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Double Potential Pulse Chronocoulometry for Detection of Plasma Membrane Cholesterol Efflux at Disk Platinum Microelectrodes

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Abstract

A double potential pulse scheme is reported for observation of cholesterol efflux from the plasma membrane of a single neuron cell. Capillary Pt disk microelectrodes having a thin glass insulator allow the 10 μ m diameter electrode and cell to be viewed under optical magnification. The electrode, covalently functionalized with cholesterol oxidase, is positioned in contact with the cell surface resulting in enzyme catalyzed cholesterol oxidation and efflux of cholesterol from the plasma membrane at the electrode contact site. Enzymatically generated hydrogen peroxide accumulates at the electrode/cell interface during a 5 s hold-time and is oxidized during application of a potential pulse. A second, replicate potential pulse is applied 0.5 s after the first potential pulse to gauge background charge prior to significant accumulation of hydrogen peroxide. The difference in charge passed between the first and second potential pulse provides a measure of hydrogen peroxide generated by the enzyme and is an indication of the cholesterol efflux. Control experiments for bare Pt microelectrodes in contact with the cell plasma membrane show difference charge signals in the range of about 7–10 pC. Enzyme-modified electrodes in contact with the plasma membrane show signals in the range of 16–26 pC.

INTRODUCTION

This research group is investigating cellular cholesterol efflux using cholesterol oxidase modified microelectrodes positioned at the plasma membrane surface of single cells and tissue. The overall utility of the methodology is the ability to distinguish disease states, which are known to exhibit altered plasma membrane cholesterol, from wild-type control samples. The thrusts of the work described in this manuscript are feasibility and advantages

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of a coulometric approach for data acquisition at microelectrodes of disk geometry. The electrode depletes cholesterol in the thin aqueous layer between the electrode surface and the plasma membrane. Enzymatic consumption of aqueous phase cholesterol disturbs the equilibrium between aqueous cholesterol and membrane cholesterol which drives net efflux of plasma membrane cholesterol at the electrode contact site. Hydrogen peroxide produced by the enzyme is electrochemically oxidized to indicate the rate of enzymatic cholesterol oxidation.

Earlier amperometric experiments demonstrated detection of membrane cholesterol in oocytes,¹ giant vesicles,² macrophages,³ human epithelial cells,⁴ and mouse nasal⁴ and tracheal tissue.⁵ In all of these studies, data acquisition involved referencing the steady-state current for cholesterol efflux to a baseline current measured in solution with the electrode positioned away from the cell surface. Measuring a change in current (relative current) was required. For example, in work by other research groups, microelectrode detection of neurotransmitter release from cells implemented the approach of stimulated exocytosis where the background current is determined prior to neurotransmitter release and detection.⁶ In such studies of neurotransmitter release from cells, the electrode is held in a fixed position during background and signal measurements, as exocytosis is triggered chemically or electrically (using additional electrodes). In our microelectrode measurements, cellular cholesterol efflux is caused (stimulated) by positioning the electrode in contact with the cell surface. Therefore, it is not possible to evaluate the background signal with the enzyme-modified electrode positioned in contact with the cell surface. As a consequence, our amperometric scheme referenced the signal measured with the electrode held in contact with the cell to the baseline current observed with the electrode positioned away from the cell (in solution). Concerns are that the baseline current observed in solution is not zero and it may change upon positioning the electrode in contact with the cell surface. The double potential pulse coulometric strategy described in this article measures a differential charge between a pair of potential pulses as a means of circumventing the impossibility of an absolute signal. In comparison with our amperometric experiments, the coulometric method does not require reference to a solution measurement and repeated movement of the electrode is not required for averaging data. A caveat is that the background contribution to the measured signal must be estimated from control experiments conducted at microelectrodes containing no immobilized enzyme.

Proof of concept for the double potential pulse experiment is provided in the group's recent work where a micro-cavity electrode geometry was used to demonstrate aqueous efflux of cholesterol from the cell plasma membrane.⁷ These micro-cavity experiments published earlier gauged the turnover rate of the enzyme by determining the amount of hydrogen peroxide that accumulated in the cavity during a 5 min hold time. The accumulated hydrogen peroxide was consumed by oxidation upon application of a 4 s potential step perturbation. To account for drift in the charge between sequential measurements, the charge passed during the potential step was referenced to the charge passed during a replicate potential step experiment conducted 30 s after the initial potential step, before significant accumulation of hydrogen peroxide. Each data point was the difference charge between a pair of potential step experiments where the first step had a 5 min hold time at the initial potential and the second step had a 30 s hold time at the initial potential. The waveform

(descriptively termed double potential pulse) was repeated to average consecutive difference charge measurements with the electrode positioned in contact with the cell plasma membrane.

There were two primary limitations of the micro-cavity electrode geometry that led to the present work described in this article where the disk electrode geometry is used. One challenge encountered for the micro-cavity was the inability to consistently prepare different electrodes with a similar Pt surface area. The differences in Pt surface area between individual micro-cavity electrodes resulted in variation in the amount of immobilized enzyme and thus different cholesterol efflux rates between experiments conducted at different electrodes. The other restriction imposed by the micro-cavity geometry was the slow rate of cholesterol extraction (efflux) from the cell plasma membrane, a consequence of the enzyme-modified electrode surface being recessed from the cell surface. The slow efflux at micro-cavity electrodes necessitated a significant hydrogen peroxide accumulation time (5 min) which dictated a long total analysis time (e.g., about 30 min. for collection of 4 difference charge measurements). Towards addressing these two issues, an optimized double potential step waveform is demonstrated at disk Pt microelectrodes.

This article describes work demonstrating the feasibility of applying the double potential pulse data acquisition scheme to microelectrodes of disk geometry for observation of cholesterol efflux from a single cell. The advantages of the disk electrode geometry over the micro-cavity are the ability to better control the surface roughness of the disk Pt electrodes and increased cholesterol efflux at the disk Pt electrodes. An electrochemical etching process was used to form the micro-cavity electrodes and it was not possible to fabricate electrodes having the same Pt electrode surface area. The disk electrodes used in the work reported in this article do not require the etching process. The disk microelectrodes are mechanically polished which leads to less variation in Pt surface area between various electrodes. Therefore, the electrode surface modification chemistries deposit a more consistent amount of immobilized enzyme on the disk electrodes than on the micro-cavity electrodes. The cholesterol efflux rate produced by the electrode is, in part, a function of the enzymatic activity exhibited by the electrode. The other significant advantage of the disk electrode geometry over the micro-cavity is that the enzyme layer is positioned closer to the cell surface and this causes larger cholesterol efflux. The smaller efflux for the micro-cavity geometry, where the electrode-immobilized enzyme was recessed 5 μm from the cell surface, was a result of a less pronounced concentration gradient of cholesterol between the cell plasma membrane surface and the enzyme-modified electrode surface. The increased efflux observed at disk electrodes, with the shorter diffusional distance and steeper cholesterol concentration gradient between the cell surface and the electrode surface, permits a shorter hydrogen peroxide accumulation time prior to electrochemical oxidation and thus a shorter total analysis time.

An exciting feature of the double potential pulse scheme applied to disk microelectrodes is that the difference charge is completely insensitive to Faradaic charge arising from redox active species that diffuse to the electrode from bulk solution during the potential pulses. Hydrogen peroxide exhibits this behavior and, as demonstrated below, the difference charge measurement is independent of bulk solution hydrogen peroxide concentration. This aspect

of the measurement provides selectivity in detecting hydrogen peroxide produced at the electrode surface by the immobilized enzyme. The difference charge is a direct measurement of the accumulation of hydrogen peroxide at the electrode surface, above any bulk solution concentration.

The selectivity of the double potential pulse measurement for species that accumulate at the electrode surface leads to a background contribution to the difference charge signal. The background arises from contaminants that adsorb on the electrode surface prior to the first potential pulse. Because the initial potential is held for a longer time before the first potential pulse (5 s) than it is for before the second potential pulse (0.5 s), the first pulse contains a larger contribution from oxidation/desorption of species adsorbed to the electrode. The background contribution to the difference charge is estimated from control experiments conducted at single cells using bare Pt electrodes containing no immobilized enzyme, which show smaller difference charge compared to enzyme-modified electrodes. The increased difference charge recorded at enzyme modified electrodes is assigned to accumulation of hydrogen peroxide at the electrode/cell contact site and is an indication of cellular cholesterol efflux.

The challenge of improving electro-oxidation of hydrogen peroxide continues to attract effort in the surface science and electroanalytical arenas. Electrodes functionalized with inorganic catalysts,⁸ nanoparticles,^{9–12} and carbon nanotubes decorated with Pt nanoparticles¹³ have been reported for hydrogen peroxide oxidation. Improvements in oxidation kinetics, increased electrode surface area, and enhanced mass transfer properties are documented in this literature. The double potential pulse scheme reported in this article could be applied in conjunction with such state-of-the-art electrode designs for enzyme-based microelectrode sensing at biological membranes.

EXPERIMENTAL SECTION

Neuron Cells

Experiments are performed using the buccal ganglia from *Aplysia californica* (Marinus Scientific, Garden Grove; CA). *Aplysia* are anaesthetized by injecting MgCl_2 (50% body mass) into the body cavity. Dissection is carried out to remove the buccal mass and buccal ganglia. Desheathing of the buccal ganglia is accomplished by pinning onto a Sylgard (0.5 cm) petri dish in *Aplysia* saline (450 mM NaCl, 10 mM KCl, 22 mM MgCl_2 , 33 mM MgSO_4 , 10 mM CaCl_2 , 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), and 10 mM glucose, pH 7.4).

Microelectrode Fabrication

For single cell studies, it is necessary for the microelectrode to have a thin insulator (small total outer diameter) so that the Pt disk electrode can be observed and positioned at the cell surface under optical magnification. Therefore, capillary Pt disk (10, 25, 50 μm diameter) microelectrodes are fabricated in-house as described earlier.² Briefly, the glass capillary (Kimax, Kimble) is pulled in a hydrogen flame and the Pt wire is inserted. The 1.3 μm diameter Pt microelectrodes are formed by electrochemically etching³ the 10 μm diameter Pt

wire prior to inserting it in the pulled capillary. Conductive epoxy (MG Chemicals) is used to make electrical contact between the Pt wire and a copper lead. The glass capillary containing the Pt wire is placed in a nickel coil and heated to pull the electrode creating a thin insulating layer of glass on the Pt wire. The microelectrode is polished (beveling machine, Narishige) to produce the capillary disk electrode geometry.

The Pt disk microelectrodes are immersed in 5 mM solution of 11-mercaptoundecanoic acid (Aldrich Chemical Co.) in hexanes for 2 h to deposit a submonolayer of the thiol. The thiol-modified electrodes are then immersed in a 0.1 M phosphate buffer (pH 7.4) aqueous solution of 2 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma Chemical Co.) and 5 mM N-hydroxysulfosuccinimide (NHS) (Fisher) for 30 minutes for conversion of the carboxylic acid end groups of the submonolayer to an NHS ester. The electrode is rinsed and immersed in 1mg/mL of recombinant cholesterol oxidase (WAKO Chemical USA, Inc., ~33 units/mg) for 3 h for covalent attachment of the enzyme to the electrode surface. Alternatively, Pt disk microelectrodes are immersed in 4 mM dimethyl sulfoxide (DMSO) solution of 3,3--dithiodipropionic acid di(N-hydroxysuccinimide ester) (DTSP; Sigma Chemical Co.) for 45 min. This thiol contains a terminal leaving group. The electrode is rinsed and immersed in cholesterol oxidase solution (as above) for 3 hours for covalent attachment of the enzyme to the electrode surface.

Data Acquisition

Chronocoulometric analysis is conducted using a two-electrode electrochemical cell and a voltmeter-amperometer (Chem-Clamp, Dagan Corp). The three-pole Bessel filter of the Chem-Clamp was set to 100 Hz. Current integration is performed digitally post-experiment or in real-time using an analog integrator built in-house. Lab View and Lab View Signal Express software were used to produce the applied potential waveform and collect the data, respectively. An Ag/AgCl (3M KCl) reference electrode was used for all experiments and all potentials are reported versus NHE.

Verification of the initial potential

The initial potential does not oxidize or reduce hydrogen peroxide (oxygen) and this is verified experimentally. The electrode is placed in a buffer solution at an applied potential of 410 mV. The buffer solution is spiked with a 4 mM stock solution of hydrogen peroxide (1 mL) to bring the buffer solution to 200 μ M hydrogen peroxide. The baseline current is observed to verify that the applied potential does not reduce or oxidize hydrogen peroxide.

RESULTS AND DISCUSSION

The described analytical scheme is to allow enzymatic generation and accumulation of hydrogen peroxide for a specified time, after which detection of the hydrogen peroxide buildup is achieved by electrochemical oxidation during a short duration potential step (termed pulse). This method is repeated for multiple cycles of hydrogen peroxide accumulation and detection with the electrode held in a fixed position in contact with the cell surface. It should be underscored that enzymatic consumption of cholesterol and the rate of cholesterol efflux from the plasma membrane are assumed to be independent of the

potential applied to the electrode. The rate of cholesterol oxidation is controlled by cholesterol efflux from the plasma membrane, the cholesterol concentration gradient between the cell surface and the electrode surface, and enzymatic activity. We assume little depletion of cholesterol in the plasma membrane adjacent to the electrode (contact site). These factors are modeled as being steady-state during sequential iterations of hydrogen peroxide accumulation and detection. Hydrogen peroxide, as a product of irreversible steady-state enzymatic cholesterol oxidation, accumulates at the electrode surface prior to application of the potential pulse and is exhaustively consumed during the potential pulse. Only hydrogen peroxide concentration (not cholesterol concentration) cycles between accumulated and depleted during repeated measurements. We model the region of plasma membrane under the electrode footprint as a cholesterol mass transport bottleneck where efflux from the membrane is a limiting step. Lateral diffusion of cholesterol in the plasma membrane to the electrode contact site is assumed to be fast. A steady-state cholesterol concentration gradient is established between the plasma membrane and the electrode surface.

The values of the initial potential and the pulse potential were strategically chosen. The pulse potential should be sufficient to drive oxidation of hydrogen peroxide. Characterization experiments for electrochemical detection of hydrogen peroxide in buffer at bare Pt microelectrodes show that the rate of hydrogen peroxide oxidation is independent of potential at about 810 mV and more positive potentials. The limiting current for hydrogen peroxide oxidation at 810 mV is believed to be largely diffusion controlled. The coulometric experiments have a 810 mV (vs. NHE) pulse potential for oxidation of hydrogen peroxide at a maximum rate. Assuming diffusion control and using the equation for flux to a disk microelectrode in a finite insulating plane (capillary microelectrode),¹⁴ the limiting current suggests a diffusion coefficient for hydrogen peroxide of 1.2×10^{-5} ($\pm 2.7 \times 10^{-7}$) cm²/s (the glass insulating capillary that contains the Pt wire is about 0.45 μ m thick). This diffusion coefficient for hydrogen peroxide is consistent with values reported in the literature (e.g., 0.66×10^{-5} cm²/s – 2.00×10^{-5} cm²/s).^{8,15}

The value of the initial potential that is applied to the electrode during the time between consecutive pulses should not oxidize or reduce hydrogen peroxide so that it will accumulate. Characterization experiments show that hydrogen peroxide does not react at 410 mV (vs. NHE), the initial potential used in this work. It is also noted that oxygen reduction is not detectable at the initial potential and that the difference charge measurement, collected at bare Pt electrodes in buffer, is unchanged between nitrogen and air purge conditions.

The analytical challenge is measuring the relatively small charge from hydrogen peroxide oxidation over the large total positive charge that passes during the potential pulse. Previous amperometric work from this research group for cellular cholesterol efflux provides an estimate of the expected contribution to the charge from hydrogen peroxide accumulation. The amperometric experiments showed steady-state current for cholesterol efflux of 1–2 pA. Based on this estimate of cholesterol efflux rate and hydrogen peroxide production rate, about 5–10 amol of hydrogen peroxide could accumulate at the electrode surface in 5 s (assuming no diffusional loss). The contribution from hydrogen peroxide oxidation to the charge that passes during a potential pulse, where the initial potential is held for 5 s, is

therefore 5–10 pC. The total positive charge that flows during a potential pulse is about 10 times larger than this estimate of the hydrogen peroxide contribution as the charge is also comprised of contributions from capacitance and oxidation/desorption of contaminants adsorbed to the electrode. The charge for hydrogen peroxide oxidation is therefore expected to be about 5–10% of the total charge passed during the potential pulse. As discussed below, the contribution to the charge from adsorbed contaminants is dependent on the hold time prior to the potential pulse (as is the hydrogen peroxide contribution). The time dependent buildup of contaminants on the electrode surface during the hold time prior to the potential pulse leads to the background component of the difference charge measurement which is a focus of this article. The background contribution to the difference charge that arises from oxidation/desorption of contaminants is estimated by control experiments conducted at bare Pt electrodes containing no immobilized enzyme.

Figure 1A is one complete cycle of the double potential pulse waveform applied to the electrode. The initial potential (410 mV) is held for 5 s allowing accumulation of hydrogen peroxide near the electrode surface, as the electrode-immobilized enzyme oxidizes cholesterol. A double potential pulse (both pulses are to 810 mV for 0.250 s) is applied to the electrode for oxidation of the hydrogen peroxide (first pulse) and for gauging the background charge (second pulse). The time between the individual potential pulses of a double potential pulse experiment is long enough (0.5 s) to allow the current to return to near baseline prior to initiation of the second potential pulse (see Figure 1B, 5.250 – 5.750 s). The basis for the measurement is the longer time allowed for hydrogen peroxide accumulation before the first pulse relative to the second pulse. So the double potential pulse scheme has two hold times; a long hold time before pulse one and a short hold time before pulse two. For clarity, the short hold time is termed the quiet-time and the long hold time is termed the hold-time.

Figure 1C shows a representative overlay of total charge traces for pulse one and pulse two. The difference charge (e.g., Figure 1D) is the subtraction of the second pulse trace from the first pulse trace. The 60 Hz noise in the difference charge signal is variable between experiments. The magnitude of the difference charge is, in part, a reflection of the greater amount of hydrogen peroxide that is oxidized in the first pulse compared to the second pulse. The other reason for pulse one charge being larger than pulse two charge is that more contaminants adsorb on the electrode prior to pulse one because the initial potential is held longer before pulse one. The difference charge signal that is observed contains two primary contributions: differential hydrogen peroxide oxidation between pulse one and pulse two, and differential oxidation/desorption of adsorbed contaminants between pulse one and pulse two. The contribution to the difference charge from contamination is referred to as the difference charge background. It is typical for the difference charge to require about six replicate double potential pulse experiments before it becomes constant (12 – 36 s) and the difference charge always decreases during stabilization. The initial drift of the difference charge may reflect a decreased contribution from the background portion of the signal. The waveform is applied for a few minutes to allow the difference charge to stabilize and the measurement is the average of at least three consecutive double pulse experiments conducted after initial stabilization.

The primary contributions to the total charge passed during application of a potential pulse are double layer capacitance, stray capacitance, oxidation of species diffusing to the electrode, and oxidation of species that adsorbed to the electrode prior to the pulse. The pulse potential is sufficient to drive oxidation of surface platinum atoms and is insufficient to drive deep oxidation of the electrode. The initial potential is insufficient to reduce the Pt-oxide and so the electrode surface remains oxidized throughout the measurements. Also, the step potential is insufficient to drive oxidative desorption of the surface bound thiol. Pseudo-capacitive charge from potential dependent adsorption/desorption of anions may contribute to the total charge.

Control experiments are conducted at bare Pt electrodes containing no immobilized enzyme, and thus provide an estimate of the difference charge background contribution. Modification of the electrode surface (thiol-linker and enzyme) tends to decrease the difference charge by a few pC. A trend in difference charge between bare platinum electrodes and thiol-modified electrodes has not been identified. However, it is noted that the sub-monolayer thiol surface coverage is likely not stable (prone to change). Complete coverage of the electrode with the thiol (forming a self-assembled monolayer) blocks electrochemical oxidation of hydrogen peroxide. In estimating the difference charge background, bare platinum electrodes are the conservative control. Recent unpublished control experiments using thiol-BSA (dummy-protein) modified electrodes, suggest a smaller background contribution to the signal (for another sample type).

A consistent and firm result is that the charge passed during pulse one is always larger than the charge passed during pulse two. The chemistry that could give rise to the difference charge background (in control experiments where no hydrogen peroxide is produced) is greater oxidation (and/or desorption) of adventitious impurities adsorbed on the electrode surface for pulse one relative to pulse two. Capacitive contributions to the total charge are expected to pass rapidly and reversibly upon potential pulse perturbation and are not expected to contribute to the difference charge.

Because the pulse duration is short relative to the quiet-time between pulse one and pulse two, charge from Faradaic reaction of species diffusing to the electrode during the pulse is expected to be identical for pulse one and pulse two. This behavior is predicted due to radial diffusion to the microelectrode. As demonstrated below, creation of a diffusion gradient during pulse one has excess time to relax prior to pulse two. This is the case even if the species being oxidized during the pulse react irreversibly. This result is expected for species that do not slowly adsorb and accumulate at the electrode surface.

To test this hypothesis, control experiments were conducted at bare Pt microelectrodes in hydrogen peroxide solution. Figure 2A shows the charge that passes during pulse one and pulse two for three consecutive double pulse experiments in buffer. Figure 2B shows the increase in total charge that passes for pulse one and pulse two upon spiking the solution with hydrogen peroxide. The increase in charge is from electrochemical oxidation of the hydrogen peroxide at the pulse potential. Because the diffusion gradient produced during pulse one has ample time to relax during the quiet-time, the increase in charge is equal for pulse one and pulse two. The difference charge is independent of bulk solution hydrogen

peroxide concentration as shown in the overlay of the difference charge with and without added hydrogen peroxide (Figure 2C). It is noted that this independence of difference charge signal from bulk solution hydrogen peroxide concentration is maintained with the electrode positioned at the cell surface. That is, despite any convoluted path for diffusion to the electrode surface from bulk solution, the relatively short pulse time compared to the quiet-time between pulses allows the bulk solution concentration to be reestablished at the electrode surface prior to pulse two.

Control experiments were conducted to investigate the source of the difference charge observed at bare Pt electrodes in buffer. The hypothesis is that contaminants adsorb to the electrode during the 5 s hold-time and are oxidized (and/or desorbed) during the first potential pulse. For the second pulse, the contribution to the charge from adsorbed species is smaller because the quiet-time (0.5 s) before the second pulse is shorter than the hold-time before the first pulse. In this model, the magnitude of the difference charge is expected to be influenced by flux of the contaminants to the electrode surface from bulk solution. Therefore, a dependence of difference charge on microelectrode diameter is predicted due to increased flux from radial diffusion at smaller diameter microelectrodes.

To test this notion, bare Pt microelectrodes of different diameter were characterized for the double potential pulse waveform in buffer. Figure 3 shows the difference charge density for Pt microelectrodes with diameters of 1.3, 10, 25 and 50 μm collected in the same buffer solution on the same day. The x-axis is increasing hold-time prior to application of each consecutive double pulse experiment. The first data points correspond to a hold-time that is equal to the quiet-time (0.5 s) and no difference charge is observed for all electrode diameters. Upon incrementally increasing the hold-time to 10 s, the smallest diameter electrode, for which the flux from radial diffusion would be the largest, shows the largest increase in difference charge density. The smallest diameter electrode also shows the most rapid plateau of difference charge density with increasing hold-time. The plateau of the difference charge density with increasing hold-time may reflect the onset of saturation in the surface coverage of the adsorbed contaminants. Qualitatively, these data suggest that the difference charge observed at bare Pt microelectrodes in buffer is a result of oxidation and/or desorption of contaminants that collect on the electrode from bulk solution. These data verify a mass transfer component of the background signal.

Based on this model where contaminants from bulk solution gradually collect on the electrode surface, the difference charge for control experiments (the difference charge background) is believed to be dependent on the rate at which the species adsorb on the electrode. The rate of adsorption is likely controlled by the flux of the species to the electrode in combination with the onset of surface coverage saturation behavior where the rate of adsorption slows as adsorption sites become occupied. Again, the difference charge background arises because more adsorption occurs during the 5 s hold-time than does during the 0.5 s quiet-time. The difference charge measured at bare Pt microelectrodes in buffer is not a reflection of the total amount (or surface coverage) of adsorbed species. Rather, the difference charge background contribution reflects the unequal amount of adsorbed material that collects on the electrode prior to pulse one relative to pulse two, and is therefore related to the rate at which species adsorb and collect on the electrode surface. The difference

charge background signal is believed to reflect the growth in surface coverage of adsorbed species over the time interval of 0.5–5 s during the hold-time. Immediately after a potential pulse, the initial rate of adsorption is relatively fast because the electrode surface contains many unoccupied adsorption sites. The second pulse includes charge associated with the initial fast adsorption phase (species that adsorb within 0.5 s after pulse one). The first pulse, however, includes charge associated with a fast initial adsorption phase as well as charge associated with a slower adsorption phase that continues over the 5 s hold-time. The 10 and 1.3 μm diameter electrodes are likely achieving a saturated coverage of contamination (at hold-times of 5 s and longer, see Figure 3). The larger charge density of the 1.3 μm diameter electrode is assigned to increased surface roughness (exposed platinum atoms), a challenge in fabricating small (e.g., 1 μm diameter and smaller) disk platinum electrodes sealed in glass.¹⁶ These data suggest that the dependence of the difference charge on hold-time is dominated by flux of contamination rather than capacity (number of platinum atoms exposed to solution) of the surface from adsorbed species. It should be understood that the magnitude of the difference charge (pC) decreases with electrode diameter despite the increased difference charge density at smaller electrodes.

Figure 4A compares the overlay of representative difference charge traces for a bare Pt electrode in contact with the cell plasma membrane (control) and for an enzyme-modified electrode in contact with the cell plasma membrane. The increased signal for the enzyme-modified electrode over the control electrode is assigned to oxidation of hydrogen peroxide originating from cellular cholesterol efflux.

The difference charge response of all fabricated electrodes is initially recorded in buffer. Bare Pt electrodes that exhibit a difference charge in the range of 7–10 pC are considered “normal” (about 80% of electrodes) and are used for cell experiments. Figure 4B shows bar graphs for bare Pt electrodes in buffer (bare solution), enzyme-modified electrodes in buffer (enzyme solution), bare Pt electrodes in contact with the cell surface (bare contact: blue), and enzyme-modified electrodes in contact with the cell surface (enzyme contact: red). All control scenarios (bare solution, enzyme solution, and bare contact) show signal of 10 pC or less. However, a clear increase in difference charge is observed for enzyme-modified electrodes in contact with the cell plasma membrane (smallest signal is 16 pC). Applying the student's *t* method to compare the mean signals from enzyme modified electrodes and bare Pt electrodes containing no immobilized enzyme (controls) shows that the larger difference charge measured at the enzyme-modified electrodes is significant at the 99.5% confidence interval ($t_{\text{calculated}}$ is 4.95 and t_{table} is 4.317). The variation in signal between different enzyme-modified electrodes in contact with different single cells (e.g., 21 ± 4 pC) is believed to be due to the magnitude of the background contribution for various electrodes (see Figure 4B - bare before touch), possible differences between the cells studied, and perhaps different enzymatic activity exhibited by each electrode.

In use of the enzyme-modified electrodes for cell and tissue comparisons (e.g., cystic fibrosis and wild-type controls), the two different samples must be evaluated using a single electrode. The ratio of the signal at disease-state sample is divided by the signal at the wild-type control sample. A positive or negative ratio is taken as increased or decreased cholesterol efflux, respectively. Importantly, different locations on an individual sample of

excised mouse trachea tissue (airway surface) show consistent responses. Also, bare platinum electrodes containing no immobilized enzyme do not show a difference between disease state samples and wild-type controls. It is noted that repeated contact experiments at a single electrode (repositioning the electrode between contact and away from the cell surface) eventually degrades response of the electrode to cholesterol. At the neuronal cells used in this paper, more than 30 repeated contact experiments can be conducted without loss of electrode response. At mouse tissue, electrode response is diminished after only 6–8 contact experiments. In general, electrode lifetime is more dependent on the number of contact experiments than it is with the time the electrode is in contact with the sample. While this observation is not fully understood, it is a motivation for the double potential pulse scheme described in this paper where movement of the electrode is not required in the data acquisition scheme.

CONCLUSIONS

Observation of cholesterol efflux from a single cell using a double potential pulse waveform applied to cholesterol oxidase modified microelectrodes is reported. The experiments for cellular cholesterol efflux show an average signal of 21 ± 4 pC while control electrodes yield an average background signal of about 9 ± 2 pC. The increased signal for the enzyme-modified electrodes is assigned to detection of hydrogen peroxide which indicates the occurrence of cholesterol efflux from the cell plasma membrane. The average increase in response for enzyme-modified electrodes over controls is about 12 pC, corresponding to oxidation of 60 amol of cholesterol during each consecutive double pulse experiment and a rate of cholesterol extraction from the plasma membrane of about $100 \text{ amol/s} \cdot \mu\text{m}^2$ which agrees with efflux rates previously reported by this research group using amperometry at disk microelectrodes. Work is in progress to further optimize the waveform and electrode surface chemistry to achieve a smaller background contribution without significant loss of hydrogen peroxide signal.

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References

1. Devadoss A, Burgess JD. Journal of the American Chemical Society. 2004; 126:10214–10215. [PubMed: 15315412]
2. Devadoss A, Palencsar MS, Jiang DC, Honkonen ML, Burgess JD. Analytical Chemistry. 2005; 77:7393–7398. [PubMed: 16285691]
3. Jiang DC, Devadoss A, Palencsar MS, Fang DJ, White NM, Kelley TJ, Smith JD, Burgess JD. Journal of the American Chemical Society. 2007; 129:11352–11353. [PubMed: 17718573]
4. White NM, Jiang D, Burgess JD, Bederman IR, Previs SF, Kelley TJ. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2007; 292:L476–L486. [PubMed: 17085523]
5. Jiang D, Fang D, Kelley TJ, Burgess JD. Analytical Chemistry. 2008; 80:1235–1239. [PubMed: 18197693]
6. Ewing AG, Wightman RM, Dayton MA. Brain Research. 1982; 249:361–370. [PubMed: 6814706]
7. Fang D, Jiang D, Lu H, Chiel HJ, Kelley TJ, Burgess JD. Journal of the American Chemical Society. 2009; 131:12038–12039. [PubMed: 19670860]

8. Hall SB, Khudaish EA, Hart AL. *Electrochim Acta*. 1998; 43:579–588.
9. Luo YC, Do JS, Liu CC. *Biosensors & Bioelectronics*. 2006; 22:482–488. [PubMed: 16908130]
10. Bahshi L, Frasconi M, Tel-Vered R, Yehezkeli O, Willner I. *Analytical Chemistry*. 2008; 80:8253–8259. [PubMed: 18841938]
11. Das J, Patra S, Yang H. *Chemical Communications*. 2008:4451–4453. [PubMed: 18802587]
12. Karam P, Halaoui LI. *Analytical Chemistry*. 2008; 80:5441–5448. [PubMed: 18543955]
13. Campbell FW, Belding SR, Baron R, Xiao L, Compton RG. *Journal of Physical Chemistry C*. 2009; 113:9053–9062.
14. Zhao G, Giolando DM, Kirchhoff JR. *Analytical Chemistry*. 1995; 67:2592–2598. [PubMed: 8849025]
15. Evans SAG, Elliott JM, Andrews LM, Bartlett PN, Doyle PJ, Denuault G. *Analytical Chemistry*. 2002; 74:1322–1326. [PubMed: 11924592]
16. Nioradze N, Chen R, Kim J, Shen M, Santhosh P, Amemiya S. *Analytical Chemistry*. 2013; 85:6198–6202. [PubMed: 23763642]

Figure 1.

(A) Potential waveform and (B) current for a double potential pulse experiment conducted at an enzyme-modified electrode positioned in contact with the cell surface. (C) Overlay of charge passed during the first pulse and second pulse, (D) the difference charge trace obtained by subtracting the charge passed during the second pulse from the charge passed during the first pulse.

Figure 2.

Data showing that the difference charge is independent of bulk solution hydrogen peroxide.

(A) The positive charge that passes during pulse one and pulse two for three consecutive double potential pulse experiments conducted at a bare Pt microelectrode in buffer, (B) the increase in positive charge for pulse one and pulse two upon addition of hydrogen peroxide (100 μM), (C) the overlay of the average difference charge with and without added hydrogen peroxide.

Figure 3.

Data showing the increased difference charge density for smaller diameter electrodes. The x-axis is hold-time prior to applications of double potential pulse perturbations. Data for a 1 s hold time are shown only for 10, 25, and 50 μm diameter electrodes. Error bars are SD of five consecutive double potential pulse experiments at each hold time.

Figure 4.

The larger difference charge for enzyme-modified electrodes over bare Pt electrodes (10 μm diameter) indicating observation of cholesterol efflux from the cell plasma membrane. (A-blue) representative difference charge trace for bare Pt electrodes in contact with the cell plasma membrane, and (red) a representative difference charge trace for enzyme-modified electrodes in contact with the cell plasma membrane. (B) Bar graphs showing the average difference charge for bare Pt electrodes in solution (bare solution; $n = 10$), enzyme-modified electrodes in solution (enzyme solution; $n = 3$), bare Pt electrodes in contact with the cell plasma membrane (bare contact; $n = 3$), and enzyme-modified electrodes in contact with the cell plasma membrane (enzyme contact; $n = 5$). Error bars are SD of three consecutive double potential pulse experiments (after stabilization).