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Experimental Investigation of Cell Membrane Nano-mechanics and Plasma Membrane-Cytoskeletal Interactions Using Optical Tweezers

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Experimental Investigation of Cell Membrane Nano-mechanics and Plasma Membrane-Cytoskeletal Interactions Using Optical Tweezers

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Mechanical Engineering

by

Nima Khatibzadeh

September 2012

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Khatibzadeh also dedicates this dissertation to the memory of Mr. Ibrahim Shahroudi (1922-2011) who will remain an inspiration for pursuing knowledge.
ABSTRACT OF THE DISSERTATION

Experimental Investigation of Cell Membrane Nano-mechanics and Plasma Membrane-Cytoskeletal Interactions Using Optical Tweezers

by

Nima Khatibzadeh

Doctor of Philosophy, Graduate Program in Mechanical Engineering
University of California, Riverside, September 2012
Professor Bahman Anvari, Chairperson

The mechanical properties of the cell components, cell plasma membrane and cytoskeleton, as well as membrane-cytoskeleton associations, determine the mechanical properties of the whole cell which is important in cellular shape change behavior and mechanical signal transduction in living cells. Examples of biologically important processes involving cellular shape changes are deformation of erythrocytes in capillaries, cell division, phagocytosis, pseudopodium and dendritic spine formation, and electromotility of the outer hair cells.

This dissertation focuses on investigating the mechanical properties of the living cell plasma membrane and the local mechanical associations of the plasma membrane with the underlying cytoskeleton.

Mechanical properties of the living cell plasma membrane are investigated by forming membrane nanotubes (tethers) from human embryonic kidney cells using optical tweezers technique. In order to analyze the role of membrane composition
on its nano-mechanical properties, the membrane cholesterol content, the major lipid component of the plasma membrane, is manipulated and the obtained membrane mechanical properties are correlated to the membrane cholesterol content. The results reveal significant effects of membrane cholesterol in specific, and membrane composition in general, on membrane nano-mechanical properties. Specifically, decreases in membrane cholesterol content were associated with increased plasma membrane equilibrium force, plasma membrane tether stiffness and plasma membrane-cytoskeleton adhesion energy per unit area. Elevation of the membrane cholesterol content was followed by lower membrane tether equilibrium force, lower stiffness values, and lower membrane-cytoskeleton adhesion energy. The membrane bending modulus was almost unchanged upon membrane cholesterol manipulations.

In order to discern the role of cell cytoskeleton on membrane mechanical properties the experiments were repeated after F-actin disruption and in control cell and cholesterol manipulated cells. The disruption of F-actin filaments showed a noticeable impact on the membrane mechanical properties and diminished the observed disparity in membrane mechanical properties upon cholesterol depletion and cholesterol enrichment.

This dissertation also focuses on local cell deformations (protrusions). These deformations occur at an intermediate region between deformations at cell plasma membrane level and whole cell deformation, and are biologically important in formation of pseudopodia and filopodia, deformation of macrophages to engulf
particles, and the surface protrusions on the cells preceding formation of membrane tethers. We used a combined optical tweezers-fluorescent microscopy technique to study cellular protrusions in adherent living cells. The mechanical properties of the protrusions were analyzed by obtaining the associated force-length plots and a Maxwell viscoelastic model is used to fit the force-length plots. The experiments are performed on adherent human embryonic kidney cells, under cholesterol depleted and cholesterol enriched conditions to examine the effects of membrane cholesterol on protrusions. The experiments indicated greater maximum protrusion forces and shorter protrusion length under cholesterol depleted conditions in addition to greater values of the protrusion stiffness. Cholesterol enrichment experiments were associated with lower values of maximum protrusion force and protrusion stiffness, and formation of longer protrusions. The observations suggest a significant contribution of the cytoskeletal F-actin filaments on the observed mechanical properties of protrusion regardless of membrane cholesterol content.
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Chapter 1

1. Introduction

This chapter presents the motivation and objective of this thesis. An outline of this dissertation is presented in the second section.

1.1 Motivation and Objective

Living cells communicate with their external environment through physical and chemical processes that take place in their plasma membrane. The plasma membrane serves as a barrier for certain molecules and ions and acts as a residing environment in which membrane components carry out their specified functions. The plasma membrane plays an important role throughout the whole life cycle of mammalian cells and participates in the most critical cell functions including adhesion, motility, endocytosis, exocytosis, and signaling.

Cell membranes are composite nanostructures containing lipids, proteins, carbohydrates and other molecules. Our collaborator, Prof. William E. Brownell at Baylor College of medicine, has recently shown that high cholesterol levels result in
auditory dysfunction while low levels of cholesterol appear to improve performance in animal studies. In vivo cochlear function analysis revealed that cochlear cholesterol loading/depletion affected the production of distortion product otoacoustic emissions in mice. Cholesterol depletion was associated with dramatic increase in cochlear electromotility of guinea pigs. Electromotility originates within the plasma membrane of outer hair cells (OHCs) in the cochlea, and is responsible for sensitivity and frequency tuning of mammalian hearing. In parallel with the in-vivo studies, we demonstrated that changes in the membrane cholesterol content modulate the membrane associated charge movement in single OHCs. In response to membrane cholesterol depletion and cholesterol loading, the peak voltage of nonlinear membrane capacitance shifted to depolarized and hyperpolarized voltages, respectively, in both OHCs and human embryonic kidney (HEK) cells expressing the transmembrane protein prestin.

While the effects of cholesterol on membrane capacitive properties have been investigated, a comprehensive analysis is needed to find out the effects of cholesterol on membrane mechanical properties. In this thesis, various nano-mechanical properties of the cell plasma membrane are investigated for control cells. To find out the effects of cholesterol on membrane mechanical properties, the membrane cholesterol concentration is manipulated and the experiments were repeated under different cholesterol manipulation conditions, including cholesterol depletion and cholesterol enrichment.
1.2 Outline of Dissertation

This dissertation is organized as follows:

Chapter 2 provides a brief background on the experimental technique used for the studies involved in this dissertation. The chapter goes through different parts of the experimental setup, different calibration procedures and protocols for measurements. The chapter continues to introduce the actual tether pulling experiments from living cells. Plasma membrane tethers are pulled and various mechanical properties of the cell plasma membranes are quantified and correlated to the cholesterol concentration of the plasma membrane. The chapter involves the investigation of the role of cytoskeleton and membrane-cytoskeleton interactions on membrane mechanical properties.

Chapter 3 describes the formation of local cellular deformations (cell protrusions) of living cells. The chapter first explains the experimental approach used to study the mechanical properties of the cellular protrusions and then involves a systematic experimental approach to investigate the effects of membrane composition on the cellular protrusions. In addition to the effects of plasma membrane composition on protrusion, the effects of the cytoskeletal F-actin on the protrusions are investigated in control cells, cells with manipulated membrane cholesterol content, for cells with intact and disrupted F-actin.
Chapter 2

2. Effects of Cholesterol on Membrane Mechanics

In this chapter, we investigated the effects of membrane cholesterol content on the mechanical properties of cell membranes by using optical tweezers. We pulled membrane tethers from human embryonic kidney cells using single and multi-speed protocols, and obtained time-resolved tether forces. We quantified various mechanical characteristics including the tether formation force, tether equilibrium force, bending stiffness, effective membrane viscosity, and plasma membrane-cytoskeleton adhesion energy, and correlated them to the membrane cholesterol level. Decreases in cholesterol concentration were associated with increases in the tether formation force, tether equilibrium force, tether stiffness, and adhesion energy. Tether diameter and effective viscosity increased with increasing cholesterol levels. Disruption of cytoskeletal F-actin significantly changed the tether formation force and tether diameters in both non-cholesterol and cholesterol-manipulated cells, while the effective membrane viscosity was unaffected by F-actin.

The materials presented in this chapter are based on our previous publication: Khatibzadeh et al., Soft Mater 8:8350-8360 @Copyright Royal Society of Chemistry 2012.
disruption. The findings are relevant to inner ear function where cochlear amplification is altered by changes in membrane cholesterol content.

2.1 Background

Cell membranes are composite structures containing lipids, proteins, carbohydrates, and other molecules. Cholesterol is an abundant lipid component of the plasma membrane of eukaryotic cells, accounting for up to 50 mol% of membrane lipids.\(^1\)\(^-\)\(^2\) It modulates the activities of membrane associated proteins, such as the kinetics of current activation in voltage-gated chloride channel CIC-2,\(^3\) current intensity in inwardly rectifying K\(^+\) channels,\(^4\) and function of G-protein-coupled receptors.\(^5\) Cholesterol also affects membrane lipid organization,\(^1\)\(^-\)\(^6\) and distribution of transmembrane proteins such as prestin in outer hair cells within the cochlea.\(^7\) Cholesterol is also a key component of lipid rafts, and the related caveolae, which are involved in signal transduction, and endocytosis.\(^8\)\(^-\)\(^10\)

Numerous studies have been conducted to investigate the effects of cholesterol on both non-biological and biological membranes. In non-biological membranes, the number of saturated hydrocarbon chains in the lipids affects the membrane thickness and membrane bending modulus.\(^11\)\(^-\)\(^12\) Specifically, membrane bending modulus increases with added cholesterol in lipids with two saturated chains such as 1,2-dimyristoyl-sn-glycero-3-phosphocholine lipids.\(^13\) However, when both chains are monounsaturated as in 1,2-dioleoyl-sn-glycero-3-phosphocholine and
1,2-dierucoyl-sn-glycerol-3-phosphocholine lipids, the bending modulus remains unchanged for cholesterol mole fractions up to 0.4.\textsuperscript{13} In giant bilayer vesicles, the bilayer cohesion increases with added cholesterol only for lipids in which both chains are saturated or monosaturated. Specifically, the area expansion modulus of stearoyloleoylphosphatidylcholine type of lipids is reported to increase by six times with addition of cholesterol up to 58 mol\%\textsuperscript{14}.

In contrast to non-biological membranes, the presence of the cytoskeleton in living cells, gives rise to a composite plasma membrane-cytoskeleton structure which is absent in synthetic vesicles. The cytoskeleton provides the cell with internal organization, and mechanical rigidity.\textsuperscript{15} Plasma membrane mechanics as well as the mechanical association of membrane with cytoskeleton are important factors that influence cell shape. Examples of biologically important shape changes are deformation of erythrocytes in capillaries, electromotility of outer hair cells (OHCs), cell division, phagocytosis, pseudopodium and dendritic spine formation. Mechanical properties of the cell membrane may be altered by modulation of the plasma membrane cholesterol content. In vitro analysis of human neutrophils with elevated membrane cholesterol content showed increased probability of firm arrest during rolling over a P-selectin coated surface.\textsuperscript{16} Neutrophil-bead collision experiments conducted with higher membrane cholesterol levels resulted in greater cell deformation during the collision, increased probability of tether formation and longer tethers compared to controls. Similar experiments conducted under cholesterol depletion conditions decreased the probability of firm arrest and
formation of tethers, and produced shorter tethers. Studies on endothelial cells with micropipette aspiration technique showed that decreased membrane cholesterol resulted in a significant increase in the stiffness of the composite membrane-cytoskeleton in bovine aortic endothelial cells. However cholesterol enrichment had no significant effect on the stiffness. In another study on the same cells, membrane tether formation experiments indicated stronger membrane-cytoskeleton association in cholesterol depleted cells, and weaker associations upon cholesterol enrichment. Results of recent experiments, obtained by nano-indentation techniques, indicate a decrease in stiffness of the lamellipodium of the fixed NIH-3T3 fibroblast cells under cholesterol depleted conditions.

We have shown that high cholesterol levels result in auditory dysfunction while low levels of cholesterol appear to improve performance in animal studies. In vivo cochlear function analysis revealed that cochlear cholesterol loading/depletion affected the production of distortion product otoacoustic emissions in mice. Cholesterol depletion was associated with dramatic increase in cochlear electromotility of guinea pigs. Electromotility originates within the plasma membrane of OHCs in the cochlea, and is responsible for sensitivity and frequency tuning of mammalian hearing. In parallel with the in-vivo studies, we demonstrated that changes in the membrane cholesterol content modulate the membrane associated charge movement in single OHCs. In response to membrane cholesterol depletion and cholesterol loading, the peak voltage of nonlinear membrane capacitance shifted to depolarized and hyperpolarized voltages, respectively, in both
OHCs and human embryonic kidney (HEK) cells expressing the transmembrane protein prestin.22

In this study, we use optical tweezers to pull cell plasma membrane nanotubes (tethers) from HEK293 cells, and investigate the effects of membrane cholesterol on membrane mechanical properties by analyzing time-resolved tether force. Membrane tethers are formed by grasping a part of the cell surface and pulling it away from the cell, using optically trapped microspheres as handles.23-25 We use the HEK cell line as a model system for cholesterol manipulation, consistent with our previous work where these cells were used to investigate the effects of cholesterol on membrane capacitive properties.22 We also present results for F-actin disrupted cells to establish the role of F-actin on plasma membrane mechanical properties, and discern if mechanical properties of the membrane in F-actin disrupted cells are also affected by membrane cholesterol levels.

2.2 Materials and Methods

2.2.1 Cell Culture

HEK293 cells (ATCC, CRL-1573, Manassas, VA) were seeded in Dulbecco’s modified eagle medium (DMEM, Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY) and 1% Penicillin/Streptomycin (Gibco). Cells were incubated in an air jacketed CO₂ incubator (NuAire, Plymouth, MN) at 37°C with 5% CO₂. When the cells became 70-80% confluent, they were passaged into glass bottom poly-d-lysine coated MatTek plates (P35GC-1.0-14C). Cells of
medium size (population distribution: 10-20 µm in diameter) were selected for measurements if they were firmly attached to the bottom of the Petri dish. All experiments were performed within 30-45 minutes of removal of cells from the incubator.

2.2.2 Plasma membrane cholesterol manipulation

A commonly used method to modify the membrane cholesterol content is incubation of cells with cyclodextrins.26 Cells were incubated in DMEM containing 3 and 5 mM methyl-β-cyclodextrin (MβCD) (Sigma-Aldrich, St Louis, MO) for cholesterol depletion. We used water-soluble cholesterol with MβCD as a vehicle (51 mg of cholesterol per one gram of cholesterol-MβCD) (Sigma-Aldrich) for cholesterol enrichment. Specifically, cells were incubated in DMEM containing 3 and 5 mM MβCD in water-soluble cholesterol (cholesterol-MβCD) for cholesterol enrichment. Incubation time was 30 minutes at 37°C and 5% CO₂ in both cholesterol depletion and cholesterol enrichment experiments.7

2.2.3 Plasma membrane cholesterol quantification

We assumed the membrane cholesterol concentrations for each cholesterol modulation condition to be the same as those reported in our previous report.22 In that study, membrane cholesterol content for control and cholesterol manipulated HEK cells was quantified using the Amplex Red Cholesterol Assay. Briefly, this colorimetric assay is based on the reaction of cholesterol with cholesterol oxidase to yield H₂O₂, which can be detected using the Amplex Red reagent.
2.2.4 Cell cytoskeleton F-actin disruption

We used Latrunculin A dissolved in DMSO (EMD Chemicals, Gibbstown, NJ) as an F-actin disrupting reagent. F-actin disruption was performed after each type of cholesterol modulation and prior to tether pulling experiments. Specifically, cells were incubated in DMEM and then Latrunculin A solution was added to DMEM to a final concentration of 2 µM Latrunculin A. Cells were incubated with Latrunculin A for 15 minutes at 37ºC and 5% CO₂.

2.2.5 Optical tweezers, displacement measurements, and calibration

We used an infrared Nd:YVO₄ diode-pumped solid state laser (1064 nm, Prisma-1064-8-V, Coherent, Santa Clara, CA) to create the optical tweezers. The optical tweezers setup consisted of an inverted microscope (Nicon Eclipse Ti-DH, Melville, NY) containing a 100X oil immersion objective with a high numerical aperture (NA=1.49) (Nikon, Apo TIRF) through which the laser beam passed and converged to form an optical trap.

The laser beam was expanded prior to entering the microscope to fill the back aperture of the microscope objective. Figure 2-1 illustrates the schematic of the experimental setup in our experiments. We will go through the details of the system in the following steps of the thesis.

We have done a systematic measurement of the laser power and have shown the laser power at different positions in the optical patch in the system in Figure 2-2.
**Figure 2-1** System layout for tether formation experiments. Experimental setup including optical tweezers, position tracking and detection, and fluorescent imaging systems. The system components are: 1- Mirror, 2- dichroic mirror (transmitting light with $\lambda<1064$ nm), 3- filter set, $\lambda_{ex}=525-560$ nm, $\lambda_{em}=570-620$ nm (TRITC TE2000, Semrock), 4- mirror, 5-beam splitter, 6- focusing lens, 7-emission bandpass filter (605±25 nm), and 8-IR (infrared) filter.

**Figure 2-2** System optical characteristics. Power measurements are done at different points in the optical path of the system versus diode current of the laser, including laser power at the laser aperture exit, before the inlet port of the microscope, at the back aperture of the objective lens and the power exit at the tip of the objective lens.
We optically trapped 4 µm diameter sulfate-modified fluorescent polystyrene microspheres (beads) (F-8858, Molecular probes, Eugene, OR), and used them as handles to pull membrane tethers from cells. The excitation spectrum of the fluorescent beads is between 480 and 590 nm with maximum fluorescence emission at 605 nm. Light from a mercury source (Nikon, Intenslight, C-HGFI) was passed through a filter set (Nikon, TRITC, TE 2000) to illuminate the trapped bead. The filter set included an excitation filter (525-560 nm), a dichroic mirror which reflected the excitation light into the objective to illuminate the trapped bead, and an emission filter (570-620 nm) which transmitted the emitted fluorescent light from bead. An emission bandpass filter (Chroma, 605±25 nm, Brattleboro, VT) was placed in front of a position-sensing quadrant photodetector (QPD) (QP50-6SD; Pacific silicon sensor, Westlake village, CA) to specifically select the fluorescent emission from the bead. Figure 2-3 shows the fluorescent image of the microspheres used in this study.

![Figure 2-3 Fluorescent image of the microspheres used as handles to pull membrane tethers.](image)
The bright field images of the free and trapped microspheres are shown in Figure 2-4. Figure 2-4A shows a bead trapped by a single trap optical tweezers system and Figure 2-4B shows two beads trapped by a dual trap optical tweezers system. The results shown in this dissertation study are based upon the use of single trap tweezers system.

![Figure 2-4 Bright field images of the free and optically trapped microspheres. A- A bead is trapped by use of a single-trap optical tweezers. B- Two beads are trapped by a dual trap optical tweezers system.](image)

The fluorescent image of the trapped bead was projected onto the center of QPD to measure the displacement of the bead from the trapping center. Using an analog-to-digital converter (Wavebook 521; IOTech, Cleveland, OH), the sum-and-difference output signals (in mV) from the QPD amplifier were digitized and subsequently recorded by WaveView software (WaveView 7.14.16; IOTech, Norton, MA). Data collection frequency was 66.66 Hz. A charge-coupled device (CCD) camera (Hamamatsu Corp., EM-CCD, C9100/13, Bridgewater, NJ) was used to visualize the objects in the field of view.

The QPD signal represented the displacement of the bead position from the trapping center, which is proportional to an external force such as the tethering
force experienced by the bead. The external force was calibrated for the output voltage signal of the QPD by the viscous drag force method utilizing Stokes’ Law. In this method, a known drag force was applied to the trapped bead while recording the resulting differential signal from the QPD. An illustrative dynamic calibration procedure is shown in Figure 2-5.

**Figure 2-5 Dynamic force calibration procedure.** First, a bead is optically trapped by laser tweezers, and then known viscous drag forces are exerted on the trapped microsphere. The output voltage of the QPD in response to the displacement of the trapped bead from the trapping center are measured and correlated with the viscous drag forces applied on the bead.

The drag force was generated by driving a piezoelectric translation-stage (PZT) (Physik Instrumente, Model P-527.C3, Waldbronn, Germany) at known velocities. The resolution of the piezoelectric stage was 10 nm in $x$ and $y$ directions, and 2 nm in $z$ directions (laser beam propagation direction). The applied force was linearly fit to the output voltage of the QPD. Figure 2-6 shows a sample dynamic
calibration curve in our experiments. In this study, calibration and force measurements were performed at laser power of 350 mW after the microscope objective. There was no evidence of thermally induced structural damages in the living cells at this power.

![Dynamic calibration curve](image)

**Figure 2-6** An example dynamic calibration curve for force measurements in our experiments. The curve correlates the known forces to the output voltage of the QPD with a linear fit.

The relationship between the bead displacement and QPD signal was determined by moving a bead immobilized on the coverslip by known displacements with the PZT while recording the QPD voltage signal. The use of fluorescent microscopy together with fluorescent beads enables us to detect the displacements as small as 20 nm in our experiments. Figure 2-7 depicts a schematic of the displacement calibration procedure.
Figure 2-7 Displacement calibration procedure. The known displacements of the fluorescent image of the bead onto the QPD are correlated with the output voltage of the QPD.

There was a linear relationship between bead displacement and QPD output signal. Figure 2-8 shows an example displacement calibration curve in our experiments at 1 µm/sec, the velocity at which tether pulling experiments were performed.

Figure 2-8 An example displacement calibration curve in our experiments. The curve correlates the known displacements to the output voltage of the QPD.
Although our experiments are done at a single velocity, the displacement calibration curves at different velocities are shown in Figure 2-9.

![Displacement calibration curves at different velocities. Each curve correlates the known displacement to the output voltage of the QPD at a specified velocity.](image)

### 2.2.6 Static tether force

A HEK cell that was firmly attached to the bottom of the Petri dish was brought into contact with an optically trapped bead by moving the PZT. After 5-10 seconds contact time, the cell was moved away by the PZT at the rate of 1 µm/s to form the plasma membrane tether. Once a tether was formed, we continued to move cell away at 1 µm/s until the tether was elongated to a desired length (10, 15 and 20 µm). Once the tether reached the desired length, laser power was then decreased gradually until the tether force exceeded the strength of the optical trap, causing the bead to escape the trap and rapidly return towards the cell. We recorded the laser
power when the bead escaped, and converted the power to force via a calibration. Figure 2-10 shows a sample calibration curve in the static tether force experiments.

![Static calibration curve](image)

**Figure 2-10** An example static calibration curve in our experiments. The curve correlates the escaping force of the trapped beads from the optical trap to the power past the objective lens. The curve is fit with a linear and a second order polynomial equation.

2.2.7 Dynamic tether force measurements: single-speed pulling protocol

After a bead was trapped, a cell which was firmly attached to the bottom of the Petri dish was identified and moved towards the bead until they were separated by \(\approx 4 \, \mu m\). Then the PZT was triggered to move the cell toward the trapped bead at 1 \(\mu m/s\) to bring them into contact. The bead and cell were in contact for 5-15 seconds to achieve plasma membrane-bead attachment. The cell was subsequently moved away from the bead at 1 \(\mu m/s\), resulting in separation of the plasma membrane from the underlying cytoskeleton to form a long, thin cylindrical membrane tube. When the tether was elongated by 20 \(\mu m\), the PZT was subsequently stopped. The tether
was maintained at this length and the relaxation of the tether force was recorded. The illustrative tether formation steps are shown in Figure 2-11.

**Figure 2-11 Three steps of tether formation from living cells in our experiments.**

Plasma membrane tethers are thin strands of the plasma membrane formed between the trapped bead and the cell. To check the existence of membrane tethers, the beads will recoil back rapidly toward the adherent cell once the laser beam is cut off, in case of formation of plasma membrane tethers (see Figure 2-12 for example of the procedure).
2.2.8 Dynamic tether force measurements: multi-speed pulling protocol

We used a multi-speed tether pulling protocol to determine the membrane effective viscosity. In this protocol, once a plasma membrane tether was formed, it was subsequently elongated at 1 µm/s for 10 seconds, followed by 2 µm/s pulling rate for three seconds, 3 µm/s for three seconds, and 4 µm/s for four seconds. The multi-speed pulling protocol is illustrated in Figure 2-13.

Figure 2-13 Multi-speed pulling protocol. A- Multiple pulling rates versus time. B- Pulling length versus time corresponding to the graph shown in A.
All tether pulling experiments were done with PZT stage providing a controlled fine movement to avoid impulsions throughout the bead-cell contact, tether formation, and tether elongation processes in our experiments. The initial bead-cell contact was detected by fluorescent microscopy technique. In this work, by use of fluorescent microspheres we were able to control the initiation of microsphere–plasma membrane physical contact by observing the appearance of a non-zero QPD differential output voltage deviation from an initial zero value (indicating bead-cell contact and plasma membrane reactive forces) rather than just attempting to visually observe and estimate the contact.

2.2.9 Membrane tether imaging

Membrane tethers were imaged in order to estimate tether diameters. For this purpose, we used the single-speed tether pulling protocol. Once tether forces reached their equilibrium values, tethers were illuminated in bright-field, and imaged by the CCD camera. Multiple (100 frames) images of the same tether were averaged, and then the background image was subtracted from the final averaged image to eliminate background noise. The background image was an image of the field of view without the cell and trapped bead. We used MATLAB Image Processing Toolbox (R2010b, The Mathworks, Natick, MA) for image processing including image averaging and background subtraction. The tether diameter was estimated from the background subtracted image using ImageJ (1.44p, National Institute of Health, USA). We calibrated the transverse (x and y) planes by measuring known
distances in the x and y directions. For this purpose, we used a microruler with 20 µm spacing. The calibration yielded 0.16 µm per pixel in both directions.

### 2.2.10 F-actin staining

We used fluorescein isothiocyanate (FITC) labeled phalloidin (Phalloidin-FITC, Sigma-Aldrich) as cytochemical marker of polymerized actin. Tethers were pulled according to the single-speed pulling protocol. Once the tether force reached its equilibrium value, the bead was immobilized by lowering the axial position of the optical trap with the PZT until the bead came in contact with the glass surface at the bottom of Petri dish. The cells were then immediately fixed with PBS-paraformaldehyde 4% for 15 minutes, treated with PBS-triton X100 0.2% for five minutes, blocked with PBS-5% BSA for 30 minutes, and incubated for one hour with PBS-phalloidin-FITC 1 µM to stain F-actin. Fluorescent emission from stained F-actin was filtered using FITC filter set (Nikon, FITC). Images were subsequently acquired by the CCD camera with 100X optical microscope, and analyzed with ImageJ software.

### 2.2.11 Statistical analysis

We performed the statistical analysis of the data using a standard two-sample Student’s t-test with unknown variances of the two data sets. Statistical significance was accepted if the P-value was <0.05.
2.3 Results and discussion

2.3.1 Dynamic plasma membrane tether force

A typical dynamic tether force plot exhibits three different regions with respect to time (Figure 2-14): (1) tether formation process which starts from the onset of pulling, reaching the maximum tether force ($F_{\text{max}}$), followed by a sudden reduction in force; (2) tether elongation, which begins after the reduction in $F_{\text{max}}$ and continues until the desired tether length is reached. We refer to the force value obtained at the end of tether elongation as $F_{\text{f}}$; and (3) tether force relaxation, which starts once the tether elongation is halted, and continues until the force reaches an equilibrium value ($F_{\text{eq}}$) (see Figure 2-14 for example force profile).

![Typical temporal plasma membrane tether force plot for a HEK cell in response to a single-speed pulling protocol. Force plot shows tether formation (TF), tether elongation (TE), and tether force relaxation (REL) regions. Tether is formed.](image)
and elongated at a constant pulling rate of 1μm/s to 20μm. \( F_{\text{max}} \)-maximum tether force, \( F_{\text{eq}} \)-equilibrium tether force, \( F_1 \)-tether force at the end of elongation.

The force profile shown in Figure 2-14 was obtained from a cholesterol-depleted HEK cell by incubating the cells in DMEM containing 5 mM MβCD for 30 minutes. In this figure, \( F_{\text{max}} \) was \( \approx 240 \) pN, and then the tether force dropped to \( \approx 100 \) pN. This sudden force drop after reaching the maximum force value indicates the separation of the plasma membrane from cytoskeleton.\(^{29}\) At the end of the tether elongation, the tether force reached a value of \( F_1 \approx 160 \) pN. Finally, at the end of the relaxation, the \( F_{\text{eq}} \) was \( \approx 70 \) pN. We sometimes observed a secondary spike during the tether formation. This type of response may be due to additional membrane pulled off from the cell after the initial tether formation.\(^{29}\)

2.3.2 Plasma membrane tether equilibrium forces (\( F_{\text{eq}} \))

The application of an external pulling force on lipid vesicles and living cell membranes can result in tether formation, depending on the value of the pulling force and the adhesion strength to the substrate.\(^{31}\) When a tether is formed and elongated, the stretched membrane induces lateral tension within the cell body plasma membrane that draws more membrane into the tether. The tether equilibrium force is a measure of the tension in the plasma membrane tether balanced against that within the cell body, and represents a zero net flow of membrane components into the tether.\(^{32}\)
The mean±s.d. values of $F_{eq}$ for control HEK cells were 39±12 pN (n=11). The values of $F_{eq}$ for control cells and cholesterol manipulated cells with intact f-actin and also cells with disrupted F-actin are shown in Figure 2-15.

![Figure 2-15](image)

**Figure 2-15 Plasma membrane tether equilibrium forces versus plasma membrane cholesterol concentration for HEK cells with intact and disrupted F-actin.**

The respective mean±s.d. value of the membrane cholesterol concentration for control cells is 7.5±0.8 pmol/µg of protein. Mean value of $F_{eq}$ increased in response to lowering the membrane cholesterol content, and decreased upon elevation of the membrane cholesterol in cells with intact F-actin. Specifically, the $F_{eq}$ value significantly increased to 66±16 (n=10) and 74±17 (n=11) ($P<0.05$) when cells were incubated in DMEM containing 3 and 5 mM MβCD for cholesterol depletion, respectively. The respective membrane cholesterol concentrations are 6.6±0.3 and 5.7±0.8 pmol/µg of protein when cells were incubated in DMEM containing 3 and 5
mM MβCD. In response to incubating the cells with intact F-actin in DMEM containing 3 and 5 mM cholesterol-MβCD for cholesterol enrichment, $F_{eq}$ significantly decreased to 25±8 (n=8) and 19.5±5 (n=6), respectively (P<0.05), where the respective membrane cholesterol concentrations are 14.5±1.9 and 17.3±0.6 pmol/µg of protein.

The fact that the equilibrium forces does not reach zero is indicative of a resistance to membrane flow from the cell body into the tether.24 Higher equilibrium forces are associated with more resistance to membrane flow, whereas lower tether equilibrium forces suggest lower resistance. The resistance could be in the form of plasma membrane-cytoskeleton adhesion and our further observations of significant decrease in $F_{eq}$ upon F-actin disruption supports this hypothesis.

In order to study the effects of cell cytoskeleton on tether equilibrium force, experiments were repeated in the presence of Latrunculin A, which affects the polymerization of F-actin. Specifically a 1:1 molar complex between Latrunculin A and G-actin forms, which lowers the available amount of free G-actin and disrupts the equilibrium between G- and F-actin.33 In control cells, the tether equilibrium forces significantly decreased from 39±12 to 24±7 pN (n=13) upon F-actin disruption (P<0.05). This reduction in $F_{eq}$ suggests the dissociation of the plasma membrane from the cytoskeleton following application of Latrunculin A as illustrated previously with other cells,34-36 and subsequently more flow of the membrane from the cell body into the tether. Similarly, disruption of F-actin in cells incubated in DMEM containing 5 mM MβCD for cholesterol depletion resulted in a
significant decrease in $F_{eq}$ from 74±17 to 26±4 pN (n=7) compared to cells with intact F-actin receiving the same cholesterol depletion ($P<0.05$). The mean±s.d. values of $F_{eq}$ obtained after F-actin disruption for cells incubated in 5 mM MβCD-cholesterol for cholesterol enrichment were 17±2 pN (n=6). Since there was no statistically significant change in $F_{eq}$ between intact and F-actin disrupted cells under cholesterol enriched condition, cholesterol enrichment may have already had a weakening effect on the plasma membrane-cytoskeleton adhesion.

### 2.3.3 Plasma membrane tether diameter

In Figure 2-16, we present images of the bead-tether-cell assembly for intact F-actin and F-actin disrupted cells with and without cholesterol manipulation. Cells were incubated in DMEM containing 5 mM cholesterol-MβCD and 5 mM MβCD for cholesterol enrichment and cholesterol depletion, respectively.

The mean±s.d. values of tether diameter ($d_{tether}$) were 290±40 nm for control HEK cells (n=5) and significantly increased to 640±90 nm (n=7) in response to cholesterol enrichment ($P<0.05$). Under cholesterol depleted conditions, tethers were not optically resolvable since their diameters were below the optical resolution limit of the imaging system. Disruption of the F-actin significantly changed $d_{tether}$ in both control cells and cholesterol-manipulated cells. Specifically, the mean±s.d. values of $d_{tether}$ in control cells and cholesterol enriched cells increased to 732±76 nm (n=8) and 890±72 nm (n=5) upon disruption of F-actin, respectively. Membrane tethers were visible with the diameter of 720±92 nm (n=5) in F-actin disrupted and cholesterol depleted cells.
Figure 2-16 Bright-field photomicrographs of HEK cell–membrane tether–bead assembly for cells with intact F-actin (a, b, c) and disrupted F-actin (d, e, f). The (b, e) images represent non-cholesterol manipulated cells. The (a, d) images represent the photomicrographs under cholesterol depletion (5.7 pmol/µg protein), and (c, f) represent the cholesterol enrichment condition (17.3 pmol/µg protein).

Measurements of tether diameter provide information on the amount of membrane which is liberated from the cell into the tether. The amount of the membrane flow can be modulated by membrane-cytoskeleton interactions in a manner that tighter interactions can cause less membrane to be liberated, resulting in thinner tethers, whereas looser interactions are associated with formation of thicker tethers. The strength of the interactions between the membrane and the cytoskeleton may be manifested in different cell types. For example, the reported diameter of tethers in neuronal growth cones,\textsuperscript{37} and human mesenchymal stem cells\textsuperscript{38} are 420 and 400 nm, respectively, whereas tethers formed from OHCs-known by their firm membrane-cytoskeleton attachment- are not-visible by bright field microscopy.\textsuperscript{39}
In the present study, we show that the membrane tether diameter varies with cholesterol content. Tethers formed from HEK cells became invisible below the optical diffraction limit of the imaging system upon cholesterol depletion. However, cholesterol enrichment resulted in a statistically significant increase in the tether diameter by 2.2-fold. The decreasing trend in tether diameters upon cholesterol depletion, and an increasing trend upon enrichment suggest stronger adhesion between the membrane and cytoskeleton in the depleted condition, and weaker adhesion upon enrichment conditions. Moreover, our tether diameter analysis revealed that increases in the equilibrium forces under cholesterol depleted condition were accompanied by decreases in tether diameters. Similarly, the lower equilibrium forces in cholesterol enriched cells were associated with larger tether diameters. We have observed a similar correlation between tether diameter and tether equilibrium force in whole cell voltage-clamped HEK cells. In those experiments, HEK cells with greater tether diameters had a smaller equilibrium tether force than OHCs with smaller tether diameters.

The measured tether diameter values for control cells and cholesterol manipulated cells, for cells with intact F-actin and F-actin disrupted cells, are shown in Figure 2-17.
2.3.4 Membrane bending modulus

To understand the effects of membrane cholesterol concentration on membrane bending modulus, we estimated the bending modulus \((B)\) based on the following equation: \(^{37,40,41}\)

\[
F_{\text{eq}} = 4\pi B/d_{\text{tether}}
\]  

where \(d_{\text{tether}}\) is tether diameter. This equation is derived with the assumption of equilibrium condition in the system, in which the tether length and tether radius both are at their equilibrium values.\(^42\) We use \(F_{\text{eq}}\) and the tether diameter values once force relaxation is reached to satisfy the equilibrium condition.

Using \(F_{\text{eq}} \approx 39\) pN and \(d_{\text{tether}} \approx 290\) nm, we estimate the mean value of \(B\) to be \(\approx 9 \times 10^{-19}\) J for control HEK cells. Reported values of \(B\) for biological membranes are in the range of \(10^{-20}-10^{-18}\) J \(^{30,43,44}\), and our estimation lies within this range. Under
cholesterol enrichment conditions and at the upper extreme of membrane cholesterol concentration, the bending modulus is estimated to be $\approx 9.9 \times 10^{-19}$ J. Although tethers were not optically resolvable under cholesterol depleted conditions, we estimate $B$ at $\approx 8.8 \times 10^{-19}$ J at the lower extreme of membrane cholesterol concentration, assuming a tether diameter of 150 nm (i.e., a diameter at optical resolution limit). The results show that $B$ is almost independent upon the cholesterol concentration within the cholesterol concentration range examined.

### 2.3.5 Plasma membrane-cytoskeleton adhesion energy per unit area

The plasma membrane-cytoskeleton adhesion energy per unit area ($W$) is a measure of membrane-cytoskeleton adhesion strength, and can be measured once the membrane tether equilibrium force and the tether diameter are known. We measured $W$ by the following equation:\textsuperscript{45, 46}

$$W = \frac{F_{eq}^2}{8\pi^2 B} \quad (2)$$

Given $F_{eq} \approx 39$ pN and $d_{tether} \approx 290$ nm, we estimate $W$ to be $\approx 21 \times 10^{-18}$ J/µm$^2$ for control HEK cells (Figure 2-18). This value is close to $\approx 30 \times 10^{-18}$ J/µm$^2$ reported for neutrophils,\textsuperscript{47, 48} and $\approx 40 \times 10^{-18}$ J/µm$^2$ for molluscan neurons.\textsuperscript{49} Enriching the cholesterol content of the membrane decreased $W$ by 75% to $5 \times 10^{-18}$ J/µm$^2$ while cholesterol depletion increased it to $\approx 70 \times 10^{-18}$ J/µm$^2$ (both at the extreme cholesterol concentrations). Higher values of $W$ upon cholesterol depletion indicate stronger membrane-cytoskeleton adhesion under cholesterol depleted conditions, while lower $W$ values in response to cholesterol enrichment indicate weaker plasma membrane-cytoskeleton adhesion.
2.3.6 Multi-speed tether pulling: tether effective viscosity ($\eta_{\text{eff}}$)

In Figure 2-19, we present the membrane tether force measured in response to the multi-speed pulling protocol. Total elongation time and total tether length were 20 seconds and 41 µm, respectively.

During each pulling interval, the membrane tether force increased exponentially and approached a specific steady-state value ($F_{\text{ss}}$). The exponential increase in membrane tether force at an increasing pulling rate was first reported in our previous publication.29 We fit the data for different pulling rates to exponential functions and obtained an estimate of $F_{\text{ss}}$ for each pulling rate. Figure 2-20 presents the curve fitting result to the measured forces for pulling rate of 3 µm/s.
Figure 2-19 Typical temporal plasma membrane tether force plot for a HEK cell in response to multi-speed pulling protocol. This plot is for a HEK cell incubated in 5 mM MβCD-cholesterol for 30 minutes in order to elevate the membrane cholesterol content. The initial negative value of the force before the beginning of tether pulling results from the cell pushing the bead in a direction opposite to that of the stage movement.

Figure 2-20 Tether force growth at a given velocity. The figure shows a single exponential curve fit to the 3rd segment of membrane tether force profile shown in Figure 2-19. R2=0.95 for this pulling rate, and >0.89 for other pulling rates (data not shown).
Values of $F_{ss}$ at each pulling rate were subsequently used to determine the effective viscosity ($\eta_{eff}$) of the membrane tether as:

$$F_{ss}(V_{pull}) = F_{ss}(0) + 2\pi\eta_{eff}V_{pull}$$

where $V_{pull}$ is pulling rate, $F_{ss}(0)$ is the steady-state force at zero pulling rate, and $\eta_{eff}$ is the membrane tether effective viscosity.

In Figure 2-21, we present illustrative values of $F_{ss}$ as a function of $V_{pull}$ based on the data shown in Figure 2-19. There is a linear relationship between $F_{ss}$ and $V_{pull}$ in the range of the utilized pulling rates where the slope of the line is divided by $2\pi$ to estimate $\eta_{eff}$.

![Figure 2-21](image)

**Figure 2-21** Steady-state membrane tether forces linearly fit to their corresponding pulling rates for the membrane force plot shown in Figure 2-19.

The mean±s.d. values of $\eta_{eff}$ for control HEK cells were $2.6±1$ pN.s/µm ($n=13$). Higher membrane cholesterol content increased $\eta_{eff}$, while lowering the membrane cholesterol content showed a tendency to reduce $\eta_{eff}$. Specifically, the mean±s.d. values of $\eta_{eff}$ were $3.2±0.85$ (n=7) and $3.6±0.73$ pN.s/µm (n=10) for cells incubated
in DMEM containing 3 and 5 mM cholesterol-MβCD for cholesterol enrichment, respectively. The mean±s.d. value of \( \eta_{\text{eff}} \) was 2.24±0.44 pN.s/µm (n=8) for cells incubated in DMEM containing 3 mM MβCD for cholesterol depletion. The measured 3.6±0.73 value of \( \eta_{\text{eff}} \) in cholesterol enriched cells was significantly different from those of control cells and cholesterol depleted cells (\( P<0.05 \)). The values of \( \eta_{\text{eff}} \) obtained in the range of examined velocities in this study versus membrane cholesterol concentration is shown in Figure 2-22.

![Figure 2-22 Effective membrane viscosity versus membrane cholesterol concentration. The results show the viscosity for control cells and cholesterol manipulated cells. The experiments were repeated for cells with disrupted F-actin.](image)

We could not determine \( \eta_{\text{eff}} \) under cholesterol depletion when cells were incubated in DMEM containing 5 mM MβCD since those tethers were broken at lengths greater than 25 µm and pulling velocities higher than 3 µm/s. Values of \( \eta_{\text{eff}} \) reported in this paper are in the range for OHCs (2.39-5.25 pN.s/µm),\textsuperscript{29} neutrophils (1.8 pN.s/µm),\textsuperscript{50} and endothelial cells (0.5 pN.s/µm).\textsuperscript{19} These values are orders of
magnitude higher than those reported for lipid vesicles which lack a “cytoskeleton”. For example, values of 5 to 13 $\times 10^{-3}$ pN.s/µm for vesicles from egg phosphatidylcholine diluted in hexane,$^{51, 52}$ and 0.071 pN.s/µm for bilayer vesicles made from a 1:1 mixture of bovine brain sphingomyelin and cholesterol have been reported.$^{53}$ The mean±s.d. value of $\eta_{\text{eff}}$ in F-actin disrupted control cells was 2.54±0.92 pN.s/µm (n=11). Similar to control cells, disruption of F-actin did not result in a significant change in the values of $\eta_{\text{eff}}$ when compared under the same cholesterol manipulation. The observation that $\eta_{\text{eff}}$ is unchanged in response to F-actin disruption supports the observation that there is no F-actin in the tethers in our experiments.

The tether effective viscosity represents the overall viscosity from three viscous sources, surface viscosity in each monolayer and the viscous slip between each of the monolayers. The third component of effective viscosity is viscous slip of the inner monolayer membrane over the cytoskeleton. Our tether equilibrium force measurements indicate weaker plasma membrane-cytoskeleton adhesion in F-actin disrupted cells. The unchanged effective viscosity upon F-actin disruption suggests that surface viscosity in each monolayer and the viscous slip between each of the monolayers have greater impact than the viscous slip of inner monolayer over the cytoskeleton at least within cholesterol concentration range examined.

Our observations are consistent with previous plasma membrane tether pulling studies on neuronal growth cones in which the apparent membrane tensions were significantly decreased upon disruption of F-actin with Cytochalasins B and D,
whereas effective membrane viscosity was not significantly decreased in those measurements. The authors suggest that cytochalasins could weaken the associations of integral membrane proteins with the cytoskeleton, specifically β₁ integrins in neuronal growth cones. Also, the application of Latrunculin A resulted in unchanged membrane effective viscosity in human brain tumor cells, consistent with our observations in HEK cells.

### 2.3.7 Example of two plasma membrane tethers pulled from a cell

We used fluorescein isothiocyanate (FITC) labeled phalloidin (Phalloidin-FITC, Sigma-Aldrich) as cytochemical marker of polymerized actin. Tethers were pulled according to the single-speed pulling protocol. Once the tether force reached its equilibrium value, the bead was immobilized by lowering the axial position of the optical trap using the PZT until it came in contact with the glass surface at the bottom of Petri dish. The cells were then immediately fixed with PBS-paraformaldehyde 4% for 15 minutes, treated with PBS-triton X100 0.2% for five minutes, blocked with PBS-5% BSA for 30 minutes, and incubated for one hour with PBS-phalloidin-FITC 1 μM to stain F-actin. Fluorescent emission from stained F-actin was filtered using FITC filter set (Nikon, FITC). The filter set included an excitation filter (525-560 nm), a dichroic mirror which reflected the excitation light into the objective to illuminate the sample, and an emission filter (570-620 nm). Images were subsequently acquired by a CCD camera with 100X optical microscope, and analyzed with ImageJ software.
To investigate the presence of F-actin in plasma membrane tethers, tethers were pulled from non-cholesterol manipulated human embryonic kidney (HEK) cells. Tethers were formed and elongated at 1 µm/s to reach a length of 20 µm, and subsequently held therein for ~2 minutes to allow for tether force relaxation and then labeled and fixed as described above. In Figure 2-23 we show the respective bright-field and fluorescent images of the same bead-tether-cell assembly. In these particular experiments, two tethers were formed from the same cell. The fluorescent image did not indicate the presence of F-actin within the tether under these pulling conditions.

![Figure 2-23 Images of cell-tether-bead assembly](image)

Figure 2-23 Images of cell-tether-bead assembly- (A) Bright-field photomicrograph of two trapped beads - two membrane tethers pulled from a control HEK cell. (B) Corresponding fluorescent image of the same beads-tethers-cell assembly shown in (A) after F-actin staining with FITC-labeled phalloidin.
2.3.8 Lipid transport from cell body plasma membrane into the tether

When a membrane tether is elongated, the stretched plasma membrane within the tether exerts a tensile force over the plasma membrane of the cell body, which draws membrane lipids of the cell into the tether causing a membrane flow. The flow of the membrane into the tether continues until the tension within the stretched tether equilibrates the oppositely oriented tension from the cell. At this state the force reaches an equilibrium value and net lipid flow into the tether equals zero. Figure 2-24 illustrates the force relaxation region of the tether force profile (Top panel) wherein the membrane flow into the tether takes place (Bottom panel).

![Tether force relaxation and membrane flow into the tether](image)

Figure 2-24 Tether force relaxation and membrane flow into the tether. Top: Tether force relaxation region of the tether force profile. The relaxation starts at the end of
tether elongation and continues until reaching an equilibrium force value. Bottom: The lipid flow from the cell into the tether during the force relaxation period.

It has been shown that gradient of surface tension between two vesicles connected by a lipid nanotube results in a two-step transport within the connecting tether. In the early stage, the surface tension gradient establishes rapidly along the tube giving rise to a Marangoni transport of lipids through the tether while the tube radius remains constant. This stage is followed by a slower stage where the tether progressively equilibrates its shape until it reaches an equilibrium radius.

In our experiments, the plasma membrane tether force relaxation (REL region in Figure 2-14) also shows a biphasic nature representing two time constants, consistent with the observations on lipid vesicles nanotubes, and our previous reports with living cell plasma membranes in HEK cells and OHCs. A single exponent decay does not fit the rapid force reduction occurring at the first stage of force relaxation whereas a bi-exponent model fits the complete force relaxation profile. Figure 2-25 represents the tether force relaxation starts at the end of pulling and continues until reaching an equilibrium value. The force relaxation is fit with a single and a bi-exponent decay equations.

Between the two force relaxation time constants obtained by bi-exponent model, the shorter relaxation time constant ($\tau_{\text{short}}$) reflects the Marangoni transport and lipid reorganization within the tether occurring at the beginning of force relaxation once the elongation is stopped.
Figure 2-25 An example tether force relaxation behavior. The membrane tether was pulled and elongated according to a single-speed pulling protocol. Time 0 represents the end of tether pulling and initiation of the force relaxation.

We estimated $\tau_{\text{short}}$ as 1.2±0.45 s for normal cells, and it remained unchanged by cholesterol modulation, indicating that cholesterol did not alter this process in our experiments within cholesterol concentration range examined. The longer relaxation time constant ($\tau_{\text{long}}$) represents the rate at which membrane tether equilibrates its radius wherein the membrane components flow from the cell into the tether in response to the tension gradient induced by tether force within the living cell body, lipid vesicles, and the vesicles partially drawn into a pipette. Our measurements show a significant effect of cholesterol on $\tau_{\text{long}}$, indicating the important role of cholesterol on tether radius relaxation. Specifically, the mean±s.d. values of $\tau_{\text{long}}$ were estimated as 11.5±1.3 s (n=12) for control cells, close to the
value reported on HEK cells previously\textsuperscript{25}; 15.5±2.5 s (n=14) for cholesterol enriched; and 8.3±1.2 s (n=7) for cholesterol depleted cells, at extreme cholesterol concentrations examined.

The tether force relaxation analysis of living cell plasma membranes, as in this study and our previous reports\textsuperscript{24, 25}, represent greater values of both times constants compared to those for lipid vesicles.\textsuperscript{55} We suggest the difference could be due to the contribution of plasma membrane-cytoskeleton interactions in living cells, which are absent in lipid vesicles. Our measurements in this study are done at a specified tether length (20 µm), and the resulting time constants may increase at longer tethers since more time is needed for establishment of surface tension gradient and lipid flow along longer tethers.\textsuperscript{55}

We analyzed the changes in tether length (dL) during force relaxation through analysis of REL region in Figure 2-14. The reduction in tether force from $F_i$ to $F_{eq}$ during the force relaxation is indicative of the displacement of the trapped bead; and hence, a change in tether length. The actual tether length at the end of force relaxation is $L+dL$, where $L$ is the initial tether length (20 µm, in single-speed pull protocol). Figure 2-26 illustrates the displacement of the bead during force relaxation region.
Figure 2-26 Illustrative displacement of the bead during tether force relaxation due to the lipid flow from the cell into the tether. The bead displaces from A at the beginning of force relaxation to B at the end of force relaxation where force reaches an equilibrium value.

The force drop from $F_i$ to $F_{eq}$ during relaxation was converted to bead displacement utilizing the force-QPD output voltage, and QPD output voltage-bead displacement relationships. The mean±s.d. values of $dL$ were 262±160 nm (n=14) for control cells and significantly decreased to 82±45 nm (n=10) ($P<0.05$) in response to incubation of the cells in DMEM containing 5 mM MβCD for cholesterol depletion. Cholesterol enrichment significantly increased the $dL$ values to 454±250 nm (n=10) ($P<0.05$) for the cells incubated in DMEM containing 5 mM cholesterol-MβCD. The increase in the tether length ($dL$) during force relaxation is an indication of membrane flow into the tether from the cell or rearrangements of components within the tether. The magnitude of $dL$ suggests a significant effect of cholesterol on this parameter. Specifically, the higher $dL$ values associated with cholesterol enrichment condition suggest less plasma membrane-cytoskeleton adhesion, and
hence, more membrane liberation from the cell into the tether. Similarly, lower $dL$ values under cholesterol depletion conditions suggest higher plasma membrane-cytoskeleton adhesion. These findings support the observed thinner membrane tethers and thicker tethers under cholesterol depletion and enrichment, respectively.

The variations in $\tau_{\text{long}}$ with cholesterol content are consistent with those in $\eta_{\text{eff}}$ which is a measure of the rate at which the membrane can flow and undergo deformations. The longer time constants upon cholesterol enrichment indicate lower deformation rates and flow rates, and are consistent with higher $\eta_{\text{eff}}$ values. Values of $\eta_{\text{eff}}$ increased under cholesterol enrichment in our measurements. Comparing with previous studies, increase in $\eta_{\text{eff}}$ upon cholesterol enrichment with cholesterol-MβCD were reported for monkey kidney fibroblast cells (COS-7)$^{57}$ and human red blood cells.$^{58}$ In those studies, no changes in viscosity were reported upon cholesterol depletion using MβCD.

2.3.9 Membrane tether stiffness estimation

In Figure 2-27, we show static tether force values as a function of tether length for three different cases: (a) control cells; (b) cells incubated in DMEM containing 3 and 5 mM concentration of MβCD to induce cholesterol depletion; and (c) cells incubated in 3 and 5 mM concentration of MβCD-cholesterol to induce cholesterol enrichment. Each discrete point indicates the mean value of static tether force from a sample size of 10-15.
In each case, the forces are higher at longer tether lengths. We follow our previously reported method and use the slope of each tether force-tether length plot as an estimation of tether stiffness ($k$). Higher $k$ was associated with cholesterol depleted cells, while cholesterol enriched cells had lower $k$ as compared with control cells. Specifically, the mean±s.d. values of $k$ for control cells were 1.2±0.19 pN/µm. The mean±s.d. values of $k$ significantly increased to 2.37±0.15 and 3.24±0.10 pN/µm for cells incubated in DMEM containing 3 and 5 mM MβCD, and significantly decreased to 0.51±0.07 and 0.28±0.01 pN/µm in response to incubation of the cells in 3 and 5 mM cholesterol-MβCD, respectively ($P<0.05$). Tether stiffness values are shown in Figure 2-28.
Figure 2-28 Plasma membrane tether stiffness. Stiffness values (k) in HEK cells versus membrane cholesterol concentrations. The values are estimated using static forces shown in Figure 2-27.

We point out that our estimated values of $k$, based on the data presented in Figure 2-28, do not take into account the effects of force relaxation that begins after tether elongation is halted. To understand the effects of force relaxation, we utilized an alternative approach based on the values of $F_{eq}$ to estimate the tether stiffness, using $dL$. The actual tether lengths at the end of force relaxation and their corresponding $F_{eq}$ were subsequently used to estimate the tether stiffness as $F_{eq}/L+dL$. We refer to this alternative method of estimating $k$ as relaxation-corrected method, and designate the corresponding tether stiffness as $k_{rc}$. The mean±s.d. values of $k_{rc}$ were 1.39±0.65 pN/µm (n=12) for control cells. Figure 2-29 represents the stiffness values obtained by the relaxation-corrected method.
Figure 2-29 Plasma membrane tether stiffness in HEK cells versus membrane cholesterol concentrations. The stiffness values are estimated using force relaxation-corrected method ($k_{rc}$).

Cholesterol depletion significantly increased the mean±s.d. values of $k_{rc}$ to 3.04±0.90 (n=5) and 3.75±0.80 (n=10) pN/µm, and cholesterol enrichment significantly decreased them to 0.98±0.56 (n=9) and 0.60±0.40 (n=8) pN/µm at the same cholesterol concentrations as $k$ values are estimated ($P<0.05$). Both $k$ and $k_{rc}$ estimations indicate the same trends: higher tether stiffness upon cholesterol depletion, and lower stiffness under cholesterol enrichment conditions. The values of tether stiffness measured for HEK cells in our experiments are in the range of the values reported for OHCs 29 and jurkat cells.59 The respective values of tether stiffness are 3.7 and 1.6 pN/µm for OHCs lateral wall and jurkat cells, respectively. We summarize the quantitative results obtained in this study in Table 2-1.
Table 2-1 Values of the quantified metrics obtained in this study for control experiments as well as measurements performed under different membrane cholesterol modulation conditions.

<table>
<thead>
<tr>
<th>Experiment/Incubation condition</th>
<th>Membrane cholesterol concentration (pmol/µg protein)</th>
<th>$F_{eq}$ (pN)</th>
<th>$\eta_{eff}$ (pN.s/µm)</th>
<th>$k$ (pN/µm)</th>
<th>$\tau_{long}$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol depletion/5mM MβCD</td>
<td>5.7±0.8*</td>
<td>73.8±16.7*</td>
<td>Not determined</td>
<td>3.2±0.1*</td>
<td>8.3±1.2*</td>
</tr>
<tr>
<td>Control</td>
<td>7.5±0.8</td>
<td>38.8±11.7</td>
<td>2.6±1</td>
<td>1.2±0.2</td>
<td>11.5±1.3</td>
</tr>
<tr>
<td>Cholesterol enrichment/5mM MβCD-cholesterol</td>
<td>17.3±0.6*</td>
<td>19.5±5*</td>
<td>3.6±0.7*</td>
<td>0.3*</td>
<td>15.5±2.5*</td>
</tr>
</tbody>
</table>

The percent changes in tether diameter and tether diameter related parameters such as the adhesion energy and bending stiffness versus membrane cholesterol concentration are shown in Table 2-2.

Table 2-2 Percent changes in tether diameter, adhesion energy and bending modulus.

<table>
<thead>
<tr>
<th>Experiment/Incubation condition</th>
<th>Membrane cholesterol concentration (pmol/µg protein)</th>
<th>$d_{tether}$ (nm)</th>
<th>$W$ ($\times10^{-18}$ J/µm²)</th>
<th>$B$ ($\times10^{-18}$ J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol depletion/5mM MβCD</td>
<td>5.7±0.8*</td>
<td>Not visible</td>
<td>+228%*</td>
<td>-2%</td>
</tr>
<tr>
<td>Cholesterol enrichment/5mM MβCD-cholesterol</td>
<td>17.3±0.6*</td>
<td>+120%*</td>
<td>-76%*</td>
<td>+10%</td>
</tr>
</tbody>
</table>

In a recent study, the plasma membrane contact stiffness decreased upon cholesterol depletion in NIH-3T3 fibroblasts, measured by a nano-indentation technique which applies local harmonically oscillating indentation forces into the cellular outermost layers (20 nm depth) above the cytoskeleton in fixed cells.20 While applying tensile forces on living cell plasma membrane and pulling it away from the cytoskeleton, we measured higher membrane stiffness upon cholesterol
depletion. The difference between these two observations could be due to different physics of the experiments (tensile versus compressive forces).

2.3.10 Plasma membrane cholesterol content and relevance to hearing

Filipin labeling of the lateral wall of OHCs revealed a lowering of membrane cholesterol content during cell maturation. Our previous OHC mechanical measurements on the plasma membrane of lateral wall showed tether equilibrium forces of $73.4\pm5.1$ pN, membrane effective viscosity as $1.7\pm0.58$ pN.s/µm, and lateral wall plasma tether stiffness as $3.71$ pN/µm. Comparing them with our results here for HEK cells, the OHC plasma membrane mechanical properties exhibit values indicative of a membrane that is depleted in cholesterol. Consistently, the membrane tethers in OHCs are not resolvable by optical microscopy, similar to the case of tethers pulled from HEK cells upon cholesterol depletion. This suggests that the plasma membrane of the lateral wall of OHCs behave as a membrane with relative low level of cholesterol in membrane. The findings of tendency to lower effective viscosities and faster time course of force relaxation under cholesterol depletion condition in HEK cell membranes suggest more rapid mechanical response of the membrane in response to an external force. These results are consistent with our recent observations, which showed an increase in electromotile response of the cochlear amplifier upon depletion of cochlear cholesterol content in guinea pigs. Also, higher effective viscosities and greater time course of force relaxation suggest slower mechanical response of the membrane under cholesterol
enrichment condition. The observed changes in mechanical properties of the membrane upon cholesterol modulation could also be useful to interpret some mechanical aspects of recent observations on the role of membrane cholesterol in regulating membrane channels and function of the OHC. 60,61

2.4 Conclusions

We have studied the effects of membrane cholesterol content on mechanical properties of the plasma membrane of a living cell with an optical tweezers force sensor. We found cholesterol depletion strengthens the plasma membrane cytoskeleton adhesion whereas cholesterol enrichment weakens the adhesion. Also, our results indicated that cholesterol depletion and cholesterol enrichment induced opposing effects on variety of mechanical properties of the membrane. Disrupting F-actin cytoskeletal filament diminished the observed disparity in the mechanical properties of the plasma membrane and membrane-cytoskeleton adhesion with cholesterol concentration. This suggests the dominant effect of cytoskeleton on plasma membrane mechanics. The dynamics of lipid flow from the cell body plasma membrane suggests there is more membrane flow into the tether under cholesterol enrichment relative to cholesterol depletion. Viscosity measurements show higher viscosities upon cholesterol enrichment and lower ones upon depletion, consistent with higher time course of lipid flow into the tether measured under cholesterol enrichment and lower rates by depletion. Our investigations help to interpret the mechanical aspect of some cholesterol dependent biological processes at membrane
and cellular level, such as the effects of cholesterol on hearing. Membrane tethers act as biological sensors to probe the plasma membrane properties, as well as plasma membrane cytoskeleton interactions (Figure 2-30).

![Figure 2-30 A plasma membrane tether pulled from an adherent cell. The membrane cholesterol, shown as yellow phospholipid molecules within the membrane, is modulated by MβCD.](image)

**2.5 References**


Chapter 3

3. Effects of plasma membrane composition and cytoskeleton actin on cellular protrusions

An optical tweezers-fluorescent microscopy technique was used to study protrusion formation in adherent human embryonic kidney cells in response to application of an external force over the cell surface. The mechanical properties of protrusions were analyzed by obtaining the associated force-length plots during protrusion formation. The plasma membrane cholesterol content modulated the protrusion formations. Specifically, lowering membrane cholesterol content by 25% increased the protrusion stiffness by 80% and resulted in formation of protrusions shorter in length by a factor of two. Enhancement of membrane cholesterol content by nearly two-fold was associated with formation of 30% longer protrusions with nearly three-fold decreased protrusion stiffness compared with control cell. The results indicated the major contribution of cytoskeleton in protrusion formations as evidenced by abolishment of membrane cholesterol effects on protrusions upon disruption of F-actin. The protrusion mechanics was interpretable by a
Maxwell viscoelastic model, and the viscoelastic behavior of protrusions was further evidenced by the observation of hysteresis effect and force relaxation. The results of the present study elucidate the coordination of plasma membrane composition and cytoskeleton in formation of protrusions.

3.1 Background

Cellular protrusions are deformations at surface of living cells formed in vivo in some biological processes such as lamellipodia, bleb [1] and pseudopodia formations [2] during cell migration and dendritic filopodia and spine protrusions in neurons [3], where the protrusion formations are driven by intracellular force generation mechanisms. Protrusions can also be formed at the surface of the cell upon application of an external pulling force, such as formation of protrusions preceding tether formations in leukocytes rolling over endothelium [4–7]. In both intra- and extracellular driven protrusions, cellular protrusions incorporate both the cell plasma membrane and cytoskeleton effects in that the dynamic interplay between membrane and cytoskeleton is essential for protrusion initiation and formation [8]. Actin cytoskeleton is the main drive of protrusions in migratory cells, wherein protrusion formation is a hierarchical process initiates with polymerization of actin monomers and elongation of single actin filaments and is followed by formation of a dendritically branched network of actin filaments at subcellular level [9]. The generated force during actin polymerization drives the membrane protrusion, and as a result of membrane-cytoskeleton interaction, the reactive forces of the plasma membrane exert a counter force to actin network which leads to
formation of a retrograde flow of actin in cellular protrusions as in lamellipodium or dendritic spines [3,10].

Actin assembly and membrane reorganization are coordinated during protrusion formation as recently evidenced by the stimulatory effects of membrane protein Exo70 on actin polymerization and branching through Arp2/3 complex [11]. The spatial coupling of actin filaments with plasma membrane in cellular protrusions makes their mechanical properties mutually inclusive in protrusion mechanics and a determining factor of changes in protrusion phenotypes and complex cellular shape changes [12,13]. The plasma membrane acts as a soft mechanical obstacle to membrane protrusions and its mechanical and geometrical properties such as membrane tension, bending stiffness and curvature are important factors in protrusion formation. Recent studies revealed the inhibitory effects of high membrane curvature on actin monomer polymerization and filament elongation, and the essential role of plasma membrane constituents in protrusion formation [9,14]. Membrane cholesterol depletion inhibited the formation of microtubule based protrusions in clostridium difficile toxin treated cells, presumably due to changes in membrane mechanical properties upon cholesterol depletion [15]. In another study, the high level of membrane cholesterol in polymorphonuclear leukocytes attenuated the pseudopodia formation in response to shear flows, indicating a link between membrane fluidity and cellular mechanotransduction [16]. Parallel to the effects of membrane properties on protrusions, cytoskeletal mechanics also revealed the effects on protrusions, cortical tension demonstrated a limiting effect on lamellipodia outgrowth in adherent cells, and acted as a determining factor for switch between the bleb and lamellipodia
types of protrusion [1]. Specifically, decreased cortical tension increased the size of the lamellipodia and elevated cortical tensions were associated with reduced lamellipodia formation.

We herein investigated the mutual effects of membrane and cytoskeletal actin on mechanical properties of cellular protrusions formed with external driving force. To this end, using optical tweezers technique protrusions were formed by grasping a part of the cell surface and retracting it away from the cell until the separation of plasma membrane from cytoskeleton. To discern the role of membrane mechanics on protrusions, the membrane lipid composition was altered in response to membrane cholesterol content manipulation, and the membrane mechanical properties were probed with optical tweezers under cholesterol depleted and cholesterol enriched conditions. We used adherent human embryonic kidney (HEK) cell line as a model for cholesterol manipulation, consistent with our previous work where these cells were used to investigate the effects of cholesterol on membrane capacitive properties. To elucidate the effects of cytoskeleton on protrusion mechanics and to understand if the protrusion mechanics is also modulated by cholesterol in F-actin disrupted cell, the experiments were repeated for cells with disrupted F-actin under cholesterol enriched and cholesterol depleted conditions.
3.2 MATERIALS AND METHODS

3.2.1 Cell Culture

Human embryonic kidney (HEK293) cells (ATCC, CRL-1573) were seeded in Dulbecco’s modified eagle medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Invitrogen) and 1% Penicillin/Streptomycin (Gibco). Cells were incubated in an air jacketed CO2 incubator (NuAire) at 37°C with 5% CO2. When the cells were 70-80% confluent, they were passaged into glass bottom poly-d-lysine coated MatTek plates (P35GC-1.0-14C). Cells of medium size (≈ 10-20 µm diameter) were selected for measurements if they were firmly attached to the bottom of the Petri dish. All experiments were performed within 30 minutes of removal of the cells from the incubator.

3.2.2 Cell membrane cholesterol manipulation

A commonly used method to modify the membrane cholesterol content is incubation of cells with cyclodextrins [17]. Cells were incubated in DMEM containing 5 mM methyl-β-cyclodextrin (MβCD) (Sigma) to reduce the membrane cholesterol content. We used water-soluble cholesterol with MβCD (51 mg cholesterol per gram of cholesterol+MβCD) (Sigma) for cholesterol enrichment. Cells were incubated in DMEM containing 5 mM water-soluble cholesterol (at 5 mM MβCD concentration) to enrich the membrane cholesterol content. Incubation time was 30 minutes at 37°C and 5% CO2 in both cholesterol depletion and cholesterol enrichment experiments [18].
3.2.3 Cell membrane cholesterol quantification

We assumed the membrane cholesterol concentrations for each cholesterol modulation condition to be the same as those reported in our previous report [19]. In that study, membrane cholesterol content for control and cholesterol manipulated HEK cells was quantified using the Amplex Red cholesterol assay. Briefly, this colorimetric assay is based on the reaction of cholesterol with cholesterol oxidase to yield $\text{H}_2\text{O}_2$ which can be detected using the Amplex Red reagent. The membrane cholesterol concentration for the control HEK cells is 7.5±0.8 pmol/µg protein. The membrane cholesterol concentration in cholesterol depleted cells, based on the use of 5 mM MβCD concentration, is 5.7±0.8 pmol/µg protein. In the case of cholesterol enriched cells, the membrane cholesterol concentration, based on the use of 5 mM water-soluble cholesterol at 5 mM MβCD concentration, is 17.3±0.6 pmol/µg protein.

3.2.4 Cell cytoskeletal F-actin disruption

We used Latrunculin-A dissolved in dimethyl sulfoxide (DMSO) (EMD Chemicals, Gibbstown, NJ) as an F-actin disrupting reagent that inhibits F-actin polymerization. Latrunculin-A affects the polymerization of F-actin; specifically a 1:1 molar complex between Latrunculin-A and G-actin forms, which lowers the available amount of free G-actin and disrupts the equilibrium between G- and F-actin [20]. F-actin disruption was performed after each type of cholesterol modulation, and prior to the deformation experiments. Specifically, cells were incubated in DMEM, and Latrunculin-A solution was then added to DMEM to a final concentration of 2 µM.
Cells were incubated in Latrunculin-A containing medium for ≈10 minutes at 37ºC and 5% CO₂.

### 3.2.5 Optical tweezers setup, force calibration, and fluorescence microscopy

We optically trapped 4 μm diameter \(d\) sulfate-modified fluorescent polystyrene microspheres (beads) (F-8858, Molecular probes, Eugene, OR), and used them as handles to induce local cell deformation, in the form of a protrusion, by pulling the cell away from the bead (Figure 3-1). An infrared Nd:YVO₄ diode-pumped solid state laser (1064 nm, Prisma-1064-8-V, Coherent) was used to create the optical tweezers [21]. The optical tweezers setup consisted of an inverted microscope (Nikon Eclipse Ti-DH) containing a 100X oil immersion objective with high numerical aperture (NA=1.49) (Nikon, Apo TIRF) through which the laser beam was passed and converged to form an optical trap. The laser beam was expanded prior to entering the microscope to fill the back aperture of the microscope objective.

We optically monitored the displacement of the bead during the protrusion formation process to measure forces associated with the protrusion formation. The excitation spectrum of the fluorescent beads is between 480 and 590 nm with maximum fluorescence emission at 605 nm. Excitation light from a mercury source (Nikon, Intenslight, C-HGFI) passed through a filter set (Nikon, TRITC, TE 2000) to illuminate the trapped bead. The filter set included an excitation filter (525-560 nm), a dichroic mirror which reflected the fluorescent excitation into the objective to illuminate the trapped fluorescent bead, and an emission filter (570-620 nm) which transmitted the
emitted light from the bead. An emission bandpass filter (Chroma, 605±25 nm) was placed in front of a position sensing quadrant photo-detector (QPD) (QP50-6SD, Pacific silicon sensor, Westlake village, CA) to specifically select the fluorescent emission from the bead.

The fluorescent image of the trapped bead was projected onto the center of the QPD to measure the displacement of the trapped bead from the trapping center. Using an analog-to-digital converter (Wavebook 521, IOTEch, Cleveland, OH), the sum-and-difference output signals (in mV) from the QPD amplifier were digitized and subsequently recorded by WaveView software (WaveView 7.14.16, IOTech). The collection frequency was 66.66 Hz. A charge-coupled device (CCD) camera (Hamamatsu Corp., EM-CCD, C9100/13, Bridgewater, NJ) was used to visualize the objects in the field of view.

The change in QPD output voltage signal represented the displacement of the bead position from the trapping center, which is proportional to an external force such as the force exerted by the cell onto the bead. The output voltage signal of the QPD was calibrated for the external force using the viscous drag force method based on Stokes’ Law [22]. In this method, a known drag force was applied to the trapped bead while recording the resulting differential signal from the QPD. The drag force was generated by driving a piezoelectric translation stage (PZT) (Physik Instrumente, Model P-527.C3, Waldbronn, Germany) at known velocities. The resolution of the piezoelectric stage was 10 nm in x and y directions, and 2 nm in z directions (laser beam propagation direction). The applied force was linearly fit to the output voltage of the QPD ($R^2=0.99$). In this
study, calibration and force measurements were performed at laser power of 350 mW after the microscope objective. There was no evidence of thermally induced structural damages in the living cells at this power. The relationship between the bead displacement and QPD signal was determined by applying known displacements to a coverslip-immobilized bead while recording the QPD voltage signal. There was a linear relationship between the bead displacement and QPD output signal ($R^2=0.97$). We measured both voltage-force and voltage-displacement relationships before each experiment.

### 3.3 Dynamic force measurements

#### 3.3.1 Formation of cell protrusion

A schematic of deformation of a local part of the cell to from cellular protrusions in our experiments is shown in Figure 3-1. Once a bead was optically trapped, using the PZT stage the bead was brought to proximity of a nearby cell, firmly attached to the bottom of the Petri dish, by \( \approx 0.5 \mu m \). We then triggered the PZT to move the cell toward the trapped bead at 10 nm steps until they were in contact, as determined by appearance of a non-zero differential QPD signal. Assuming the indentation depth ($\Delta h$) of a 4 \( \mu m \) diameter microsphere in the plasma membrane to be \( \leq 10 \) nm, the patch radius is estimated as $r_p \leq \sqrt{d.\Delta h}$ or $r_p \leq 0.2 \mu m$ [23]. Therefore, with patch area defined as $A_p = \pi r_p^2$, it was estimated as $A_p \leq 0.126 \mu m^2$.

The bead and the cell were in contact for \( \approx 5-10 \) seconds to achieve plasma membrane-bead attachment. The cell was subsequently moved away from the bead at 1
£m/s, forming a protrusion (Figure 3-1). Cell movement was continued to the extent that the protrusion resulted in separation of the plasma membrane from the cytoskeleton as determined by a sudden reduction in force from its maximum value. Fluorescence imaging of the trapped microsphere eliminated the force measurement artifact that arises under bright-field illumination when the microsphere is in proximity of the cell [23,24].

Figure 3-1 Schematic of a cellular protrusion formed on an adherent living cell. The protrusion formed in response to application of a tensile force (F) by a laser trapped microsphere (bead) in Y direction (drawn not to scale). A quadrant photodetector records the instantaneous displacement of the trapped microsphere from the trapping center for force measurements.

3.3.2 Measurement of cell protrusion length

Length of the cell protrusion ($x_{pt}$) was determined by using the instantaneous piezoelectric stage displacement ($x_{PZT}$), and the transverse displacement of the trapped bead from the trapping center ($x_{bead}$) in the pulling direction (3-1):

$$x_{pt}(t) = x_{PZT}(t) - x_{bead}(t)$$

3-1
The schematic of a bead-cell system is shown in Figure 3-2 wherein the cell and the bead are initially in physical contact prior to formation of the protrusion. This stage is shown by dashed lines on the figure, and is followed by a subsequent stage where a protrusion of length $x_{pt}$ is formed by moving the cell away from the bead by moving the PZT stage over a distance $x_{PZT}$. This stage is shown by solid lines where the bead is displaced by $x_{bead}$ from the trapping center.

![Figure 3-2 Schematic of bead-cell contact system. Dashed lines show initial cell-bead contact where there is no external force applied on the bead, and the solid lines show the bead-cell system once the cell is moved away from the trapped bead by $x_{pzt}$ when the bead is displaced from the trapping center by $x_{bead}$ and the length of the protrusion is $x_{pt}$.](image)

**3.3.3 Physical model and data analysis**

The instantaneous force-length plots ($F-x_{pt}$) were obtained from optical tweezers experiments under constant retraction velocity. We examined three standard viscoelastic models (Voigt, Maxwell, and Kelvin bodies) to fit the measured force-length profiles of the cell protrusion [25–27]. We present an illustrative force-length profile where the data are fit with Maxwell model in Figure 3-3.
Figure 3-3 Force-length plot during protrusion formation. An example force-length (F-x_{pt}) plot associated with protrusion formation fit with Maxwell model (blue solid circles). $F_{max}$ is the maximum force and $l_{pt-max}$ is the maximum protrusion length at plasma membrane-cytoskeleton separation.

According to Figure 3-3, force increases with protrusion length until reaching a maximum value ($F_{max}$) before the plasma membrane becomes separated from the underlying cytoskeleton. Among the three models, the Maxwell body provided the best fit to the data in our experiments with the governing equation as [25]:

$$\dot{f}(t) + \frac{k}{\eta} f(t) - k\ddot{x}(t) = 0$$  \hspace{1cm} 3-2

where $f(t)$ is the instantaneous force that induces cell protrusion, and $k$ and $\eta$ are the spring stiffness and dashpot coefficient of viscosity of the Maxwell body, respectively.

With $t = \frac{x}{V}$ and $f(x=0)=F_0$, Equation 3-2 can be converted to Equation 3-3 as:

$$f(x_{pt}) = \eta V + (F_0 - \eta V) \exp(-\frac{k}{\eta V} x_{pt})$$  \hspace{1cm} 3-3

$V$ is the deformation (pulling) rate. We fitted Equation 3-3 to the measured force-length profiles to extract the $k$ and $\eta$ values of the Maxwell model. We excluded from our
analyses the cases where there was a rupture event during the protrusion formation experiments as evidenced by an abrupt force drop in the force-length profiles.

### 3.3.4 Reverse pull experiments

In the reverse pull experiments, a local part of the cell was elongated using the trapped microspheres as handles at 1 µm/sec by 3 µm as “pull” process, and then immediately pushed back in the reverse direction at 1 µm/sec by -3 µm as “push” process. The reverse pull experiments were performed without delay between pull and push processes. A force-time plot of an example reverse pull experiment is shown in Figure 3-4A, representing two different regions: 1- pull (AB segment) where the cell is moved away by 3 µm at 1µm/sec, and 2- push (BC segment) where the cell is moved back by 3 µm at 1µm/sec. At the end of the push process at state C, force value is slightly smaller than that of before pull process at state A. This could be due to the local protrusion formed during the pull process which pushes against the bead at the end of push process (state C). The cell restored its shape as the protrusion disappeared in several seconds and the force reached state D where the value of the force was nearly the same as that at the beginning of the pull process at state A. Inserts in Figure 3-4A, show the corresponding $x_{PZT}$, $x_{bead}$, and $x_{pt}$ in that experiment. The force-time profiles of the reverse pull experiments were converted to force-length profiles based on the method described earlier in the Materials and Methods, and the one corresponding to the force-time plot in Figure 3-4A is presented in Figure 3-4B.
Figure 3-4 Hysteresis effect in cellular protrusion.

Figure 3-4A, shows an example force-time plot associated with reverse-pull experiments involving two subsequent “pull” and “push” processes. The inset shows the $x_{\text{bead}}$, $x_{\text{PZT}}$, and $x_{\text{pt}}$ plots corresponding to bead's displacement, piezoelectric stage displacement and local deformation length in pull and push processes of a reverse-pull experiment. Figure 3-4B shows the corresponding force-displacement plot of
the force-time plot shown in Figure 3-4A. The plot indicates the existence of a hysteresis effect as the lag of push curve below the pull one during the reverse-pull experiments.

### 3.3.5 Statistical analysis

We used a standard two-sample student’s t-test with unknown variances of the two datasets in our statistical analysis of the data. Statistical significance was accepted if the $P$-value was <0.05.

### 3.4 RESULTS

#### 3.4.1 Maximum protrusion force ($F_{\text{max}}$)

The values of $F_{\text{max}}$ in control HEK cells and cells under different cholesterol manipulation conditions are shown in Figure 3-5, for cell with intact F-actin and F-actin disrupted cells. Cholesterol depletion resulted in significant increase in the values of $F_{\text{max}}$, whereas cholesterol enrichment decreased it. Specifically, mean±standard deviation (s.d.) values of $F_{\text{max}}$ for control HEK cells was 130±28 pN (n=16), and significantly increased to 218±32 pN (n=7) when cell were incubated in DMEM containing 5 mM MβCD for cholesterol depletion. In response to incubation of the cells in DMEM containing 5 mM cholesterol-MβCD for cholesterol enrichment, the mean±s.d. values of $F_{\text{max}}$ significantly decreased to 96.72±29 (n=13) ($P<0.05$).

Disruption of F-actin by Latrunculin-A resulted in a statistically significant reduction in $F_{\text{max}}$ values from 130±40 to 72.44±25 pN in control cells. Consistent with control cells,
values of $F_{\text{max}}$ decreased in response to disruption of F-actin in cholesterol manipulated cells; specifically from 218±45 to 90±26 pN ($P<0.05$) in cholesterol depleted cells, and from 96.72±29 to 55±28 pN ($P<0.05$) for the cells under cholesterol enrichment conditions. Comparing the forces among the cells with disrupted F-actin, we found no statistically significant difference between control and cholesterol enriched cells, nor between control and cholesterol depleted cells.

![Figure 3-5 Maximum force of protrusion. Maximum protrusion force ($F_{\text{max}}$) versus membrane cholesterol concentration, for cells with intact F-actin and disrupted F-actin. The $F_{\text{max}}$ is the force at plasma membrane-cytoskeleton separation.](image)

### 3.4.2 Maximum protrusion length ($l_{\text{pt-max}}$)

The maximum protrusion length ($l_{\text{pt-max}}$) is the length above which the plasma membrane becomes separated from the underlying cytoskeleton. For control cells, this length was 1.95±0.3 µm, and decreased upon cholesterol depletion to 1.1±0.3 µm, and elevated to 2.5±0.28 µm in response to cholesterol enrichment. The $l_{\text{pt-max}}$ data are shown in
Figure 3-6. The Disruption of F-actin in control cells increased the $l_{\text{pt-max}}$ to 2.68±0.33 µm, and to 2.45±0.35 in cholesterol depleted cells. The value of $l_{\text{pt-max}}$ in cholesterol enriched cells after disruption of F-actin was 2.75±0.3 µm.

![Maximum protrusion length ($l_{\text{pt-max}}$). Maximum length of protrusion versus membrane cholesterol concentration, for cells with intact F-actin and disrupted F-actin.](image)

**3.4.3 Stiffness and Viscosity**

The values of stiffness and coefficient of viscosity associated with the local deformations were obtained by fitting the force-displacement plots with the Maxwell model (Eq. 3) and the results are presented in Figure 3-7 and Figure 3-8, respectively. The mean±s.d. value of the stiffness ($k$) for control HEK cells is measured ≈306±92 pN/µm (Figure 3-7). There is a statistically significant increase in stiffness to 550±120 pN/µm for cells incubated in DMEM containing 5mM MβCD for cholesterol depletion.
Figure 3-7 protrusion stiffness. Protrusion stiffness versus membrane cholesterol concentration, for cells with intact F-actin and disrupted F-actin.

The stiffness parameters decreased significantly to 105±75 pN/μm for cells incubated in DMEM contacting 5 mM cholesterol-MβCD for cholesterol enrichment.

Figure 3-8 Protrusion viscosity. Viscosity associated with protrusions versus membrane cholesterol concentration, for cells with intact F-actin and disrupted F-actin.
Disruption of the F-actin resulted in significant decrease in the values of the stiffness for normal and cholesterol depleted cells to $\approx 131\pm 72$ and $204\pm 45$ pN/µm, respectively ($P<0.05$).

The values of viscosity coefficient ($\eta$) were $\approx 183\pm 32$ and $103\pm 36$ pN.s/µm for control and cholesterol-enriched cells, respectively (Figure 3-8), and did not change significantly in cholesterol-depleted cells with respect to normal cells. Disruption of F-actin polymerization significantly decreased the viscosity values compared to cells with intact F-actin, specifically to $82\pm 26$ and $43\pm 23$ pN.s/µm in control and cholesterol-enriched cells and to $116\pm 29$ pN.s/µm in cholesterol depleted cells.

3.5 DISCUSSION

3.5.1 Plasma membrane composition modulates cellular protrusion formation

Membrane lipid composition was modulated by manipulating the membrane cholesterol content and the formation of protrusions was analyzed for cholesterol depleted and cholesterol enriched cells. Membrane cholesterol manipulation modulated the protrusion formation and elongation in cholesterol treated cells; specifically, cholesterol depletion required more forces for formation of protrusions associated with shorter protrusions, while protrusions were formed by less force at longer lengths under cholesterol enriched conditions. The results indicate the effects of membrane cholesterol content on protrusions at least within the cholesterol concentration examined. The modulation of protrusion force and length upon membrane cholesterol manipulation
could be arising from the changes in mechanical properties of the plasma membrane in cholesterol treated cells.

We have probed the mechanical properties of the plasma membrane under different cholesterol modulation conditions, and the results revealed significant effects of cholesterol on plasma membrane mechanical properties [21]. Specifically, the membrane stiffness significantly increased upon cholesterol depletion and decreased by cholesterol enrichment in cell with intact F-actin network. The increased membrane stiffness could be manifested as more force required for protrusion formation in our experiments under cholesterol depletion, and similarly less stiffness in cholesterol enriched cells corresponds to less protrusion forces. The changes in membrane mechanical properties were also recently propose to be an important factor in the formation of microtubule-based protrusion where cholesterol depletion induced by MβCD inhibited the formation of protrusions [15]. The plasma membrane tension is another important parameter in protrusion formation, and measured as a parameter dependent upon membrane cholesterol concentration in our experiments, within the examined cholesterol range. Specifically, the membrane tension was elevated by cholesterol deletion and decreased as a result of cholesterol enrichment. The apparent membrane tension measured for control cells and cholesterol manipulated cells, for both cell with intact and disrupted f-actin, are shown in Figure 3-9.

The higher membrane tension in cholesterol depleted cells is a potential contributing factor in high forces required for formation of protrusions in so is the lower tensions in cholesterol enriched cells. The recent studies revealed that increased cortical
tension in migratory cells reduced the lamellipodia extension in migratory cells, and the size of lamellipodia significantly increased under low cortical tension conditions [1].

**Figure 3-9** Apparent membrane tension. Apparent membrane tension versus membrane cholesterol concentration for cells with intact F-actin and F-actin disrupted cells.

Since the protrusion formation is associated with deforming a part of the membrane to a curved shape, curvature elastic modulus (bending stiffness) is important in protrusion formation. The recent studies revealed that high membrane curvature demonstrated a membrane curvature-dependent inhibitory effect on actin elongation, in that higher curvature decreased the number of filaments pointing toward the membrane resulting in decreased efficiency of actin elongation [9,14]. Besides the geometrical effects of curvature on protrusion formation, the bending stiffness could be another contribution factor in protrusions as it resists membrane deformation. We measured the membrane bending stiffness via tether formation experiments, and found it remained
almost unchanged upon cholesterol manipulation in our experiment within the concentration examined. This observation indicates that membrane bending modulus may not be a significant contributor to the observed changes in protrusion formation under different cholesterol manipulation conditions.

3.5.2 Effects of membrane composition on protrusion is cytoskeleton dependent

The observed reduction of $F_{\text{max}}$ for control cells upon disruption of F-actin is indicative of the role of actin filaments in protrusion formation. The reduction in $F_{\text{max}}$ could be either due to fewer actin filaments-plasma membrane connections, or weakened structural rigidity of actin network within the F-actin disrupted cells, both resulting in less force required for formation of protrusion with a corresponding reduction in $F_{\text{max}}$.

To investigate the relationship between the presence of an intact F-actin network and membrane cholesterol content, we formed protrusions in cholesterol depleted and cholesterol enriched cells after disrupting the cytoskeleton F-actin network. Interestingly, disruption of F-actin diminished the disparity observed between the values of $F_{\text{max}}$ in cholesterol manipulated cells and control cells. This indicates that membrane cholesterol content is only significant in the presence of an intact F-actin network in the concentration range examined, suggesting that F-actin-cytoskeleton interactions have more dominant effect than membrane cholesterol content on protrusion formation. The effects of membrane-cytoskeleton interactions on protrusions could be manifested as the membrane-cytoskeleton adhesion energy which is altered upon membrane cholesterol manipulation such that cholesterol depletion elevated the adhesion energy rather
cholesterol enrichment had corresponding reducing effects as reported in our previous paper. Also, the protrusion stiffness \( (k) \) decreased significantly upon disruption of F-actin in control cells, showing the importance of the membrane-cytoskeleton associations on protrusion stiffness regardless of membrane cholesterol manipulations. Consistently, in intact F-actin cells membrane cholesterol modulations of cells was not associated with significant alteration in stiffness values of cholesterol manipulated cells with respect to control cells with disrupted F-actin.

### 3.5.3 Effects of cholesterol are beyond the plasma membrane in protrusions

Comparing the protrusion stiffness values \( (k) \) of control cells with intact F-actin \((\approx 306\pm 92\ \text{pN/µm})\) with that of plasma membrane \((\approx 2\ \text{pN/µm} \) for control HEK cells [21], 3.4 \text{pN/µm} for jurkat cells [27], and 3.7 \text{pN/µm} for outer hair cells [22]), the noticeably greater values of protrusion stiffness implies the major contribution of cytoskeleton in these protrusions. Therefore, the enhancing effect of cholesterol depletion on \( k \) values is possibly attributed to its effects on the cytoskeleton F-actin within the protrusion (comparing value of \( k \) under cholesterol depletion \( \approx 550\pm 120 \) to the plasma membrane stiffness under the same condition \( \approx 3.24\pm 0.10\ \text{pN/µm} \)). Likewise, values of \( k \) under cholesterol enrichment condition \( \approx 105\pm 75 \) are greater than the plasma membrane stiffness under the same cholesterol manipulation condition \( \approx 0.3\ \text{pN/µm} \).

The observed effects of cholesterol on underlying cytoskeleton in protrusions is in harmony with the observed stiffening effect of membrane cholesterol depletion on bovine aortic endothelial cells where greater aspiration pressures were required to locally
deform cells compared with control cells [28,29]. Yet, neutrophils with elevated membrane cholesterol level showed enhanced whole cell deformability in neutrophil-microbead collision experiments [30]. The values of stiffness associated the cell protrusions in this study are close to the values of ≈311±180 pN/µm previously reported for HEK cells [23], ≈190 pN/µm for jurkat cells [27], and 150-300 pN/µm for neutrophils [31,32].

Disruption of F-actin resulted in significant reduction of $k$ values in control cells upon F-actin disruption (≈131±72), presumably due to attenuated contribution of F-actin. Latrunculin-A specifically disrupts cytoskeleton F-actin by binding to monomeric G-actin to inhibit F-actin assembly without impacting cell viability or interfering with microtubules and intermediate filaments [20,33–35]. Therefore, the non-zero values of $k$ in cells with disrupted F-actin indicate the effects of other cytoskeletal filaments, microtubules and intermediate filaments, on the observed resultant protrusion stiffness. The presence of a statistically significant difference between $k$ values of cholesterol depleted and cholesterol enriched cells is indicative of the contribution of the effects of cholesterol manipulation on membrane mechanical properties.

### 3.5.4 Viscoelastic behavior of protrusion formation

The force-length profiles associated with protrusion formation demonstrated a transition from a linear to a nonlinear relationship (Figure 3-3) in response to the application of a tensile force over a patch area of the cell and retracting it away from the cell at a constant pulling rate. This is a consistent observation with those reported in cell micropipette aspiration experiments [36,37], plasma membrane indentation experiments
with AFM [38,39], tether formation experiments [23,31,32,40–42], and recent modeling studies performed on cell surface protrusions and tether formation [7,43,44]. The transition from a linear to a nonlinear regime is arising from breakage of the bonds between plasma membrane and cytoskeleton and subsequent flow of membrane lipids into the protrusion [40], with additional contributions due to plastic deformations of the plasma membrane-cytoskeleton system during deformation. Lateral segregation of lipids from integral membrane proteins, shearing, breaking and extraction of molecular bonds during deformation as well as the viscous effects could be other possible sources of the observed nonlinearity [45–47].

The force-length response of surface protrusions were well fit by a Maxwell model for intact and F-actin disrupted cells indicating the viscoelastic behavior of these protrusions in our experiments within the cholesterol concentration examined. To further investigate the viscoelasticity of the protrusions, the hysteresis effect and the force relaxation behavior of the protrusions were examined, as these two phenomena were observed concomitantly in neutrophil surface protrusions showing that the surface protrusions in those experiments are essentially viscoelastic [42]. The hysteresis effect was manifested by the hysteresis loop area enclosed within pull and push processes in the reverse-pull experiments (Figure 3-4B).

The hysteresis effect was quantified by using the hysteresis loop area to determine the normalized energy loss \( W_{loss} \) in protrusion formation as \( (W_{pull} - W_{push})/W_{pull} \), where \( W_{pull} \) and \( W_{push} \) are the energies associated with pulling and pushing the protrusions, respectively. The \( W_{pull} \) and \( W_{push} \) energy parameters were determined as
the area under the force-length curves during the associated processes. To discern the effects of cytoskeleton on the hysteresis effect, the experiments were repeated for F-actin disrupted cells, resulting in contraction of hysteresis loop area and decrease in $W_{\text{loss}}$ (data shown in Figure 3-10-Top), presumably due to the weakened plasma-membrane cytoskeleton interactions and diminished effects of cytoskeletal. The lessened plasma membrane-cytoskeleton interactions upon disruption of F-actin could be stemming from the breakage of the bonds between transmembrane proteins and the cytoskeleton, diminished direct connections of membrane to the cytoskeleton [48], or loss of membrane bonds to the cytoskeletal F-actin filaments via phosphatidylinositol bi-phosphate (PIP2) [49]. Further observations of the decreased $W_{\text{loss}}$ values in cholesterol enriched cells and the increase of $W_{\text{loss}}$ under cholesterol depletion conditions suggests a correlation between the hysteresis effect and the plasma membrane-cytoskeleton associations in that stronger interaction could be associated with more pronounced hysteresis effect and weaker interactions with less hysteresis. These are in concert with the observations in lipid vesicles with no cytoskeletal components and membrane-cytoskeleton interactions and in neuronal growth cones with weak membrane-cytoskeleton adhesion, marked by their low $F_{\text{max}} \approx 8$ pN [45] compared to that of in other cells such as $\approx 130$ pN in HEK cells [21]. Neuronal growth cones exhibited no hysteresis effect in tether pulling experiments in that “out” experiments were not different from the “back” experiments, while using similar approach in red blood cells with relatively strong membrane-cytoskeleton interaction $F_{\text{max}} \approx 50$ pN, the hysteresis effect was observable [47,50,51]. In a more recent investigation, using a “loading” and “unloading” approach similar to the “pull” and
“push” processes in the present study, the hysteresis effect was observed in human neutrophils ($F_{\text{max}} \approx 65$ pN) [42]. In that study, $W_{\text{loss}}$ estimated $\approx 53\pm 15\%$ in control experiments, which is close to the measurement of $\approx 67\pm 6\%$ for $W_{\text{loss}}$ in control HEK cells in our experiments. In vesicles, there is no hysteresis effect reported in such “push” and “pull” experiments to our knowledge, and nor the hysteresis was observable in tether forces, and angle between two elongated tethers in tube coalescence/splitting cycles performed on giant unilamellar vesicles [52].

To examine the presence of force relaxation during protrusion formation, and discern if protrusion formation was accompanied by both hysteresis and force relaxation effects, the PZT movement was stopped after the pull process in reverse pull experiments in order to examine force relaxation. The protrusion force began to relax exponentially once the cell movement was stopped and continued until reaching an equilibrium value (Figure 3-10-Bottom). The association of hysteresis effect with force relaxation in protrusion formation in our experiments supported the viscoelastic behavior in these protrusions, consistent with previous observations in surface protrusions of neutrophils [42].
Figure 3-10 Energy loss Protrusion formation. Top: The ratio of energy loss per total energy during reverse puling experiments versus membrane cholesterol concentration, for intact and disrupted F-actin cells. Bottom: Force relaxation in a reverse pull experiment. Inset shows the exponential force decay between push and pull experiments starts once the pull process is halted and continues until reaching an equilibrium value.

Another contributing factor to the observed hysteresis effect might be the transition point between linear and nonlinear regimes. As the stiffness associated with cellular protrusion becomes smaller, the force-displacement response turns to a more linear one with less nonlinearity (Figure 3-11, left), while the greater stiffness values shifts this
transition point towards higher force values and lower displacement region, resulting in a prolonged nonlinear regime. While changes in the stiffness seems to affect the low-displacement regime of the force-displacement profiles, changes in the coefficient of viscosity influences the high-displacement regime; i.e. the increase in viscosity moves the transition point to the higher forces and higher displacement region (Figure 3-11, Right).

![Figure 3-11 Protrusion formation behavior predicted by Maxwell model. The effects of changes of stiffness (Left) and viscosity (Right) on behavior of local deformations, predicted by Maxwell model. The higher stiffness values shifts the transition between linear and nonlinear regime to lower displacements, similar to the case in decreased values of viscosity.](image)

The viscosity coefficient seems to be not effective on the force-displacement behavior at small displacements (<100 nm) based on Figure 3-11,Right, where it shows that at lower displacements the changes in the viscosity are not accompanied by a change in force. Cholesterol depleted cells demonstrated higher stiffness values and unchanged coefficient of viscosity in our experiments, shifting the transition point towards lower displacement regime resulting in longer nonlinear regime. Cholesterol enrichment yielded decreased values of stiffness and viscosity, both of these parameters shift the transition point toward the higher deformation lengths, and hence make the effects of nonlinearity
less pronounced. Similarly, cells with disrupted F-actin with decreased stiffness and viscosity showed smaller hysteresis effect. These observations suggest a correlation between the transition point and the hysteresis effect in our experiments such that transition point in the shorter displacement regime is associated with greater hysteresis effect and in the longer regime to lower hysteresis.

Other common models of cell viscoelasticity are based on power-law relationships for the cell complex modulus vs. frequency [53–55]. The power law models are suited for cases of probing cells under different frequencies (from $10^{-3}$ Hz [53] up to $10^5$ Hz [54]). In our analysis, we focus on a snapshot of time since the pulling times are nearly constant around 3 sec (a timescale relevant to tether formation experiments [21–23,56]), and the Maxwell model became effective in the description of membrane-cytoskeleton behavior on the considered early stages of the tether pulling experiment.

### 3.6 Conclusions

Protrusion formations were studied in adherent HEK cells with an optical tweezers-fluorescence microscopy technique. Modification of the membrane composition, specifically membrane cholesterol content, modulated the protrusion formations indicating the effects of plasma membrane on protrusions. Specifically, cholesterol depletion increased the maximum protrusion force, protrusion stiffness, and decreased the maximum protrusion length. Cholesterol depletion demonstrated less maximum protrusion force, and less protrusion stiffness, while higher maximum protrusion length. The protrusion formations were associated with
energy loss during formation, and the membrane cholesterol content modulated the percent of energy loss during formation of protrusions. Specifically, depletion of cholesterol was associated with more energy loss and cholesterol enrichment was associated with decreased percent of energy loss. The protrusions should viscoelastic behavior, first evidenced by the applicability of Maxwell viscoelastic model to the force-length plots, and second by the observations of hysteresis effect and force relaxation in these protrusions. The cytoskeleton F-actin demonstrated significant effect on the observed effects of the cholesterol on the protrusion mechanics, as the observed disparities between cholesterol depletion and enrichment were diminished upon disruption of f-actin.

3.7 References


Chapter 4

4. Conclusion

Cellular mechanics can be studied at different levels, including at cell plasma membrane level, at the whole cell level, and also at an intermediate range between plasma membrane and whole cell which is in the form of cellular protrusions involving deformation of a local part of the cell. In contrast to synthetic vesicles containing only membranes, the presence of cytoskeleton in living cells gives rise to the complexity involved in their mechanical properties. The presence of the cytoskeleton and its association with plasma membrane is important in many cellular processes. Mechanical properties of the plasma membrane, and mechanical properties of cytoskeleton, as well as local plasma membrane-cytoskeleton mechanical associations, are important in regulating the mechanical properties of the cell and cellular shape change and mechanotransduction in living cells.

We have used a modified optical tweezers technique by combining optical tweezers with fluorescence microscopy to create an optical force sensor in order to study the properties of the cell plasma membrane, and its local interactions with the underlying cytoskeleton. The combined optical tweezers-fluorescence microscopy yielded the force
measurement resolution as small as ≈5 pN and position detection resolution in the order of ≈20 nm. The membrane mechanical properties are investigated by pulling plasma membrane nanotubes (tethers) from adherent living human embryonic kidney (HEK) cells using fluorescent microspheres as pulling probes. Time-resolved tether force plots are obtained by monitoring the displacement of the microspheres in response to application of external forces on the microsphere. The plasma membrane tether force plots shows three different regions in response to the application of an external axial tensile force yielding information about plasma membrane mechanical properties as well as membrane-cytoskeleton interactions.

In order to investigate the effects of membrane composition on membrane nanomechanical properties, the membrane lipid composition is modulated in our experiments. Specifically, the membrane cholesterol content of the cell plasma membrane is modified and the membrane mechanical properties are quantified by tether forming experiments from cells with the plasma membrane cholesterol levels higher than that of in normal cells (cholesterol enrichment condition), and also for cells with the cholesterol content lower than the normal cells (cholesterol depleted conditions). The mechanical properties of the plasma membrane are quantified and correlated with membrane cholesterol content for serial concentrations of membrane cholesterol.

The changes in membrane cholesterol content showed significant effects on membrane mechanical properties. The depletion of membrane cholesterol content was associated with higher membrane equilibrium forces, and higher membrane stiffness and the membrane viscosity showed a tendency to decrease upon depletion of the membrane
cholesterol content. Elevating membrane cholesterol content resulted in lower equilibrium forces, lower stiffness, and higher membrane viscosity. The membrane tethers were elongated by different length and the stiffness of the membrane is calculated by measuring the membrane tether force at different tether lengths and using the tether force-tether length relationship. Lowering the membrane cholesterol concentration by 25% increases the membrane stiffness by approximately a factor of 2.6, whereas enriching the membrane cholesterol concentration by two-fold lowers the membrane stiffness nearly four times. The time course of force relaxations in tethers pulled from living cells revealed two phenomena in the force relaxation. First, the force relaxation starts with a rapid reorganization of membrane lipids along the tether and our measurement showed that this step is not affected by membrane cholesterol concentration. This first step is followed by a second rapid step wherein the lipids flow from the cell body plasma membrane into the membrane tether due to tether force. The time course of lipid flow showed dependency on membrane cholesterol, in that the cholesterol enrichment increased the time course of force relaxation (lipid flow) consistent with higher viscosities upon cholesterol enrichment. Cholesterol depletion decreased the time course of lipid flow which is a consistent observation with lower viscosity values under cholesterol depletion.

Tether geometry (diameter) also analyzed in addition to tether force measurements, yielded quantification of membrane bending stiffness and plasma membrane-cytoskeleton adhesion energy as a measure of local membrane-
cytoskeleton interactions. The results indicated the independency of plasma membrane bending modulus to membrane cholesterol content. Membrane cholesterol concentration significantly modulated plasma membrane-cytoskeleton adhesion energy. Specifically, lowering the membrane cholesterol concentration by 25% increased plasma membrane-cytoskeleton adhesion energy by approximately a factor of 3.5, and enriching the membrane cholesterol concentration by two-fold lowers the membrane stiffness nearly four times.

Our measurements at cellular plasma membrane leveled insights into understanding the effects of cholesterol on some biological phenomena involving the cellular shape change, specifically the effects of cholesterol on hearing. Comparing our results here for HEK cells with those of at the OHC plasma membrane, plasma membrane of the OHCs behave as a membrane with relative low level of cholesterol membrane. The findings of tendency to lower effective viscosities and faster time course of force relaxation under cholesterol depletion condition in HEK cell membranes suggest more rapid mechanical response of the membrane in response to an external force. These results are consistent with our recent observations, which showed an increase in electromotile response of the cochlear amplifier upon depletion of cochlear cholesterol content in guinea pigs. Also, higher effective viscosities and greater time course of force relaxation suggest slower mechanical response of membrane. These observations provide insight to understanding the correlation between the previously observed increase of hearing
thresholds and hypercholesterolemia in rabbits, chinchillas, guinea pigs, and human subjects.

The cytoskeleton F-actin integrity showed significant effect on the plasma membrane mechanical properties of the cholesterol treated cells. The opposing effects of cholesterol on membrane mechanics were diminished upon disruption of F-actin demonstrating that the effects of plasma membrane-cytoskeleton connections are more significant than the effects of cholesterol on membrane mechanics. This indicates that as a plasma membrane-cytoskeleton composite, the mechanical properties of the plasma membrane and cytoskeleton needs to be studied together and emphasized on the importance of the plasma membrane-cytoskeleton interactions on plasma membrane mechanics.

Plasma membrane tether formation is preceded by the cellular protrusions at cell surface. The protrusion formation and protrusion mechanics are demonstrated to be dependent upon membrane cholesterol concentration. Lower cholesterol concentration values were associated with shorter protrusions and higher maximum deformation forces whereas cholesterol enrichment showed the longer protrusions and less maximum forces of protrusions. Similar to the observations in tether studies, the effects of cholesterol on protrusion was diminished upon disruption of cytoskeleton F-actin. This indicates that the effects of membrane cholesterol content on cellular protrusions are effective in the presence of an intact F-actin. The cellular protrusions demonstrated viscoelastic properties in that their force-length behavior in response to an external tensile force was
associated with force-relaxation and hysteresis effect. Taken together, plasma membrane and cytoskeleton are mutually contributing to the viscoelastic behavior of the cellular protrusions. Effects of plasma membrane mechanical properties are relatively minor in comparison with cytoskeleton effects, however surprisingly modulation of membrane mechanics resulted in significant changes in protrusion mechanics. Manipulation of membrane composition by membrane cholesterol depletion and enrichment showed dissimilar effects on various mechanical properties of protrusion while disruption of F-actin diminished the observed disparities. The observations suggest a correlation between membrane and protrusion mechanical properties through modifications in plasma membrane-cytoskeleton connections and underlying cytoskeleton properties in response to changes in membrane mechanical properties. The results of the study provide insight into understanding the membrane-induced modulations of cellular protrusions and mechanotransduction.