readily sensed when they electrostatically attach to the nanopipette surface. Indeed, the addition of poly-L-lysine (PLL) caused a marked modulation of current amplitude similar to an ELISA system, providing a wide variety of target molecules that could be captured and detected. In both strategies, we applied a voltage between the electrode inside the nanopipette and the reference electrode, and focused on changes in the measured current over time. The measured current represents the flow of mobile ions affected by molecular interactions in the nanopipette tip region.

Sensing Biotin-Streptavidin Interaction on the Nanopipette Tip Surface. Obviously, target molecules with less or no charge would be hard to detect by electrostatic binding. Even with sufficiently charged molecules, a problem remains in how to specifically identify current changes in samples that contain multiple molecular species. Therefore, our next step was to introduce a recognition element into the nanopipette biosensing system that would result in a distinguishable signal upon binding of specific target molecules.

The first and most straightforward test was the biotinylation of the nanopipette surface in 2 steps: (1) PLL coating of the outside walls of the nanopipette tip followed by a baking process; and (2) physisorption of biotin-BSA onto the baked PLL. The hydrophilic surface allowed the reactions to occur on the outer walls as well as the tip. The resulting biotinylated tip surface was primed to interact with streptavidin molecules.

Fig. 3 depicts a typical result of the biotin-streptavidin binding assay. To evaluate whether each measurement indeed reflects biotin-streptavidin binding events, we used streptavidin conjugated with fluorescein isothiocyanate (FITC) to enable optical confirmation of binding. On the addition of streptavidin-FITC to the bath solution, the biotin-BSA-functionalized nanopipette measured slightly higher negative current than before the addition, whereas the control nonbiotinylated BSA nanopipette did not (Fig. 3A and Fig. S2). The difference in current before and after the streptavidin-FITC addition reached as high as 20%. 

Fig. 2. Modulation of the current rectification property caused by polyelectrolytes. (A) Onto the negatively charged quartz surface, free poly-L-lysine (PLL) added to the bath (10^{-4} wt/vol) inverted the polarity of current rectification to positive values. Rinsing with buffer (hatched areas) canceled this effect, reviving the original negative rectification (which became less prominent, however). Applied voltage, ±500 mV. (B) Current recorded at t = 0 at the onset of the first PLL addition (indicated by an arrow in panel A).

Fig. 1. Nanopipette-based biosensing platform. (A) Measurement set up. Up to 2 nanopipette electrodes were used to record the current amplitude, in which case one nanopipette (labeled channel 1) was designed to detect antigens specific to the antibodies immobilized on its tip surface, whereas the other nanopipette (channel 2) served as a control. (B) Schematic representation of sensing strategies. Interaction between free analytes in solution and the nanopipette tip modulates the ionic flow through the 50-nm pore. Electrostatic interaction occurs when the analytes and the surface are both charged, affecting the rectification property of a quartz nanopipette. Specific interaction occurs when a recognition element immobilized on the nanopipette tip surface distinguishes its target molecules from others present in the sample solution.
However, although an increased negative current was repeatedly measured with biotin-BSA-functionalized nanopipettes, there were additional cases in which changes in current were not large enough to be distinguished from the normal fluctuation levels. We discriminated such “false negatives” by confirming that the degree of the current increase was beyond the standard deviation from the baseline level (Fig. S3). This variability can be explained by low coverage of streptavidin-FITC molecules at the very tip region and/or large lot-to-lot variations in the physisorbed layers. These electrical measurements were followed by complementary optical observations that always confirmed that the intended biotin-streptavidin binding did occur. The probe nanopipette functionalized with biotin-BSA immediately after the electrical measurement showed a much higher fluorescence intensity than the control nanopipette with BSA alone (Fig. 3B). This indicates that, although the very tip of the nanopipette cannot be visualized because of the diffraction limit, biotin-streptavidin-specific binding can be differentiated from nonspecific adsorption of streptavidin-FITC, thus confirming that the specifically bound streptavidin-FITC plays a major role in causing the measured difference of ionic current.

Chemical Functionalization of the Nanopipette Tip Surface with Antibodies. To demonstrate an improved approach to specific detection with a more stable surface, we covalently immobilized antibodies onto the tip surface using 4 main steps: (1) PLL coating and baking; (2) incubation in a linking carboxylate solution followed by surface activation by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) aided by N-hydroxysulfosuccinimide (NHS); (3) protein A/G conjugation; and (4) IgG immobilization. Protein A/G, a commercially available chimeric protein that captures the Fc region of an IgG molecule, was covalently immobilized on the nanopipette surface to capture IgG with its antigen-binding ability maintained by exposing its antigen-binding sites in the correct orientation.

The successful functionalization of the nanopipette surface with IgG was optically verified (Fig. 4). A standard protocol was always performed with 2 nanopipettes: one with biotinylated IgG (biotin-IgG) and the other with nonbiotinylated IgG. The 2 nanopipettes were then exposed to free streptavidin-FITC molecules and rinsed. The high fluorescence intensity from the biotin-IgG-immobilized nanopipette tip indicates the presence of biotin-IgG and the effective biotin-streptavidin binding on the surface. The nonbiotinylated IgG surface, however, showed fluorescence intensity as low as the background level, indicating that nonspecific binding of streptavidin-FITC was minimal.

Sensing the Specific Binding of Cancer Biomarker Proteins to the Immobilized IgG Molecules. After validating the successful functionalization with IgG, the nanopipette probes were applied to protein sensing. We chose 2 protein species known to be important human colorectal cancer biomarkers, interleukin-10 (IL-10) (22) and vascular endothelial growth factor (VEGF) (23), which would lay the groundwork in applying the system to detecting other proteins related to different cancer types (24).

The added antigens (IL-10 or VEGF) instantly reduced both the positive and negative current amplitude, but a similar reduction was not observed in the control nanopipette with an immobilized nonspecific antiferritin IgG, confirming the specific detection of the cancer biomarkers (Fig. 5 and Fig. S4). In general, the current reduction dynamics followed a transient curve where the maximal reduction appeared in minutes or tens of minutes. The degree of reduction was typically larger in negative than in positive peaks (10 of 14 measurements; Table S1) and varied from 3% up to about 20%. The recorded current amplitude was relatively stable compared with that when probe molecules were physisorbed to the nanopipette surface (Fig. 3, for example), providing evidence of a more stable functionalized surface in these experiments. By further improving and optimizing protocols, we will be able to minimize lot-to-lot variations in the functionalized surface and perform a more quantitative analysis of the observed current reduction.

Discussion

We showed that 2 sensing strategies can be effective in detecting nonlabeled target molecules present in a liquid environment using nanopipettes. The first strategy relies on electrostatic interaction of charged polymers with the glass nanopipette surface, and the second strategy uses biotin-streptavidin and antibody-antigen interactions as models of specific binding events. For protein assays, the antibody-antigen scheme is particularly useful in that it potentially allows us to build multiple nanopipette biosensing “modules” that can be implemented for highly multiplexed detection of different targets.

The modulation of current amplitude caused by PLL and PAA can be interpreted as the effect of surface charge present on the
nanopipette tip surface. The native, negatively charged quartz surface attracts positively charged molecules, especially when they have high surface-charge density and a favorable shape amenable to stacking on the glass surface. Lysines on a PLL strand are protonated at neutral pH, resulting in a condition in which the PLL molecules bind to the glass surface and form a layer or stack of layers, changing the polarity of the surface charge to positive. This notion corresponds to previous reports on the rectification effect of PLL and polyamines coating the glass (19), gold-plated (25), and carboxylated (26) surface of a nanoporous structure, although the effect here seems less prominent because the polycations are attracted from outside the nanopipette tip. The reversible transition between negative and positive r values (Fig. 2) may indicate that PLL molecules leave and attach to the surface depending on thermodynamic equilibrium. Similarly, the transition of r values from positive to negative caused by PAA additions (Fig. S1) could be due to the resulting net negative surface charge. The effect of PAA appears more complicated, however, because it could be explained by either the formation of a PLL/PAA polyelectrolyte multilayer or the exposure of the glass surface after entire polymer layers peeled off under a strong electric field generating nonequilibrium conditions.

Specific binding of proteins on either the biotinylated or IgG-functionalized surface affected the current amplitude but not the rectification polarity. We speculate that the rectification can only be inverted by exceptionally highly charged polymers, such as PLL. Proteins in general would mainly have an effect on the current because of their physical size (typical Stokes radius ranges several nanometers) when binding to the functionalized surface and partially blocking the current path. The observed reduction in current (Fig. 5) may have been caused by this partial blockade of the ionic flow through the pore, whereas stochastic binding events that provide a different surface coverage by the target proteins could explain the variability in the degree of the current reduction. However, when target molecules significantly change nanopipette surface charge, the effect of charges gained by their binding to the tip surface may add up to or surpass the effect of the physical narrowing of the pore. The results with streptavidin-FITC, which showed an increase in current rather than a decrease, could be due to the effect of negative charges carried by FITC molecules conjugated to the streptavidin molecule in the 4:1 ratio as described by the manufacturer. However, understanding of such a combined effect would require more variations in experimental conditions and more stable functionalization layers, which will be the focus of future studies. Quantitative discussion on the measured changes in current also requires more statistically rigorous validation. We stress here that our main objective in the current study is to show that 2 nanopipette sensing strategies (i.e., electrostatic and specific binding), can be used for electrical detection of biomolecular interactions.

The sensitivity of the nanopipette biosensor depends on conditions of the functionalized surface. In principle, the limit of detection could be determined by the number of molecules that generate a detectable signal at the 50-nm nanopipette tip. In practice, however, consumption of target molecules on the nanopipette sidewalls prevents us from achieving this theoretical limit. In this study, we tested the saturated amount of target molecules introduced to the bath solution, but efforts are underway to determine the actual limit of detection of the nanopipette biosensing system. Improvements can be made, for example, by limiting the functionalized surface area to prevent the capture of targets on the sidewalls away from the sensing region. Another option could be the use of secondary antibodies, which also showed the same current reduction effect as IL-10 and VEGF (Table S1), to enhance signals by the formation of larger complex. For particular targets, accumulation of molecules in and around the tip region (27) may also enhance the sensitivity.

The features of the nanopipette biosensor shown in this study can potentially lead to the development of a fast, compact, and easily multiplexed biosensing platform surpassing its optical counterparts. Unlike ELISA, which requires considerable incubation time and colorimetric fluorescent or optical readout, our strategies can be performed in real time and are label free. The fully electrical molecular detection is also unique compared with technologies such as Biacore, a surface plasmon resonance-based bioanalyzer (28), in that it works without optical signal readout. For such electrical detection, a nanoporous structure is known to be suitable, especially when it is used in resistive-pulse mode (29, 30). In this study we showed another practical option using functionalized glass nanopipettes in which antibodies of interest were immobilized on the surface of individual nanopipettes rather than dispersed in the bulk solution. This approach will eventually provide a biosensor that not only enables realtime label-free biomolecule detection, but can also be precisely positioned with submicron accuracy using a micromanipulator, an ideal tool for investigating dynamic processes of single cells. The movable sensor approaches an attached cell, in contrast to a fixed sensor detecting responses from floating cells (31).

In conclusion, we introduced a label-free, real-time protein assay using functionalized nanopipette electrodes. We showed 2 sensing strategies that yielded proof-of-principle results of charge-based and affinity-based biosensing. Charge-based biosensing was demonstrated by PLL and PAA interacting with the glass nanopipette surface, which modulated the rectification property. Affinity-based biosensing was tested in 2 schemes (physisorbed biotin-BSA and

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**Fig. 5.** Cancer biomarker detection by the nanopipette probe functionalized with corresponding IgG molecules. (A) IL-10 detection (4 μg/ml). Applied voltage, ±200 mV. (B) VEGF detection (4 μg/ml). Applied voltage, ±200 mV. In both cases, an immediate decrease in current was recorded by the probe nanopipette (antiferritin bound, dashed lines).
covalently immobilized IgG), in both of which the ionic current was affected by the specific interaction. Easily fabricated and tailored at the bench, the nanopipette probes could be used for biomedical and biological applications, including clinical diagnostics and single-cell studies.

Materials and Methods

Reagents. Poly-L-lysine (PLL) was purchased from Electron Microscopy Sciences (19320-A). Biotinylated BSA was purchased from either Rockland Immunochemicals (001-0633) or Pierce Biotechnology (29130). Streptavidin-FITC, EDC, NHS, and protein A/G were from Pierce Biotechnology (21224, 22981, 24500, and 21186, respectively). Recombinant human IL-10 and VEGF were from R&D Systems (430-VE and 2347-VE), and the purified IgG fraction of their respective part antibody was purchased from R&D Systems (anti-VEGF [goat], AF-293-NA) and BioLegend (anti-IL-10 [rat], 501407). Antiferritin (rabbit IgG) was purchased from Rockland Immunochemicals (100-401-090) and used as a control to the other antibodies. All other chemicals used were ACS grade or higher. Aqueous solutions were made with Milli-Q water and purified with a 0.1-μm syringe filter before use.

Nanopipette Fabrication. Nanopipettes were fabricated from quartz capillaries with filaments, with an outer diameter of 1.0 mm and an inner diameter of 0.2 mm. Each module was applied between the nanopipette and reference electrodes in the ionic measurement setup. The datasets were further processed to quantitatively compare results from multiple experiments. To discuss the current rectification effect, we defined a parameter \( r \), rectification ratio, as \( r = \log(l^+ / l^-) \), where \( l^+ \) and \( l^- \) are the positive and negative peak current amplitude, respectively. A positive, negative, or zero value means a positive, negative, or the absence of rectification.

Surface Functionalization: Antibody Immobilization. Antibodies were immobilized through the following steps. After PLL coating as previously described, the nanopipette tip was incubated in a linking carboxylate solution (32) for 10 min to cover the surface with functional carboxylate groups. Following rinsing with water 3 times, an EDC/NHS mixture solution (50 mg/ml each) was applied for 1 h at room temperature to activate the surface, and rinsed with water afterward. The activated surface was immediately exposed to a 0.1-mg/ml protein A/G solution and kept still at 4°C for overnight. During this incubation, a stable amide linkage was formed between a protein A/G molecule and the nanopipette surface. The protein A/G-coated tip was transferred to and kept in a 40-μg/ml IgG solution for 1 h at room temperature to allow the protein A/G to capture the Fc region of the IgG molecules. In this study, either anti-IL-10 or anti-VEGF was immobilized to form a probe nanopipette, and antiferritin was used as a control.

Preparation of the Fluorescent Nanopipette Tip. Streptavidin-FITC was diluted with working buffer to 40 μg/ml and immediately used. Biotinylated and nonbiotinylated nanopipette tips were incubated in the streptavidin-FITC solution for 30 min at room temperature. Upon completion, the nanopipette tips were extensively rinsed with water to remove any nonspecifically bound streptavidin-FITC molecules.

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Fig. S1. Modulation of the current rectification property caused by polyanion molecules. (A) Of the sporadic additions of polyacrylic acid (PAA) at increasing final concentrations in the bath (10⁻⁸%, 10⁻⁷%, 10⁻⁶%, and 10⁻⁴%, all wt/vol), the first addition caused a delayed response that quickly inverted the polarity of current rectification, whereas subsequent PAA additions only slightly enhanced the negative rectification effect. Note that the nanopipette used was initially coated with poly-L-lysine, showing positive rectification. Applied voltage, ±500 mV. (B) Current recorded at t = 0 and 13 (indicated by arrows in panel A).
Fig. S2. Current recorded by the biotinylated probe nanopipette (Top) and the nonbiotinylated control nanopipette (Bottom) at t = 0 and 12 of Fig. 3A, which shows streptavidin-FITC detection.
Fig. S3. Signal-to-noise ratio analysis on the current increase observed in the biotin-streptavidin assays. To filter out changes caused by biotin-streptavidin interaction from those by other effects (e.g., nonspecific binding onto the surface, evaporation from the bath solution leading to drifting current), we calculated and evaluated a parameter $\Delta I' = [I'(\text{probe}) - I'(\text{control})]$, the difference between the probe and control nanopipettes in negative current peaks. In each graph shown here, the mean ± SD of $\Delta I'$ values are represented as horizontal lines to visualize the signal ($t$ = 5 to 20 min) and the baseline ($t$ = -20 to 0 min) levels. Among the results there were cases in which the current increase was large (A), those in which the increase was less obvious but still above the baseline level (B), and “false negatives” in which the current increase was not large enough to be distinguished from the baseline (C).
Fig. S4. Current recorded by the probe nanopipette (Top), which was functionalized with either anti-IL-10 (A) or anti-VEGF (B) IgG, and the antiferritin-immobilized control nanopipette (Bottom) at t = 0 of Fig. 5A and Fig. 5B, respectively. These figures illustrate cancer biomarker detection.
Fig. 55. Description of the data analysis procedures. A raw data file consists of multiple (typically 20) sweeps. During each sweep, 50 cycles of a sinusoidal voltage was applied and corresponding peaks in current were measured and averaged. Normalization was done by dividing a given current value by the average immediately before the addition of target molecules for the experiment.
Table S1. Summary of results from biosensing experiments using nanopipette probes functionalized with immobilized IgG

<table>
<thead>
<tr>
<th>Target</th>
<th>No.</th>
<th>c, ng/ml</th>
<th>V, mV</th>
<th>(\Delta I, \text{ normalized}, %)</th>
<th>t, min</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>((-5,0))</td>
<td>5</td>
</tr>
<tr>
<td>IL-10</td>
<td>1 (Fig.5A)</td>
<td>(4 \times 10^3)</td>
<td>200</td>
<td>+</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(4 \times 10^3)</td>
<td>200</td>
<td>-</td>
<td>0 ± 0</td>
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<tr>
<td></td>
<td>3</td>
<td>(4 \times 10^3)</td>
<td>200</td>
<td>-</td>
<td>1 ± 1</td>
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<td></td>
<td>4</td>
<td>(1 \times 10^3)</td>
<td>500</td>
<td>+</td>
<td>0 ± 0</td>
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<td></td>
<td>5</td>
<td>(1 \times 10^3)</td>
<td>500</td>
<td>+</td>
<td>1 ± 1</td>
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<tr>
<td>Anti-goat IgG</td>
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<td>200</td>
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Individual datasets obtained by a single biosensing experiment were analyzed and listed with a number assigned to each of them. In addition to IL-10 and VEGF, also shown are results from experiments using the probe nanopipettes functionalized with anti-VEGF (goat IgG) and the control nanopipettes with antiferritin (rabbit IgG) aiming to sense the secondary antibody anti-goat IgG (KIB004; Rockland Immunochemicals). The experimental procedures were identical to those of the IL-10 and VEGF experiments as described in the main text. c is the final target molecule concentration in the bath solution. V is the amplitude of the applied sinusoidal voltage. Positive and negative current peaks were analyzed separately and the results were shown in the 2 rows labeled + and −, respectively. Defined as \(\Delta I^+ = I^+(\text{probe}) − I^+(\text{control})\) and \(\Delta I^- = I^-(\text{probe}) − I^-(\text{control})\), the \(\Delta I\) values become positive when the current was increased, and becomes negative when the current was decreased. \(\Delta I\) in time interval \((-5,0)\) min is intended for comparison with \(\Delta I\) at various time points after the target molecule addition. Within each experimental result, the positive or negative signs and the maximum \(|\Delta I|\) are highlighted as bold to emphasize how much current reduction was observed at which polarity.