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

by

DANJUN FANG

Submitted in partial fulfillment of the requirements

For the degree of Doctor of Philosophy

Dissertation Adviser: Professor James. D. Burgess

Department of Chemistry

CASE WESTERN RESERVE UNIVERSITY

January 2010
We hereby approve the dissertation of

Danjun Fang

candidate for the PH.D. degree *.

(signed)            Daniel Scherson
                   (chair of the committee)

                        Alfred Anderson

                        Carlos Crespo

                        Thomas Kelley

                        James Burgess

(date)                07/07/09

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

MY FAMILY
TABLE OF CONTENTS

TABLE OF CONTENTS .................................................................................................................... I

LIST OF TABLES .......................................................................................................................... III

LIST OF FIGURES ....................................................................................................................... IV

LIST OF ABBREVIATIONS ......................................................................................................... XIII

ACKNOWLEDGEMENTS ............................................................................................................. XV

ABSTRACT ................................................................................................................................. XVI

CHAPTER 1: INTRODUCTION ................................................................................................. 1

1.1 MECHANISM OF CHOLESTEROL EFFLUX FROM THE PLASMA MEMBRANE......................................................................................................................... 1

1.2 CHEMICAL POTENTIAL OF CHOLESTEROL IN THE PLASMA MEMBRANE .................................................................................................................. 5

1.3 CHOLESTEROL TRAFFICKING IN CYSTIC FIBROSIS (CF) ......................... 10

1.4 REFERENCES .................................................................................................................. 12

CHAPTER 2: OBSERVATION OF CELLULAR CHOLESTEROL EFFLUX AT MICRO-CAVITY ELECTRODES ................................................................................................................. 15

2.1 INTRODUCTION ............................................................................................................. 15

2.2 EXPERIMENTAL METHODS ...................................................................................... 16

2.3 RESULTS AND DISCUSSION ..................................................................................... 23

2.4 ACKNOWLEDGEMENTS .......................................................................................... 45
LIST OF TABLES

Table 2-1. Charge at 1 s for seven electrodes in buffer, the increase in charge at 1 s for the electrodes in 100 μM cholesterol solution, and the % relative charge increase. .......................................................... 27
LIST OF FIGURES

Figure 1-1. Mechanisms of efflux of unesterified cholesterol from peripheral cells. A. Aqueous diffusion. Cholesterol molecules move from the plasma membrane into the aqueous solution and are subsequently incorporated into lipidation of ApoAI. B. SR-BI-mediated efflux. Cells expressing SR-BI at the plasma membrane enhances cholesterol efflux. C. ABCA1 mediated efflux. ABCA1 promotes the unidirectional efflux of cholesterol and phospholipids to lipid-free or lipid-poor apolipoproteins. HDL: high density lipoprotein. ABCA1: ATP-binding cassette transporter A1.

Figure 1-2. Models for the chemical potential of cholesterol as function of cholesterol content in a lipid membrane. A. The condensed complex model predicts a jump in cholesterol content at a stoichiometric composition. B. The superlattice model predicts dips in free energy at superlattice compositions, which also implied sharp spikes in chemical potential. C. The umbrella model predicts a cascade of jump in chemical potential, and each jump corresponds to a stable cholesterol distribution.

Figure 2-1. Scheme of micro-cavity electrode positioned in contact with the cell plasma membrane. The average molecular area of cholesterol in the plasma membrane is estimated at 40 Å².
Figure 2-2. A. Photograph under optical magnification of the 10 μm diameter micro-cavity electrode with a 5 μm cavity depth. B. Cyclic voltammetry of ferrocyanide (5 mM, 100 mM phosphate buffer, pH 7.4) at a bare Pt disk microelectrode and after etching to form the micro-cavity electrode. Scan rate: 100 mV/s.

Figure 2-3. Characterization of electrode modification. Cyclic voltammetry of ferrocyanide (5 mM, 100 mM phosphate buffer, pH 7.4) at a bare micro-cavity electrode, after modification with a sub-monolayer of 11-mercaptoundecanoic acid, and after covalent attachment of cholesterol oxidase to the sub-monolayer. Scan rate: 1000 mV/s. The decrease in limiting current after functionalization of the electrode surface with 11-mercaptoundecanoic acid and oxidase is consistent with partial deposition of a sub-monolayer of enzyme that impedes reaction of ferrocyanide at the electrode surface.

Figure 2-4. Circuit for charge integration.

Figure 2-5. A. The photograph of *Aplysia Californica*. B. The pinned buccal ganglia in *Aplysia* saline. C. The ganglia are desheathed to expose the neuron cells. D. The micro-cavity electrode is in contact with a neuron cell.

Figure 2-6. Consecutive potential step experiments (200 mV to 600 mV) with a 5 min hold time between experiments in buffer and buffered 100 μM Cholesterol solution. 1- 4 is the chronological order.
Figure 2-7. The relationship of the charge increase and double layer charge when the electrode is exposed to 100 µM cholesterol. The linear fit includes (0, 0).

Figure 2-8. Background subtracted analog coulometry collected for oxidase modified disk electrode in 1 mM cholesterol solution.

Figure 2-9 Analog coulometry collected for an enzyme-modified micro-cavity electrode before (1, 2) and during (3) contact with the cell.

Figure 2-10. A. Applied voltage; B. Overlaid charge for each step to 600 mV.

Figure 2-11. A. Difference charge for enzyme-modified electrode and bare electrode at the cell (1, 2, 3: chronological order); B. Three determinations of the difference charge at the cell and cyclodextrin (500 µM; 12 hours) treated cell using the enzyme-modified electrode and bare electrode at 1 second.

Figure 2-12. Background subtracted analog coulometry collected for oxidase modified disk electrode in 1000 µM cholesterol solution after one min holding time.

Figure 2-13. A. Difference charge collected for enzyme-modified (1) and bare (2) micro-cavity electrodes with small capacitance (double layer charge of 748 pC at 1 second for the second potential step) and large capacitance (double layer charge of 2056 pC at 1 second for the second potential step).
Relative charge increase before and after enzyme modification for both electrodes. .................................................................38

Figure 2-14. Action potential observed after the consecutive four detections of accumulated peroxide in the cavity.................................................................39

Figure 2-15. A. Difference charge for enzyme-modified electrode in 300 μM cholesterol and buffer solution.  B. Dependence of average (n = 3) difference charge (1 s) at the enzyme-modified electrode on cholesterol concentration.................................................................41

Figure 2-16. Data for consecutive difference charge determinations at a bare micro-cavity electrode. ..............................................................................42

Figure 2-17. Amperometric data for contacting (↑) a single cell with an enzyme modified Pt disk microelectrode and withdrawing (↓) the microelectrode from the contact position. .................................................................44

Figure 3-1. Fluorescent assay to determine cholesterol concentration in saturated solution at room temperature after stirring for three hours using the Amplex® Red cholesterol assay kit (Invitrogen).  The produced hydrogen peroxide can react with Amplex® Red reagent with HRP to give fluorescence.  Fluorescence is measured with a fluorescence microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm.  The square points are collected using standard cholesterol solution.
The value with the mark (×) is obtained from saturated cholesterol solution.

(This experiment was performed by Richard West.).................................50

Figure 3-2. Models of cholesterol initial efflux and influx from/to the plasma membrane in A. culture medium, B. medium without cholesterol and C. medium with saturated cholesterol. ...........................................................52

Figure 3-3. Picture of the 100 μ enzyme modified Pt electrode touching the cell......55

Figure 3-4. Three fold current response observed on the cell exposed to saturated cholesterol solution for 30 min. .................................................................56

Figure 3-5. No current responses are observed when the electrode touches the dead cell before and after the exposure to saturated cholesterol solution..............57

Figure 3-6. Current response of enzyme modified electrode exposed to saturated cholesterol solution. (↓) indicates the time for flow injection of saturated cholesterol. ..............................................................................................59

Figure 3-7. Current responses of enzyme modified electrode in contact position with the cell cultured in different cholesterol concentrations. ......................60

Figure 3-8. Current responses of bare electrode in contact position with the cell cultured in different cholesterol concentrations.................................62

Figure 3-9. Current responses of three enzyme modified disk electrode with different enzyme activity contacted with the cell cultured in the cholesterol solutions with different concentration..............................................63
**Figure 3-10.** Current response ratio at cholesterol concentration of 990 nM over 875 nM on seven cells.................................................................64

**Figure 3-11.** Current responses of three enzyme modified disk electrodes with different enzyme activity in contact position with the cell treated with cyclodextrin overnight cultured in different cholesterol concentrations. .........................66

**Figure 4-1.** Electrochemical determination of membrane cholesterol content from mouse nasal epithelium.  A. Representative traces of membrane cholesterol determination in excised nasal epithelium from Cfr R117H/R117H (R/R) mice, compared to sibling Cfr +/- (wt) mice.  B. Quantification of responses.  Responses are reported relative to WT response (response ratio) to indicate the fold-increase in response.  Error bars represent SEM, n = 4 for each.  *p < 0.001…………………………………………………………..75

**Figure 4-2.** Electrochemical determination of membrane cholesterol content from mouse nasal epithelium.  A. Representative traces of membrane cholesterol determination in excised nasal epithelium from Cfr ΔF508/ΔF508 (ΔF/ΔF) mice, compared to sibling Cfr +/- (WT) mice.  B. Quantification of responses.  Responses are reported relative to WT response (response ratio) to indicate the fold-increase in response.  Error bars represent SEM, n = 4 for each.  *p < 0.001…………………………………………………………..76

**Figure 4-3.** Effect of acute (24h) CFTR inhibition and chronic (72 h) CFTR inhibition with CFTRinh-172 (20 μM) on membrane cholesterol content.  A.
Representative traces of membrane cholesterol determination in 9/HTEo-cells after treatment with the CFTR inhibitor CFTRinh-172 for either 24 h or 72 h with fresh inhibitor placed on cells every 24 h or cells with no treatment (NT). B. Quantification of responses. Responses are reported relative to NT response (response ratio) to indicate the fold-difference in response. Error bars represent SEM, n = 4 for each. *p < 0.001

Figure 4-4. Specificity of CFTR inhibition in regulating membrane cholesterol content.

A. Representative traces of membrane cholesterol determination in 9/HTEo- pCEPR (CF) cells after treatment with the CFTR inhibitor CFTRinh-172 for 24 h (red line) or cells with no treatment (NT, black line).

B. Quantification of responses. Responses are reported relative to NT response (response ratio) to indicate the fold-difference in response. Error bars represent SEM, n = 4 for each.

Figure 4-5. Specificity of CFTR inhibition in regulating membrane cholesterol content.

A. Representative traces of membrane cholesterol determination in 9/HTEo- pCEP (wt) cells after treatment with Inactive-CFTRinh-172 for 24 h (red line) or cells with no treatment (WT, black line). B. Quantification of responses. Responses are reported relative to WT response (response ratio) to indicate the fold difference in response. Error bars represent SEM, n = 4 for each.
Figure 4-6. Comparison of nasal epithelium membrane cholesterol content in Cftr +/+ and Cftr +/− mice. A. Representative traces of membrane cholesterol determination in excised nasal epithelium from Cftr +/+ (wt) mice and Cftr +/− mice. B. Quantification of electrochemical membrane cholesterol determination. Responses are reported relative to WT response (response ratio) to indicate the fold-difference in response. Error bars represent SEM, n = 4 for each. *p = 0.008. ................................................................. 83

Figure 4-7. De novo cholesterol synthesis in CF mouse tissue compared to matched controls. Deuterium incorporation into newly synthesized tissue cholesterol was measured by GC/MS. Data are normalized to each tissue WT control and reported as fold increase of % newly synthesized cholesterol/8 h. Filled bars represent ΔF/ΔF mice and open bars represent R/R mice. The number of replicates is shown in parenthesis above each bar and represents individual assay on multiple tissue samples obtained over 3 experiments. *p < 0.05, #p < 0.01. ................................................................. 85

Figure 4-8. Increased SRE response in INH-172 treated control epithelial cells. 9/HTEo-pCEP (wt) cells were incubated in serum free conditions for 24 h with or without 20 µM CFTR inhibitor (INH-172) or 5 µg/mL U18666a (U18), a known cholesterol transport inhibitor, in serum free media for an additional 24 h. Data are normalized to serum free NT control levels over 3 experiments. Number (n) of samples is in parenthesis above each bar.
Significance was determined by t test. Error bars represent SEM. * p < 0.0001.

Figure 4-9 A&B. $^{11}$C-acetate incorporation into the lungs of CF mouse models. A. Ratio of the average SUV in the lungs of the R117H or ΔF508 mouse to the average SUV in the lungs of the WT control mouse. The number of pairs used is indicated in parentheses. The average SUV from both lungs 25 min after injection of $[1-^{11}$C]acetate was used in the calculations. B. Percentage of the injected dose normalized by the weight of the lungs as determined by well counting.

Figure 4-9 C&D. $^{11}$C-acetate incorporation into the lungs of CF mouse models. C. PET images of the R117H (left) and WT control (right) mice taken 25 minutes after injection of $[1-^{11}$C]acetate overlaid on the transmission images. The lungs are indicated by the arrows. D. PET images of the ΔF508 (left) and WT control (right) mice taken 25 minutes after injection of $[1-^{11}$C]acetate overlaid on the transmission images. The lungs are indicated by arrows.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>celsius degree</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>DC</td>
<td>direct current</td>
</tr>
<tr>
<td>fA</td>
<td>femtoampere</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>i</td>
<td>current</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>min.</td>
<td>minute</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>mV</td>
<td>millivolt</td>
</tr>
<tr>
<td>NHE</td>
<td>normal hydrogen electrode</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>ns</td>
<td>nanosecond</td>
</tr>
<tr>
<td>pA</td>
<td>picoampere</td>
</tr>
<tr>
<td>pM</td>
<td>picomolar</td>
</tr>
<tr>
<td>Pt</td>
<td>platinum</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

First, I would like to thank my PhD adviser Professor Jim Burgess for his inspirational and patient guidance. Jim is not only a great scientist, but also a friend. I am grateful for all I’ve learned from Jim.

I also thank our great collaborators, Professor Tom Kelley and Professor Hillel Chiel. My thesis work would not have been possible without their help.

I remain grateful to my committee, Professor Dan Scherson, Professor Alfred Anderson, Professor Carlos Crespo, Professor Cather Simpson and Professor Tom Kelley. This group helps me to critically view my work and provide insightful comments.

I thank all my colleagues, Dr. Anando Devadoss, Simona Palencsar, Dr. Dechen Jiang, Susu Yuan, Mary Manson, Richard West and Hui Lu, for the help with the experiments and many enjoyable scientific discussions.

I thank all support from Department of Chemistry, Case Western Reserve University.

I would also thank all the help and support I have received from my friends, seniors and juniors during my stay in Cleveland.

Last but not least, I would like to thank my family, my grandmother, my parents, my parents in law, my aunt, my little sister, my little brother, my lovely daughter Keke and my dear husband Dechen Jiang.
ELECTROCHEMICAL MEASUREMENT OF PLASMA MEMBRANE

CHOLESTEROL IN LIVE CELLS AND MOUSE TISSUES

ABSTRACT

By

DANJUN FANG

In this study, cholesterol oxidase-modified platinum micro-cavity electrodes are used to measure cholesterol efflux from the plasma membrane surface of a single neuron in the buccal ganglion of Aplysia at room temperature. Background subtraction analog chronocoulometry is used to measure hydrogen peroxide accumulation resulting from cellular cholesterol efflux and enzymatic oxidation in the micro-cavity volume. The data are consistent with the aqueous diffusion model for cellular cholesterol efflux where plasma membrane cholesterol undergoes exchange with solution phase cholesterol.

The notion that the cellular cholesterol “set-point” can be modeled as a thermodynamic state (i.e., activity; chemical potential) exhibited at the cell surface is explored. A titration assay is developed, using the electrode measurements as an indicator, where the solution cholesterol concentration required to drive net cholesterol influx into the cell is determined. When cholesterol influx to the cell occurs, plasma
membrane cholesterol increases and larger electrode responses are observed. Because the experiments are equilibrium based, data are discussed assuming that the end-point estimates obtained reflect a thermodynamic cholesterol state that tracks the concentration of cholesterol in the membrane.

Previous observations demonstrate that CFTR (cystic fibrosis transmembrane conductance regulator) -null cells and tissues exhibit an increase in plasma membrane cholesterol content compared to wild type (WT) controls. The hypothesis of this study is that these alterations in cholesterol processing will correlate with Cfr genotype. Membrane cholesterol contents are elevated in both R117H and ΔF508 mouse nasal epithelium compared to age-matched sibling WT controls demonstrating a genotype correlation to membrane cholesterol content. With the advent of new CFTR correctors and potentiators, determining membrane cholesterol content could prove to be an important biomarker of CFTR function and the cellular responses to various levels of CFTR function.
CHAPTER 1: INTRODUCTION

Cholesterol is an amphipathic lipid molecule that is known to play essential roles in cellular membrane structure and function.\textsuperscript{1-3} Macrophage cells can efflux excess cholesterol to HDL to protect against the onset of arteriosclerosis.\textsuperscript{4} A better understanding of the mechanisms of cholesterol efflux is important to develop clinical strategies for treating atherosclerosis and other cholesterol related diseases.

1.1 Mechanism of Cholesterol Efflux from the Plasma Membrane

Peripheral cells release cholesterol to an extracellular acceptor, high-density lipoprotein (HDL) which then mediates cholesterol delivery to the liver for excretion.\textsuperscript{5} This process is believed to be the primary mechanism by which HDL (and its major protein component Apolipoprotein A-I: apoAI) protect against atherosclerosis.\textsuperscript{6} Cholesterol efflux from cells to HDL can occur by several mechanisms, including unmediated aqueous diffusion\textsuperscript{7} and specific receptor-mediated processes, such as ATP-binding cassette transporter A1 (ABCA1),\textsuperscript{8} scavenger receptor class B type I (SR-BI),\textsuperscript{9} and ABCG1,\textsuperscript{9} e.g., Figure 1-1.
**Figure 1-1.** Mechanisms of efflux of unesterified cholesterol from peripheral cells.  

A. Aqueous diffusion. Cholesterol molecules move from the plasma membrane into the aqueous solution and are subsequently incorporated into lipidation of ApoAI.  


C. ABCA1 mediated efflux. ABCA1 promotes the unidirectional efflux of cholesterol and phospholipids to lipid-free or lipid-poor apolipoproteins.\(^3\)  

In SR-BI-mediated efflux, cells contain SR-BI at the plasma membrane and the cholesterol efflux occurs via an HDL receptor mechanism. Expression levels of SR-BI are correlated to the rate of efflux to acceptor.\(^9\) SR-BI binds lipoproteins, including LDL, and promotes the bidirectional exchange of cholesterol between cells and the extracellular acceptor. In ABCA1 and ABCG1 mediated efflux, ABCA1 and ABCG1 promote the unidirectional efflux of cholesterol and phospholipids to lipid-free or lipid-poor apolipoproteins. ABCA1 is also involved in the lipidation of apolipoprotein AI in the formation of nascent HDL.\(^7,\)\(^8\)

The aqueous diffusion mechanism was proposed by Rothblat and coworkers. They observed a maximum plateau in the rate of cellular cholesterol efflux with increasing acceptor concentration.\(^7\) 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.

Recently, they knocked out SR-BI, ABCA1 and ABCG1 in cholesterol-normal peripheral macrophage and found little or no effect on cholesterol efflux.\(^10\) This suggests that the dominant mechanism for efflux from cholesterol-normal macrophage is aqueous diffusion. Enriching the cell with cholesterol stimulated increased cholesterol efflux and 30% (in vitro)\(^10\) : 35% (in vivo)\(^11\) of cholesterol efflux was found to be attributable to aqueous diffusion.
Despite the enormous body of literature concerning cellular cholesterol efflux, no direct evidence has been reported showing cholesterol efflux to aqueous solution by diffusion. This study is missing because the solubility of cholesterol in aqueous media is very low. The solubility, perhaps low nM, is below the sensitivity limits of common methodologies (e.g., fluorescence or radio-labeling). To increase cholesterol content in the cell medium, cyclodextrin is commonly added. 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) to cyclodextrin can be assigned to that from the plasma membrane.\textsuperscript{5} However, the continued implementation of this method by others using fluorescence or radio-labeling detection does not regard this stipulation.

In chapter 2, cholesterol oxidase modified platinum micro-cavity electrodes are fabricated for detection of plasma membrane cholesterol efflux. The ring end of the glass capillary, extending beyond the enzyme-modified surface of the recessed platinum electrode, is placed in contact with the plasma membrane. This geometry creates a cylindrical compartment isolated from the rest of the solution, into which cholesterol efflux from the membrane occurs and is detected through oxidation of accumulated hydrogen peroxide. The rate of enzymatic consumption is decreased at the recessed electrode surface compared with that at the disk electrode. This decrease results in a decreased rate of unidirectional efflux of cholesterol from the plasma membrane to solution in the cavity. Thus depletion of plasma membrane cholesterol at the efflux site is minimized.
1.2 Chemical Potential of Cholesterol in the Plasma Membrane

The rate of cholesterol efflux from the cell plasma membrane is believed to be dependent on the chemical potential of membrane cholesterol as predicted by classic kinetic theory. A better understanding of the chemical potential of membrane cholesterol has significance for the study of asymmetric cholesterol distribution between cellular compartments. Three conceptual models, including the condensed complex model, the superlattice model, and the umbrella model, have been proposed that all have different predictions for the chemical potential of cholesterol in lipid membranes (Figure 1-2).

The condensed complex model was initially proposed by McConnell based on their study of cholesterol efflux from a lipid monolayer at the air-water interface. In this model, low free-energy stoichiometric cholesterol-lipid complexes (molecular ratio 1:2) are hypothesized to exist in the plasma membrane. At this stoichiometric composition, a sharp jump in cholesterol chemical potential is calculated based on the observation of the change in surface tension of lipid membrane and on the observed efflux rate. For membranes with cholesterol content below the 1:2 composition, most of cholesterol is complexed and efflux is slow. Increasing the membrane cholesterol content above the 0.5 cholesterol/phospholipid molar ratio leads to the formation of a high free-energy cholesterol pool that is not complexed with phospholipid. It has also been suggested that a cholesterol superlattice and lipid rafts are examples of the proposed condensed complexes. Our previous electrochemical experiments on giant vesicles revealed that the electrochemical response to cholesterol
Figure 1-2. Models for the chemical potential of cholesterol as function of cholesterol content in a lipid membrane.\(^{15}\) A. The condensed complex model predicts a jump in cholesterol content at a stoichiometric composition. B. The superlattice model predicts dips in free energy at superlattice compositions, which also implied sharp spikes in chemical potential. C. The umbrella model predicts a cascade of jump in chemical potential, and each jump corresponds to a stable cholesterol distribution.
in the membrane increased sharply only when the cholesterol content was greater than 0.5 molar ratio of cholesterol/phospholipid. These data support the existence of a condensed cholesterol-phospholipid complex.

The superlattice model was proposed to explain drops in fluorescence intensity of dehydroergosterol (DHE) in liquid crystalline dimyristoyl phosphatidylcholine (DPPC) bilayers at specific cholesterol mole fractions. The phenomenon is consistent with the presence of a hexagonal superlattice in the fluid membranes. This model suggests that the difference between the cross-sectional area of cholesterol and other lipid molecules can cause long-range repulsive forces between cholesterol molecules that maintain superlattice distributions. At the cholesterol mole fractions where superlattices form, the free energy decreases. Because chemical potential is the derivative of the free-energy, this model implies that the chemical potential should have sharp spikes for mole fractions that form superlattices.

The umbrella model suggests that the presence of the small polar hydroxyl group and the larger nonpolar body of the cholesterol molecule results in preferential association of cholesterol with other membrane molecules. In a bilayer, the small polar hydroxyl group of cholesterol is covered by the larger polar headgroups of the neighboring phospholipids. Because it costs much more free energy to cover a cholesterol cluster than a single cholesterol molecule, cholesterol molecules have a strong tendency not to cluster or at least not to form large clusters. The simulations based on this interaction showed that cholesterol can form a ‘‘hexagonal’’ pattern at a cholesterol mole fraction of 0.50, a ‘‘dimer’’ pattern at 0.571, and a ‘‘maze’’ pattern at
The simulation also showed that the formation of a stably regular distribution is always accompanied by a jump in chemical potential.\\(^{19}\)

These three models that have different predictions for the chemical potential of cholesterol in lipid membranes are based on different experimental measurements. All the methods can be applied for the study of lipid monolayers or bilayers but are not suitable for cell plasma membranes. Recently, the estimation of chemical potential of cholesterol in the cell plasma membrane has been obtained by exposing cells to cholesterol oxidase solution.\\(^{15,20}\) 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. The overall problem is that a kinetic measurement is used to infer information about the thermodynamic state prior to initiation of the cholesterol efflux.

The electrochemical measurement of plasma membrane cholesterol using oxidase modified disk electrodes assumes there is a thin aqueous hydration layer between the electrode surface and the plasma membrane.\\(^{20-25}\) The electrode consumes the aqueous phase cholesterol between the electrode surface and the plasma membrane that has already effluxed from the plasma membrane. The early results suggest that the measured electrochemical response correlates with chemical potential of cholesterol in the cellular plasma membrane without making significant interruption of membrane cholesterol distribution. However, the further application of this methodology is limited by the varied current densities with electrode areas, oxidase enzyme activity or loading on the electrode. No effective standard calibration membrane is available, and
it is impossible to achieve quantitative information of chemical potential of cholesterol in the plasma membrane with this method (or any other method so far).

DHE, a cholesterol analog, has been introduced into the cell plasma membrane in the absence of receptors. It is loaded in the plasma membrane by exposing the cell to a saturated DHE solution. In chapter 3, when the cell is cultured in media, the equilibrium of cholesterol influx and efflux to and from plasma membrane is achieved. At equilibrium, the chemical potential of cholesterol in the plasma membrane is equal to that of solution cholesterol chemical potential. Increasing the concentration of cholesterol in solution above the equilibrium value loads cholesterol into the plasma membrane, which increases the chemical potential of membrane cholesterol so that the efflux rate of cholesterol increases. The enhanced efflux rate gives a larger current response in the electrochemical experiments.

Experimentally, the cell is exposed to a series of cholesterol solutions ranging from zero to saturated concentration. A gradual increase in current response is observed for cholesterol concentrations above 875 nM cholesterol. Constant current responses are obtained when the cholesterol concentrations are below this value. The endpoint of titration is independent of the electrode activity and is proposed to related to the chemical potential of membrane cholesterol. A shift of the endpoint to lower solution concentration is observed when the cell is incubated in cyclodextrin solution overnight to remove cellular cholesterol. This experimental result provides proof of concept for the titration assay for tracking the chemical potential of membrane cholesterol.
1.3 Cholesterol Trafficking in Cystic Fibrosis (CF)

CF is an autosomal recessive monogenic disorder affecting the exocrine (mucous) glands of the lungs, liver, pancreas, and intestines, causing progressive disability due to multisystem failure. The main hallmarks of cystic fibrosis are salty tasting skin, appetite but poor growth and weight gain, excess mucus production, and coughing and shortness of breath. Often, symptoms of CF appear in infancy and childhood. Cystic fibrosis is one of the most common life-shortening genetic diseases. In the United States, 1 in 4,000 children are born with CF. It is most common among western European populations; one in twenty-two people of Mediterranean descent is a carrier of one gene for CF, making it the most common genetic disease in these populations.

CF is caused by the lost function of the cAMP-dependent chloride channel cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is known to be correctly localized at the apical membrane of epithelial cells. CFTR directly regulates other ion channels, such as the outwarding 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. 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. The experimental results suggest that CF epithelial cells posses an inherent flaw in cholesterol regulation due to the loss of CFTR activity or expression, which results in 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. The full identification of the relationship between cholesterol accumulation and CFTR function will help determine if our measurements of membrane cholesterol can be utilized as a biomarker of CF.

ABCA1 is known to mediate cholesterol transport across the plasma membrane to high-density lipoprotein (HDL). Although the structure of CFTR is similar to that of ABCA1, there is evidence that CFTR is incapable of directly modulating cholesterol movement\(^{32}\). In chapter 4, CFTR function is shown to correlate with plasma membrane cholesterol content and with measurements of de novo cholesterol synthesis. Data demonstrate a clear \(Cftr\) genotype correlation with \(\Delta F508\) CFTR mice exhibiting higher membrane cholesterol content and increased de novo cholesterol synthesis relative to R117H CFTR mice. Acute inhibition of CFTR function results in a significant depletion of membrane cholesterol. However, prolonged inhibition of CFTR leads to a reversal of this phenotype, suggesting that a secondary response is responsible for the observed increase in membrane cholesterol in CF cells and tissues. Nasal epithelium from \(Cftr\ +/-\) mice exhibit a slight but statistically significant drop in membrane cholesterol content compared to sibling \(Cftr\ +/+\) mice. These data suggest that being heterozygous for CFTR mutation is sufficient to prevent any feedback responses associated with the regulation of cholesterol processing. Taken together, these results demonstrate that membrane cholesterol content is a sensitive marker to indirectly assess CFTR function and likely a direct assessment of cellular responses to changes in CFTR function. The ability to detect these secondary-signaling events may be a way to answer the elusive question of how much CFTR function is enough.
1.4 References


CHAPTER 2: OBSERVATION OF CELLULAR CHOLESTEROL EFFLUX
AT MICRO-CAVITY ELECTRODES

2.1 Introduction

A fundamental uncertainty in cellular cholesterol homeostasis is the role of aqueous diffusion in cholesterol mass transport between hydrophobic lipid membranes such as the cell plasma membrane and lipoprotein in extra-cellular space.\textsuperscript{1-5} Cholesterol transport from cells can also occur through specific receptor-mediated processes, such as ATP-binding cassette transporter A1 (ABCA1), scavenger receptor class B type I (SR-BI), and ABCG1.\textsuperscript{1-5} The poor understanding of the contribution of aqueous diffusion to cholesterol efflux is hampered by the low solubility of cholesterol in aqueous solution.\textsuperscript{6} This missing information has resulted in an unclear understanding of the significance of cholesterol mass transfer by aqueous diffusion to the kinetics of specific biochemical (or active) cholesterol trafficking pathways. This chapter reports cholesterol oxidase modified platinum micro-cavity electrodes for detection of plasma membrane cholesterol efflux. The data are consistent with the notion that cholesterol in the cell plasma membrane undergoes exchange with cholesterol in solution (as discussed in chapter 3). Additionally, the micro-cavity electrode geometry results in slow efflux relative to the expected rate of intracellular cholesterol movement to the plasma membrane. Thus, the depletion of plasma membrane cholesterol at the efflux site is minimized.
The experimental arrangement employed for the micro-cavity measurements\textsuperscript{7-10} involves placing the ring end of the glass capillary, extending beyond the enzyme-modified surface of the recessed platinum electrode, in contact with the plasma membrane (Figure 2-1).\textsuperscript{11} This geometry creates a cylindrical compartment ca. 0.4 pL in volume, isolated from the rest of the solution, into which cholesterol efflux from the membrane occurs and is detected through oxidation of accumulated hydrogen peroxide.

Enzymatic consumption of cholesterol at the recessed electrode surface results in a decreased rate of unidirectional influx of cholesterol from solution in the cavity to the plasma membrane. Net efflux of cholesterol from the plasma membrane to solution in the cavity is, thus, driven by enzymatic consumption of cholesterol at the electrode surface.

\subsection*{2.2 Experimental Methods}

\textit{Cavity platinum microelectrode fabrication.}

The fabrication of the 10 µm diameter platinum capillary disk electrodes has been described earlier.\textsuperscript{12, 13} Platinum micro-cavity electrodes (10 µm diameter) were fabricated by electrochemically etching the disk electrodes.\textsuperscript{14} The etching procedure used a 15\% CaCl\textsubscript{2} aqueous solution. The etching voltage perturbation was a sinusoidal waveform (2.5 V peak to peak) of 60 Hz applied between the Pt wire and a counter electrode (large area Pt electrode). After etching, the electrode was sonicated
Figure 2-1. Scheme of micro-cavity electrode positioned in contact with the cell plasma membrane. The average molecular area of cholesterol in the plasma membrane is estimated at 40 Å². ¹¹
in ethanol and water for 10 s to remove some remnant platinum in the cavity. Etching of a 5 µm deep cavity was achieved in 10 s and verified under optical magnification (Figure 2-2A). Cyclic voltammetry in ferrocyanide (5 mM, 100 mM phosphate buffer, pH 7.4) was used to check the faradaic current of cavity electrode and only ~ 40 % of current should be observed,\textsuperscript{15} compared with that on disk electrode (Figure 2-2B).

**Electrode modification**

Immediately after etching and characterization, the micro-cavity electrodes were immersed in a 5 mM hexane solution of 11-mercaptoundecanoic acid (Aldrich Chem. Co.) for 4 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 5 mM N-hydroxysulfosuccinimide (NHS) (Fisher) for 30 min for conversion of the carboxylic acid end groups of the sub-monolayer to an NHS ester.\textsuperscript{16} This activated surface was immersed in a 1 mg/mL solution of recombinant cholesterol oxidase (Wako Chemicals USA, Inc., ca. 33.0 units/mg) overnight for covalent attachment of the enzyme to the electrode surface. The NHS esters of the sub-monolayer serve as leaving groups when amide bonds form with lysine residues on the surface of the enzyme. The partial modification was qualitatively indicated by ferrocyanide voltammetry (Figure 2-3).
Figure 2-2. A. Photograph under optical magnification of the 10 μm diameter micro-cavity electrode with a 5 μm cavity depth. B. Cyclic voltammetry of ferrocyanide (5 mM, 100 mM phosphate buffer, pH 7.4) at a bare Pt disk microelectrode and after etching to form the micro-cavity electrode. Scan rate: 100 mV/s.
Figure 2-3. Characterization of electrode modification. Cyclic voltammetry of ferrocyanide (5 mM, 100 mM phosphate buffer, pH 7.4) at a bare micro-cavity electrode, after modification with a sub-monolayer of 11-mercaptoundecanoic acid, and after covalent attachment of cholesterol oxidase to the sub-monolayer. Scan rate: 1000 mV/s. The decrease in limiting current after functionalization of the electrode surface with 11-mercaptoundecanoic acid and oxidase is consistent with partial deposition of a sub-monolayer of enzyme that impedes reaction of ferrocyanide at the electrode surface.
Electrodes were sonicated for 10 seconds after enzyme modification to remove enzyme that may be adsorbed to the glass wall of the micro-cavity.

**Preparation of cholesterol solution and detection**

Cholesterol was dissolved in chloroform and dried under nitrogen before preparing the solutions of the 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 were collected in quiet buffer after spiking aliquots of cholesterol solution.

**Data Acquisitions**

The chrono-coulometric analysis was conducted using a two-electrode cell and a voltammeter-amperometer (Chem-Clamp, Dagan Corp.). Analog coulometry was collected using a built-in charge integrator. The integration was calculated using the following equations.

\[
\frac{\delta}{t_{in}} = \frac{V_{in}}{R} \quad (1)
\]

\[
V_{out} = \frac{1}{RC} \int V_{in} \, dt \quad (2)
\]

\[
\frac{\delta}{t_{out}} = C \frac{dV_{out}}{dt} = i_{in} \quad (3)
\]

The circuit is shown in Figure 2-4. The three-pole Bessel filter of the Chem-Clamp was set to 100 Hz. An Ag/AgCl (1 M KCl) reference electrode was used for all experiments and series of two potential steps were applied to the electrode.
Figure 2-4. Circuit for charge integration.
Single cell from Aplysia californica

Aplysia californica weighing 200-450 g (Marinus Scientific, Garden Grove, CA) were maintained in an aerated aquarium containing artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) kept at 18 ± 1 °C. To get single cells, animals were anaesthetized and the buccal ganglia were dissected out. The buccal ganglia were pinned caudal side up in Aplysia saline. Ganglia were desheathed to expose the cell bodies of the neurons. The whole process is shown in Figure 2-5.

For electrochemical analysis of plasma membrane cholesterol efflux, the cells are kept in an isotonic saline (460 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM glucose, 27 mM MgCl₂, 27 mM MgSO₄, and 10 mM CaCl₂; pH 7.6) at room temperature during the experiments.

2.3 Results and Discussion

Significant measurement error caused by single potential step method

Initial characterization experiments for detection of cholesterol solution using the micro-cavity electrode were conducted by a single potential step method with analog integration. With the electrode in buffer containing no cholesterol, sequential potential step experiments were performed at 5-min intervals. The charge passed for replicate potential step experiments decreases and this background
Figure 2-5. A. The photograph of *Aplysia Californica*. B. The pinned buccal ganglia in *Aplysia* saline. C. The ganglia are desheathed to expose the neuron cells. D. The micro-cavity electrode is in contact with a neuron cell.
drift varies between different electrodes and can be pronounced as shown in Figure 2-6 (Buffer: points 1-4). Injection of cholesterol solution to bring the bulk concentration to 100 μM shows an increase in charge (Figure 2-6, difference between Buffer, point 4 and 100 μM Cholesterol solution, point 1). Background drift varies between different electrodes and over time at a single electrode. This method has significant error. However, the charge increases from point 4 in buffer to point 1 in cholesterol solution on different electrodes which suggests that there is variation in enzymatic activity exhibited between different electrodes. Table 2-1 shows the charge at 1 s for seven electrodes in buffer (point 4 in buffer, estimated as double layer charge), the increase in charge at 1 s for the electrodes in 100 μM cholesterol solution, and the % relative charge increase for the electrodes in 100 μM cholesterol solution (charge increase/double layer charge × 100%). These data (Figure 2-7) suggest a correlation between electrode surface area and enzymatic activity. Electrodes with higher surface area (indicated by larger capacitive charge) allow immobilization of more enzyme molecules on the electrode surface and the electrodes exhibit higher enzymatic activity. No accumulation of peroxide could be created on a disk microelectrode for the radial diffusion and no response could be observed, as shown in Figure 2-8.

To address the error of background drift variation, a background subtraction method is employed for single cell analysis, as discussed in what follows.
Figure 2-6. Consecutive potential step experiments (200 mV to 600 mV) with a 5 min hold time between experiments in buffer and buffered 100 μM Cholesterol solution. 1-4 is the chronological order.
Table 2-1. Charge at 1 s for seven electrodes in buffer, the increase in charge at 1 s for the electrodes in 100 μM cholesterol solution, and the % relative charge increase.

<table>
<thead>
<tr>
<th></th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
<th>E4</th>
<th>E5</th>
<th>E6</th>
<th>E7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charge Increase (pC)</td>
<td>23</td>
<td>15</td>
<td>16</td>
<td>89</td>
<td>18</td>
<td>93</td>
<td>45</td>
</tr>
<tr>
<td>Background Charge (pC)</td>
<td>687</td>
<td>802</td>
<td>656</td>
<td>2782</td>
<td>996</td>
<td>2906</td>
<td>2043</td>
</tr>
<tr>
<td>Relative Increase (%)</td>
<td>3.3</td>
<td>1.9</td>
<td>2.4</td>
<td>3.1</td>
<td>1.8</td>
<td>3.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>
Figure 2-7. The relationship of the charge increase and double layer charge when the electrode is exposed to 100 µM cholesterol. The linear fit includes (0, 0).
Figure 2-8. Background subtracted analog coulometry collected for oxidase modified disk electrode in 1 mM cholesterol solution.
**Single cell analysis using two sequential potential step method**

Placing the micro-cavity electrode in contact with the cell surface results in about a 10% decrease in the charge passed for the potential step. The data in Figure 2-9 indicate that the faradaic background is smaller when the electrode is in contact with the cell (compare the slopes of the charge traces between 1 and 4 seconds) and decreased electrode capacitance may also occur.

Two sequential potential step experiments are performed for analog chronocoulometric analysis of the accumulated hydrogen peroxide (Figure 2-10). The first potential step (Figure 2-10A, step 1) is from 200 mV (the hydrogen peroxide collection potential) to 600 mV for complete mass transfer controlled oxidation of the hydrogen peroxide confined in the cavity volume (Figure 2-10B; trace labeled step 1). The potential is stepped back to 200 mV for 30 s (to allow the current to reach a constant value so that the integrator can be reset) and a replicate of the first potential step is conducted (Figure 2-10A, step 2) to gauge the background charge (Figure 2-10B, trace labeled step 2). Subtraction of the background charge measured in the second potential step from the charge past in the first potential step is an estimate of the accumulated hydrogen peroxide in the cavity volume from presumably steady-state enzymatic oxidation of cholesterol during the hold time (Figure 2-11A, trace labeled Enzyme modified). The background charge can drift over minutes and this sequential potential step method allows the background to be continuously tracked and subtracted to give charge from hydrogen peroxide oxidation. Additionally, data acquisition does not require physical movement (repositioning) of the electrode.
Figure 2-9 Analog coulometry collected for an enzyme-modified micro-cavity electrode before (1, 2) and during (3) contact with the cell.
Figure 2-10. A. Applied voltage; B. Overlaid charge for each step to 600 mV.
Figure 2-11. A. Difference charge for enzyme-modified electrode and bare electrode at the cell (1, 2, 3: chronological order); B. Three determinations of the difference charge at the cell and cyclodextrin (500 μM; 12 hours) treated cell using the enzyme-modified electrode and bare electrode at 1 second.
The background charge measured in step 2 must contain a contribution from hydrogen peroxide being generated during the 30 s quiet time between the first and second potential step experiments as enzymatic oxidation of cholesterol is continuous. However, the electrode is held with the cell with one minute and no charge increase could be observed, as shown in Figure 2-12. If the 30 s hold time between the two steps could create measurable peroxide in the cavity, this 1 min hold time before step 1 and then 30 s quiet time should give a similar charge change after the subtraction. Also, the rate at which hydrogen peroxide escapes from the cavity is uncertain and is assumed to be a systematic error.

In addition, the difference charge at one second reflects faradaic reactions of other species. This faradaic background charge is estimated using the bare platinum micro-cavity electrode (electrode prior to enzyme modification) positioned in contact with the same cell (Figure 2-11A, trace labeled Bare). Within one second, the difference charge from the enzyme-modified electrode reaches a maximum value relative to the difference charge from the bare cavity electrode (Figure 2-11A, 1-3 s). Larger difference charge for the enzyme-modified electrode is assigned to hydrogen peroxide oxidation. Hydrogen peroxide oxidation is driven at a mass transfer controlled rate. The controlled rate occurs at 600 mV and the cavity volume is quickly depleted. Enzyme-modified and bare electrode difference charge traces are compared for analysis at 1 s as the background subtraction can introduce noise at short time. Comparing averaged data from three sequential and replicate experiments conducted at the bare and enzyme modified electrode surfaces suggests that about 75% of the
Figure 2-12. Background subtracted analog coulometry collected for oxidase modified disk electrode in 1000 μM cholesterol solution after one min holding time.
The difference charge past at the enzyme-modified electrode at 1 s (Figure 2-11B, bars 1 and 2, respectively) is from oxidation of hydrogen peroxide. The average efflux rate (lower limit) is calculated to be 2 pmol/cm² s, where the efflux site (contact area of the cavity mouth: ca. 80 μm²) is 0.25% of the total area of the cell surface.

The distance between the cell surface and the electrode surface (i.e., the 5 μm cavity depth) is one factor controlling the cholesterol concentration gradient at the cell surface and the resulting rate of cholesterol efflux. The other major factor controlling the efflux rate is the rate of enzymatic consumption of cholesterol at the recessed platinum electrode surface. The measurements are therefore taken as a qualitative estimate of plasma membrane cholesterol concentration. The total estimated amount of cholesterol extracted from the cell over 5 min. is about 400 amol; 0.25 times the amount contained at the contact site (plasma membrane outer leaflet covering the cavity mouth)¹⁹ and 0.06% of the estimated total cholesterol in plasma membrane outer leaflet.

The aqueous efflux model for the micro-cavity electrode measurements assumes no direct contact between the plasma membrane and the electrode surface. It is also assumed that no enzyme is immobilized on the glass walls of the cavity or at the perimeter of the cavity mouth. This issue and the consequences on the physical contact between the electrode tip and the cell surface are uncertain. The charge measurement is a step closer to probing the unperturbed homeostatic plasma membrane cholesterol content of natural living cells.
Cells partially depleted of cholesterol using cyclodextrin solution are studied to verify that the measurements are cholesterol dependent. After cholesterol depletion, the electrode showed a smaller difference charge indicating a decreased rate of cholesterol oxidation during the hold time (Figure 2-11B, bar 3) and, thus, slower efflux of cholesterol from the cell plasma membrane. Exposure of the cells to cyclodextrin solution with higher concentration results in an additional decrease in cholesterol efflux rate.

Figure 2-13 compares the micro-cavity electrode cell response (1: enzyme-modified) and control charge (2: no immobilized enzyme) for a relatively small capacitance (low surface area) electrode (also shown in Figure 2-10B) with the cell response (1: enzyme-modified) and control charge (2: no immobilized enzyme) for a relatively large capacitance electrode. For both electrodes, the estimated faradaic background contribution (2) to the signal (1) is 20-30%. Normalizing the data to double layer charge, as an estimate of enzymatic activity, shows that both electrodes exhibit a relative cell response of 17%.

The hydrogen peroxide concentration in the cavity volume may be as high as 1 mM assuming that all of the difference charge assigned to hydrogen peroxide (75%) is from hydrogen peroxide contained in the cavity volume. An action potential is observed on the cell after the potential step experiment and cyclodextrin experiment, as shown in Figure 2-14, which proves the cell viability.
Figure 2-13. A. Difference charge collected for enzyme-modified (1) and bare (2) micro-cavity electrodes with small capacitance (double layer charge of 748 pC at 1 second for the second potential step) and large capacitance (double layer charge of 2056 pC at 1 second for the second potential step). B. Relative charge increase before and after enzyme modification for both electrodes.
Figure 2-14. Action potential observed after the consecutive four detections of accumulated peroxide in the cavity.
Characterization of micro-cavity electrodes for enzymatic activity

Characterization of micro-cavity electrodes for enzymatic activity is conducted in cholesterol/Triton X-100 solution. Figure 2-15A shows the difference charge for an enzyme modified micro-cavity electrode in buffer and in 300 μM cholesterol solution. Three sequential determinations of the difference charge are shown overlaid for the buffer and cholesterol solution. Figure 2-15B is a bar graph including average data for 100, 300 and 600 μM cholesterol solutions.

Figure 2-16 shows control experiments indicating no response to cholesterol solution at bare micro-cavity electrodes containing no immobilized enzyme. This electrode exhibited a relatively large apparent surface area (double layer charge) and thus the faradaic background is also relatively large.

While the solution data provide a measure of enzymatic activity, it is not possible to directly compare the solution data with data collected at the cell surface. For the cell experiments, the plasma membrane likely acts as a barrier slowing escape of hydrogen peroxide from the cavity volume. For the solution experiments, the mouth of the cavity is open and hydrogen peroxide can readily escape. Additionally, for the solution experiments, the cholesterol concentration gradient at the electrode surface extends beyond the cavity mouth into solution. For the cell experiments, the plasma membrane gates cholesterol mass transport to the cavity volume and, therefore, it is proposed that the cavity depth largely dictates the thickness of the diffusion layer.
Figure 2-15. A. Difference charge for enzyme-modified electrode in 300 μM cholesterol and buffer solution. B. Dependence of average (n = 3) difference charge (1 s) at the enzyme-modified electrode on cholesterol concentration.
Figure 2-16. Data for consecutive difference charge determinations at a bare micro-cavity electrode.
A consequence of the cavity electrode geometry and the defined diffusion distance between the electrode surface and the plasma membrane is that cholesterol efflux occurs at a relatively slower rate compared to disk electrode measurements conducted in this laboratory, as shown in Figure 2-17 (ca. 1.6 times slower). Slower efflux at the micro-cavity electrode, while maintaining the small size of the efflux site, leads to a measurement that is expected to be more reflective of the efflux step and to be less influenced by depletion of cholesterol at the electrode contact site.

The degree to which the micro-cavity measurement locally depletes plasma membrane cholesterol is of concern regarding the relevance of the measured efflux rate to the unidirectional efflux rate occurring at pseudo-equilibrium. The literature regarding cholesterol efflux from cells and model lipid membranes is consistent with the existence of two pools of plasma membrane cholesterol. Most cholesterol molecules in the plasma membrane efflux slowly to cyclodextrin solution ($t_{1/2}$: ca. 20 min)\textsuperscript{20} and are likely associated with phospholipids.\textsuperscript{21, 22} A smaller amount of the cholesterol, however, effluxes rapidly to cyclodextrin solution ($t_{1/2}$: ca. 15 s)\textsuperscript{20} and likely reflects excess cholesterol above the stoichiometric amount that are able to complex by cell surface phospholipids.\textsuperscript{21, 22} This amount of “excess” cholesterol probably serves as a switch in cell signaling for cholesterol trafficking.\textsuperscript{21, 22}
Figure 2-17. Amperometric data for contacting (↑) a single cell with an enzyme modified Pt disk microelectrode and withdrawing (↓) the microelectrode from the contact position.
2.4 Acknowledgements

This work was supported by the Cystic Fibrosis Foundation and the Department of Chemistry, Case Western Reserve University. We thank Professor Barry Miller, Dan Scherson, and Ted Steck for helpful review of this manuscript. The author gratefully thanks Dechen Jiang, Hui Lu, Hillel J Chiel, Thomas J. Kelley for their contributions to this chapter.

2.5 References


CHAPTER 3: TITRATE THE CELL TO DETERMINE CHEMICAL POTENTIAL OF CHOLESTEROL IN THE PLASMA MEMBRANE

3.1 Introduction

Cholesterol is distributed unevenly between cellular organelles such as the plasma membrane and intracellular membranes.\(^1\)-\(^3\) This asymmetry is likely maintained, in part, by the interaction of cholesterol with other lipids in the membrane. This complication resulting from cholesterol lipid interactions has resulted in use of the term chemical potential in referring to membrane cholesterol. Three competing conceptual models, including the condensed complex model,\(^4\) the superlattice model,\(^5\) and the umbrella model,\(^6\) have different predictions for chemical potential of cholesterol in lipid membranes. This uncertainty is partially caused by the lack of methodology to determine the chemical potential of membrane cholesterol. The methodologies developed need either an solution acceptors (cyclodextrin\(^7\) or oxidase\(^8\)) to extract membrane cholesterol or fluorescent labeling of cholesterol,\(^9\) which changes membrane cholesterol content and thus chemical potential of membrane cholesterol. In addition, these methods have limited sensitivity for this application and thus, can not be applied to determine the chemical potential of cholesterol in individual cells. In this chapter, a titration method is developed where zero net efflux of cholesterol from a plasma
membrane occurs so that it becomes reasonable to infer information concerning the chemical potential of cholesterol in the plasma membrane of the cell.

Previously, we demonstrated detection of plasma membrane cholesterol using disk microelectrodes in direct contact with the cell plasma membrane. It is believed that cholesterol oxidase on the electrode consumes cholesterol that has effluxed from the plasma membrane. It is believed that this efflux rate is controlled by the chemical potential of cholesterol in the membrane. Increasing plasma membrane cholesterol content elevates the chemical potential of cholesterol and thus creates a higher cholesterol efflux rate, which gives a larger current response at the electrodes. Although this method can give information about chemical potential of cholesterol without significantly perturbing membrane cholesterol content, the current output varies with electrode area and oxidase activity or loading on the electrode. Because no effective standard calibration membrane is available, it is impossible to apply this method to achieve measurements of the chemical potential of cholesterol in the plasma membrane. Continuing to develop this method should focus on the measurement of the chemical potential without utilizing the absolute electrode responses.

When the cell is cultured in medium, the net flux of cholesterol from the plasma membrane to medium is zero. The chemical potential of solution cholesterol, which is determined by solution cholesterol concentration, should be equal to that of membrane cholesterol. Cholesterol has a limited solubility in water, perhaps around 1 μM, when determined using a fluorescent assay shown in Figure 3-1. The solution cholesterol
**Figure 3-1.** Fluorescent assay to determine cholesterol concentration in saturated solution at room temperature after stirring for three hours using the Amplex® Red cholesterol assay kit (Invitrogen). The produced hydrogen peroxide can react with Amplex® Red reagent with HRP to give fluorescence. Fluorescence is measured with a fluorescence microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm. The square points are collected using standard cholesterol solution. The value with the mark (×) is obtained from saturated cholesterol solution. (This experiment was performed by Richard West.)
concentration in cell medium can not be larger than this saturated value. If the solution cholesterol concentration increases, the elevated chemical potential of solution cholesterol should increase the cholesterol influx rate, and thus cholesterol is loaded into the plasma membrane. This increase in membrane cholesterol increases the measured cholesterol efflux rate (at the point of electrode contact), which gives a larger current response. The hypothesis is outlined in Figure 3-2. By titrating the cell with cholesterol solution with increasing concentrations, a gradual increase in current response is observed once a certain cholesterol concentration is reached. The endpoint of the titration, which appears just prior to the onset of the electrode response increase, corresponds to a zero net efflux condition. The solution cholesterol concentration at the endpoint thus give information about the chemical potential of cholesterol in plasma membrane.

3.2 Experimental Methods

Electrode fabrication and modification

The fabrication of the 100 μm diameter platinum capillary disk electrodes has been described earlier.12,13 The electrodes 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...
Figure 3-2. Models of cholesterol initial efflux and influx from/to the plasma membrane in A. culture medium, B. medium without cholesterol and C. medium with saturated cholesterol.
Chem. Co.) and 5mM N-hydroxysulfosuccinimide (NHS) (Fisher) for 30 min for conversion of the carboxylic acid end groups of the sub-monolayer to an NHS ester.\textsuperscript{14} This activated surface was immersed in a 1 mg/mL solution of recombinant cholesterol oxidase (Wako Chemicals USA, Inc., ca. 33.0 units/mg) overnight for covalent attachment of the enzyme to the electrode surface. The NHS esters of the sub-monolayer serve as leaving groups when amide bonds form with lysine residues on the surface of the enzyme. The modification was qualitatively indicated by cyclic voltammetry of ferrocyanide.

\textit{Preparation of cholesterol solution}

Cholesterol is dissolved in chloroform and dried under nitrogen before preparing the solutions of required concentration. The dried cholesterol is dissolved in an aliquot of saline buffer by stirring for 3 h at room temperature. The solution was filtered using 0.22 μm filter.

\textit{Single cell experiment}

The process to get single cells from \textit{Aplysia} has been described in Chapter 2. The electrode was positioned around 500 μm away from the cell to acquire baseline data. The electrode was repositioned so that it was in physical contact with single cell for cholesterol analysis. Electrode contact was defined as the force required for physically altering the shape of the cell as gauged visually under optical magnification.
3.3 Results and Discussion

*Evidences for the loading of cholesterol in membrane*

The hypothesis that cholesterol can be loaded to the cell directly from solution without lipoprotein as a is tested. To confirm this loading, a single neuron in the buccal ganglion of *Aplysia* is exposed to saturated cholesterol solution for half an hour (to reach equilibrium) and then contact is made with oxidase modified electrode, as shown in Figure 3-3. A 3-fold current response is observed in Figure 3-4, which indicates higher cholesterol content in the plasma membrane. To exclude the possibility that this current increase is contributed by concentrated cholesterol between the electrode and plasma membrane during the measurement, a cell stays in the medium for three days until no current response is observed during the electrode contact. At this point, an intact shape of the cell is kept. If the previous current increase is caused by concentrated cholesterol between plasma membrane and electrode, a similar current increase should be observed when this cell is exposed to saturated cholesterol solution. However, no current response is observed in Figure 3-5, which implies that the previous current increase should be contributed by increased cholesterol efflux from plasma membrane.
Figure 3-3. Picture of the 100 μ enzyme modified Pt electrode touching the cell
Figure 3-4. Three fold current response observed on the cell exposed to saturated cholesterol solution for 30 min.
Figure 3-5. No current responses are observed when the electrode touches the dead cell before and after the exposure to saturated cholesterol solution.
Also, the response of the electrode exposed to flow-injected saturated cholesterol is less than 1 pA (as shown in Figure 3-6), it is impossible that saturated cholesterol solution between the electrode and plasma membrane could create ~ 30 pA current increase. Although the mechanism of cholesterol loading into the plasma membrane is not clear, it is confirmed that concentrated cholesterol can be loaded to cell plasma membrane. A similar loading is also observed in a fluorescence study, which loads a fluorescent cholesterol analog, dehydroergosterol (DHE), into the cellular plasma membrane without lipoproteins.\textsuperscript{15}

\textit{Titrating the cell with cholesterol solution}

For the titration experiment, the cell is cultured in the medium for at least four hours and then contact is made with the electrode to get a current response. Then, the cell is exposed to cholesterol solutions ranging from zero to saturated concentration. At each concentration, the cell is cultured for half an hour before measurements are carried out with the electrode. Figure 3-7 shows that the current response starts to increase gradually when the cholesterol concentration is above 875 nM (87.5% of saturated concentration: assuming 1000 nM for the saturated solution). This increase suggests that the elevated extracellular chemical potential of cholesterol drives influx increasing the cholesterol content of the cell plasma membrane. The endpoint is estimated at 875 nM.
Figure 3-6. Current response of enzyme modified electrode exposed to saturated cholesterol solution. (↓) indicates the time for flow injection of saturated cholesterol.
Figure 3-7. Current responses of enzyme modified electrode in contact position with the cell cultured in different cholesterol concentrations.
Figure 3-8 using bare Pt electrodes containing no immobilized enzyme do not show any current increase in the titration process.

When the concentration of solution cholesterol is less than 875 nM, constant current responses are observed. These constant responses show that the small amount of cholesterol lost from the cell on reaching equilibrium does not change the chemical potential of membrane cholesterol as cholesterol is replenished to the membrane (the set point is maintained) by internal cholesterol stores and transport to the plasma membrane.

An aim is to develop a new methodology to exclude a dependence of the assay on the absolute current response of the electrodes. Different EDC and NHS concentrations are used to immobilize different amounts of cholesterol oxidase on three different electrodes to regulate electrode response, as shown in Figure 3-9. The same endpoints (875 nM) are observed for all three electrodes, which confirms that the endpoint is not dependent on the absolute electrode response.

Although cholesterol could be loaded into the plasma membrane, the amount of cholesterol loaded after half an hour is varied on the cell. Figure 3-10 shows the response ratio for 990 and 875 nM cholesterol solutions collected on seven cells.
Figure 3-8. Current responses of bare electrode in contact position with the cell cultured in different cholesterol concentrations.
**Figure 3-9.** Current responses of three enzyme modified disk electrode with different enzyme activity contacted with the cell cultured in the cholesterol solutions with different concentration.
Figure 3-10. Current response ratio at cholesterol concentration of 990 nM over 875 nM on seven cells.
These data reflect the increase in membrane cholesterol. The ratios range from 1.5 to 3, suggesting the existence of cell heterogeneity to physically loading cholesterol. Continuing to increase the loading time does not increase the current response suggesting that equilibrium has been reached.

Cells partially depleted of cholesterol by cyclodextrin (CD) solution have decrease membrane cholesterol content and thus we should observe a decrease in the chemical potential as reflected by the titration end point. To test our methodology for the determination of chemical potential of membrane cholesterol, cells are cultured in medium with 10 μM cyclodextrin overnight. Figure 3-11 shows the current responses obtained from three different electrodes for the titration process. The endpoint for cells with partially depleted cholesterol is 750 nM. This left-shift of the endpoint supports the principle that this methodology can be used to investigate the chemical potential of membrane cholesterol. A detailed kinetic study of cholesterol loading and the application of this method for cells in disease states is planned.
Figure 3-11. Current responses of three enzyme modified disk electrodes with different enzyme activity in contact position with the cell treated with cyclodextrin overnight cultured in different cholesterol concentrations.
3.4 Acknowledgements

This work was supported by the Cystic Fibrosis Foundation and the Department of Chemistry, Case Western Reserve University. The author gratefully thanks Dechen Jiang, Richard West, Hillel J Chiel, Thomas J. Kelley for their contributions to this chapter.

3.5 References


CHAPTER 4: CHOLESTEROL SYNTHESIS AND MEMBRANE

CHOLESTEROL CONTENT ARE INDIRECT BIOMARKERS OF CFTR FUNCTION

4.1 Introduction

Recent studies have identified alterations in cholesterol processing associated with cystic fibrosis (CF).\textsuperscript{1-4} The hypothesis of this study is that two aspects of altered cholesterol processing in CF cells, plasma membrane cholesterol content, and \textit{de novo} cholesterol synthesis will correlate with \textit{Cftr} genotype. Identifying this relationship between cholesterol measurements and CFTR function will help determine if these measurements can be used as biomarkers of CF. With the development of new therapies targeting CFTR function, new methods of identifying efficacy are needed that are reliable and non-invasive.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a-cAMP-activated chloride channel of the ATP binding cassette (ABC) family, the only ion channel within the ABC superfamily.\textsuperscript{5,6} A structurally similar protein within this family, ABCA1, is known to mediate cholesterol transport across the plasma membrane to high-density lipoprotein (HDL).\textsuperscript{7,8} The role of CFTR function in regulating cholesterol transport is unclear, particularly with respect to how membrane cholesterol content is regulated. It has been observed that cultured CF cells, as well as nasal and tracheal epithelium from CFTR null mice, exhibit a significant increase in
plasma membrane cholesterol content\(^3\). Although structurally similar to ABBA1, there is evidence according to Boujaoude et al that CFTR is incapable of directly modulating cholesterol movement.\(^9\) It is possible that elevated membrane cholesterol content in CF cells is due to the random diffusion of cholesterol accumulated in the perinuclear region to the plasma membrane for storage. If passive diffusion were the mechanism, then it would be expected that Niemann-Pick type C (NPC) cells would also exhibit this passive diffusion. However, evidence suggests that membrane cholesterol content is reduced in NPC cells, suggesting that NPC1 protein actively regulates cholesterol transfer between membranes.\(^10\)

Elevated membrane cholesterol content could have a number of influences related to CF characteristics. Several studies have demonstrated increased \(\Delta F508\) CFTR recycling, which is consistent with elevated membrane cholesterol content.\(^11, 12\) Lim et al have recently reported that membrane cholesterol depletion with methyl-\(\beta\)-cyclodextrin significantly increases \(\Delta F508\) CFTR retention.\(^13\) Also, the development of ceramide rich rafts has been demonstrated to be important to epithelial responses to \textit{Pseudomonas aeruginosa} and for the localization of CFTR after bacterial challenge.\(^14, 15\) Consistent with this model, recent reports demonstrate a CF-related deficiency in membrane ceramide content that is corrected by fenretinide treatment,\(^16-18\) although there are competing views on ceramide content in CF.\(^19\) Megha and London have shown that ceramide can displace cholesterol from membrane fractions.\(^20\) It is possible that ceramide deficiency fails to displace plasma membrane cholesterol in CF.
resulting in elevated cholesterol content and influencing inflammatory responses characteristic of CF lung disease.

Regardless of whether cholesterol processing changes observed in CF cells and tissues are directly involved in the pathology of CF, these cholesterol changes are potentially good, accessible indirect markers of CFTR function. The goal of this study is to determine if CFTR function correlates with plasma membrane cholesterol content and with measurements of de novo cholesterol synthesis. Data demonstrate a clear Cftr genotype correlation with ΔF508 CFTR mice exhibiting higher membrane cholesterol content and increased de novo cholesterol synthesis relative to R117H CFTR mice. R117H mice exhibit a slight response to forskolin in the nasal potential difference assay and near WT survival and no intestinal obstruction (personal communication, M. Drumm, CWRU). Surprisingly, it is found that acute inhibition of CFTR function results in a significant depletion of membrane cholesterol. However, prolonged inhibition of CFTR leads to a reversal of this phenotype suggesting a secondary response is responsible for the observed increase in membrane cholesterol in CF cells and tissues.

4.2 Experimental Methods

Cell culture

Human epithelium 9/HTEo- cells over expressing the CFTR R domain (pCEPR) and mock-transfected 9/HTEo- cells (pCEP), the wild type phenotype, were a generous
gift from the lab of Dr. Pamela B. Davis (Case Western Reserve University). Cells were cared for as previously described. Niemann-Pick fibroblasts (NPC) (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 at 37 °C in 95% O2 -5% CO2 on Falcon 10 cm diameter tissue culture dishes in MEM Eagle with 2 mM L-glutamine containing 15% fetal bovine serum (FBS) and 1 u/mL penicillin/streptomycin.

Mice

Mice homozygous for the ΔF508 CFTR mutation were described previously, as were mice carrying the R117H CFTR mutation. Mice heterozygous for CFTR expression (Cfrtm1Unc) were obtained from Jackson Laboratories (Bar Harbor, MA). All mice were provided by the CF Center animal core facility at Case Western Reserve University. CFTR wild-type mice were siblings of Cfr +/− mice. All mice were used between six and eight weeks of age and were back-crossed over ten generations onto a C57Bl/6 background. Mice were cared for in accordance with the Case Western Reserve University IACUC guidelines by the CF Animal Core Facility.

Electrochemical measurements of cholesterol

Platinum microelectrodes were fabricated in house (4 μm diamter wire for cell work and 100 μm diameter wire for tissue measurements, Goodfellow Corp.) as
described.\textsuperscript{24} Platinum wire was inserted into glass capillaries (Kimax-51, Kimble products) and placed inside a heated platinum coil. The glass was pulled to create a thin insulating layer on the platinum wire. The capillary microelectrodes were polished using a beveling machine (WPI, Inc.) to produce a disk electrode. The microelectrodes were immediately immersed in a 5 mM hexane solution of 11-mercaptoundecanoic acid (95%, Aldrich Chem. Co) for 2 h to form a carboxylic acid terminated monolayer on the electrode surface. Then, the microelectrodes were treated with 2 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma Chem. Co.) in 100 mM phosphate buffer solution (PBS) (pH 7.4) for 30 min. to activate the carboxyl groups to an acylisourea intermediate. The modified electrode was immersed in 1 mg/mL recombinant cholesterol oxidase (WAKO Chemical USA, Inc., 42.0 units/mg) solution for 3 h allowing this intermediate to react with amine immobilizing the enzyme on the electrode surface.

Amperometric measurements were conducted using a two-electrode cell and a voltammetry-amperimeter (Chem-Clamp, Dagan corp.). The three-pole bessel filter in the voltammetry-amperimeter was set to 100 Hz. The output was further processed using a noise-rejecting voltmeter (model 7310 DSP, Signal Recovery Inc.) to digitally filter 60-Hz noise. A Ag/AgCl (1 M KCl) reference electrode was used for all experiments, and the applied potential is 780 mV versus NHE for all experiments. All experiments were performed in 100 mM PBS (pH 7.4) at 36 °C.

Single cells and excised tissue were captured by a capillary prepared in house using an IM-6 microinjector (Narishige International USA, Inc.). The electrode was
initially positioned about 50 μm from the cell surface or tissue inner edge for acquisition of baseline data. The electrode was repositioned for contacting the biological sample and acquisition of electrode response.

4.3 Results and Discussion

Membrane cholesterol genotype comparison

Previous work has demonstrated that cultured cell models of CF and nasal and tracheal epithelium from Cftr -/- mice exhibit an increase in membrane cholesterol content.3, 25 In order to determine if membrane cholesterol content correlated with Cftr genotype, membrane cholesterol content was measured in nasal epithelium isolated from mice homozygous for either the R117H (R/R) or the ΔF508 (ΔF/ΔF) CFTR mutations. Membrane cholesterol content as measured by response ratio is elevated in both R/R and ΔF/ΔF nasal epithelium (1.64 ± 0.09 (R/R), p < 0.001; 2.14 ± 0.10 (ΔF/ΔF), p = 0.01) compared to age-matched sibling WT controls (Figure 4-1 and Figure 4-2). These data demonstrate that either a mild or severe disease-related CFTR mutation will result in an increase in membrane cholesterol, with a larger magnitude increase in the ΔF/ΔF tissue. The magnitude increase in membrane cholesterol content in ΔF/ΔF mouse nasal tissue is identical to what is observed in Cftr -/- nasal tissue.3 These data demonstrate that membrane cholesterol measurements can differentiate between genotypes.
Figure 4-1. Electrochemical determination of membrane cholesterol content from mouse nasal epithelium. A. Representative traces of membrane cholesterol determination in excised nasal epithelium from *Cftr R117H/R117H* (R/R) mice, compared to sibling *Cftr +/+* (wt) mice. B. Quantification of responses. Responses are reported relative to WT response (response ratio) to indicate the fold-increase in response. Error bars represent SEM, n = 4 for each. *p < 0.001.
Figure 4-2. Electrochemical determination of membrane cholesterol content from mouse nasal epithelium. A. Representative traces of membrane cholesterol determination in excised nasal epithelium from *Cftr* ΔF508/ΔF508 (ΔF/ΔF) mice, compared to sibling *Cftr* +/- (WT) mice. B. Quantification of responses. Responses are reported relative to WT response (response ratio) to indicate the fold-increase in response. Error bars represent SEM, n = 4 for each. *p < 0.001.
The effect of pharmacological inhibition of CFTR on membrane cholesterol content

Multiple CF models, both cultured and mouse, have consistently demonstrated an elevation in plasma membrane cholesterol content, and the above data indicate a genotype relationship. Since mouse nasal epithelium from ΔF/ΔF mice exhibit significantly larger membrane cholesterol content than R/R mice, the question of how CFTR function influences membrane cholesterol content was examined. 9/HTeo-epithelial cells were treated with the CFTR selective inhibitor CFTRinh-172 (20 μM) for 24 h and membrane cholesterol content measured electrochemically.26,27 CFTR inhibition under these conditions with this compound has been reported to reproduce cell regulation profiles associated with CF inflammation.28 Contrary to cellular and in vivo CF models, acute CFTR inhibition resulted in a dramatic reduction in membrane cholesterol content (Figure 4-3). This finding suggested that increased membrane cholesterol content in CF is actually a secondary response. To test this hypothesis, 9/HTeo- cells were exposed to CFTR_{inh}-172 (20 μM) continuously for 72 h being replenished with fresh drug every 24 h. Chronic CFTR inhibition results in a rebounding of membrane cholesterol that begins to exceed baseline levels, although not statistically significant at this time point (Figure 4-3). These data suggest that alterations in cholesterol processing in CF may be due to feedback mechanisms triggered by initially diminished membrane cholesterol content in response to lost CFTR function. However, membrane cholesterol does not significantly exceed baseline levels after 72 h. Pharmacological inhibition of CFTR with CFTR_{inh}-172 does not recapitulate the whole process of CF alterations in cholesterol processing and
Figure 4-3. Effect of acute (24 h) CFTR inhibition and chronic (72 h) CFTR inhibition with CFTRinh-172 (20 μM) on membrane cholesterol content. A. Representative traces of membrane cholesterol determination in 9/HTEo-cells after treatment with the CFTR inhibitor CFTRinh-172 for either 24 h or 72 h with fresh inhibitor placed on cells every 24 h or cells with no treatment (NT). B. Quantification of responses. Responses are reported relative to NT response (response ratio) to indicate the fold-difference in response. Error bars represent SEM, n = 4 for each. *p < 0.001.
the source of increased membrane cholesterol content still needs to be determined.

To assure that CFTRinh-172 was likely mediating the drop in membrane cholesterol via CFTR inhibition, two controls were performed. First, the influence of CFTRinh-172 on CF-model pCEPR 9/HTEo- cells was examined. The pCEPR cells are lacking CFTR function due to the over expression of the regulatory (R) domain and have been shown to exhibit the phenotype of increased membrane cholesterol content compared to wt controls.\textsuperscript{3,21} If the initial drop in membrane cholesterol content is due to CFTR inhibition, CFTRinh-172 should have no effect in pCEPR cells. Exposure of CF-model pCEPR cells to CFTRinh-172 (20\textmu M) for 24 h indeed has no impact on membrane cholesterol content (Figure 4-4). These data support the finding that the initial drop in membrane cholesterol is due to acute CFTR inhibition, and further suggest that the subsequent increase in membrane cholesterol content in CF cells is due to a secondary feedback response. A second control consisted of treating 9/HTEo-cells with an inactive analog of CFTRinh-172 (inactive-CFTRinh-172) to verify that some nonspecific drug interaction was not responsible for the decrease in membrane cholesterol. As shown in Figures 4-5, inactive-CFTRinh-172 had no influence on membrane cholesterol content. These data strongly support the findings that acute inhibition of CFTR function leads to decreased membrane cholesterol content.

\textit{Heterozygote effect}

The finding that acute CFTR inhibition leads to significant membrane cholesterol
Figure 4-4. Specificity of CFTR inhibition in regulating membrane cholesterol content.

A. Representative traces of membrane cholesterol determination in 9/HTEo- pCEPR (CF) cells after treatment with the CFTR inhibitor CFTRinh-172 for 24 h (red line) or cells with no treatment (NT, black line).

B. Quantification of responses. Responses are reported relative to NT response (response ratio) to indicate the fold-difference in response. Error bars represent SEM, n = 4 for each.
Figure 4-5. Specificity of CFTR inhibition in regulating membrane cholesterol content.

A. Representative traces of membrane cholesterol determination in 9/HTEo- pCEP (wt) cells after treatment with Inactive-CFTRinh-172 for 24 h (red line) or cells with no treatment (WT, black line).  
B. Quantification of responses. Responses are reported relative to WT response (response ratio) to indicate the fold difference in response. Error bars represent SEM, n = 4 for each.
depletion, coupled with the observations that both mild and severe CFTR mutations result in elevated membrane cholesterol content, prompted the examination of nasal epithelium isolated from *Cftr +/-* mice. The goal of the study was to determine if there is a CFTR dose effect with *Cftr +/-* mice having elevated membrane cholesterol intermediated between WT and CF models, or if there was actually a loss of membrane cholesterol. As shown in Figure 4-6, *Cftr +/-* nasal epithelium exhibits a relatively slight, but significant loss of membrane cholesterol (0.87 ± 0.04 fold compared to WT, p < 0.01). These data suggest that CFTR function impacts cholesterol movement to the plasma membrane. Loss of CFTR function actually leads to reduced membrane cholesterol content and that drop triggers a feedback response to replenish membrane cholesterol. The slight drop in membrane cholesterol content in *Cftr +/-* nasal tissue is likely insufficient to trigger the feedback mechanism.

*Increased cholesterol synthesis in two different mouse models of CF*

Another aspect of cholesterol processing found to be altered in CF mice is an increase in *de novo* cholesterol synthesis in *Cftr -/-* mice. This increase is a predicted consequence of decreased cholesterol reaching the ER due to perinuclear accumulation causing activation of sterol response element (SRE) binding proteins to increase intracellular cholesterol levels as indicated. The goal of this study is to determine if *de novo* cholesterol synthesis also correlates with *Cftr* genotype. Deuterium incorporation into cholesterol of specific tissue was determined by GC/MS analysis. Results reveal a 1.6 ± 0.2 fold (p = 0.009) increase in % new cholesterol
Figure 4-6. Comparison of nasal epithelium membrane cholesterol content in $Cftr^{+/+}$ and $Cftr^{+-}$ mice. A. Representative traces of membrane cholesterol determination in excised nasal epithelium from $Cftr^{+/+}$ (wt) mice and $Cftr^{+-}$ mice. B. Quantification of electrochemical membrane cholesterol determination. Responses are reported relative to WT response (response ratio) to indicate the fold-difference in response. Error bars represent SEM, n = 4 for each. *p = 0.008.
synthesis in the lung of ΔF/ΔF mouse compared to controls and a 1.6 ± 0.3 fold (p = 0.04) in the liver (Figure 4-7). However, there was no change in cholesterol synthesis in the small intestine. Similar results were found in the R/R mouse with a 1.2 ± 0.1 fold (p = 0.04) and 1.7 ± 0.2 fold (p = 0.008) respectively in the lung and liver. Small intestine of R/R mice showed a 1.2 ± 0.1 fold (p = 0.04) increase compared to matched controls (Figure 4-7). Increased cholesterol synthesis in two other CF mouse models validates our previous findings in Cftr -/- mouse tissue, and a more severe CFTR mutation correlates with greater increases in the rate of cholesterol synthesis in the lung, supporting the importance of CFTR function in regulating cholesterol synthesis. Why there is no significant difference between genotypes in liver and intestine regarding cholesterol synthesis is unclear. These tissues may have properties that lessen the relative importance of CFTR in influencing cholesterol synthesis.

Increased sterol response element (SRE) activation in response to CFTR inhibition

The above data suggest that CFTR function influences the regulation of de novo cholesterol synthesis. To test directly whether CFTR function impacts SRE activation, the effect of the CFTR inhibitor CFTRinh-172 on SRE activation was examined utilizing an SRE-luciferase construct. The construct contains SRE binding sites of the promoter region of HMG-CoA synthase, the rate-limiting enzyme regulating de novo cholesterol synthesis. 9/HTEo- cells were treated with 20 μM CFTRinh-172 for 24 hours and assayed for SRE responsiveness. Control epithelial cells treated with CFTRinh-172 showed a significant increase of 2.8 ± 0.3 fold
Figure 4-7. *De novo* cholesterol synthesis in CF mouse tissue compared to matched controls. Deuterium incorporation into newly synthesized tissue cholesterol was measured by GC/MS. Data are normalized to each tissue WT control and reported as fold increase of % newly synthesized cholesterol/8 h. Filled bars represent ΔF/ΔF mice and open bars represent R/R mice. The number of replicates is shown in parenthesis above each bar and represents individual assay on multiple tissue samples obtained over 3 experiments. *p < 0.05, #p < 0.01.
(p < 0.0001) above control levels (Figure 4-8). As a positive control, cells were treated with a known cholesterol transport inhibitor, U18666a (U18). Control cells treated with 5 μg/mL U18 also had an increase in SRE gene responsiveness of 2.6 ± 0.3 fold (p < 0.001) above control levels. A similar response in U18 and CFTRinh-172 treated cells indicate activation of selective pathways. These data are consistent with the in vivo data above that loss of CFTR function leads to an increase in de novo cholesterol synthesis.

\( ^{11}C \)-acetate incorporation in mouse models of CF

The ability to evaluate de novo cholesterol synthesis utilizing small animal positron emission tomography (microPET) scanning technology was assessed to determine if this phenotype could be monitored in a non-invasive manner. \( ^{11}C \)-acetate incorporation and imaging is widely used to monitor tumor progression due to increased lipid synthesis.\textsuperscript{29} Mice received 200 μCi of \( ^{11}C \)-acetate via tail vein injection. ΔF/ΔF and R/R mice were examined by this technique and compared to respective wild-type littermate controls to determine if alterations of cholesterol homeostasis could be detected using another method in live mice. Data are standard uptake values (SUV) within the lungs. These data are presented as a SUV ratio comparing CF mouse models and respective WT controls. As shown in Figure 4-9A, ΔF/ΔF mice exhibit increased \( ^{11}C \)-acetate uptake compared to sibling WT mice. However, R/R mice do not demonstrate any increased uptake compared to respective controls. To verify these results, lungs were excised from individual mice and percentage of injected dose
Figure 4-8. Increased SRE response in INH-172 treated control epithelial cells. 9/HTEo-pCEP (wt) cells were incubated in serum free conditions for 24 h with or without 20 µM CFTR inhibitor (INH-172) or 5 µg/mL U18666a (U18), a known cholesterol transport inhibitor, in serum free media for an additional 24 h. Data are normalized to serum free NT control levels over 3 experiments. Number (n) of samples is in parenthesis above each bar. Significance was determined by t test. Error bars represent SEM. * p < 0.0001.
Figure 4-9  A&B. $^{11}$C-acetate incorporation into the lungs of CF mouse models.  A. Ratio of the average SUV in the lungs of the R117H or ΔF508 mouse to the average SUV in the lungs of the WT control mouse. The number of pairs used is indicated in parentheses. The average SUV from both lungs 25 min after injection of [1-$^{11}$C]acetate was used in the calculations. B. Percentage of the injected dose normalized by the weight of the lungs as determined by well counting.
Figure 4-9 C&D. $^{11}$C-acetate incorporation into the lungs of CF mouse models. C. PET images of the R117H (left) and WT control (right) mice taken 25 minutes after injection of [1-$^{11}$C]acetate overlaid on the transmission images. The lungs are indicated by the arrows. D. PET images of the ΔF508 (left) and WT control (right) mice taken 25 minutes after injection of [1-$^{11}$C]acetate overlaid on the transmission images. The lungs are indicated by arrows.
normalized to tissue weight (% I.D./g) was analyzed. Again, no difference in R/R mice was observed compared to wt controls, while ΔF/ΔF mice exhibit a 1.7-fold increase compared to controls (Figure 4-9B). Representative PET scans are shown (Figure 4-9C, D). These data demonstrate a significant increase in acetate incorporation in ΔF/ΔF mice, but do not show a difference in R/R compared to WT controls as direct measurement of cholesterol synthesis shown above, suggesting that this assay is less sensitive. However, acetate is incorporated into several lipids, dampening the cholesterol specific signal. PET imaging of labeled acetate incorporation is a viable method to monitor cholesterol synthesis in vivo in relation to CFTR function, although sensitivity is lost.

Discussion on the role of CFTR in the cholesterol trafficking

Membrane cholesterol content is capable of modulating a variety of cellular functions including the regulation of ion channels such as ENaC and the formation of signaling complexes. In addition to intracellular perinuclear accumulation of free cholesterol, our previous work has identified a CF-related increase in membrane cholesterol content and an increase in de novo cholesterol synthesis. Excess cholesterol can be stored in membrane fractions and initially it was thought that excess membrane cholesterol content in CF cells was the result of passive diffusion of excess cholesterol to the membrane. Given the potential importance of chronic alterations in membrane cholesterol content and de novo synthesis, the goal of this chapter is to determine if CFTR genotype and function influence these outcome measures.
Regardless if cholesterol-processing alterations prove to be critical to CF pathogenesis, cholesterol measurements could prove to be important biomarkers for CFTR function.

Presented data demonstrate that membrane cholesterol content is particularly responsive to CFTR genotype/function. R/R mice exhibit a 1.6-fold increase in membrane cholesterol content in nasal epithelium compared to sibling WT mice, whereas, ΔF/ΔF mice exhibit a 2.1-fold increase. Interestingly, acute 24 h CFTR inhibition with CFTRinh-172 results in a decrease in membrane cholesterol content. However, 72 h exposure to the CFTR inhibitor (replenished every 24 h) demonstrates a rebound in cholesterol content suggesting a secondary cellular response to lost CFTR function is influencing membrane cholesterol regulation. The rebound effect does not lead to elevated membrane cholesterol content as seen in CF cells and tissues, indicating that the conditions tested here are not completely replicating the CF situation. The acute inhibition of CFTR function with CFTRinh-172 does have an in vivo correlate with membrane cholesterol content. Nasal epithelium from Cfr +/− mice exhibit a slight but statistically significant drop in membrane cholesterol content compared to sibling Cfr +/+ mice. These data suggest that being heterozygous for Cfr mutation is sufficient to prevent any feedback responses associated with the regulation of cholesterol processing. Taken together, these results demonstrate that membrane cholesterol content is a sensitive method to indirectly assess CFTR function and likely a direct assessment of cellular responses to changes in CFTR function.

In addition to membrane cholesterol content, de novo cholesterol synthesis also correlates to Cfr genotype. Direct measurement of cholesterol synthesis reveals that
lungs from R/R mice make significantly less cholesterol than ΔF/ΔF mouse lungs, although cholesterol synthesis in both genotypes is elevated compared to respective WT controls. As a more viable, non-invasive in vivo analysis, PET imaging of $^{11}$C-acetate incorporation into lung tissue was examined. Acetate incorporation was similar between lungs of R/R mice and sibling WT controls, whereas, ΔF/ΔF mice exhibit a significant increase compared to their controls. This imaging study is non-invasive, but is unable to examine cholesterol directly likely accounting for reduced sensitivity compared to direct cholesterol measurement. However, this technique is used in humans for tumor monitoring\textsuperscript{31} and should be capable of monitoring correction of ΔF508 CFTR by pharmacological means.

The conclusion of this study is that in vivo membrane cholesterol content and de novo cholesterol synthesis correlate with Cftr genotype and can be potentially exploited as a biomarker for CFTR function. Membrane cholesterol content is a particularly sensitive measure with its ability to distinguish between WT and Cftr heterozygous mice and would not be any more invasive than a nasal potential difference assay. In addition to merely being an indirect measure of CFTR function, measurement of cholesterol processing variations may be a measure of important cellular feedback responses to lost CFTR function. We recently reported that elevated cAMP-mediated signaling is a likely cause for perinuclear cholesterol accumulation.\textsuperscript{1} Whether the cAMP pathway is also responsible for cholesterol synthesis and membrane cholesterol content variations characteristic of CF is currently being explored. The ability to detect these secondary-signaling events may be a way to answer the elusive question of
how much CFTR function is enough. By measuring the cellular response to CFTR correction and not just transepithelial chloride transport, a stronger indication of how CF disease pathology could be influenced can be achieved.

4.4 Acknowledgements

This work is supported by a grant from the Cystic Fibrosis Foundation and by NIH/NHLBI grant HL080319. Technical support for this project was provided by core facilities of the cystic fibrosis center (P30 DK 27651). The authors thank Dr. Pam Davis (CWRU) for providing cell lines necessary for the completion of this study and to P.Bead for technical assistance. The author gratefully thanks Dechen Jiang, Mary Manson and Thomas J. Kelley for their contributions to this chapter.

4.5 References


13. Lim, C.H.; Bijvelds, M.J.; Nigg, A.; Schoonwerd, K.; Houtsmuller, A.B.; de Jonge, H.R.; Tilly, B.C. “Cholesterol depletion and genistein as tools to promote...


BIBLIOGRAPHY


