Viscoelastic behavior, deformation, and rupture of cells

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VISCOELASTIC BEHAVIOR, DEFORMATION, AND RUPTURE OF CELLS

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Glory be to God, the Creator of heaven and earth.

Dedicated to my ever loving and supportive God-sent wife, Deng Huiling.

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Abstract

In this study, the effects of tensile stress on single cells were examined at both the cellular and subcellular levels in terms of global and local deformation, based on the application of micropipette aspiration. At the cellular level, the global deformation behavior of human mesenchymal stem cells (hMSCs) was studied in relation to temperature effect and the structural integrity of actin filaments. hMSCs were found to possess the characteristics of a viscoelastic solid material which can be described by a sudden increase in length at the initial aspiration time before gradually reaching an equilibrium length over time. In addition, there were three other types of non-typical viscoelastic behaviors. Based on the three-parameter viscoelastic model, the instantaneous and equilibrium Young's moduli at 20°C were calculated as 886 ± 289 Pa and 372 ± 125 Pa, respectively while the apparent viscosity of hMSCs was recorded as 2700 ± 1600 Pa.s. By disrupting the F-actin filaments of hMSCs using cytochalasin D with a concentration up to 20 μ M, the stiffness of hMSCs decreased drastically by up to 84% and the viscosity experienced a large increase by up to 255%. Compared to the results obtained at 20°C, the stiffness of hMSCs at 37°C was found to be significantly reduced by 42 – 66% while there was a 95% increase in viscosity. These findings demonstrate that the stiffness and viscosity of hMSCs are dependent on the structural integrity of F-actin filaments and temperature.

At the subcellular level, the local deformation of microtubules during micropipette aspiration was investigated with the aid of TubulinTracker™ (TT), a fluorescent dye for labelling microtubules. Results showed that microtubules buckled and straightened at four different regions of single NIH 3T3 fibroblasts, indicating that cells exhibit anisotropic behavior. To establish the relationship between the global and local deformations quantitatively, the change in the average bending energy of microtubules over time was calculated over the work done by the aspiration pressure. The percentage of this ratio was found to be not more than 0.001%, signifying that the elastic energy transferred from the work done is largely used to deform other cell components such as nucleus, actin and intermediate filaments and to detach cells from the substrate. Surprisingly, the change in bending energy of microtubules over time was not dependent on the magnitude of applied pressure. This provides a strong

evidence that microtubules are capable of rearranging themselves to maintain cell structural integrity through a negative feedback mechanism induced by external tensile stress.

Finally, the rupture mechanics of plasma membrane were also studied at the subcellular level. Single NIH 3T3 fibroblasts experienced rupture at the cell apex over a large range of membrane tension. Three different types of rupture modes were found. The measured mean tensile strengths of plasma membrane were 3.83 ± 1.94 and 3.98 ± 1.54 mN/m for control cells and cells labeled with TubulinTracker™, respectively. Interestingly, the plasma membrane strength of fibroblasts can be described by Weibull distribution. Based on the Weibull distribution plots, the probability for TT-stained cells and nucleus-aspirated cells to rupture were found to be lower than control cells. This shows that material homogeneity affects the strength of membrane. Assuming cell membrane is a composite material consisting of lipids and transmembrane proteins (TMPs), the average TMP-lipid strength was estimated to be 2.64 ± 0.64 mN/m by using the result of the average plasma membrane strength and the random sampling of volume ratio of TMPs in cell membrane. It was showed that the variation in TMP-lipid strengths gave rise to the Weibull distributed plasma membrane strength. Simulations using finite element models at the initial, necking and rupture stages were performed to provide a qualitative analysis of stress distribution within a cell. Based on the experimental results, a quantitative rupture model was proposed to establish the relationship between global and local deformation in terms of the percentage ratio of critical membrane surface energy and work done by aspiration pressure. Large amount of energy (up to 99%) was transferred from work done by aspiration pressure to deform cell membrane. The membrane toughness for 3T3 fibroblasts was found to be $0.53 \pm 0.47 \times 10^{-3}$ J/m², which is comparable to lipid vesicles.

Chapter 1 Introduction

In a normal living body of a human or an animal, cells experience both external and internal mechanical forces that are generated within the cells such as the actin-myosin contractile stress [1]. The examples of externally applied stresses include the shear stress exerted on endothelial cells through blood flow, tensile and compressive stresses experienced by smooth muscle cells and bone cells. The deflection of hair cells located in the inner ear by acoustic waves entering the ear is another good example. It has been found that cell fate such as proliferation, migration, differentiation and apoptosis very much depends on the cell mechanical properties and the external mechanical stimuli that are exerted on the cells [2, 3]. For example, a recent study revealed that mechanical stress generated by muscle is necessary for the development of joints in mouse embryos [4]. These findings have led to the need to understand the effects of mechanical stresses on cells and their corresponding response, which defines the field of cell mechanics. As a result, many methods such as micropipette aspiration, optical tweezer, atomic force microscopy (AFM), micropatterned substrates and magnetic twisting cytometry have been employed to characterize the mechanical properties of cells under various conditions. These methods for studying living cells have been extensively reviewed in Refs. 5-7.

An attractive outcome of studying cell mechanics is found in the possibility of establishing the connection in relating the change in mechanical properties of cells to certain diseases. Many studies have proven that cancerous and disease-inflicted cells possess significantly different stiffness as compared to healthy cells. For example, red blood cells (RBCs) infected with malaria parasite appears to be stiffer than healthy RBCs [8]. In contrast, cancerous human breast epithelial cells were observed to more deformable or fluidly as compared to healthy cells [9]. The variation in cell stiffness may be directly linked to the change in cytoskeleton content as cancerous fibroblasts were found to contain 40% lesser actin cytoskeleton [10] which may explain the lower contractility as compared to healthy fibroblasts [11]. Besides, the presence of structural defect in nucleus due to genetic disorder, such as seen in Hutchinson–Gilford progeria syndrome, led to a brittle-like nucleus as the nuclear lamina failed to rearrange swiftly under mechanical stress [12]. A comprehensive understanding of the mechanical

signature in various diseases would eventually bring about major breakthroughs in the development of new strategies and diagnostic approaches to detect and treat cancerous or diseased-inflicted cells.

The external mechanical stimuli exerted on a cell are sensed by the cell membrane and transmitted through the cell cytoskeleton to the nucleus where changes to the DNA and protein conformation would trigger and alter gene expression of the cell [2, 13]. The process of converting mechanical signals into biochemical signals is known as mechanotransduction. Many reviews on the development of the field of mechanotransduction and cell mechanics are available in Refs. 14-21 and 13, 22-27, respectively. However, mechanotransduction pathways are yet to be fully understood due to the complexity involved at different spatial and temporal scales. Thus, it is necessary to dissect the study of cell mechanics into separate scales or levels. Apart from probing the effect of mechanical stimuli at cellular level, cell mechanics study has to be conducted at subcellular level for it is a well-established fact that internal microstructure determines the overall or macrostructure properties and behavior of a material. The investigation at subcellular level will provide crucial information in understanding the intricate roles of various cytoskeleton components, such as microtubules and actin filaments, in remodelling and maintaining the cell structure under the influence of mechanical force.

In order to analyze the response of cell and the stress propagation within its structure due to the application of external force quantitatively, computational modeling is needed. Depending on the length scales of interest, there are two different approaches, namely the continuum and nanoscale approaches [27]. At the cellular level where the smallest length scale of interest is larger than the internal structure such as the cytoskeleton and proteins, continuum-based computational models such as finite element model are used to predict and interpret the results obtained from a specific experiment such as micropipette aspiration. On the other hand, nanoscale approaches, which involve the use of molecular simulations, are required when the length scale of interest is comparable to the internal structure of cell. The results obtained for the mechanical properties of cells such Young's modulus varies according to the types of experiments adopted [28]. It is undeniable that modeling cell mechanics is a demanding task because of the complexity of cell and the technical limitation of

computational power and software efficiency. As a result, there is no perfect model as assumptions have to be made to simplify these models. Hence, a suitable model to represent the structure of a cell or its internal components has to be chosen based on the length scale of interest.

1.1 Overall objective

The overall objective of this study is to investigate the time-dependent behavior of single cells under external tensile stress at both cellular and subcellular levels using the micropipette aspiration technique. At the cellular level, the focus is on studying the global deformation of human mesenchymal stem cells (hMSCs) in terms of viscoelastic properties while at the subcellular level, the focus is on analyzing the local deformation of microtubules and plasma membrane rupture. The flow chart showing the thesis objectives is shown in Figure 1.1.

Figure 1.1 The overview of the thesis structure.

Micropipette aspiration is a well-established active measurement method in studying viscoelastic properties of cells through the application of suction pressure on cell periphery [29-35], which is recognized in terms of validity and suitability. So far,

majority of micropipette aspiration studies are restricted to understanding cell mechanics of suspended cells based on various continuum models at the cellular level. Even though this method is considered “classical”, the novelty in this research can be found in:

- a) Generating new data for the viscoelasticity of hMSCs under different conditions: temperature and disruption of F-actin which is the key cytoskeleton component in maintaining cell stiffness. The elastic moduli for hMSCs obtained from this study have been cited by other works [36-44] in the scientific community as the baseline data.
- b) Observing microtubules deformation in semi-suspended cells, which provides both clear cytoskeleton formation and sufficient height for aspiration, over time. None of the previous micropipette studies based on fully suspended cells were able to achieve this because of the inability of suspended cells to generate distinct formation of cytoskeleton as seen in adherent cells [35].
- c) Studying the rupture mechanics of cell over time. Apparently, there is no detailed study on the rupture mechanism of living cell plasma membrane under tensile stress. Previous studies were only performed mainly on lipid vesicles [45-47].

1.2 Hypotheses and specific aims

At the cellular level, we hypothesized that the behavior of hMSCs under load is viscoelastic and dependent on the structural integrity of actin filaments and temperature. At the subcellular level, it is believed that microtubules exhibit different types of deformations at different locations of cell upon application of force which may demonstrate that a cell has anisotropic mechanical properties. In addition, we hypothesized that plasma membrane strength can be statistically described and is affected by the flexibility of microtubules and nucleus presence.

To test these hypotheses, four specific aims were developed for this thesis:

- a) To characterize the viscoelastic properties and behavior of hMSCs based on the homogenous viscoelastic solid model. The model was then tested for its validity based on the results obtained from the experiments. Actin filament network, which is the key cytoskeleton component in maintaining cell stiffness [48, 49], was disrupted using suitable chemical while the experiment was performed at room and physiological temperature to study the roles of actin filament and the effects of temperature in determining the viscoelastic properties hMSCs.
- b) To analyze the morphological changes of microtubules qualitatively and quantitatively during micropipette aspiration with the aid of a fluorescent microscope and a high speed image acquisition system. The bending energy of microtubules were calculated based on the measurement of microtubules curvature over time.
- c) To determine the strength of plasma membrane quantitatively through the calculation of membrane tension required for rupture based on experimental observation. Weibull distribution was used and tested for its suitability to represent membrane rupture data. Using the average membrane strength obtained, the transmembrane protein-lipid rupture strength was analyzed through the random sampling of transmembrane protein volume ratio in membrane.
- d) To map the local stress distribution with relate to the overall cell mechanical behavior during rupture, qualitative analysis was performed using finite element modeling. In addition, the critical membrane surface energy is calculated based on a proposed quantitative plasma membrane rupture model.

1.3 Thesis outline

In the next chapter (Chapter 2), the definition of viscoelasticity and the previous works that have been done in the area of stem cell mechanics are presented. Besides that, a brief introduction of microtubules and plasma membrane with their respective structures and roles in cell mechanics is provided. Subsequently, in Chapter 3, the viscoelastic properties of hMSCs in terms of Young's modulus and viscosity calculated from the results of micropipette aspiration are presented. The effects of disruption of actin filaments and temperature are also analyzed. In Chapter 4, we turn our attention to the subcellular level deformation which involves microtubules. The variation of bending energy of microtubules over the different regions of cells is examined. In Chapter 5, the characteristics of plasma membrane rupture and the applicability of Weibull distribution are studied. The quantitative and qualitative results obtained from the calculation of average membrane strength and finite element model simulations, respectively, are also presented. At the end of the chapter, a quantitative rupture model of plasma membrane is proposed to calculate and analyse the critical membrane surface energy. Chapter 6, the last chapter of the thesis, contains the overall conclusions gathered from the studies together with the suggestions for future work.

Chapter 2 Literature Review

In this chapter, a brief definition of cell viscoelasticity is provided. The effects of mechanical and chemical stimuli at the cellular and cytoskeleton levels, particularly hMSCs and microtubules, respectively, are reviewed. The roles and mechanical properties of plasma membrane in cell mechanics together the membrane protein-lipid interactions are also described at the end of the chapter.

2.1 Definition of viscoelasticity

Materials that possess both viscous and elastic properties, such as most engineering plastics and cells, are known as viscoelastic materials. Viscoelastic behavior can be represented by a combination of spring and dashpot to describe the elasticity and viscosity of the material, respectively. The spring represents the resistance of cell to deformation due to its elasticity, where deformation energy is stored [50]. The resistance to fluid flow due to the viscosity of cell, is represented by the dashpot (shock absorber), where deformation energy is lost [50]. The degree of deformation of a viscoelastic material depends on both of the rate and magnitude of applied stress [50]. A sudden application of large stress would lead to the stiffening of the material and vice versa [51].

Numerous experiments have shown that cells behave like a viscoelastic material, which deform slowly under a constant stress [50]. There are various types of viscoelastic models for cells, such as the standard linear solid (Figure 2.1) and the Newtonian liquid drop models (Figure 2.2), which have been extensively reviewed by Hochmuth [31] and Lim et al. [52]. Through the use of micropipette aspiration and corresponding continuum-based models, the viscoelastic properties of many types of cells in terms of Young's modulus and viscosity including endothelial cells [49], chondrocytes [29], smooth muscle cells [53], aortic valve interstitial cells [54] and even cell nucleus [30] have been characterized.

Here, we examine the viscoelasticity of human mesenchymal stem cells (hMSCs) to provide baseline data for future characterization of hMSCs differentiation. The characteristics of hMSCs and the effects of chemical and mechanical stimuli on hMSCs differentiation will be discussed in the next section.

Figure 2.1 The standard linear solid model. (a) The cell behavior is represented by two springs (k_1 and k_2) and a dashpot (μ) which describe the elasticity and viscosity of the material, respectively. (b) The corresponding creep response or strain, γ over time plot of the model resulted from the application of a constant stress, σ , at $t = 0$. Upon the release of the load at $t = 200$, the material recovers to its initial shape (Reproduced from Ref. 52).

Figure 2.2 The Newtonian liquid drop model. (a) The cell behavior is represented by a dashpot (μ) to describe cytoplasm viscosity and a cortical layer with constant tension, T_0 . (b) The corresponding creep response or strain, γ over time plot of the model resulted from the application of a constant stress, σ , at $t = 0$. No initial jump in γ is observed. Upon the release of the load at $t = 1$, the material does not recover to its initial shape (Reproduced from Ref. 52).

2.2 Mesenchymal stem cells

2.2.1 Characteristics

In recent years, stem cells have captured the attention of many scientists throughout the world due to their promising use in patient-specified gene therapy [55] and tissue engineering related applications [56-58]. For example, stem cells combined with a smart biomaterial can be used to generate bone graft [59] while heart muscle cells or cardiac myocytes differentiated from bone marrow stem cells have the potential to be used as biopacemakers [60]. Generally, stem cells can be divided into two main categories, that is, adult and embryonic stem cells. Adult stem cells can be obtained from mature tissues such as bone marrow, umbilical cord blood, fat, muscle and skin, while embryonic stem cells are harvested from fetal or embryo tissues which are still very much controversial ethically. To avoid the ever-sensitive ethical issues [61], many have decided to focus their research work on adult stem cells.

Mesenchymal stem cells (MSCs) which are also known as marrow stromal stem cells can be extracted from bone marrow and other sources such as liver, umbilical cord, placenta, adipose tissue, synovial membrane, amniotic fluid and even teeth [55]. MSCs have the ability to grow and differentiate into different cell lineages such as fibroblasts, osteoblasts, chondrocytes and neuron cells both *in vitro* and *in vivo*. Compared to the well-studied hematopoietic stem cells (HSCs) which can also be obtained from bone marrow, MSCs are immunologically inert [62], immune suppressive [63, 64], exhibits antithrombogenic property [65] and able to migrate to sites of injury, making MSCs the better substitute for tissue repair or wound healing [66] and for cell engraftment or transplantation purposes [67].

Like any other living cells, the vitality and function of stem cells also rely on the mechanical stimuli exerted by their surrounding. The mechanical, biochemical and structural properties of stem cells, however, are different from other cells [68]. Direct mechanical stimuli such as compression and tension have been identified to affect the differentiation MSCs into specific lineages. However, very few studies have been

performed to comprehend the underlying structural and functional changes in MSCs due to the external mechanical forces.

2.2.2 Effects of mechanical and chemical stimuli on MSCs

MSCs differentiation towards a specific cell lineage can be induced through two major approaches. First, biochemical cues which include soluble and insoluble growth factors. Various soluble growth factors such as transforming growth factor-beta (TGF- β) and bone morphogenetic proteins (BMPs) have been successfully used to direct the differentiation of MSCs into chondrocytes and osteoblasts, respectively [58, 69]. Extracellular matrix (ECM) proteins such as fibronectin [70] and collagen [71], which are categorized as insoluble growth factors, have also been found to play a major role in MSC differentiation.

The second approach involves physical cues which can be provided by substrate topography and external mechanical stimuli. Based on the topographical cues given by the different sizes of micropatterned fibronectin 'islands', the cells which exhibit round shape and the cells which are allowed to spread on the 'islands' lead to the differentiation of MSCs into adipose cells and osteoblasts, respectively [72]. Scaffold structure and properties are also known to generate topographical cues for MSC differentiation. Poly(ethylene glycol) diacrylate (PEGDA) hydrogel was found to provide suitable three-dimensional (3D) environment for the differentiation of MSCs into chondrocytes under the influence of the chondrogenic-supplemented medium [73]. Continuous chondrogenic and osteogenic differentiation were observed when MSCs were cultured on electrospun PLGA nanofiber scaffolds [74]. More recently, it has been shown that stem cell lineage specification is also directed by matrix elasticity [75].

The effects of direct mechanical stimulus such as compressive, tensile, and shear forces on MSC differentiation have been experimented both *in vivo* and *in vitro*. Using a specially made bone conduction chamber, a compressive hydrostatic stress of 2 MPa on mesenchymal tissue was found to promote the formation of chondrocytes [76]. Compressive and tensile stress applied on the midpalatal suture cartilage in rats

using orthodontic wire apparatus led to chondrogenic and osteogenic differentiation, respectively [77, 78]. The cyclic compressive load applied by a electromagnetic cell exciter on MSCs cultured on alginate hydrogel was able to stimulate the differentiation of chondrocytes without the presence of TGF- β 3 [79]. Studies combining various mechanical stimuli were also found to direct the differentiation of MSCs into certain lineages [80, 81]. In Table 2.1, the various types of external mechanical stimulus used to induce MSC differentiation into specific cell lineages and ECM proteins are summarized.

Table 2.1 The effects of various mechanical stimuli on MSC differentiation.

Type of mechanical stimulus	MSC source	Cell lineages/ ECM proteins	Reference
<i>In vivo</i>			
Hydrostatic pressure	Tibia of rats	Chondrogenic	[76]
Compressive stress	Midpalatal suture cartilage in rats	Chondrogenic	[77]
Tensile stress	Midpalatal suture cartilage in rats	Osteogenic	[78]
<i>In vitro</i>			
Electromagnetic compressive force	Rabbit bone marrow	Chondrogenic	[79]
Compressive stress	Mouse embryonic limb bud	Chondrogenic	[82]
Stretching/ Tensile force	Human bone marrow	Collagen	[83]
Uniaxial and equiaxial strain	Human bone marrow	Smooth muscle cells	[84]
Combination of flexure and shear stress	Sheep bone marrow	Collagen and endothelial cells	[80]
Shear and compressive stress	Rat bone marrow	Smooth muscle cells	[81]

These studies, however, did not provide a quantitative explanation to the results obtained in terms of global deformation, cytoskeletal reorganisation and protein conformations of the MSCs during mechanical force application. These three key components are crucial in uncovering the mystery of the mechanotransduction pathways that exist in MSCs due to external mechanical stimuli. A recent study shows that rheological character of stem cell nucleus is closely related to differentiation [33]. Therefore, we seek to understand and quantify the mechanical behavior MSCs as it is the first step towards providing a useful insight on how to correlate the mechanical properties with the state of differentiation of stem cells and modelling stem cell behavior in a systematic way.

2.3 Microtubules

2.3.1 Characteristics

Cell cytoskeleton is composed of three biopolymer components, that is actin filaments, microtubules and intermediate filaments which contribute to the overall mechanical properties of cell. Each of them has specific properties and they are interconnected through protein cross-linkers [85-88]. Compared to actin and intermediate filaments, microtubules are known to be the most rigid cytoskeleton component. Each microtubule is like a hollow tube that is made of tubulin subunits, commonly with 13 protofilaments (Figure 2.3) with a diameter of 25 nm and cross sectional area of 200 nm^2 [89]. Microtubules are nucleated at their minus end from a focal point called the microtubule-organizing centre (MTOC) [90] or centrosome which is located near the nucleus (Figure 2.4) and at the Golgi apparatus [91] (Figure 2.5). Dyneins and kinesins are the two types of motor proteins that operate along microtubules to transport cellular vesicles.

Figure 2.3 (a) The classical model of microtubule with 13 protofilaments. (b) Cryoelectron microscopy image of single microtubules (Both (a) and (b) are reproduced from Ref. 89). (c) 3D reconstruction of a microtubule based on the combination of single particle and crystallographic image processing methods (Reproduced from Ref. 92).

Figure 2.4 Illustration of a single adherent fibroblast with microtubules originating from the centrosome in a star-like pattern.

Figure 2.5 Microtubules can be nucleated at centrosome and/or Golgi apparatus as shown by the black and red lines, respectively (Reproduced from Ref. 91).

Using the thermal fluctuation method [93], the flexural rigidity of microtubules was found to be $2.1 \times 10^{-23} \text{ Nm}^2$ while its Young's modulus was obtained as 1.2 GPa. The tensile failure strength for microtubules is approximately 10 times larger than of actin filaments. Interestingly, purified microtubules network has the highest deformability as compared to actin and intermediate filaments [94] (Figure 2.6). The various elastic properties of the two major cytoskeleton components, that is, microtubule and actin filament are presented in Table 2.2. It is to be noted that different methods used to study the individual cytoskeleton components would lead to different results. For example, optical trap method reveals a much lower flexural rigidity of $7.9 \times 10^{-24} \text{ Nm}^2$ and lower Young's modulus of 0.46 GPa for microtubules [95]. Recently, a comparison has been made between microtubules and carbon nanotubes (CNTs) and it was found that they are similar in terms of their hollow tube structure, high resilience to deformation and capability to form large bundles [96]. It is therefore very exciting and promising that the understanding of microtubule structure

and deformation may eventually lead to the development of a new generation of nanomaterials based on CNTs.

Figure 2.6 The deformation behavior of actin, tubulin, vimentin and fibrin polymer networks (Reproduced from Ref. 94).

Table 2.2 Comparison of estimated mechanical properties between individual microtubules and actin filaments.

Mechanical properties	Microtubule	Ref.	Actin Filament	Ref.
Diameter (nm)	25	[89]	6	[97]
Persistence length (μm)	6000	[98]	15	[99]
Young's modulus (GPa)	1.2	[93]	2.6	[93]
Flexural rigidity (Nm^2)	2.1×10^{-23}	[93]	7.3×10^{-26}	[93]
Tensile failure strength (pN)	1000	[100]	108	[101]
				]

2.3.2 Roles of microtubules and the effects of chemical stimuli

Due to its high rigidity, microtubules play an important role in providing mechanical strength and support to cells. In addition, they direct cell polarization during migration process [102] and provide a track for motor proteins such as kinesin and dynein to transport membrane-enclosed organelles [103]. These motor proteins are found to influence the dynamics of microtubules [104]. Microtubules are required for dendritic extensions but not for cell spreading [105]. Besides that, microtubules are actively involved in wound healing as observed from the recruitment and elongation of microtubules at wound site [106]. Microtubules undergo dynamic instability [107] due to the different rate of polymerization and depolymerization at the opposite ends [103].

The structural integrity of microtubules are affected by various drugs such as nocodazole, colchicine and alicin which are known to cause rapid depolymerization of microtubules [108] while Taxol promotes polymerization and stabilizes microtubules against depolymerization [109]. Taxol and nocodazole are found to inhibit cell polarisation while cytochalasin D, which disrupt actin cytoskeleton, prevents cell spreading [105]. The two-dimensional (2D) morphological changes of microtubules after the application of platelet-derived growth factor (PDGF), nocodazole (Noc), Taxol and cytochalasin D (Cyto D) are shown in Figure 2.7. This knowledge of the effect of the drugs, especially Taxol, on microtubules and the entire cell would be very valuable for our study as the fluorescent dye used to stain the microtubules for live cell imaging contains Taxol.

Figure 2.7 The effects of various drugs on microtubules (green) and actin (red). PDGF - platelet-derived growth factor, Noc - nocodazole, Cyto D - cytochalasin D (Reproduced from Ref. 105).

2.3.3 Interconnectivity with actin filaments

Microtubule dynamics are affected when the tension in the actin cytoskeleton is altered. Depolymerization of microtubules occur at the local site where the contractility of actin cytoskeleton is inhibited [110]. By applying a radial stress on the cell edge, it was observed that microtubules grow in length (Figure 2.8) while focal adhesions, which are connected to actin filaments [111], grow in size (Figure 2.9) in the region of increased stress [112]. This result points to the existence of crosstalk between microtubules and actin cytoskeleton which involves a stress-dependent feedback system [102] to regulate the microtubule dynamics and growth towards the adhesion sites of cells.

Figure 2.8 The growth of microtubules after a transient tensile force is applied on the cell periphery. Arrows represent the direction of tensile force. (a) Brightfield images. (b) Fluorescent images of microtubules (Reproduced from Ref. 112).

Figure 2.9 The polymerization of microtubules and the enlargement of focal adhesions during the application of transient tensile stress at cell periphery. Arrows represent the direction of tensile force. (a) Brightfield images. (b) Fluorescent images of microtubules (Reproduced from Ref. 112).

The crosstalk is further confirmed by a study utilizing the total internal reflectance microscopy (TIRFM) [113], where polymerizing microtubule ends were found to be very close (within 50 nm) to the substrate (Figure 2.10). Other studies performed using the multimode fluorescent speckle microscopy (FSM) to examine the relationship between microtubules and actin filaments showed that microtubules polymerization is directed by actin filaments [114, 115]. These studies presented solid evidence of the interdependence between microtubules and actin filaments which may be explained by the presence of microtubule actin cross-linking factor (MACF) proteins that provide the physical linkage [116]. Thus, it is very likely the observed morphological changes on one of the cytoskeleton components under the influence of mechanical force may result in the change of another component due to their interconnectivity.

Figure 2.10 (a) Confocal images of fluorescent-stained focal adhesions and microtubules. The tips of three microtubules moving towards an adhesion complex are indicated with different arrowheads. (b) A schematic diagram showing the dynamics of microtubules in the vicinity the cell-substrate interface. EW refers to the evanescent wave, FA is the focal adhesion, a is actin, TCx is the tip complex proteins MT is the microtubule, Cr is the coupler complex and ECM is extracellular matrix (Reproduced from Ref. 113).

2.3.4 The effects of mechanical stimuli on microtubule dynamics

Even though microtubules are considered the most rigid cytoskeletal element, buckling of microtubules can be observed within a normal cell. A recent study shows that driving force behind microtubule bending comes from the anterograde microtubule transport [117]. An earlier quantitative study conducted by Brangwynne et al. [118] to explore this phenomenon found that microtubules experienced short-wavelength buckling at three different situations, that is, first, when they touched the plasma membrane, second, when a compressive force was applied by a microneedle on the cell periphery and third, when the cell contracted due to the action of myosin (Figure 2.11). From this study, it was concluded that microtubules can withstand large-compressive forces due to the support given by the surrounding cytoskeleton which led to the hypothesis that the region with large buckling wavelength would demonstrate weak cytoskeleton [118].

Figure 2.11 Buckling of microtubules observed at 3 different situations: (a) microtubule contacting the plasma membrane, (b) application of force by microneedle at cell edge, and (c) contractile motion of a cardio myocyte. (Reproduced from Ref. 118).

However, McGrath pointed out that instead of weak cytoskeleton, the short wavelength buckling may be due to weak microtubules as a result of defects [119]. In addition, Brangwynne et al. was not able to account for the magnitude of the external

compressive stress applied on the cell periphery due to their experimental method which involves the application of random transient force. As compared to Brangwynne et al., we have the added advantage in our study in terms of the ability to quantify the magnitude of applied tensile stress and subsequently, the possibility to establish the relationship between the amount of external stress and the local deformation of microtubule observed. At the same time, we would like to find whether microtubule buckling would still occur under the application of constant tensile stress using micropipette aspiration.

2.4 Plasma membrane

2.4.1 Characteristics

Like a barrier, plasma membrane, which is a lipid-protein composite material formed through non-covalent interactions, separates the cytosol from the extracellular environment but allows exchange of ions (Ca^{2+} , K^{+}) and fusion/budding of vesicles. The schematic diagram of a phospholipid molecule is shown in Figure 2.12. The kink that is present on the phospholipid molecule is caused by the *cis*-double bond at the hydrophobic tail, making it difficult for the phospholipids to be closely packed leading to the fluid nature of bilayers. Each phospholipid is free to flex and rotate along its long axis while collectively, they are free to diffuse laterally [103]. It is estimated that the mass ratio for lipid to protein is 50% [103].

Figure 2.12 A phospholipid molecule, which is phosphatidylcholine in this example. The schematic, chemical structure, space-filling model and symbol of this lipid molecule is shown in (a), (b), (c) and (d), respectively. The kink on the one of the hydrophobic tails is due to the presence of cis-double bond (Reproduced from Ref. 103).

Structurally, the lipid membrane, which is only 5 nm thick, consists of a continuous asymmetrical double monolayer of lipid molecules, or better known as lipid bilayer, bonded by van der Waals forces [103, 120]. In eukaryotic cells, cholesterol is embedded in lipid bilayer, up to as many as one molecule per phospholipid molecule, making cell membrane more resistant to deformation due to its high rigidity as compared to phospholipids [103]. The plasma membrane does not exactly have constant thickness throughout the plane as there exists lipid rafts (~ 70 nm in diameter) which contain clusters of sphingolipids, cholesterol and some other membrane proteins [103].

Membrane proteins serve as the extracellular signal sensor, ion channels, and the bridge linking cytoskeleton to extracellular matrix or a neighbouring cell. There are two types of membrane proteins: peripheral and integral proteins. Peripheral membrane proteins are bound to the membrane surface or transmembrane proteins through noncovalent bonds. The membrane proteins that are permanently attached to the membrane such as transmembrane proteins are categorized as integral membrane proteins. Transmembrane proteins are embedded in the plasma membrane via

hydrophobic matching [121], spanning across the entire membrane thickness and are connected to the cytoskeleton through protein linkers such as the ezrin, radixin, and moesin (ERM) family [103]. The distribution and binding of membrane proteins are determined by the lipid bilayer material properties and electrostatic charge [122]. The various types of membrane protein configurations are shown in Figure 2.13. It has been found that membrane curvature is governed by five factors: (1) lipid composition, (2) integral membrane proteins, (3) cytoskeletal proteins and microtubule motor activity, (4) peripheral membrane proteins and (5) amphipathic helices [123]. In our study, we will focus on the roles of cytoskeletal proteins and transmembrane proteins in determining plasma membrane strength under external mechanical stress, which is another major factor that determines the shape of the membrane.

Figure 2.13 Membrane proteins can interact with the lipid bilayer in many ways. (a) Single-pass transmembrane protein or α helix. Multipass transmembrane proteins are made up of (b) multiple α helices, or (c) a β barrel. Membrane proteins may be attached to the membrane surface through (d) an amphipathic α helix, (e) covalent bonding, (f) an oligosaccharide linker or (g, h) noncovalent bonding with other membrane proteins for the case of peripheral membrane proteins (Reproduced from Ref. 103).

2.4.2 Membrane tension and rupture

A number of studies have been performed to analyze the membrane tension of cells, which is an important feature of cell membrane in controlling cell shape during migration and determining the action of endocytosis or exocytosis [124-126]. Membrane tension can be calculated from the measured tether force required to pull a

tether, 15 - 500 nm in diameter [126], from cell periphery using optical tweezers (Figure 2.14). It is defined as the in-plane stress resulting from the membrane bilayer-cytoskeleton adhesion or due to cell osmotic swelling [126].

Figure 2.14 Illustration of the tether formation based on optical tweezer. (a) A suction pressure (ΔP) is applied to generate bilayer tension, T_m . This results in the formation of tether force, f_t which is balanced by the force on bead, f_b . (b) Membrane skeleton is separated from the fluid tether due to the tether force (Reproduced from Ref. 126).

It has been established that membrane tension is controlled by cytoskeleton, which is confirmed by the observation of actin filaments rearrangement upon the alteration of membrane tension [127, 128] and the decrease in tether force measured from bleb, which is void of cytoskeleton, as compared to normal cell without bleb [125]. In addition, it is shown that cytoskeleton is separated from the tether membrane during tether pulling due to the strong adhesion between cytoskeleton and cell membrane [126, 129, 130]. As a result, the energy difference created between the tether (which is at a higher energy state due to the detachment from cytoskeleton) and the membrane with cytoskeleton adhesion, would lead to the retraction of tether towards the main cell body [126].

For red blood cells, the tether force and membrane tension can reach up to 50 pN and 0.29 mN/m, respectively [131]. In comparison, the tether force and membrane tension obtained from neuronal growth cone membrane are much lower, at 8 pN and 0.003 mN/m, respectively [132]. In the case of cancerous skin cells or melanoma cells, the tether force and membrane tension range from 11 – 14 pN and 0.011 – 0.012 mN/m, respectively [125]. Even though the membrane tension is successfully characterized through these studies, however, none of these cell membrane studies, including the single cell creep tests such as Ref. 133, provide detailed information on the membrane tension for plasma membrane rupture.

Cell survival depends on the integrity of cell membrane. Loss of plasma membrane integrity due to external force is linked to change in cellular function and morphology. For example, damaged membrane of neuronal rat cells after subjected to spinal cord trauma leads to blebbing [134]. The inability of muscle cells to reseal and repair damaged membrane due to the presence of mutated cytoskeletal proteins is the main reason of muscular dystrophy, where muscles become weak progressively [135]. As an example, rupture on plasma membrane of striated muscle due to repetitive strain is known to be an early warning of Duchenne and Becker muscular dystrophy [136].

In normal functioning cells, membrane rupture would trigger membrane resealing process naturally to prevent the outflow of cell internal components to external environment and the inflow of Ca^{2+} , which is ironically needed for resealing to take place, and other foreign objects that can be toxic to cells [137]. This phenomenon is one of the nature's best examples of mechanotransduction. A study by Needham et al. showed that membrane lipid with cholesterol ruptures once its area is stretched slightly beyond 2 - 5%. [45]. In addition, lipid membrane is only able to withstand bending and stretching but not in-plane shear [138]. However, there is no available data on live cell membrane rupture strength. Thus, it is of our interest to quantify the plasma membrane strength and analyze its rupture mechanism under tensile stress.

2.4.3 Protein-lipid structure and interaction forces

Membrane proteins and lipid bilayer are closely connected and influence each other in terms of shape and function. Even though the presence of transmembrane proteins lead to a more stable lipid bilayers [139], the hydrophobic mismatch between the different lengths of transmembrane proteins and the neighbouring lipids gives rise to the variation in membrane thickness [121, 140]. As proposed by Mouritsen et al. [140], the presence of a transmembrane protein which has a longer hydrophobic domain than the lipid bilayer would result in an increase in the membrane thickness at the protein region (Figure 2.15(a)). On the contrary, a shorter transmembrane protein than lipid bilayer would lead to a reduction in the membrane thickness (Figures 2.15(b) and (c)). The theoretical model based on this proposal is also known as the mattress model, which is useful in analyzing the phase the phase behavior of lipid membrane embedded with transmembrane proteins [140]. This is an important step in understanding the lipid-protein interaction as the physiological functions of membrane proteins are found to be dependent on the physical properties and phase behavior of lipid membrane which tend to induce membrane protein conformational changes [120] and eventually, trigger mechanotransduction pathways such as the ion channel opening (Figure 2.16) [141].

Figure 2.15 Schematic diagram of hydrophobic mismatch between transmembrane protein and lipid bilayer. Transmembrane protein is (a) longer and (b) shorter than the lipid bilayer thickness (Reproduced from Ref. 140). (c) The initial and end images taken from the molecular dynamics simulation of hydrophobic mismatch due to the presence of a short α -helix transmembrane protein in a lipid bilayer (Reproduced from Ref. 142).

Figure 2.16 Possible mechanotransduction mechanism due to the deformation of transmembrane proteins in the membrane. (a) The native state of a transmembrane protein (green). (b) The transmembrane protein experiences a structural change during the rearrangement of neighbouring lipids (red patch) as a result of a change in the lateral pressures of the lipid bilayer (Reproduced from Ref. 141).

Few studies have been done to understand and analyze the interaction forces between membrane proteins and lipids. Cross et al. reported that the force required to extract glycosylphosphatidylinositol (GPI) anchored proteins from the lipid membrane is 103 ± 17 pN (Figure 2.17) [143]. Besides that, Desmeules et al. obtained the adhesion force between recoverin, a calcium–myristoyl switch protein and lipid bilayers as 48 ± 5 pN in the presence of Ca^{2+} [144]. However, these AFM-based studies were conducted using reconstituted membrane and not living cells. Furthermore, the membrane proteins selected in those studies were under the category of peripheral membrane proteins.

Figure 2.17 Illustration of pulling membrane proteins out from membrane using AFM. (a) An AFM tip covered with GPI-anchored alkaline phosphatase (AP_{GPI}), through amylose spacer, is lowered onto the dipalmitoylphosphatidylcholine (DPPC) bilayer coated on glass. (b) Upon retraction of tip, there is a steady increase in force signal until the rupture due to the adhesion between one or more proteins and the DPPC bilayer (Reproduced from Ref. 143).

Another AFM study conducted by Afrin et al. revealed that the average force required to pull transmembrane proteins out from living cell membrane is within the range of 400 – 600 pN [145], which is much larger than the rupture force of peripheral proteins mentioned earlier. Even though this particular study has the advantage of analyzing protein-lipid rupture strength in living cells, it is not able to provide the crucial information on the lateral protein-lipid rupture strength during plasma membrane rupture due to external tensile force. Thus, we attempt to uncover the lateral transmembrane protein-lipid rupture strength, which is not yet available in the literature, quantitatively through statistical analysis based on the plasma membrane rupture strength data obtained from our single cells study.

2.5 Summary

From our discussion earlier, it is clear that many works have been done to study the influence of direct mechanical forces and chemical cues on MSCs differentiation into a specific lineage. However, these studies did not offer quantitative data on MSCs mechanical properties. At the subcellular level, the studies of microtubules deformation due to mechanical stimuli have yet to establish the relationship between the amount of external stress and the local deformation of microtubule observed. Besides that, there is little information on the failure mechanism and strength of plasma membrane under tensile stress. Based on these gaps in understanding the effects of external mechanical stimuli on cell mechanics at the perspectives of global and local deformation, we attempt to develop the relevant materials and experimental methods to bridge those gaps and accomplish the objectives of this thesis. In the next chapter, we will present and discuss the results obtained from the study of viscoelastic behavior of hMSCs.

Chapter 3 Viscoelastic Behavior of hMSCs^{*}

In this chapter, the experimental procedures and setup used to characterize and model the viscoelastic properties of human mesenchymal stem cells based on micropipette aspiration method are presented. The quantitative results in terms of Young's modulus and viscosity of hMSCs are determined. The roles of F-actin cytoskeleton and temperature on the viscoelastic properties of hMSCs, were analyzed using cytochalasin D to disrupt F-actin network and raising the temperature from room temperature to 37°C, respectively. With these results, the comparison between the viscoelastic properties of hMSCs found at room temperature (control), 37°C and under the chemical influence of cytochalasin D is made. A detailed discussion on the results obtained is presented at the end of the chapter.

3.1 Material and methods

3.1.1 Cell culture and disruption of actin cytoskeleton

Human mesenchymal stem cells (hMSCs), derived from human bone marrow, were purchased from Cambrex Bio Science Walkersville, Inc., USA. hMSCs were cultured at 4% CO₂, 37°C using MSCs growth medium (MSCGM) purchased from Cambrex which contains the mixture of MSC basal medium (MSCBM) and supplements such as MSC Growth Supplement (MCGS), L-glutamine, and penicillin/streptomycin. Once reached 80% confluence after about 5 days of incubation, hMSCs were harvested with 0.25% Trypsin/EDTA (Invitrogen, USA) which was added for 5 minutes. After adding appropriate amount of stem cell medium to neutralize trypsin (with the volume ratio of 2:1), the suspended cells were allowed to recover for 1 minute before they were plated on a coverslip (0.17 mm thick) for micropipette aspiration experiments. hMSCs used in the study did not exceed passage number 9 as hMSCs may lose its multi-lineage potential above this or later passage number [146].

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Cytochalasin D (Sigma, USA) was added to the cell culture at three different concentrations, that is, 0.2 μM , 2.0 μM and 20 μM to disrupt the F-actin filaments [35, 147]. These chemical-induced cells were incubated for 3 hours prior to detachment by trypsin for micropipette aspiration. The total number of cells analyzed for the respective experimental conditions is shown in Table 3.1.

Table 3.1 The corresponding cell number of hMSCs under the different experimental conditions (Reproduced from Ref. 148).

Treatment	Concentration of cytochalasin-D, (μM)	Cell number, n
20°C (control)	0	24
Low	0.2	11
Medium	2.0	13
High	20.0	17
37°C	0	13

3.1.2 Micropipette aspiration

Micropipette aspiration technique was deployed to study the viscoelastic behavior of hMSCs by applying a constant suction pressure to the surface of single hMSCs which were suspended in hMSCs growth medium plated on a glass coverslip. For the experiments conducted at 37°C, the coverslip was placed on a heating stage (Heatable Universal Mounting Frame M-H, Zeiss, Germany) which was connected to a temperature regulator, Tempcontrol 37-2 digital (Zeiss, Germany). The actual experimental setup and schematic diagram are shown in Figures 3.1 and 3.2, respectively. Micropipettes were fabricated and refined by drawing borosilicate glass capillary tubes (Sutter Instrument Co., CA, USA.) using a horizontal pipette puller (FLAMING/BROWN Micropipette puller, Model P-97, Sutter Instrument Co., CA, USA.) and a microforge (Narishige, Japan), respectively, to obtain a range of inner diameter from 7 μm to 11 μm for maintaining a constant cell-diameter to inner-micropipette-diameter ratio. Micropipettes were coated with Sigmacote chemical (Sigma, USA) to prevent cell adhesion on the wall of the micropipettes during aspiration.

Figure 3.1 The experimental setup for micropipette aspiration.

Figure 3.2 Schematic diagram of the micropipette aspiration setup. Teflon FEP tubing is represented by the blue lines.

Before fixing a micropipette to the metal holder, air bubbles, which affect the pressure reading, were removed from the 2 mm outer diameter $\times$ 1 mm inner diameter Teflon FEP (fluorinated ethylene propylene) tubing (Upchurch Scientific, USA) connecting the micropipette system. This can be easily done by turning on the valve and the water pump to push the distilled water out from the reservoir, which eventually expels air bubbles, if any, from the tubing. The micropipette was filled with distilled water through a flexible needle, also known as MicroFil (World Precision Instruments, USA) that was attached to a syringe before placing the micropipette into the holder. The suction pressure and the position of the micropipette were controlled by a micromanipulator (Narishige, Japan). The amount of pressure exerted on the cells was measured by an in-line pressure transducer (Model DP15–30, Validyne Engineering Corp., Northridge, CA, USA) with a resolution of 1 Pa and was converted to voltage reading using a demodulator (Model CD280-2C, Validyne Engineering Corp., Northridge, CA, USA) and a digital multimeter (Fluke 115). Each experimental session was limited to within 1 hour to maintain consistent cell viability.

The initial diameter of the target cell was calculated by using the ellipse tool provided by the SPOT software version 4.5 (Diagnostic Instruments Inc., USA). The micropipette tip was lowered gently towards the coverslip surface to ensure that the front portion of micropipette is in focus with the target cell. Once this was done, the initial diameter of the target cell and the equilibrium pressure in terms of voltage (V_{eq}), where the single cells are neither aspirated nor pushed away, were obtained before the start of each aspiration to minimize the error incurred by any drift to the measurement of the pressure. A constant step pressure, ranging from 500 – 1000 Pa, was then applied and the aspiration process was recorded for 350 s using an inverted brightfield microscope (Zeiss Axiovert S100, Germany) equipped with a CCD camera (RT Monochrome, Diagnostic Instruments Inc., USA) through a 40 $\times$ objective lens. The length of the aspirated cell was measured using the line tool provided by SPOT software at regular intervals and with an accuracy of $\pm 0.3 \mu\text{m}$.

To obtain the correlation between the measured voltage and pressure, a calibration graph was obtained by placing the micropipette holder into a long plastic measuring cylinder. At each fill of water in the cylinder, the height of the water and

the output voltage were recorded. The actual pressure, P , corresponding to the voltage obtained can be calculated using the following equation

$$P = \rho gh \quad (3-1)$$

where ρ is the density of water (1000 kg/m³), g is the gravitational acceleration (9.81 m/s²) and h is height of water. The curve fit of pressure versus voltage is shown in Figure 3.3 where the linear relationship between pressure and voltage can be written as $P = 531.2 \times V + 1156.5$ with a very high mean correlation coefficient of $R^2=0.99$. The gradient of the graph refers to the ratio between the change of pressure, ΔP , and change of voltage, ΔV , which is 513 Pa/V for our study. With this value, the voltage reading can be converted to pressure reading. The actual pressure, P , exerted on the cell is therefore,

$$P = (V_{eq} - V_{ap}) \times 513 \quad (3-2)$$

where V_{ap} is the applied voltage.

Figure 3.3 The calibration graph for the micropipette aspiration.

3.1.3 Modeling of cell viscoelastic behavior

Two mechanical models have been developed for cell study based on micropipette aspiration, i.e., cortical shell–liquid core (liquid drop) model and solid model [52]. Based on our study, hMSCs were observed to reach equilibrium length after the application of suction pressure, which is the characteristic of a solid model [52]. Therefore, the solid model is chosen to represent the behavior of hMSCs instead of liquid drop model. Within the solid model, there are 2 specific models i.e., the linear elastic solid model and the linear viscoelastic solid model [52]. Unlike the elastic model, viscoelastic model includes the time factor [52], making it more accurate in representing the mechanical behaviour of cell. As such, the viscoelastic solid model is chosen in this study.

A homogenous half-space linear, three-parameter viscoelastic solid model [49] or the standard linear solid (SLS) model [101] was used in this study to determine the mechanical properties of hMSCs. The schematic of SLS model is shown in Figure 3.4.

Figure 3.4 Theoretical model of the micropipette aspiration test. (A) The cell or nucleus was represented as a homogeneous half-space under small deformation with an applied uniform axisymmetric pressure from the micropipette (Reproduced from Ref. 49). (B) A three-parameter viscoelastic model (standard linear solid) was used to represent the material behavior of the cell (Reproduced from Ref. 30). Its configuration is a combination of a spring, k_1 (1) and Maxwell model (2), i.e., a spring, k_2 and a dashpot, μ , in series.

The assumptions for this model are:

- a) The pressure applied on cell is uniform and axisymmetric [149].
- b) The cell has intrinsic incompressibility and isotropy (Poisson's ratio = 0.5) [49].
- c) The cell membrane surface is smooth [150].
- d) The corners of the micropipette tip are square-shaped [149].

The main advantage of using SLS model is that its elastic and viscosity parameters are comparable to those in the other mechanical models for cells [52]. Besides that, it is an established continuum model in capturing the deformation behaviour of the whole cell [151], which is the focus of this chapter. However, there are also some limitations. The SLS model provides no direct information on the behaviour of cytoskeleton as it considers the cell as a homogenous material where the cytoskeleton is uniformly distributed [149, 151]. In addition, the model is more accurate for small deformation (strain of 5%) analysis [149, 152]. As it is a half-space model, it does not account for the finite boundaries of cell [149].

The force applied on the first spring in Figure 3.4B is $F_{s1}(t) = k_1 x_s(t)$, where k_1 is the spring constant and $x_{s1}(t)$ is the extension of spring. In the case of the dashpot, the force applied is $F_d(t) = \mu \dot{x}_d(t)$ where μ is the apparent viscosity (or damping coefficient) and $\dot{x}_d(t)$ is the velocity of deflection. For the spring and dashpot in series (equivalent of Maxwell model) as shown in Figure 3.4B, the sum of displacement, $x_2(t)$ and force relationship, $F_2(t)$ are

$$x_2(t) = x_{s2}(t) + x_d(t) \quad (3-3)$$

$$F_2(t) = F_{s2}(t) = F_d(t) \quad (3-4)$$

where s_2 refers to the spring with elasticity constant, k_2 . Differentiating Equation 3-3 with respect to time,

$$\dot{x}_2(t) = \dot{x}_{s2}(t) + \dot{x}_d(t) = \frac{1}{k_2} \dot{F}_{s1}(t) + \frac{1}{\mu} F_d(t) \quad (3-5)$$

Substituting Equation 3-4 into Equation 3-5, the force-displacement relationship for Maxwell model is

$$\dot{x}_2(t) = \frac{1}{k_2} \dot{F}_2(t) + \frac{1}{\mu} F_2(t) \quad (3-6)$$

For SLS model, i.e. the combination of a spring and the Maxwell model, the force equilibrium is,

$$F_T(t) = F_1(t) + F_2(t) \quad (3-7)$$

and the overall displacement,

$$x_T(t) = x_1(t) = x_2(t) \quad (3-8)$$

where $F_1(t) = F_{s1}(t)$ and $x_1(t) = x_{s1}(t)$.

Substituting Equations 3-7 and 3-8 into Equation 3-6,

$$\begin{aligned} \dot{x}_T(t) &= \frac{1}{k_2} [\dot{F}_T(t) - \dot{F}_1(t)] + \frac{1}{\mu} [F_T(t) - F_1(t)] \\ &= \frac{1}{k_2} [\dot{F}_T(t) - k_1 \dot{x}_T(t)] + \frac{1}{\mu} [F_T(t) - k_1 x_T(t)] \end{aligned} \quad (3-9)$$

Rearranging Equation 3-9,

$$\frac{\mu}{k_1} \dot{F}_T(t) + F_T(t) = k_1 \left[x_T(t) + \frac{\mu}{k_1} \dot{x}_T(t) \left(1 + \frac{k_1}{k_2} \right) \right] \quad (3-10)$$

By applying a constant step force, F_0 to the model, then $F_T(t) = F_0$ and $\dot{F}_T(t) = 0$ for $t > 0$. With these conditions, Equation 3-10 becomes,

$$F_0 = k_1 [x_T(t) + \tau \dot{x}_T(t)] \quad (3-11)$$

where $\tau = \mu \frac{k_1 + k_2}{k_1 k_2}$, which is defined as the strain relaxation time (or the time to

reach equilibrium length) at constant stress.

The initial condition at time $t = 0^+$ is $\dot{x}_d(0^+) = 0$, i.e., the dashpot has zero displacement. As a result, the force equilibrium at $t = 0^+$ is

$$F_T(0^+) = k_1 x_s(0^+) + k_2 x_s(0^+)$$

With these conditions and by solving the differential Equation 3-11 for $x(t)$, the force-displacement of SLS model can be obtained [153], i.e.,

$$x(t) = \frac{F_0}{k_1} \left[1 - \frac{k_2}{k_1 + k_2} \exp\left(-\frac{t}{\tau}\right) \right] \quad (3-12)$$

According to Ref. 49, $F_0 = \frac{\Phi(\eta)a\Delta P}{\pi k_1}$. Therefore, the final expression for the displacement of cell into the micropipette as a function of time, $L(t)$, which is a creep function, can be expressed as [49],

$$L(t) = \frac{\Phi(\eta)a\Delta P}{\pi k_1} \left[1 - \frac{k_2}{k_1 + k_2} \exp\left(-\frac{t}{\tau}\right) \right] H(t) \quad (3-13)$$

where inner radius of the micropipette and the applied aspiration pressure are represented by a and ΔP , respectively. The critical pressure, ΔP_c , occurs at $L/a = 1$, that is when the cell shape within the micropipette is hemispherical [31]. $H(t)$ is the unit step function, also known as the Heaviside step function, where $H(t) = 0$ for $t < 0$ and $H(t) = 1$ for $t > 0$. $\Phi(\eta)$ is the wall function which is related to the ratio of micropipette wall thickness to inner radius of micropipette, a , or known as the wall parameter, $\eta = \frac{b-a}{a}$, where b is the outer radius of the micropipette [149]. Based on the data of $\Phi(\eta)$ generated in Ref. 149, $\Phi = 2.0$ for entire range of micropipette inner and outer diameter used in this study. The detailed derivations of Equation 3-13 can be found in Ref. 149.

From Equation 3-11, the apparent viscosity, μ , of the cell (represented as the dashpot in Figure 3.4B) is given by

$$\mu = \frac{\tau k_1 k_2}{k_1 + k_2} \quad (3-14)$$

where the elastic constants, k_1 and k_2 can be found by solving Equation 3-13 using nonlinear regression method.

Based on Refs. 150, 154, there are two expressions for shear modulus, G :

$$G_0 = \frac{k_1 + k_2}{2}, \quad G_\infty = \frac{k_1}{2} \quad (3-15)$$

As the relationship between shear modulus, G and Young's (elastic) modulus, E is

$$E = 2G(1 + \nu) \quad (3-16)$$

where ν is the Poisson's ratio. Assuming that the cell is incompressible, $\nu = 0.5$, Equation 3-16 becomes

$$E = 3G \quad (3-17)$$

Substituting Equation 3-17 into Equation 3-15, we obtain the following equations [30],

$$E_0 = \frac{3}{2}(k_1 + k_2), \quad E_\infty = \frac{3}{2}k_1 \quad (3-18)$$

where E_0 is the instantaneous Young's modulus upon the application of constant stress at $t = 0$ and E_∞ is the equilibrium Young's modulus obtained at adequately long time where the aspiration length is more or less constant (for this study, the time to reach equilibrium aspiration length is about 3 - 5 minutes).

Graph plotting was performed using SigmaPlot version 8.02 software (SYSTAT Software, USA). Unpaired two-sample (with unequal variance) t -test was used to examine whether the means calculated are significantly different.

3.2 Results

3.2.1 Characteristic behavior of hMSCs

Upon the application of a step pressure to a single hMSC at the control temperature of 20°C, the cell aspiration length showed an initial jump, followed by a gradual increase, and eventually reached its equilibrium length after 100 s (Figure 3.5). This observation demonstrates that hMSCs exhibit a typical monotonic viscoelastic behavior as do many other engineering materials, which was confirmed by a very high mean correlation coefficient of $R^2 = 0.98$ when the aspiration length-time data were fitted with a viscoelastic solid model (Figure 3.6). This justifies the use of the viscoelastic solid model to represent the deformation behaviour of hMSCs. The instantaneous Young's modulus, E_0 , the equilibrium Young's modulus, E_∞ , and the apparent viscosity, μ , of hMSCs obtained at 20°C were: 886 ± 289 Pa, 372 ± 125 Pa and 2700 ± 1600 Pa·s, respectively (Figures 3.7 and 3.8).

Figure 3.5 Micropipette aspiration of hMSCs at room temperature. Images (A-D) are displayed at time $t = 1$ s, 15 s, 100 s and 200 s after the application of step aspiration pressure, respectively. An initial jump of cell protrusion (A) into the micropipette in response to a step aspiration pressure is followed by an asymptotic creep behavior and eventually reaching equilibrium (Reproduced from Ref. 148).

Figure 3.6 Various patterns of aspiration length at different conditions with respect to time. hMSCs treated with 20 μM of cytochalasin D at 20°C achieve the longest aspiration length. hMSCs reach the equilibrium aspiration length faster at 20°C than 37°C (Reproduced from Ref. 148).

Figure 3.7 Effects of temperature and cytochalasin D on the instantaneous and equilibrium Young's modulus of hMSCs. Mean and standard deviation are shown for each experiment. The stiffness of hMSCs decreases with increasing temperature and increasing concentration of cytochalasin D. * $p < 0.001$ as compared to control. (1) $p < 0.05$ as compared to cytochalasin treatment at 0.2 μ M. (2) $p < 0.05$ as compared to cytochalasin treatment at 2.0 μ M. (3) $p < 0.05$ as compared to cytochalasin treatment at 20 μ M. (4) $p < 0.05$ as compared to cells at 37°C (Reproduced from Ref. 148).

Figure 3.8 Dependence of viscosity of hMSCs on temperature and the integrity of actin cytoskeleton. Mean and standard deviation are shown for each experiment. * $p < 0.05$ as compared to control. (1) $p < 0.05$ as compared to cytochalasin treatment at 0.2 μM . (2) $p < 0.05$ as compared to cytochalasin treatment at 2.0 μM . (3) $p < 0.05$ as compared to cytochalasin treatment at 20 μM . (4) $p < 0.05$ as compared to cells at 37°C (Reproduced from Ref. 148).

3.2.2 Effect of disruption of F-actin

The F-actin network of hMSCs was disrupted to certain degree as a result of the treatment with cytochalasin D before the cells were aspirated at 20°C (Figure 3.9). Under such condition, the hMSCs no longer displayed the typical viscoelastic behavior of solid materials. The cells experienced a much slower rate of increase in aspiration length with no obvious initial jump, and rose towards a much higher equilibrium length compared to the cells tested under the control condition (Figure 3.6). The addition of cytochalasin D at various concentrations caused E_0 and E_∞ to decrease significantly by 67% to 84% from the control ($p < 0.001$) (Figure 3.7). There was no significant change in E_0 and E_∞ ($p > 0.1$) when the concentration was increased from 0.2 μM to 2.0 μM , which is the half maximal inhibitory concentration or IC_{50} value for hMSC. The apparent viscosity, μ , increased significantly by up to 255% at the higher concentration of 2.0 μM and 20 μM as compared to the control condition and low concentration of cytochalasin D (0.2 μM), respectively ($p < 0.05$) (Figure 3.8).

Figure 3.9 Micropipette aspiration of hMSCs treated with the highest concentration (20 μM) of cytochalasin D (A – D). Images are displayed at $t = 1$ s, 15 s, 100 s and 200 s, respectively, after the application of step aspiration pressure. The aspiration length of the cell increased significantly due to the disruption of F-actin filaments.

3.2.3 Temperature effect

hMSCs aspirated at physiological temperature of 37°C also exhibited typical viscoelastic behavior. However, the initial jump of the aspirated length was much shorter than the cells examined at 20°C (Figure 3.6). The rate of increment of aspiration length decreased gradually with time before it reached the equilibrium aspiration length, which was longer than the control cells, but lower than those treated with cytochalasin D. Both E_0 and E_∞ of hMSCs determined at 37°C were significantly reduced from the control, to 518 ± 280 Pa and 126 ± 81 Pa, respectively ($p < 0.001$) (Figure 3.7), which correspond reduction of 42% and 66%, respectively. The apparent viscosity, μ , was increased significantly from the control by 95% to 5290 ± 3026 Pa·s ($p < 0.05$) (Figure 3.8).

3.2.4 Non-typical standard viscoelastic behaviors

During the experiment at 37°C, three other types of non-standard viscoelastic behavior of hMSCs that constitute about 55% of total observations, represented by the aspiration length-time curves, were observed (Figure 3.10). Instead of reaching a stable equilibrium aspiration length, close to 34% of the hMSCs displayed further increase of aspiration length with time (departure 1). 14% of the hMSCs reached maximum aspiration length before decreasing to a lower equilibrium length (departure 2). A small amount of hMSCs (7%) demonstrated a step-wise viscoelastic behavior in the aspiration length-time curve (departure 3), where the first step of aspiration length increment occurred at $t = 50$ s while the second step at $t = 150$ s as seen in Figure 3.10.

Figure 3.10 The different patterns of non-typical viscoelastic creep response of hMSCs. After reaching equilibrium length, 34% of the hMSCs experienced a further increase of length with time (departure 1) while 14% of the hMSCs reached a peak aspiration length before dropping to a lower equilibrium length (departure 2). A small amount of hMSCs (7%) exhibited a step-wise deformation behavior (departure 3). Lines connecting data point indicate data trend (Reproduced from Ref. 148).

3.3 Discussion

3.3.1 Viscoelastic behavior of hMSCs

A liquid-like cell with a constant cortical tension will flow easily into a micropipette when the suction pressure exceeds the critical pressure [31]. In contrast, a solid-like cell will reach an equilibrium length of projection instead [31] which was observed from the aspiration results of hMSCs. The viscoelastic properties of hMSCs in terms of the calculated equilibrium Young's modulus in this study falls in the same order of 0.5 kPa as compared to porcine endothelial cells and human chondrocytes, which are classified as viscoelastic solid materials (Table 3.2). Therefore, based on

previous studies on other cell types as well as the results gathered in this study, it can be concluded that hMSCs possess the characteristics of solid-like viscoelastic cells.

Table 3.2 The comparison of mechanical properties obtained for various cells (Reproduced from Ref. 148).

Cell types	Mechanical properties			Reference
	E_0 (Pa)	E_∞ (Pa)	μ (Pa·s)	
Porcine endothelial	429	114 ± 35	8300 ± 4000	49
Human chondrocyte	640	270	2100	30
hMSC	886 ± 289	372 ± 125	2700 ± 1600	This work

E_0 is the instantaneous Young's modulus, E_∞ is the equilibrium Young's modulus and μ is the apparent viscosity. Note that the results compared are generated from the same viscoelastic model used in our study.

3.3.2 Effect of disruption of F-actin

As expected, the disruption of F-actin by cytochalasin D has the highest impact on the stiffness of hMSCs, leading to the lowest Young's modulus registered. Actin, the major protein component in eukaryotic cells, plays an important role in cell motility and in maintaining cell shape [155]. For suspended cells, actin filaments are found to concentrate on the cell periphery or cortex [35]. When administered to the cells, cytochalasin D degrades the actin network into shorter filaments in a dose-dependent manner [35]. As a result, the reduced density of the cortical actin network renders the cell softer, more viscous, and more liquid-like, leading to a higher equilibrium aspiration length (Figure 3.6).

3.3.3 Temperature effect

Temperature difference also affected the viscoelastic behavior of hMSCs. The increase in viscosity of cells is attributed to changes in the mechanical integrity of the

cell body spanned by the cytoskeleton. Microtubule, one of the major components of cytoskeleton that provides support for compressive load on cells [118], are known to be temperature-sensitive. It has been found that the bending stiffness or Young's modulus of microtubules decreases with increasing temperature [156], possibly leading to a lower overall cell modulus at higher temperature. Besides that, a decrease in temperature suppresses microtubules structural instability by slowing down the rate of catastrophe or shrinkage [157, 158]. However, lowering temperature may also imply a reduction in the net amount of assembled microtubules. That is to say, higher temperature promotes microtubule instability as more of them are forming network. From the results obtained, it seems that the former is dominating the two competing effects, which may explain the drop in cell stiffness when the experiments were performed at 37°C, compared to the data at 20°C.

3.3.4 Departures from standard viscoelastic behavior

Interestingly, three types of departures from the typical viscoelastic behavior were observed during the study (Figure 3.10). The first departure (departure 1) involves a further increment of aspiration length after reaching a seemingly equilibrium state, which could be attributed to mechanical disruption of the cytoskeleton towards the later stage of the aspiration process, causing the cell to turn liquid-like. It is possible that the cytoskeleton of cell breaks apart into smaller fragments or depolymerizes due to the tensile stretch on cell surface. This kind of behavior is indeed very similar to the so-called tertiary state in creep behavior of materials, when a material elongates abruptly due to internal damage and eventually ruptures. The second departure (departure 2), which is characterized by a peak before reaching the equilibrium length, may arise due to either global or localized cell contraction, by rearrangement of cell cytoskeleton (comprises of actin filaments, microtubules and intermediate filaments) after reaching the maximum aspiration length. It is likely that the cytoskeleton are reorganised in such a way to resist the applied external tensile force as part of a feedback mechanism to protect the cell structural integrity. The third departure (departure 3) with a step-wise pattern could be interpreted as the result of a combination of large cell deformation and contraction during aspiration. Cell may contract even though there is an external tensile stress

applied due to its inherent elasticity as shown in Figure 3.11. These three departure patterns, however, were not observed in the hMSCs treated with cytochalasin D, suggesting that the disruption of actin directly affects the deformation behavior of cells. It should be mentioned that the mechanisms for the latter two departures are still unclear at present. The results obtained from all types of departures were excluded in the calculation of Young's modulus and viscosity.

Figure 3.11 Contraction of cell observed during aspiration. Arrow shows the direction of aspiration pressure.

It is important to note that the spatial location of nucleus may contribute to the overall deformation of a cell in an aspiration experiment and thus influence the aspiration length-time curve. For instance, if the stiff nucleus is near the tip of the micropipette, it is anticipated that the aspiration length would be longer compared to the case where the nucleus is away from the tip. However, quantitative and systematic analyses of the effect of nucleus can only be revealed by a combination of experimental and computational methods. As such, the influence of nucleus on cell fluidity in terms of the maximum aspiration length attainable before rupture will be examined in Chapter 5.

3.4 Summary

In summary, our findings validated the hypothesis that hMSCs behave like a viscoelastic solid material, consistent with other cell types such as chondrocytes and endothelial cells. In addition to typical viscoelastic behavior, three other types of non-typical viscoelastic behaviors of hMSCs were also seen. We have also demonstrated that both instantaneous and equilibrium Young's modulus, as well as the apparent viscosity of hMSCs were sensitive to the structural integrity of F-actin filaments and temperature. The baseline data for hMSCs obtained in this study is expected to facilitate future studies in understanding the relationship between mechanical stimuli and phenotypic alterations of hMSCs, and quantitative description of hMSC behavior.

Moving on from the study on the global deformation of single cells, we will present and discuss the results obtained from the analysis of local deformation of single NIH 3T3 mouse fibroblasts undergoing aspiration, in terms of the change in microtubule morphology which can be quantified through the calculation of bending energy, in the following chapter. The results and discussion generated will provide a better understanding and answer to what exactly happens at the cytoskeleton level of a cell as the external force propagates from the single cell level into its subcellular structure.

Chapter 4 Microtubules Deformation

In this chapter, the procedures for staining microtubules with TubulinTracker™ Green reagent is described together with the imaging and measurement technique applied to observe and quantify the deformation of microtubules during aspiration. Subsequently, two major observations obtained during the experiments performed to investigate the microtubules morphological changes are presented. The bending energy of the microtubules are calculated based on the microtubules curvature measured from the 4 different regions of the cell main body. With this data, the relationship between global and local deformation can be characterized quantitatively. At the end of this chapter, a comprehensive explanation and discussion on the findings that shed new light in the area of microtubules mechanics under the influence of external tensile load are presented.

4.1 Material and methods

4.1.1 Cell culture

Instead of using hMSCs, NIH 3T3 mouse fibroblasts were chosen and used to explore and observe the deformation of microtubules during micropipette aspiration. This decision was made after numerous attempts to stain the microtubules of hMSCs with TubulinTracker™ (TT) Green reagent (Invitrogen, USA) and eGFP-tubulin yielded a very poor staining efficiency. This may be due to the fact that hMSC is unstable as it is prone to differentiation initiated by various factors such as passage number and external environment [146]. For the few hMSCs successfully stained, the cells tended to grow and spread larger than the viewing range of the 100× microscope lens.

In contrast, NIH 3T3 fibroblasts stained with TT showed good staining efficiency, enabling sharp and clear microtubules images to be captured within the microscope viewing limit. We did explore the use GFP-tubulin staining which has the main advantage of not affecting microtubules dynamics [159]. Unfortunately, we

found that the transfection efficiency of eGFP-tubulin on the fibroblasts was also low and its photobleaching rate was even higher than TT. As a result, we decided to use TT.

NIH 3T3 mouse fibroblasts were purchased from ATCC (USA) and cultured using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-Glutamine, 10% fetal bovine serum and 5% penicillin/streptomycin in a 35 mm Petri dish at 37°C, 5% CO₂.

4.1.2 Microtubule fluorescence staining

TubulinTracker™ Green reagent (T34075, Molecular Probes™) which is also known as Oregon Green 488 Taxol bis-acetate is a non-fluorescent and uncharged compound that permeates easily into plasma membrane to stain the polymerized tubulin of live cells. This is done with the help of Taxol (Figure 4.1) which is also known as paclitaxel, a natural anticancer drug [160, 161] obtained from the tree bark of *Taxus brevifolia* [162] that attaches to and stabilizes microtubules (Figure 4.2). As a result, there is a net increase in the polymerization of tubulin which translates into longer and less dynamic microtubules [162]. In addition, microtubules appear to be assembled in thick bundle [163, 164]. However, there is a disadvantage when using this reagent, that is, cell division will be inhibited as the G2 and M phases of cells are blocked by Taxol [165]. The excitation and emission wavelengths for the reagent are 494 nm and 522 nm, respectively.

Figure 4.1 Chemical structure of Taxol (Reproduced from Ref. 162).

Figure 4.2 Schematic representation of microtubule stabilisation by Taxol (Reproduced from Ref. 162).

The staining protocol given by the manufacturer was followed closely during the staining process. To prevent unnecessary loss of fluorescent activity, the tube and Petri dish that contained the dye were covered with a thin layer of aluminium sheet. Instead of using the usual phosphate buffered saline (PBS) buffer, Hanks balanced salt solution (HBSS) purchased from Invitrogen, USA was used to maintain physiological pH for cells as recommended by the manufacturer. The optimum concentration for the fluorescent dye was obtained at 250 nM for this experiment. Any concentration higher than that was found to disrupt the cell morphology. It is important to note that freshly made staining solution will give the best result in terms of fluorescent intensity.

Upon reaching 70 - 80% confluence, the cells cultured in a 35 mm Petri dish were stained with TT at an optimum concentration of 250 nM after removing the culture medium and rinsing the cells with HBSS. The concentration of 250 nM of TT was obtained by diluting 20 μ l of 5 μ M of TT stock solution with 380 μ l of HBSS. After 30 minutes of incubation, the cells were rinsed with HBSS buffer for 3 times to remove excess of TT dye which may give rise to unnecessary background fluorescence during imaging process. 500 μ l of 0.25% Trypsin/EDTA was added for a duration of 1 minute to detach the cells. 1000 μ l of cell culture medium was added to neutralize trypsin and cells were transferred into a 15 ml plastic test tube for centrifugation at 1500 rpm for 5 minutes. The mixture of medium and trypsin were then carefully disposed without disrupting the cells accumulated at the bottom of the test tube. 500 μ l of HBSS was added to disperse the cells. 10 μ l (2% of total volume) of this mixture of cells in HBSS was then transferred into a black low-wall Petri dish (Catalogue no. FD3510B-100, World Precision Instruments, Inc.) which contained 200 μ l of HBSS and a 10 mm diameter coverslip (0.17 mm thick) coated with 0.05 mg/ml fibronectin

(F4759, Sigma-Aldrich) and was incubated for 10 minutes. To prevent photobleaching, the Petri dish that contained the stained cells was covered with a thin layer of aluminium foil.

Special precautions must be exercised when preparing the stock solution for TT as dimethyl sulfoxide (DMSO) has to be added. DMSO is very harmful as it can penetrate the skin easily. Proper chemical gloves, lab coat and chemical hood must be used when mixing the solution. User must read and understand the material safety datasheet (MSDS) for DMSO provided by Sigma Aldrich before using the chemical. Due to its strong amphiphilic nature, it has been found that 2% of DMSO is enough to degrade the lipid membrane, leading to changes in the elastic properties of cell membrane [166]. This is the disadvantage that arises from using this chemical to allow the penetration of the TT dye into the cells. However, in our study, only 0.025% of DMSO was used to prepare the TT dye which would be further diluted upon mixing with HBSS buffer solution. We expect that this small percentage of DMSO will not affect the mechanical properties of lipid membrane significantly.

4.1.3 Micropipette aspiration

To find the best state of cell for displaying clear microtubules network and providing the minimum thickness for aspiration, the TT-stained cells were harvested and incubated at 3 different conditions. First, the fully suspended or non-adherent cells, which were observed immediately after being harvested with trypsin, did not show any defined microtubules structure (Figure 4.3). Second, the adherent cells which were incubated for 12 hours showed good spread and excellent fluorescent images of distinct microtubules (Figure 4.4). However, it was very difficult to aspirate these cells as they were very thin. Lastly, the 'semi-suspended' or 'semi-adherent' cells that were obtained after the process of harvesting and incubating the stained cells for 10 minutes and seeded on a 0.05 mg/ml fibronectin coated coverslip, produced the optimum result in terms of clearly defined microtubules network and cell thickness for aspiration (Figure 4.5). Thus, only the semi-suspended cells were selected for our study in order to observe the deformation of microtubules more effectively during micropipette aspiration.

Figure 4.3 Microtubules structure is not clearly defined for fully suspended cells. (a) Brightfield image of suspended cells (b) The corresponding fluorescent image showing the TT-stained microtubules of the suspended cells. Scale bar represents 20 μm.

Figure 4.4 Cell stained with TubulinTracker™ after 12 hours of incubation. (a) Brightfield image of the well spread cell. (b) The corresponding fluorescent image of TT-stained microtubules. Scale bar represents 20 μm .

Figure 4.5 A single semi-suspended cell displaying distinct microtubules network after 10 minutes of incubation on a fibronectin-coated coverslip. (a) Brightfield image. (b) The corresponding fluorescent image of the TT-stained microtubules. Scale bar represents 10 μm .

After completing the staining procedure as mentioned earlier in Section 4.1.2, semi-suspended TT-stained cells were aspirated for 5 minutes at a constant pressure of 1 - 9 kPa using 1.5 - 4 μm diameter micropipette in real-time at 37°C (Figure 4.6). The same micropipette aspiration system equipped with heating stage described earlier in Chapter 3 was used. The only difference in the current setup was that the aspiration process was viewed under another inverted fluorescence microscope, Axiovert 40 MAT (Carl Zeiss, Germany) using 100 $\times$ oil immersion lens and a high speed camera, AxioCam Hsm (Carl Zeiss, Germany). The images and video streaming during aspiration were captured by AxioVision software version 4.6 (Carl Zeiss, Germany). The front tip of the micropipette, which is flexible, was bent by lowering the micropipette until it was in contact with the coverslip (Figure 4.6). This was to allow the tip to be in focus with the cell target during image capturing process as well as to enable the suction pressure to be applied horizontally on the cell periphery.

Figure 4.6 A schematic diagram showing the side view of a semi-suspended cell aspirated with pressure, P. The flexible micropipette is bent in such a way that the front tip of the micropipette is in contact with the coverslip.

During aspiration, it was found that TT bleached rapidly under continuous exposure to fluorescent light. To overcome the photobleaching effect, the fluorescent light was activated manually for approximately 3 – 5 seconds through a slider at every 1 minute interval during aspiration. However, this step must be done gently and carefully to avoid moving the micropipette which may end up poking and damaging the target cell.

A total of 30 cells with clear microtubules images were selected for the bending energy analysis. Unpaired two-sample (with unequal variance) *t*-test was used to examine whether the change in average bending energy, calculated at various regions of the single cells, are significantly different.

4.1.4 Image processing

The fluorescent images gathered from the experiment were processed using Image Processing and Analysis in Java or better known as ImageJ, a free software provided by National Institutes of Health, USA for public use [167]. It is a powerful and user-friendly software that is loaded with many useful functions for analyzing images. It has the convolution function which is not available in AxioVision software used for capturing the timelapse images during aspiration. Convolution filtering is a method of substituting a pixel value by moving and multiplying a square kernel to each

pixel across the image [168]. When activated, this function makes the microtubules clearer and sharper in terms of quality, enabling more accurate measurement of their properties such as radius and angle as shown in Figure 4.7. The only downside of this method is that the background noise is increased.

Figure 4.7 The effect of convolution method to enhance the visibility of fluorescent stained microtubules. (a) The fluorescent image before convolution. (b) The corresponding image after convolution based on the default kernel of 5×5 matrix.

4.1.5 Measurement techniques

Diameter of microtubules bundle

Diameter of microtubules was measured and averaged to check for any bundle formation for TT-stained cells. This was done by comparing the EGFP-stained microtubules with TT-stained microtubules. It was found that single EGFP-stained microtubules have an average diameter of $0.39 \mu\text{m}$ (Figure 4.8) while the TT-stained microtubules have average diameter in the range of $0.39 - 0.68 \mu\text{m}$, indicating that bundling of microtubules does occur. We counter check our results with the diameter of EGFP-stained microtubules from Brangwynne et al. work [118] and found that the diameter of single microtubule measured from their fluorescent images are also in the range of $0.25 - 0.40 \mu\text{m}$. It is clear now that the diameter of a single microtubule measured from fluorescent images is much larger than the known diameter of a single

microtubule which is 25 nm. It is very likely that the huge discrepancy is due to dispersion of fluorescent light emitted by the stained microtubules, making them look much larger than the actual diameter. In addition, the image and measurement tool resolution limited to 0.19 μm per pixel may also contribute to the overestimation. As a result, we decided to use 0.39 μm as the baseline for the diameter of a single microtubule in our study for consistency in comparing with the diameter of TT-stained microtubules.

Figure 4.8 3T3 fibroblast with EGFP-stained microtubules. (a) Fluorescent image of the stained microtubules. (b) The image of microtubules taken from an enlarged section of the cell in (a) for diameter measurement purpose. Scale bar represents 2 μm .

Buckling wavelength

The buckling wavelength of microtubules, λ , is essential for the calculation of buckling force required to deform the microtubules. The wavelength of buckled MT is the distance measured between two peaks along a MT bundle (Figure 4.9). The wavelengths were measured over the entire cell based on the fluorescent images captured at various times by using the distance tool provided in the AxioVision software as shown in Figure 4.10.

Figure 4.9 Schematic diagram showing the wavelength of a buckled MT.

Figure 4.10 Calculation of the wavelengths of buckled microtubules using AxioVision software.

Radius of curvature

The radius of curvature, R , which is required for the calculation of bending energy, can be obtained by fitting a circle to the curve found along microtubules while the angle of curvature, θ , can be calculated by using the *3-point method* which involves the centre point of the fitted circle and the two end points of the curve (Figure 4.11). These measurements were done using the circle and angle tools provided by the AxioVision software with an accuracy of $\pm 0.19 \mu\text{m}$. The values of R and θ recorded by the software were exported into an Excel spreadsheet.

Figure 4.11 The 3-point method used to obtain the radius and angle of selected microtubule segment. Blue line represents the circle generated to match the curvature of the microtubule segment and subsequently the radius of the curvature. Red line refers to the 3-point method used to extract the angle of the segment of interest. Point 1 is the centre of the circle while Points 2 and 3 are the start and end points of the segment.

4.2 Results

4.2.1 Buckling of microtubules

The aspiration process of a semi-suspended cell captured using a brightfield microscope is shown in Figure 4.12. The portion of the semi-suspended cell outside the micropipette was adhered to the fibronectin-coated coverslip as observed through the formation of lamellipodium, a sign of cell spreading, and yet still having sufficient height for aspiration purpose. This is the main experimental difference between the aspiration of semi and fully suspended cells. For this particular cell, there was no immediate flow of cell into the micropipette until after 3 minutes of aspiration. After 4 minutes of aspiration, the main bulk of cytoplasm including cell nucleus was aspirated into the micropipette, which can be seen from its corresponding brightfield image of Figure 4.12(f).

Figure 4.12 The timelapse of brightfield images (a) – (f) extracted from the micropipette aspiration experiment using a micropipette of the diameter of $5\ \mu\text{m}$ at a constant pressure of $6.9\ \text{kPa}$ (direction indicated by yellow arrow) over a period of 5 minutes. Significant length of cell can be observed at (c) in the micropipette as indicated by the white arrow. The corresponding fluorescent images of the same cell are shown in Figure 4.13. Time shown is based on $hh : mm : ss$ format, where h is hour, m is minute, s is second. Scale bar represents $10\ \mu\text{m}$.

Microtubules of the semi-suspended cell appeared to be accumulated around the cell periphery (Figure 4.13), which is consistent with the fluorescent images obtained for majority of semi-suspended cells. It was observed that the microtubules at the opposite side of the micropipette tip start to buckle at $t = 162$ s as shown in Figure 4.13(d). Consistent with the last brightfield image Figure 4.12(f), most of the microtubules are being pulled towards the micropipette at $t = 256$ s in Figure 4.13(f) resulting from the extensive flow of cytoplasm into the micropipette. Interestingly, no substantial amount of fluorescence was observed in the aspirated cell portion within the micropipette (Figure 4.13), suggesting that microtubules were either not available or lacking in that region. To facilitate analyzing microtubules deformation, the same fluorescent images of Figure 4.13 were processed using the convolution method making the microtubules to appear much clearer as shown in Figure 4.14. From the overlapped image of the first and last time frame presented in Figure 4.14(f), it is confirmed that microtubule buckling occurs at the region away from the micropipette entrance.

Figure 4.13 Close-up fluorescent images (a) – (f) of the same cell in Figure 4.12 during micropipette aspiration. Buckling effect of microtubules at the opposite end of the aspirated cell can be observed in (d) as indicated by the white arrow. Time shown is based on $hh : mm : ss$ format, where h is hour, m is minute, s is second. Scale bar indicates $10\ \mu\text{m}$. The video recording of the observation is attached in the supplementary CD in Appendix F.

Figure 4.14 Convoluted images (a) – (e) that show the buckling effect of microtubules at the region away from the micropipette entrance can be observed in (d) as indicated by the red arrow. The direction of aspiration pressure is indicated by the black arrow in (a). The traces of microtubules during the initial (red) and final (blue) frames overlapping each other are presented in (f). Time shown is based on *hh : mm : ss* format, where *h* is hour, *m* is minute, *s* is second. Scale bar represents 10 μm .

Buckling wavelength & critical buckling force

It was observed that the buckling wavelength of microtubules of the same cell shown previously in Figure 4.12 decreases steadily with time (Figure 4.15). The average buckling wavelength calculated over a duration of 4 minutes was $5.1 \pm 1.8 \mu\text{m}$. The distribution of microtubules buckling wavelength within the same cell was found to change at different time intervals (Figure 4.16). For example, the microtubules exhibited long buckling wavelength of 8 - 18 μm at $t = 99 \text{ s}$ while at a later stage, $t = 263 \text{ s}$, majority of the microtubules were seen to buckle at a much shorter wavelength of 3 - 5 μm .

Figure 4.15 The mean of microtubule buckling wavelength with standard deviation error bars observed over time for the cell shown in Figure 4.12 and the corresponding non-linear regression curve ($R^2 = 0.91$). n refers to the number of microtubule segments measured at each data point.

Figure 4.16 Statistical distribution of microtubules buckling wavelength of the cell shown in Figure 4.12 at various time intervals (a) – (d).

As the microtubules in this study tend to combine and form bundles due to the effect of Taxol, the flexural rigidity or bending stiffness of microtubule bundle, which is required for the calculation of buckling force, has to be estimated. Assuming that the individual microtubules are closely packed, the number of individual microtubule within a bundle, N_{MT} , can be estimated by dividing the bundle circular cross sectional area, A_b , with the single microtubule circular cross sectional area, A_s , as follows,

$$N_{MT} = \frac{A_b}{A_s} = \frac{(d_b)^2}{(d_s)^2} \quad (4-1)$$

where N_{MT} is a positive integer, d_b is the diameter of bundle, which can be measured from fluorescent images of microtubules, and d_s is the diameter of a single microtubule. The flexural rigidity of a microtubule, κ_s , is given by

$$\kappa_s = E \times I_s \quad (4-2)$$

where E is Young's modulus and I_s is the area moment of inertia for a circular rod, that is, $\frac{\pi}{64}d^4$. For a bundle with N numbers of rods, the area moment of inertia,

$$I_b = \sum_{j=1}^N I_{s,j} = N_{MT} \times I_s. \text{ Therefore, flexural rigidity of a bundle, } \kappa_b, \text{ consisting of } N$$

number of microtubules, can be approximated by

$$\begin{aligned} \kappa_b &= E \times I_b \\ \kappa_b &\approx E \times N_{MT} \times I_s \approx N_{MT} \times \kappa_s \end{aligned} \quad (4-3)$$

where the flexural rigidity for a single Taxol-stabilised microtubule, κ_s , obtained at 37°C is $2.54 \times 10^{-24} \text{ Nm}^2$ [95] and the average diameter of a single microtubule measured from the fluorescent images, d_s , is 0.39 μm , as mentioned earlier in Section 4.1.5.

To obtain the minimum or critical force, f_c , required for microtubules buckling to occur during aspiration, we used the modified version of Euler buckling that accommodate the short wavelength buckling of microtubules as proposed by Brangwynne et al. [118], which is as follows

$$f_c = 8\pi^2 \frac{\kappa}{\lambda^2} \quad (4-4)$$

where κ refers to the flexural rigidity of microtubule while λ refers to the buckling wavelength. Based on the result shown in Figure 4.14 and at 2:42 in Figure 4.14(d) where buckling of microtubules was first observed, it was found that the critical buckling force, $f_c \approx 24 \text{ pN}$ using Equation 4-4 and the calculated average buckling wavelength of $\lambda \approx 5 \mu\text{m}$. The estimated flexural rigidity, κ , of $7.62 \times 10^{-24} \text{ Nm}^2$ was obtained from Equation 4-3 for a microtubule bundle made up of based on the average number of 3 individual microtubules calculated from Equation 4-1.

4.2.2 Straightening of microtubules

This section presents the 2nd type of MT deformation observed during aspiration, i.e., the straightening of MT at the cell region near the micropipette

entrance under the same experimental condition as Section 4.2.1. The brightfield images captured in Figure 4.17 are somewhat similar to Figure 4.12 in terms of cell morphology, except that the cell together with its nucleus still remained largely outside the micropipette even after 4 minutes of aspiration. However, by observing the fluorescent images of the cell in Figure 4.18 and the convoluted images in Figure 4.19, it can be seen clearly that microtubules are pulled and straightened at the vicinity of the micropipette entrance in the direction of suction pressure at $t = 165$ s (Figure 4.19(c)). The straightening effect is confirmed by analyzing the overlapping image of the first and last time frame of microtubules traces as shown in Figure 4.19(f). Again, no significant and distinct microtubules were observed within the aspirated portion of cell (Figure 4.18). Similar observations of microtubule straightening were observed in many of the other cells studied which are presented in Appendix A.

Figure 4.17 The timelapse of brightfield images (a) – (e) extracted from the micropipette aspiration experiment over a period of 5 minutes at a constant pressure of 3.6 kPa (direction indicated by yellow arrow). The nucleus remained in the host cell throughout the aspiration. The corresponding fluorescent images are shown in Figure 4.18. Time shown is based on $hh : mm : ss$ format, where h is hour, m is minute, s is second. Scale bar represents 10 μm .

Figure 4.18 Close-up fluorescent images (a) – (e) of the same cell in Figure 4.17 during micropipette aspiration. Straightening of microtubules is observed at the micropipette entrance (white arrow) as indicated in (c). Time shown is based on $hh : mm : ss$ format, where h is hour, m is minute, s is second. Scale bar represents $10\ \mu\text{m}$. The video recording of the observation is attached in the supplementary CD in Appendix F.

Figure 4.19 Convoluted images (a) – (e) of the same cell found in Figure 4.18 show clearer microtubules structure deformation during aspiration. The traces of the microtubules during the initial (red) and final (blue) frames overlapping each other are shown in (f). Time shown is based on $hh : mm : ss$ format, where h is hour, m is minute, s is second. Scale bar represents $10\ \mu\text{m}$.

4.2.3 Bending energy variation over time

To analyze the local stress state of a cell in terms of change in bending energy of microtubules, each cell was split into 4 rectangular regions as shown in Figure 4.20. Region 1 is located nearest and perpendicular to the micropipette entrance followed by Region 2 which is located parallel to the micropipette axis of symmetry. Region 3 is situated directly opposite the micropipette entrance. Region 4 is positioned above Region 2. Both Regions 2 and 4 are extended equally to cover the middle region of cell. The regions were chosen based on deformation patterns of MT (straightening near micropipette entrance and buckling away from the entrance). The size and location of each region were considered to be sufficient in dividing the cell for local deformation analysis.

Figure 4.20 The 4 different regions marked out for the analysis of microtubule bending energy.

Microtubules can be represented as elastic semi-flexible rods due to their physical configurations. The local bending energy, U_b , for such rods is expressed as

$$U_b = \frac{\kappa}{2} \int_L \left(\frac{\partial \vec{t}}{\partial s} \right)^2 ds \quad (4-5)$$

where κ is the flexural rigidity of the material, $\partial \vec{t} = \vec{t}_2 - \vec{t}_1$ is the change in the tangent vector to the arc, $\partial s = \Delta s$ is the segment length and $\left(\frac{\partial \vec{t}}{\partial s} \right)$ is the curvature of the rod, C

[169] (Figure 4.21). This expression of U_b is also known as the Kratky-Porod or worm-like chain model [170]. For a rod with an intrinsic curvature, $C_0 = \frac{1}{R_0}$, that bends

into a circular shape with radius, R , $\frac{\partial \vec{t}}{\partial s} = (C - C_0) \vec{n} = \left(\frac{1}{R} - \frac{1}{R_0} \right) \vec{n}$ where $\vec{n}$ is the unit

normal vector to the arc. The expression of U_b in Equation 4-5 becomes

$$U_b = \frac{\kappa}{2} \int_L \left(\frac{1}{R} - \frac{1}{R_0} \right)^2 \vec{n} ds \quad (4-6)$$

Figure 4.21 The tangent (red) and normal (blue) vectors, i.e., $\vec{t}_1, \vec{t}_2$ and $\vec{n}_1, \vec{n}_2$, respectively on an arc length, Δs with an angle, θ and radius, R . (Reproduced from Ref. 169).

As arc length, $s = R\theta \Rightarrow ds = R d\theta$, therefore, Equation 4-6 can be solved by integration with respect to $d\theta$ giving

$$U_b = \frac{\kappa}{2} \int_{\theta} \left(\frac{1}{R} - \frac{1}{R_0} \right)^2 R d\theta = \frac{\kappa}{2} \left(\frac{1}{R} - \frac{1}{R_0} \right)^2 R \int_{\theta} d\theta = \frac{\kappa}{2} \left(\frac{1}{R} - \frac{1}{R_0} \right)^2 R \theta \quad (4-7)$$

To obtain U_b per unit length of microtubule measured,

$$\frac{U_b}{L} = \frac{U_b}{s} = \frac{U_b}{R\theta} \quad (4-8)$$

Combining Equations 4-7 and 4-8,

$$\frac{U_b}{L} = \frac{\kappa}{2} \left(\frac{1}{R} - \frac{1}{R_0} \right)^2 \quad (4-9)$$

where this expression of U_b per unit length will be used in calculating the average bending energy of microtubules during aspiration. The radius of curvature, R and flexural rigidity of microtubule bundle, $\kappa = \kappa_b$, were obtained from experimental results as mentioned earlier in Section 4.1.5 and using Equation 4-3 from Section 4.2.1, respectively. For the purpose of comparison with others, the unit of the calculated U_b / L is converted to $kT/\mu\text{m}$ using the thermal energy scale, kT where k is the Boltzmann's constant, 1.38×10^{-23} J/K and T is the absolute temperature. As the temperature set for our study was at 37°C or 319.15 K, kT is equivalent to 4.28×10^{-21} J.

It is important to understand how the global deformation induced by pressure and the local deformation of microtubules are linked quantitatively. This analysis can be done by determining the percentage of energy transferred from the work done by aspiration pressure, dW , to the change in bending of microtubules at a specific time and region, that is,

$$\frac{d\bar{U}_b}{dW} \times 100\% \quad (4-10)$$

where $d\bar{U}_b$ is the change in average bending energy of microtubules, which can be expressed as $d\bar{U}_b = \bar{U}_f - \bar{U}_i$, where $\bar{U}_i$ and $\bar{U}_f$ are the average bending energy calculated at the start and end of aspiration for a specific region of a cell. The average bending energy is

$$\bar{U}_b = \frac{\sum \bar{U}_b}{N} \quad (4-11)$$

where $\sum \bar{U}_b$ is the total average bending energy of MT consolidated from a bundle of MTs located in the same region, and N is the total number of MT curvature located in the specific region of the cell. The reason for using *average* U_b is because there is often a mixture of both straightening and buckling behaviors within the same region of cell.

Work done by the aspiration pressure, dW , in displacing cell volume into micropipette can be expressed as

$$dW = P dV = AP dx \quad (4-12)$$

where P is the applied constant suction pressure, V is the cell volume in micropipette, A is the cross-section area of micropipette, and dx is the length of aspirated cell into micropipette. dW varies with time as it depends on length of the aspirated cell, which also changes with time.

For microtubules that buckle during aspiration, the corresponding bending energy, $d\bar{U}_b$, will increase over time. This can be explained by the fact that since $d\bar{U}_b \propto \frac{1}{R^2}$ as shown in Equation 4-9, there will have an increase in $d\bar{U}_b$ when a microtubule bends over time, with a decrease in R . In contrast, $d\bar{U}_b$ decreases as a microtubule straightens over time.

Taking the previous cell shown in Figure 4.14 as an example, its U_b increased from $\sim 6 \text{ kT}/\mu\text{m}$ at $t = 66 \text{ s}$ to $\sim 240 \text{ kT}/\mu\text{m}$ at $t = 191 \text{ s}$ as witnessed in Region 3 during buckling process (Figure 4.22(a)). For Regions 2 and 4, where both U_b trends are very similar, there is a slight increase in the bending energy over time reflecting the minor occurrence of microtubules buckling (Figure 4.22(a)). However, there was a sudden decrease in bending energy in Region 1 from $\sim 22 \text{ kT}/\mu\text{m}$ to $\sim 1 \text{ kT}/\mu\text{m}$ near the final stage of aspiration, indicating that the microtubules at that region experienced straightening (Figure 4.22(a)). No data points were available after 4 minutes of aspiration as the microtubules images were not clear enough for analysis purpose. The number of MT segments processed at each region is shown in Table 4.1. Note that within a specific region of interest, the number of MT segments generally increases as MT buckles over time and decreases for a MT that straightens over time. The main reason for the large deviation in the average bending energy of MT is found in the large variations in the MT curvatures within each region of the cell. This points to the heterogeneity nature of cells. For the same cell, the percentage of $d\bar{U}_b/dW$ decreased with time as shown in Figure 4.22(b). This is mainly because the aspiration length for this cell increased over time leading an overall increase in dW , which was more significant in terms of magnitude as compared to the increase in $d\bar{U}_b$. The highest percentage of $d\bar{U}_b/dW$ was found to be close to 0.0003% at Region 3 (Figure 4.22(b)) where the microtubules experienced the highest range of average bending energy.

Figure 4.22 Microtubules bending energy plots for the same cell displayed in Figure 4.14. (a) The fluctuation of average bending energy of microtubules at each of the 4 designated regions with respect to time. Region 3 displays buckling phenomena as the average bending energy increases over time. (b) The variation in percentage of average bending energy of microtubules over the work done by aspiration pressure with respect to time. Error bars represent standard deviation.

Table 4.1 The number of MT segments processed at each data point (time) per region for the bending energy plot shown in Figure 4.22.

Time (s)	Number of MT segments			
	Region 1	Region 2	Region 3	Region 4
4	11	3	9	7
66	11	3	11	8
119	10	6	13	8
162	12	10	18	11
191	6	8	17	12

Taking another cell presented earlier in Figure 4.19 where its deformation was dominated by microtubule straightening as an example, it was observed that there was a sharp decrease in the bending energy in all 4 regions at the early stage of aspiration as shown in Figure 4.23(a). This result demonstrates that all of its microtubules were straightened upon the application of pressure. However, from $t = 98$ s to 165 s, there was an increase in $d\bar{U}_b$ for both Regions 1 and 3 which implies that the microtubules experienced buckling (Figure 4.23(a)). There was no significant fluctuation detected in the bending energy of Region 4 after $t = 98$ s as compared to other regions (Figure 4.23(a)). The number of MT segments processed at each region is shown in Table 4.2. Analyzing the same cell, the percentage of $d\bar{U}_b/dW$ exhibited a trend which is similar to the variation of $d\bar{U}_b$ over time plot of each region. The reason behind this similarity is that the increase in the magnitude of $d\bar{U}_b$ was much more significant than the increase in dW with respect to time. As a result, the change in the percentage of $d\bar{U}_b/dW$ was dominated by the change in $d\bar{U}_b$. The percentage of $d\bar{U}_b/dW$ by the aspiration pressure was found to be lower than 0.0002% (Figure 4.23(b)). From the entire population of the 30 cells studied, the highest percentage of $d\bar{U}_b/dW$ recorded was approximately 0.0008% which was located at Region 4. The remaining plots of bending energy variation and percentage of $d\bar{U}_b/dW$ for each of the remaining 28 cells can be found at Appendix A.

Figure 4.23 Microtubules bending energy plots for the same cell displayed in Figure 4.19. (a) The fluctuation of average bending energy of microtubules at the 4 designated regions with respect to time. All 4 regions display straightening phenomena as the average bending energy decreases over time at the beginning of aspiration. (b) The variation in percentage of average bending energy of microtubules over the work done by aspiration pressure with respect to time. Error bars represent standard deviation.

Table 4.2 The number of MT segments processed at each data point (time) per region for the bending energy plot shown in Figure 4.23.

Time (s)	Number of MT segments			
	Region 1	Region 2	Region 3	Region 4
17	13	6	10	7
98	12	7	7	5
165	12	10	9	4
231	13	12	6	4
277	10	6	5	5

As mentioned earlier, the change in average bending energy, $d\bar{U}_b$, can be expressed as

$$d\bar{U}_b = \bar{U}_f - \bar{U}_i \quad (4-13)$$

where $\bar{U}_f$ refers to the average bending energy calculated at the end of the aspiration and $\bar{U}_i$ is the average bending energy obtained at the start of the aspiration. A positive $d\bar{U}_b$ indicates that microtubules experience buckling while a negative $d\bar{U}_b$ represents the straightening of microtubules over time. Remarkably, it was found that there was no apparent relationship between the $d\bar{U}_b$ and the applied aspiration pressure in each of the 4 designated regions after combining the data obtained from all the cells studied. This finding was deduced from the widely scattered points of data shown in Figure 4.24.

Figure 4.24 The change in average bending energy, $d\bar{U}_b$ in all 4 regions, (a) – (d), is independent of the applied aspiration pressure. Each data point represents a cell.

In order to have an overall view of how the microtubules behave at each of the 4 regions, the results of the $d\bar{U}_b$ of all the cells tested were combined into a bar chart as shown in Figure 4.25. Region 1 recorded the value of $d\bar{U}_b$ at $-6.4 \text{ kT}/\mu\text{m}$ while the $d\bar{U}_b$ for Region 2 and 4 were $8.3 \text{ kT}/\mu\text{m}$ and $3.3 \text{ kT}/\mu\text{m}$, respectively. Region 3 has the highest value of $d\bar{U}_b$ at $9.4 \text{ kT}/\mu\text{m}$. All regions, except Region 1, had positive $d\bar{U}_b$. This result demonstrates that, generally, microtubules have the tendency to straighten at Region 1 and buckle at Region 2, 3 and 4 even though there was no significant change across all regions based on the statistical t -test ($p > 0.1$).

Figure 4.25 Change in average bending energy at the 4 regions of cells. Positive value refers to microtubule buckling while negative value refers to straightening of microtubules. Error bars represent standard deviation. Inset shows the 4 designated regions of a cell, where Region 1 is the nearest to the micropipette entrance.

4.3 Discussion

4.3.1 Morphology of microtubules in semi-suspended cells

With our experimental methods, we were able to view the formation of distinct microtubule bundles on semi-suspended cells which are suitable for the purpose of observing microtubules deformation during aspiration. The fact that microtubules could still be observed in the host cell even when most of the cytoplasm was aspirated in the micropipette confirms that microtubules were in close contact with the focal adhesion sites which link the cell and extracellular matrix which, in our case, is fibronectin. This is consistent with the work done by Krylyshkina et al. [113], where the presence of microtubules was detected near the focal adhesions. Thus, cell adhesion may be the driving factor in the formation of well-defined microtubules at the semi-suspended cell body located outside the micropipette, where the cell was in contact with fibronectin-coated surface.

We speculate that the lack of microtubules observed at the cell apex within the micropipette, similar to the work done on actin cytoskeleton by Discher et al. [171], may be due to the breaking of microtubules into smaller fragments upon entering the micropipette. In addition, the strong interconnectivity between microtubules and other cytoskeleton components such as actin and intermediate filaments through protein cross-linkers and motor proteins [85, 87], may prevent the microtubules from moving into the deeper portion of the aspirated cell. Therefore, it is reasonable to assume that in general, majority of cytoskeleton are separated from the aspirated membrane, similar to the effects of tether pulling experiments [126, 129, 130]. On the other hand, the cytoskeleton-membrane adhesion remained intact at the main cell body, which was partially adhered to fibronectin, as indicated by the distinct microtubules presence. This is because the bonds between membrane lipids and cytoskeletal proteins at the cell-extracellular matrix contact region is much stronger than non-contact region, such as the aspirated cell region [172].

4.3.2 Microtubule buckling and straightening

The observation of microtubule buckling away from the micropipette entrance (Region 3) may indeed occur due to cell contraction, resulting from the reduction of cell volume during aspiration, which led to the increase in compressive load generated by the cytoplasm on microtubules. This result is similar to a study done on cardiac muscle cell, where microtubules were found to buckle throughout the cytoplasm during the natural contraction of the cell [118]. On the contrary, straightening of microtubules at the micropipette entrance (Region 1) indicates that the microtubules were in tension and concentrated at the entrance region as the target cell was drawn into the micropipette, which is similar to the effect of pulling a bundle of ropes through a hole.

4.3.3 Buckling wavelength and critical buckling force

The volume of cell outside the micropipette reduces over time during aspiration due to the net flow of cell volume into the micropipette. As a result, MTs experience an increasing compressive load over time due to compression from surrounding cytoplasm, leading to the observed time-dependent change of MT buckling length. At present, there is no similar cell-based data from the literature for direct comparison. The closest data for comparison is found in Ref. 173 where the wavelength of thick MT bundle (polymerized from tubulin solution) decreases steadily over a long period of time (88 minutes) as compared to the shorter time period (5 minutes) in this study. This shows that the force generated by MT polymerization has considerably low impact on the buckling wavelength of MT bundle as MT elongates during polymerization compared to the compressive load generated by the reduction in cytoplasm volume. It is to be noted that the MTs in our study are stabilized by Taxol and does not polymerize freely unlike in Ref. 173.

It has been established using atomic force microscopy (AFM) that the local stiffness of a cell changes with the location of measurement [174]. However, we showed that the stiffness of cell may not just vary with respect to location but also time. The shift of microtubules buckling wavelength from larger to smaller wavelength

shows that the local stress field within a cell changes with time, giving rise to the fact that a cell exhibits anisotropic behavior of ever-changing mechanical properties throughout the aspiration process. As a result, the viscoelastic solid model [49], which assumed that the cytoskeletal filaments and organelles are uniformly distributed throughout a cell [149], does not reflect the true heterogeneity nature of cell.

Based on the optical trap study conducted by Kikumoto et al., the flexural rigidity of Taxol-stabilised microtubules is lower than the normal microtubules [95]. This implies that Taxol weakens the binding of the protofilaments of microtubules, making them more flexible. However, Taxol-stabilised microtubules, which generally appear in bundle formation compared to the separated individual normal microtubules, are stiffer collectively. This is confirmed by the study done by Nishimura et al. [175] who found that Taxol-stabilised cardiomyocytes have higher shear stiffness than control cells. This explains the seemingly contradictory effect of Taxol on the stiffness of microtubules and subsequently, cell as a whole. From our study, it was observed that the bundle thickness varied from cell to cell. Thus, the calculated flexural rigidity of microtubule bundle is different for each cell.

Though in the same order, our result of the critical buckling wavelength is higher than the value found by Brangwynne et al. [118] (Table 4.3). As the microtubules in our study were in bundle formation, it would be harder to bend these bundles, which has a higher flexural rigidity as compared to single microtubules. As a result, the buckling wavelength of microtubule bundle will be larger than single microtubules. On the other hand, the small difference between Kawaguchi et al. [176] and our study is expected as both studies involved the use of Taxol-stabilised microtubules which are less dynamic than the normal microtubules (Table 4.3). However, Kawaguchi et al. performed their study using reconstituted microtubules prepared by polymerizing tubulin extracted from porcine brain in a cocktail of chemical solution. In contrast, our Taxol-stabilised microtubules were contained within a living cell. As a result, the deformation of other cytoskeleton components such as actin and intermediate filaments during aspiration may lead to the slightly lower buckling wavelength than those reconstituted microtubules.

Table 4.3 Comparison of microtubules critical buckling force and wavelength

Cell types	N_{MT}	κ (Nm ²)	λ (μm)	f_c (pN)	Reference
Cos7 epithelial	1	2.0×10^{-23}	2.8	100	[118]
Porcine brain*	1	2.54×10^{-24}	6.6	2.4	[176]
NIH 3T3 fibroblast*	3	7.62×10^{-24}	5.1	24	This study

*Taxol-stabilised microtubules. N_{MT} is the number of microtubules, κ is the flexural rigidity, λ is the buckling wavelength and f_c is the critical buckling force.

The value of the critical buckling force calculated in this study performed at 37°C is about 4 times lower than the previous result obtained by Brangwynne et al. at 100 pN for a single microtubule [118] (Table 4.3). Apart from the number of microtubules involved, the difference between these two values is attributed to the use of larger flexural rigidity of single microtubules, that is, 2.0×10^{-23} Nm² [93] by Brangwynne et al. for their work which was performed at room temperature. However, in a more recent study by Kawaguchi et al., they found that the flexural rigidity of single microtubules extracted from porcine brain cells remained relatively constant at 2.54×10^{-24} Nm² within the temperature range of 20 - 35°C [176]. As a result, we decided to use this flexural rigidity instead as it was obtained at a temperature range closer to our study, which was at 37°C. Compared to Kawaguchi et al. [176], the critical buckling force in our study is about 10 times larger (Table 4.3). This difference is mainly due the presence of microtubule bundles in our cells which essentially leads to a higher force threshold for buckling to occur.

4.3.4 Bending energy variation of microtubules

According to Liu et al. [177], the average bending energy of a single microtubule with a large wavelength of 600 μm is 0.14 $kT/\mu m$ based on the relationship of $U_b \propto \kappa/\lambda^2$ where the flexural rigidity, κ , used is 34×10^{-24} Nm² [178]. Using the same calculation method as Liu et al. and by proportion, the bending energy for a single microtubule with a wavelength of 5 μm is found to be 2016 $kT/\mu m$.

Based on our result shown in Figure 4.15 and Figure 4.22(a), it required approximately $184 \text{ kT}/\mu\text{m}$ to bend microtubule bundle consisting of an average of 3 single microtubules into a wavelength of $5 \mu\text{m}$ (Region 3). Effectively, it was about $61 \text{ kT}/\mu\text{m}$ per microtubule, which is about 33 times lower than the value determined earlier using the same method as Liu et al.. This may be explained by the fact that Liu et al. used a larger flexural rigidity of single microtubules in their calculation, which is about 13 times larger than ours. Furthermore, their study was conducted using reconstituted microtubules and at a lower temperature, 30°C as compared to ours at 37°C . The temperature difference is another contributing factor as it affects the thermal energy scale, kT used in the calculation.

The minimal net change in average bending energy over time exhibited in some of the regions may imply that the microtubules are capable in reorganising themselves through both straightening and buckling to counter the effect of external load on cell. This strongly suggests that there exists a negative feedback control system within the microtubule network. The external force sensed by the microtubules through structural deformation, may trigger a biochemical signal that turns on a certain mechano-sensitive switch in microtubules to resist the force and maintain the cell structural integrity. One example of this feedback system is seen in the study performed by Kaverina et al. [112], who found that tensile stress induces the polymerization of microtubules towards adhesion sites.

The lack of a significant relationship between the change in the average bending energy of microtubules and the aspiration pressure may point to the presence of yet another sophisticated negative feedback mechanism that involves not just microtubules, but other cytoskeleton components such as actin and intermediate filaments acting in concert to resist external load [1], regardless of magnitude. It has been shown that actin filaments respond to external force by clustering at the local site of applied load [179] or to guide microtubules to the adhesion sites while intermediate filaments are speculated to provide lateral support to microtubules to minimise buckling effect [180, 181]. Clearly, actin and intermediate filaments besides microtubules are involved in maintaining the structural integrity of cell under stress.

The total elastic deformation energy, dU_{total} , converted from the work done, dW , by aspiration pressure at a specific time was assumed to be the sum of the energy required to deform each of the components within a cell, dU_i and the energy required to break the adhesion between the cell and the substrate, dU_a . In short, $dW = dU_{total} = \sum_i^n dU_i + dU_a$, where i refers to each of cell components such as the nucleus, cytoplasm, plasma membrane and cytoskeleton. Based on our study, the low percentage of microtubule bending energy over the total work done on cell, $d\bar{U}_b/dW$, which was not more than 0.001% at each of the 4 regions, indeed reflects the significant contribution of other cell components in providing mechanical support to cells. Apart from deforming cell structures, it is likely that a substantial amount of the work done by aspiration pressure is transferred to the energy required to detach the semi-adherent cells from the fibronectin-coated coverslip during aspiration. In comparison, this small percentage of microtubules contribution appears to be much lower than the result obtained by Stamenovic et al. where microtubules were found to support 13% of the total cell contractile stress [181]. The reason for the large difference is because their result was obtained by disrupting the entire microtubule network. As for our study, the microtubule deformation was studied in a two-dimensional (2D) focus plane, possibly neglecting the remaining microtubules which were not visible. This may lead to an underestimation of the actual energy needed to deform the entire population of microtubules within a cell.

4.3.5 Overall microtubules deformation pattern

Microtubules at Region 1 experience the highest local strain as it is the only region with the negative change in average bending energy (Figure 4.25). Indeed, this is consistent with the theoretical calculation which shows that the cytoskeleton network at the micropipette entrance sustains the highest strain [169]. Intuitively, this is also the region with the highest stiffness and strength as it contains the highest density of microtubules accumulated from the pulling and straightening of microtubules together with other cytoskeleton components in the direction of suction pressure. The tendency for the microtubules in Region 2 and 4 to experience buckling

may be due to compressive stress generated by the bulk cytoplasm moving from each region towards the micropipette entrance in the direction of aspiration. Meanwhile, we expected the values obtained for the change in average bending energy in Region 2 and 4 to be more or less similar because both regions can be considered as mirror image of each other separated by the symmetrical axis along the micropipette and cell axis. However, the result shown in Figure 4.25 proved otherwise. This may be due to the variation in the alignment of individual microtubules at both regions which demonstrates that cell is heterogeneous in terms of internal structure. Moreover, not all cells are exactly symmetrical. The lack of clear microtubules at Region 4 in some cells tested may be another contributing factor to the difference in result.

In certain cells tested, buckling of microtubules were not observed in Region 3. This could arise from the different positioning of centrosome, the origin for microtubules [182], with respect to the micropipette entrance before aspiration begins (Figure 4.26). It is likely that microtubule buckling tends to occur when the centrosome is located at Region 3 as longer microtubules are present. Once the centrosome is positioned at Region 1, there may be more microtubule ends at Region 3 instead, leaving shorter segments of microtubules which is non-favourable for buckling to take place. It has been established that the centrosome in animal cells is dynamic and attached to the nucleus by dynein dependent linkage [183]. It is possible that this linkage is broken as microtubules were pulled towards the micropipette, causing the centrosome to be detached from the nucleus and moves freely together with the microtubules. In addition, it is possible for the microtubules to break away from the centrosome [117, 184, 185], forming loose individual fragments as seen from the fluorescent images. Both factors, that is, the loose centrosome and microtubule fragments may prevent microtubule buckling from occurring at Region 3. Instead, these factors may increase the likelihood of microtubule strengthening.

Figure 4.26 Schematic diagram of two possible positioning of centrosome before aspiration ($t = 0$). (a) Centrosome, which is attached to the nucleus through dynein dependent linkage, is located away from the micropipette entrance leading to microtubules buckling upon the application of aspiration pressure, P ($t > 0$). (b) Centrosome is located near the micropipette entrance resulting in no buckling of microtubules.

Combining both microtubules buckling and straightening results obtained, the microtubule distribution within a cell during micropipette aspiration may be illustrated through a simple diagram as shown in Figure 4.27. Region 1 represents the area near the entrance of the micropipette where microtubules are stretched and straightened while the regions away from the micropipette entrance are labelled as Region 2, 3, 4 where buckling of microtubules may (or may not) occur, depending on factors such as the positioning of centrosome as discussed earlier. According to a compression study of microtubules [118], short wavelength of buckling is attributed to the weak surrounding cytoskeleton arises from the disruption of actin filaments using cytochalasin D. From that study, Brangwynne et al. concluded that cytoplasm elasticity and surrounding cytoskeleton provide additional support to microtubules. In the same perspective, microtubule buckling that occurs in Region 3 may point to the lack of structural support from surrounding cytoskeleton for the microtubules due to the shifting of main bulk of cytoplasm towards the micropipette during aspiration.

Figure 4.27 Illustration of the overall microtubule distribution during micropipette aspiration where microtubule straightening occurs in Region 1, 2, 4 while microtubule buckling appears in Region 3. Cell nucleus is omitted from the diagram for better readability.

The finding of local-dependent stress field will be useful in providing the critical parameters, such the energy transmitted from the applied stress to the cytoskeleton level, elastic and viscous constants, for generating a more complete and accurate viscoelastic model. The new viscoelastic model may be formed based on a collective of different regions of different material properties (for example, spring and damping constants) within the cell [28]. This is possible as shown by a recent study in Ref. 186 which incorporated individual components of a cell (nucleus, cytoskeleton, membrane etc.) and cellular processes (mitosis, growth etc.) into the viscoelastic model of cell to understand the effects of biomechanical properties of single cells at the tissue level.

Even though cell behaviour is dependent on specific regions as found in this chapter, the standard linear viscoelastic model used in Chapter 3, which considers the cell as a homogenous material where the cytoskeleton is uniformly distributed [149, 151], is still valid. This is because it is an established continuum model for capturing the deformation behaviour and calculating the elastic and viscosity parameters of the whole cell [151] (the focus of chapter 3), which are comparable to those in the other

mechanical models for cells [52]. The suggestions to overcome the limitation of the current viscoelastic model are provided in Section 6.3.

4.4 Summary

We found that the global deformation experienced by single cells leads to local deformation, which was shown by the buckling and straightening effects of microtubules at 4 designated regions of the single cells. In addition, we gained new insights from the results on how external force propagates into the cytoskeleton level of cells, mainly through the quantification of microtubule bending energy. As observed from the microtubules deformation patterns which varied over time and space, it is evident that cell exhibits anisotropic behavior. The deformation patterns may be affected by certain factors such as the support provided by other cytoskeleton components and the position of centrosome with respect to the micropipette entrance. From the lack of correlation between the change in bending energy and aspiration pressure, we speculate that microtubules are capable of rearranging themselves through a negative feedback loop mechanism to maintain the cell structural integrity and prevent the cell from being damaged by the external force. It is very likely that the involvement of other cell components such as nucleus and the process of breaking cell adhesion contacts during aspiration contribute to the low percentage of the energy transferred to deform microtubules.

In the following chapter, we will study the rupture mechanics of plasma membrane under tensile stress at local deformation level. We will explore the effect of microtubules flexibility and nucleus elasticity in determining the strength of plasma membrane and critical membrane surface energy. The results obtained will provide a useful insight into how the plasma membrane responds, remodels and ultimately fails under the application of external force.

Chapter 5 Plasma Membrane Rupture^{*}

In the first section of this chapter, the experimental and finite element methods performed to examine the plasma membrane rupture mechanism during aspiration are described. Following that, the results of the rupture analysis of control and TubulinTracker™ (TT) stained cells in terms of non-nucleus and nucleus-aspirated cells are presented. Results were analyzed by the Weibull model, a statistical model that has been used to describe the strength distribution of a wide range of engineering materials, to determine the characteristics of plasma membrane rupture and the roles of microtubules dynamics and nucleus elasticity in affecting membrane rupture strength. The simulation results created using ANSYS, which has an important application in making qualitative comparison with the experimental results, are also presented. The calculation and results of critical membrane surface energy based on the proposed model for rupture mechanics of cells are introduced at the end of this chapter.

5.1 Material and methods

5.1.1 Cell culture and micropipette aspiration

NIH 3T3 mouse fibroblasts were prepared using the same cell culture and fluorescence staining methods as described previously in Chapter 4. In addition, the same micropipette aspiration setup and experimental conditions, including the use of fibronectin to coat the coverslip to enable the cells to be semi-suspended, were used in this study. The only difference is that the focus here was on the behavior of aspirated cell length before and after rupture. The results were compared with the aspiration of control cells, which were not stained with TT but were prepared in similar condition, to understand the effects of Taxol in the TT-stained cells in terms of plasma membrane strength. The position and presence of nucleus was also observed from the recorded brightfield images throughout the aspiration to ascertain its impact on the probability of plasma membrane rupture.

^{*} A large portion of this chapter is published in *Journal of Biomechanics*, April 2011, volume 44, pages 1361-1366.

A total of 133 cells were tested, of which 60 were control (without TT) and 73 were TT-stained. Unpaired two-sample (with unequal variance) *t*-test was used to examine whether the mean critical length, calculated at various pressure, are significantly different.

5.1.2 Finite element modelling and simulation

The main purpose for the finite element modelling is for qualitative analysis purposes, specifically:

- a) to obtain the stress distribution and locate region in the cell with the highest stress during deformation,
- b) to observe the necking process of cell,
- c) to explain the reason for the cell to rupture at the tether region, and
- d) to understand the effect of ‘defects’, i.e., the material inhomogeneity resulted from transmembrane proteins, on stress distribution in the cell.

In this section, the parameters and steps taken to generate three finite element models using ANSYS version 11 for the cell deformation simulations during micropipette aspiration are presented. These models are (1) initial stage, (2) necking stage and (3) rupture stage models. There are two main reasons for using three separate models to represent the shapes of cell during the three different stages of aspiration. First, we were unable to apply the actual experimental pressure at 1000 Pa and above to the initial stage model. The problem arises from the corner of micropipette which tends to severely distort the element mesh of contacting cell upon simulation. The best solution is to refine the mesh at that region. However, due to the limited number of nodes available (only a maximum of 10,000 nodes for education version of ANSYS), this method was not successful to allow higher pressure to be applied on the cell model. The maximum pressure that can only be applied for the initial stage model to be simulated successfully was found to be 50 Pa which is 20 times lower than the actual pressure.

Second, as a result of the first reason, we decided to generate two additional models that represent the cell shape just before necking and rupture, respectively. The

purpose is to obtain the stress distribution in the cell at that particular instance and understand qualitatively which portion of cell experiences the highest stress, which may lead to necking and rupture. Now, the maximum pressure that can be applied for the necking stage is two times higher than the initial stage, that is 100 Pa, though still 10 times lower than the experimental value of pressure. However, the maximum pressure allowable for rupture stage remained at 50 Pa. As there was already a small portion of aspirated cell within the micropipette in these models, the problem related to the contact point with the micropipette corner is minimised, possibly leading to the increase in applied pressure. The detailed steps to create the finite element models and perform the corresponding simulations using ANSYS including the full programming codes for each model can be found in Appendices B and F (supplementary CD), respectively.

Assumptions

A two-dimensional (2D) axisymmetric homogenous isotropic model was used and analyzed to reduce the complexity of the model and to save computational time. Only half of the 2D model is analyzed assuming that the model is symmetrical at the centre line along the micropipette longitudinal axis. A three-parameter viscoelastic model (standard linear solid) shown previously in Figure 3.4(b) was chosen to represent the material behavior of the cytoplasm and nucleus. As the model used is only 2D (x- and y-axis), cell adhesion on the substrate (z-axis) located at the cell portion outside the micropipette was neglected. It was assumed that there was no friction between the cell and the micropipette wall.

Geometry of models

The dimensions used for the initial stage model were taken from the work of Jean et al. [187] (Table 5.1). For necking and rupture stages, the cell diameter was approximated to be half the size of the initial stage. This is because the cell portion outside the micropipette is expected to shrink in size upon entering the micropipette as observed experimentally. The original line and area geometries of the three models used to represent the initial, necking and rupture stage are shown in Figures 5.1, 5.2 and 5.3, respectively.

Table 5.1 The respective components of the model and their dimensions.

	Actual dimension (μm)	Dimension in simulation (μm)	
		Initial stage	Necking and rupture stages
Diameter of cell	20	20	10
Diameter of nucleus	8	8	5
Cell membrane thickness	0.005	0.1*	
Micropipette wall thickness	1	5*	
Micropipette fillet radius	0.1	0.1	

*Note that the thickness of cell membrane was exaggerated by 20 times to allow the simulation to run smoothly as the actual membrane thickness at 5 nm [103] is too small compared to the rest of the cell components for the simulator to operate. In addition, the micropipette wall thickness was also much larger than actual size to provide the cell with more contact line at the micropipette entrance during aspiration, preventing unwanted deformation that may lead to failure in simulation.

Figure 5.1 Initial stage model. (a) The lines and (b) areas created for modelling the initial stage of cell with nucleus (blue), cytoplasm (purple) and cell membrane (red).

Figure 5.2 Necking stage model. (a) The lines and (b) areas created for modelling the necking stage of cell with nucleus (blue), cytoplasm (purple) and cell membrane (red).

Figure 5.3 Rupture stage model with tether connecting cell host and cell apex before breaking away. (a) The lines and (b) areas created for modelling the rupture stage of cell with nucleus (blue), cytoplasm (purple) and cell membrane (red).

Material properties

According to previous works, the NIH 3T3 fibroblasts cytoplasm instantaneous Young's modulus, E_0 is 600 Pa [188] while the nucleus the elastic spring constants k_1 and k_2 for nucleus are equal at 0.75 kPa [30]. Based on the chosen experimental data, we calculated the remaining parameters required, that is, the apparent viscosity, μ , of cytoplasm and nucleus, the values for the exponential time constant, τ , and the equilibrium Young's modulus, E_∞ , using Equations 3-14 and 3-18, respectively, from Chapter 3, assuming that k_1 and k_2 are equal. Subsequently, with these data, the values for shear modulus, $G(t)$ and bulk modulus, $K(t)$ were calculated using

$$G(t) = \frac{k_1}{2(1+\nu)} + \frac{k_2}{2(1+\nu)} \exp\left(-\frac{t}{\tau}\right) \quad (5-1)$$

$$K(t) = \frac{k_1}{3(1-2\nu)} + \frac{k_2}{3(1-2\nu)} \exp\left(-\frac{t}{\tau}\right) \quad (5-2)$$

where the Poisson's ratio, ν for all cell components (nucleus, cytoplasm and membrane) is chosen to be 0.49, which is widely used in modeling cell [189-193], assuming that cell materials are nearly incompressible. Note that cell is commonly assumed as a perfectly incompressible material ($\nu = 0.5$) [30, 49, 52]. Mathematically, $K(t)$ tends to infinity when $\nu = 0.5$, giving rise to numerical difficulties during the simulation. However, this is not relevant because in reality, no materials have $\nu = 0.5$ [194]. Viscoelastic materials which have ν close to 0.5 is considered to be incompressible [194]. In addition, it has been determined experimentally that the Poisson ratio for cell membrane is in the region of 0.4 to 0.5 [188]. The effects of the difference in ν , for example, 0.48, 0.499 or 0.4999 were not investigated in this study because it has been shown that only a few percent change in stress field when comparing between cell materials with $\nu = 0.45$ and $\nu = 0.49$ [192]. So, by using $\nu = 0.49$, our simulation is valid. The calculated viscoelastic material properties are presented in Table 5.2.

Table 5.2 Parameters used to define the viscoelastic material properties of cytoplasm and nucleus.

Cell component	k_1 (Pa)	k_2 (Pa)	μ (Pa · s)	τ (s)	ν	t (s)	E_0 (Pa)	E_∞ (Pa)	Shear modulus, $G(t)$ (Pa)	Bulk modulus, $K(t)$ (Pa)
Cytoplasm	200	200	5000	25	0.49	0	600	300	216	3335
Cytoplasm	200	200	5000	25	0.49	∞	600	300	67	3333
Nucleus	750	750	500	0.667	0.49	0	2250	1125	810	12505
Nucleus	750	750	500	0.667	0.49	∞	2250	1125	252	12500

k_1 and k_2 are the elastic spring constants, μ is the apparent viscosity, τ is the exponential time constant, ν is the Poisson ratio, t is the time, E_0 is the instantaneous Young's modulus, E_∞ is the equilibrium Young's modulus.

To represent the nucleus and cytoplasm with the corresponding viscoelastic data, we used the axisymmetric VISCO88 elements which are 2D 8-node viscoelastic solid elements (Figure 5.4). The axisymmetric PLANE183 solid elements, also with 8 nodes (Figure 5.4), were used to represent an isotropic and elastic cell membrane with Young's modulus of 500 Pa [195] and Poisson ratio of 0.49.

Figure 5.4 The geometry of VISCO88 and PLANE183. Both elements are 2-D and have the same number of nodes, that is, 8, indicated as I - P (Reproduced from the ANSYS help file).

Mesh generation

To prevent severe distortion of mesh during simulation, the elements located at sharp corners of each model are further refined using a smaller mesh size. The completed meshed models are presented in Figure 5.5.

Figure 5.5 The mesh generated for the respective models. (a) Initial stage, (b) necking stage and (c) rupture stage.

Contact pair

Since the aspirated cell will be in contact with the micropipette wall, the creation of a contact pair between the cell surface and micropipette wall is required. The direction of contact normal for each surface has to point towards each other as shown in Figure 5.6.

Figure 5.6 The directions of the contact normal of the target (micropipette) and contact surface (cell membrane) are indicated by the red and yellow arrows, respectively. The actual contact normal for both surfaces are shown by the thin green lines.

Constraints and load application

Each model has to be appropriately constrained. Micropipette is constrained in all direction to prevent displacement while the symmetrical line on the cell is defined as shown in Figure 5.7. Pressure is applied on the cell line that is located within the micropipette (Figure 5.7).

Figure 5.7 The boundary conditions applied on the (a) initial stage, (b) necking stage and (c) rupture stage models. S is the symmetry boundary condition, red arrow is the pressure direction and the blue triangles represent the displacement constraint. Note that the direction of the pressure shown for all three models does not reflect the true general direction, which is in the positive direction of y-axis. This is because by software default, the direction of pressure will be tangent to the line of interest.

Addition of defects to cell membrane for necking stage

To understand the influence of transmembrane proteins in terms of membrane deformation and stress propagation during the necking of cell, defects were added along the cell membrane model to represent the cluster of transmembrane proteins. We expect the region where transmembrane proteins are embedded and linked with neighbouring lipid membrane to be stiffer than pure lipid membrane due to the stronger molecular interaction between lipid molecules and transmembrane proteins. As a result, we applied a variation of Young's modulus (750, 1000 and 1500 Pa) for the defects, which is at a higher stiffness than the plasma membrane (500 Pa), and Poisson ratio of 0.49. The size of each defect is $0.1 \mu\text{m} \times 0.1 \mu\text{m}$ and the number of defects added on separate simulations was 1, 2, 3 and 4 at different locations along the cell membrane. The reason for using $0.1 \mu\text{m} \times 0.1 \mu\text{m}$ to represent a defect is to apply geometric similitude by scaling the embedded TMP clusters to have the same thickness as the cell membrane model i.e. $0.1 \mu\text{m}$.

5.2 Results

5.2.1 Characteristics of plasma membrane rupture

Under negative constant pressure, which ranges from 1 – 10 kPa, cell extends along the micropipette in the direction of suction and rupture may (or may not) occur subsequently, which can be as short as a second or as long as a few minutes. The characteristics of the rupture modes of single cells observed through time lapse images can be described first by the necking of cell membrane at the region close to the cell apex (Figure 5.8), followed by membrane tether formation with a thickness that ranges from $0.55 - 0.95 \mu\text{m}$ and a length that spans as long as $50 \mu\text{m}$ in some cells. Subsequently, within a few seconds, a small portion of cell breaks off at the interface between the tether and cell apex to form a separate enclosed membrane in the shape of a spherocylinder or pill-like shape, while the remaining tether retracts slightly towards the host cell due to intrinsic cell elasticity [196]. Interestingly, there were two distinct regions existed within the portion of aspirated cell. The first region, A, which is the nearest to the micropipette entrance, contained what seems to be cytoplasmic particles

originating from main cell body while the second region, B, where the break-off occurred, was lighter in color and more transparent, indicating that it contained less cytoplasmic contents (Figure 5.8a). This is expected as formation bleb may occur as a result of the aspiration pressure on the cell surface. Low or no fluorescence intensity of stained MTs were seen at the cell apex region (Figure 5.8b).

Figure 5.8 (a) The time-lapse sequence of apical cell breaking away from its main body at the cell apex region in the direction of suction pressure (white arrow) by forming a tether (dashed red arrow) and an enclosed membrane (red arrow). (b) Straightening of microtubules labeled with TubulinTracker™ (TT) of the same cell shown in (a) at the micropipette entrance (arrow) over aspiration time. It is to be noted that no fluorescence was observed in the region of cytoplasm located further into the

micropipette (dotted lines). Scale bars indicate 10 μm (Reproduced from Ref. 197). The video recording of the observation is attached in the supplementary CD in Appendix F.

Three different types of rupture modes of the cells, that do not seem to relate to the magnitude of the applied pressure (load-independent), were observed during aspiration represented by aspiration length versus time plots shown in Figure 5.9. In Mode 1 rupture, cells experienced a single break during aspiration, reflected by a drop of the aspiration length, after which the aspiration length from the host cell continued to increase. In Mode 2, cells underwent multiple ruptures (5 such breaks are shown in Figure 5.9) at the initial stage of the aspiration process (typically within 100 s), followed by an increase in aspiration length. In Mode 3, cells were subjected to multiple ruptures throughout the aspiration process (8 such breaks are seen in Figure 5.9). The brightfield images of a cell undergoing multiple breaks with its fragments of enclosed membrane are shown in Figure 5.10. For cells ruptured in Mode 3, the gradient of the aspiration length versus time graph decreases after each break. The percentage of cells with aforementioned rupture modes is tabulated in Table 5.3.

Figure 5.9 The various rupture modes and viscoelastic behavior of 3T3 fibroblasts (Reproduced from Ref. 197).

Figure 5.10 Timelapse images showing a cell undergoing multiple breaks during aspiration. Yellow arrows point to the enclosed membrane that have broken away from the cell apex. Scale bar represents 20 μm .

Table 5.3 Percentage of cells in different failure modes (Reproduced from Ref. 197).

Rupture Mode	Control (%)		TT-stained (%)	
	NNAC	NAC	NNAC	NAC
1	15	17	3	34*
2	10		21	
3	50		34	
No rupture	8		8	

NNAC stands for non-nucleus aspirated cell while NAC is for nucleus-aspirated cell.

*Mode 2 not seen.

For 34% TT-stained and 17% control cells, their nuclei were aspirated together with the cytoplasm, possibly due to the close proximity of the nuclei to the micropipette entrance. These cells are referred to as nucleus-aspirated cells (NACs), to be distinguished from non-nucleus aspirated cells (NNACs) described earlier. NACs also experienced all of the three aforementioned rupture modes, except for TT-stained cells where Mode 2 rupture was not seen. A small percentage of cells, 8% for both control and TT-stained cells (of NNACs and NACs), did not experience any rupture during aspiration. Instead, they exhibited the standard viscoelastic behavior characterized by the steady increase in aspiration length which tends toward equilibrium after a certain time (Figure 5.9) [49], which is similar to result for the aspiration of viscoelastic cellular aggregates [198]. Results for NACs and NNACs were treated separately for critical length and statistical analysis of tensile strength.

5.2.2 Effects of microtubules flexibility

From observations of failure modes, it is assumed that the suction in the micropipette is primarily sustained by plasma membrane, the membrane tension (in force/unit length), τ , experienced by the aspirated cell is simply

$$\tau = \frac{P r}{2[1 - (r/R_s)]} \quad (5-3)$$

which is derived from Laplace formula [199, 200] where P is applied suction pressure, r is the inner radius of micropipette and R_s is the radius of cell portion outside the micropipette. τ is calculated for the first break of each cell and taken as the tensile strength of the plasma membrane.

The critical length of membrane rupture, which is defined as the maximum length of cell measured from the entrance of micropipette just before it breaks completely from the cell for the first time, and indication of “failure strain”, was plotted in Figure 5.11, against membrane tension. NNACs(TT) generally have longer critical lengths compared to the NNACs(control). There was no strong correlation between critical length and the membrane tension range for both NNACs(control) ($p > 0.1$) and NNACs(TT) ($p > 0.1$).

Figure 5.11 The comparison of the mean critical length with its respective standard deviation obtained between control and TT-stained 3T3 fibroblasts at various range of membrane rupture tension. * $p < 0.05$ as compared to NNACs(control) at 4 – 6 mN/m range. ** $p < 0.05$ as compared to NNACs(TT-stained) at 2 – 4 and 4 – 6 mN/m range, respectively. *** $p < 0.01$ as compared to NACs(control) at 2 – 4 mN/m range. n refers to the number of cells tested (Reproduced from Ref. 197).

5.2.3 Contributions of cell nucleus

In general, NACs have longer critical lengths compared to NNACs (Fig. 3). The critical length of NACs(control) displays a significant increase of 160% compared to NNACs(control) at the 4 – 6 mN/m range ($p < 0.05$). NACs(TT-stained) experience a significant jump in critical length by 284% and 161% from the NNACs(TT-stained) at the 2 – 4 mN/m and 4 – 6 mN/m range, respectively ($p < 0.05$). There is a significant increase of 331% in the critical length of NACs(TT-stained) as compared to NACs(control) at 2 – 4 mN/m range ($p < 0.01$). Similar to NNACs, there is no significant correlation between the critical length and the membrane tension applied, for both NACs(control) ($p > 0.1$) and NACs(TT-stained) ($p > 0.1$). The trend shown in Figure 5.11 may be governed statistically due to the scattering of defects (holes) in the

membrane and may be represented by Weibull distribution, which will be explored in Section 5.2.5.

5.2.4 Time to first rupture

To further understand the cell rupture mechanism during aspiration, another series of graphs of membrane tensions in various rupture modes were plotted with respect to time to first rupture. The trend for Mode 1 NNACs(control) was inconclusive as only two cells from the entire population exhibited this rupture mode (Figure 5.12(a)). Majority of Mode 2 NNACs(control) ruptured within the first 10 seconds over a wide range of membrane tension (0.5 – 6 mN/m) as shown in Figure 5.12(b). It was observed in Figures 5.11(c) and (d) that Mode 3 NNACs(control) and NACs(control) had similar trend except that the minimum membrane tension required to rupture NACs(control) was higher.

Figure 5.12 The time taken for the first rupture to occur at various membrane tension during aspiration of different rupture modes for NNACs(control) as indicated by (a) – (c) and NACs(control) in (d). Each data point represents a cell.

For Mode 1 NNACs(TT), there was no obvious trend as the data points are widely scattered (Figure 5.13(a)). The time to first rupture seemed to increase with the membrane tension for Mode 2 NNACs(TT) (Figure 5.13(b)). Remarkably, Mode 3 NNACs(TT) (Figure 5.13(c)) had similar trend as Mode 3 NNACs(control) in terms of the observation of cell rupture within the first 10 seconds, which was independent of membrane tension. For NACs(TT), the time to first rupture was inversely proportional to the membrane tension (Figure 5.13(d)).

Figure 5.13 The time taken for the first rupture to occur at various membrane tension during aspiration of different rupture modes for NNACs(TT) as indicated by (a) – (c) and NACs(TT) in (d). Each data point represents a cell.

Taking all rupture modes into account, the individual plots of various rupture modes from Figures 5.12 and 5.13 were combined using a consistent time frame as shown in Figures 5.14 and 5.15. The purpose of generating this combined plot is to compare between both control and TT-stained cells in two different conditions (with and without nucleus aspiration) on how membrane tension influences the first rupture time. With these combined plots, the rupture trend is now clearer, confirming that there are two main trends of cell rupture. The first trend is where majority of the cells experience the first rupture within 10 s regardless of membrane tension applied which was observed for both NNACs and NACs of control and TT-stained cells. In the second trend where $t > 10$ s, time to first rupture decreases with membrane tension, which indicates that higher membrane tension leads to shorter time required to rupture

a cell and vice versa. However, the second trend was not observed in NNACs(control) as shown by the linear regression line with a positive gradient (Figure 5.14). The low mean correlation coefficient ($R^2 < 0.05$) for the linear regression lines of both NNACs and NACs is mainly due to the wide scattering of data points over the plot.

Figure 5.14 The combined results of the time taken for the first rupture to occur during aspiration for both NNACs(control) and NNACs(TT) where all rupture modes are included. Shaded region represents the rupture that occurs within first 10 s. The linear regression lines of NNACs(control) and NNACs(TT) are fitted using the data located at $t > 10$ s with the mean correlation coefficients, R^2 , of 0.017 and 0.046, respectively.

Figure 5.15 The combined results of the time taken for the first rupture to occur during aspiration for both NACs(control) and NACs(TT) where all rupture modes are included. Shaded region represents the rupture that occurs within first 10 s. The linear regression lines of NACs(control) and NACs(TT) are fitted using the data located at $t > 10$ s with the mean correlation coefficients, R^2 , of 0.45 and 0.0004, respectively.

For NACs, the minimum membrane tension required to inflict first rupture of membrane was found to be approximately 2 mN/m (Figure 5.15), which is approximately four times higher than the NNACs (Figure 5.14). In addition, the distribution of data for NACs was less scattered as compared to NNACs. The slope for NACs(control) linear regression line was steeper than NACs(TT) (Figure 5.15), indicating that the magnitude of membrane tension will affect the lifetime of the membrane of NACs(control) more significantly. Even though the mean correlation

coefficient for the linear regression line of NACs(control) was much higher than NACs(TT) (Figure 5.15), both values of R^2 were still relatively low, that is, $R^2 < 0.5$.

5.2.5 Statistical analysis using Weibull distribution

Weibull model, an established probability model that is commonly used to test the reliability of engineering materials [201] and some biological materials such as dentin [202] and fibrillar collagens [203], is based on weakest link concept where the strength of the material is controlled by the most significant defect, i.e., a material fails when the weakest chain fails [204]. The variations in distribution of strength are due to the random distribution of defects in the material. In our case, defects (holes) could be initiated at the TMP-lipid sites and subsequently evolved into holes in the membrane from the sustained tensile stress. To describe the tensile strength distribution of the plasma membrane, two-parameter Weibull distribution is used:

$$P_f = 1 - \exp \left[- \left(\frac{\tau}{\tau_o} \right)^m \right] \quad (5-4)$$

where P_f is the cumulative density function (cdf) of rupture, τ_o is known as the characteristic strength while m is the Weibull modulus that governs the shape of the distribution or the scattering of data. Materials with uniform or homogenous structure have higher Weibull modulus which is indicated by smaller scattering of tensile strength data. Regardless of the value of m , 63.2% of the population fail upon reaching τ_o [205]. P_f at specific applied stress, τ , can be approximated by using the Median Rank Method, also known as the Benard's approximation [206], which is a widely used estimate for the cdf of ordered failure data,

$$P_f = \frac{i - 0.3}{N + 0.4} \quad (5-5)$$

where i is the rank of τ among all of the strength data in ascending order and N is the total number of strength data [207]. Here, i is the rank of a specific τ value among all of the strength (τ) data in ascending order, and n is the total number of strength data. Transforming Equation 5-4 into linear form, that is,

$$\ln[-\ln(1 - P_f)] = m(\ln \tau - \ln \tau_o) \quad (5-6)$$

then, τ_o and m can be estimated by linear regression after plotting $\ln[-\ln(1-P_f)]$ against $\ln\tau$ using the values of P_f calculated from Equation 5-5 and the respective values of membrane tension, τ , obtained from the experiment.

The Weibull probability density function, P_d , can be obtained by differentiating Equation 5-4 with respect to τ , giving

$$P_d = \frac{m}{\tau_o} \left(\frac{\tau}{\tau_o} \right)^{m-1} \exp \left[- \left(\frac{\tau}{\tau_o} \right)^m \right] \quad (5-7)$$

For continuous distributions, the probability of failure between τ_1 and τ_2 , is represented by the area under the probability density function, P_d , or simply,

$$P(\tau_1 < \tau < \tau_2) = \int_{\tau_1}^{\tau_2} P_d(\tau) d\tau \quad (5-8)$$

Linear regressions for the linear form of Weibull plots for both control and TT-stained cells resulted in high coefficient of correlation ($R^2 > 0.93$), implying that the tensile strength of cell membrane is adequately described by the Weibull model (Figure 5.16). For NNACs(control), τ_o and m were obtained as 3.57 mN/m and 1.90, respectively, while for the NNACs(TT), τ_o and m were higher and calculated as 3.80 mN/m and 2.76, respectively. For NACs(control) and NACs(TT), τ_o was 4.34 mN/m and 4.65 mN/m, and m was 4.01 and 3.49, respectively, which were higher than the values obtained for non-nucleus aspirated cells. Anderson-Darling (A-D) test [208, 209] was applied to verify whether Weibull distribution is a good fit for the data obtained from each cell group (refer to Appendix C for calculation method). The A-D test is commonly used to perform goodness-of-fit tests on Weibull distributions because it is more powerful compared to Chi-square and Kolmogorov-Smirnov tests [205, 210]. As shown in Table 5.4, the test statistic, A^2 , for all cell groups are much lower than the critical value, A^2_{crit} , at a significant level of $\alpha = 0.05$, implying that the tensile strength of cell membrane is adequately described by Weibull model.

Figure 5.16 Linear plot of Weibull distribution for NNACs(control) and NNACs(TT) where both show a high mean of correlation coefficient, $R^2 = 0.985$ and 0.983 , respectively. For NACs(control) and NACs(TT), $R^2 = 0.933$ and 0.938 , respectively. n refers to the number of cells tested. LR refers to linear regression line (Reproduced from Ref. 197).

Table 5.4 Results of Anderson-Darling test.

	NNACs (control)	NNACs (TT-stained)	NACs (control)	NACs (TT-stained)
Sample size	38	62	22	11
Calculated value of A^2	0.3459	0.1920	0.3740	0.3833
$A^2_{crit,0.05}$	2.492	2.492	2.492	2.492
Reject H_0 if $A^2 > A^2_{crit}$	No	No	No	No

A^2 is the test statistic value and $A^2_{crit,0.05}$ is the critical value at a significant level of 0.05 (Reproduced from Ref. 197).

NNACs(control) and NNACs(TT) have the weighted average or mean membrane tension to rupture, τ_{avg} of 3.83 ± 1.94 mN/m and 3.98 ± 1.54 mN/m, respectively, while for NACs(control) and NACs(TT), τ_{avg} was higher, that is, 4.98 ± 1.35 mN/m and 5.12 ± 1.44 mN/m, respectively. For the control cells, τ_{avg} increased by 1.15 mN/m from non-nucleus to nucleus-aspirated cells. Interestingly, the change in τ_{avg} for TT-stained cells from non-nucleus to nucleus-aspirated cells was 1.14 mN/m, similar to the change in τ_{avg} for control cells. By comparison, τ_o and τ_{avg} for the NNACs(control) were lower than NNACs(TT), and the same trend applies for NACs. Results including the weighted average membrane strength, τ_{avg} , are tabulated in Table 5.5.

Table 5.5 Results of Weibull statistics and critical length (Reproduced from Ref. 197).

Cell group	m	τ_o (mN/m)	R^2	τ_{avg} (mN/m)	Critical length
NNACs (control)	1.86	4.40	0.986	3.83 ± 1.94	lowest
NNACs (TT-stained)	2.68	4.48	0.987	3.98 ± 1.54	low
NACs (control)	3.85	5.50	0.942	4.98 ± 1.35	high
NACs (TT-stained)	3.23	5.73	0.927	5.12 ± 1.44	highest

m is the Weibull modulus, τ_o is the characteristic strength, R^2 is the coefficient of correlation and τ_{avg} is the weighted average membrane strength.

Substituting these parameters into Equation 5-4, the cumulative probability of rupture graph for each set of cell group was plotted as shown in Figure 5.17. It was observed that the probability of rupture for NNACs(TT) was lower than the NNACs(control) for membrane tension less than 4.8 mN/m. Above this level of tension, NNACs(TT) have a higher probability of breaking instead. Both NACs(control) and NACs(TT) exhibited similar trend at low membrane tension. However, NACs(control) have a higher chance of fracturing as compared to the NACs(TT) when membrane tension goes beyond 4.4 mN/m. In general, the probability

curve shifts to the right from non-nucleus to nucleus-aspirated cells for both the control and TT-stained cells.

Figure 5.17 Cumulative Weibull distribution for control and TT-stained cells, with and without nucleus aspiration. n refers to the number of cells tested.

As shown in Figure 5.18, the Weibull density plot for NNACs(TT) was located more to the right of NNACs(control) with a higher peak and narrower distribution, which refers to lower variance. Again, these results suggest that NNACs(TT) were able to withstand higher membrane tension before breaking as compared to control cells. From Figure 5.18, it was observed that the probability distributions of NACs were located at higher membrane tension as compared to NNACs, which imply that they have the likelihood to rupture at a higher range of membrane tensions as compared to non-nucleus involved aspiration. Consistent with the cumulative Weibull distribution shown earlier, the probability distribution of TT-stained cell translated horizontally to the right from non-nucleus to nucleus-aspirated cells, at a lower height of peak. This indicates that the presence of nucleus has a direct impact on the plasma membrane strength.

Figure 5.18 Weibull probability density plot for control and TT-stained cells, with and without nucleus aspiration. n refers to the number of cells tested.

5.2.6 Transmembrane proteins-lipid rupture strength

To determine the transmembrane proteins-lipid rupture strength, we assumed that the cell membrane is a composite material consisting of lipids and proteins mixture, where the total strength of the cell membrane (force/unit length), τ_m can be expressed in a “rule of mixture” type formulation:

$$\tau_m = \frac{A_l}{A} \tau_{l-l} + \frac{A_p}{A} \tau_{p-l} \quad (5-9)$$

where A_l and A_p the cross-sectional areas of lipid and transmembrane proteins (TMP), respectively while the total cross-sectional area of cell membrane, $A = A_l + A_p$. τ_{l-l} is the lipid-lipid rupture strength and τ_{p-l} is the TMP-lipid rupture strength. Using $\alpha = A_p/A$ to represent the volume fraction of TMP within the cell membrane, Equation 5-9 becomes

$$\tau_m = (1 - \alpha)\tau_{l-l} + \alpha\tau_{p-l} \quad (5-10)$$

Rearranging Equation 5-10, the TMP-lipid rupture strength can be rewritten as

$$\tau_{p-l} = \frac{\tau_m - (1 - \alpha)\tau_{l-l}}{\alpha} \quad (5-11)$$

100 random numbers within the range of 0.3 to 0.8 were generated using a true random number generator to serve as the number set for random sampling of α . Substituting the experimental weighted average membrane strength, $\tau_m = \tau_{avg} = 3.83 \pm 1.94$ mN/m (from present study), $\tau_{l-l} = 4.9 \pm 1.6$ mN/m [46] and the random sampling of α into Equation 5-11, we obtained a series of τ_{p-l} , with a mean value of 2.64 ± 0.64 mN/m, which is not available yet in the literature, as far as we know.

Verification of membrane strength distribution based on random sampling of transmembrane proteins volume fraction

To explore whether τ_m based on random sampling of α follows a Weibull distribution, a series of τ_m (sample size of 100) was computed using Equation 5-10 by randomly assigning α between 0.1 – 0.8 together with the values obtained and used earlier for τ_{p-l} and τ_{l-l} , that is ~ 2.64 mN/m and ~ 5 mN/m, respectively. The calculated membrane strengths were then fitted to the Weibull probability density function using Equation 5-7 with $m = 8.89$ and $\tau_o = 4.11$, which were obtained from maximum likelihood estimation (MLE). The derivation of MLE for a two-parameter Weibull distribution is presented in Appendix D.

As seen from Figure 5.19, the fitted Weibull distribution of plasma membrane strength based on the random sampling of α resembles a normal distribution. Thus, it is very much different from the Weibull distribution determined from experimental data. Based on the Wilcoxon rank sum test [211, 212] in

Table 5.6, there is no significant difference between the strength of NNACs(control) and the simulated data with random α at the significant level of 0.05 (refer to Appendix E for the test method). This means that it is likely that the simulated data were from the same Weibull distribution as the experimental data. However, as seen from Figure 5.19, the *simulated* Weibull distribution has a much narrower distribution, i.e., lower scattering of membrane strength as the Weibull modulus, m , is about 5 times larger than NNACs(control). Based on this result, it can be concluded that the statistically distributed membrane strength extracted from experimental data is very unlikely to be dominated by the randomness of α . Instead, we hypothesized that the Weibull characteristics of plasma membrane strength were likely to be originated from the different TMP-lipid strengths due the various types of TMPs interacting with lipids [213].

Figure 5.19 A comparison between the probability density function of membrane strength, τ_m , calculated from experimental data, random sampling of TMP volume fraction, α , and TMP-lipid rupture strength, τ_{p-l} (Reproduced from Ref. 197).

Table 5.6 Results of Wilcoxon rank sum test (Reproduced from Ref. 197).

Test samples	A vs B	A vs C
Calculated value of Z	-0.481	0.700
Reject H_0 if $Z < -1.96$ or $Z > 1.96$ at significant level of 5%	No	No
Sample A is NNACs(control), sample B is the membrane strength at random $\alpha = 0.1 - 0.8$, and sample C is the TMP-lipid cleavage strength at $\alpha = 0.5$ where α is volume fraction of TMP.		

Verification of membrane strength distribution based on lipid-transmembrane proteins strength distribution

To validate our hypothesis, τ_{p-l} was calculated using Equation 5-11 using a widely accepted value of α at 50% [103], $\tau_{l-l} = 5$ mN/m [46] and the experimental data of τ_m . The series of τ_{p-l} obtained were then fitted to the Weibull probability density function using Equation 5-7 with $m = 1.35$ and $\tau_o = 4.75$, obtained using MLE (Figure 5.19). Similarly, there is no significant difference between the strength of NNACs (control) and the simulated data of TMP-lipid strength with $\alpha = 0.5$ based on Wilcoxon rank sum test at the significant level of 0.05 (Figure 5.6). Remarkably, τ_{p-l} exhibited very similar Weibull distribution as compared to the experimental data of τ_m (Figure 5.19). Therefore, the TMP-lipid rupture strength is indeed Weibull distributed, acknowledging that the underlying variation of TMP-lipid interactions is the major contributing factor to the Weibull characteristic of membrane strength.

5.2.7 Qualitative analysis using finite element simulations

In this section, we present the results generated from the simulation of the three stages of the cell during aspiration with the emphasis on the stress distribution.

1) Initial stage

At the initial stage, the cell model was simulated at $t = 0$, under the aspiration pressure of 50 Pa which resulted in a small portion of cell being aspirated into the micropipette (Figure 5.20(a)). No necking was observed at the cell apex in this stage, which may be directly linked to the low pressure applied. It is shown in Figure 5.20(b) that the cell experienced the highest stress of 296 Pa at the cell contact point with the corner of the micropipette entrance. In terms of direction, the high stress was transmitted from the corner of micropipette towards the symmetrical axis of the cell. However, only a small amount of stress, in the range of 0.01 – 33 Pa, was transferred to the nucleus and the cytoplasm region located further away from the micropipette entrance. The von Mises stress distribution on the corresponding element mesh is shown in Figure 5.20(c). As expected, the cell apex, where the pressure was applied, has the largest displacement vectors, in the range of 0.64 – 0.72 μm , which also means that this region has the fastest flow of material into the micropipette during deformation (Figure 5.20(d)).

Figure 5.20 Computational simulation of an axisymmetric 2D cell at the initial state ($t = 0$) after the application of aspiration pressure of $P = -50$ Pa. (a) von Mises stress distribution contour. Red color refers to the region with the highest stress while blue refers to the lowest stress. MX and MN indicate the location of maximum and minimum von Mises stress, respectively. (b) The enlarged section of the area indicated

by the red box in (a). (c) von Mises stress contour across elements boundaries. (d) Structural displacement vector contour. Red arrows refer to the highest displacement while blue refers to the lowest displacement. The video for the simulation shown can be found in the supplementary CD in Appendix F.

2) Necking stage

Without defects

As mentioned earlier in Section 5.1.2, we decided to design a post-aspirated cell model to observe the stress distribution during necking directly because the model at the initial stage was not able to reach the necking stage at the maximum pressure of 50 Pa. For this model, the cell portion within the micropipette was initially in contact with the micropipette wall. During the simulation, the same portion of aspirated cell started to form necking upon the application of constant pressure at 100 Pa as seen from the inward-curving shape of the aspirated portion of cell in Figures 5.20(a) and (b). Stress distribution, ranging from 83 – 124 Pa, was considerably high, as indicated by the lighter blue contour, at the necking region of cell as compared to the other regions of the cell model (Figure 5.21(b)). The stress distribution on the elements of the model is shown in Figure 5.21(c). Similar to the initial stage, the highest stress concentration was found at the contact point between cell and the micropipette entrance wall (Figure 5.21(b)) while the largest displacement vectors were located at the region of applied pressure (Figure 5.21(d)). However, quantitatively, the necking stage model has a higher range of von Mises stress and displacement vectors at 330 – 372 Pa and 0.8 – 0.9 μm , respectively, as compared to the initial stage model.

Figure 5.21 Computational simulation at the necking stage of an axisymmetric 2D cell aspirated at 100 Pa. (a) von Mises stress distribution contour. (b) The enlarged section of the area indicated by the red box in (a) where the apparent necking is indicated by the red ellipse. Red color refers to the region with the highest stress while blue refers

to the lowest stress. MX and MN indicate the location of maximum and minimum von Mises stress, respectively. (c) von Mises stress contour across elements boundaries. (d) Structural displacement vector contour. The video for the simulation shown can be found in the supplementary CD in Appendix F.

With defect(s)

A cell model embedded with a $0.1\ \mu\text{m} \times 0.1\ \mu\text{m}$ ‘defect’ of a Young’s modulus of 750 Pa near the cell apex is shown in Figures 5.22(a) and (b). The defect was used to represent clusters of transmembrane proteins, which are expected to be stiffer than membrane lipids. As compared to the model without a defect in Figure 5.21, the necking region of this model did not show any significant change in terms of deformation size and shape. The stress distribution (Figures 5.22(b) and (c)) and vector displacement (Figure 5.22(d)) were also similar to the model without any defect, except that the defect experienced higher stress than the neighbouring cell membrane as indicated by the lighter blue stress contour in Figure 5.22(b). In addition, the maximum range of von Mises stress was slightly lower at 323 – 364 Pa and there was a small decrease in displacement vector range by approximately $0.0005\ \mu\text{m}$ as compared to the model without a defect.

Figure 5.22 Computational simulation at the necking stage of an axisymmetric 2D cell aspirated at 100 Pa with a square 'defect' embedded near the top of the cell apex. (a)

von Mises stress distribution contour. Red color refers to the region with the highest stress while blue refers to the lowest stress. MX and MN indicate the location of maximum and minimum von Mises stress, respectively. (b) The enlarged section of the area indicated by the red box in (a) where the square ‘defect’ is shown by the red circle and arrow. (c) von Mises stress contour across elements boundaries. (d) Structural displacement vector contour.

The other positions and clustering of defects are shown in Figure 5.23 and Figure 5.24. Generally, the maximum von Mises stress and vector displacement decrease as the number of defects added to the membrane increases. For the same number of defects, the model with closely packed defects has a higher maximum von Mises stress and vector displacement. In terms of defect location, the maximum von Mises stress and vector displacement decreased when a defect was placed nearer to the micropipette entrance. There was no significant change maximum von Mises stress and vector displacement as the defects stiffness was increased. However, when both the stiffness of cell membrane and defects were doubled to 1000 Pa and 1500 Pa from the initial stiffness of 500 Pa and 750 Pa, respectively, the maximum von Mises stress is increased by about 70% and the maximum vector displacement dropped by 0.1 μm . The results in terms of maximum von Mises stress and displacement for the different types of defects configurations and stiffness are presented in Table 5.7. It should be noted that the maximum von Mises stress remained at the contact point of cell with the corner of micropipette entrance for all various conditions.

Figure 5.23 The simulated necking region of a cell with defects indicated by red circles. (a) von Mises stress distribution on a cell model with 2 square defects aspirated at 100 Pa. (b) von Mises stress distribution on a cell model with 4 square defects aspirated at 100 Pa.

Figure 5.24 The simulated necking region of a cell with densely packed defects where each defect is separated by a distance equivalent to 1 defect. (a) von Mises stress distribution on a cell model with 4 square defects closely packed near the top of the cell apex (categorized as top) as shown by the red ellipse. (b) von Mises stress distribution on a cell model with 4 square defects closely packed near the micropipette entrance (categorized as bottom) as shown by the red ellipse.

Table 5.7 Parameters used to analyze the effects of defects presence in the cell membrane at the necking stage model and the corresponding results of maximum von Mises stress and node displacement in y-axis. The applied pressure is fixed at 100 Pa for all cases.

No. of defects	Position of defects along cell membrane	Young modulus of defect (Pa)	Young modulus of membrane (Pa)	Maximum von Mises stress (Pa)	Maximum displacement of the peak node in the y-axis (μm)
0	NA	NA	500	372	0.908
1	top	750	500	364	0.907
1	middle	750	500	362	0.907
1	bottom	750	500	360	0.907
2	evenly distributed	750	500	366	0.906
3	evenly distributed	750	500	354	0.906
4	evenly distributed	750	500	352	0.905
4	top (dense)	750	500	358	0.906
4	top (dense)	1000	500	358	0.904
4	bottom (dense)	750	500	354	0.904
0	NA	NA	1000	613	0.808
1	top	1500	1000	614	0.807
1	bottom	1500	1000	614	0.807
2	evenly distributed	1500	1000	613	0.806
4	top (dense)	1500	1000	594	0.805
4	bottom (dense)	1500	1000	608	0.803

NA – not applicable

The arrangement of defect clusters representing TMP is approximately 50% (a general surface density of TMPs [103]) for a specific region along membrane as shown in Figure 5.24 where 4 defects (grey square) are separated by an equal gap (white square) of 0.1 μm , which represent the portion of lipid membrane as illustrated in

Figure 5.25. Since we are only interested to study the effects of the presence of defects on cell membrane properties qualitatively, only a maximum of 4 TMP clusters were added. However, geometric similitude is still applied for this particular section of cell membrane since the surface density of TMP is 50%.

Figure 5.25 Illustration showing the surface density of TMPs cluster at 50%.

3) Rupture stage

The cell model deformation at this stage is characterized by the thinning and elongation of membrane tether (Figure 5.26(a)). In contrast to the results for initial and necking stages, the stress distribution is now concentrated at the tether region. The maximum von Mises stress was located at the bottom tether region indicated by 'MX' in Figure 5.26(b). This was the point where the cell tether was expected to rupture. However, based on the observation from experimental result, the rupture point of majority of tethers were located at the top tether region instead, nearer to the cell apex. The maximum von Mises stress and the maximum displacement vector were obtained as 1012 Pa (Figure 5.26(b)) and 4.59 μm (Figure 5.26(d)), respectively, which were approximately three and four times larger than the results from the initial and necking stage models. As observed from Figure 5.26(c), the elements at the tether region were stretched significantly.

Figure 5.26 Computational simulation at the rupture stage of an axisymmetric 2D cell aspirated at 50 Pa. (a) von Mises stress distribution. MX and MN indicate the location

of maximum and minimum von Mises stress, respectively. (b) The enlarged section of the area indicated by the red box in (a) where MX is located at the lower portion of the tether (red circle). (c) von Mises stress contour across elements boundaries. (d) Structural displacement vector contour. The video for the simulation shown can be found in the supplementary CD in Appendix F.

5.2.8 Critical membrane surface energy based on proposed cell rupture model

To quantify the relationship between the external stress on cell membrane, it is crucial to obtain the membrane surface energy which comprises of both bending and stretching energy. Here, we provide the formulation of membrane surface energy based on our proposed cell rupture model which accounts for cell shapes observed before and after rupture during aspiration.

Bending surface energy of plasma membrane

The bending energy density of a particular surface (bilayer membrane), E_d , can be expressed as

$$E_d = \frac{\kappa_b}{2} (C_1 + C_2 - C_0)^2 + \kappa_G C_1 C_2 \quad \text{Unit} = \text{J/m}^2 \quad (5-12)$$

where C_0 is the spontaneous curvature, while C_1 and C_2 are the local principal curvatures of the surface [169, 214]. κ_b is the bending rigidity and κ_G is the Gaussian bending rigidity. Based on Equation 5-12, the theoretical formula for the membrane bending energy at the various stages of cell shape before and after rupture are presented below:

i) Initial shape (before aspiration)

The initial shape of a semi-adherent cell before aspiration (Figure 5.27) can be represented by a combination of a hemisphere (A) with radius, R_0 , that is,

$C_1 = C_2 = \frac{1}{R_0}$ and a pancake (B), which is in contact with the coverslip. The

hemispherical surface area is $A_{h0} = \frac{1}{2} \times 4\pi R_0^2 = 2\pi R_0^2$. Assuming that the bilayer structure is homogenous, that is, $C_0 = 0$ [215], the surface bending energy for the hemispherical shape of cell, U_{h0} , is obtained by integrating Equation 5-12 over the surface area, i.e.,

$$U_{h0} = \int E_d dA = E_d \times A_{h0} = \frac{1}{R_0^2} (2\kappa_b + \kappa_G) \times 2\pi R_0^2 = 2\pi (2\kappa_b + \kappa_G) \quad (5-13)$$

Note that U_{h0} is independent of the radius of the hemispherical surface. Based on Ref. [169], the surface bending energy of a pancake is

$$U_p = \pi\kappa_b (8 + \pi\rho_0) + 4\pi\kappa_G \quad (5-14)$$

where $\rho_0 = \frac{R_{p0}}{r_p} \gg 1$, $2R_{p0}$ is the major axis of pancake at the initial shape and the

height of pancake, $r_p = 0.08 \mu\text{m}$ [216], which is assumed to be constant throughout the aspiration process for simplicity. Note that the surface area of the curved section of a pancake is $A_p = 2\pi r_p^2 (\pi\rho_0 + 2)$ [169]. Combining Equations 5-13 and 5-14, the total bending surface energy for the initial configuration of cell is

$$\begin{aligned} U_0 &= U_{h0} + U_p = 2\pi (2\kappa_b + \kappa_G) + \pi\kappa_b (8 + \pi\rho) + 4\pi\kappa_G \\ \Rightarrow U_0 &= \pi\kappa_b (12 + \pi\rho_0) + 6\pi\kappa_G \end{aligned} \quad (5-15)$$

Figure 5.27 The initial shape of a semi-adherent cell before aspiration.

ii) Aspirated cell shape

Assuming that shape of a semi-adherent cell located outside the micropipette during aspiration (Figure 5.28) can also be considered as a combination of a hemisphere and a pancake similar to Equation 5-15, then, its surface bending energy is,

$$U_{hl} = \pi\kappa_b (12 + \pi\rho) + 6\pi\kappa_G \quad (5-16)$$

where $\rho = \frac{R_p}{r_p} \gg 1$

Figure 5.28 The top and side view of an aspirated semi-adherent cell.

The cylinder (C) has the characteristics of $C_1 = \frac{1}{r}$, $C_2 = \frac{1}{\infty} = 0$ and the surface area of $A_c = 2\pi rL$. Substituting C_1 and C_2 into Equation 5-12 and similarly assuming $C_0 = 0$, the energy density for a cylinder, $E_d = \frac{\kappa_b}{2r^2}$. Therefore, the surface bending energy for the cylinder, U_c , is

$$U_c = E_d \times A_c = \frac{\kappa_b}{2r^2} \times 2\pi rL = \frac{\kappa_b \pi L}{r} \quad (5-17)$$

where r is the inner diameter of micropipette.

For hemispherical cap labeled as Hemisphere 2 (D) as shown in Figure 5.28, its surface area, $A_{h2} = 2\pi r^2$. Subsequently, the surface bending energy for the hemisphere, U_{h2} , is

$$U_{h2} = E_d \times A_{h2} = \frac{1}{r^2} (2\kappa_b + \kappa_G) \times 2\pi r^2 = 2\pi (2\kappa_b + \kappa_G) \quad (5-18)$$

Combining Equations 5-15 – 5-18, the change in the total surface bending energy, dU_{sb} , for the configuration of aspirated cell shown in Figure 5.28 can be represented by,

$$\begin{aligned}
 dU_{sb} &= (U_{h1} + U_c + U_{h2}) - U_0 \\
 &= \pi\kappa_b (12 + \pi\rho) + 6\pi\kappa_G + \frac{\kappa_b \pi L}{r} + 2\pi(2\kappa_b + \kappa_G) - \pi\kappa_b (12 + \pi\rho_0) - 6\pi\kappa_G \\
 &= \pi\kappa_b (\rho - \rho_0) + \frac{\kappa_b \pi L}{r} + 2\pi(2\kappa_b + \kappa_G) \quad (5-19)
 \end{aligned}$$

Note that L changes with aspiration time and it is assumed that the bending energy for the small surface area found in between Hemisphere 1 (A) and Cylinder (C) at the micropipette entrance is negligible.

iii) Aspirated cell just before the first rupture (critical length)

Similarly, the surface bending energy for an aspirated cell with tether formation (just before rupture) can be found by assuming that cell shape is formed with a combination of 4 hemispheres and 3 cylinders as indicated in Figure 5.29. Using the surface bending energy derived in Equations 5-13 – 5-18, the change in the total surface bending energy, dU_{sb} , for the aspirated cell with tether formation can be expressed as,

$$\begin{aligned}
 dU_{sb} &= (U_{h1} + U_{c1} + U_{c2} + U_{c3} + 3U_{h2}) - U_0 \\
 &= \pi\kappa_b (\rho - \rho_0) + \frac{\kappa_b \pi L_C}{r} + \frac{\kappa_b \pi L_E}{r_E} + \frac{\kappa_b \pi L_F}{r} + 3[2\pi(2\kappa_b + \kappa_G)] \\
 &= \pi\kappa_b (\rho - \rho_0) + 6\pi(2\kappa_b + \kappa_G) + \kappa_b \pi \left(\frac{L_C}{r} + \frac{L_E}{r_E} + \frac{L_F}{r} \right) \quad (5-20)
 \end{aligned}$$

where r_E is the radius of the tether (E). Note that the surface bending energy at the corners along the tether connecting the cell main body and apex are assumed to be negligible and thus, not considered in the calculation, similar to the previous studies in Refs. 132, 217. Based on experimental data, $L_E = 0$ for some cells. Equation 5-20 represents the maximum (critical) surface bending energy configuration achievable before rupture.

Figure 5.29 The top and side view of an aspirated semi-adherent cell with tether formation before rupture.

iv) Aspirated cell after rupture

After rupture occurs, the total surface energy will be reduced as the cell apex breaks away from the tether to form a separate enclosed membrane (Figure 5.30). Based on Equations 5-15 – 5-18, the change in the total surface bending energy, dU_{sb} , for the aspirated cell (excluding the separated portion) after rupture can be expressed as,

$$\begin{aligned}
 dU_{sb} &= (U_{h1} + U_{c1} + U_{h2} + U_{c2}) - U_0 \\
 &= \pi\kappa_b (\rho - \rho_0) + \frac{\kappa_b \pi L_C}{r} + 2\pi(2\kappa_b + \kappa_G) + \frac{\kappa_b \pi L_E}{r_E} \\
 &= \pi\kappa_b (\rho - \rho_0) + 2\pi(2\kappa_b + \kappa_G) + \kappa_b \pi \left(\frac{L_C}{r} + \frac{L_E}{r_E} \right)
 \end{aligned} \tag{5-21}$$

Figure 5.30 The representative shape of cell upon rupture at the tether region.

Surface stretching energy

The membrane surface stretching energy is expressed as [218]

$$U_{st} = \frac{K_A}{2A_0} (A - A_0)^2 = \frac{K_A}{2} A_0 \alpha^2 \quad \text{Unit: J} \quad (5-22)$$

where K_A is the apparent area expansion modulus, A_0 is the initial membrane surface area before aspiration and $\alpha = \frac{A - A_0}{A_0}$ is the change in membrane surface area. Since

the membrane tension, $\tau = K_A \alpha$ [45], Equation 5-22 becomes

$$U_{st} = \frac{\tau}{2} A_0 \alpha = \frac{\tau}{2} (A - A_0) \quad (5-23)$$

Critical membrane surface energy at rupture

In this study, we are interested in obtaining the total critical membrane surface energy at rupture, which can be expressed as [218]

$$dU_{sc} = dU_{sb} + dU_{st} \quad (5-24)$$

Substituting Equations 5-20 and 5-23 into Equation 5-24,

$$dU_{sc} = \pi\kappa_b(\rho_c - \rho_0) + 6\pi(2\kappa_b + \kappa_G) + \kappa_b\pi\left(\frac{L_C}{r} + \frac{L_E}{r_E} + \frac{L_F}{r}\right) + \frac{\tau_c}{2}(A_c - A_0) \quad (5-25)$$

where $\rho_c = \frac{R_c}{r_p}$, $2R_c$ is the major axis of the pancake at the critical length stage, $2r_p$

is the height of the pancake, $\kappa_b = 2.0 \times 10^{-19}$ J [130] and $\kappa_G = -6.4 \times 10^{-20}$ J [219]. The initial membrane surface area before aspiration is

$A_0 = A_{h0} + A_p = 2\pi R_0^2 + 2\pi r_p^2(\pi\rho_0 + 2) = 2\pi[R_0^2 + r_p^2(\pi\rho_0 + 2)]$, where A_c is the membrane surface area at critical membrane tension, τ_c , i.e.,

$$\begin{aligned} A_c &= A_{h1} + A_p + A_{c1} + A_{c2} + A_{c3} + 3A_{h2} \\ &= 2\pi[R_1^2 + r_p^2(\pi\rho_C + 2)] + 2\pi r L_C + 2\pi r_E L_E + 2\pi r L_F + 3(2\pi r^2) \\ &= 2\pi\left\{[R_1^2 + r_p^2(\pi\rho_C + 2)] + r L_C + r_E L_E + r L_F + 3r^2\right\} \end{aligned}$$

All the parameters from Equation 5-25 can be measured and calculated from the experimental data.

From Table 5.8, it is observed that the critical stretching energy of membrane is about 4 order of magnitude larger than the critical bending energy of membrane for all cell groups. This shows that membrane stretching energy is much more significant than membrane bending energy for large deformation due to external tensile stress which is expected [169]. Therefore, the membrane bending energy in this case can be neglected in the calculation of the total critical membrane surface energy, dU_{sc} (Equation 5-24). NNACs(control) have the lowest critical membrane surface energy at $2.48 \pm 2.14 \times 10^{-13}$ J while NACs(TT) has the highest at $8.41 \pm 4.50 \times 10^{-13}$ J (Figure 5.31). The critical membrane surface energy for NACs(control) increased significantly by about 105% as compared to NNACs(control) ($p < 0.001$) as seen in Figure 5.31. Similarly, there is a significant increase of 167% in the critical membrane surface energy of NACs(TT) as compared to NNACs(TT) ($p < 0.001$) (Figure 5.31).

Table 5.8 Critical membrane bending and stretching energies for the 4 different cell groups.

Cell groups	Critical Membrane Bending Energy, $dU_{sb} \times 10^{-17} \text{ J}$	Critical Membrane Stretching Energy, $dU_{st} \times 10^{-13} \text{ J}$
NNACs(control)	1.58 ± 1.09	2.48 ± 2.14
NNACs(TT)	2.41 ± 2.17	3.15 ± 2.35
NACs(control)	3.18 ± 3.44	5.08 ± 4.95
NACs(TT)	4.40 ± 2.22	8.41 ± 4.50

Figure 5.31 The total critical surface energy (mean and SD) of the 4 cell groups. n refers to the total number of cells tested. * $p < 0.001$ as compared to NNACs(control) and ** $p < 0.001$ as compared to NNACs(TT).

To analyse the relationship between the applied pressure and the local deformation of cell at the membrane level, we calculated the percentage ratio of total critical membrane surface energy, dU_{sc} and work done by aspiration pressure, dW (Equation 4-12), for each cell group (Figure 5.32). NNACs(control) has the largest dU_{sc}/dW at 98.8 ± 45.6 %. There is a significant drop of percentage for NNACs(TT) by $\sim 19\%$ to 80.2 ± 35.1 % as compared to NNACs(control) ($p < 0.05$). The dU_{sc}/dW for NACs(control) is 93.0 ± 105.5 %. As shown in Figure 5.32, the nucleus-aspirated cells, i.e., both NACs(control) and NACs(TT) experience a decrease in the dU_{sc}/dW as compared to their respective NNACs. Among the 4 cell groups, NACs(TT) has the lowest dU_{sc}/dW at 59.3 ± 22.2 % ($p < 0.01$).

Figure 5.32 Percentage ratio of total critical membrane surface energy, dU_{sc} and work done by aspiration pressure, dW , in the form of mean $\pm$ standard deviation. * $p < 0.05$ and ** $p < 0.01$ as compared to NNACs(control).

5.3 Discussion

5.3.1 Characteristics of plasma membrane rupture

Necking, which is observed as the reduction in cross section area, starts to form as the cell apex becomes mechanically unstable due to the high concentration of strain. The necking process leads to the formation of tether in some cells. Once the tether elongates until it reaches zero cross-sectional area or the maximum local stress allowable for the cell membrane, the cell apex breaks off from the tether, also known as rupture, to form a separate enclosed membrane. From the material science point of view, the necking and elongation of tether observed in a single cell deformation during micropipette aspiration is very similar to the behavior of polymer materials during tensile creep tests performed using a mechanical tensile tester such as Instron. Referring to Figure 5.9, aspirated cells of our study did follow the general trend of a typical creep curve except that the cells ruptured at the early stage of tertiary creep without experiencing a sharp increase in strain. This unique creep trend, which marks the difference between cell and non-biological material, is consistent with the work done by Desprat et al. [133] on studying the creep function of single cells using the uniaxial stretching rheometer.

Cell body rupture involves essentially plasma membrane and the encapsulated cytoplasm. Because low or no fluorescence intensity of stained MTs were seen at the cell apex region (Figure 5.8b), no substantial microtubules (and other cytoskeleton components) were believed to be involved at the site of rupture, although trivial amount, not being visible, may still present in the extended cell body in the micropipette. In addition, it was observed that the microtubules were pulled and straightened in the direction of suction at the micropipette entrance (Figure 5.8b) similar to the results in Chapter 4, forming a region of higher microtubules density. Intuitively, this is a region with higher stiffness and strength, which agrees with the observations that all ruptures had occurred away from the micropipette entrance.

The mechanisms behind the three rupture modes are still unclear. It is likely that nucleation (initiation) and growth (propagation) of ‘holes’ on the membrane due to

the tensile stress lead to membrane rupture [199, 220]. Upon the application of aspiration pressure, nucleation of a hole occurs on the surface of membrane. Above a certain critical hole size, the hole grows irreversibly, tearing deeper into the membrane which eventually rupture [220]. After each break, the cell becomes stiffer (as seen from the decrease of gradient in Figure 5.9) due to the loss of the fluid-like cytoplasmic contents, leaving behind the nucleus and intact cytoskeleton structure that are more solid-like. This rupture-induced stiffening response may be the result of the cell experiencing strain hardening [51, 221-225], where the cell becomes more resistant (increase in cell elasticity or stiffness) to the applied tensile stress as they are stretched beyond its elastic range or yield point. This is consistent with the study done by Trepal et al. [226] who showed that cell stiffening occurs due to sustained stretch. In the case of multiple ruptures, the cell experiences a repeated cycle of nucleation and growth of ‘holes’ after each break. It is speculated that the occurrence of each rupture mode is dependent on the scattering or variation in the size and the quantity of holes formed during the sustained stretch. More experiments in the future are required to clarify the proposed mechanism.

5.3.2 Roles of microtubules & nucleus

Because of the effect of TT and blockage by the nucleus, it is believed that the “material” structure of the portion of the cell body within the micropipette are different in the four different groups, that is, TT-stained and control for both NNACs and NACs. TT contains Taxol, a chemical that stabilizes microtubules by making them *less* dynamic [162] and cluster in the form of bundles [227], which may lead to stiffer microtubules, and thus, more resistant to fragmentation during the application of aspiration pressure. As a result, it is anticipated that there were less mobile MT fragments in the aspirated section of TT-stained cells than in the control cells. This is evident from Figure 5.8 where the TT-stained MTs did not flow deeper into the micropipette together with the aspirated cytoplasm over time. In contrast, the presence of randomly distributed mobile MT fragments in control cells, which was observed as bright fluorescence in the aspirated section of cells with enhanced green fluorescent protein (eGFP) labeled MT (Figure 5.33), may be viewed from the perspective of material science as defects (or inclusions) that render the aspirated section of control

cells to be less homogeneous than the TT-stained cells. As eGFP does not affect the MT dynamics [159, 228], eGFP-stained cells are considered to be good representation of our control cells. The lack of cytoskeleton fragments may also result in a lower cytoskeleton-membrane adhesion in the aspirated region, which translates into higher membrane fluidity and longer critical length for TT-stained cells. This reasoning is in line with the finding of Remy-Kristensen et al. where the depolymerization of microtubules network induced by chemical agents led to an increase in membrane fluidity [229].

Figure 5.33 A cell with eGFP-stained MT undergoing aspiration. White arrow indicates the portion of cell within micropipette (dotted lines).

For NACs, because of blockage by the nucleus, less cytoskeleton components or network were entering the micropipette such that the cell content in the micropipette was more fluidic and homogeneous than those of NNACs. Taken together, the degree of homogeneity for the four different cell groups in descending order is: NACs(TT), NACs(control), NNACs(TT), and NNACs(control). Alternatively, this ordering is also indicative of decreasing fluidity of the cell body in the micropipette.

Assuming that the same load (aspiration pressure) was experienced by the nucleus and cytoplasm for NACs, their relationship can be view as two different viscoelastic materials (nucleus in black, cytoplasm in red) in series to each other in a ‘circuit’ model as shown in Figure 5.34. Theoretically, the stiffer material will deform lesser compared to a softer material when both materials are under the same load. This can be proven as follows:

$$\sigma_n = \varepsilon_n E_n \quad (5-26)$$

$$\sigma_c = \varepsilon_c E_c \quad (5-27)$$

where E_n is the Young's modulus of nucleus and E_c is the Young's modulus of cytoplasm. Based on this assumption, the total load applied on the cell is $\sigma = \sigma_n = \sigma_c$ and Eqs. 5-26 and 5-27 can be combined to become

$$\varepsilon_n = \varepsilon_c \frac{E_c}{E_n} \quad (5-28)$$

Since the nucleus is known to be 3 - 4 times stiffer than cytoplasm [30], therefore $\frac{E_c}{E_n} < 1$, which resulted in $\varepsilon_n < \varepsilon_c$. This is supported and confirmed through visual observation during aspiration where the nucleus indeed deforms much lesser as compared to the cytoplasm.

Figure 5.34 Schematic diagram showing the relationship between nucleus and cytoplasm under load.

The results from critical length measurements seem to support the proposed cell body homogeneity discussed earlier: the more fluidic the cell content, the longer the critical length (Table 5.5). The lack of nucleus-aspirated cells data in the membrane tension of 0 – 2 mN/m may point to the fact that this range of membrane tension is not sufficient to dislodge the nucleus from its original position for aspiration to occur. Based on the results, it is very likely that the nucleus of NACs(TT), which requires a higher membrane tension range (2 – 4 mN/m) than NACs(control) for aspiration to occur, were better secured by the stiffer network of TT-stained microtubules. In addition, the position of nucleus with respect to the micropipette entrance may be another factor that determines whether the nucleus can be aspirated together with the cytoplasm: the nearer the nucleus to the micropipette, the higher the chance for it to be aspirated.

5.3.3 Time to first rupture

The two separate rupture trends observed during aspiration (Figures 5.13 and 5.14) are likely to be governed by two different rupture mechanisms. It is speculated that in the first trend of rupture process, where rupture occurred almost immediately (within 10 s) after aspiration pressure was applied, the rupture involved the breaking of loosely held and thin membrane of lamellipodium, which is the cell protrusion formed by actin network. The lamellipodium is considered to be a weaker segment of cell as compared to the central region of cell that contains nucleus integrated with cytoskeleton network, endoplasmic reticulum, Golgi apparatus and other cytoplasmic components. The sudden surge of pressure at the initial stage of aspiration may shock this weaker portion of cell causing it to rupture. Minor damage due to poking on the cell membrane during the positioning of micropipette close to the edge of cell may yet be another possibility for the rapid rupture that takes place at the start of aspiration.

The second trend or the decreasing trend of membrane tension as a function of time to first rupture observed is similar to the rupture behavior of engineering materials such as metal alloys under intergranular, creep-controlled rupture mechanism [230]. This rupture mechanism can be described by the sliding of grain boundary due to sustained tensile stress leading to the formation of grain and boundary voids capable of growing into larger voids through diffusion [230]. In the same perspective, higher membrane tension or tensile stress would speed up the growth of ‘voids’ within the cell, which ultimately leads to shorter time required for the cell to rupture. In cells, the ‘voids’ may be created through the breaking of intermolecular bonds between lipid-lipid, lipid-membrane protein and lipid-cytoskeleton molecules located within and along the plasma membrane.

Based on the membrane tension versus time to rupture regression plots in Figure 5.14, it is interesting that the gradient for NNACs(control) is positive, meaning that the higher the membrane tension, the longer is the time to rupture. In contrast, NNACs(TT) has the opposite trend. The unconventional trend observed for NNACs(control) may be the result of lack of experimental data, giving rise to the inaccuracy of the linear regression line. As seen in Figure 5.15, NACs(TT), which

have a more gentle decrease in membrane tension with respect to the time to first rupture, would rupture at a much later time compared to NACs(control) under the same membrane tension. This supports the consensus mentioned earlier in Section 5.3.2 that TT-stained cells are more fluidic than control cells. It is surprising that there was a minority of cells which did not rupture even over a wide range of membrane tension which were not featured in the time to first rupture plots.

5.3.4 Weibull distribution analysis

τ_o and τ_{avg} for NNACs (control) are lower than NNACs (TT-stained), and the same trend applies for NACs (Table 5.5). This shows that the rupture strength for control cells, which contain more dynamic MTs, is essentially lower than TT-stained cells. The range of τ_{avg} for each cell group (3.8 – 5.1 mN/m) corresponds to the region of 2 – 6 mN/m where the maximum critical length is found (Figure 5.11). For NNACs, the fact that the Weibull modulus is below 3.0 for both control and TT-stained cells suggest that the membrane tensile strength has large scattering [231, 232] and the material structure is inhomogeneous. For NACs, the scattering is lesser, reflected by the higher Weibull modulus ($m > 3$) and narrower peak of probability density distribution as the hindrance imposed on cytoskeleton flow by nucleus lead to a more homogeneous cytoplasm. It is interesting to see that τ_o and τ_{avg} follow the order of material homogeneity discussed earlier, that is, in the order of NACs(TT), NACs(control), NNACs(TT), and NNACs(control), the last one being the least homogeneous.

The Weibull cumulative distribution and probability density plots demonstrated that, generally, TT-stained cells and nucleus-aspirated cells are able to withstand higher membrane tension before breaking as compared to control and non-nucleus aspirated cells. Overall speaking, the probability of rupture at a specific tension (τ) follows essentially the opposite trend for material homogeneity: the higher the material homogeneity, the lower is the probability of membrane rupture (Table 5.9). This provides a direct evidence that the level of material homogeneity, which is dependent

on the presence of aspirated nucleus and microtubule flexibility, determines the plasma membrane strength under tensile stress.

Table 5.9 The relationship between material homogeneity and probability of plasma membrane to rupture from the 4 different cell groups.

Cell group	Material homogeneity	Probability of failure
		at a specific membrane tension, τ
NNAC(control)	Lowest	Highest
NNAC(TT)	Low	High
NAC(control)	High	Low
NAC(TT)	Highest	Lowest

The average membrane tensile strengths calculated in this study are generally lower, even though within the same order of magnitude, as compared to the tensile strength of a pure egg phosphatidylcholine membrane [47], polyunsaturated lipid bilayers [46] and electroporated SOPC liposomes [233], which are 9.4 mN/m, 5 mN/m and 1 – 2.8 mN/m, respectively. The difference may be due to the fact that plasma membrane in living cells is highly dynamic in nature as a result of the vesicles movement in and out of the membrane [234]. Apart from that, plasma membrane is heterogeneous in structure [234], composing not just of lipid bilayers, but also cholesterol [235], lipid rafts [236] and transmembrane proteins [234], which may act like defects in a material. Defects are known to weaken a material as a result of the tendency of stress concentration building up at the defect regions during the application of tensile stress. In the same way, transmembrane proteins embedded may increase the membrane susceptibility to rupture as compared to pure membrane vesicles which have homogenous structural composition. It has been determined that the presence of peptides in membrane leads to lower rupture tension of lipid vesicles [220]. In contrast, the presence of cholesterol [237] and lipid rafts [238] increase the membrane resistance to rupture. Therefore, it is clear that the strength of the plasma membrane is determined by the strength of the interaction between lipids and their

surrounding components. The comparison of the membrane tensile strength made between the several studies mentioned earlier and our study is presented in Table 5.10.

Table 5.10 Membrane tensile strength obtained from various micropipette-based studies conducted on different types of lipid bilayers and cells.

Cell types	Location of measurement	Membrane tensile strength (mN/m)	Reference
Pure egg phosphatidylcholine	Bilayer vesicle periphery	9.4	47
Mono-unsaturated lipid bilayers	Bilayer vesicle periphery	5	46
Electroporated SOPC liposomes	Bilayer vesicle periphery	~1 – 2.8	233
NIH 3T3 fibroblasts	Cell periphery	3.83 ± 1.94	This study

SOPC = 1-stearoyl-2-oleoylphosphatidylcholine

5.3.5 Transmembrane proteins-lipid rupture strength

According to Afrin *et al.*, the average force required to pull membrane proteins out from cell membrane is 0.5 nN [145]. The line force of pullout, f , is $F/2\pi r$, where F is the pullout force, and r is the radius of the TMP spanning the lipid bilayer. Based on the results of Afrin *et al.* (2003) and an estimated value of $r = 5$ nm [239], the corresponding TMP-lipid adhesion strength is ~ 16 mN/m. This value, representing TMP-lipid interaction along the normal of the membrane surface, is about six times larger than estimated result for τ_{p-l} from the present study. The difference could be attributed to the fact that much larger pulling force is needed to dissociate the individual TMPs in the through-the-thickness direction, both from the sea of lipids and the connected, underlying cytoskeleton network [240] (Figure 5.35). In contrast, the TMPs in our study were separated laterally from the lipid membrane at the location of rupture, parallel to direction of aspiration pressure through the breaking of hydrogen

bonds while remaining intact on the other side of lipid surface, as illustrated in Figure 5.36. The membrane rupture process also involves the dissociation between the lipid-lipid domains along the line of separation. Immediately after the separation of TMPs from the lipids, the surrounding lipids rearrange themselves to prevent the exposure of hydrophobic tails to water and thus, returning the lipids to their energetically favorable state. It should be noted that the adhesion strength between membrane and cytoskeleton was not considered in the calculation of membrane strength. The reason is that in our study, plasma membrane rupture occurs at the aspirated cell region, which lacks cytoskeleton as the result of the separation of membrane from the cytoskeleton during the initial stage of aspiration.

Figure 5.35 An illustration showing the transmembrane proteins being pulled out by force, F , using AFM method as described in Ref. 145. Transmembrane proteins are linked to actin cytoskeleton through protein linkers.

Figure 5.36 Illustrations of a small section of plasma membrane consisting of lipids (grey) and transmembrane proteins (green), (a) before and (b) after rupture due to external tensile stress, P (Reproduced from Ref. 197).

From the results of probability density plots in Figure 5.19, it is now clear that the Weibull characteristics of plasma membrane strength originated from the different TMP-lipid strengths and not from the variation of volume fraction of TMPs in cell membrane. This finding confirms the existence of different types of TMP-lipid interactions. One main reason for the variation in the interaction between TMPs and lipid bilayer is due to the fact that there are many different configurations of TMPs, in terms of 3D structure, embedded within cell membrane. This is because individual TMPs can pass through the membrane single (α -helix) or multiple times (α -helices and β -sheet) [103], as mentioned in Chapter 2. Taking integrins, a type of TMPs required for cell adhesion, as an example, it has been found that there are at least 3 different types of integrins [241], that is, $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_4$, for fibroblasts to adhere to extracellular matrix proteins such as collagen, fibronectin and laminin. Furthermore, the orientation of each TMP is unique [103] as it may be tilted to an angle due to hydrophobic mismatch, which occurs as a result of the unequal thickness between the hydrophobic segments of the TMP and lipid bilayer [213]. Thus, it is evident that the underlying variation of TMP-lipid strengths determines the overall rupture characteristics of plasma membrane.

5.3.6 Qualitative ANSYS analysis

As the maximum allowable pressure for the finite element model is 100 Pa, which is 10 times lower than the actual experimental pressure used, the results from the simulations can only be analyzed and compared to the experimental results qualitatively. The models used at the necking and rupture stages were good representations of the actual cell in terms of deformation as necking and thinning of tether phenomena were observed, respectively. However, the major difference was found in the simulated necking stage, where the necking region, as shown by the inward-curving of cell membrane away from micropipette wall, stretched over a long distance from the micropipette entrance to the cell apex (Figure 5.21). In contrast, necking only occurred over a considerably short distance near the cell apex based on actual experimental observations, such as the one shown in Figure 5.8. This discrepancy may arise from the inability of the material models used to fully mimic the true dynamic behavior of cytoplasm and membrane undergoing tensile stress. In addition, it is likely that the low pressure applied on the model may contribute to the discrepancy.

From the simulation results, it is clear that the local stress distribution within the cell is time-dependent as it varies at different stages of aspiration. Consistent with the finite element modelling studies performed by Drury et al. [242] and Vaziri et al. [243], the highest stress location was observed at the contact point of the cell with micropipette entrance for the initial stage model, which corresponds to the straightening of microtubules (local deformation) as observed in Chapter 4. Combining the simulation results obtained from the three models of different deformation stages, it is evident that the location of the high stress distribution shifted from the contact point to the aspirated portion of cell, which led to necking. Subsequently, the high stress distribution was transferred to a small localised region on the tether. As a result, the tether cross-sectional area was reduced significantly and eventually ruptured at the location of highest stress which was at a distance away from the micropipette entrance, consistent with experimental results. The analysis of stress transmission from the applied pressure to the cell based on the simulations is another important step in

providing a comprehensive explanation for the mechanism behind the necking and rupture phenomena observed experimentally.

Consistent with materials engineering, addition of defects representing transmembrane proteins weakens the material. This was observed from the drop in maximum von Mises stress, which is the yield stress that characterizes material strength, for every increment of defect quantity in our model. However, there was no major change in stress propagation or physical deformation during necking as compared to the model without defects. One possible reason for the lack of influence by the defects on the stress distribution is that the effect of the collective stiffness of the rest of the interconnected cell components in the model was more dominant than the effect of the inserted defects as they are much smaller in size. Another possibility is that the maximum amount of defects inserted was only limited to four, which may lead to the underestimation of the effect of defects on membrane deformation as there should be much more clusters of transmembrane proteins distributed throughout the heterogenous cell membrane.

5.3.7 Critical membrane surface energy based on proposed cell rupture model

The critical membrane surface energy, dU_{sc} , follows the same order of material homogeneity discussed earlier in Section 5.3.4, i.e., in the order of NACs(TT), NACs(control), NNACs(TT), and NNACs(control), the last one being the least homogeneous and having the lowest dU_{sc} . This may imply that the more fluidic the cell content, the larger the critical membrane surface energy. As mentioned in Section 4.3.4, the total elastic deformation energy, dU_{total} , converted from the work done, dW , by aspiration pressure at a specific time can be expressed as is the sum of the energy required to deform each of the components (such as the nucleus, cytoplasm, plasma membrane and cytoskeleton), dU_i , within a cell, and the energy required to break the adhesion between the cell and the substrate, dU_a . Based on study of critical membrane surface energy, we found that dU_{sc} is within the same order as the dW as dU_{sc} / dW ranges from 60 - 99%. This implies that majority of energy transferred to

deform membrane than the cytoskeleton which in the case of microtubule deformation (Chapter 4), its $d\bar{U}_b/dW$ is less than 0.001%. The observations of the decrease in dU_{sc}/dW for NACs as compared to NNACs may indicate that more energy is transferred to deform nucleus than membrane. This reflects the significant contribution of nucleus in providing mechanical support to cells. Note that the large variation in data may be due to the overestimation of cell surface area for the cell portion situated outside the micropipette.

Membrane toughness, T_m , which represents the work done per unit area on the membrane up to the point of rupture [45], can be expressed as

$$T_m = \frac{K_A}{2} \alpha_c^2 = \frac{1}{2} \tau_c \alpha_c \quad \text{Unit: J/m}^2 \quad (5-29)$$

Using Equation 5-29, we found that the average membrane toughness for NNACs(control) and NNACs(TT) are $0.53 \pm 0.47 \times 10^{-3} \text{ J/m}^2$ and $0.72 \pm 0.51 \times 10^{-3} \text{ J/m}^2$, respectively. Statistically, there is no significant difference between the average membrane toughness for NNACs(control) and NNACs(TT) ($p > 0.05$). The average T_m obtained is in the same order as the results obtained for cholesterol-coated and streptavidin-coated SOPC vesicles as shown in Table 5.11. This shows that the formulation used to calculate the critical membrane surface energy based on the proposed cell rupture model in this study is valid and can be applied for cells in general.

Table 5.11 Comparison of membrane toughness between lipid bilayers and cell membrane.

Membrane type	Membrane toughness $\times 10^{-3} \text{ J/m}^2$	References
Cholesterol-coated SOPC vesicles	0.08 – 0.65	[45]
Streptavidin-coated SOPC vesicles	0.11	[244]
NNACs(control)	0.53 ± 0.47	This work
NNACs(TT)	0.72 ± 0.51	This work

SOPC = 1-stearoyl-2-oleoylphosphatidylcholine

5.4 Summary

In summary, we have shown that single 3T3 fibroblasts experience rupture at the cell apex during the aspiration process over a range of membrane tension. Three different types of rupture modes were observed and it was found that tensile strength of the plasma membrane can be described by the Weibull distribution. Based on the Weibull distribution, we found that TT-stained cells were able to withstand higher membrane tension as compared to control cells, while the presence of nucleus near the micropipette decreased the probability of plasma membrane rupture. Thus, it is evident that microtubules dynamics and nucleus elasticity affect the mechanical behavior of cells, where less dynamic microtubules lead to more fluid-like cells.

Based on the result of the average plasma membrane strength and the random sampling of volume ratio of TMPs in cell membrane, the average TMP-lipid strength was estimated. We showed that the variation of TMP-lipid rupture strength is the underlying reason for the plasma membrane strength to be characterized by Weibull distribution. The qualitative results obtained from the finite element simulation allowed us to examine and understand the stress distribution within a cell during the initial, necking and rupture stages. From the proposed cell rupture model, the relationship between global and local deformation was established quantitatively in terms of the percentage ratio of critical membrane surface energy and work done by aspiration pressure. Large amount of energy (up to 99%) was transferred from work done by aspiration pressure to deform cell membrane. The membrane toughness for NNACs(control) was found to be comparable to lipid vesicles.

In the next chapter, we will present the conclusions gathered from all the work presented in this thesis together with the recommendations for future work.

Chapter 6 Conclusions and Future Work

The framework for the entire project is built upon the main objective of establishing the relationship between external mechanical stimuli and cell behavior. To achieve the objective, a comprehensive study on the time-dependent behaviour of cells under the application of tensile stress was performed using micropipette aspiration at two different levels, that is, the single cell level (global deformation) and the subcellular level (local deformation). At the single cell level, we have studied the viscoelastic behavior of hMSCs (Chapter 3) while at the subcellular level, we have analyzed the microtubules deformation of NIH 3T3 fibroblasts (Chapter 4) and the rupture mechanism of plasma membrane of NIH 3T3 fibroblasts (Chapter 5). In general, this study reveals the response of cell, both globally and locally, to external stress. The global deformation of cells is very much governed by its internal microstructure i.e., the cytoskeleton, membrane and nucleus.

In Chapter 3, it was found that hMSCs possess the characteristics of a viscoelastic solid material similar to chondrocytes and endothelial cells based on the agreement between experimental data and the theoretical viscoelastic model. Experiments involving F-actin demonstrated the importance of cytoskeleton in controlling the viscoelastic characteristics of hMSCs where the disruption of F-actin cytoskeleton led to a major drop in cell stiffness in terms of Young's modulus and a significant increase in cell viscosity. Moreover, the viscoelastic behavior of hMSCs is temperature-dependent. hMSCs studied at a physiological temperature (37°C) have lower stiffness and higher viscosity as compared to the ones at room temperature (20°C). Interestingly, we also observed three other types of non-typical viscoelastic behaviors of hMSCs during the study. These departures are likely to be governed by a combination of changes within the cell internal structure, such as the fragmentation and rearrangement of cytoskeleton due to the sustained external tensile stress. The study of viscoelasticity of hMSCs has generated new data in terms of the elastic moduli of hMSCs. The results obtained have been increasingly cited by other works [36-44] as the baseline data.

With the aid of fluorescence staining using TubulinTracker™, we were able to observe the local deformation of microtubules at four different regions of single cells during aspiration process in Chapter 4. Microtubules were observed to be straightened at the region closest to the micropipette entrance. Remarkably, microtubules have the tendency to buckle at the region away from the micropipette entrance. These observations were supported by the quantitative data gathered from the calculation of the change in microtubule bending energy over time, assuming that microtubules are represented by elastic semi-flexible rods. It is clear that the time and region-dependent local deformation give solid evidence to the anisotropic behavior of cells. Interestingly, there is a lack of correlation between the change in bending energy and aspiration pressure. In addition, low percentage of energy is transferred from the work done by aspiration pressure to microtubule bending. These findings point to the contribution of other cell components such as the nucleus, actin and intermediate filaments working together in unity to maintain cell structural integrity through a unique feedback mechanism that involves cytoskeleton rearrangement. The finding of local-dependent stress field within the cell will be useful in providing the critical parameters, such the energy transmitted from the applied stress to the cytoskeleton level, elastic and viscous constants, for generating a more complete and accurate viscoelastic model. The new viscoelastic model may be formed based on a collective of different regions of different material properties (for example, spring and damping constants) within the cell [28]. This is possible as shown by a recent study in Ref. 186 which incorporated individual components of a cell (nucleus, cytoskeleton, membrane etc.) and cellular processes (mitosis, growth etc.) into the viscoelastic model of cell to understand the effects of biomechanical properties of single cells at the tissue level.

In Chapter 5, we have presented the results of plasma membrane rupture under tensile stress, which can be described by Weibull distribution. We found that the Weibull distribution originated from the variation in transmembrane protein-lipid strength and not the volume fraction of transmembrane proteins to lipids. At the same time, we explored the effects of microtubule flexibility and nucleus presence on the rupture mechanics of plasma membrane. Cells with stiffer microtubules have lower probability to rupture. The same result is true for nucleus-aspirated cells. From these results, we concluded that the extent of cell homogeneity plays a major role in

determining plasma membrane strength. A cell, which contains stiffer microtubules and with nucleus aspirated, is considered to have a high level of homogeneity at the aspirated portion of cell as there would be lesser amount of cytoskeleton fragments. As a result, the cell has higher membrane fluidity, which translates into longer critical length. Computational simulations based on finite element models enabled us to understand and analyze the qualitative changes of local stress propagation within a cell under tensile stress at three different stages, that is initial, necking and rupture stages. The proposed quantitative cell rupture model sheds new light on the intricate relationship between the global and local deformation due to external tensile force in terms of critical membrane surface energy.

Apparently, there is no detailed study on the rupture mechanism of living cell plasma membrane under tensile stress. Previous studies were only performed mainly on lipid vesicles [45-47]. Thus, the results obtained from our study will definitely benefit the bioengineering community in terms of providing new data based on living cells. In addition, the results from study of plasma membrane rupture based on the fibroblasts, which are commonly found in connective tissues [103], have a potential for comparing and differentiating between the healthy and diseased fibroblasts. Since fibroblasts are known to play a major role in wound healing [103], this study can be extended to analyze the wound healing process of fibroblasts under sustained stretch.

The results obtained from the study of plasma membrane rupture would be useful in comparing with the membrane rupture data obtained from electroporation, a process that involves the transient opening of cell pores through intense electric field and has many important applications such as in transfection (gene transfer) [245], drug delivery [246], electro-chemotherapy [247]. The comparison of experimental data may lead to the development of a unified theoretical membrane rupture model for living cells. In addition, our study is possibly a good stepping stone for the future development of an automated multi-channel suction system to analyze cell membrane strength. This system may consist of a disposable and transparent ‘biochip’ that allows the sorting of individual cells into multi-parallel compartments where suction pressure would be applied and the cell deformation captured by optical microscope. Compared to micropipette aspiration, this system would be able to generate more data for the same period of time. It would be very useful for testing the influence and effectiveness

of drugs on a population of cells or differentiating normal and diseased cells in terms of mechanical properties. This biochip may benefit the pharmaceutical industry in testing new drugs or serve as a disposable diagnostic kit for clinical use.

In the following sections, the recommendations for future work are presented.

6.1 Viscoelastic properties of stem cells

For the study of viscoelastic behavior of hMSCs, further clarification regarding the two competing effects, that is the reduction in the rate of microtubule depolymerization and net amount of assembled microtubules due to a decrease in temperature, is needed. One way is to stain microtubules with enhanced green fluorescent protein (eGFP) in order to measure and compare the rate of polymerization and depolymerization at both room and physiological temperature, respectively. This method of comparison may give a better insight into which one of the effects is more dominant. As we have tested and found that the efficiency of lipid-based transfection of eGFP-tubulin for hMSCs is very low, an alternative eGFP transfection method called nucleofection may be used so that microtubules deformation within hMSCs during aspiration can be observed and compared with the results obtained from NIH 3T3 fibroblasts. This method may be more effective as it has the ability to transfect fluorescent gene directly into cell nucleus and does not rely on cell division [248].

Systematic disruption of other cytoskeletal components such as microtubules and intermediate filaments using relevant chemicals would be a natural step forward to provide a comprehensive understanding of the roles of the other cytoskeletal components apart from actin filaments, in relation to the viscoelastic behavior of hMSCs. Another attractive follow-up to this study would be to observe and analyze the cytoskeletal changes during the differentiation process of hMSCs induced by specific growth factors such as TGF- β 1. This may provide crucial information on what exactly transpire within the cell internal structure during differentiation. It would also be interesting to study the viscoelastic properties of induced pluripotent stem (iPS) cells which are deemed to be one of the most important breakthroughs in stem cell engineering as they may overcome the ethical issues related to the use of embryonic

stem cells. iPS cells are derived from human somatic cells and possess the same characteristics as human embryonic stem cells (hESCs) [249]. The result obtained would be useful in indentifying the differences in viscoelastic properties, if any, between iPS cells, native hESCs (data available from Ref. 250) and hMSCs (data available from our study).

6.2 Cytoskeletal deformation

At the cytoskeleton level, it would be interesting to establish the relationship between microtubules and actin filaments during cell deformation. One suggestion would be to disrupt actin cytoskeleton using cytochalasin D and observe its effect on microtubules deformation using the same experimental setup. On the other hand, micropipette aspiration experiments could be carried out separately to analyze the deformation patterns of actin filaments tagged with eGFP through bending energy calculation and compare with the microtubules deformation found in this study. Similarly, the structural integrity of microtubules could be disrupted using nocodazole to examine its effects on actin filaments deformation. Meanwhile, centrosome could be labeled with GFP-centrin [251] to understand and confirm the effect of centrosome position with respect to micropipette entrance on microtubule deformation.

Cells may be grown on gelatin substrate embedded with fluorescent beads, which is commonly used to study cell traction force [252]. This method would be useful in establishing the relationship between cytoskeleton deformation and the displacement force at the cell-matrix interface for single cells undergoing aspiration. The change in the magnitude and direction of displacement forces of the labeled beads can be tracked by using custom software such as the one created by Dembo et al. [253] and Marganski et al. [254].

To provide a new dimension in performing tensile loading onto cells, the micropipette aspiration system could be fitted onto advanced imaging systems such as the confocal microscopy and total internal reflectance fluorescence microscope (TIRFM) [255] as our current experimental setup is only limited to 2D image acquisition. Confocal microscope would be an excellent tool for capturing the 3D

distribution and deformation of labelled cytoskeleton in cells. On the other hand, TIRFM is highly capable of providing crucial information at the single molecule level during cell deformation, specifically the distribution of the focal adhesions at the cell-substrate interface by labeling one of the intracellular anchor proteins such as talin, vinculin, α -actinin, or filamin [256, 257]. This would be very useful in understanding the de-adhesion process that may occur during the aspiration of semi-adherent cells.

A more complete energy balance is required to fully understand the relationship between external load and the local deformation of microtubules. This may be done by applying a small pressure change, ΔP , after setting the pressure to the desired value at P_0 . The change in the microtubules curvature, say Δx , can be measured during the change in pressure. By plotting P against x , the slope of the graph $\Delta P/\Delta x$ at P_0 , which provides an important information on energy balance, can be obtained. Alternatively, Δx can be measured during the increase of pressure from zero to P_0 .

6.3 Plasma membrane rupture

It has been established that cytoskeleton is separated from membrane during the formation of thin tether with a diameter that ranges from 15 to 500 nm [126]. However, in our case, the portion of cell aspirated is much larger than the tether. Thus, to follow up and confirm whether the detachment of cytoskeleton from the lipid membrane did occur during the aspiration, fluorescence resonance energy transfer (FRET) technique could be performed to measure the dynamic interaction between membrane lipid and cytoskeleton in the range of 2 – 8 nm [258]. This technique involves labeling the proteins of interest with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), respectively. In our study, the donor protein tagged with CFP could be one of the cytoskeleton-associated anchor proteins linking actin filaments to the membrane lipids while the acceptor protein could be the hydrophilic head of the lipid membrane. If there is binding between the cytoskeleton and membrane, the fluorescence of the acceptor protein would be observed upon excitation of donor protein. However, when there is no binding, only the donor protein would fluoresce upon excitation indicating the separation of the membrane from the cytoskeleton.

It would be beneficial to observe the changes that occur on the surface morphology of cell membrane before and after rupture in analyzing the process of membrane resealing. This can be performed by using scanning electron microscope. The results obtained may be compared with the cell surface morphology obtained in the cell resealing study by McNeil et al. [259] to see if there is any difference between cell membrane that rupture under constant tensile stress and transient stress. In addition, simulation of transmembrane proteins-lipid rupture using molecular dynamics (MD) may be used to obtain the rupture strength for comparison with our calculated data. The transmembrane protein-lipid model can be created by utilizing common transmembrane proteins for fibroblasts which can be found from the Protein Data Bank (PDB) [260] and inserting the selected protein into a lipid bilayer model.

As the current cell finite element model is based on the continuum mechanics which neglects the presence of cytoskeleton [28], we were not able to use it to explain the effect of cytoskeleton fragments on cell fluidity during membrane rupture. Apart from that, the current model is not able to provide accurate quantitative data on the local stress experienced by the cell due to its inability to accept the pressure range used in the experimental study. It is a fact that the variation or difference in length scale and timescale posed a major problem in relating experimental and simulation results [261]. Therefore, a new theoretical model based on our finding at both global and local deformation levels that incorporates the cell heterogeneity, cell adhesion, and cytoskeleton dynamics would be needed to overcome the shortcoming found in the current model.

One possible way is through a multiscale approach [27] which involves the use of MD simulation to elucidate the interaction and deformation that occur at the atomic level of cytoskeleton and adhesion interface under tensile stress. The results obtained from the atomistic simulation, such as the material elastic constants, could be utilized as an input to mesoscopic computational models of cytoskeleton and cell adhesion. Similarly, the data obtained from the mesoscopic computational models could then be used to determine the properties and behavior of cell at continuum scales or cellular level. This approach has been successfully applied in various studies such as modelling cell adhesion to extracellular matrix [262] and lipid bilayer system [263].

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Appendix A Convoluted images of microtubules and bending energy plots

The following are the convoluted images showing the microtubule deformation of the remaining 28 cells tested in Chapter 4, arranged in the order of increasing applied pressure (Figures A.1 – A.28) . The corresponding plots of bending energy and the ratio of average bending energy over work done with respect to time for each cell are also presented. The analysis of microtubule deformation was performed over 4 different regions of each cell as shown in Figure 4.20.

Figure A.1 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 2745$ Pa. (a) Convolved timelapse images of microtubules. Scale bar represents $10 \mu m$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.2 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 3026$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.3 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 3086$ Pa. Scale bar represents $10 \mu\text{m}$. (a) Convoluted timelapse images of microtubules. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.4 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 4118$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10 \mu m$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.5 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 4719$ Pa. (a) Convolved timelapse images of microtubules. Scale bar represents $10 \mu m$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.6 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 4733$ Pa. (a) Convolved timelapse images of microtubules. Scale bar represents $10 \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.7 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 4887$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10 \mu m$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.8 Microtubule deformation of a cell undergoing aspiration at $P = 5182$ Pa. (a) Convolved timelapse images of microtubules. Scale bar represents $10 \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.9 Microtubule deformation of a cell undergoing aspiration at $P = 5879$ Pa. (a) Convolted timelapse images of microtubules. Scale bar represents $10 \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

(a)

(b)

(c)

Figure A.10 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 5953$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.11 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 6057$ Pa. (a) Convolved timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.12 Microtubule deformation of a cell undergoing aspiration at $P = 6265$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10 \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.13 Microtubule deformation of a cell undergoing aspiration at $P = 6427$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.14 Microtubule deformation of a cell undergoing aspiration at $P = 6468$ Pa. (a) Convolved timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.15 Microtubule deformation of a cell undergoing aspiration at $P = 6590$ Pa. (a) Convolved timelapse images of microtubules. Scale bar represents $10 \mu m$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.16 Microtubule deformation of a cell undergoing aspiration at $P = 6797$ Pa. (a) Convolutional timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.17 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 6948$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.18 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 7019$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.19 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 7063$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.20 Microtubule deformation of a cell undergoing aspiration at $P = 7085$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.21 Microtubule deformation of a cell undergoing aspiration at $P = 7113$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.22 Microtubule deformation of a cell undergoing aspiration at $P = 7503$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10 \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.23 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 7912$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10 \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.24 Microtubule deformation of a cell undergoing aspiration at $P = 8574$ Pa. (a) Convolved timelapse images of microtubules. Scale bar represents $10 \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.25 Microtubule deformation of a nucleus-aspirated cell undergoing aspiration at $P = 8603$ Pa. (a) Convolutional timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.26 Microtubule deformation of a cell undergoing aspiration at $P = 8650$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10 \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.27 Microtubule deformation of a cell undergoing aspiration at $P = 9267$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10\ \mu\text{m}$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Figure A.28 Microtubule deformation of a cell undergoing aspiration at $P = 9797$ Pa. (a) Convoluted timelapse images of microtubules. Scale bar represents $10 \mu m$. (b) The average bending energy over time plot for each of the 4 designated regions. (c) The variation in percentage of average bending energy of microtubules, dU_{MT} , over the work done, dW by aspiration pressure with respect to time. Error bars represent standard deviation.

Appendix B Steps to generate ANSYS models for plasma membrane rupture simulations

In this appendix, the detailed steps for creating the three models (initial, necking and rupture stage) in Chapter 5 are provided.

Geometry

The following are the general steps taken to draw and create the geometry of a model:

1. Based on the dimension, the coordinates for each keypoint of the model were calculated before being transferred into the software by *Main Menu> Preprocessor> Modeling> Create> Keypoint*.
2. Lines were created to link all the keypoints according to the shape of the model using *Main Menu> Preprocessor> Modeling> Create> Lines*.
3. Subsequently, areas for nucleus, cytoplasm, membrane and micropipette were generated based on the lines created by using *Main Menu> Preprocessor> Modeling> Create> Areas*.
4. All the generated areas were glued together using Boolean function to ensure connectivity or no slippage between the different parts of the model during deformation through *Main Menu> Preprocessor> Modeling> Operate> Booleans> Glue> Areas*. The no slippage assumption is based on the presence of the protein-protein interactions along the interface of each cell component.

Element types and material properties

The detailed steps to select and input the material properties data are shown below:

1. Cytoplasm and nucleus were represented by VISCO88 using *Main Menu > Preprocessor > Element Type > Add/Edit/Delete*. The element is set as axisymmetric under the options as shown in Figure B.1.

Figure B.1 Setting the behavior of VISCO88 to be axisymmetric.

2. The values calculated for each parameter (Table 5.2) were inserted accordingly to the material data table by using *Main Menu > Preprocessor > Material Props > Material Models > Structural > Nonlinear > Viscoelastic > Maxwell* as shown in Figures B.2, B.3 and B.4. The definition for each of the boxes in the viscoelasticity table in ANSYS are highlighted in Table B.1.

Figure B.2 The viscoelastic material model for cytoplasm and nucleus can be selected from the pre-defined models available in ANSYS.

Figure B.3 Viscoelastic material properties of cytoplasm were inserted into this table where each box has its specific definition as defined in Table B-1.

Viscoelasticity for Material Number 2

Viscoelasticity for Material Number 2

	1	2	3	4	5
C1-C5	0	0	0	0	0
C6-C10	0	0	0	0	0
C11-C15	0	0	0	0	0
C16-C20	0	0	0	0	0
C21-C25	0	0	0	0	0
C26-C30	0	0	0	0	0
C31-C35	0	0	0	0	0
C36-C40	0	0	0	0	0
C41-C45	0	0	0	0	0
C46-C50	810.43	251.68	12505	12500	1
C51-C55	1	0	0	0	0
C56-C60	0	0	0	0	0
C61-C65	0.66667	0	0	0	0
C66-C70	0	0	0	0	0
C71-C75	1	0	0	0	0
C76-C80	1	0	0	0	0
C81-C85	0	0	0	0	0
C86-C90	0.66667	0	0	0	0
C91-C95	0	0	0	0	0

Graph

OK Cancel Help

Figure B.4 Viscoelastic material properties of nucleus were inserted into this table where each box has its specific definition as defined in Table B-1.

Table B.1 Definitions of each box located in the table of viscoelastic properties

Constant	Definition
46	$GXY(0)$ (shear modulus at time = zero (the full shear modulus)).
47	$GXY(\infty)$ (shear modulus at time = infinity (the residual shear modulus after the full decay)). If no relaxation of the shear modulus, use $GXY(\infty) = GXY(0)$.
48	$K(0)$ (bulk modulus at time = zero).
49	$K(\infty)$ (bulk modulus at time = infinity). If no relaxation of the bulk modulus, use $K(\infty) = K(0)$.
50	No. of Maxwell elements (10 max) used to approximate the shear modulus ($GXY(0) - GXY(\infty)$) relaxation.
51-60	Up to ten values of C_{smi} (coefficients for shear modulus relaxation using Maxwell elements, $\sum C_{smi} = 1.0$ if shear modulus relaxes).
61-70	Up to ten values of λ_{smi} (relaxation times for shear modulus relaxation using Maxwell elements).
71	No. of Maxwell elements (10 max) used to approximate the bulk modulus ($K(0) - K(\infty)$) relaxation.
76-85	Up to ten values of C_{bmi} (coefficients for bulk modulus relaxation using Maxwell elements, $\sum C_{bmi} = 1.0$ if bulk modulus relaxes).
86-95	Up to ten values of λ_{bmi} (relaxation times for bulk modulus relaxation using Maxwell elements).

3. The PLANE183 element of cell membrane can be selected by repeating steps 1 and 2. The cell membrane material properties were inserted as shown in the screenshots obtained at each step in Figures B.5, B.6 and B.7.

Figure B.5 Setting the element behavior of PLANE183 to axisymmetric.

Figure B.6 The linear isotropic material model for membrane can be selected from the pre-defined models available in ANSYS.

Figure B.7 The Young's modulus (EX) and Poisson ratio (PRXY) of membrane are inserted using the table provided.

Mesh generation

1. After assigning the appropriate element and properties to each areas, all 3 areas (cytoplasm, nucleus and membrane) were meshed using smart size level 5, 3 and 2, respectively, through *Main Menu > Preprocessor > Meshing > Mesh Tool*. It should be noted that the lower is the smart size level, the finer is the mesh.
2. Meshed elements of membrane and cytoplasm located around the sharp bend of micropipette region were selected for mesh refinement at level 3. This is to ensure that the simulation of the elements at that sharp region run smoothly without extreme distortion which would render the simulation unsuccessful. The final meshed elements for each model are shown in Figure 5.5.
3. The defect was meshed using 'mapped element mesh' to generate a uniform shape of defect. The element size for the defect was set at 0.1 which is the same as the thickness of membrane using *Mesh tool > Size controls > Area > Set* as shown in Figure B.8.

Figure B.8 Element edge length was set at 0.1 for the defect.

Contact pair

1. Once meshing was completed, contact pair was created between the cell line (contact) and the micropipette wall (target) using the contact pair wizard at *Main Menu > Preprocessor > Modeling > Create > Contact Pair*. The detailed steps are shown in Figures B.9, B.10, and B.11.

Figure B.9 The line of the inner micropipette wall was selected to be the rigid target surface.

Figure B.10 The line of the cell membrane was selected as the contact surface while the surface-to-surface contact element type was chosen.

Figure B.11 Initial penetration was excluded as it was not applicable for the models. It is assumed that there is no friction between the micropipette wall and cell membrane.

2. The contact conditions were set using the Contact Properties form as shown in Figures B.12 and B.13.

Figure B.12 Under the Basic tab, exclude everything is selected for the initial penetration while close gap is chosen for the automatic contact adjustment. Close gap is needed to ensure that there is no gap between the contact and target surface at the start of the simulation.

Figure B.13 Under the Misc tab, the element time increment is maintained at reasonable increment.

3. Once the contact pair is created, it is important to check and ensure that the orientation of the contact normal on the contact (membrane) and target (micropipette) lines is pointing towards each other as shown in Figure 5.6. If it is in the wrong direction, the contact normal can be flipped using the function provided in the wizard.

Constraints and load application

1. Reference temperature is required for viscoelastic element simulation and was set at 37°C which is similar to actual experimental temperature using *Main Menu > Preprocessor > Loads > Define Loads > Settings > Reference Temp.*
2. All degree of freedom (DOF) of x- and y-axis (UX and UY) were constrained on the micropipette lines using *Main Menu > Solution > Define Loads > Apply > Structural > Displacement > On Lines* (Figure 5.7).

3. The y-axis or the long axis of the cell model was assigned with the symmetry boundary condition using *Main Menu> Solution> Define Loads> Apply> Structural> Displacement> Symmetry B. C.> On Lines* (Figure 5.7) to reflect the axisymmetric nature of the cell model.
4. The aspiration pressure was applied on the upper most line of the cell membrane within the micropipette as shown in Figure 5.7 using *Main Menu> Solution> Define Loads> Apply> Structural> Pressure> On Lines*.
5. To ensure that the displacement during simulation is using true scaling, the displacement scale factor was set to 1 using *PlotCtrls> Style> Displacement Scaling*.

Solution

As it is our interest to observe the final outcome of the simulation under a steady load, static analysis was used to obtain the solution for the model under the application of a constant step pressure, neglecting the damping and inertia effects generated by time-varying loads. The steps are shown as follows:

1. The analysis type was defined as static using *Main Menu> Solution> Analysis Type > New Analysis > Static*.
2. The solution controls were set using *Main Menu> Solution > Analysis Type > Sol'n Control* with the following parameters (Figure B.14):
 - a) The analysis was set to small displacement static mode. It is to be noted that large displacement mode is not applicable for VISCO88 element.
 - b) The time at the end of the loadstep was set to 1 which means that the total time for the entire simulation is 1 sec. However, this has no significant meaning for static analysis.
 - c) Automatic time stepping was switched on to allow ANSYS to divide the load steps into appropriate sizes.

- d) Number of substeps was set to 100. This will configure the initial substep to 1/100 of the total loadstep.
- e) Maximum number of substeps was set to 1000. This terminates the simulation if the solution does not converge after 1000 steps.
- f) Minimum number of substeps was set to 20.
- g) Frequency was set to write every Nth substep. With this function, all solution items will be written to a result file.
- h) Under the 'Nonlinear' tab in Figure B.15, the maximum number of iterations was set to 100.

Figure B.14 Basic parameters set up for solution controls.

Figure B.15 Parameters used for nonlinear analysis setup

3. Finally, the model simulation is solved using *Main Menu> Solution> Solve> Current LS*.

Results

1. The software automatically generates the Graphical Solution Tracking (GST) plot after each simulation which can be used to verify whether the simulation is converged. As an example, the solution according to the GST plot shown in Figure B.16 is considered converged as the L2 force residual line (purple) is lower than the force convergence criterion line (blue) at the end of the load step.

Figure B.16 A sample Graphical Solution Tracking (GST) plot of a completed simulation.

2. The equivalent stress, σ_e or better known as the von Mises stress, which can be calculated from the 3 principal stresses (σ_1 , σ_2 , σ_3) of an element,

$$\sigma_e = \left[\frac{1}{2} (\sigma_1 - \sigma_2)^2 + (\sigma_2 - \sigma_3)^2 + (\sigma_3 - \sigma_1)^2 \right]^{\frac{1}{2}}$$

was used to generate the stress distribution of the simulated cell model. The von Mises stress distribution for the nodes and elements of the model were plotted using *General Postproc>Plot results>Contour Plot>Nodal Solu>Stress>von Mises stress* and *General Postproc>Plot results>Contour Plot>Element Solu>von Mises stress*, respectively. The video format of the von Mises distribution over time can be acquired using *Utility Menu> PlotCtrls> Animate> Deformed Results*.

3. The vector diagram showing the displacement direction on each nodes was generated using *General Postproc>Plot results>Vector Plot>Predefined>DOF Solution> Translation U*.

Appendix C Anderson-Darling test

The hypothesis for Anderson-Darling (A-D) test is defined as,

H_0 : The data follows Weibull distribution

H_a : The data do not follow Weibull distribution

The test statistic, A^2 , for A-D test can be calculated using

$$A^2 = -\sum_{i=1}^N A_i - N$$

where,

$$A_i = \frac{2i-1}{N} \left[\ln P_f(\tau_i) + \ln(1 - P_f(\tau_{N+1-i})) \right]$$

N is sample size and $P_f(\tau_i)$ is the cumulative Weibull distribution (Equation 5-4) for the rupture tension, τ , at rank i . At a significant level of 0.05 (5%), H_0 is rejected if A^2 is more than the critical value, $A_{\text{crit},0.05}^2$, which can be obtained from the corresponding table in statistics textbooks such Ref. 209.

Appendix D Derivation of Maximum Likelihood Estimation

The following is the mathematical derivation of Maximum Likelihood Estimation (MLE) for a two-parameter Weibull distribution used in Chapter 5.

For a two-parameter Weibull distribution, the probability density function is

$$P_d = \frac{m}{\tau_o} \left(\frac{\tau_i}{\tau_o} \right)^{m-1} \exp \left[- \left(\frac{\tau_i}{\tau_o} \right)^m \right] \quad (\text{C.1})$$

The corresponding likelihood function is

$$L(\tau_i, m, \tau_o) = \left(\frac{m}{\tau_o} \right)^n \prod_{i=1}^n \left(\frac{\tau_i}{\tau_o} \right)^{m-1} \exp \left[- \sum_{i=1}^n \left(\frac{\tau_i}{\tau_o} \right)^m \right] \quad (\text{C.2})$$

Then,

$$\ln L = n \ln \left(\frac{m}{\tau_o} \right) + (m-1) \sum_{i=1}^n \left(\frac{\tau_i}{\tau_o} \right) - \sum_{i=1}^n \left(\frac{\tau_i}{\tau_o} \right)^m \quad (\text{C.3})$$

Differentiating Equation C.3 with respect to m and τ_o ,

$$\frac{\partial \ln L}{\partial m} = \frac{n}{m} + \sum_{i=1}^n \ln \left(\frac{\tau_i}{\tau_o} \right) - \sum_{i=1}^n \left(\frac{\tau_i}{\tau_o} \right)^m \ln \left(\frac{\tau_i}{\tau_o} \right) \quad (\text{C.4})$$

$$\frac{\partial \ln L}{\partial \tau_o} = \frac{-m \times n}{\tau_o} + \frac{m}{\tau_o} \sum_{i=1}^n \left(\frac{\tau_i}{\tau_o} \right)^m \quad (\text{C.5})$$

By solving Equations C.4 and C.5 simultaneously,

$$\frac{\partial \ln L}{\partial m} = 0 \text{ and } \frac{\partial \ln L}{\partial \tau_o} = 0,$$

the Weibull parameters m and τ_o can be obtained.

Appendix E Wilcoxon Rank Sum Test

The hypothesis for Wilcoxon rank sum test [212] is defined as

$$H_0: F = G$$

$$H_a: F \neq G$$

where F and G is two independent populations with sample size n_1 and n_2 , respectively.

For large sample size ($n_1 + n_2 > 30$), the critical values can be obtained using the normal approximation [211]:

The test statistic, $Z = \frac{R - \mu}{\sigma}$

where R is the smaller sum of rank between the two samples while μ is mean and σ is the square root of variance which can be expressed as,

$$\mu = \frac{n_1(n_1 + n_2 + 1)}{2} \text{ and } \sigma = \sqrt{\frac{n_1 n_2 (n_1 + n_2 + 1)}{12}}$$

At the significant level of 0.05, reject H_0 if $Z < -1.96$ or $Z > 1.96$ [264].

Appendix F Supplementary CD

The supplementary CD is attached on the back cover of the thesis. The contents are compiled into two folders as follows:

Under the folder “Chapter 4 Microtubule Deformation”,

1. Microtubule buckling of the cell presented in Figure 4.13.
(Filename: Fig 4-13 Microtubule buckling.avi)
2. Microtubule straightening of the cell presented in Figure 4.18.
(Filename: Fig 4-18 Microtubule straightening.avi)

Under the folder “Chapter 5 Plasma Membrane Rupture”,

1. The time-lapse sequence of apical cell breaking away from its main body in the direction of suction pressure shown in Figure 5.8.
(Filename: Fig 5-8 Cell rupture.avi)
2. The finite element simulation of of the initial stage presented in Figure 5.20.
(Filename: Fig 5-20 Initial stage.avi)
3. The finite element simulation of of the necking stage presented in Figure 5.21.
(Filename: Fig 5-21 Necking stage - No defect.avi)
4. The finite element simulation of of the rupture stage presented in Figure 5.26.
(Filename: Fig 5-26 Rupture stage.avi)
5. ANSYS programming codes for each of the three simulation models, that is, initial stage, necking stage and rupture stage models.
(Filename: ANSYS programming codes for plasma membrane fracture simulations.doc)

Appendix G Biographical sketch

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Research interests: Cell mechanics

Education:

2004 Bachelor of Engineering, School of Mechanical & Production Engineering, Nanyang Technological University, Singapore.

Professional Experience:

2010 – Present Process Engineer, GlaxoSmithKline Biologicals, Singapore.

2004 – 2006 Project Officer, Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore.

Awards:

2006 – 2009 NTU-EDB Nanocluster Research Scholarship.

2004 SPIE (International Society of Photonics) Best Poster Award (Merit).

1998 – 1999 MOE Pre-U ASEAN Scholarship.

Publications:

1. **S.C.W. Tan**, T. Yang, Y. Gong, and K. Liao, *Rupture of plasma membrane under tension*. Journal of biomechanics, April 2011, vol. 44 (7), p. 1361-1366.
2. Cai, N., C.C. Wong, Y.X. Gong, **S.C.W. Tan**, V. Chan, and K. Liao, *Modulating Cell Adhesion Dynamics on Carbon Nanotube Monolayer*

- Engineered with Extracellular Matrix Proteins*. ACS Applied Materials & Interfaces, April 2010, vol. 2 (4), p. 1038-1047.
3. H. Yu, C. Y. Tay, W. S. Leong, **S.C.W. Tan**, K. Liao, L. P. Tan, *Mechanical behavior of human mesenchymal stem cells during adipogenic and osteogenic differentiation*. Biochemical and Biophysical Research Communications, February 2010, vol. 393 (1), p. 150-155.
 4. N. Cai, C. C. Wong, **S.C.W. Tan**, V. Chan and K. Liao, *Temporal effect of functional blocking of $\beta 1$ integrin on cell adhesion strength under serum depletion*. Langmuir, July 2009, vol. 25 (18), p. 10939–10947.
 5. **S.C.W. Tan**, W.X. Pan, G. Ma, N. Cai, K.W. Leong and K. Liao, *Viscoelastic behaviour of human mesenchymal stem cells*. BMC Cell Biology, July 2008, vol. 9, p. 40-46.
 6. **S.C.W. Tan**, G. Ma, N. Cai and K. Liao, *Viscoelastic properties of human mesenchymal stem cells*. 15th International Conference on Mechanics in Medicine and Biology, Singapore, December 2006, p. 503-505.

Contributed Presentation:

March 2008	S.C.W. Tan , Y. Gong, and K. Liao, Microtubules deformation patterns during micropipette aspiration of semi-adherent NIH 3T3 fibroblasts, Poster and oral presentation at NTU-WASEDA Joint Symposium in Chemical and Life Sciences.
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