

# Membrane Tethers Formed from Blood Cells with Available Area and Determination of Their Adhesion Energy

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**ABSTRACT** Fundamental to all mammalian cells is the adherence of the lipid bilayer membrane to the underlying membrane associated cytoskeleton. To investigate this adhesion, we physically detach the lipid membrane from the cell by mechanically forming membrane tethers. For the most part these have been tethers formed from either neutrophils or red cells. Here we do a simple thermodynamic analysis of the tether formation process using the entire cell, including tether, as the control volume. For a neutrophil, we show that the total adhesion energy per unit area between lipid membrane and cytoskeleton depends on the square of the tether force. For a flaccid red cell, we show that the total adhesion energy minus the tension in the spectrin cytoskeleton depends also on the square of the tether force. Finally, we discuss briefly the viscous flow of membrane. Using published data we calculate and compare values for the various adhesion energies and viscosities.

## INTRODUCTION

The membrane of vertebrate eucaryotic cells consists of two basic components: a phospholipid bilayer and an underlying cytoskeleton. The lipid membrane acts as a sealer and gate-keeper between the cytosol and extra cellular fluid whereas the cytoskeleton gives the cell its shape, strength, and motility. Fundamental to the life of the cell is the adhesion of the lipid bilayer to the underlying membrane associated cytoskeleton. The two adhere to each other according to some characteristic adhesion energy per unit area. A determination of this adhesion energy requires that the lipid bilayer be separated from the cytoskeleton in some way. Our purpose here is to show how this adhesion energy for the case of neutrophils and red cells can be calculated from a measurement of the force required to form a lipid membrane tether.

Lipid membrane tethers are created by exerting a force at a point on the surface of a cell. The lipid bilayer separates from the cytoskeleton and forms a long, thin cylindrically shaped tether. Because of its very small diameter it is likely that the tether is devoid of cytoskeleton (Hochmuth et al., 1982, 1983), although there is evidence that actin monomer exists in tethers formed from neutrophils (Zhelev and Hochmuth, 1995). Early on, tethers were formed from human red cells (Hochmuth et al., 1973, 1982; Waugh, 1982). Later work continued with studies of tethers from red cells (Hwang and Waugh, 1997), nerve growth cone (Dai and Sheetz, 1995), neurons (Dai et al., 1998) and neutrophils (Shao and Hochmuth, 1996). Because the lipid membrane is capable of only a minimal expansion in area on the order of 4% or less (Waugh and Evans, 1979; Needham and Nunn, 1990), the material for the tether must come from the cell body. Thus, a tether is formed with the

constraint that the total (cell body plus tether) surface area of the lipid bilayer remains constant. In addition, because the cytosol is incompressible, a tether is formed at constant total cell volume. If the cell is spherical in shape, such as a neutrophil, the lipid membrane for the tether must come from microvilli on the surface of a cell. If the cell is disk shaped, such as a human red cell, then the lipid membrane comes from a gradual change in shape of the cell until, in the limit, the cell would become a sphere with an extraordinarily long tether. In fact, tethers are formed from spherically shaped red cells created by aspirating swollen, slightly flaccid cells into pipettes, but in this case most of the excess lipid membrane comes from that portion of the cell in the pipette (Hochmuth and Evans, 1982; Hochmuth et al., 1983; Hwang and Waugh, 1997). When a cell runs out of lipid area during the tether formation process, the force on the tether steadily increases (Raucher and Sheetz, 1999).

Two analyses of tethers formed from cells have been done, but each has its limitations. Hochmuth et al. (1996) postulated some unspecified “far-field” tension in the lipid component of the membrane. To calculate the adhesion energy from their analysis requires that the far-field tension be known a priori, which it is not. As we will show, knowledge of the far-field tension is not necessary as long as the thermodynamic system used for the analysis is the entire cell including the tether. Hwang and Waugh (1997) imposed a far-field tension by aspirating a portion of a nearly flaccid red cell into a small pipette until the external part of the cell forms a sphere. Nevertheless, in general cells are not aspirated into pipettes and do not have a tension imposed on the membrane by mechanical means.

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## ADHESION ENERGY FROM TETHER FORCE

### Cells with microvilli such as neutrophils

Consider a cell with microvilli stuck to a surface or adhering to a pipette as illustrated in Fig. 1 *a*. The pressure around the entire surface of the cell is uniform, and a tether is formed

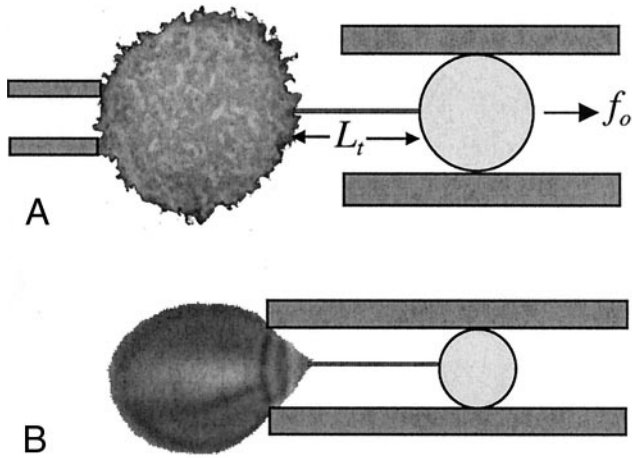


FIGURE 1 (a) Membrane tether extracted from a neutrophil stuck to a pipette. The tether force at equilibrium (zero velocity) is  $f_o$  and the tether length is  $L_t$ . (b) Membrane tether extracted from a red cell stuck to a surface.

from the cell by some means. The excess lipid area is represented by the microvilli on the cell's surface. In this case, the equilibrium work  $dW$  at the boundary of the cell is

$$dW = f_o \times dL_t, \quad (1)$$

in which  $f_o$  is the equilibrium force on the tether and  $dL_t$  is the change in length of the tether. The isothermal work at the boundary increases the total free energy of the entire cell (cell body plus tether) in a number of ways, but several of these will be neglected in favor of the following three major contributions to the free energy of a neutrophil. 1) The bending energy of the tether increases as more lipid membrane is drawn onto the highly curved tether. Here we assume that the stress free state is a surface with a significantly smaller curvature than the tether; e.g., a flat surface or one with the typical curvature of a microvillus. 2) The surface free energy increases as more lipid membrane is separated from the cytoskeleton. 3) The surface free energy increases as mobile lipid membrane proteins unable to diffuse freely between cell body and tether are retained on the cell body. Taking all these factors into account allows us to write in the order given above:

$$f_o dL_t = \left[ \frac{B}{2} \left( \frac{1}{R_t^2} \right) \right] (2\pi R_t dL_t) + \gamma (2\pi R_t dL_t) + \Pi (2\pi R_t dL_t) \quad (2)$$

in which  $B$  is the bending modulus for a two-dimensional surface (with units of energy),  $\gamma$  is the adhesion energy per unit area between lipid membrane and cytoskeleton,  $\Pi$  is a surface osmotic pressure difference created by the accumulation of mobile membrane components in the cell body, and  $R_t$  is the (constant) tether radius. Because the adhesion energy and the osmotic pressure difference depend in the

same way on the tether length, these two terms are added together and called the total adhesion energy  $\gamma_t$ :

$$\gamma_t = \gamma + \Pi. \quad (3)$$

Thus, Eq. 3 is substituted into Eq. 2 and the result divided by  $2\pi R_t dL_t$  to give

$$\frac{f_o}{2\pi R_t} = \gamma_t + \frac{B}{2R_t^2}. \quad (4)$$

An independent relation between the tether force and tether radius was given by Waugh and Hochmuth (1987):

$$f_o = \frac{2\pi B}{R_t}. \quad (5)$$

Eqs. 4 and 5 can be combined in such a way as to eliminate either the bending modulus or the tether radius. In the later case we solve for  $\gamma_t$  to obtain

$$\gamma_t = \frac{f_o^2}{8\pi^2 B}. \quad (6)$$

Eq. 6 shows simply that for a closed system with available membrane area the square of the tether force is proportional to the total adhesion energy per unit area between lipid membrane and cytoskeleton.

An assumption in the derivation given above is that no energy is stored in the cell due to the so-called "nonlocal bending" (Waugh et al., 1992). This is an appropriate assumption because of the short tethers formed in most experiments and the large lipid membrane surface area of the cells that have been studied so far. A second assumption is that the lipid membrane is incompressible in these experiments and, thus, does not store energy by expanding or contracting its area. A third assumption is that the work that goes into compressing the membrane associated cytoskeleton is small compared with the other terms in Eq. 2. Because of the constant volume constraint and the availability of excess surface area in the microvilli, the surface area compression of the cytoskeleton compared with the surface area increase of the tether is on the order of the ratio of the tether radius to cell radius,  $R_t/R_c$ , which is small and can be neglected. A fourth assumption is that the bending energy per unit area stored in the microvilli is small compared with that in the tether because  $(R_t/R_v)^2$  is small, in which  $R_v$  is a typical value for the radius of the microvillus. Our unpublished measurements of 100 microvilli give  $R_v = 0.15 \mu\text{m}$ . Eq. 5 with  $B = 0.2 \text{ pN}\cdot\mu\text{m}$  and  $f_o = 45 \text{ pN}$  (a measured value) gives  $R_t = 0.03 \mu\text{m}$ . Thus,  $(R_t/R_v)^2 = 0.04$ . Finally, we assume that the shape of the cell does not change as the tether is formed.

### Disk-shaped cells with a smooth lipid membrane surface

Here we consider a disk-shaped cell such as a red cell. The cell is either stuck to a surface and a tether attached to a

bead is extracted with a pipette as shown in Fig. 1 *b* or the end of the tether is stuck to the surface and the cell is subjected to a fluid shear (Hochmuth et al., 1973). In either case, the total work on the cell is given by Eq. 1. In this case, however, there are two constraints: constant overall volume, as before, and, because of the smooth lipid surface area, constant surface area. Because of this additional constraint, there is a significant decrease in the surface area of the cell body given by  $-2\pi R_t dL_t$  and, thus, the tension in the cytoskeletal component of the membrane,  $T_{cm}$ , can be important. Now an energy balance gives

$$f_o dL_t = \left[ \frac{B}{2} \left( \frac{1}{R_t^2} \right) \right] (2\pi R_t dL_t) + \gamma (2\pi R_t dL_t) + \Pi (2\pi R_t dL_t) - T_{cm} (2\pi R_t dL_t) \quad (7)$$

or

$$\frac{f_o}{2\pi R_t} = \gamma_t - T_{cm} + \frac{B}{2R_t^2}. \quad (8)$$

The negative sign on the last term in Eq. 7 comes from the equal and opposite change in area of the cell body and tether,  $dA_c = -2\pi R_t dL_t$  and, therefore,  $T_{cm} \cdot dA_c = -T_{cm} \cdot 2\pi R_t dL_t$ . It is interesting to note that the adhesion energy and the stress in the cytoskeleton both scale in the same way with an increase in the tether length. When we use Eq. 5 to eliminate the tether radius from Eq. 8, we obtain

$$\gamma_t - T_{cm} = \frac{f_o^2}{8\pi^2 B}. \quad (9)$$

For a red cell, if the “prestress” (the value for  $T_{cm}$ ) in the spectrin cytoskeleton is significant, then the tether force cannot be attributed exclusively to the total adhesion between the lipid component of the membrane and the cytoskeleton. This is unlike the neutrophil, as shown by Eq. 6, whereby the cytoskeletal membrane tension in the white cell, even if such a tension exists, will make only a negligible contribution to the tether force.

### Spherical cells with a smooth lipid membrane surface

Hwang and Waugh (1997) swelled human red blood cells in a hypotonic medium, aspirated the cells into a pipette with a suction pressure  $\Delta P$ , and extracted tethers from these cells. This adds another term to the work at the boundary in addition to the work done in forming the tether. With this additional term, the total work done on the cell body becomes

$$dW = f_o \times dL_t - \left[ \frac{R_p \Delta P}{2(1 - R_p/R_c)} \right] 2\pi R_t dL_t \quad (10)$$

in which  $R_c$  is the radius of the swollen cell outside the pipette and  $R_p$  is the radius of the pipette. To obtain Eq. 10,

we assume that the overall volume and surface area of the cell remains constant as the tether is formed and we neglect terms of  $O(R_t/R_p)$ . For the case of a swollen cell aspirated into a pipette, the available surface area for the tether comes mainly from the portion of membrane in the pipette (Hochmuth and Evans; 1982; Hochmuth et al., 1983). As we discuss in the next section, the term in the square brackets is simply the overall tension  $T$  imposed on the cell membrane caused by the suction pressure in the pipette. This (negative) work term and the tension that results from it alters Eq. 9 as follows:

$$\gamma_t - T_{cm} = \frac{f_o^2}{8\pi^2 B} - T \quad (11a)$$

or

$$\frac{f_o^2}{8\pi^2 B} = T + \gamma_t - T_{cm}. \quad (11b)$$

The “energy per unit area of association” or the “separation work per unit area,”  $W_{sk}$ , defined by Hwang and Waugh (1997), is equivalent to the term  $\gamma_t - T_{cm}$  in Eq. 11. For a simple phospholipid bilayer,  $\gamma_t - T_{cm} = 0$ , and Eq. 11b reduces to the one given by Heinrich and Waugh (1996) when we ignore their nonlocal bending term.

### OVERALL MEMBRANE TENSION

As noted above, the membrane consists of two basic components: a phospholipid bilayer and an underlying cytoskeleton. Together they create an overall membrane tension  $T$ , which is sometimes called the “cortical” tension. For cells with liquid interiors and spherical shapes, such as white cells and swollen red cells that are partially aspirated into a pipette, a value for  $T$  is determined with the law of Laplace as applied to these cells (Waugh and Evans, 1979; Evans and Yeung, 1989):

$$T = \frac{\Delta P \times R_p}{2 \left( 1 - \frac{R_p}{R_c} \right)} \quad (12)$$

in which  $\Delta P$ , as before, is the suction pressure. In the case of the white cell, the tension exists naturally. Because of this tension all passive white cells are spherically shaped when not acted upon by an external force. For swollen red cells with their smooth lipid bilayer, the suction pressure in the pipette creates the membrane tension. In both cases, we assume that this overall membrane tension is the sum of the tensions in the two separate components of the membrane (Schmid-Schönbein et al., 1995):

$$T = T_{lm} + T_{cm} \quad (13)$$

in which  $T_{lm}$  is the tension in the lipid component of the membrane at a point where the membrane is tangent to the

spherical surface and  $T_{cm}$ , as already defined, is the tension in the cytoskeletal component of the membrane. For a neutrophil, a measured value for  $T$  is between  $\sim 24$  pN/ $\mu\text{m}$  (Needham and Hochmuth, 1992) and  $35$  pN/ $\mu\text{m}$  (Evans and Yeung, 1989). For a red cell, the value for  $T$  depends on the suction pressure according to Eq. 12. If the red cell is flaccid, as it is in its natural state, then  $T = 0$  and, thus,  $T_{cm} = -T_{lm}$ . That is, the lipid and cytoskeletal membrane tensions balance each other so they are equal and opposite in sign.

## CALCULATION OF ADHESION ENERGY

### Neutrophils

By measuring the force required to continuously form a tether at different velocities and then by extrapolating to zero velocity, Shao and Hochmuth (1996) showed that the minimal force required to form a tether from a neutrophil was  $45$  pN. Although a value for the bending modulus for a neutrophil membrane has not been measured, typical values for a lipid bilayer are on the order of  $0.2$  pN $\cdot\mu\text{m}$  (Hwang and Waugh, 1997). Thus, Eq. 6 gives

$$\gamma_t = \frac{(45\text{pN})^2}{8\pi^2 \times 0.2\text{pN} \times \mu\text{m}} = 130 \frac{\text{pN}}{\mu\text{m}}$$

### Red cells

There has been no published direct measurement of the force required to extract a tether from a flaccid red cell. Hochmuth et al. (1973) measured the wall shear stress required to keep a preexisting tether stuck to a surface at constant length when it was extracted from a flaccid cell. Hwang and Waugh (1997) measured the force required to extract a tether from a pressurized red cell with a known membrane tension. In the first case a lower bound for the tether force can be estimated from the shear stress at the wall by multiplying the apparent surface area of the upper membrane surface of the cell by the shear stress at the wall. The shear stress required to maintain a tether at equilibrium was  $1.5$  dynes/cm<sup>2</sup> ( $0.15$  pN/ $\mu\text{m}^2$ ) (Hochmuth et al., 1973). For a surface area of  $60$   $\mu\text{m}^2$ , the lower bound for the tether force is  $9$  pN. The analyses of Hyman (1972) indicate that a hemisphere will have a force that is approximately 5 times this value. Splitting the difference between a flat surface and a hemisphere gives an estimate for the force of  $\sim 30$  pN. With this value, Eq. 9 gives

$$\gamma_t - T_{cm} = \frac{f_o^2}{8\pi^2 B} = \frac{(30\text{pN})^2}{8\pi^2 \times 0.2\text{pN} \times \mu\text{m}} = 60 \frac{\text{pN}}{\mu\text{m}}.$$

In the experiments of Hwang and Waugh (1997), they did not determine a value for the tether force at zero membrane tension. (The typical measured value in their experiments

for the force to form a tether from pressurized red cells was on the order of  $50$  pN.) However, they did determine a value for the ‘‘separation work per unit area’’ of  $0.06$  mJ/m<sup>2</sup>, which is  $60$  pN/ $\mu\text{m}$ . This separation work is equal to the total work to form a tether minus the work that goes into bending the tether and the work against the micropipette-imposed suction pressure. It is equivalent to the value of  $60$  pN/ $\mu\text{m}$  calculated from Eq. 9 above. Recent work presented in an abstract by Butler et al. (2001) indicates a value for the ‘‘separation energy’’ (equivalent to  $\gamma_t - T_{cm}$ ) of  $90$  pN/ $\mu\text{m}$ . (Note that their standard deviation was  $\pm 50$  pN/ $\mu\text{m}$ .) This value, in turn, predicts a critical tether force for a flaccid cell of  $35$  pN when their measured value for the bending modulus of  $0.17$  pN $\cdot\mu\text{m}$  is used in Eq. 9.

### Comparison of adhesion energy for neutrophils and red cells

We see that the value for the adhesion energy for neutrophils is  $130$  pN/ $\mu\text{m}$ , whereas that for red cells is  $60$  to  $90$  pN/ $\mu\text{m}$ . Nevertheless, these values cannot be compared because of the possible importance of the cytoskeletal membrane tension,  $T_{cm}$ , when tethers are extracted from red cells (compare Eqs. 6 and 9). Boey et al. (1998) and Discher et al. (1998) estimate a value for  $T_{cm}$  of  $15$  pN/ $\mu\text{m}$ . Thus for a red cell,

$$\begin{aligned} \gamma_t &= 60 - 90 \text{ pN}/\mu\text{m} + 15 \text{ pN}/\mu\text{m} \\ &= 75 - 105 \text{ pN}/\mu\text{m} . \end{aligned}$$

Even with the larger values obtained by Butler et al. (2001), the value for the lipid membrane-cytoskeleton adhesion energy (separation energy) for a red cell is still somewhat less than that for a neutrophil.

## VISCOUS DRAG OF LIPID MEMBRANE ON CYTOSKELETON

The analyses given above assume that the process of tether formation is thermodynamically reversible. We note here that when tethers are formed at a finite velocity, the tether force increases relative to the tether force at equilibrium (or zero velocity),  $f_o$ . This increase in tether force comes from irreversible processes, the primary one being the viscous drag between lipid membrane and cytoskeleton (Hochmuth et al., 1996). If this viscous shear stress between them is linearly related to the velocity, then in general we can write the tether force  $f$  at a finite velocity as (Hochmuth et al., 1996)

$$f = f_o + 2\pi\eta_{eff}U_t, \quad (14)$$

in which  $U_t$  is the velocity at which a tether is formed.

## Neutrophil

For a range of tether velocities from 0 to  $\sim 10 \mu\text{m/s}$ , the result of Shao and Hochmuth (1996) gives a value for the effective viscosity of  $1.8 \text{ pN}\cdot\text{s}/\mu\text{m}$ . This can be compared with the much smaller value for nerve growth cone of  $0.14 \text{ pN}\cdot\text{s}/\mu\text{m}$  (Hochmuth et al., 1996).

## Red cell

In the work of Hochmuth et al. (1973), when the shear stress and thus the tether force is double that of the shear stress at zero velocity, the velocity of the tether is  $\sim 0.18 \mu\text{m/s}$ . This gives an effective viscosity of  $27 \text{ pN}\cdot\text{s}/\mu\text{m}$ . Results from Hwang and Waugh (figure 5, 1997) give an effective viscosity of  $(214 \text{ pN}\cdot\text{s}/\mu\text{m})/2\pi = 34 \text{ pN}\cdot\text{s}/\mu\text{m}$ . This value is in reasonable agreement with the original result of Hochmuth et al. (1973), especially when we note that the result from Hwang and Waugh came from a study of 38 cells, whereas that from Hochmuth et al. (1973) as calculated here came from only one cell.

## Comparison of the effective viscosity for neutrophils and red cells

Although the adhesion (separation) energy for neutrophils and red cells is similar (compare  $130 \text{ pN}/\mu\text{m}$  to  $75\text{--}105 \text{ pN}/\mu\text{m}$ ), the effective viscosity for the two is different by more than a factor of 10. Although it is slightly easier to detach the lipid membrane of the red cell from its underlying cytoskeleton, it is significantly more difficult to pull it off at a finite rate when compared with that for a neutrophil. We have no simple explanation for this.

## DISCUSSION

Membrane tethers have been extracted from different kinds of cells, but mainly red and white cells, for many years although it is only recently that we have begun to understand what the force on a tether means. Initially, it was thought that the tether force was simply the overall tension in the membrane produced by a pipette suction pressure (Eq. 12) times the circumference of the tether (Hochmuth and Evans, 1982; Waugh, 1982). Of course, this does not explain the dynamics of tethers extracted from flaccid red cells (Hochmuth et al., 1973) because in that case the membrane tension is zero and yet the tether force is clearly finite. Later work showed that the bending of a tether made a significant contribution to the force on a tether (Bo and Waugh, 1989). In the more recent work of Hochmuth et al. (1996) and Hwang and Waugh (1997) as well as the work presented in this paper we see the important influence on the tether force of the total adhesion energy per unit area between membrane and cytoskeleton. Finally, we see that for a red cell the total adhesion energy minus the prestress in the spectrin

cytoskeleton contribute to the tether force for a flaccid cell. Thus, to compare the adhesion energy  $\gamma_t$  for neutrophils and red cells, we must account for a prestress  $T_{\text{cm}}$  in the spectrin cytoskeleton of the red cell (Eq. 9). No such prestress term exists when tethers are formed from neutrophils (Eq. 6). In all cases the adhesion energy with or without a prestress depends on the square of the tether force.

Modeling the total membrane tension as the sum of the membrane tensions in the lipid and cytoskeleton (Eq. 13) leads to interesting result for the case of a flaccid red cell. Here the two tensions are equal and opposite in sign to each other because the total membrane tension on a flaccid cell is essentially zero. (Thermal fluctuations could produce a small residual tension.) Thus, a compressive stress in the spectrin cytoskeleton (Boey et al., 1998) causes a positive tension in the lipid component of the membrane.

In summary we have shown that the adhesion energy per unit area between lipid membrane and cytoskeleton is proportional to the square of the force required to separate the two and form a tether. Our earlier work on this subject (Hochmuth et al., 1996) confounded this important relation with an undefined "far-field tension." However, a thermodynamic analysis on the entire cell eliminates this term altogether and reveals the fundamental relations given by Eqs. 6 and 9.

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