Background-subtraction of fast-scan cyclic staircase voltammetry at protein-modified carbon-fiber electrodes

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Abstract

Background-subtraction techniques were applied to the voltammetry of nicotinamide adenine dinucleotide (NADH) at protein-modified carbon-fiber microelectrodes. The background currents at carbon-fiber electrodes were stable and voltammetric scans immediately before or after the analyte were effectively used for background subtraction. Digital step-potential waveforms were used to excite these carbon-fiber electrodes, where the resulting voltammetric analysis assessed the optimal switching and initial potentials and the electrochemical response time was determined. The initial potential was 0.0 V and the switching potential 1.1 V (versus Ag/AgCl) and the response time was approximately 300 ms. Some sensitivity to NADH was lost and voltammetric prescans were required at protein-modified electrodes to obtain a stable baseline. Current versus time was assessed by the average current of the faradaic region from each voltammogram and by differential current; the average current minus the current from a non-faradaic potential range. Differential current assessments discriminated against artifacts caused by pH (as high as 1.0 pH unit) and ionic strength flux (100 mM). These background-subtraction techniques allowed the faradaic information to be obtained quickly and conveniently while maximizing sensitivity and maintaining selectivity. © 1998 Elsevier Science S.A. All rights reserved.

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1. Introduction

Fast-scan cyclic staircase voltammetry (FSCSV) at microelectrodes has become a well established technique. This technique has led to an exceptional degree of spatial (≤ 5 μm) and temporal (≤ 20 ms) resolution, especially for monitoring stimulated release of easily oxidized neurotransmitters and metabolites in vivo (O’Neil, 1974; Wightman et al., 1988). One complication of a fast scan rate is the concomitant increase in the background capacitive current (Bard and Faulkner, 1980). However, this background current at carbon electrode surfaces is remarkably stable. The stability of this current from scan to scan (given a consistent local buffer environment) offers a convenient and simple source of background scans for subtraction schemes. This scheme leads to the improvement of detection limits (Wiedemann et al., 1991; Kawagoe et al., 1993b). In this paper, this method is applied to nicotinamide adenine dinucleotide (NADH) voltammetry associated with enzyme-modified carbon-fiber electrodes. This class of modified electrodes provides a method to monitor non-electroactive species in physiologically relevant time-scales and volumes (Kuhr et al., 1993). The enzymes that use NADH as a co-factor include some 200 species and each may be coupled to this class of biosensors via an avidin–biotin linkage system (Kuhr et al., 1993). NADH/NAD + -linked electrochemical probes offer new types of enzyme-based biosensor for a large number of analytes, but the fundamental operating parameters must be investigated.

Background-subtraction can digitally minimize or remove the background current. For a FSCSV experiment, the processed data is described as a background-subtracted cyclic voltammogram (BSCV) in which a full cyclic voltammogram is generated each 200 ms...
(Wightman and Wipf, 1990; Kuhr et al., 1993). For this background-subtraction procedure, the analytical performance of the FSCSV measurement is enhanced in numerous ways, most notably by addressing the trade-off between temporal resolution and current signal-to-noise ratio (while retaining qualitative information). Digital background-subtraction techniques permit sensitive measurements over a short time course without decreasing the scan rate to diminish background capacitive currents. Separation of the faradaic information of the analyte from the background ensures that the inherent selectivity of the FSCSV measurement is also retained. This selectivity is quantitated by the position of the oxidative and/or reductive peak potential which differentiates the species on the basis of their electron transfer kinetics. This technique allows this qualitative information to be recorded in a fast, sensitive manner (Wightman and Wipf, 1990).

Fast-responding, enzyme-modified carbon-fiber microelectrodes, which transduce the non-electroactive analytes into an electroactive species through the interaction of cofactors have been developed (Pantano and Kuhr, 1993). This microelectrode utilizes avidin–biotin interaction to immobilize enzymes onto the electrode surface. The dehydrogenase family of enzymes is particularly attractive for this purpose since their activity is linked to the electroactive cofactor, NADH. The cofactor generated in this manner acts as an electron-transfer mediator that can be monitored by FSCSV at carbon-fiber surfaces. While the FSCSV for the oxidation of NADH produces high faradaic currents and low overpotential at bare carbon-fiber microelectrodes, the response at an enzyme-modified surface is diminished (Pantano and Kuhr, 1993; Hayes and Kuhr, 1998a). The diminished FSCSV response occurs because the carbon-fiber surface is both the site of electron transfer and of enzyme-immobilization. A quantitative balance between these two tasks has been attempted and, because these are competing activities, some voltammetric performance has necessarily been sacrificed (Pantano and Kuhr, 1993; Hayes and Kuhr, 1998a, b).

Factors were evaluated that control the quality of a BSCV generated at a dehydrogenase-modified carbon-fiber microelectrodes to allow full interpretation of the available information. Near the detection limit for NADH the results of these background-subtraction parameters were most pronounced.

A digitally generated potential-step waveform was used to excite carbon-fiber electrodes to generate a BSCV. The carbon-fiber electrodes required an electrochemical pretreatment to provide low overpotential and high faradaic currents for the oxidation of NADH. These pretreated electrodes were used to characterize the background-subtraction technique. First, the switching and initial potentials were determined and the response time was then characterized. Background currents were found to drift more at protein-modified electrodes than freshly polished electrodes, but stable background currents were obtained after a series of voltammetric prescans were performed. To obtain qualitative voltammetric information, the scans used for background subtraction must be chosen, both in number and position. These choices were characterized for sensitivity and stability. Improvement of the signal-to-noise ratio was obtained by both full-scan summing and averaging, and averaging each three adjoining data points within a single scan. Current artifacts caused by pH flux and ionic strength changes were eliminated by using information available within each scan. Current arising in non-faradaic potential regions was used to compensate those within faradaic, or information-containing, regions. These data manipulations reduced sensitivity, but could compensate for pH flux up to 1.0 pH units and ionic strength changes of 100 mM. These digital excitation and data manipulation techniques provide a convenient and powerful method to obtain information quickly and with high sensitivity for the electrochemical measurement of NADH.

2. Experimental

2.1. Chemicals

Glutamate dehydrogenase (GDH, 40 units/mg, E.C. 1.4.1.3), NADH, ExtrAvidin, and 1-ethyl-3-((dimethylamino)propyl) carbodiimide (EDC) (Sigma Chemical Co., St Louis, MO, USA); sulfo-NHS-LC-biotin (Pierce Chemical Co., Rockford, IL, USA), and poly(oxyalkylene)diamine (Jeffamine™ ED-2001; Texaco Chemical Co., Houston, TX, USA) were used as they were received. Phosphate buffer (PBS; 0.15 M NaCl, 0.10 M Na₂HPO₄, pH 8.5) was prepared with reagent grade chemicals in water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA). All FSCSV measurements were conducted in pH 8.5 phosphate buffer.

2.2. Carbon-fiber microelectrodes

The fabrication of carbon-fiber microelectrodes has been described previously (Pantano and Kuhr, 1993). All 10 μm diameter carbon-fiber microelectrodes (Thornel P-55S; Amoco Performance Products, Greenville, SC, USA) were bevelled at a 30° angle for 10 min on a polishing wheel covered with 1 μm diamond paste (Metadi II; Buehler, Lake Bluff, IL, USA). Residual polishing materials were removed by sonicating the electrodes in hot toluene and then in de-ionized water for 10 s. Unless otherwise noted, all 10 μm diameter carbon-fiber microelectrodes were electrochemically treated in 1.0 M HCl by a 3 s, 50 Hz cyclic potential waveform generated between − 0.2 V and 1.8 V (versus Ag/AgCl). All 32
2.3. Instrumentation

Fast-scan cyclic-staircase voltammetry was performed with an EI-400 potentiostat (Cypress Systems, Lawrence, KS, USA) which was designed to accommodate placement of the working electrode pre-amplifier inside a faraday cage. All staircase cyclic voltammetric waveforms were generated, and currents acquired, with an 80486 PC microcomputer utilizing a 12-bit, 20 kHz A/D–D/A interface (Labmaster DMA; Scientific Solutions, Solon, OH, USA). All potentials reported were referenced to a 0.4 mm o.d. Ag/AgCl electrode. A 400 kHz digital oscilloscope (Nicolet instruments Model 310; Madison, WI, USA) was used to acquire the staircase waveform shown in Fig. 1(a); the waveform was acquired with 1 ms (Fig. 1(a) inset) and 200 ms (Fig. 1(a)) oscilloscope time constants. All currents were recorded with a faraday cage with a flow-injection analysis (FIA) system previously described, where buffer (1.5 ml/min) was now controlled by a peristaltic pump (Model 203; Scientific Industries, Bohemia, NY, USA) (Kuhr et al., 1993). All FSCSV measurements acquired were filtered within a 1–3 kHz range by the two-pole, low pass filter (3 db between 1.5 Hz and 15 kHz) of the EI-400 potentiostat.

2.4. Data manipulation and presentation

FIA data may be presented in a variety of formats. Qualitative data for BSCV in cyclic voltammetric-format (CV) was obtained by subtracting background scans from analyte scans. The background scans may be chosen from data sets before or after the analyte plug.

Time versus current information is presented in two distinct formats: average current and differential current. The average current versus time plots is the average current of a potential range from each cyclic voltammogram-format data set (typically including the peak potential \(E_p\) of the analyte). This potential range for averaged current may be varied and was investigated. Differential current plots subtracted the average current from a potential range where faradaic analyte current is absent from the average current data. This data manipulation allows changes in buffer composition which influence electrode capacitive currents to be compensated. The current changes caused by capacitive influence will be reflected equally in faradaic and non-faradaic regions. Therefore, this non-faradaic current region provides a background-subtraction source for compensation of these artifact currents. Plots may also be created in a three-dimensional view. Some data was exported as an ASCII file and manipulated in Lotus 1-2-3 (Lotus Development Corp., Cambridge, MA, USA) or Excel (Microsoft, Redmond, WA, USA).

3. Results and discussion

The exceptional spatial and temporal resolution of a fast-scan voltammetric measurement is made possible by the rapid electrochemical response of microelectrodes (approximately a 1 \(\mu s\) time constant) (Wightman and Wipf, 1990). An experiment may be performed at a scan rate of 100 V/s, when the oxidative and reductive scans across a 1 V region are completed in 20 ms. When these scans are repeated at 100 Hz, the time course of the measurement provides sub-second temporal resolution (approximately 200 ms), where selective and sensitive data can be recorded in the same measurement (Wightman et al., 1988).

Digitally generated step-potential waveforms are instrumental for these measurements (Bilewicz et al., 1989; Murphy et al., 1989; Karpinski and Osteryoung, 1993). With fast linear scan rates, the capacitive (residual) current \(i_c\) increases linearly with an increasing scan rate, whereas the faradaic current \(i_f\) follows only a square root dependence. At high scan rates, the capacitive current is dominant. The use of step-potential waveforms and time-delay digital data acquisition minimize the contributions of capacitive currents at fast scan rates, thus allowing the relative amount of faradaic current to increase.

A cyclic staircase potential waveform (Fig. 1(a)) is described by its potential step-height and its potential step-width, in which the scan rate \(V/s\) is determined by their ratio (Howell et al., 1986). The advantage of this technique arises from the temporal control of the current measurement. Digital sampling provides for acquisition of the current at any point along the potential step width. Since the decay of capacitive currents are fast \((e^{-t/Rc})\), where \(t\) is time, \(R\) is the electrode resistance and \(C\) is the electrode capacitance), and the decay of the faradaic current follows a slower time dependence \((t^{1/2})\), the current is recorded at the end of the potential step (arrows, Fig. 1(a) inset).

The background current at a carbon-fiber microelec-
Fig. 1. Fast-scan cyclic staircase voltammetry (100 V/s, 100 prescans) at a polished and electrochemically pretreated, 10 μm diameter carbon-fiber microelectrode. (a) Cyclic staircase potential waveforms, in which the oxidative and reductive scans across a 1.2 V region (τ = 22 ms) were repeated every 200 ms. (a, Inset) An individual potential step was 18.30 mV high and 0.18 ms long. All currents were sampled at the end of each potential step (arrows). (b) Three-dimensional view of the oxidative portion, plotted in an upward direction, of the FSCSV experiment. (c) Average oxidative current versus time when the time course for the appearance of NADH is ascertained by monitoring the oxidative current between 714 and 934 mV versus Ag/AgCl. (d) Cyclic staircase voltammograms (average of 10 scans) acquired during (boxes) and after (triangles) the 4 s FIA injection of 100 μM NADH. (e) BSCV created by the digital subtraction of the two voltammograms from (c).
trode contains not only capacitive components but also faradaic components. The faradaic component of the residual background current stems from the carbon-oxygen moieties that are localized on the carbon surface, most notably quinones and hydroquinones (Wiedemann et al., 1991; Kawagoe et al., 1993a). While the use of digital staircase waveforms discriminates against capacitive currents, the background current is significantly affected by these surface-bound faradaic processes (Howell et al., 1986; Kawagoe et al., 1993b). Since the background current is only partially discriminated against with this technique, further improvements are needed.

Far greater results for improving \(i/i_0\), and the signal-to-noise ratios have been reported with background-current subtraction (Howell et al., 1986). For this technique, FIA provides the stable background-currents because the environment of the microelectrode surface is constant, except for the introduction of the sample bolus (Engstrom et al., 1988). This stable background current is necessary to prevent distortion of the faradaic information (Howell et al., 1986; Kawagoe et al., 1993a, b). A well-formed electrochemical response to a 100 \(\mu\)M bolus of NADH and a stable background current is shown in Fig. 1(b and c) at a polished carbon-fiber microelectrode. This demonstrates a consistent background current, and the introduction of analyte does not produce artifacts (odd peak shape, hysteresis, etc.).

To optimize FSCV further for the electrochemical measurement of NADH when performed at carbon-fiber microelectrodes, several electrochemical pre-treatments were explored. These were examined for their ability to optimize the quality and reproducibility of the NADH response for FSCV. A mild anodic treatment in HCl produced low overpotential and high faradaic currents for oxidation of NADH (Pantano and Kuhr, 1993) (Fig. 1(d and e)) at 10 \(\mu\)M diameter carbon-fiber electrodes, whereas 32 \(\mu\)M carbon fibers required treatment in pH 8.5 PBS to produce similar results (Hayes and Kuhr, 1998a). While low overpotential and high faradaic currents can be observed at freshly polished carbon-fiber surfaces, there was considerable variability from electrode to electrode. The BSCV shown in Fig. 1(e) was generated at a pretreated electrode by subtracting voltammetric scans acquired after the FIA injection (Fig. 1(c), triangles) from the voltammetric scans acquired during the injection (Fig. 1(c), boxes). This result demonstrates a BSCV where the facile electron-transfer information is retained through the subtraction process.

Measurement of the response time of this system was accomplished by monitoring the current from 800 to 1100 mV (faradaic current from oxidation of NADH) versus time (Fig. 1c). The response observed to this square-wave input was an electrochemical measurement characteristic of the temporal response of the sensing system. The response of a pretreated carbon-fiber microelectrode to the injection of NADH in a FIA-FSCSV experiment is quite rapid (Fig. 1(c)); the time required to reach 63% of the steady-state current is approximately 300 ms.

3.1. Selection of initial and switching potentials

While FSCSV of the NADH at a polished, electrochemically pretreated carbon-fiber microelectrode typically exhibits an oxidative peak potential at 800 mV versus Ag/AgCl (Fig. 1(e)), this anodic wave is shifted more than 300 mV positive following the enzyme-modification procedure (Pantano and Kuhr, 1993; Hayes and Kuhr, 1998b). A switching potential of 1100 mV (versus Ag/AgCl) was used because it was the maximum potential allowed without significant interference from the oxidation of the background buffer. The possibility of using the reductive NADH current to improve sensitivity is eliminated since oxidation of NADH at carbon electrodes is chemically irreversible (Moiroux and Elving, 1979, 1980).

The choice of an initial potential is influenced by other factors that are associated with the use of these probes in vivo. In previous in vivo FSCSV determinations for stimulated release of catecholamines, a – 400 mV (versus Ag/AgCl) initial potential was required (Wightman et al., 1988). This value was chosen to ensure the complete reduction of the oxidized quinone present on numerous biological molecules of interest generated during the oxidative voltammetric scan. Fluctuations in the concentrations of these species could interfere with the analysis of NADH. Nonetheless, even if no detectable quinone/hydroquinone redox couple were present in solution, quinone and hydroquinones on the carbon-fiber surface itself would always be present (Kawagoe et al., 1993a). An initial potential of 0.0 V (versus Ag/AgCl) was chosen to eliminate the quinone/hydroquinone redox couple, thus avoiding possible artifacts from this process altogether.

3.2. Background current drift

Repetitive cycling of a potential waveform will improve the stability of the background current, when this cycling allows the electrode surface to approach a steady state (Kinoshita, 1988). In vivo voltammetry experiments typically require a 10 min cycling of the potential waveform after implantation before any data is acquired (Wightman et al., 1988). The majority of this voltammetric degradation occurs within the first few minutes after implantation. With carbon-fiber surfaces, the sensitivity is diminished, but the background currents will remain stable for the remainder of the experiment and therefore background-subtraction techniques may be employed.

A carbon-fiber surface with immobilized enzymes
also requires cycling of the voltammetric waveform to use the background-subtraction technique. Quantitative evaluation of the background current upon repetitive cycling of the voltammetric waveform was investigated (Fig. 2). The number of conditioning prescans to eliminate or minimize the drift observed at an enzyme-modified electrode surface was determined. A flat baseline was produced with approximately 700 prescans, and 1000 prescans were used before analytical voltammograms were recorded (Fig. 2(b)). This number of prescans can be generated in 2–3 min in a FSCSV experiment depending on the exact conditions.

3.3. Selection of voltammograms for BSCV

The sensitivity of FSCSV for NADH was degraded by the fabrication procedure for enzyme-modified electrodes. The initial faradaic response for the NADH oxidation was diminished by more than 84%, and the oxidative peak potential shifted. This reduces the clarity with which the NADH oxidative signal can be interpreted and requires that all measures be examined to maximize the signal-to-noise ratio.

The reduction in sensitivity caused by these voltammetric shifts led to a quantitative investigation of the selection of CVs used for the background subtraction. Consistent results were obtained whether the subtraction CVs were obtained immediately before or immediately after the analyte plug. At high concentrations, there is no significant difference between using the background scans from before or after the analyte plug. For this set of eight electrodes exposed to 100 µM NADH in a 4 s plug, there was only one difference (Table 1), an increase of 8% (only 0.59 nA) in \( i_p \). Due to this slight increase, background scans were used from after the analyte plug. However at lower concentrations even small differences are important. Thus, the slight under-subtraction observed when the background is taken before injection of sample can severely affect the observed response. Therefore, it is generally best to use background obtained after the injection of sample, since this is more representative of the electrode condition during the sample.

A single electrode was used to investigate another aspect of the background scans used for subtraction. Background scans were taken from long before (0–2 s, Fig. 2(a)) and long after (15–17 s, Fig. 2(a)) the analyte bolus and subtracted. The resultant CVs had \( i_p \) of 6.0 nA; \( E_p \) of 930 and \( i_p \) of 6.6 nA; \( E_p \) of 930, respectively. While the background currents generated at the carbon-fiber surface remained remarkably stable throughout the 17 s (85 scans) of the FIA measurement, it is notable that the electrode does continue to change with time. Therefore, it is best to minimize the time allowed between sample and background scans. The optimum subtraction is obtained by using the background immediately following the removal of the sample.

The number of scans to be used for both the analyte and the background data sets were also investigated. The number of scans for both the analyte data set and the background data set were kept equal and increased from 1 to 16 (Fig. 3). The noise from the resultant BSCV was plotted versus number of scans averaged, when the noise was reduced by an approximate square root-dependence with the number averaged. However, no significant S/N enhancement was found after 10 scans were averaged, indicating that unknown factors other than random noise contribute significantly to the noise for these electrodes.

Noise reduction was also performed by averaging...
Table 1
Position of scans used for background subtraction for BSCV

<table>
<thead>
<tr>
<th>Electrochemical parameter</th>
<th>Before (1-3 s)</th>
<th>After (9-11 s)</th>
<th>Change</th>
<th>Percentage change</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_p$ (mV)</td>
<td>856 ± 17</td>
<td>862 ± 16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$i_p$ (nA)$^b$</td>
<td>6.50 ± 0.42</td>
<td>7.09 ± 0.66</td>
<td>+ 0.59 ± 0.30</td>
<td>+ 8 ± 4.6</td>
</tr>
</tbody>
</table>

$^a$All errors quoted are SEM.

$^b$Average current from 800 to 1100 mV minus average current from 200 to 400 mV versus time for a 4 s FIA injection of 100 µM NADH.

Signal optimization for time course measurements was examined first. The signal potential range ($R_{sig}$) was varied and the signal intensity recorded (Fig. 4(a)). For this particular electrode ($E_p = 860$ mV, Fig. 4(a)), the maximum signal intensity occurs when the voltage range encompasses $E_p$ and greater, while minimizing other potential ranges. Current was collected from ~50 mV less than $E_p$ to 1100 mV for the differential current versus time plots.

Signal optimization for differential time course measurements examined the background potential range ($R_{bkgd}$) for the subtractive current. The background capacitance currents caused by these processes were minimized by an additional quantitative data manipulation. Current obtained in a voltage range where no relevant faradaic current occurred was subtracted from the oxidative faradaic current. Capacitive effects are independent of the value of the potential, depending only on the scan rate. So, the capacitive currents in the voltammogram which overlap the faradaic signal are equal to those in the non-faradaic region.

3.4. Differential current versus time measurements

Electrochemical detection is not immune to its local environment: surface changes in pH, ionic strength, etc., especially near detection limits may cause artifacts (Wiedemann et al., 1991; Kawagoe et al., 1993a). Background capacitance currents caused by these processes were minimized by an additional quantitative data manipulation. Current obtained in a voltage range where no relevant faradaic current occurred was subtracted from the oxidative faradaic current. Capacitive effects are independent of the value of the potential, depending only on the scan rate. So, the capacitive currents in the voltammogram which overlap the faradaic signal are equal to those in the non-faradaic region.

Fig. 3. Noise of background-subtracted cyclic voltammogram versus number of scans averaged. Both the background and signal scan numbers were increased equally. Data taken from FIA experiment similar to that shown in Fig. 1(c).

Fig. 4. Potential range optimization for differential current versus time data. (a) Potential range versus current intensity for average current plots. $E_p$ for NADH at this carbon-fiber electrode was 860 mV. Range used was from the data point value to 1.1 V. (b) Potential range used for subtraction current versus current intensity for differential current plots. Average current values were obtained from 800 to 1100 mV.
potential range was varied from 0–100 mV to 0–1100 mV (Fig. 4(b)), when there was no loss in sensitivity until the subtracted current potential range exceeded 400 mV. This maximum range (400 mV) was optimal for the compensation of buffer-caused capacitive changes to the carbon-fiber electrode. This differential method produced a quantitative digital subtraction procedure, which allows these experiments to be performed in areas of pH flux and ionic strength shifts (Kawagoe et al., 1993a).

Special consideration must be given to large pH or ionic strength shifts created with the FIA analyte bolus. Although a reduction in sensitivity occurs, pH shifts up to 1.0 pH units and ionic strength changes of 100 mM were compensated with differential current data treatments. The need for alternative data treatment arises due to an oxidative wave at 100 mV for pH shifts (Fig. 5(a and b)). In addition, large changes in ionic strength resulted in significantly different currents arising across the potential range due to these ionic effects alone. To compensate for these additional variable currents across the potential range, which are not due to the oxidation of NADH, the differential potential ranges were shifted to 800–1000 mV minus 700–800 mV. With this data manipulation (Fig. 5(c)), the standard deviation of the oxidative current for NADH, while changing the bolus pH from 7.5 to 9.0, improved from 9.6 nA (average current) to 0.15 nA (differential current, 6400% reduction) with a 64% loss in sensitivity for the static-pH bolus (pH 8.5) experiment. For similar experiments with shifts in ionic strength, the standard deviation

Fig. 5. Effects of pH on background-subtracted cyclic voltammetry and resulting current intensity quantitated with the average current and differential current. (a) Background-subtracted cyclic voltammograms for 100 μM NADH at an enzyme-modified carbon-fiber microelectrode where the analyte bolus was at the same pH (8.5) as the background buffer. (b) Same conditions as (a), except bolus pH was 8.0. (c) Current intensity for differential current (●, current from 800 to 1000 mV minus current from 700 to 800 mV) and average current (■, current from 800 to 1000 mV) versus bolus pH. Background buffer pH was 8.5.
improved from 6.24 to 0.70 nA (890% reduction) with a 67% loss in sensitivity at the static ionic strength bolus (250 mM) experiment.

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