Technical Note

A Method for Low Nanomolar Concentration Analyte Sensing Using Electrochemical Enzymatic Biosensors

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A Method for Low Nanomolar Concentration Analyte Sensing Using Electrochemical Enzymatic Biosensors

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ABSTRACT

We introduce a new electrochemical measurement method compatible with enzymatic biosensors that is capable of analyte sensing down to the low nanomolar concentration regime. This method is termed accumulation mode sensing and utilizes an immobilized redox polymer mediator wired to an oxidoreductase enzyme to store charge during a pre-measurement charge concentration step, followed by a measurement step in which this accumulated charge is quantified. We demonstrate this new method using a model glucose sensor and show how the sensitivity of a sensor can be modified simply by adjusting the time duration of the charge concentration step. We achieve a limit of detection of 4.7 ± 1.4 nM using accumulation mode sensing, which represents a 25-fold improvement over traditional amperometry.

INTRODUCTION

Enzymatic biosensors, which utilize enzyme associated with a transducer as a biorecognition element for a target analyte, have been developed and utilized extensively.[1,2] While many different signal transduction methods have been used, the most common has been electrochemical.[3,4,5] This is due in part to the benefit of the biological event being directly converted to an electrical signal, which obviates the needed for complex instrumentation, giving electrochemical biosensors an advantage in terms of size, cost, and portability. Among the electrochemical techniques used for signal transduction, amperometry is often used. In an amperometric measurement, the working electrode of the sensor is held at a constant potential while the current flowing through the sensor is measured. The sensor is designed such that this current is dependent upon analyte concentration. A well-known example of an enzymatic biosensor utilizing amperometry is the continuous glucose sensor, which is a wearable, *in vivo* device designed to provide frequent blood glucose concentration measurements to the user.[6] These devices utilize a glucose oxidoreductase enzyme, typically glucose oxidase (GOx), immobilized on a working electrode as the glucose-sensing element. Electrons are first passed from glucose to the enzyme via enzymatic oxidation, and then to the working electrode through a redox mediator, such as O_2 or an Os-containing redox polymer.

While amperometry has proven viable for measuring analytes such as glucose, which is present at relatively high physiological concentrations (~5 mM), it may not be suitable for measuring analytes present at lower concentrations. Many of these analytes are both useful and could conceivably be measured using an electrochemical enzymatic biosensor, such as cortisol (approximate physiological concentration = 100 nM; useful for stress monitoring)[7] or creatinine (approximate physiological concentration = 100 μM; useful for renal monitoring).[8] We note that glucose has been successfully detected down to femtomolar levels via a strategy utilizing a second enzyme to completely remove oxygen, a redox interference species, from the sample solution.[9] However, additional methods that can measure low analyte concentration, even in the presence of potential interference species, are still

desirable. With this in mind, we deemed it prescient to investigate new electrochemical techniques compatible with enzymatic biosensors that are capable of high-sensitivity analyte detection.

One of these techniques, which we introduce here, is termed "accumulation mode sensing." This method draws inspiration from anodic stripping voltammetry (ASV), a high-sensitivity electrochemical technique most commonly used for trace metal ion detection.[10,11,12] In ASV, metal ions are first concentrated by electrodeposition onto the working electrode for a period of time and then detected via stripping with an anodic potential sweep. The key to the high sensitivity of ASV is the pre-measurement concentration of the analyte onto the sensing electrode. We sought to apply to enzymatic biosensors this same principle of a concentration step in order to increase the sensor sensitivity. Our method utilizes an immobilized redox polymer mediator, which "wires" the enzyme to the working electrode[13] by serving as an electron-transfer relay between the oxidoreductase enzyme and the working electrode, to store (or "accumulate") charge from the analyte during a concentration (or "accumulation") step. This accumulation step is followed by a measurement step in which the accumulated charge is measured. It is important to note that typical high-sensitivity voltammetry methods, such as normal pulse, differential pulse, and square wave voltammetry, are rendered ineffective in a sensor utilizing a redox mediator, as the potential pulses would result in large current peaks due to the electrochemical reaction of the redox mediator, thus obscuring the analyte signal.

Here, we demonstrate the principle of accumulation mode sensing using a previously-developed, model glucose sensor.[14] This sensor consists of GOx enzyme "wired" to a working electrode using an immobilized Os-containing redox polymer. During the accumulation step, electrons derived from GOx-catalyzed glucose oxidation are stored in the redox polymer via Os^{3+} reduction. In the subsequent measurement step, the accumulated charge is measured by oxidizing the excess Os^{2+} back to Os^{3+} at the working electrode. We first describe and demonstrate how this electrochemical sensing technique works and show that the sensitivity of a measurement can be increased simply by increasing the time duration of the accumulation step. We next demonstrate a sensing experiment and show that accumulation mode sensing gives a linear response over a range of analyte concentrations. Finally, we show that under

optimized conditions, accumulation mode sensing has a limit of detection of 4.7 ± 1.4 nM, a 25-fold improvement over amperometry. While this report uses a glucose sensor as a model enzymatic biosensor to demonstrate this new technique, we believe accumulation mode sensing should be broadly applicable to any biosensor utilizing an oxidoreductase enzyme and an immobilized redox mediator.

EXPERIMENTAL SECTION

Materials and Sensor Fabrication

Materials and the procedure for sensor fabrication can be found in the supporting information.

Electrochemical Measurements

All electrochemical measurements were made using a conventional three-electrode cell with the glucose sensor as the working electrode, a Ag/AgCl reference electrode (in 3M KCl; Bioanalytical Systems, Inc.), and a screen-printed carbon counter electrode. The current vs. time trace for a sensor was measured throughout the course of an accumulation mode experiment using a potentiostat. For an accumulation mode measurement, the working electrode was electrically disconnected from the potentiostat for a set amount of time (the accumulation time), after which point it was reconnected to the circuit. **Figure S1** of the supporting information shows a scheme of the electrode diagram. When the working electrode of a sensor was electrically connected, it was poised at +40 mV. For the experiments shown in **Figures 1**, **2**, and **S2** a BASi Petit Ampere potentiostat (model LC-3D; Bioanalytical Systems, Inc., West Lafayette, IN) was used for current measurements. A 0.5 s sampling interval and 0.03 Hz filter were used, and the current signal was recorded using in-house LabView (National Instruments) software. For all other experiments, an increased time resolution was desired. Therefore, a potentiostat with higher time resolution was used (model 1030C; CH Instruments, Inc., Austin, TX). This potentiostat was used with a 0.1 s sampling interval and a 3.2 Hz filter except for those shown in **Figures S3** and **S5** of the supporting information. For those experiments, this potentiostat was used with a 0.1 s sampling interval and either a 3.2 Hz filter or a 0.032 Hz filter, as indicated. This signal was recorded using manufacturer-provided software. Measurements of peak area, peak height, and amperometric current in the resulting current vs.

time traces were made using Graphpad Prism 6 software. All experiments were carried out in 100 mM PBS buffer (pH = 7.4, 100 mM NaCl) and at 33 °C.

RESULTS AND DISCUSSION

Demonstration and Sensitivity Advantage of Accumulation Mode Sensing

Figure 1a and **1b** show a conceptual overview of accumulation mode sensing with an electrochemical enzymatic biosensor. This sensing strategy relies on having an oxidoreductase enzyme (AOx) electrically "wired" to the working electrode of the sensor through a redox polymer.[13] During normal amperometric sensing, the electrode is poised at a potential so that the analyte is reacted at a constant rate, which is proportional to analyte concentration. For an analyte oxidation reaction ($A \rightarrow A^{+}$), as shown in **Figure 1a** and **1b**, this means that electrons will flow from the analyte to the enzyme to the redox polymer to the working electrode at a constant rate, producing a steady-state current. If the working electrode is then electrically disconnected from the circuit, the flow of electrons from the redox polymer to the working electrode will stop, resulting in no current flow through the circuit. However, the analyte will still undergo enzymatic oxidation, which in turn results in reduction of the redox polymer ($Os^{3+} \rightarrow Os^{2+}$). This results in a buildup of the reduced form of the redox polymer (Os^{2+}) over time, as electrons from the analyte are stored in the redox polymer. When the working electrode is reconnected to the circuit so that it is poised at its original potential, the buildup of the reduced form of the redox polymer will be oxidized, resulting in a large current spike. The current will then decay back to the original amperometric current as the redox system reaches steady-state once again. This two-step process forms the basis for accumulation mode sensing: one in which the working electrode of the sensor is disconnected from the circuit for a set amount of time (termed the accumulation time), enabling charge from the analyte to "accumulate" in the redox polymer, and a second in which the working electrode of the sensor is reconnected back to the circuit, enabling the accumulated charge to be measured as a sharp peak.

To demonstrate accumulation mode sensing, we used a developed glucose sensor consisting of a glucose-specific sensing reagent deposited onto a screen-printed carbon electrode. The glucose sensing

reagent consists of glucose oxidase enzyme cross-linked to an Os redox polymer. This reagent has already been demonstrated for use in glucose biofuel cells[15,16] and both self-powered[17] and potentiostat-powered[14,18,19] continuous glucose sensors. **Figure 1c** and **1d** demonstrate how accumulation mode sensing can be used to increase the sensitivity of an electrochemical measurement. In this experiment, a glucose sensor was placed in a solution of 2 μM glucose and 100 mM PBS and several accumulation mode measurements were made while the sensor current was monitored. For each measurement, the sensor was initially poised at +40 mV to drive steady-state glucose oxidation, then the working electrode was electrically disconnected for a set time (the accumulation time) to allow for charge accumulation, and then the working electrode was reconnected to measure the accumulated charge. **Figure 1c** shows the current vs. time traces measured for five different accumulation times. As seen, the size of the oxidative current spike increases with an increasing accumulation time. This shows that by simply increasing the accumulation time, the sensitivity of the measurement is increased. The amperometric signal, which was measured as the steady-state sensor current, as well as the peak height and peak area of the current spikes measured in **Figure 1c** are plotted vs. accumulation time in **Figure 1d**. As expected, the amperometric current shows no dependence on accumulation time and remains constant. However, both the height and the area of the current spike show a linear dependence upon accumulation time, highlighting the advantage accumulation mode sensing has over traditional amperometry: the sensitivity of the sensor can be tuned by altering an easily adjustable parameter of the measurement technique, the accumulation time.

Accumulation Mode Sensing in a Calibration Experiment

We next sought to demonstrate accumulation mode sensing in practice with a calibration experiment to show the resulting signal over a range of analyte concentrations. **Figure 2** shows an example of a calibration experiment using a model glucose sensor for glucose concentrations up to 100 μM. A 60 s accumulation time was used for each detection. **Figure 2a** shows the resulting current vs. time trace for this experiment. As seen, both the steady-state amperometric current and the size of the accumulation mode current peaks increase with an increasing glucose concentration. **Figure 2b** shows plots of the amperometric current and the peak height and peak area of the current spikes as a function of

glucose concentration, with all three signals exhibiting a linear dependence upon analyte concentration. This is important, as it shows that accumulation mode sensing yields linear calibration curves and can be utilized for sensing in a manner analogous to traditional amperometry. Since the peak height obtained from accumulation mode sensing is measured in units of current, the sensitivity of this measurement method can be quantitatively compared to the sensitivity of amperometry. This can be done by comparing the slopes of the calibration curves, such as those shown in **Figure 2b**. In this case, amperometry has a sensitivity of 0.44 nA/μM, while accumulation mode (using the peak height measurement) has a sensitivity of 1.69 nA/μM. Therefore, with an accumulation time of 60 s, accumulation mode sensing increased the sensitivity of the electrochemical measurement by a factor of nearly 4. The supporting information shows an additional experiment in which calibrations were run using accumulation mode sensing with different accumulation times on sensors containing a flux-limiting outer membrane (**Figure S2**). This experiment shows that accumulation mode sensing still performs as expected when an outer polymer membrane is added to the sensor and gives another example of how the sensitivity of the sensor can be tuned by altering the accumulation time.

High Sensitivity Measurements Using Accumulation Mode Sensing

We next sought to demonstrate how the sensitivity of accumulation mode sensing could be tuned to give superior detection performance over amperometry at low analyte concentrations. Two important steps were taken to help enhance the accumulation mode signal. The first was modifying the frequency at which the current signal was recorded. In order to maximize the peak height measured during the accumulation detection current spike, the signal was recorded at a faster rate (0.1 Hz) and filtered at a higher frequency (3.2 Hz) than the previously discussed experiments (0.5 Hz sampling rate, 0.03 Hz filter). The effect of the filtering frequency on the current peak shape is further discussed in the supporting information (**Figure S3**). The second change was adding carbon nanotubes to the sensing reagent.[20,21,22] This has the effect of making the dispensed sensing reagent film more uniform (**Figure S4**) and electrically conductive. This is important, as it increases the kinetics of the redox polymer oxidation step, which results in the accumulation mode current spike having a larger peak height. The supporting

information contains further discussion on the effect of the signal filtering frequency and addition of carbon nanotubes on the resulting accumulation mode signal (**Figure S5**). The important takeaway, however, is that changing the filtering frequency from 0.032 Hz to 3.2 Hz was found to increase the peak height signal by a factor of 2-3, while the addition of CNTs to the sensing reagent was found to increase the peak height signal by a factor of 5-6.

By using these idealized detection parameters along with an increased accumulation time, accumulation mode sensing can significantly out-perform traditional amperometry at low concentration analyte sensing. **Figure 3** shows the results of an accumulation mode detection experiment at glucose concentrations from 0 to 200 nM using these idealized detection parameters and an accumulation time of 30 min. One feature clearly seen is the presence of a negative background signal. This negative background, which is constant over time, is eliminated upon deoxygenation of the sample solution, indicating it is due to the oxygen reduction reaction. This shows that oxygen acts as a redox interference species and that its concentration in the sample should be kept constant for accurate analysis. This is further discussed in the supporting information (Figure S6). The resulting calibration curves, which are the average response of 8 sensors, are shown in **Figure 3b**. As seen, the currents associated with the amperometric measurements are exceedingly small (< 50 pA) and lose linearity below 100 nM, while the signals for accumulation mode sensing are much larger and retain linearity well below 100 nM. **Table 1** shows the sensitivity, limit of detection (LOD) (calculated as 3σ/slope, utilizing standard approach 1[23]), and linear detection range associated with these measurements. The linear detection range is discussed further in the supporting information (**Figure S7**). As seen, with an accumulation time of 30 min accumulation mode sensing using the peak height measurement gives an 800-fold increase in sensitivity over amperometry. In terms of detection limit, accumulation mode sensing using the peak area measurement is the superior measurement method, giving an LOD of 4.7 ± 1.4 nM, a 25-fold improvement over amperometry. While the linear range for accumulation mode sensing is more limited than for amperometry, it should be noted that this range can be shifted to higher concentrations by using a

shorter accumulation time. Overall, this data demonstrates that accumulation mode sensing can be utilized to give superior detection over amperometry at low analyte concentrations.

CONCLUSION

We have demonstrated a new, high-sensitivity electrochemical measurement technique for use with enzymatic biosensors. This technique, termed "accumulation mode sensing," utilizes a concentration step in which analyte charge is accumulated in an immobilized redox polymer to increase the sensitivity of the measurement. The accumulated charge is then quantified in a measurement step. To demonstrate this new technique, we used a model glucose sensor, consisting of glucose oxidase enzyme co-immobilized on a working electrode with an Os-containing redox polymer. While we used a model glucose biosensor here, we emphasize that the key components of accumulation mode sensing are an oxidoreductase enzyme co-immobilized with a redox mediator. Therefore, accumulation mode sensing will be applicable to any electrochemical biosensor using this structure. Importantly, we found that both the peak height and peak area of the detection signal scale linearly with analyte concentration. Additionally, we demonstrated that the sensitivity of a sensor could be increased simply by increasing the accumulation time. This is an advantage over amperometry, which has no measurement-related parameter that can be altered to change the sensitivity of the measurement. Under the optimized conditions of an increased signal filtering frequency and the addition of CNTs, we showed that with an accumulation time of 30 min, accumulation mode sensing has a >800-fold higher sensitivity than amperometry, resulting in a 25-fold improvement in the limit of detection.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Included are a materials section, description of sensor fabrication, diagram of the electrode setup, an experiment demonstrating accumulation mode sensing with polymer-coated sensors, experiments showing

optimization of the accumulation mode signal for high sensitivity detection, an experiment investigating the background signal, and an experiment demonstrating the linear detection range.

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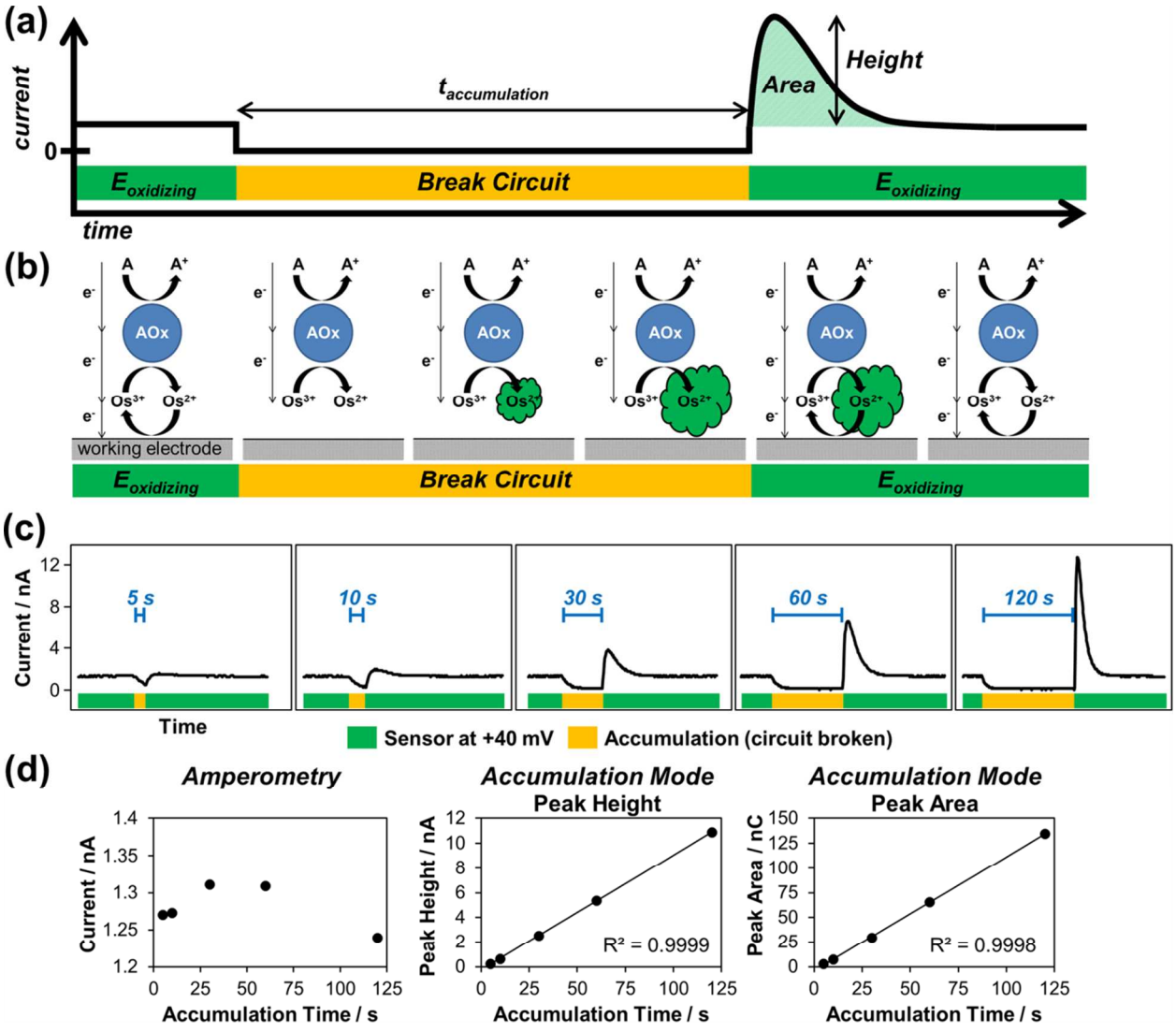

Figure 1. (a) The expected current vs. time signal and quantitative parameters (accumulation time, peak area, and peak height) of accumulation mode sensing. **(b)** A scheme of the redox reactions occurring during accumulation mode sensing of an oxidizable analyte (analyte A) using an oxidase enzyme (AOx) co-immobilized with an osmium redox polymer. **(c)** The current vs. time traces obtained for accumulation mode sensing of 2 μM glucose using a model glucose sensor and different accumulation times. **(d)** A comparison of the amperometry and accumulation mode signals measured for the accumulation times shown in (c).

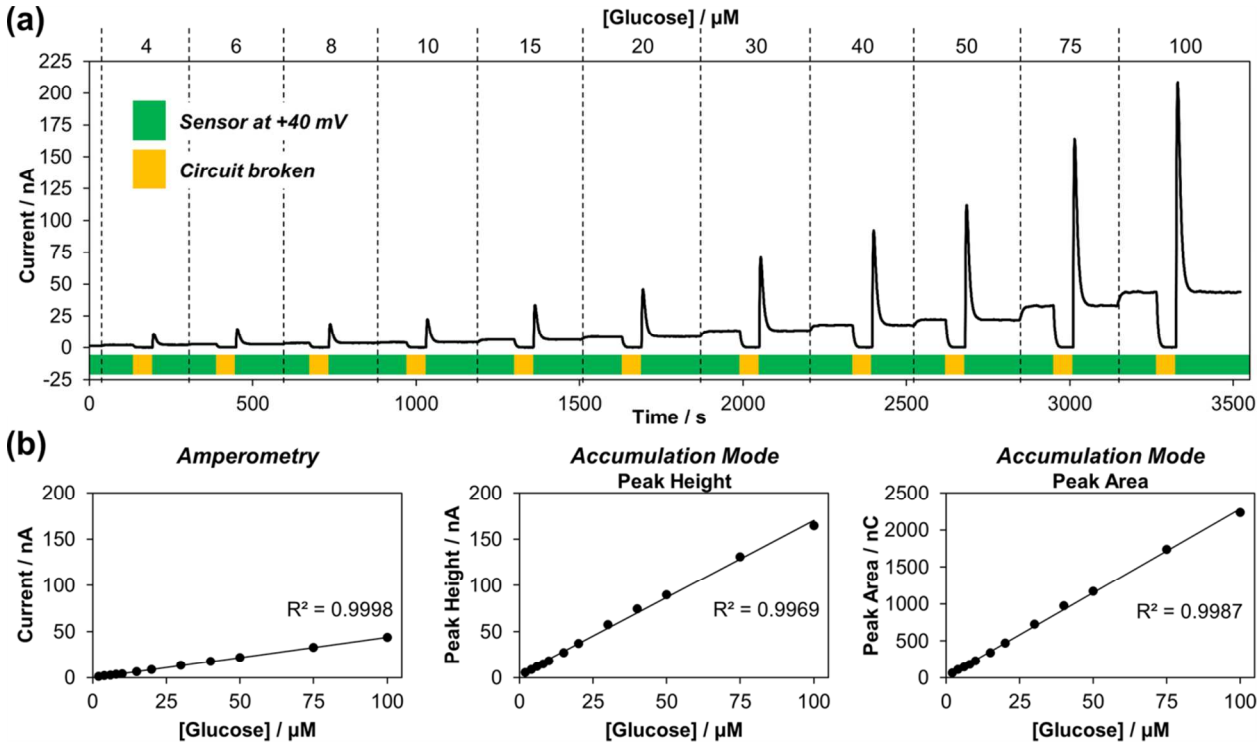

Figure 2. (a) A representative current vs. time trace for a calibration experiment using accumulation mode sensing with a model glucose sensor and a 60 s accumulation time for each detection. **(b)** A comparison of calibration curves resulting from the amperometry and accumulation mode signals measured for the sensing experiment shown in (a).

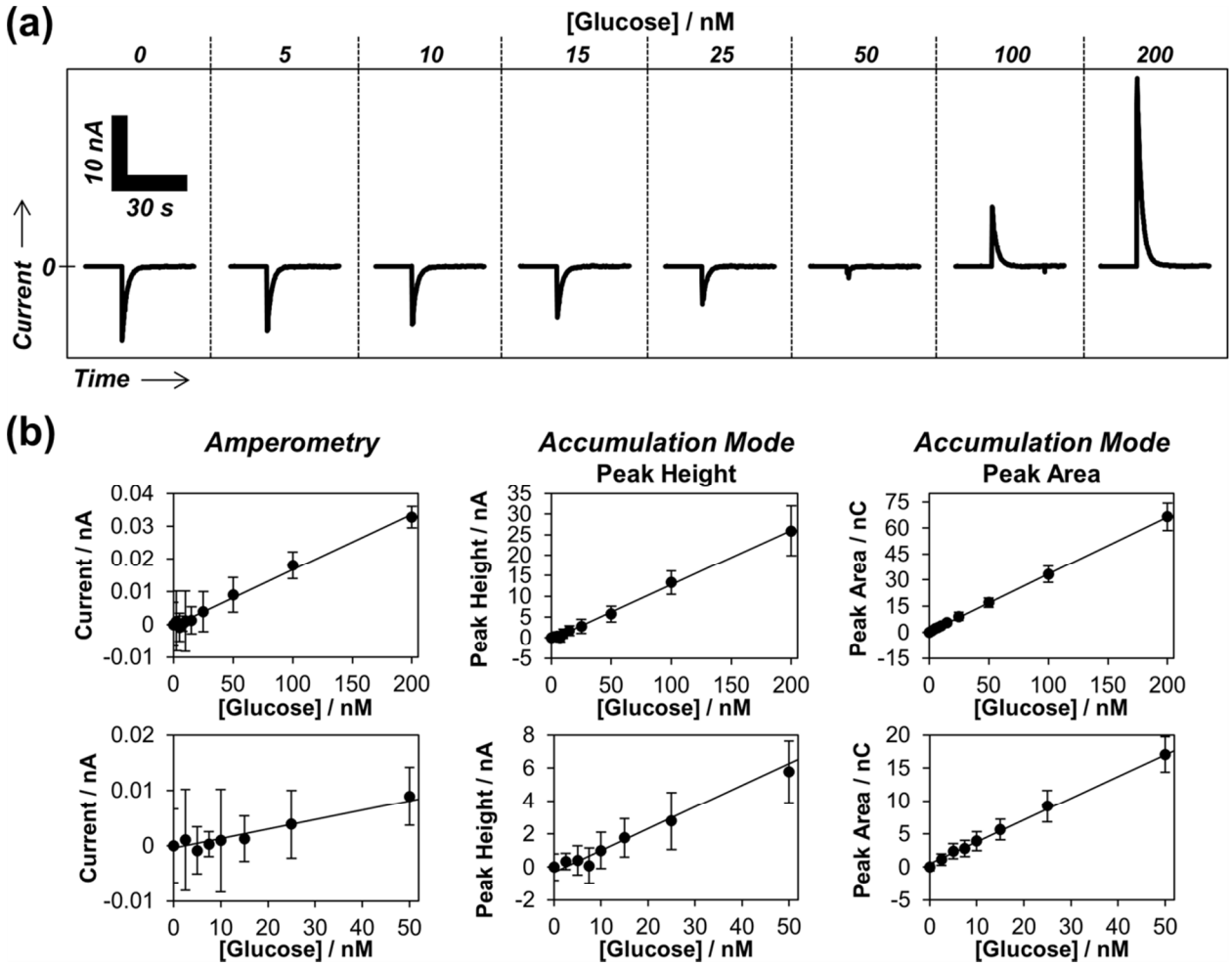

Figure 3. (a) Accumulation mode signals obtained for a representative glucose sensor during a calibration experiment using glucose concentrations from 0-200 nM. A 30 min accumulation time was used for each detection. The signal was filtered at 3.2 Hz and CNTs were added to the sensing reagent. **(b)** Calibration curves with corresponding linear fit resulting from the amperometry and accumulation mode signals measured for the sensing experiment shown in (a). Each signal is the background-subtracted mean of 8 sensors, with error bars representing the standard deviation. The bottom row of plots is a zoom-in showing glucose concentrations from 0-50 nM.

Measurement Method	Sensitivity	LOD / nM	Linear Range / μM
Amperometry	0.00017 ± 0.00001 nA/ nM	120 ± 42	0.12 - >100
Accumulation Mode-Peak Height	0.14 ± 0.03 nA/nM	20 ± 16	0.02 - 2
Accumulation Mode-Peak Area	0.33 ± 0.04 nC/nM	4.7 ± 1.4	0.004 - 5

Table 1. Comparison of sensitivity, limit of detection, and linear range for amperometry and accumulation mode sensing using an accumulation time of 30 min.

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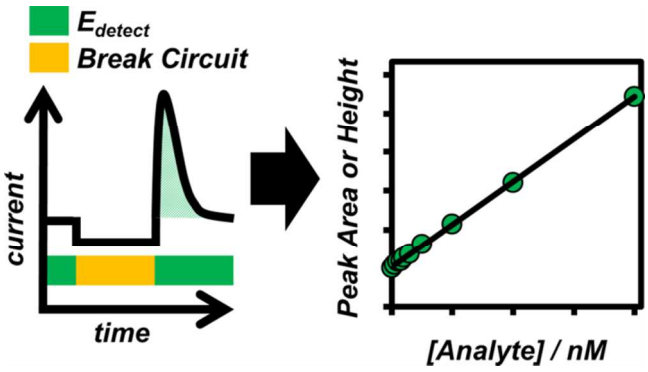
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