ELECTROCHEMICAL EVALUATION OF PLASMA MEMBRANE
CHOLESTEROL IN LIVE CELLS AND MOUSE TISSUES

by

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For the degree of Doctor of Philosophy

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August 2008
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03/28/08  

*We also certify that written approval has been obtained for any proprietary material contained therein.*
DEDICATED TO

MY WIFE AND MY PARENTS
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<th>Abbreviation</th>
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<tr>
<td>AC</td>
<td>alternating current</td>
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<tr>
<td>°C</td>
<td>celsius degree</td>
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<td>cm</td>
<td>centimeter</td>
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<td>DC</td>
<td>direct current</td>
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<td>NHE</td>
<td>normal hydrogen electrode</td>
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<td>ns</td>
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<td>picomolar</td>
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<tr>
<td>Pt</td>
<td>platinum</td>
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<tr>
<td>QCM</td>
<td>Quartz crystal microbalance</td>
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<td>Symbol</td>
<td>Description</td>
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<td>-------------------------------------</td>
</tr>
<tr>
<td>TM-SFM</td>
<td>Tapping mode-scanning force microscopy</td>
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<td>μg</td>
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Electrochemical Evaluation of Plasma Membrane Cholesterol in Live Cells and Mouse Tissues

Abstract

by

DECHEN JIANG

In this work, microelectrodes covalently modified with cholesterol oxidase were implemented for evaluation of the cholesterol content in the plasma membrane of single cells at physiological temperature. Cholesterol is a tightly regulated structural component of the cell plasma membrane. Electrochemical data indicated that transport of intracellular cholesterol to the plasma membrane was active in an atherosclerotic macrophage model and was inhibited in a human fibroblast model of Niemann-Pick disease. Electrochemical detection of plasma membrane cholesterol at the surface of excised mouse trachea tissue was reported. Experiments involving direct contact between the cholesterol oxidase-modified electrode and the surface of excised trachea tissue at 37 °C indicated steady state responses that were largely independent of the contact position on the tissue surface and the contact force. Trachea tissue excised from a mouse model of cystic fibrosis showed an electrode response that was about 40% larger than the response observed for wild-type mouse trachea tissue. Electrochemical studies of cholesterol chemical activity in a 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine
(DPPC) lipid monolayer formed at the air-water interface were also performed. The current responses showed an increase when the molecular ratio of cholesterol to lipid was above 0.6, suggesting an existence of the stoichiometric condensed cholesterol-phospholipid complex in lipid monolayers.

Further study in cystic fibrosis showed excess cholesterol stored in lysosomes and elevated cholesterol content in the plasma membrane. This suggested a possibility of increased Niemann-Pick disease, type C1 (Npc1) - driven membrane cholesterol transport in the absence of CFTR function. An application of an oxidase-modified electrode in the research of germ cell migration led to the observation of an elevated cholesterol content at the surface of genital ridge. The data supported a model where cholesterol was required for primordial germ cells development, but can be supplied either via uptake or synthesis.

Scanning force microscopic images on mica exhibited features assigned to *Streptomyces Sp.* Cholesterol oxidase (class I) of 1-2 nm in height immobilized on membrane plateaus and 3-4 nm in height in *Pseudomonas Sp.* Cholesterol oxidase (class II). This phenomenon could be reasonably explained for the larger ternary crystal structure of Class II cholesterol oxidase.
INTRODUCTION

Cholesterol is an amphipathic lipid molecule that is known to play essential roles in cellular membrane structure and function. The plasma membrane is the largest sub-cellular cholesterol pool and complex trafficking pathways exist within cells to maintain this distribution. Excess cholesterol is toxic and contributes to several diseases, notably atherosclerotic vascular disease. A better understanding of cholesterol homeostasis can be attained if the cholesterol content of the plasma membrane is monitored in a live cell under physiological conditions. Developing direct electrochemical methods to track the cholesterol content of the plasma membrane of cells is a goal of this work.

Common methodologies for cellular cholesterol analysis such as fluorescent labeling and isolation of sub-cellular compartments do not yield precise determination of the physiological cholesterol content of the plasma membrane relative to that of internal compartments. In chapter 1, we developed an electrochemical method that allowed direct real time measurements of the plasma membrane cholesterol content in live mammalian cells at physiological temperature. Our state-of-the-art assay to estimate plasma membrane cholesterol was the method developed by Rothblat and coworkers where cellular radio-labeled cholesterol was removed by a cyclodextrin solution.¹ Although Rothblat has reported that the initial rate of cellular cholesterol efflux within ca. 15 s can be assigned to that from the plasma membrane, variations of this method have been implemented without regard to this stipulation. Our electrochemical measurements are rapid and localized, and thus do not significantly perturb the total amount of cholesterol in the plasma membrane. Our microelectrode data demonstrated that transport of
cholesterol to the plasma membrane from stores inside the cell was inhibited in a human fibroblast model of Niemann-Pick disease type C (NPC) and was active in atherosclerotic macrophage foam cells. A model of electrochemical detection and the chemical activity of cholesterol in the plasma membrane is also discussed.

The electrochemical measurements have proven useful in evaluating the effect of specific disease state models on intracellular trafficking of cholesterol to the plasma membrane. Extension of cholesterol electroanalysis for evaluation of cell plasma membrane cholesterol at the surface of primary tissue will allow more clinically relevant studies of animal disease state models and the efficacy of drug treatments. Chapter 2 reports an electrochemical method for gauging cholesterol at the airway surface of mouse trachea tissue. Experiments were performed to investigate cholesterol distribution at the tissue surface and the electrode contact mode. The cholesterol content in the plasma membrane of cystic fibrosis (CF) tissue was studied. The electrochemical data indicated that transport of cholesterol to the plasma membrane from stores inside the cell was upregulated in CF airway epithelial cells.

Our previous representative electrochemical experiments on giant vesicles revealed that the electrochemical responses to cholesterol in the membrane increase sharply only when the cholesterol content increased above 0.5 cholesterol/phospholipids. The data supported the existence of a condensed cholesterol-phospholipid complex in the membrane of giant vesicles, as proposed by McConnell and colleagues. However, our vesicle experiments could not exclude the possibility that cholesterol present in the inner leaflet of the plasma membrane and/or ion transport in bilayer membrane significantly contributes to cholesterol activity in the outer leaflet and thus electrode responses. In
chapter 3, a simplified lipid monolayer system at the air-water interface was fabricated and the relation of current response and cholesterol content in lipid monolayer was investigated. The electrochemical data showed that the current response increased when the molecular ratio of cholesterol and lipid was above 0.6. This phenomenon also supported the condensed cholesterol-lipid complex model in lipid monolayer membranes.

CF is an autosomal recessive monogenic disorder caused by lost function of the cAMP-dependent chloride channel cystic fibrosis transmembrane conductance regulator (CFTR). It is proposed that a loss of CFTR function leads to increased cholesterol synthesis and this perturbation in cholesterol regulation contributes to the inflammatory response present in CF. In chapter 4, the anomalies in cholesterol-related regulation in both cultured cell models and in primary tissue of CF origin that exhibit intracellular cholesterol accumulation and elevated plasma membrane cholesterol content were investigated. It is concluded that CF epithelial cells possess an inherent flaw in cholesterol regulation due to the loss of CFTR activity or expression, which results in increased cholesterol synthesis. The plasma membrane cholesterol level in an Npc1-inhibited cell was also measured and a possible Npc1-driven membrane cholesterol transport model in CF was proposed in this chapter.

Primordial germ cells (PGCs) are the embryonic precursors of gametes. They migrate through or around diverse tissues in order to find the site of the developing gonads. Hydroxymethylglutaryl coenzyme A reductase (HMGCR) was originally identified in a forward genetic screen for genes required for PGC migration in Drosophila. In chapter 5, the role of HMGCR in PGC migration in mammals was tested. The study demonstrated that HMGCR was required for PGC survival and motility
and that both isoprenoids and cholesterol were required downstream of this enzyme in mammals. Electrochemical data exhibited elevated cholesterol levels within the developing genital ridge in embryonic mouse tissue, which suggested a unique role for cholesterol in PGC guidance.

Chapter 6 reports a tapping mode-scanning force microscopy study of a lipid bilayer membrane incorporating cholesterol oxidase on cleaved mica. The images obtained are significant to understand the structure of lipid membranes formed by vesicle fusion at solid surfaces and the nature of cholesterol oxidase binding to the membrane. Two classes of oxidase enzymes with different crystal structures were embedded in lipid bilayer membranes for the study. Topographic images were consistent with the existence of cholesterol oxidase molecules as monomers and aggregates partially immersed in the membrane.

To better understand the mechanism of intracellular cholesterol trafficking, the continued development of oxidase-modified biosensors with better sensitivity and larger current density is critical. In chapter 7, two promising electrode architectures were proposed and some preliminary data were reported on solution cholesterol detection and single cell analysis.

REFERENCES


CHAPTER 1

DIRECT ELECTROCHEMICAL EVALUATION OF PLASMA MEMBRANE

CHOLESTEROL IN LIVE MAMMALIAN CELLS*

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1.1 INTRODUCTION

The plasma membrane is the largest sub-cellular cholesterol pool. Complex trafficking pathways exist within cells to maintain this distribution. Common methodologies for cellular cholesterol analysis such as fluorescent labeling do not yield precise determination of the physiological cholesterol content of the plasma membrane relative to that of internal compartments. Furthermore, the isolation of sub-cellular compartments is prone to contamination, and cholesterol may get transferred between compartments during separation. This lack of spatial resolution in traditional cholesterol analysis of sub-cellular compartments has led to questions regarding how plasma membrane cholesterol is affected under conditions of altered intracellular cholesterol transport. This chapter reports microelectrodes that allow direct real time measurements of plasma membrane cholesterol content in live mammalian cells at physiological temperature. The measurements are rapid (less than 10 s) and localized, and thus do not perturb significantly the total amount of cholesterol in the plasma membrane. The microelectrode data demonstrated that transport of cholesterol to the plasma membrane from stores inside the cell was inhibited in a human fibroblast model of NPC disease and was active in atherosclerotic macrophage foam cells. Because both of these disease state models result in intracellular accumulation of cholesterol, the ability to measure directly
a decrease in plasma membrane cholesterol for the NPC cells and an increase in plasma membrane cholesterol for atherosclerotic foam cells reveals the ability to track the plasma membrane cholesterol pool irrespective of cholesterol build-up inside the cell.

A state-or-the-art assay to estimate plasma membrane cholesterol was the method developed by Rothblat and coworkers where cellular radio-labeled cholesterol was removed by a cyclodextrin solution. 4 Although there is a question as to what cellular cholesterol pools are removed, Rothblat has reported that the initial rate of cellular cholesterol efflux (within ca. 15 s) can be assigned to that from the plasma membrane. Variations of this method have been implemented without regard to this stipulation. Estimates of plasma membrane cholesterol have also been obtained by exposure of cells to cholesterol oxidase solution. A concern with this method is that the enzyme may gain access to internal cholesterol through endocytosis, membrane breakage, and movement of cholesterol during the assay. 2 Our direct electrochemical measurements of plasma membrane cholesterol are localized to the cell surface and require only about 5-10 s thus addressing these caveats.

The thermodynamic activity of cholesterol in the plasma membrane is not thought to be linear with cholesterol content (cholesterol-to-phospholipid ratio) of the membrane. The thermodynamic term “cholesterol activity”, rather than cholesterol content, is used in this chapter. Cholesterol activity represents the tendency of cholesterol to flee, and the quality is usually approximately equal to that of cholesterol content. To explain how the cell is able to maintain stringent regulation of plasma membrane cholesterol, it has been proposed that the activity of cholesterol in the membrane changes drastically as cholesterol content was subtly altered from the normal physiological value. 5
Radhakrishnan and McConnell proposed the formation of a complex between one cholesterol molecule and two phospholipid molecules to interpret this behavior that was observed in a lipid monolayer model. This stoichiometry suggested a sharp increase in the activity coefficient for cholesterol in the membrane centered at a cholesterol-to-phospholipid mole ratio of 0.5. Our earlier data for microelectrode analysis of cholesterol in giant unilamellar vesicle membranes were consistent with this model in that a larger increase in response was observed between mole ratios of 0.5 and 0.66 than between 0.33 and 0.5. Also, no response was measured for ratios of \( \leq 0.33 \) indicating that a threshold cholesterol content was required to achieve a measurable activity. For the cell studies described here, it is hypothesized that a large deviation in cholesterol activity with relatively smaller fluctuations in actual membrane cholesterol content permits electrochemical determination of increases and decreases in plasma membrane cholesterol activity that occur in disease states. This notion was also supported by studies conducted by Steck and co-workers where increasing plasma membrane cholesterol above the physiological level was found to increase the endoplasmic reticulum (ER) cholesterol pool.

1.2 EXPERIMENTAL

**Platinum microelectrode fabrication**

Platinum disk microelectrodes (4 µm diameter) were fabricated in-house. The end of platinum wire (10 µm diameter, Goodfellow Inc.) was electrochemically etched to a tip of less than 1 µm in diameter. The etching procedure involved inserting the platinum wire into a 15% CaCl\(_2\) aqueous solution covered with a layer of acetone. The
layer of acetone prevented the generation of foam at the liquid/air interface as gas was produced at the platinum wire during electrochemical etching. The etching voltage perturbation was a sinusoidal waveform (2.5V peak to peak) of 60 Hz applied between the Pt wire and a counter electrode (large area Pt electrode). Etching was complete when electrical contact between the etched platinum wire and the solution was broken as indicated by termination of bubble production. The sharpened Pt wire (or as purchased 10 µm diameter Pt wire for 10 µm diameter electrodes) was washed with H₂O, dried in a steam of nitrogen, and inserted into glass capillaries (Kimax-51, Kimble products). The glass capillary was heated in a platinum coil and pulled (using an attached weight) to form a thin insulating layer of glass on the platinum wire. The tip of the pulled capillary containing the sharpened platinum wire was polished using a beveling machine (WPI, Inc.) to produce a 4 µm diameter Pt disk electrode.

**Electrode modification**

Immediately after polishing, the platinum disk microelectrodes were immersed in a 5 mM hexane solution of 11-mercaptoundecanoic acid (Aldrich Chem. Co.) for 2 h. These reaction conditions produced a sub-monolayer coverage of 11-mercaptoundecanoic acid on the platinum electrode surface. The sub-monolayer modified platinum surface was exposed to a 100 mM sodium phosphate aqueous solution (PBS, pH 7.4) containing 2 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma Chem. Co.) and 5mM N-hydroxsulfosuccinimide (NHS) (Fisher) for 30 min for conversion of the carboxylic acid end groups of the sub-monolayer to an NHS ester. This activated surface was immersed in a 1 mg/ml solution of recombinant cholesterol oxidase (Wako Chemicals USA, Inc., ca. 33.0 units/mg) for 2 h for covalent attachment
the noise (peak-to-peak) in the electrochemical experiments was dependent on the electrode used and no speculation was offered for this observation. Approximately half of the prepared electrodes exhibited noise (for as collected data prior to digitally smoothing) of $\leq 0.05$ pA and only such electrodes were used in single cell experiments.

Preparation of cholesterol solution and detection

Cholesterol was dissolved in chloroform and dried under nitrogen before preparing the solutions of required concentration. The dried cholesterol was dissolved in an aliquot of PBS containing 1% (v/v) Triton X-100 by sonication. Electrode responses to cholesterol solution at 37 °C were collected in quiet buffer after spiking with aliquots of cholesterol solution. The spiked aliquots were preheated for the 37 °C experiments. Injection times were controlled manually.

Data Acquisition

Amperometric measurements were conducted using a two-electrode cell and a voltammetry-amperometer (Chem-Clamp, Dagan corp.). The three-pole Bessel filter of the Chem-Clamp was set to 100 Hz. The signal was further processed using a noise-rejecting voltmeter (model 7310 DSP, Signal Recovery Inc.) to digitally filter 60 Hz noise and to provide a DC voltage output with a time constant of 0.1 s. Collected data were smoothed using a moving boxcar average (5-20 data points). A commercial Ag/AgCl (1 M KCl) reference electrode was used for all experiments, and the applied potential was 820 mV versus NHE for all experiments. For electrochemical evaluation of
plasma membrane cholesterol, the cells were rinsed with 37 °C Dulbecco's Phosphate Buffered Saline (D-PBS) (Invitrogen) and kept in D-PBS at 37 °C during the experiment.

**AcLDL-loaded macrophage cells**

RAW264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). On day 1, the cells were plated in 35 mm diameter Petri dishes. On day 2, the cells were cholesterol loaded by incubation in serum-free DMEM supplemented with 50 mM glucose, 2 mM glutamine, and 0.2% BSA (DGGB) containing 0.1 mg/ml acetylated low density lipoprotein (acLDL) overnight.

**AcLDL-loaded macrophages with inhibited ACAT**

AcLDL-loading RAW264.7 cells were treated overnight in DGGB in the presence or absence of 10 µg/ml acyl-coenzyme A: cholesterol O-acyltransferase (ACAT) inhibitor (58-035).

**AcLDL-loaded macrophages with Nocodazole**

AcLDL-loaded macrophages were treated with DGGB in the presence or absence of 10 µg/ml nocodazole (Calbiochem) for one hour at 0 °C and one hour at 37 °C.

**NPC fibroblasts**

Niemann-Pick fibroblasts (GM03123A) containing two missense mutations in the Npc1 gene were obtained from Coriell Cell Repository (Camden, NJ). Control human fibroblasts (CRL-2076) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown on Falcon 35 mm diameter tissue culture dishes in MEM Eagle with 2 mM L-glutamine containing 15% FBS and 1µg/ml penicillin/streptomycin.
1.3 RESULTS AND DISCUSSION

We recently reported electrochemical detection of cholesterol in the lipid bilayer membrane of a giant vesicle\textsuperscript{6} and at the surface of a single oocyte\textsuperscript{12} at room temperature using Pt microelectrodes (10 µm diameter) modified with a lipid bilayer membrane containing cholesterol oxidase. However, this surface architecture is not stable at 37 °C. Here we report direct covalent attachment of cholesterol oxidase to Pt microelectrodes (4 µm diameter) to achieve a surface structure that is stable at physiological temperature. Characterization studies were conducted to track modification of the electrode surface with cholesterol oxidase. Platinum electrodes were functionalized with a sub-monolayer of 11-mercaptoundecanoic acid. The terminal carboxyl groups of the sub-monolayer were chemically activated to allow covalent attachment of cholesterol oxidase through amide bonds with lysine residues on the surface of the enzyme.\textsuperscript{11} Electrochemical heterogeneous electron transfer of solution phase ferrocyanide at the electrode surface was used to qualitatively confirm modification of the platinum surface with 11-mercaptoundecanoic acid, and subsequently, with cholesterol oxidase. Figure 1-1 shows ferrocyanide voltammetry at a bare platinum microelectrode (trace a), after functionalization with a sub-monolayer of 11-mercaptoundecanoic acid (trace b), and after attachment of cholesterol oxidase to the sub-monolayer (trace c). The decrease in the limiting current after functionalizing the electrode surface with 11-mercaptoundecanoic acid is consistent with deposition of a sub-monolayer that partially blocks the electrode surface. It is noted that deposition of a sub-monolayer coverage (as supposed to a full monolayer) is critical so that regions of the platinum electrode surface remain unmodified after attachment of the enzyme. Unmodified regions of the platinum
Figure 1-1. Characterization of electrode modification. Cyclic voltammetry of a solution of potassium ferrocyanide (5 mM, 100 mM PBS, pH 7.4) at a bare 10 μm diameter Pt microelectrode (a), after modification with a sub-monolayer of 11-mercaptopoundecanoic acid (b), after covalent attachment of cholesterol oxidase to the sub-monolayer (c), and after incubation at 37 °C for 30 min (d, dash). The reference electrode is Ag/AgCl (1 M KCl) and the scan rate is 100 mV/s.

electrode surface allow electrochemical oxidation of hydrogen peroxide for signal transduction during enzymatic oxidation of cholesterol. Exposure of cholesterol oxidase solution to the activated sub-monolayer modified platinum electrode resulted in additional blocking of ferrocyanide reaction qualitatively indicating covalent attachment of the enzyme to the electrode. Attachment of cholesterol oxidase to the electrode surface did not completely block reaction of ferrocyanide indicating that bare platinum sites remained unmodified for oxidation of hydrogen peroxide in cholesterol detection experiments. Also shown in Figure 1-1 trace d is the ferrocyanide voltammogram taken after incubation of the cholesterol oxidase modified electrode in 37 °C buffer for 30 min.
The unchanged limiting current measured before and after incubation at 37 °C suggests that no enzyme is lost from the electrode surface.

The cholesterol oxidase modified microelectrodes were characterized for detection of cholesterol solution at 37 °C. Figure 1-2A shows representative amperometric responses for cholesterol solution (25 μM to 680 μM). Data from three replicate experiments is reflective of Michaelis-Menten enzyme kinetics (Km is 150 μM) as shown in Figure 1-2B. These data indicate that electrode-immobilized cholesterol oxidase retains activity for catalyzing the oxidation of cholesterol by molecular oxygen at physiological temperature. The electron transfer reaction scheme for cholesterol detection is shown in scheme 1-1.

Membrane: \[ \text{Cholesterol} + \text{O}_2 \rightarrow \text{Cholestenone} + \text{HOOH} \]

Electrode: \[ \text{HOOH} \rightarrow \text{O}_2 + 2e^- + 2\text{H}^+ \]

**Scheme 1-1.** In the membrane, the enzyme catalyzes the oxidation of cholesterol to cholestenone, and the reduction of molecular oxygen to hydrogen peroxide. At the electrode surface, hydrogen peroxide is electrochemically oxidized, yielding oxygen and steady state current responses.

Placing the electrode surface in contact with the plasma membrane (Figure 1-3A) resulted in enzymatic oxidation of cholesterol at the cell surface. The plateau current increase observed in the first several seconds upon positioning the electrode in contact with the cell surface (e.g., Figure 1-3B) is believed to be limited by the rate of enzymatic catalysis and is proposed to be a measure of plasma membrane cholesterol content. Control experiments using bare Pt electrodes containing no immobilized enzyme do not show responses (Figure 1-4).
Figure 1-2. Characterization of cholesterol oxidase modified electrode (4 µm diameter) in quiescent solution at 37 °C. (A) Representative amperometric responses for exposure to cholesterol solution. (↑) indicates the times at which cholesterol solution was injected. (B) Plot of steady state current responses vs. cholesterol concentration for the oxidase modified microelectrodes (■) and bare Pt microelectrodes (○). Error bars (mean ± SD) reflect three independent experiments using three different electrodes.
Figure 1-3. Electrochemical analysis of cell plasma membrane cholesterol. (A) Optical photograph showing contact of a captured macrophage with a microelectrode. (B) Representative (as collected) amperometric data (dotted line) for contacting (↑) a single macrophage cell with an enzyme modified Pt microelectrode (4 μm in diameter) for 20 s and withdrawing (↓) the microelectrode from the contact position. The solid line is smoothed data indicating electrode response on contact.
**Figure 1-4.** Control experiments for contacting different types of cells with three bare Pt electrodes (E1, E2 and E3) containing no immobilized enzyme. A) RAW 264.7 macrophage B) AcLDL-loaded RAW264.7 macrophage and C) AcLDL-loaded RAW264.7 macrophage treated with an ACAT inhibitor. (A-C) reflect three independent cell preparations and five sequential contact experiments per cell. The (↑) arrow indicates the time of contact between the electrode (4 μm in diameter) and cell plasma membrane. (D) Bar graph constructed for each electrode, error bars (mean ± SD) reflect five replicated contacts shown in a-c. (E) Averaged data from three electrodes, error bars are mean ± SD.
Our model for electrochemical detection of plasma membrane cholesterol invokes the aqueous diffusion mechanism proposed by Rothblat for cellular cholesterol efflux to solution phase acceptors such as lipoproteins, vesicles, and cyclodextrin. The aqueous diffusion mechanism is based on studies that show a maximum plateau in the rate of cellular cholesterol efflux with increasing acceptor concentration. The zero order dependence of efflux on acceptor concentration (above $1.6 \times 10^{16}$ phosphatidylcholine vesicles/ml) indicates that the rate of cholesterol removal from the plasma membrane is independent of collision frequency between acceptor and the plasma membrane. Thus, movement of cholesterol out of the plasma membrane and solvation in the aqueous phase is proposed as the initial step in efflux of cellular cholesterol to solution phase acceptors. Our idealized physical model for microelectrode detection of cholesterol at the cell surface has a thin aqueous hydration layer between the electrode surface and the plasma membrane.

The cholesterol oxidase modified electrode consumes the aqueous phase cholesterol that exists between the electrode surface and the plasma membrane. This consumption of aqueous phase cholesterol at the cell surface causes further cholesterol efflux from the plasma membrane as cholesterol mass transport occurs from the high concentration in the plasma membrane to the lower concentration at the electrode surface. It is hypothesized that the rate of cholesterol exchange between the plasma membrane and the aqueous layer is fast relative to the rate of cholesterol oxidation at the electrode surface. Mass transport of cholesterol to the electrode contact site likely involves lateral diffusion of cholesterol in the plasma membrane, flip-flop (transbilayer movement) of cholesterol between the membrane leaflets, and delivery of cholesterol to the plasma membrane.
membrane from stores inside the cell. As discussed below, electrochemical data collected at cells with increased cholesterol content indicate a correlation between electrode response and plasma membrane cholesterol content.

Dysfunctions in intracellular cholesterol transport have been linked to several disease states including atherosclerosis, where plaque formation in artery walls is triggered by accumulation of LDL-cholesterol ("bad cholesterol") in macrophage cells (i.e., foam cell formation). Dysfunctions in intracellular cholesterol transport have been linked to several disease states including atherosclerosis, where plaque formation in artery walls is triggered by accumulation of LDL-cholesterol ("bad cholesterol") in macrophage cells (i.e., foam cell formation).2 Figure 1-5 shows a direct electrochemical comparison of plasma membrane cholesterol content of RAW264.7 macrophages with and without prior incubation with AcLDL, a ligand for the scavenger receptor A. Figure 1-5A, B and C show data collected at a single oxidase-modified electrode for five sequential contacts at a macrophage followed by five sequential contacts at an AcLDL-loaded macrophage. The variation in electrode response (Figure 1-5 D) for sequential contact experiments is random, which might due to noise and possibly differences in the nature of the physical contact. The bar graph (Figure 1-5 E) represents averaged data comparing the cells with three different electrodes (five responses each) and three different cell preparations. The average electrode response is 50 ± 8 fA for the macrophage and 80 ± 10 fA for the AcLDL-loaded macrophage. Relative to the control cells, the AcLDL-loaded cells have an increased electrochemical response of more than 60%. This trend is independent of the order in which the cells are studied. The faster rate of enzymatic cholesterol oxidation indicates a higher concentration of cholesterol at the electrode surface under steady state turnover and an increase in plasma membrane cholesterol content. These data provide direct observation of an increase in plasma membrane cholesterol upon accumulation of AcLDL derived cholesterol in macrophages, as suggested by Maxfield.14
Figure 1-5. Electrochemical comparison of plasma membrane cholesterol content of macrophages and AcLDL-loaded macrophages. (A) Electrode 1 (E1), (B) Electrode 2 (E2), (C) Electrode 3 (E3). (A-C) reflect three independent cell preparations and five sequential contact experiments per cell. The (↑) arrow indicates the time of contact between the electrode (4 μm in diameter) and cell plasma membrane. (D) Cluster plot showing all electrode responses. (E) Bar graph constructed for each electrode, error bars (mean ± SD) reflect five replicated contacts shown in A-C.
Accumulation of cholesterol in macrophage cells activates ACAT, which generates cholesterol esters that can be stored intracellularly in lipid droplets. Treatment of macrophages with an ACAT inhibitor prevents conversion of cholesterol to cholesterol esters and results in an increase of intracellular unesterified cholesterol. Rothblat has reported that the increased accumulation of intracellular unesterified cholesterol, caused by inhibition of ACAT, also results in an increase in plasma membrane cholesterol content. We conducted electrochemical measurements on AcLDL-loaded macrophage cells with and without treatment with an ACAT inhibitor (Figure 1-6). A comparison of these cells at a single electrode shows an average electrode response of 80 ± 12 fA for the AcLDL-loaded macrophage and 130 ± 20 fA for the ACAT inhibited AcLDL-loaded macrophage. These data independently verify that additional cholesterol is transported to the cell plasma membrane when cholesterol storage through esterification is compromised. This agreement between our electrochemical measurements and those of Rothblat on the same cellular system where cellular radio-labeled cholesterol is removed and measured by a cyclodextrin solution, provides validation for both methods in addressing the inadequately understood transport mechanisms involved in movement of cholesterol between internal stores and the plasma membrane.

Control experiments on the three cell types were performed using bare Pt microelectrodes containing no immobilized cholesterol oxidase (Figure 1-4). Bare Pt electrodes show no response or a small apparent reduction current. It is hypothesized that the negative shift in baseline current observed for many of the control experiments reflects blocking of the bare Pt electrode surface (upon positioning the electrode in contact with the cell surface) from solution phase contaminants that are oxidized at the
Figure 1-6. Electrochemical comparison of plasma membrane cholesterol content of AcLDL-loaded macrophages and AcLDL-loaded macrophages with ACAT inhibitor. (A) Electrode 1 (E1), (B) Electrode 2 (E2), (C) Electrode 3 (E3). (A-C) reflect three independent cell preparations and five sequential contact experiments per cell. The (↑) arrow indicates the time of contact between the electrode (4 μm in diameter) and cell plasma membrane. (D) Cluster plot showing all electrode responses. (E) Bar graph constructed for each electrode, error bars (mean ± SD) reflect five replicated contacts shown in A-C.
applied potential. No difference in electrochemical response to various cell types was detected using bare Pt microelectrodes.

Because the rate of cholesterol flip-flop between the plasma membrane leaflets is reported to be varied from seconds to days, we assume that the electrode response is a measure of the cholesterol content in the outer leaflet of the plasma membrane. Excessive contact experiments (e.g., 20 or more) conducted at the same cell led to altered electrode response. Most often, excessive contact experiments conducted using the same electrode and the same cell resulted in diminished electrode responses. While the reason for altered electrode response upon excessive cell contact experiments is unclear, the data presented here reflect a maximum of five contacts experiments per cell and a maximum of ten contact experiments per electrode (five experiments per cell type for the comparison).

There is ample cholesterol contained in the outer leaflet to produce the electrochemical responses observed. For the plasma membrane with a cholesterol-to-phospholipid ratio of 0.5, the molecular area for cholesterol is 40 Å². The number of cholesterol molecules present in the region of the plasma membrane (outer leaflet only) that is directly adjacent to the 4 µm diameter microelectrode (i.e., electrode footprint) is estimated to be 16 amoles. This amount of cholesterol gives 3.2 pico-coulombs. An electrode response of ~ 50 fA suggests that ~ 15% of this amount of cholesterol is oxidized over 10 s. It is estimated that ca. 4% of the total cholesterol contained in the outer leaflet of a macrophage (~ 8 µm in diameter) is consumed during each contact experiment. Cholesterol may be replenished to the electrode contact site between experiments by lateral diffusion of cholesterol in the plasma membrane during the several
second interval between each contact of the electrode to the cell. Flip-flop (transbilayer movement) of cholesterol between the membrane leaflets, as well as delivery of cholesterol to the plasma membrane from stores inside the cell, may also be involved in replenishment of cholesterol to the electrode contact site between contact experiments.

Intracellular microtubule networks appear to be required for transport of internal cholesterol to the plasma membrane. In fibroblasts, the filament depolymerizing agent, nocodazole, causes accumulation of cholesterol inside the cell with a concurrent decrease in plasma membrane cholesterol.\textsuperscript{18} This redistribution of intracellular cholesterol suggests that transport to the plasma membrane from inside the cell is dependent on the existence of microtubules and that movement of cholesterol from the plasma membrane to the endosomal/lysosomal region is evidently not. Our electrochemical data for treatment of AcLDL-loaded macrophages with nocodazole indicate a decrease in plasma membrane cholesterol content (Figure 1-7) such that the cholesterol content or activity is below our electrochemical detection limit (discussed above). These data are consistent with previous reports on fibroblasts\textsuperscript{18} and thus support the hypothesis that transport of intracellular cholesterol to the plasma membrane of macrophages is also dependent on the microtubule network. The data also support the notion that cholesterol activity decreases sharply when the plasma membrane cholesterol content falls below the basal level.
Figure 1-7. Electrochemical detection of altered plasma membrane cholesterol content in cells treated with nocodazole. (A) Nocodazole treatment of AcLDL-loaded RAW 264.7 cells leads to a decrease in plasma membrane cholesterol content compared to AcLDL-loaded control cells. Each of the three comparative experiments shown is conducted using a single cholesterol oxidase modified electrode (4 μm in diameter). The three replicate experiments for each comparative data set represents data collected at three independent preparations of cells and three different electrodes. (B) Bar graph constructed for each electrode, error bars (mean ± SD) reflect three replicated contacts shown in Figure 1-7A.
NPC is a neurological disease characterized by endosomal and lysosomal accumulation of intracellular cholesterol directly resulting from impaired cholesterol transport due to gene mutations in either Npc1 or Npc2, which are cholesterol transport proteins located in endosomes and lysosomes.\textsuperscript{19} Despite the in depth analysis of cholesterol movement within NPC cells, there is still a question as to what the effect of these transport defects is on plasma membrane cholesterol. Liscum and coworkers,\textsuperscript{20,21} studying Chinese hamster ovary (CHO) cell mutants that carry Npc1 defects, have reported that transport of LDL-derived cholesterol from lysosomes to the plasma membrane is defective, resulting in decreased membrane cholesterol. Others have reported that NPC fibroblasts exhibit no change in plasma membrane cholesterol compared to wild type (WT) fibroblasts.\textsuperscript{18,22} Additionally, an increase in plasma membrane cholesterol has been reported in hepatocytes from mouse models of NPC.\textsuperscript{23} Differences in the results of these studies could be due to the different model systems studied and to the relatively indirect methods used for cholesterol analysis. To address the question of plasma membrane cholesterol content in the NPC phenotype, we performed microelectrode experiments that compared human NPC fibroblasts, containing two missense mutations in the Npc1 gene, with WT human fibroblasts. While significant plasma membrane cholesterol activity was observed for the WT fibroblast, that of the NPC cells was markedly low (Figure 1-8) and cholesterol activity was below our electrochemical detection limit. This result is consistent with the work of Liscum et al. on CHO mutants where defects in Npc1 impede transport of lysosomal cholesterol to the plasma membrane.\textsuperscript{20,21} These data further suggest a relatively small activity coefficient for cholesterol when plasma membrane cholesterol content is below the wild type level.
Figure 1-8. Electrochemical detection of altered NPC cell plasma membrane cholesterol content. (A) NPC fibroblasts compared to wild type (WT) fibroblasts (control) have decreased plasma membrane cholesterol content. Each of the three comparative experiments shown is conducted using a single cholesterol oxidase modified electrode (10 μm in diameter). The three replicate experiments for each comparative data set represent data collected at three independent preparations of cells and three different electrodes. (B) Bar graph constructed for each electrode, error bars (mean ± SD) reflect three replicated contacts shown in Figure 1-8A.
1.4 CONCLUSION

Platinum microelectrodes covalently modified with cholesterol oxidase have been fabricated to allow direct, real time measurements of plasma membrane cholesterol activity in live cells at physiological temperature. The electrochemical determinations of plasma membrane cholesterol activity indicate that transport of cholesterol to the plasma membrane from stores inside the cell is inhibited in a human fibroblast model of NPC and is active in atherosclerotic macrophage foam cells. The results reveal that a threshold plasma membrane cholesterol content is required to achieve a cholesterol activity at the cell surface that is detectable at the microelectrodes. Increases in plasma membrane cholesterol content above the threshold level result in electrochemically resolvable increases in the thermodynamic activity of cholesterol at the cell surface. Taken together, these observations support the notion that cholesterol activity sharply tracks plasma membrane cholesterol content when it is altered from the physiology set point.

1.5 ACKNOWLEDGEMENTS

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1.6 REFERENCES


CHAPTER 2

ELECTROCHEMICAL ANALYSIS OF CELL PLASMA MEMBRANE

CHOLESTEROL AT THE AIRWAY SURFACE OF MOUSE TRACHEA*

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2.1 INTRODUCTION

Our group recently reported a microelectrode strategy to track plasma membrane cholesterol in single living mammalian cells at 37 °C.¹ The measurements employ Pt electrodes modified with a sub-monolayer of covalently attached cholesterol oxidase. The sub-monolayer functionalization allows the Pt electrode surface to be placed directly at the membrane/solution interface so that measurements of cholesterol as a component of the plasma membrane can be resolved. Because the rate of cholesterol consumption at the electrode is low, analysis can be performed without significant depletion, as mass transfer of cholesterol in living cells to the electrode contact site sustains the apparent steady state amperometric response. The electrochemical measurements have proven useful in evaluating the effect of specific disease state models¹ on intracellular trafficking of cholesterol to the plasma membrane. Extension of cholesterol electroanalysis for evaluation of cell plasma membrane cholesterol at the surface of primary tissue will allow more clinically relevant studies of animal disease state models and the efficacy of drug treatments. This chapter reports an electrochemical method for gauging cholesterol at the airway surface of mouse trachea tissue.

Cholesterol is an essential structural component of mammalian cell membranes. For example, the fibroblast plasma membrane is estimated to contain ~ 60-80% of the
total cellular cholesterol, and complex trafficking pathways exist within cells to maintain this distribution. Dysfunctional intracellular cholesterol transport can lead to accumulation of cellular cholesterol and is thus believed to be inherently involved in the onset of atherosclerosis and Niemann-Pick disease. Work by Kelley and co-workers, has demonstrated elevated cellular cholesterol in mouse and cell line models of cystic fibrosis (CF). CF is a genetic disease caused by the lost function of the cyclic adenosine monophosphate (cAMP)-dependent chloride channel cystic fibrosis transmembrane conductance regulator (CFTR). Although the mechanism leading to the aggressive inflammatory signaling in CF is still unclear, it is believed that improper cholesterol processing is a trigger for altered inflammatory signaling responses. Our recent electrochemical studies on plasma membrane cholesterol of cell line models of CF and of excised nasal tissue from CF mouse models indicated that plasma membrane cholesterol was elevated in the CF models relative to the basal physiological level observed in WT cells and nasal tissue.

Here, we demonstrate the methodology for electrochemical cholesterol analysis of animal tissue using excised mouse trachea, a tissue sample that provides a relatively large homogeneous and well defined (airway) surface for study by direct contact with cholesterol oxidase modified electrodes. Data are reported for contacting a single tissue sample at multiple positions on the airway surface to verify cholesterol content that is spatially homogeneous. Tissue samples were mounted on quartz crystal microbalance (QCM) electrodes to qualitatively estimate the contact force between the electrode and the tissue surface. The elevated cholesterol content of CF airway epithelial cells, including those of the trachea, provides the ability to demonstrate electrochemical
evaluation of plasma membrane cholesterol in cells at the natural surface of tissue samples. The data indicate that the CF trachea surface exhibits increased plasma membrane cholesterol. This result further verifies that transport of cholesterol to the plasma membrane from stores inside the cell is upregulated in CF airway epithelial cells.

2.2 EXPERIMENTAL

Mice

Mice lacking CFTR expression (CFTR<sup>tm1Unc</sup>) were obtained from Jackson Laboratories (Bar Harbor, MA). CFTR and WT mice were siblings of <i>cflr<sup>−/−</sup></i> mice. All mice were used between 6 and 8 week of age and were backcrossed over 10 generations onto a C57Bl/6 background. CF mice were fed a liquid diet as described by Eckman et al. Mice were cared for in accordance with the Case Western Reserve University Institutional Animal Care and Use Committee guidelines by the CF Animal Core Facility. Tracheas of mouse epithelium were obtained from WT and CF animals. The Animal Care and Use Committee of Case Western Reserve University approved all animal procedures. Trachea tissue samples were surgically excised using established procedures.

Electrode modification

The preparation and modification of platinum disk electrodes (100 µm diameter) are described in chapter 1 and refs 1, 8 and 9.

Preparation of cholesterol solutions

Cholesterol was dissolved in chloroform and dried under nitrogen before preparing the solutions of required concentration. The dried cholesterol was dissolved in an aliquot of PBS containing 1 % (v/v) Triton X-100. Solution flow-injection analyses
type experiments were conducted using a flow cell constructed in-house. Briefly, the electrochemical flow cell was a microscope glass slide with a circular silicone barrier in the middle to hold a buffer volume of ca. 200 \( \mu l \). The flow rate was 500 \( \mu l/\text{min} \). The flow was controlled using a six-way valve (Rheodyne Model 5020, Supelco Corp.) and two syringe pumps (Harvard Apparatus) to direct buffered Triton X-100 or buffered Triton X-100 containing cholesterol over the electrode. Injection times were controlled manually. Electrode responses to cholesterol solution at 37 °C were collected in quiet buffer after spiking with aliquots of cholesterol solution. The spiked aliquots were preheated to 37 °C and the solution was kept at 37 °C during the experiments.

**Data acquisition**

Amperometric measurements were conducted using a two-electrode cell and a voltammetric-amperometer (Chem-Clamp, Dagan corp.) as described in chapter 1. Collected data were smoothed using a moving boxcar average (10 data points). An Ag/AgCl (1 M KCl) reference electrode was used, and the applied potential was 820 mV versus NHE for all experiments. For electrochemical evaluation of plasma membrane cholesterol, the excised tissue samples were rinsed with 37 °C D-PBS and kept in D-PBS at 37 °C during the experiment. Prior to the electrochemical experiments, the tissue sample was captured using a pulled glass capillary connected to an IM-6 microinjector and positioned around 500 \( \mu m \) from the electrode to acquire baseline data. The electrode was repositioned so that it was in physical contact with the airway surface of the tissue sample for cholesterol analysis. Electrode contact was defined as the force required to physically alter the position of the tissue as gauged visually under optical magnification.
Contact force estimates

To investigate the dependence of contact force between the electrode and the tissue surface on electrode response, the trachea tissue was spread on a QCM electrode (10 MHz, International Crystal Manufacturers). Increased force applied between the tissue sample and the electrode produced a decrease in QCM resonant frequency providing a qualitative measure of contact force. No model was presented to quantify the absolute force applied.

2.3 RESULTS AND DISCUSSION

Microelectrode detection of cholesterol in solution

The cholesterol oxidase-modified electrodes were characterized for detection of room temperature cholesterol solution in flow-injection type experiments. Figure 2-1 shows typical amperometric responses for flow exposure of cholesterol solution (25 μM to 1mM) to an oxidase-modified electrode (Figure 2-1A). Plots of electrode response (oxidase-modified electrodes and bare Pt control electrodes) vs. cholesterol concentration are shown in Figure 2-1B. The control electrodes show no response for cholesterol solution and the three oxidase electrodes show standard derivations in the range of 5% - 20% for all concentrations (see error bars in Figure 2-1B). These data are consistent with a covalent scheme for immobilization of the enzyme on gold.\textsuperscript{10,11} The plateau behavior shown in Figure 2-1B could reflect enzyme saturation and/or molecular oxygen becoming a limiting reactant. The electron transfer reaction scheme for cholesterol detection is shown in Scheme 1-1.
Figure 2-1. Characterization of cholesterol oxidase modified electrodes (100 μm in diameter) for solution cholesterol at room temperature. (A) Flow-injection analysis data for cholesterol solution at indicated concentrations. (↑) indicates the times for injecting cholesterol solution and (↓) indicates the times for reverting the flow to buffer. (B) Plot of steady state current response vs. cholesterol concentration for the oxidase-modified electrodes (squares) and bare Pt control electrodes (circles). Error bars are SD for three electrodes.
The oxidase electrodes were characterized for detection of cholesterol solution at 37°C (Figure 2-2). Amperometric data were collected for spiking quiescent solutions with aliquots of cholesterol solution (Figure 2-2A). Plots of electrode response (three oxidase-modified electrodes and three bare Pt control electrodes) vs. cholesterol concentration are shown in Figure 2-2B. The standard derivations for electrode response to solution cholesterol at 37 °C are in the range of 8% - 20%. These data demonstrate that the surface structure is stable at physiological temperature.

**Tissue cholesterol analysis**

Experiments for detection of plasma membrane cholesterol at the surface of excised trachea were conducted with the tissue sample submerged in buffer so that the electrode response could be resolved by comparing the baseline current (no contact) to the steady state response observed with the electrode in direct physical contact with the airway tissue surface. Figure 2-3A shows the steady state current responses for duplicate contacts at the same position on the tissue surface. About five sequential contact experiments can be conducted with a given oxidase-modified electrode before response is diminished gradually. Diminished electrode response is likely due to the adsorption of cellular material to the electrode surface that blocks hydrogen peroxide oxidation or that causes loss of enzyme activity. Control experiments (Figure 2-3B) using bare Pt electrodes do not show oxidative responses. These data suggest that the current observed at oxidase-modified electrodes is a response to plasma membrane cholesterol in cells at the tissue surface. The average response for six replicate experiments conducted using different electrodes and different tissue samples is 27 ± 4.3 pA. The relative error (16%) is similar to the variation between electrodes in solution characterizations.
Figure 2-2. Characterization of cholesterol oxidase modified electrodes (100 μm in diameter) for solution cholesterol at 37 °C. (A) Amperometric data collected at a cholesterol oxidase modified electrode in quiet cholesterol solution as labeled. (↑) indicates the times for injecting cholesterol solution. (B) Plot of steady state current response vs. cholesterol concentration for oxidase-modified electrodes (squares) and bare Pt control electrodes (circles). Error bars are SD for three electrodes.
Figure 2-3. (A) Amperometric responses of a cholesterol oxidase modified electrode for two consecutive contact experiments at the same position on trachea tissue surface. ↑ indicates the time of contact and ↓ indicates the times of withdrawal. (B) Control experiment conducted using a bare Pt electrode to contact the same position on the trachea tissue surface.
Our mechanistic model for detection of plasma membrane cholesterol is that previously reported for detection of cholesterol at the surface of single macrophage cells, i.e., the aqueous diffusion model. The aqueous diffusion mechanism was based on studies that showed a maximum plateau in the rate of cellular cholesterol efflux to solution phase acceptors with increasing acceptor concentration. The physical model for contact of the tissue surface with the electrode assumes an aqueous hydration layer between the electrode surface and the plasma membrane tissue surface. Cholesterol in the aqueous phase between the electrode and tissue surface is consumed at the electrode surface resulting in mass transfer of cholesterol from the plasma membrane to the aqueous phase. Based on the electrode response at the tissue samples, cholesterol is estimated to be consumed at a rate of about 2 pmol/s cm² (a similar calculation process has been shown in chapter 1). This rate of cholesterol extraction is in agreement with reported rates of cholesterol removal from a lipid monolayer at the air/water interface using cyclodextrin (a well documented aqueous phase cholesterol acceptor of cellular efflux) as the solution phase acceptor (1.3 ~ 6.6 pmol/s cm² for various mole fractions of cholesterol in the lipid monolayer).

Based on this model, cholesterol detected by the electrode is aqueous phase cholesterol originating as cholesterol efflux from the cell plasma membrane. It is proposed that the rate of cholesterol efflux from the plasma membrane to the aqueous layer between the electrode and tissue surface influences the magnitude of the steady state response. The efflux rate is dependent on the cholesterol content of the plasma membrane. The number of cholesterol molecules initially present in the plasma membrane of cells directly adjacent to the 100 μm diameter electrode (e.g., electrode
footprint) is estimated to be $2 \times 10^{10}$, which gives an equivalent of approximately 6.7 nC (assuming cholesterol-to-phospholipid ratio of 0.5 in the plasma membrane and 100% oxidation of the generated hydrogen peroxide). This calculation suggests that about 10% of the available cholesterol is consumed over 20 s. Lateral diffusion of cholesterol in the plasma membrane, transbilayer movement (flip-flop) of cholesterol in the plasma membrane, and delivery of cholesterol to the plasma membrane from stores inside the cell all likely contribute to replenishing cholesterol to the electrode contact site. The mass transfer rate of cholesterol to the electrode contact site, caused by the local depletion, likely also contributes to the steady state electrode response. Other factors possibly affecting electrode response are ion transport and enzyme kinetics. No speculation is provided here regarding these uncertainties.

Our objective of this study was to establish the tissue cholesterol measurements as a diagnostic tool. Thus, experiments were conducted to determine the dependence of electrode response on contact position at the airway surface of the tissue. The responses of an individual oxidase-modified electrode collected at three different positions on the same trachea tissue sample are shown in Figure 2-4A. The standard derivation of the three responses is less than 10% for two replicate experiments (two electrodes and two tissue samples; Figure 2-4A and B). These data show that the cholesterol content of the tissue sample is homogeneous across the surface of the tissue suggesting that a single oxidase electrode can be used to compare the plasma membrane cholesterol content at the surface of two different tissue samples.
Figure 2-4. Data from two cholesterol oxidase modified electrodes (A and B) for contacting the trachea tissue at different locations (1, 2, and 3) on the airway surface. (↑) indicates the time of contact.
We speculate that the trachea airway surface has some topography that could change upon contact by the electrode. The nature of the electrode tissue contact (e.g., average thickness of the aqueous layer and area of plasma membrane under the electrode footprint) could be altered under varied contact forces. To verify that such behavior does not significantly affect the electrode response, trachea tissue samples were placed on a QCM electrode surface so that contact force applied between the cholesterol oxidase modified electrode and the tissue surface could be qualitatively tracked. Applying mechanical force to the QCM electrode caused a decrease in resonant frequency providing a real time estimate of the contact force. While no model is given to quantify the QCM response to altered electrode contact force, the experiments did provide a relative measure of contact force with respect to the amperometric response of the oxidase-modified electrode.

Figure 2-5 shows the QCM resonant frequency and the amperometric oxidase electrode response for sequentially increasing the applied contact force in step functions with the first step being initial contact. Steady state responses are shown for three different contact forces as verified by the decrease in QCM resonant frequency. When the force was manually increased by moving the electrode in the direction of the tissue (Figure 2-5A), no resolvable change in steady state current was observed (Figure 2-5B). This indicates that complications from changes in tissue surface topography upon contact by the electrode do not significantly affect the measured steady state response. No speculation is given regarding the current transients during and immediately after increasing the contact force. It is noted, however, that the electrode forces applied in these experiments are greater than those applied in all other experiments shown in this
chapter. In all other experiments, the contact force was indicated and limited by physical movement of the tissue sample. These data demonstrate the ability to reliably assay tissue surface cholesterol without strictly controlling the applied force of electrode contact.

Figure 2-5. (A) QCM data for increasing in the force applied between the cholesterol oxidase-modified electrode and the tissue surface. (B) Amperometric response of the cholesterol oxidase-modified electrode with increased contact force. (↑) indicates the time of contact and (↑↑) indicates the application time of increased force.
Comparing different tissue samples

As a means of demonstrating the ability to evaluate the relative plasma membrane cholesterol content of different tissue samples using a single cholesterol oxidase modified electrode, electrode responses at trachea tissue excised from a WT mouse to that excised from a CF mouse model were compared. Figure 2-6 shows two independent experiments (A and B) comparing WT and CF tissue samples. While there was variation in response between different electrodes, the individual electrodes showed nearly the same relative increase in response for the CF samples. An increased current response of more than 40% was observed at the CF trachea tissue and these data were consistent with our earlier work comparing WT and CF cells and WT and CF nasal tissue.6

2.4 CONCLUSION

Electrochemical analysis of plasma membrane cholesterol at the airway surface of mouse trachea was reported. Experiments for the exposures of cholesterol-oxidase-modified electrodes to 37 °C cholesterol solution show steady state responses. The data show that electrode response correlates with plasma membrane cholesterol content at the trachea surface and that the steady state measurements are not significantly dependent on contact position or contact force. Cholesterol is elevated in the plasma membrane of cells at the airway surface of CF mouse trachea. This work rationalizes our goal of implementing electrochemical analysis of tissue surface cholesterol as a clinical tool for CF diagnostics and management. Sensor optimization studies involving varying electrode size and amount of enzyme immobilized on the electrode surface are underway.
Figure 2-6. Two independent electrochemical experiments (A and B) using two oxidase-modified microelectrodes to compare CF and WT tissue samples. (↑) indicates the time of contact.
2.5 ACKNOWLEDGEMENTS

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2.6 REFERENCE


CHAPTER 3

ELECTROCHEMICAL ANALYSIS OF THE CHEMICAL ACTIVITY OF CHOLESTEROL IN A LIPID MONOLAYER AT THE AIR-WATER INTERFACE

3.1 INTRODUCTION

Previously, our group reported a microelectrode strategy to detect cholesterol in the lipid bilayer membrane of a giant vesicle and at the surface of a single living oocyte cell at room temperature.\textsuperscript{1,2} The measurements implemented Pt microelectrodes modified with a lipid bilayer membrane incorporating cholesterol oxidase. Recently, to achieve a thermally-stable surface structure at physiological temperature, direct covalent attachment of cholesterol oxidase to a Pt microelectrode was demonstrated to track plasma membrane cholesterol in single living mammalian cells and at the surface of mouse tissue at 37 °C.\textsuperscript{3,4} Our electrochemical detection of the plasma membrane cholesterol is presumed to follow an aqueous diffusion model that assumes cholesterol efflux from the plasma membrane to an aqueous hydration layer between the electrode surface and the surface of the plasma membrane.\textsuperscript{5} Cholesterol in the aqueous phase is consequently consumed at the electrode surface resulting in a steady state current response. The magnitude of the steady state current response is influenced by the rate of cholesterol efflux from the plasma membrane. A detailed discussion of the model has been presented in chapter 1 and 2.

The direct relationship between the cholesterol efflux rate and the cholesterol content at the plasma membrane is not known. The lipid monolayer model has been
investigated by McConnell and colleagues.\textsuperscript{6} It was found that the cholesterol efflux rate to cyclodextrin (aqueous solution acceptor) showed a sharp increase when the cholesterol content exceeded 0.5 cholesterol/phospholipids. To explain this phenomenon, a condensed complex model of cholesterol and phospholipid in plasma membrane was proposed.\textsuperscript{6,7} A thermodynamic term “cholesterol activity”, rather than cholesterol content, is used in this model (see the definition of “chemical activity” in chapter 1). For membranes with cholesterol content below the composition (0.5 cholesterol/phospholipids) corresponding to the stoichiometry of a presumed condensed complex, complex formation consumes most of cholesterol. Increasing the membrane cholesterol content above 0.5 cholesterol/phospholipid leads to the formation of a high free energy cholesterol pool. Membrane cholesterol activity is increased, as gauged by the increased cholesterol efflux rate. A series of experimental results and theoretical calculations on the intracellular cholesterol distribution have been reported to support this model.\textsuperscript{7,8,9}

Our previous electrochemical experiments on giant vesicles revealed that the electrochemical response to cholesterol in the membrane increases sharply only when the cholesterol content was greater than 0.5 cholesterol/phospholipid.\textsuperscript{2} The data support the existence of a condensed cholesterol-phospholipid complex in the membrane of giant vesicles. However, this vesicle experiment could not exclude the possibility that cholesterol present in the inner leaflet of the membrane and/or ion transport in bilayer membrane significantly contributes to cholesterol activity in the outer leaflet and thus electrode responses.\textsuperscript{10} To clarify this point, a simplified lipid monolayer system at the air-water interface was fabricated for further investigation of cholesterol activity in the
lipid membranes by microelectrodes. The electrochemical data presented in this chapter further support the existence of the condensed cholesterol-lipid complex in lipid monolayer.

3.2 EXPERIMENTAL

Chemicals

Phosphatidylthioethanol and 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Avanti Polar Lipids Inc.), cholesterol oxidase (Wako Chemicals USA, Inc., 3.6 units/mg), cholesterol (Sigma) and chloroform (Fisher). All water used was deionized.

Modification of platinum microelectrode

Platinum disk microelectrodes (10-µm diameter) were constructed in-house as described in chapter 1. The platinum disk microelectrodes were immersed in ethanol with 6 µM phosphatidylthioethanol for 2 h, and 1mM DPPC solution with 100 mM PBS (pH 7.4) for half an hour. Finally, the microelectrodes were reacted with 3 mg/ml cholesterol oxidase (enzyme) overnight. The modification process was monitored by heterogeneous electron transfer of Fe (II) in 5mM K₄[Fe (CN)₆] solution using a Bioanalytical System CV-50 potentiostat.

Electrochemical Measurements

The experimental setup for contacting the lipid monolayer at the air-water interface is shown in Figure 3-1. A Teflon container (cross section area of 9 cm²) was filled with 100 mM PBS (pH 7.4). The microelectrode was fixed to the container so that the electrode surface is normal to the air-water interface. A 30 µL chloroform mixture of DPPC and cholesterol at a controlled molar ratio was added on the surface of the solution.
Formation of the lipid monolayer by chloroform evaporation was confirmed by the change in surface pressure, measured using a Wilhelmy surface balance (KSV instruments) and LB 5000 software. The surface pressure values obtained for the lipid monolayers were in the range of 21-23 mN/m.

The distance between the monolayer and microelectrode surface was adjusted by adding or removing the bulk buffer solution using an injection syringe with a resolution of 1 μm/μL. To attain contact between the lipid membrane and the electrode, the interface was moved towards the electrode until electrical contact was lost (Figure 3-2). When the monolayer contained cholesterol, just before losing electrical contact, a steady state current was established. This point, around 1μm away from the position losing the electrical contact, was considered as the contact position for the electrode with the lipid monolayer. Amperometric measurements were conducted using a two-electrode cell and a voltammeter-amperometer (Chem-Clamp, Dagan corp.). The three-pole Bessel filter of the Chem-Clamp was set to 100 Hz. An Ag/AgCl (1 M KCl) reference electrode was used, and the applied potential was 820 mV versus NHE for all experiments.

3.3 RESULTS AND DISCUSSION
Contact of DPPC/cholesterol monolayer at the air-water interface using microelectrodes

Figure 3-3 showed the characteristic steady state current response when an oxidase-modified electrode was positioned in contact with the lipid monolayer containing cholesterol. Here the electrode response of 0.3 pA should be dependent on the efflux rate
**Figure 3-1.** Electrochemical setup for contacting the lipid monolayer at the air-water interface using oxidase-modified Pt microelectrode (W). An AgCl/Ag electrode is used as the reference electrode (R).

**Figure 3-2.** Current trace to define “contact” position for the electrode with a lipid monolayer. The contact position is the point where the current drops dramatically during removing the bulk buffer. The current can be restored to the baseline after 1 µL buffer is added back to the bulk.
and the enzyme turnover rate. A calculation estimates that the number of cholesterol molecules present in the contact region is 400 amoles. An electrode response of ~ 0.3 pA suggests that ~ 10% of this amount of cholesterol is oxidized over ten seconds. Figure 3-4 shows the typical current change for the control experiments using enzyme modified electrodes contacted with a lipid monolayer containing no cholesterol. No current increase was observed at the contact position.

Figure 3-3. Typical electrochemical data for the oxidase-modified electrode upon contacting a lipid monolayer containing cholesterol (the cholesterol to lipid ratio is 0.70). (↑) indicates the position for the contact and (↓) indicates the position for the loss of the contact.
**Detection of chemical activity of cholesterol in lipid monolayer**

DPPC monolayers with different cholesterol content were formed to investigate the activity of the cholesterol in lipid monolayer membrane. When the cholesterol-to-lipid ratio reached 0.60, a current was observed that increases as a function of cholesterol-DPPC ratio (Figure 3-5A); whereas, no responses appear when the ratios were below this ratio. As discussed above, the response magnitude is indicative of the cholesterol efflux rate to the aqueous diffusion layer controlled by the chemical activity of cholesterol in lipid monolayer and the rate of consumption at electrode. The sharp increase in current response (Figure 3-5B) with cholesterol content greater than 0.6 is consistent with the condensed complex model. The stoichiometry of the condensed
Figure 3-5. (A) Amperometric responses obtained at an oxidase-modified electrode for contacting lipid monolayers containing cholesterol to lipid ratios of (1) 0.70, (2) 0.65, and (3) 0.60. (B) Amperometric responses as a function of cholesterol content in lipid monolayer at air-water interface. Values shown are means ± the standard error of the mean (s.e.m) for triplicate measurements on one oxidase-modified microelectrode. No error bar calculation on non-response range.
complex is not the same as the value reported by McConnell (0.5 cholesterol/DPPC). This shift might be due to mass transfer of cholesterol to the electrode or different measurement methods.

3.4 CONCLUSION

Electrochemical studies of cholesterol chemical activity in DPPC lipid monolayers formed at the air-water interface have been presented. Experiments for contact between the microelectrode and the lipid monolayer containing cholesterol indicate steady state currents that reflect the chemical activity of cholesterol in the monolayer. The current response shows an increase when the molecular ratio of cholesterol and lipid is increased above 0.6. These behaviors suggest the formation of stoichiometric condensed cholesterol-phospholipid complexes in the lipid monolayer is existed. Future work should be done to investigate the influence on cholesterol activity of lipid structure and the mechanism of cholesterol efflux to the electrode surface using different electrode sizes and amounts of immobilized enzyme.

3.5 ACKNOWLEDGEMENTS

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3.6 REFERENCES


CHAPTER 4

ALTERED CHOLESTEROL HOMEOSTASIS IN CULTURED AND IN VIVO MODELS OF CYSTIC FIBROSIS*

*The fluorescence data in this chapter were provided by Professor. Thomas J. Kelley; and this following text have been stated in our published paper (reference 5) with some revision.

4.1 INTRODUCTION

CF is an autosomal recessive monogenic disorder caused by the lost function of the cAMP-dependent chloride channel cystic fibrosis transmembrane conductance regulator (CFTR).⁴ CFTR is known to be correctly localized at the apical membrane of epithelial cells.² CFTR directly regulates other ion channels, such as the outwardly rectifying chloride channel and the amiloride-sensitive sodium channel, and thus plays an important role in the control of ions and water in body tissues.³ Previous work demonstrated that cultured models of CF epithelial cells exhibited intracellular accumulation of unesterified cholesterol in a manner similar to what was observed in cells from patients with NPC disease.⁴ It was determined that NPC fibroblasts shared a number of cell signaling alterations previously identified in CF cells, including reduced inducible nitric oxide synthase (NOS2) expression, increased Ras homolog gene member A (RhoA) and signal transducer and activator of transcription-1 (STAT1) protein expression, and reduced mothers against decapentaplegic homolog 3 (SMAD3) protein expression.⁴,⁵ These data suggest that improper cholesterol processing may be a trigger for altered inflammatory signaling responses in CF cells.

Further support for the importance of cholesterol-related pathways in CF cell signaling regulation has been demonstrated previously by the impact of
isoprenoid/cholesterol synthesis on these signaling cascades. Treatment with the 3-
hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor mevastatin
resulted in the correction of STAT1 and NOS2 signaling in both cultured cell models and
mouse models of CF.5-8 Similarly, inhibition of isoprenoid transferase activity resulted in
correction of SMAD3 expression and transforming growth factor-1 (TGF-1) signaling in
CF cells.5,9 The above findings suggest a relationship between cholesterol accumulation
and the isoprenoid/cholesterol synthesis pathway in CF. Kelley and colleagues speculate
that cholesterol processing in CF models is a potential cause of alterations in CF
inflammatory pathways.5 Their results showed that a loss of CFTR function led to
increased cholesterol synthesis. This perturbation in cholesterol regulation is proposed to
contribute to the inflammatory response present in CF.

In this chapter, the anomalies in cholesterol-related regulation in both cultured
cell models and in primary tissue of CF origin, including intracellular cholesterol
accumulation and elevated plasma membrane cholesterol content, are investigated. It is
concluded that CF epithelial cells possess an inherent flaw in cholesterol regulation due to
the loss of CFTR activity or expression, which results in the increased cholesterol
synthesis. The aberrations in cholesterol homeostasis are believed to be a CF-related
phenotype that potentially influences a number of relevant cell signaling events.

4.2 EXPERIMENTAL

Cell culture

IB3–1 cells, human epithelial with the delta F508 mutation (CF phenotype), and
S9 cells, IB3–1 cells stably transfected with the full-length WT CFTR (control), were a
generous gift from Pamela L. Zeitlin (Johns Hopkins University, Baltimore, MD). Human epithelium 9/HTEo-cells overexpressing the CFTR R domain (pCEPR) and mock-transfected 9/HTEo-cells (pECP2), the WT phenotype, were a generous gift from the lab of Dr. Pamela B. Davis (Case Western Reserve University). Cells were cared for as previously described.¹⁰

Mice

Mice lacking CFTR expression (CFTRtm1Unc) were obtained from Jackson Laboratories (Bar Harbor, MA). CFTR WT mice were siblings of cfr⁻/⁻ mice. All mice were used between 6 and 8 wk of age and were backcrossed over 10 generations onto a C57Bl/6 background. CF mice were fed a liquid diet as described by Eckman et al.¹¹ Mice were cared for in accordance with the Case Western Reserve University Institutional Animal Care and Use Committee guidelines by the CF Animal Core Facility. Nasal scrapings of mouse epithelium were obtained from both WT and CF animals. The Animal Care and Use Committee of Case Western Reserve University approved all animal procedures.

Electrochemical measurements of cholesterol

The fabrication and modification of platinum microelectrodes (4, 11.5 and 100 μm-diameter wire, Goodfellow) are described in chapter 1 and ref 13 and 14. The electrochemical measurement on single cells and excised tissues, and the data acquisitions are exhibited in chapter 1 and 2.

Note: the experimental descriptions of NBD-cholesterol staining, flow cytometry analysis and filipin staining can be referred from reference 5.
4.3 RESULTS AND DISCUSSION

In vivo accumulation of unesterified cholesterol

Previous observations of unesterified cholesterol accumulation in CF were limited to two cultured cell models.\(^4\) To determine whether this phenotype was conserved in vivo and relevant to human disease, cholesterol content in sections from CF and non-CF trachea and upper lung airways was determined by filipin staining. Filipin is a protein from the bacteria Streptomyces filipinensis that binds to unesterified cholesterol, becoming detectable in the ultraviolet range.\(^{15}\) Compared with control tissue, CF trachea exhibits increased intracellular cholesterol content and more intense staining (Fig. 4-1A). The same pattern of accumulation observed in the CF trachea is also present in the epithelium of the upper airway of the lung (Fig. 4-1B). These data support previous findings of increased intracellular unesterified cholesterol content in cultured CF models and demonstrate that primary tissue of CF origin exhibits the aberrant cholesterol transport phenotype.

NBD-cholesterol accumulation in CF cell models

Filipin staining demonstrates accumulation of unesterified cholesterol in cell and tissue models of CF. To determine whether these findings are due to a disruption of transport or simply due to deficient reesterification, the transport of a fluorescently labeled cholesterol analog was examined. With the use of a fluorescent cholesterol probe, 25-[N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino]-27-norcholesterol (NBD-cholesterol), cholesterol trafficking in cultured cell models was measured. After \(\sim\)24 h of incubation with NBD-cholesterol, cells were placed in fresh media for 4 h before being fixed. Confocal images clearly demonstrate accumulation of NBD-cholesterol in two
Figure 4-1. Unesterified cholesterol accumulation in cystic fibrosis (CF) tissue. (A) filipin staining of non-CF and CF trachea epithelium tissue. (B) filipin staining of non-CF and CF of epithelium from upper airway tissue. At right of each filipin stain is a transmitted image to indicate tissue structure. Images are representative of multiple sections of each sample. Trachea and lung tissue is from separate individuals. Bar = 30 μm.

Note: This figure is obtained from ref 5.
different cultured CF cell models (9/HTEo-pCEPR and IB3) compared with respective controls (Fig. 5-2A). Quantification of NBD-cholesterol accumulation was accomplished using flow cytometry analysis. Cells were treated similarly as before but allowed to process cholesterol in fresh media for longer periods of time (approximately overnight). There was a significant increase in mean fluorescence present in both CF-like cells; 9/HTEo-pCEPR had a 1.4 ± 0.2-fold (P = 0.003) increase compared with 9/HTEo- pCEP controls, and IB3 cells had a 2.0±0.2-fold (P = 0.005) increase compared with S9 controls (Fig. 5-2B). These data confirm by separate technique the observation of cholesterol accumulation in CF cells initially determined by filipin staining and suggest that the observation is due to a flaw in lipid transport mechanisms.

On the basis of the increased content of free cholesterol in CF cells and tissues, it is postulated that cholesterol is accumulating in late endosomes and lysosomes, similar to NPC. To determine whether the cholesterol accumulation phenotype could be reversed, 9/HTEo-pCEPR (CF phenotype) cells were treated with gly-phe-B-naphthylamide (GPN), a cathepsin C substrate that causes lysosomal disruption. The pattern of cholesterol accumulation was examined by visualization of NBD-cholesterol. 9/HTEo-pCEPR cells were treated with 50 μM GPN and 5 μg/ml NBD-cholesterol for ~24 h. GPN treatment reduces cholesterol accumulation in pCEPR to near control (pCEP) levels (Fig. 4-3A). NBD-cholesterol content was objectively determined by flow cytometry analysis. Flow cytometry analysis reveals a significant decrease in NBD-cholesterol fluorescence in GPN-treated cells compared with untreated 9/HTEo-pCEPR cells (Fig. 4-3B). GPN treatment significantly reduces mean fluorescence in pCEPR (CF phenotype) cells ~34% to control (pCEP) levels. These data suggest that cholesterol is at least in part being
Figure 4-2. NBD-cholesterol accumulation in 2 CF cell culture systems. (A) cells are incubated for 24 h with NBD-cholesterol, a fluorescent cholesterol probe, and then placed in fresh media for 4 h before being fixed. 9/HTEo-pCEP and S9 (wild type; WT) and pCEPR and IB3 (CF phenotype) images are representative of average confocal images found over 5 experiments. Bar =8 μm. (B) NBD-cholesterol accumulation is quantified using flow cytometry analysis. Significance is determined by t-test. Error bars represent SE (n=8 for each). *P =0.002, **P=0.005.

Note: This figure is obtained from ref 5.
Figure 4-3. Lysosomal storage of NBD-cholesterol in CF cells. (A) cells are treated for 24 h with 50 μM GPN and 5 μg/ml NBD-cholesterol. Images are average projections of z-stacks representative of results from 3 experiments. Bar = 45 μm. (B) quantification of decreased NBD-cholesterol accumulation in GPN-treated cells is shown by flow cytometry analysis. Solid bars represent 9/HTEo-pCEPR (CF) cells, and open bars represent 9/HTEo-pCEP (WT) cells. Significance is determined by t-test comparing nontreated (nt) pCEPR and GPN-treated pCEPR. Error bars represent SE (n = 3). *P = 0.008.

Note: This figure is obtained from ref 5.
accumulated in lysosomal compartments. The use of NBD-cholesterol for trafficking studies must be viewed with some caution, as the hydrophobic NBD moiety can potentially interact with the membrane itself and interfere with processing.

**Increased cholesterol content in the plasma membrane of CF model systems**

Intracellular cholesterol accumulation in cultured cell and in vivo CF models may indicate flaw in cholesterol transport mechanisms. NPC cells exhibit reduced plasma membrane cholesterol as a result of impaired Npc1 function (data shown in chapter 1). Membrane cholesterol content is potential important in immune responses to bacterial challenge\(^\text{17}\) and in cell signaling through lipid raft formation. Therefore, we measured plasma membrane cholesterol content in 9/HTEo-pCEP and pCEPR cells and in excised nasal epithelium from WT and cftr\(^{\text{-/-}}\) mice using oxidase-modified electrodes. In both cultured cell models (Figure 4-4) and nasal epithelial tissue (Figure 4-5), CF samples exhibit an approximate two-fold (\(P < 0.01\)) increase in detectable current, indicating increased membrane cholesterol content. These results are consistent with earlier work demonstrating increased cholesterol content in plasma membranes of CF lymphoblast\(^{\text{21}}\) and our previous result on CF trachea tissues.

The current study suggests that free cholesterol accumulation is a CF-related phenotype relevant to the in vivo condition. The existence of higher cholesterol content in the plasma membrane in mice null for CFTR expression further indicates that a loss of CFTR function, as opposed to a trafficking defect, is responsible for altered cholesterol movement. Whether the increase in membrane cholesterol content in CF cells is due to passive diffusion of lipid droplets to the membrane or to an increase in active transport
Figure 4-4. Microelectrode determination of cellular plasma membrane cholesterol content. (A) representative traces of membrane cholesterol determination in 9/HTEo- pCEPR (CF) and pCEP (WT) cells. Control trace consists of an examination of pCEPR (CF) cells with a platinum electrode without incorporation of cholesterol oxidase to determine cholesterol specificity of current response. (B) quantification of responses between 9/HTEo- pCEPR (CF) and pCEP (WT) cells. Responses are reported relative to WT response (response ratio) to indicate the fold increase in response. Error bars represent SE; n = 5 for each. Significance is determined by t-test. *P< 0.01.
Figure 4-5. Microelectrode determination of membrane cholesterol content at the surface of tissues. (A) representative traces of membrane cholesterol determination in excised nasal epithelium from cftr<sup>−/−</sup> (CF) and sibling cftr<sup>−/−</sup> (WT) mice. Control trace consists of an examination of cftr<sup>−/−</sup> (CF) nasal tissue with a platinum electrode without incorporation of cholesterol oxidase to determine cholesterol specificity of current response. (B) quantification of responses between cftr<sup>−/−</sup> (CF) and sibling cftr<sup>−/−</sup> (WT) nasal tissue. Responses are reported relative to WT response (response ratio) to indicate the fold increase in response. Error bars represent SE; n = 5 for each. Significance is determined by t-test. *P < 0.01.
mechanism is unclear. Passive transport is unlikely, since NPC cells exhibit an extreme deficiency in the plasma membrane cholesterol. As we have discussed previously, both CF cells and NPC cells exhibit similar accumulation of free cholesterol. If passive diffusion of excess cholesterol is responsible for elevated membrane cholesterol content, it would be expected that NPC cells would also exhibit a similar increase. However, NPC cells do not display this increase suggesting a specific role of Npc1 in modulating cholesterol transport to the membrane. Inhibition of Npc1 with the compound U18666a in WT cells (Figure 4-6) eliminates membrane cholesterol content verifying a role for Npc1 in the function in CF cells. Previous results demonstrated increased Npc1 mRNA content in CF cells, also supporting the possibility of increased Npc1-driven membrane cholesterol transport in CF.

4.4 CONCLUSION

In this chapter, how the regulation of cellular cholesterol process is impacted in CF is determined to understand disease pathology. Unesterified cholesterol accumulation is observed in lung and trachea sections obtained from CF patients compared with non-CF tissues, suggesting an inherent flaw in cholesterol processing. The intracellular cholesterol is likely improperly deposited in lysosomes in CF cells. Electrochemical data suggest excess cholesterol manifested in the plasma membrane of cultured CF cells and nasal tissue excised from cftr−/− mice. This altered intracellular cholesterol movement indicates a possibility of increased Npc1-driven membrane cholesterol transport in CF. This perturbation of cholesterol homeostasis represents a potentially important process in CF pathogenesis.
Figure 4-6. Microelectrode (4μm in diameter) determination of cell membrane cholesterol content treated with U18666a. (A) representative traces of membrane cholesterol determination in pCEP (WT) cells and WT cells treated with 2.5 μM U18666a for 24h. (B) quantification of responses between WT cells and WT cells treated with 2.5 μM U18666a. Error bars represent SE; n = 5 for each. Significance is determined by t-test. *P< 0.01.
4.5 ACKNOWLEDGEMENTS

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4.6 REFERENCES


CHAPTER 5

CHOLESTEROL ACTS DOWNSTREAM OF HMGCoA REDUCTASE DURING PGC MIGRATION IN MAMMALS*

* The fluorescence and PCR data in this chapter were provided by Prof. Kathleen Molyneaux; and the mass spectroscopy data are provided by Prof. Nicholas Winograd. The following text has been stated in our submitted manuscript (ref 9).

5.1 INTRODUCTION

Primordial germ cells (PGCs) are the embryonic precursors of gametes. In most model systems, these cells are migratory and navigate through or around diverse tissues in order to find the site of the developing gonads. PGCs in different organisms take diverse paths to reach their targets.1 Despite this, PGCs in different organisms are thought to share conserved features for migration indicating the process might have arisen in a common eukaryotic ancestor. A recent study demonstrates that zebrafish PGCs, like Drosophila PGCs, required hydroxymethylglutaryl coenzyme A reductase (HMGCR) activity for normal migration.2

HMGCR is the rate limiting enzyme in isoprenoid and cholesterol biosynthesis (see Figure 5-1). HMGCR was originally identified in a forward genetic screen for genes required for PGC migration in Drosophila.3 In this system, HMGCR activity is required that is non-cell autonomous for PGC guidance. HMGCR mRNA is elevated in the somatic gonadal precursors, the target tissue for PGC migration. Additionally, ectopic expression of HMGCR is able to lure PGCs away from their normal migration route.3

How HMGCR provides guidance cues is still a mystery although the process appears to involve an isoprenoid intermediate and not cholesterol. Flies lack several of
the post-squalene enzymes required for de novo cholesterol synthesis and are consequently cholesterol auxotrophes.\textsuperscript{4} Santos and Lehmann have shown that mutations in genes required specifically for isoprenoid biosynthesis (farnesyl-diphosphate synthase and geranylgeranyl diphosphate synthase) caused germ cell migration defects. Additionally, mutations in the geranylgeranyl transferase 1 subunit also cause migration defects suggesting that the relevant target is a geranylgeranylated protein. Candidates include small GTPase in the Ras, Rac, or Rho families that may act by controlling secretion of hedgehog\textsuperscript{5} or other putative PGC attractants.

Thorpe et al. used statins to inhibit HMGCR activity in whole zebrafish embryos demonstrating that this enzyme was required for PGC migration in vertebrates.\textsuperscript{2} They further demonstrated that addition of isoprenoids, but not cholesterol precursors can rescue the statin-induced migration defects. However, unlike in fly, HMGCR mRNA is ubiquitously expressed during PGC migration in zebrafish. In this system, HMGCR is likely to act cell autonomously perhaps by controlling isoprenylation of G-protein subunits required for PGCs to respond to chemoattractants. In \textit{Drosophila}, geranylgeranylation of a G subunit was recently shown to be required downstream of HMGCR for cardiac development.\textsuperscript{6,7}

HMGCR is the only gene shown to be required for PGC migration in both vertebrate and invertebrate systems. Consequently, we decide to test its role in mammals with the hope of providing further evidence that PGC migration is a conserved process. Loss of HMGCR is early embryonic lethal in mice.\textsuperscript{8} This highlights the importance of HMGCR and its products in normal development, but unfortunately precludes analysis of PGC migration in this line. Instead, we test the role of HMGCR in PGC migration by
inhibiting its activity in organ culture. Our study demonstrates that HMGCR is required for PGC survival and motility and that both isoprenoids and cholesterol are required downstream of this enzyme in mammals. In this chapter, only the role of cholesterol in PGC migration is discussed. The information about the role of isoprenoids can be found in reference 9. The study exhibits elevated cholesterol levels within the developing genital ridges in embryonic mouse tissue, which suggests a unique role for cholesterol in PGC guidance.

**Figure 5-1.** The HMGCR pathway. HMGCR is the rate-limiting enzyme that converts HMG-CoA into mevalonate. Mevalonate serves as a precursor for the synthesis of both cholesterol and isoprenoids. Enzymes inhibited in this study are indicated in red. *Note: This figure is obtained from ref 9.*
5.2 EXPERIMENTAL

Organ culture experiments

Embryos heterozygous for the Oct4: PE: GFP germ cell marker were generated by crossing Oct4: PE: GFP males with CD1 females (Charles River). Embryonic day 0.5 (E0.5) was assumed to be noon on the day on which a cervical plug was seen. On E9.5, pregnant females were sedated with isoflurane and sacrificed by cervical dislocation. The uterus was removed and placed into PBS. Embryos were dissected from the uterus using forceps and then transferred via pipette into DMEM/F-12 media (Invitrogen) supplemented with 100 U of penicillin, 100 μg streptomycin (Invitrogen) and 0.04% lipid free BSA (Sigma) (culture media). Transverse slices approximately 2 somites thick were cut from the trunk region using a scalpel. Dissected tissue was placed into organ culture chambers (MiliCel).

Cholesterol measurements

For electrochemical detection of cholesterol on fresh dissected embryo tissue, the fabrication of oxidase-modified microelectrodes (10 μm and 100 μm in diameter) and the amperometric measurements are described in chapter 1 and 2.11

Note: The other experimental descriptions, including the confocal microscopy to quantify changes in germ cell number, filipin staining, secondary ion mass spectroscopy images and RT-PCR for HMGCR mRNA can be referred from reference 9, 12 and 13.
5.3 RESULTS AND DISCUSSION

Cholesterol levels are elevated in the developing genital ridge

*Drosophila* HMGCR mRNA is elevated in somatic gonadal precursors supporting the model that HMGCR acts non-cell autonomously to guide PGC migration. However, zebrafish HMGCR2 is uniformly expressed in cleavage stage and gastrulating embryos raising the possibility that HMGCR has both non-autonomous and autonomous effects. Like in zebrafish, mouse HMGCR is ubiquitously expressed in E10.5 embryos based on *in situ* hybridization. However, *in situ* are not sensitive enough to reveal modest changes in gene expression. As a more sensitive technique, quantitative RT-PCR was used to compare HMGCR mRNA levels within the genital ridge and non-ridge tissue during PGC migration (E9.5) (Figure 5-2 A and B). Mouse HMGCR was uniformly expressed at this stage confirming the previous *in situ* hybridization results.

HMGCR activity is controlled by transcriptional and post-transcriptional mechanisms. For example, cholesterol and its derivatives oxysterols feedback and inhibit HMGCR expression at the transcriptional level, however isoprenoids can inhibit HMGCR activity by inducing degradation of the HMGCR protein. Therefore, the absolute amount of HMGCR mRNA is unlikely to be a very useful indicator of the activity of the pathway. The distribution of cholesterol is examined to provide an indirect measure of HMGCR pathway activity (Figure 5-3 and 5-4). In initial experiments, filipin staining was used to map the distribution of cholesterol during PGC migration (Figure 5-3). Filipin staining was uniform at E9.5; however we are concerned that fixation and processing (e.g. permeabilization) might have caused diffusion of cholesterol in the samples.
Figure 5-2.  (A) Example of a transverse tissue slice dissected from an E9.5 mouse embryo. PGCs are green because of the presence of the Oct4 PE: GFP transgene. To isolate genital ridge and non-ridge tissues, cuts are performed as shown. (B) Quantitative RT-PCR demonstrating that HMGCR is uniformly expressed. In three independent experiments, the mRNA levels in non-ridge tissue are compared to mRNA levels in ridge tissue which is set to an arbitrary value of 100. The average ± s.e.m. for the three experiments is shown on the right.  

Note: This figure is obtained from ref 9.
Figure 5-3. (A) Filipin staining is used to compare total cholesterol levels in E9.5 tissue slices. For each slice, the pixel intensity is averaged in three midline and three lateral regions (colored spots) and the background signal is subtracted (-Filipin signal). For each slice the average midline signal is normalized to the average ridge signal (set to 100%). (B) Filipin distribution is compared in midline and genital ridge tissue. The average ± s.e.m. is displayed. n indicates the number of slices.

Note: This figure is obtained from ref 9.
To avoid processing artifacts, we take advantage of both an electrochemical method developed to measure plasma membrane cholesterol levels in living cells\textsuperscript{17,18} as well as time of flight secondary ion mass spectrometry (TOF-SIMS)\textsuperscript{19}. A cholesterol oxidase modified electrode (10\textmu m diameter) was used to compare surface cholesterol levels within the genital ridge and midline tissues (Figure 5-4). Current generated by the electrode was proportional to the cholesterol level at the contact site of the electrode. The oxidase-modified electrode detected a moderate, but consistent elevation of cholesterol within the genital ridge relative to the midline (gut mesentery). TOF-SIMS analysis was performed on tissue slices that were snap frozen immediately after dissection and no consistent elevation of cholesterol was detected in the ridges (Figure 5-5A). However, localized accumulation of cholesterol was detected in tissue that had been incubated for 30 min. in soluble cholesterol prior to freezing (Figure 5-5B). This suggests that cells within the genital ridges accumulate high levels of cholesterol via uptake instead of \textit{de novo} synthesis. We propose that localized uptake of cholesterol within the ridge modulates the signaling interactions required for early development of the gonad.

**Statins decrease cholesterol level by the inhibition of HMGCR**

To test the role of HMGCR in PGC migration, tissue slice dissected from E9.5 embryos were cultured in the presence of statins. Dose response curves were established for simvastatin, mevinolin and atorvastatin. All three statins caused a dose dependent increase in PGC and somatic apoptosis after 18 h in culture. The experimental data and a full discussion are presented in ref 9.

Recent evidence suggests that statins can elicit responses that are independent of their effects on HMGCR.\textsuperscript{19} Additionally, the effects of inhibiting HMGCR itself may be
Figure 5-4. Cholesterol uptake is elevated in the genital ridges investigated by electrochemical method. (A) Optical photograph showing contact of genital ridge and non-ridge tissue with a microelectrode. (B) A cholesterol oxidase modified microelectrode (10 μm diameter) is used to compare surface cholesterol levels in genital ridge and non-ridge tissue. (C) Control experiment is performed using a bare Pt electrode to see current response during the contact. (D) The oxidase-modified electrode gives a consistently higher current response when touched to the genital ridge as compared to midline tissue. To summarize data from multiple slices, the midline response is normalized to the ridge response (100%). Data presented is the average ± s.e.m. N is the number of slices.
Figure 5-5. Cholesterol uptake is elevated in the genital ridge investigated by mass spectroscopy technology. (A) Scanning TOF-SIMS of an E9.5 slice imaged for phosphatidylcholine (m/z =184). (B) Slice imaged for cholesterol (m/z=369). (C) Scanning TOF-SIMS of an E9.5 slice pre-treated for 30 min. with soluble cholesterol imaged for phosphatidylcholine. (D) Scanning TOF-SIMS of the slice imaged for cholesterol. Arrowheads indicate the position of the genital ridges. 

Note: This figure is obtained from ref 9.
complex, involving modulation of isoprenoids, cholesterol or both. To test whether our treatment is effective at inhibiting HMGCR, we used filipin staining and the cholesterol oxidase modified microelectrode to compare cholesterol levels in atorvastatin-treated and untreated tissue (Figure 5-6). Atorvastatin-treatment caused a dose dependent reduction in both filipin staining and current response. 10 μM atorvastatin was the lowest dose capable of causing a statistically significant drop in cholesterol levels. It is interesting to note that 1 μM atorvastatin had little effect on cholesterol levels despite causing a significant reduction in germ cell numbers. This indicates that germ cell survival is likely more sensitive to isoprenoid levels and may not depend upon the cholesterol branch of the HMGCR pathway.

How might cholesterol accumulation within the genital ridges or somatic gonadal precursors impact PGC behavior? Cholesterol, like isoprenoids has a large number of possible roles in development. Changes in cholesterol levels can impact the distribution of cell surface proteins by altering the formation of lipid rafts. Cholesterol can be further converted into oxysterols which signal directly by binding and activating nuclear receptors. Finally, cholesterol modification of members of the Hedgehog growth factor family either restricts or facilitates diffusion of these secreted proteins. The exact role of cholesterol in this system is being evaluated.
Figure 5-6. Plasma membrane cholesterol analysis in living cells treated with statin. (A) Summary of filipin staining data from three experiments. Filipin staining intensity is quantified for each slice, the background signal is subtracted and the raw signal normalized to control values (set to 100%). The average staining intensity for each treatment group is shown. Error bars are s.e.m. N indicates the number of slices. (F<0.05 Analysis of Variance from Walpole and Meyer with Fishers least significance post test). (B) In a single experiment, a cholesterol oxidase modified microelectrode (100 µm diameter) is used to measure surface cholesterol at a random position in control and atorvastatin treated slices. (C) Control experiment is performed using a bare electrode contacting with the tissue. (D) The current response of the treated slices is normalized to the controls (set to 100%). The average response for each group is shown. Error bars are s.e.m.

Note: This figure (A) is obtained from ref 9.
5.4 CONCLUSION

In this study, unlike in flies, HMGCR mRNA is not elevated in the target tissue for PGC migration in mouse embryos. However, using a novel cholesterol oxidase modified electrode, we detect elevated cholesterol at the surface of the genital ridge. Additionally, scanning time of flight mass spectroscopy confirmed increased uptake of cholesterol in the ridges. Statin treatment of embryonic tissue in culture reduced total cholesterol levels, slowed primordial germ cell migration, and induced both germ cell and somatic cell apoptosis. It is concluded that HMGCR produces both cholesterol and isoprenoids that are necessary for PGC survival and movement in mice. Similar to the zebrafish system, we propose that HMGCR activity within PGCs produces isoprenoids required for PGC survival and motility. However, our study suggests a unique non-cell autonomous role for cholesterol during PGC migration in mammals.

5.5 ACKNOWLEDGEMENTS

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5.6 REFERENCE


CHAPTER 6

SCANNING FORCE MICROSCOPIC IMAGES OF CHOLESTEROL OXIDASE IMMOBILIZED IN A SUPPORTED LIPID BILAYER MEMBRANES*

*Figures 6-1 and 6-4 are equally contributed by Dr. Anando Devadoss and the author.

6.1 INTRODUCTION

A goal of our research group is the further development and implementation of microelectrodes containing immobilized cholesterol oxidase for detection of cell plasma membrane cholesterol.\textsuperscript{1-5} Compared with our direct covalent attachment of cholesterol oxidase to Pt microelectrodes,\textsuperscript{4,5} immobilization of the oxidase in supported lipid bilayer membranes is believed to retain a more active enzyme conformation by mimicking the natural interaction of the enzyme with the cell plasma membrane. In the membrane, the oxidase converts cholesterol to cholestenone. Analysis of enzymes with this activity has shown that two classes of cholesterol oxidase can be defined. Enzymes belonging to class I contain noncovalently bound flavin adenine dinucleotide (FAD), whereas the class II enzymes contain FAD covalently bound to an active-site histidine.\textsuperscript{6,7} Despite catalyzing the same chemical reaction, class I and class II enzymes show no sequence similarity and have a different molecular architecture. In this chapter, a tapping mode-scanning force microscopic (TM-SFM) study aimed at characterizing the state (e.g., monomers vs. aggregates) of two classes of enzymes associated with supported lipid bilayer membrane is presented.

The enzyme immobilization scheme is supported by structural studies of cholesterol oxidase (class I), as well as reports indicating that the enzyme associates with
lipid bilayer vesicles and the cell plasma membrane during oxidation of membrane-
resident cholesterol.\(^8\) A detailed description of this research background can be found in
Dr. Anando Devadoss’s thesis.\(^9\)

6.2 EXPERIMENTAL

Sample Preparation

*Streptomyces Sp.* Cholesterol oxidase (class I) from N. Sampson’s laboratory and
*Pseudomonas Sp.* Cholesterol oxidase (class II) from Wako Pure Chemical Industries,
Ltd. were chosen for the study. The cholesterol oxidase (2mg/ml) was dissolved in 50
mM PBS with a pH 7.4. DPPC (Avanti Polar Lipids, Inc.) dissolved in chloroform was
dried under nitrogen and sonicated in buffer until clear to obtain a 1mM vesicle solution.
A freshly cleaved mica surface was exposed to the vesicle solution for 30 min. The
substrate with a DPPC membrane was exposed to cholesterol oxidase solutions for 15
min and dried to image cholesterol oxidase embedded on lipid membrane. The samples
were rinsed with water and dried before imaging.

Instrumentation

All images were acquired in air using MAC mode-Scanning force microscope
(Molecular Imaging, Inc.). The tapping frequencies of the probes (Type II MAClevers)
were in the range of 60-85 kHz. The images were scanned at a range of 4 – 15 μm/s.
6.3 RESULTS AND DISCUSSION

It is well documented that vesicle fusion can be used to deposit lipid bilayer membranes on cleaved mica and there is much scanning probe microscopy data available in the literature on this system.\textsuperscript{10-13} Figure 6-1 shows a typical topographical image of DPPC deposited on cleaved mica by vesicle fusion. The topography of bilayer membrane consists of plateaus that differ in the height associated with the thickness of lipid bilayer ($5.9 \pm 0.6$ nm). This thickness is consistent with the heights observed from other groups using AFM\textsuperscript{14,15} and is comparable to the results from X-ray diffraction measurements.\textsuperscript{16,17} The thickness observed indicates a single bilayer on the mica surface with, presumably, a thin layer of water between the mica surface and the bilayer. It is noticed that bilayer regions of the surface are basically flat without any feature and large defects are existed on unmodified regions on the mica.\textsuperscript{14}

After exposure of the lipid membrane to \textit{Streptomyces Sp.} cholesterol oxidase (class I) solution, much of the surface showed lipid bilayer plateaus decorated with hemispherical features that appear to be 1-2 nm in height and 30-50 nm in diameter (e.g., Figure 6-2). It is noted that lateral diminutions of small particles can appear to be more than double in size due to the aspect ratio of the tip.\textsuperscript{18} Thus, a single cholesterol oxidase molecule partially inserting into the lipid membrane and having dimensions of 7.3 x 6.3 x 5.1 nm (taken from the crystal structure of the \textit{Streptomyces} Sp. Enzyme)\textsuperscript{19} may appear as a feature that is ca. 10-20 nm in diameter. Previously, Sampson’s group has determined the cholesterol oxidase inserts 8 Å into lipid bilayer using the fluorescence parallax method of London.\textsuperscript{20} They speculated that the enzyme could undergo a conformational change during membrane binding that causes it to flatten. This explains
Figure 6-1. TM-SFM images of a lipid bilayer membrane on mica. (A) topographical image. (B) Z-axis height cross section.
Figure 6-2. TM-SFM images of *Streptomyces* *Sp.* cholesterol oxidase immobilized in a lipid bilayer membrane on mica. (A) topographical image of cholesterol oxidase in lipid bilayer membrane. (B) Z-axis height cross section.
1-2 nm height observed for the oxidase in this study. Figure 6-3 exhibits features with larger lateral dimension (100-200nm), which is likely small islands of enzyme molecules that are laterally spaced in a bilayer structure as aggregates. Thus, the size of the features imaged on lipid plateau regions of the surface is consistent with dimensions of the enzyme monolayer aggregate and its predicted insertion in the lipid membrane.

Interestingly, the topographical image (Figure 6-4) of *Pseudomonas Sp.* cholesterol oxidase (class II) shows the hemisphere on the lipid membrane appearing to be 3-4 nm in height and 30-50 nm in diameter, which indicates that class II enzyme has a larger height above the surrounding bilayer membrane compared to class I enzymes. This phenomenon could be reasonably explained by the larger crystal structure of *Pseudomonas Sp.* cholesterol oxidase (12.0 x 12.0 x 10.1 nm).

The data observed support that these two classes of oxidase sit on the supported lipid bilayer likely as aggregates. TM-SFM images for longer exposure of the lipid membrane to enzyme solution suggest that the surface becomes covered by aggregates of cholesterol oxidase and it is not possible to clearly discern the existence of lipid membrane plateaus.
Figure 6-3. TM-SFM images of *Streptomyces Sp.* cholesterol oxidase aggregates immobilized in a lipid bilayer membrane on mica. (A) topographical image of cholesterol oxidase in a lipid bilayer membrane (B) Z-axis height cross section.
Figure 6-4. TM-SFM images of *Pseudomonas Sp.* cholesterol oxidase immobilized in a lipid bilayer membrane on mica. (A) Topographical image of cholesterol oxidase in a lipid bilayer membrane. (B) Z-axis height cross section.
6.4 CONCLUSION

TM-SFM images of a bilayer lipid membrane on cleaved mica show membrane plateaus and step edges that are one lipid bilayer thicknesses in height. Exposure of cholesterol oxidase solution to the lipid membrane results in immobilization of enzyme in lipid membrane plateaus. Based on the size of the features assigned to immobilized enzyme, it is proposed that cholesterol oxidase exists as monomers and aggregates partially inserted in the lipid membrane. The images pictorially support the proposed model for interaction of the bacterial enzyme with the plasma membrane of mammalian cells.

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6.6 REFERENCES


CHAPTER 7

FUTURE DIRECTIONS

7.1 INTRODUCTION.

The goals of this group are to implement electrochemical analysis of cell plasma membrane cholesterol to better understand the mechanism of intracellular cholesterol trafficking; and to develop amperometric analysis of cholesterol at the epithelial surface of the human nose as a diagnostic tool in management of CF. Our previous work has demonstrated that modification of Pt electrodes with a sub-monolayer of covalently attached cholesterol oxidase allowed cholesterol sensing directly at the plasma membrane surface of single living cells and at tissue surfaces. The electrochemical data suggest that the excess cholesterol inside the cell can up-regulate the intracellular cholesterol trafficking from lysosomes to the plasma membrane, which is likely controlled by Npc1 expression.1-4 Further investigation in this study will enhance our understanding of the molecular and cellular basis of lipid-associated disorders such as Tangier disease, Niemann–Pick disease type C, and atherosclerosis.5 Also, study in cystic fibrosis has identified that CF cells and tissues have an inherent flaw in their ability to process cholesterol.3,4,6 It is hypothesized that electrochemical measurements of nasal epithelial cholesterol can be utilized as a non-invasive, reproducible biomarker to assess treatment effectiveness and disease severity. To realize these goals, some technological challenges should be overcome and are discussed in detail in this chapter.
Chapter 1 shows that direct covalent attachment of cholesterol oxidase to a bare platinum surface yields electrodes that exhibit an utilizable (moderate) enzymatic activity at 37 °C. This covalent immobilization limits the number of enzyme molecules immobilized on the electrode surface and gives a relatively small demand for cholesterol. Therefore, a novel approach to gradually increase electrode-demand for cholesterol is to crosslink additional cholesterol oxidase molecules to the cholesterol oxidase sub-monolayer attached to the Pt electrode surface using glutaraldehyde chemistry. The initial experiment used the electrode containing a sub-monolayer of enzyme. This electrode was exposed to 100 mM PBS containing 5 wt % glutaraldehyde for 0.5 h to allow reaction of residues (lysine or arginine) on the protein surface with the carbonyl group of glutaraldehyde. Exposure of this surface to enzyme solution allowed crosslinking of additional enzyme to the electrode surface through covalent bonds. It was possible to repeat the procedure to cross-link additional enzyme molecule. The surface architecture is shown in Figure 7-1. Figure 7-2 shows preliminary data using the modified electrodes for the detection of cholesterol solution. The current responses increase with each additional layer of the enzyme up to three layers. More enzyme loading results in a larger number of cholesterol molecules reacted. For the electrode modified with four layers of enzyme, a slight decrease in the response was observed. This phenomenon might be explained that the increased diffusion path of H₂O₂ back to the electrode surface through the interconnected protein layer and the lost of enzyme activity during the modification. Figure 7-3 exhibits an increase in current response from
Figure 7-1. Idealized structure of covalent architecture to link multi-layer oxidase to the electrode surface.

Figure 7-2. Amperometric response of Pt microelectrode modified with one (1), two (2), three (3) and four (4) layers of oxidase. The cholesterol concentration is 1mM in 100mM PBS containing 1% triton X-100.
the plasma membrane for Pt microelectrodes modified with additional enzyme. This increase is attributed to the faster consumption rate of cholesterol at the electrode surface causing faster efflux of cholesterol from the plasma membrane. Continued studies on this electrode architecture should focus on the quantitative analysis of enzyme activity, the investigation of the electrode sensitivity and thermally-stability, and the application of this electrode to the single cell and tissue cholesterol detection at physiological temperature.

The other possible way to improve the electrode sensitivity is the introduction of Pt nanoparticles into the oxidase membrane on the electrode. In our study, polycrystalline Pt electrodes are used for the detection of H$_2$O$_2$ produced upon cholesterol oxidation. However, deactivation of electrode surface for H$_2$O$_2$ oxidation due to the

Figure 7-3. Amperometric response of Pt microelectrode (10 μm diameter) modified with one and four layers of oxidase contacting with the plasma membrane of *Aplysia* neuron cell.
formation of a surface oxide is one problem.\textsuperscript{8,9} Pt nanoparticles have exhibited an excellent performance in glucose detection.\textsuperscript{10} Here, sol-gel chemistry combined with Pt nanoparticle incorporation, developed by Dr. Retna Raj’s group, is introduced to improve the electrode sensitivity for cholesterol detection.

Briefly, 24 μl (γ-methacryloxypropyl) trimethoxysilanes (MPTS) and 10 μl (0.1 M) HCl in 2 ml water are stirred vigorously for 30 min. The MPTS sol (0.5 ml) prepared is mixed with 0.5 ml oxidase in PBS (pH 7.4) and stirred for 2-3 min to encapsulate oxidases into the silicate network. Then, the cleaned electrode is soaked in MPTS sol for 10 min to allow the formation of 3-D silicate network. The colloidal Pt nanoparticles are synthesized according to the reported procedure with little modification.\textsuperscript{11,12} 25 ml aqueous solution of H\textsubscript{2}PtCl\textsubscript{6} (0.02 mM) and 1 mM D-glucose are mixed and stirred for 2 min. Then the pH of the solution is adjusted to 8 with NaOH. A 400 μl of aqueous NaBH\textsubscript{4} (0.05 M) is then added dropwise to the stirred solution and the stirring is continued for 15 min. The formation of the Pt nanoparticles can be followed by UV-visible spectroscopy. The precursor H\textsubscript{2}PtCl\textsubscript{6} solution has showed a peak at 200-300 nm, corresponding to the ligand-to-metal charge transfer (LMCT) transition. The peak of the solution at 200-300 nm disappears upon the addition of reducing agent, indicating the reduction of the PtCl\textsubscript{6}\textsuperscript{2-} ions to metallic Pt. The resulting MPTS sol modified electrode is immersed into colloidal Pt nanoparticles prepared for 24 hrs. Because Pt has affinity for thiols, Pt nanoparticles chemisorb on the thiol groups of silicate network. A similar electrode architecture using gold nanoparticles to detect NADH has been reported\textsuperscript{11}. Pt nanoparticles have exhibited a high catalytic activity on H\textsubscript{2}O\textsubscript{2} oxidation,\textsuperscript{10} hence a better sensitivity for cholesterol detection is predicted. More concerns in the study are the
thickness and the thermal-stability of oxidase-gel film and the application of the surface chemistry for plasma membrane cholesterol detection.

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