

**The Effects of Membrane and Cytoskeletal Mechanics
On Cell Adhesiveness**

by

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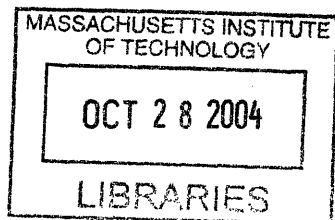
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Submitted to the Department of Mechanical Engineering
on May 7, 2004 in Partial Fulfillment of the
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ABSTRACT

The cytoskeleton, the internal network of filaments that regulates cell shape and structure, has been implicated in several critical aspects of cell adhesion. Its role, however, has primarily been demonstrated as a component of the numerous intracellular signaling events that regulate cell surface receptors. In this study we sought to gain insight into how the mechanical properties of the cytoskeleton could affect adhesion. Specifically, we addressed how membrane deformability, which we demonstrate is critically dependent upon tethering to the cytoskeleton, could impact a whole cell adhesion assay. To modify membrane deformability, we treated cells with the pharmacological agents phalloidin, Latrunculin B, Cytochalasin D, colchicine, and Paclitaxel, which have varying effects on microfilaments and microtubules, two of the main constituents of the cytoskeleton. We found that dissolution of the actin cytoskeleton could reduce the number of adherent cells to a callogen-coated substrate by over 85%. We theorize that this is due largely to the inhibition of signaling events associated with the cytoskeleton, but it may also be affected by changes in shape and deformability. To truly understand the implications of this experiment, we believe further study using high-resolution force technology such as atomic force microscopy or magnetic bead microrheometry is necessary.

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1. Introduction

Cellular adhesion is a critical function that impacts physiological processes ranging from wound repair to tumor cell migration. Although it is widely accepted that the cytoskeleton, the internal network of filaments that gives the cell structure, has a role in determining the adhesiveness of the cell, the mechanisms of this effect have yet to be fully elucidated in a qualitative or quantitative manner. The goal of this study is first to establish the relevance of the cytoskeleton in cell adhesion using experimental techniques, then propose a model of membrane and cytoskeletal deformation and predict their impact on overall cell adhesiveness.

1.1 Cell Adhesion Molecules

Forces are transmitted from the outside of the cell to the membrane and cytoskeleton by glycoprotein receptors embedded in the membrane. These molecules characteristically have an outer domain that binds membrane receptors on other cells, extracellular matrix proteins, and various other soluble ligands. Additionally, a cytoplasmic domain links the protein to the core structure of the cell, the cytoskeleton. An illustration of this configuration is shown in Figure 1. In this study, we will focus on cell adhesion molecules that specifically bind to the extracellular matrix, the network of secreted proteins that occupy the space between cells and form a monolayer substrate for epithelial cells known as the basal lamina. The fibrous protein collagen is believed to be the primary structural component of the ECM, while fibronectin and laminin play a greater role in adhesion to cells. The varying levels of these and other proteins provide a means of regulating the stiffness of the matrix, which in turn is believed to influence cell differentiation, adhesiveness, and motility. The ECM further impacts these processes due to the specificity of its composite proteins in adhering to certain cell surface receptors and their

domains as well as a variety of growth factors and other hormones.

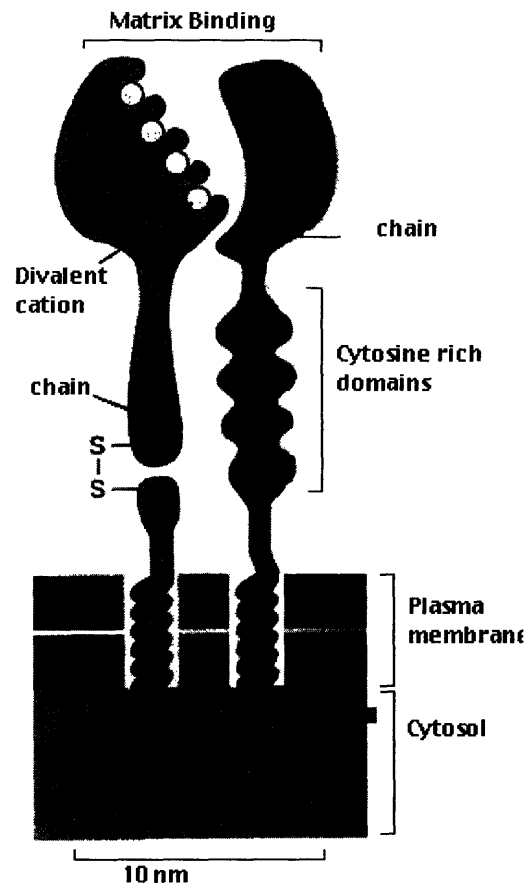


Figure 1: The Integrin Heterodimer [1]

The largest and most thoroughly studied receptor that specifically binds the extracellular matrix is integrin, a heterodimeric protein consisting of an alpha and a beta subunit. There are currently sixteen known alpha subunits and eight beta subunits, which, through the numerous combinations, allow the cell to express receptors that preferentially bind any one or more of the proteins comprising the ECM. For example, $\alpha 5 \beta 1$ integrin binds mainly fibronectin, while $\alpha 1 \beta 1$ binds both laminin and callogen.

The regulation of integrin adhesiveness is generally placed in one of two categories: affinity regulation and avidity regulation. Affinity regulation describes regulating adhesion by

modification of the integrin binding domain in a manner that either increases or decreases its affinity for ligand. The most widely accepted model is a mechanism in which the integrin extracellular domain “folds” to inhibit regulation and “unfolds” to increase affinity. This conformational change has been shown to occur due to the presence or absence of certain divalent cations and more recently the attachment of the focal adhesion proteins talin and α -actinin to the integrin cytoplasmic domain.

Avidity is a term that has been widely used to describe any regulation other than affinity regulation, but it is more specifically regulation of adhesiveness through modification of receptor density or geometry of the cell surface. In either case, adhesiveness is increased due to formation of parallel bonds, rather than by modifying the strength of bonds between particular molecules. By migration of receptors through the cell surface, clusters may be formed, thereby increasing total adhesion. These clusters have been shown in prior work using electron microscopy with another cell adhesion molecule, α -dystroglycan, in response to the laminin G4-5 domain, a dystroglycan specific ligand.

1.2 The Membrane

Cell membrane structure and assembly is a result of the hydrophobic effect, which dictates that the long carbon chains in lipid molecules attract one another and repel water. The lipids' hydrophilic “heads” form both the inner and outer surfaces of the membrane, while the hydrophobic “tails” form a core layer impermeable to polar molecules like water. This is shown in Figure 2. Roughly 100 different types of lipid molecules form the membrane bilayer, which is embedded with various proteins such as ion channels, hormone amplifiers, and more importantly for this discussion, cell surface receptors like integrin. Proteins freely diffuse two-

dimensionally through the bilayer unless they are linked to the cytoskeleton. This is indicative of the fluid-like nature of the membrane; however, upon deformation, it also exhibits the elastic characteristics associated with solids. Although these viscoelastic properties make rheologic studies of the cell infinitely more difficult, both fluid- and solid-like properties are essential to the membrane's function. A detailed analysis of membrane deformation will follow in section 2.

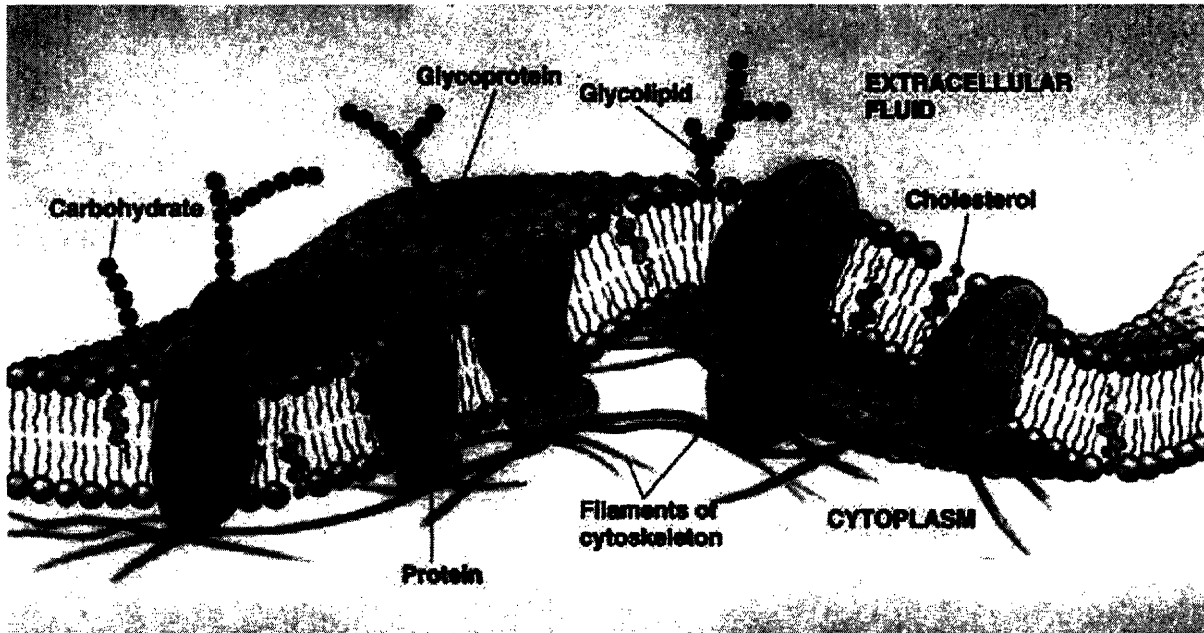


Figure 2: The Cell Membrane and Associated Structures [2]

1.3 Focal Adhesions and Cytoskeletal Tethering

Sites on the cell membrane in which a receptor clustering is present are often associated with an assembly of several proteins in the cytoplasm that link the membrane receptors to the underlying actin cytoskeleton, a formation termed a focal adhesion. Although numerous proteins have been associated with focal adhesions, among the important structural proteins are vinculin, α -actinin, talin, and paxillin.¹² The focal adhesion is believed to have two principal roles. First, it serves as a mechanical link between the relatively rigid cytoskeleton and the receptor, which

otherwise migrates unhindered in a highly deformable membrane. Second, the focal adhesion is comprised of many important signaling proteins essential to adhesion regulation. Certain focal adhesion proteins may activate integrin directly as described above, while others may be involved in promoting clustering. For these reasons, the dynamic regulation of focal adhesion assembly and disassembly is critical to both membrane deformability and cell adhesion. One of the critical questions addressed in this research is the degree to which these downstream effects are related, if at all.

1.4 The Cytoskeleton

Cells maintain shape and rigidity through the cytoskeleton, a network of filaments in the cytoplasm constantly undergoing rearrangement. In addition to its role in adhesion and motility, the cytoskeleton aids transport through the cell, helps divide cells during mitosis, and performs a variety of other specific functions unique to certain cell phenotypes. There are three main categories of filaments, which are differentiated by size and function: microfilaments, intermediate filaments, and microtubules. All types are composed of a chain of repeating units, which in the case of microfilaments is the protein actin. Individual microfilaments are about 3-6nm in diameter, 20um in length, and have very little resistance to bending. However, in vivo the filaments crosslink to form a relatively a rigid network beneath the membrane several hundred nanometers in thickness, providing the cell with structure and rigidity. Short cross-linking proteins like α -actinin and fimbrin cause filaments to bundle, whereas longer proteins like spectrin and filimin allow more spacing between filaments, thereby forming a less dense, crisscrossing network.⁵ In addition to the structural role of the actin cortex beneath the plasma membrane, the actin cytoskeleton occupies the cytosol, providing a guide for intracellular

movements.

Polymerized actin is called F-actin, while the actin monomer is known as G-actin. Because G-actin adds more rapidly to one end of growing microfilaments than the other, the ends of the F-actin polymer are denoted plus and minus. Polymerization of these ends proceeds until equilibrium is reached with the G-actin in solution. The equilibrium G-actin concentration is called the critical concentration (C_c). C_c at the plus and minus ends is approximately 0.1 and 0.8, respectively, indicating the higher concentration of G-actin required for polymerization to occur at the minus end.

Because actin does not permeate the plasma membrane, concentrations of G-actin available for polymerization must be modified in other ways. Thymosin B4 (TB4) is a protein that sequesters unbound G-actin, lowering the cytosolic concentration of G-actin and preventing polymerization. The binding of TB4 is in turn regulated by proteins like profilin, which is believed to be activated at the cell membrane by extracellular signals. Activated profilin displaces TB4 and allows G-actin to freely polymerize.

Microtubules are comprised of the protein heterodimer tubulin, which forms a hollow rod-like structure when polymerized. This is shown below in figure 3. Like microfilaments, microtubules bind and unbind tubulin more rapidly at the plus end than at the minus end, which each have a characteristic critical concentration. However, unlike microfilaments, microtubules grow in discrete lengths called protofilaments. Therefore, protofilaments grow until equilibrium is reached whereupon they bind to one another and form the wall of the microtubule.

Microtubules play a somewhat different role in the cell compared to microfilaments, but many of their functions overlap. They can cause protrusions in the cell membrane, control cellular movements like the beating of cilia and flagella, and separate chromosomes prior to cell

division during meiosis and mitosis. Furthermore, the long polymers provide a means of transport throughout the cell for membrane vesicles. Although it was originally proposed that this transport occurred through the hollow center of microtubules, it is now known that these and other molecules travel only along the outer wall of the microtubule tracks.

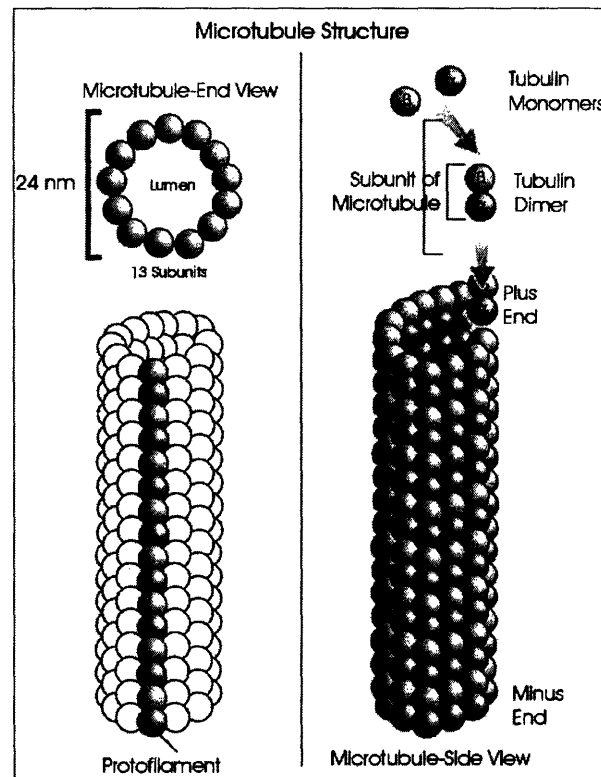


Figure 3: Microtubule Structure and Subunits [3]

Intermediate filaments (IF) are comprised of a variety of proteins from six distinct families. Although they are analogous in many ways to actin microfilaments, they tend to be more stable polymers that anchor the cell membrane to the IF cytoskeleton. The protein subunits are thin, rod-shaped proteins that bundle to form protofilaments, which form successive bundles of protofilaments followed by protofibrils and finally the intermediate filament with a diameter of ~10nm. Although intermediate filaments are beyond the scope of this study, it is important that they be addressed in future study.

2. Theoretical Analysis

Rheology, the study of deformation and flow of matter, has made immense progress in its application to cell mechanics in recent decades. However, layer upon layer of complexity, from the dynamic nature of cell structure to the diversity of contributing interactions, has prevented development of an accurate quantitative model of cell deformation. In this section the basic theories of viscoelastic mechanics will be described as they relate specifically to the problem of membrane deformation.

2.1 Viscoelasticity

Elastic deformation of a solid is characterized by a spring-like return to its original shape. This phenomena is quantified by Hooke's Law, which describes a linear relationship for the force per unit area applied to a solid to the ratio of change in length to original length. Force per unit area is called stress, while the ratio of lengths is known as strain, and they are denoted by σ and ε , respectively. The following stress-strain equation governs small deformations of most solids:

$$\sigma_E = E \cdot \varepsilon, \quad (1)$$

which can also be written

$$\frac{F}{A} = E \cdot \frac{L - L_0}{L_0}, \quad (2)$$

where F is force, A is the area perpendicular to the force, and L and L_0 are the new and original lengths of the material, respectively. The Young's modulus is a characteristic of the material that quantifies the stiffness and is commonly denoted by E . A second important characteristic of solids is the Poisson's ratio, ν , which quantifies the expansion or contraction of a solid

perpendicular to an applied force.

Liquids do not exhibit the restorative properties that characterize solids. Rather, they resist flow with shear stress proportional to the velocity that particles flow past one another. If the relationship between shear stress and the velocity gradient is linear, it is called a Newtonian fluid and described by the following equation:

$$\tau = \eta \cdot \frac{dv}{dx}, \quad (3)$$

where τ is the shear stress, dv/dx is the velocity gradient, and the coefficient η characterizes the viscosity of the fluid. Thus, elasticity is resistance proportional to change in length, while viscous resistance is proportional to the rate of change in length.

To combine the elastic and viscous components of a given system, it is important to recognize the physical relationship between the two properties. This may be accomplished by drawing mechanical equivalent circuits, which are analogous to electrical circuits. The elastic stiffness of the system is represented by a spring, while the viscous resistance is shown as a dashpot. The unique arrangement of springs and dashpots in series or parallel determines the response of the system to a force input. Examples of viscoelastic equivalent circuits are the Maxwell body, Voigt body, and Kelvin body, which are shown in Figure 4A.

More recently, a model has been proposed that more accurately embodies membrane characteristics observed by experimentation. It is essentially a dashpot in series with a Kelvin body, which is shown in figure 4B along with the response of this model to a step input. The displacement of this model along a coordinate x in response to a force input F is shown below.

$$x(t) = F \left[\frac{1}{k_0} \left(1 - \frac{k_1}{k_0 + k_1} \exp\left\{ -\frac{t}{\tau} \right\} \right) + \frac{t}{\gamma_0} \right], \quad (4)$$

where the time constant for relaxation is:

$$\tau = \frac{\gamma_1(k_0 + k_1)}{k_0 k_1}. \quad (5)$$

Initially (as $t \rightarrow 0$), deformation is proportional to $1/(k_0 + k_1)$, and is therefore a purely elastic response. However, at long time scales ($t > \tau$), the elastic component of deformation diminishes and the molecules that constitute the membrane flow according to the last component in the equation, t/γ_0 . Thus, there are three domains in the deformation of this model: I) elastic

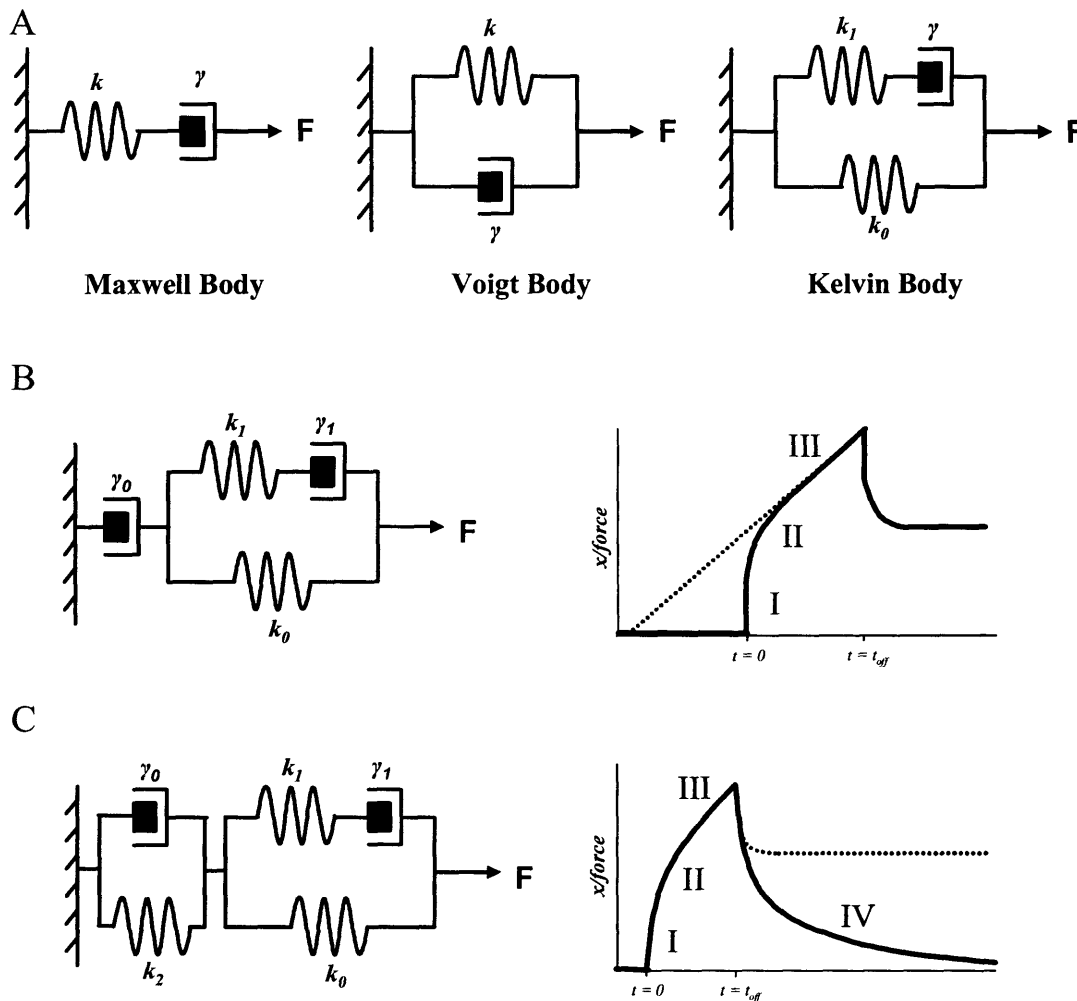


Figure 4: Equivalent Mechanical Circuits. (A) The early models proposed for viscoelasticity. (B) A Kelvin body in series with a second dashpot; response of this system to a step input. (C) Kelvin body in series with a Voigt body; response of this system to a step input.

deformation, II) relaxation as elastic deformation reaches its maximum and III) viscous flow. This is shown in figure 4B. The time constant τ represents the approximate elapsed time when elastic resistance weakens and flow begins i.e. Domain II.

2.2 Deformation of Erythrocytes

Because most cells have highly heterogeneous membrane stiffness due to irregularities in the three-dimensional cytoskeleton and the dynamic nature of tethering, quantitative studies of membrane deformation in most cells are difficult. To simplify somewhat, the majority of membrane research has been performed in red blood cells, which have a relatively uniform internal structure composed of a thin actin-spectrin cortex beneath the membrane and a liquid interior.⁸ The cortex structure is formed by the long fibrous protein spectrin which is joined by short actin filaments and the proteins adducin, tropomyosin, and tropomodulin, which collectively constitute a junctional complex. Spectrin and the proteins forming the junctional complex are permanently linked to the erythrocyte membrane by Ankyrin and Band 4.1 proteins, respectively.⁵ This intracellular arrangement exhibits more consistency in stiffness in the cell as a whole, allowing the use of lower resolution techniques to study membrane deformation.

Numerous methods have been employed to study the mechanical characteristics of the erythrocyte membrane. The most effective in terms of quantitative results have been microaspiration¹³ and microinterferometry.¹⁴ Microaspiration utilizes a pipette to induce negative pressure in the cell while simultaneously measuring the height of the resulting bulge.

The surface stress is given by the following relationship:

$$T = \frac{(x_0^2 + d^2 / 4)^2}{d^2 - 4x_0^2} S_0 \quad (6)$$

where x_0 is the initial height of the bulge, d is the diameter of the pipette, and S_0 is the slope of the pressure-deformation curve. The coefficient for the elasticity of the membrane can then be calculated by relating the change in surface stress to deformation.

Microinterferometry or flicker spectroscopy uses the harmonic modes generated by thermal excitation to determine the membrane bending modulus. For a small area L , where $L \ll R_{cell}$, the amplitudes of membrane deflection u_q can be related to the bending modulus κ and membrane tension σ by the following equation:

$$L^2 \langle u_q^2 \rangle = \frac{k_B T}{\kappa * q^4 + \sigma * q^2}, \quad (7)$$

where q is the wave vector, k_b is Boltzmann's constant and T is the temperature. Using these methods, bending module of 1.8×10^{-19} J for microaspiration and $1.3-3 \times 10^{-20}$ J for microinterferometry have been estimated.

2.3 High resolution mechanical assays

Technological advances in the last two decades have facilitated higher-resolution studies in relatively complex cell membrane composite structures. Two techniques in particular, magnetic bead microrheometry (MBM) and atomic force microscopy (AFM) have revealed important membrane characteristics. While both methods allow measurement of membrane deformation at the resolution of tens of nanometers, MBM generally applies forces tangential to the membrane whereas AFM applies it perpendicularly as shown in figure 5. For this reason, each technique has unique advantages and disadvantages in analyzing membrane mechanics, which will be elaborated upon in the following sections.

2.3.1 Magnetic Bead Microrheometry

This technique utilizes a magnetic bead approximately 4.5 μm in diameter to apply forces along the plane of the cell membrane. To measure the resultant displacement field, smaller non-magnetic beads made from latex are adhered to the cell and tracked with microscopic imaging technology to a resolution of $\sim 10\text{nm}$.¹⁵ Recently this technique was used to expand upon the viscoelastic model of membrane deformation described in Equation 4 to account for tethering of receptors to the actin cytoskeleton. This tethering anchors the receptors in the membrane, meaning there is complete elastic recovery once stress is released. The equivalent circuit that embodies this system along with the resulting step response is shown in Figure 4C. The circuit is essentially a Voigt body in series with a Kelvin body, and may be described by the following equation:

$$x(t) = F \left[\frac{k_1}{k_0(k_0 + k_1)} \exp\left\{-\frac{t}{\tau}\right\} - \frac{1}{k_2} \left(1 - \exp\left\{-\frac{t}{\tau_{decay}}\right\}\right) + \frac{T}{\gamma_0} \right] \quad (8)$$

where τ is the same as Equation 4 and a second relaxation constant τ_{decay} is equal to η/k_2 . Though less clear with the addition of the “tethering” spring, the three domains observed in the previous model remain in the response shown in Figure 4C. The main difference is a fourth domain in which the membrane springs back to its original position due to cytoskeletal tethering.

2.3.2 Atomic Force Microscopy

Atomic force microscopy is used to map force at the piconewton resolution by measuring the deflections of a thin, triangular tip of known stiffness. This capability may be translated into the ability to generate force maps of a variety of materials to measuring the strength of individual receptor-ligand bonds in vitro. In studies of membrane deformation, the majority of work has utilized indentation of the cell membrane rather than pulling due to the relative simplicity of the

resulting deformation. However, for this discussion we will address both cases, which are illustrated in Figure 5B-C.

One distinct advantage of applying force perpendicular to the membrane is that there is no net force on the receptor in the plane of the membrane. Therefore, regardless of whether the receptor is tethered to the cytoskeleton, it should not flow through the membrane. This

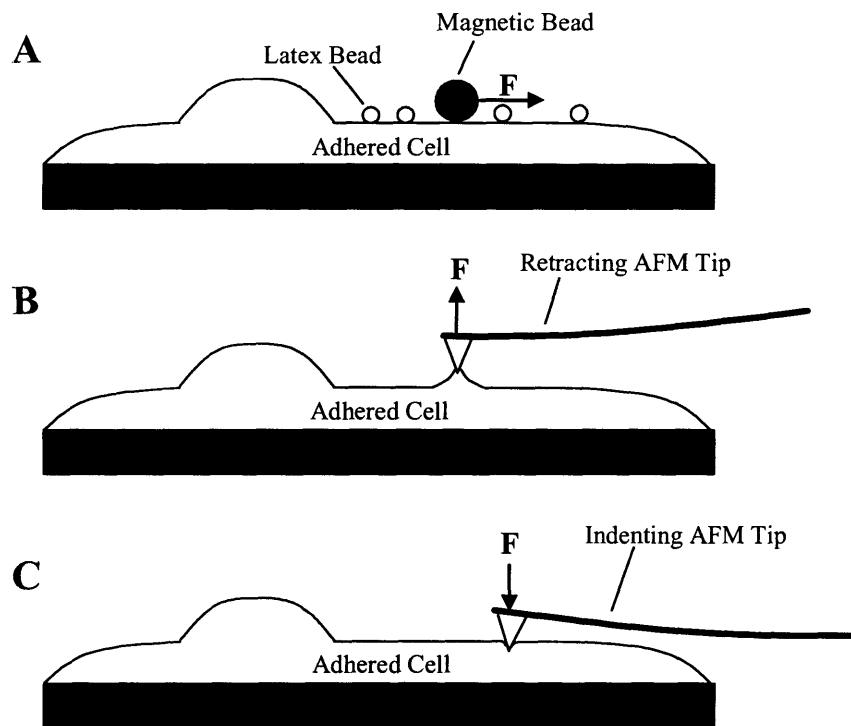


Figure 5: Advanced studies of membrane elasticity (not necessarily to scale). (A) Magnetic bead microinterferometry. (B) Atomic force microscopy membrane pulling. (C) Indentation with atomic force microscopy

effectively simplifies the model to a Kelvin body. Unfortunately, this simplification is far overshadowed by the added complexity of the deformation of the membrane. For that reason, viscous effects in the Kelvin model are neglected, and as a first approximation deformations are considered completely elastic. In the case of indentation studies, an additional assumption is made that the membrane deforms to the shape of the indenter. Therefore, the geometry of the

deformation is known and is a function of the indentation depth. The resulting force is a function of the strains required to obtain the given geometry. For example, in the case of a membrane indented by a stiff cone, the relationship between force F and depth δ is:

$$F = \frac{E}{1-\nu^2} * \frac{2 \tan \alpha}{\pi} * \delta^2, \quad (9)$$

where α is the half-angle, E is the Young's modulus and ν is the Poisson's ration. In order to calculate E , a value must be estimated for ν , which is typically 0.3-0.5. The series of assumptions necessary to utilize this model from neglecting viscous effects to estimating the Poisson's ratio makes the resultant error very high.

Due to the complexity of deformation resulting from pulling the membrane, few models have been proposed. However, a model proposed by Karunasena⁶ for an exact solution to plate deformation was utilized in an AFM pulling experiment by Scheffer, et al.⁷ The relationship between force F and displacement z predicted by this model is shown below:

$$z = \frac{F * C}{\kappa}, \text{ where } C = 10^{-4} * R^2 (b_1 + b_2 r^2 + b_3 \ln r + b_4 r^2 \ln r) / 2\pi, \quad (10)$$

κ is the bending modulus and the coefficients b_1 - b_4 are tabulated in the reference by Karunasena. In addition to elastic deformation, this solution assumes that the ratio the small radius (R) of deformation to the large radius (r) remains constant. This is illustrated in Figure 6 below.

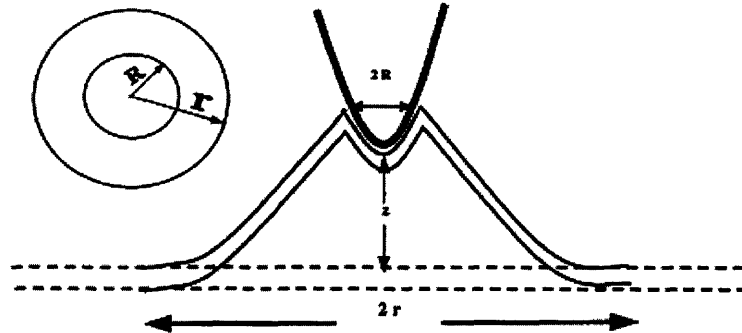


Figure 6: Relevant radii in atomic force pulling

2.4 Passive versus Active Response

An additional layer of complexity that must be considered in studies of membrane microrheology is the phenomenon of active response. Passive response refers to the elastic or viscous response of the cell resulting from stretching and breaking of intermolecular bonds in the membrane and cytoskeleton. Active response, on the other hand, is a result of rearrangement that occurs within the cytoskeleton and membrane at longer time scales, which can cause membrane deformation in the absence of externally applied force.

2.5 Thesis Objectives

In the above introduction and analysis we have established in both qualitative and quantitative terms the characteristics of the membrane-cytoskeleton composite structure. In the experiment described below, we modified this structure using chemical treatments and tested for the resulting effects on adhesion. We will later discuss the potential mechanistic causes of the observed effects.

3. Materials and Methods

3.1 Cell Culture

SW620 cells were maintained using standard cell culture techniques as prescribed by Basson et al.¹⁰ Media used for cell culture during this study was 45% RPMI, 45% Dulbecco's modified Eagle's medium (DMEM), and 10% Fetal Bovine Serum.

3.2 Cell Adhesion Assay

Control cells (100,000 cell/well) were allowed to adhere to collagen I coated 6-well

plates for 30 minutes at 37°C. After 30 minutes, non-adherent cells were gently washed away with warm phosphate-buffered saline (PBS), and adherent cells were formalin-fixed, hematoxylin-stained, and counted in 20 or more random high power fields per well using an Olympus microscope. Modified cells were treated with phalloidin (10 µmol/L), Cytochalasin D (5 µmol/L), Latrunculin B (500 nmol/L), paclitaxel (10 µmol/L), or colchicine (10 µmol/L).

4. Results

4.1 Adhesion in Actin-Modified Cells

There were varying degrees of reduction in adhesion resulting from the cytoskeletal inhibitors used in this assay due to their unique effects on the cell. Phalloidin binds F-actin and inhibits depolymerization, making it a convenient probe for actin filaments when conjugated with fluorescent labels.⁴ We found in this case that the inability to depolymerize F-actin to G-actin resulted in a small reduction (15%) in the number of adhering cells.

Two chemicals that have been shown to dissolve the actin network, Latrunculin B and Cytochalasin D, resulted in 45% and 85% reduction in adhering cells, respectively. It is believed that latrunculin causes depolymerization by binding G-actin, effectively reducing the concentration of unpolymerized actin which in turn induces depolymerization. Cytochalasin D on the other hand, binds the (+) end of F-actin and prevents addition of G-actin subunits, thereby achieving the same effect.⁵ It is important to note the concentration of Cytochalasin D used is well above the accepted minimum to completely dissolve the actin network, while the lower concentration of Latrunculin B would only partially disrupt actin filaments.¹

4.2 Adhesion in Cells with Modified Microtubules

Colchicine is a potent inhibitor of microtubule polymerization. By forming a complex with the B-subunit of the tubulin heterodimer, it destabilizes the microtubule because of a steric clash between the α -tubulin and the newly added monomer. Although low concentrations of colchicines only inhibit polymerization, the high concentrations used in this experiment cause complete disassembly of the microtubule polymer. This resulted in over 50% reduction in adhered cells.

Paclitaxel, or taxol, is a strong stabilizer of microtubules, acting in the much the same mechanism as colchicine but achieving the opposite effect. One subtle mechanistic difference is that taxol only binds the formed polymer, rather than binding the free monomer. Once attached, the taxol-tubulin complex stabilizes the microtubule structure and prevents depolymerization. This effect caused a 35-40% reduction in overall cell adhesion. These results are shown in the tables below.

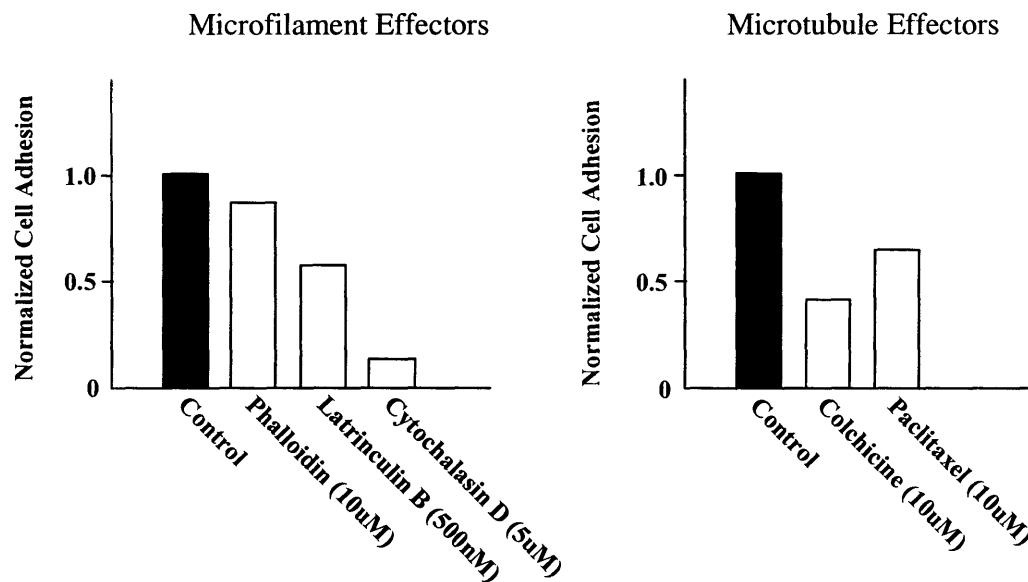


Figure 7: Results of cell adhesion assay expressed as adherent cells normalized to control

5. Discussion

Understanding the implications of this experiment for the role of the cytoskeleton in adhesion is not trivial. Disruption of the various filaments that comprise the cytoskeleton, which would also disrupt the stability of focal adhesions, could potentially alter several key components of adhesive structure, from the integrin-collagen bond to the deformability of the underlying actin skeleton. To aid in analysis somewhat, we will assume loss of cytoskeletal function has two major impacts on overall cellular function. First, the membrane is no longer tethered, resulting in a highly deformable membrane with unanchored receptors. Second, all intracellular signaling pathways involving the cytoskeleton are interrupted. Our discussion of the results observed in this experiment will therefore be divided into these two categories.

5.1 Intracellular Signaling

Within this category, there are a few ways intracellular signaling via the cytoskeleton could affect the adhesiveness of the cell. First, it could directly alter the affinity of the receptor-ligand bond as described in Section 1.2. Because focal adhesion proteins such as talin and α -actinin have been implicated in integrin activation, disruption of cytoskeleton could lead to deactivation of integrin binding, thereby reducing adhesive force. Second, the cytoskeleton could be involved in recruitment of receptors to an initially bound site, which would form clustered parallel bonds. Third, the cytoskeleton could alter cell shape, which could facilitate the formation of additional parallel bonds.

5.2 Membrane Deformability

In prior work we have shown that bond force recorded in live cells using atomic force

microscopy retraction is highly dependent upon deformability of the plasma membrane.¹⁷ We theorize this is a result of the effective rate of force loading perceived by the receptor-ligand bond. To analyze this effect quantitatively the plate deformation theory shown in Equation 10 will be utilized. Rearranging to show force F as a function of displacement z , it is

$$F = \frac{\kappa * z}{C}, \text{ where } C = 10^{-4} * R^2 (b_1 + b_2 r^2 + b_3 \ln r + b_4 r^2 \ln r) / 2\pi . \quad (7)$$

Thus, the loading rate is:

$$\frac{dF}{dt} = \frac{\kappa}{C} \frac{dz}{dt}, \quad (8)$$

where dF/dt is the rate of force loading, dz/dt is approximately the AFM tip retraction rate, and C is a constant. The bending modulus of the membrane is κ , which we will assume is variable depending on the degree of tethering to the cytoskeleton. Therefore, because the tip retraction rate is a constant, an increase in the bending modulus caused by the presence of a focal adhesion will result in a higher rate of force loading. Conversely, an untethered membrane will have a lower bending modulus and force loading will be slower. This is an important result because a direct correlation has been shown between loading rate and bond break force, which is consistent with our results.¹⁸

Unfortunately, these findings are not directly applicable to this experiment, which analyzed the ability of a cell to transform from a suspended state to spread and adhered to a substrate. In AFM, the cell is firmly adhered to a substrate and the membrane is deformed. In this case it is the opposite; the membrane is adhering to the membrane while the cell is subjected to force. Furthermore, a whole cell assay addresses the interactions of hundreds of parallel interactions, whereas AFM is a high-resolution single molecule force assay. The dynamics of parallel bonds under heterogeneous loading on a non-uniform surface is infinitely more

complicated. Additionally, although bond formation and breakage is nearly reciprocal on the single bond level, they are very different processes on the whole cell scale. However, there are a few ways we can theorize how the cytoskeleton could mechanically impact the whole cell adhesion assay performed in this study in the context of a proposed whole cell adhesion model.

5.3 Whole Cell Adhesion Model

As a suspended cell initially comes in contact with a substrate, it is possible that only a few bonds form between the cell and substrate. These few bonds may initiate intracellular signaling pathways that result in the recruitment and activation of other cell adhesion molecules. It is critical that the proteins that form the initial bonds do not break, which would likely interrupt the signaling process in addition to allowing the cell to drift from the substrate. If the membrane is rigid in this scenario, any force transmitted to the cell will be directly transmitted to the receptor, which would likely rupture. If, however, the membrane is untethered for the period prior to formation of several parallel bonds, forces transmitted to the cell will not be transmitted completely to the bound receptor. This suggests an important role for the untethered membrane.

After clustering has occurred and the cell is more firmly anchored to the substrate, it is important that the cell change shape to assume a more hydrodynamic profile. Otherwise, viscous flow past the cell could remove it from the substrate. If true, this would suggest another possible way in which cytoskeletal effectors could impact this adhesion assay. This is especially true of phalloidin and taxol, which stabilize the cytoskeleton polymers rather than disrupt them. Without the ability to depolymerize, the filaments would be unable to rearrange and change cell shape, which could, as described, reduce the number of adhered cells.

6. Conclusions

The critical role of the cytoskeleton in cell adhesion was confirmed by this study. Upon treatment with a variety of chemical effectors of microfilaments and microtubules, we demonstrated up to 85% reduction of gross cell adhesion. Furthermore, we demonstrated that inability of the cytoskeleton to rearrange by depolymerization had a negative impact on adhesion.

In depth analysis of membrane deformability illustrated the important effects of focal adhesion complexes on membrane dynamics. However, we did not provide definitive evidence of a link between loss of membrane tethering and decreased ability of cells to adhere. Without additional experiments, the assay used in this experiment was simply insufficient to solve for the plethora of variables associated with adhesion.

7. Recommendations

A variety of experiments could be used to effectively supplement the ones performed in this study. Notably, atomic force microscopy and magnetic bead microrheometry would provide force distance curves that could provide extensive insight to the models discussed here. AFM studies of membrane deformation in conjunction with bond break force could provide a fundamental basis for the role of the cytoskeleton in adhesion.

Several other topics for future work have emerged from this study. The time scales in which focal adhesions form is poorly understood, yet this is critical in understanding the boundaries of active and passive response. Pulling studies with AFM that attempted to correlate surface dwell time to increased membrane tethering could shed light on this problem.

Additionally, the current models of plate deformation are inadequate to describe soft,

viscoelastic materials such as the membrane and cytoskeleton. These models were often developed for use with much harder materials with negligible viscous properties.

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Figures

[1] <http://ntri.tamuk.edu/homepage-ntri/lectures/protein/junction1.html>

[2] http://fig.cox.miami.edu/Faculty/Dana/105F00_4.html

[3] <http://www.erin.utoronto.ca/~w3bio315/background%20MTs.htm>