Constitutive Model of Erythrocyte Membranes with Distributions of Spectrin Orientations and Lengths

Zhe Feng,1,2 Richard E. Waugh,3 and Zhangli Peng1,*

1Department of Bioengineering, University of Illinois at Chicago, Chicago, Illinois; 2Department of Aerospace and Mechanical Engineering, University of Notre Dame, Notre Dame, Indiana; and 3Department of Biomedical Engineering, University of Rochester, Rochester, New York

ABSTRACT We present an analytical hyperelastic constitutive model of the red blood cell (erythrocyte) membrane based on recently improved characterizations of density and microscopic structure of its spectrin network from proteomics and cryo-electron tomography. The model includes distributions of both orientations and natural lengths of spectrin and updated copy numbers of proteins. By applying finite deformation to the spectrin network, we obtain the total free energy and stresses in terms of invariants of shear and area deformation. We generalize an expression of the initial shear modulus, which is independent of the number of molecular orientations within the network and also derive a simplified version of the model. We apply the model and its simplified version to analyze micropipette aspiration computationally and analytically and explore the effect of local cytoskeletal density change. We also explore the discrepancies among shear modulus values measured using different experimental techniques reported in the literature. We find that the model exhibits hardening behavior and can explain many of these discrepancies. Moreover, we find that the distribution of natural lengths plays a crucial role in the hardening behavior when the correct copy numbers of proteins are used. The initial shear modulus values we obtain using our current model (5.9–15.6 pN/μm) are close to the early estimates (6–9 pN/μm). This new, to our knowledge, constitutive model establishes a direct connection between the molecular structure of spectrin networks and constitutive laws and also defines a new picture of a much denser spectrin network than assumed in prior studies.

INTRODUCTION

The mechanical properties of the membranes of red blood cells (RBCs or erythrocytes) have been extensively studied, but there remains a lack of integrated understanding from the molecular level to the continuum level. On one hand, empirical continuum constitutive laws have been well established, such as the ones described in the monograph by Evans and Skalak (1). In this approach, a two-dimensional hyperelastic constitutive model is derived from thermodynamics using invariants based on assumptions such as isotropy. In early work, Skalak et al. derived a strain energy function of red blood cell membrane based on the invariants of Green’s strain tensor (2). Subsequently, to better separate the shear deformation and the area deformation, Evans and Skalak provided another strain energy function in terms of the area invariant $\alpha = \lambda_1\lambda_2 - 1$ and shear invariant $\beta = (\lambda_1/\lambda_2 + \lambda_2/\lambda_1 - 2)/2$, where $\lambda_1$ and $\lambda_2$ are the principal stretch ratios (1). For simplicity, the derivatives of free energy with respect to invariants were assumed to be constants and, as such, define the shear modulus and mean stress resultant (isotropic tension) in the material. In this formulation, the area modulus was defined as the second derivative of the free energy with respect to area, and this was also assumed to be a constant.

Subsequently, the accumulation of experimental evidence indicates that the shear modulus and area modulus are certainly not constants, as would be expected based on the highly nonlinear behaviors of biopolymers with mixed entropic and energetic contributions to free energy (3–5). Interestingly, Dimitrakopoulos found that the original Skalak...
law (2), which was supplanted by thermodynamically based models, does a reasonable job of explaining the discrepancy in the shear modulus measurements due to its hardening behavior (6), but connections between such models and molecular events are not defined.

From the molecular perspective, several coarse-grained molecular dynamics models have been developed to study the cytoskeletal cortex of RBCs, but no systematic effort has been made to extract the mechanical properties of these coarse-grained molecular dynamics models to reconstruct corresponding continuum strain-dependent constitutive models that could be used for whole-cell simulations of in vivo biological processes or in vitro experiments. One exception is the work of Dao et al., who derived shear and areal moduli for small deformations using a spectrin-level model based on the virial stress (7). Another exception is the work of Svetina et al. (8), who used an energy method based on a random network of ideal springs to make macroscopic predictions of membrane behavior, including micropipette aspirations, and derived expressions for the deformation dependence of the area and shear moduli, although explicit stress-strain relationships were not derived. Thus, although several microscopic models of RBC membranes have been developed, including spectrin-level models (9–11), with few exceptions, these have not been linked to mechanical behaviors at large deformation via continuum models. In addition, there are recent proteomic data from two independent sources (12,13) showing that whereas the stoichiometry of spectrins and junctional complex proteins are consistent with earlier estimates, copy numbers of RBC proteins such as spectrin are two- to threefold higher than well-accepted values in the literature (14). The predictions of any molecularly based model of red cell membrane behavior need to be re-examined in light of these new data.

In this study, we present a novel, to our knowledge, constitutive description for arbitrarily large deformation that is based on, and connects directly to, spectrin-level behavior as a worm-like chain. In addition, we also derive a simplified version of the constitutive law and give the corresponding analytical solution of the pressure-length relation in micropipette aspiration. Our approach accounts for two important characteristics not found in earlier constitutive models: namely, it exhibits strain-hardening behavior, and it allows for local changes in cytoskeletal density. This latter feature is in keeping with fluorescence imaging experiments showing that the cytoskeleton density does change significantly when cells are aspirated into micropipettes (15), becoming compressed near the pipette entrance and expanded near the tip of the membrane projection. We will explore the effect of the area change on the aspiration curve and measured shear modulus using the microstructure-based model. With microstructure-based strain hardening and cytoskeletal area change, good comparisons with our experiments and those in the literature are demonstrated.

**METHODS**

**Constitutive model of the RBC cytoskeleton with orientation distributions of spectrins**

We consider the relationship between the molecular free energy of the molecules of the cytoskeletal network and the free energy per unit area of the skeleton. We follow the development of Discher and colleagues (9), which was based on a molecular network model developed by Boey et al. (16). The membrane skeleton was treated as a triangular elastic network in which the springs connecting network nodes have the characteristics of an elastic, worm-like polymer modeled after erythrocyte spectrin. The energy of the triangular network took the form (9)

\[ E_{\text{net}} = \sum_{\text{bonds}} \left[ V_{\text{eff}}(s) + C/\lambda \right], \]  

where \( \lambda \) is the area per molecule and \( C \) is a coefficient preventing network collapse. The attractive potential \( V_{\text{eff}}(s) \) of the “bonds” that represent the spectrin molecules was derived from the force-extension relationship of a worm-like chain (WLC) with both ideal and divergent behavior (17). The approximate interpolation formula of the WLC potential (17) for a bond with node-to-node distance \( s \) and maximal separation \( s_{\text{max}} \) is

\[ V_{\text{eff}}(s) = \frac{k_{\text{B}}T s_{\text{max}}}{4p} \left( \frac{s}{s_{\text{max}}} \right)^2 \left( 3 - \frac{2}{1 - s/s_{\text{max}}} \right), \]

where \( p \) is the persistence length of the chain segments, \( k_{\text{B}} \) is Boltzmann’s constant, and \( T \) is the absolute temperature. The corresponding force of the effective WLC potential is given as

\[ f(s) = -\frac{\partial V_{\text{eff}}}{\partial s} = -\frac{k_{\text{B}}T}{p} \left[ \frac{1}{4\left(1-s/s_{\text{max}}\right)^2} - \frac{1}{4} + s/s_{\text{max}} \right]. \]

Discher and colleagues applied these molecular models to obtain predictions both for the distribution of skeletal density in pipette aspirated cells and for the projection length of a biconcave disk being aspirated into a micropipette at different pressures (9). There are three unknown molecular parameters in the model: the maximal molecular length \( s_{\text{max}} \), the persistence length \( p \), and the area coefficient \( C \). Writing the molecular lengths relative to their stress-free natural length (\( s/s_0 \)) and imposing the condition that the system must be in a minimal energy state at rest, one can solve for one of these parameters \( (C) \) in terms of the other two, leaving two coefficients to adjust to vary the mechanical stiffness of the network, \( p \) and \( s_{\text{max}} = s_{\text{max}}/s_0 \).

Taking \( s_0 \) as the resting molecular length, the molecular extension \( s/s_0 \) is

\[ \frac{s}{s_0} = |F \cdot t_0| = \sqrt{t_0 \cdot C \cdot t_0}, \]

where \( t_0 \) is the filament unit orientation in the reference configuration,

\[ C = F^T F = \begin{bmatrix} \lambda_1^2 & 0 \\ 0 & \lambda_2^2 \end{bmatrix}, \]

is the right Cauchy-Green deformation tensor, and \( F \) is the deformation gradient. The molecular extension \( s/s_0 \) is related to the material stretch ratios by

\[ \left( \frac{s}{s_0} \right)^2 = \lambda_1^2 \cos^2 \theta_{0,i} + \lambda_2^2 \sin^2 \theta_{0,i}, \]
where \( \theta_{0,i} = i\pi/n, i \in (1, n) \) is the angle between the molecular vector for orientation \( i \) and the principal axis of extension in the resting state. The energy per unit area must be summed over molecular orientations. Note that in this formulation, \( n \) is the number of distinct molecular orientations in the resting state, not the number of elements in a network unit. For example, in a regular hexagonal array, \( n = 3 \), but in a random network, \( n \to \infty \).

In Appendix B in the Supporting Materials and Methods, we show the detailed derivation of the stress resultants from the energy. The resulting constitutive equations for the skeleton take the form

\[
\tau_{i} = c_{\beta} \left[ \frac{2}{n} \lambda_{1} \left( \sum_{i=1}^{n} \cos^{2} \theta_{0,i} P_{i}(s / s_{0}) \right) - \frac{c_{a}}{\lambda_{1}^{2} \lambda_{2}^{2}} \right],
\]

and

\[
\tau_{2} = c_{\beta} \left[ \frac{2}{n} \lambda_{2} \left( \sum_{i=1}^{n} \sin^{2} \theta_{0,i} P_{i}(s / s_{0}) \right) - \frac{c_{a}}{\lambda_{1}^{2} \lambda_{2}^{2}} \right],
\]

where

\[
c_{a} = \frac{6 \lambda_{max}^{2} - 9 \lambda_{max} + 4}{(\lambda_{max} - 1)^{2}},
\]

\[
c_{\beta} = \frac{k_{B} T \rho_{0}}{8(p/s_{0}) \lambda_{max}},
\]

and

\[
P_{i}(s / s_{0}) = \frac{6 \lambda_{max}^{2} - 9 \lambda_{max}(s / s_{0}) + 4(s / s_{0})^{2}}{(\lambda_{max} - (s / s_{0}))^{2}}.
\]

An important reference point for comparison with other theoretical developments is the value of the modulus at the resting state \( \mu_{0} \). Taking the limit at constant area for \( \lambda_{1} \approx 1 + e \) and \( \lambda_{2} \approx 1 - e, e \to 0 \), we obtain

\[
\mu_{0} = c_{\beta} \left[ c_{a} + \frac{3 \lambda_{max}^{2} - \lambda_{max}}{4(\lambda_{max} - 1)^{3}} \right] = \frac{k_{B} T \rho_{0}}{8(p/s_{0}) \lambda_{max}} \frac{6 \lambda_{max}^{2} - 9 \lambda_{max} + 4}{(\lambda_{max} - 1)^{2}} + \frac{3 \lambda_{max}^{2} - \lambda_{max}}{4(\lambda_{max} - 1)^{3}}.
\]

This expression is identical to one obtained previously by Dao et al. (7) for the specific case of \( n = 3 \). After substituting \( \lambda_{0} = \lambda_{max} = s / s_{0} \), recognizing that for a triangular network \( \rho_{0} = 2/3 / s_{0}^{2} \) and applying algebraic manipulation, we find

\[
\mu_{0} = \sqrt{3} k_{B} T \frac{3}{4(1 - x_{0})^{2}} + \frac{3}{4} + 4 x_{0} + \frac{x_{0}}{2(1 - x_{0})^{3}},
\]

Note that

\[
\frac{1}{\lambda_{max}} \left[ \frac{6 \lambda_{max}^{2} - 9 \lambda_{max} + 4}{(\lambda_{max} - 1)^{2}} + \frac{3 \lambda_{max}^{2} - \lambda_{max}}{4(\lambda_{max} - 1)^{3}} \right] = \frac{3}{4(1 - x_{0})^{2}} + \frac{3}{4} + 4 x_{0} + \frac{x_{0}}{2(1 - x_{0})^{3}}.
\]

Remarkably, no assumptions about \( n \) were made in deriving Eq. 14, demonstrating that it is in fact accurate for all \( n \) with the same chain density.

Similarly, from the definition of area modulus \( K_{e} = (\rho_{0} / \rho_{e})_{a} \) and as detailed in Appendix C in the Supporting Materials and Methods, the area modulus can be expressed as the following forms

\[
K_{e} = \frac{c_{\beta}}{2n(1 + \alpha)^{2}} \left[ \sum_{i=1}^{n} \left( \frac{s}{s_{0}} \right) \frac{3 \lambda_{max}^{2} - \lambda_{max}}{(\lambda_{max} - (\lambda_{max} / n))^{3}} \right] + \frac{2 c_{a} c_{\alpha}}{(1 + \alpha)^{3}},
\]

and

\[
K_{e,a} = \frac{2 c_{a}}{x_{0}} \left( \frac{3 \lambda_{max}^{2} - x \lambda_{max}}{(\lambda_{max} - x)^{3}} \right),
\]

There are two forms of special interest. One is the isotropic case \( \lambda_{i} = \lambda_{2} = s / s_{0} = x = \sqrt{\alpha + 1} \),

\[
K_{e,a} = \frac{2 c_{a}}{x_{0}} \left( \frac{3 \lambda_{max}^{2} - x \lambda_{max}}{(\lambda_{max} - x)^{3}} \right),
\]

and the other is the limit of no deformation at the resting state \( (s / s_{0})_{a} \to 1 \).
Comparing Eq. 17 with Eq. 13, we find that the initial area modulus is twice the initial shear modulus, \( K_0^{el} = 2\mu_0 \).

**Simplified shear stress expression**

For specific molecular orientations, a simplified expression can be derived to approximate the material behavior to facilitate analytical solutions to certain problems, such as micropipette aspiration. As detailed in Appendix B in the Supporting Materials and Methods, the shear stress resultant in Eq. 10, if the area is incompressible, can be approximated as

\[
\tau_{sk}^{el} = \frac{2C_\beta}{3x_0} \left[ c_0 + c_1 (\lambda_1 - 1) + \frac{\lambda_1}{4(1 - \lambda_1 x_0)^2} \right],
\]

where \( c_0 = \frac{1}{4(1 - x_0)} \) and

\[
c_1 = \frac{48x_0^4 - 153x_0^3 + 171x_0^2 - 71x_0 + 1}{4(x_0 - 1)^3}.
\]

The shear modulus is given as

\[
\mu = \frac{2\tau_{sk}^{el}}{\lambda_1 - \lambda_2} = \frac{4C_\beta}{3x_0 (\lambda_1 - \lambda_2)} \left[ c_0 + c_1 (\lambda_1 - 1) + \frac{\lambda_1}{4(1 - \lambda_1 x_0)^2} \right].
\]

This equation also works for arbitrary \( n > 2 \). The detailed derivation and the case of area compressible cytoskeleton can be found in Appendix B in the Supporting Materials and Methods.

**Constitutive model of RBC cytoskeleton with natural length distributions of spectrin**

When we derived the expressions of the shear stress and energy function, we applied a single value for the natural end-to-end length \( s_i \) in the unstressed state. Recent tomographic studies demonstrate that the resting end-to-end distance of spectrin is not single valued but distributed over some range \((18)\). In this case, we postulate that the energy of the skeleton and corresponding force resultants can be considered to be a superposition of networks with different resting end-to-end distances, weighted according to the frequency at which a given end-to-end distance occurs (at rest). In this case, the shear stress becomes

\[
\tau_{sk}^{el} = \sum_i \varphi_i \tau_{sk,i}^{el},
\]

where \( \varphi_i \) is the fractional occurrence of a given end-to-end distance \( s_{ij} \). Cryo-electron microscopy (cryo-EM) data \((18)\) show that for mouse erythrocytes, the initial length distribution is given as \( x_0 = [20, 30, 40, 50, 60, 70, 80, 90, 97] \) nm, with frequencies of \( f = [1, 17, 30, 38, 19, 17, 6, 1, 1] \). Then the fractional weight is \( \varphi = [0.00769, 0.13077, 0.23077, 0.29231, 0.14615, 0.13077, 0.046154, 0.00769, 0.00769] \). We can also write

\[
\mu_0 = \sum_i \varphi_i \mu_{0,i}, \quad \mu = \sum_i \varphi_i \mu_i,
\]

where \( \mu_{0,i} = (k_0 T \rho_0 / 3(\rho_0 s_{max,i})^2)(s_{max,i} - 9(s_{max,i} - 1)) + 4(s_{max,i}^3 - \lambda_{max,i}^3/4(s_{max,i} - 1)^3) \) \( \lambda_{max,i} = s_{max,i} \rho_0 \), and \( \mu_{i} \) is given in Eq. 12 for specific \( s_{0,i} \). Note that the maximal contour length \( s_{max} \) is assumed to be the same for all chains regardless of \( s_{0,i} \).

Recent efforts have successfully localized junctional complexes on the red cell membrane using super-resolution microscopy \((19)\), but because this approach requires sparse labeling, not all junctions are labeled, and interjunctional distances cannot be reliably determined. For example, the length distributions in this super-resolution imaging study were calculated as counts/\( \mu \text{m}^2 \), and the summation of the total counts in their figures gives \( \sim 110 \) junctions/\( \mu \text{m}^2 \). This is much lower than the value of \( \sim 730 \) junctions/\( \mu \text{m}^2 \) estimated from proteomic data \((13)\) and even significantly lower than the traditional number of \( \sim 270 \) junctions/\( \mu \text{m}^2 \) \((14)\).

**Analysis of micropipette aspiration**

**Closed form solution using an analytical model with the simplified constitutive model**

Using the simplifying approximation in Eq. 18, a closed form prediction for the aspirated projection length of the cell as a function of aspiration pressure can be obtained. The cell deformation is approximated as the formation of a cylinder plus hemispherical cap from an infinite membrane plane (Fig. 1 A: (1)). For each projection length, the corresponding meridional tension at the pipette tip \( \tau_{sk,tip} \) is determined, and this in turn is directly related to the predicted aspiration pressure \( P_{R_{tip}} \) by \( \tau_{sk,tip} = 2\pi r \tau_{sk,tip} \). In this way, a prediction for the projection length as a function of aspiration pressure is generated, in this case from the equilibrium equation in cylindrical coordinates of the flat membrane

\[
\tau_1^{sk} - \tau_2^{sk} + r \frac{d\tau_1^{sk}}{dr} = 0.
\]

In the limit as \( r \to \infty \), we take \( \tau_1^{sk} \to 0 \). In this case, the stress at the tip \( \tau_{sk,tip} \) can be obtained by integrating Eq. 22,

\[
\tau_{sk,tip}^{el} = \int_{R_p}^{\infty} \frac{2\tau_{sk}^{el}}{r} \, dr,
\]

where \( \tau_{sk}^{el} = (\tau_1^{sk} - \tau_2^{sk})/2 \). To evaluate the integral, we make the simplifying assumption that the cytoskeleton is laterally incompressible. From the definition of the principle stretch, we have \( \lambda_2 = r/r_0 \). In the area incompressible case, we can relate the instantaneous position of a material element \( r \) to its initial location in the undeformed plane \( r_0 \) by equating the areas:

\[
\pi r_0^2 = \pi (r^2 + 2L_p R_p - R_p^2) \Rightarrow \lambda_1 = r = R_p \sqrt{\frac{r^2 + 2L_p R_p - R_p^2}{r_0^2}} \Rightarrow\lambda_1 \approx \frac{r}{r_0} \sqrt{\frac{r^2 + 2L_p R_p - R_p^2}{r_0^2}}.
\]

At the lower limit of the integration, \( r = R_p \), we have \( \lambda_2 = \lambda_1 \) \( r = R_p \) \( \sqrt{2L_p R_p - R_p^2} \). With these simplifications, recognizing that \( (dr/d\lambda_1) = (\lambda_1 r / \lambda_2^2) \) and using the expression for the stress given in Eq. 18, we evaluate the integral (Eq. 23):

\[
\tau_{sk,tip}^{el} = \frac{4C_\beta}{3x_0} \left[ D_0(x_0) + D_1(x_0) \lambda_A + D_2(x_0) \ln \left( \frac{\lambda_A + 1}{2} \right) \right]

\[+ D_3(x_0) \ln \left( \frac{1 - x_0}{1 - x_0 \lambda_A} \right) \] + D_4(x_0) \ln \left( \frac{1 - x_0}{1 - x_0 \lambda_B} \right),
\]

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Microstructure-Based RBC Membrane Model

\[
D_1(x_0) = c_1,
\]

\[
D_2(x_0) = -\frac{x_0^2 + 1}{4(1 + x_0)^2(1 - x_0)^2} - c_1,
\]

\[
D_3(x_0) = \frac{x_0}{2(1 + x_0)^2(1 - x_0)^2},
\]

and

\[
D_4(x_0) = \frac{1}{4(1 - x_0)^2}.
\]

Because \(R_p \Delta P = 2 r_{1,tip}^s\), we have the aspiration pressure \(\Delta P\) vs. length \(L_p\) relation as

\[
R_p \Delta P = \frac{8c_{\beta,i}}{3x_0} \left[ D_0(x_0) + D_1(x_0)\lambda_L + D_2(x_0)\ln \left( \frac{\lambda_L + 1}{2} \right) \right. \\
+ D_3(x_0)\ln \left( \frac{1 - x_0}{1 - x_0\lambda_L} \right) + \left. D_4(x_0) \right].
\]

\[\text{(25)}\]

**Effect of natural length distributions of spectrin**

With \(\varphi_i\) as the fractional weight of contributions for the end-to-end distance \(s_{0,i}\), we have

\[
R_p \Delta P = \sum_i \left\{ \varphi_i \frac{8c_{\beta,i}}{3x_0} \left[ D_0(x_{0,i}) + D_1(x_{0,i})\sqrt{\frac{2L_p}{R_p}} \right. \\
+ D_2(x_{0,i})\ln \left( \frac{\sqrt{2L_p/R_p} + 1}{2} \right) \right. \\
+ D_3(x_{0,i})\ln \left( \frac{1 - x_{0,i}}{1 - x_{0,i}\lambda_L} \right) \right. \\
+ \left. D_4(x_{0,i}) \right].
\]

\[\text{(26)}\]

where \(c_{\beta,i} = k_B T p_{0,\lambda_{0,i}}/k(p/s_{0,i})\) and \(x_{0,i} = s_{0,i}/s_{max}\). Note that the maximal contour length \(s_{max}\) is assumed to be the same for all chains regardless of \(s_{0,i}\).

**Effect of adhesion energy between cell membranes and pipette walls**

In a previous study (8), we have provided evidence of an attractive energy between the cell membrane and the micropipette, \(\sigma_{m}\). The effect of such an attractive interaction is to shift the predicted curves for \(L_p/R_p\) as a function of \(\Delta P_{R_p}\) along the horizontal axis. Effectively, this transforms \(L_p/R_p = f(\Delta P_{R_p})\) to \(L_p/R_p = f(\Delta P_{R_p} + \sigma_{m})\). We introduce \(\sigma_{m}\) as a fitted constant for a given pipette on a given day of experiments.

**Effect of cytoskeletal area change**

We cannot calculate the distribution of cytoskeletal area change analytically because of mathematical difficulties, but if the cytoskeletal area change of

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**FIGURE 1**  (A) The analytical model for analyzing micropipette aspiration by assuming an infinite flat membrane outside the pipette is shown. (B) The ODE model for analyzing micropipette aspiration by assuming a biconcave shape outside the pipette is shown. A cross section of the axisymmetric cell contour used in the analysis showing coordinate directions is given. Key experimental parameters are the pipette radius \(R_p\) and the length of the aspirated membrane projection \(L_p\). The meridional distance along the surface is \(s\), and the radial coordinate is \(r\). The radius of the outer disk \(R_d\) is calculated to conserve total surface area. Integration proceeds from the tip of the projection and the center of the disk outside the pipette. The starting stretch ratios \(\lambda_0\) and \(\lambda_d\) are found such that the membrane stress resultant at the pipette tip \(r_{1,tip}^s\) is continuous and the total mass of the membrane-associated cytoskeleton is conserved.

where

\[
\lambda_L = \sqrt{2L_p/R_p},
\]

\[
D_0(x_0) = \frac{-1}{4(1 + x_0)^2(1 - x_0)^2} - c_1,
\]
Feng et al.

the flat membrane is assumed to be uniform, a modified analytical solution can be obtained as

\[ R_p \Delta P = \frac{8c_0}{3D} \left[ D_0' + D_1\lambda_L' + D_2' \ln \left( \frac{\lambda_L + 1}{2} \right) \right. \\
\left. + D_3\ln \left( \frac{1 - \lambda_L}{1 - \lambda_L D_4} \right) + \frac{D_4}{1 - \lambda_L D_4} \right] + 2T, \tag{27} \]

where detailed expressions for the coefficients can be found in Appendix C in the Supporting Materials and Methods.

**Numerical solution using an ODE model with the general constitutive model**

For the general expressions (Eqs. 5 and 6), we use a numerical approach to solve an ordinary differential equation (ODE) model to obtain predictions for the projection length as a function of aspiration pressure for a micropipette-aspirated biconcave cell (Fig. 1B). We denote the principal stretches and stress resultant in the meridional direction as \( \lambda_1 \) and \( \tau_1 \), and in the circumferential as \( \lambda_2 \) and \( \tau_2 \). By symmetry, we see that the principal stretch ratios at the tip of the membrane projection in the pipette \( \lambda_0 \), and the center of the disk opposite the pipette \( \lambda_d \) must be isotropic. For a given cell shape (projection length \( L_0 \), and outer biconcave disk diameter \( R_0 \)), we find values for \( \lambda_0 \) and \( \lambda_d \) that satisfy the conditions that the meridional tension at the tip of the pipette \( \tau_{1p} \) is continuous and the total mass of the membrane cytoskeleton is conserved (see Appendix A for details of this ODE model in the Supporting Materials and Methods).

**Experimental measurements**

The projection length of a portion of the red cell membrane aspirated into a micropipette was measured as a function of the aspiration pressure. Measurements were obtained on three different days using large (~0.85 \( \mu \)m inside diameter) and small (~0.56 \( \mu \)m inside diameter) micropipettes. Cells were obtained from human donors by simple finger stick under a protocol approved by the Office of Human Subject Protection at the University of Rochester. Cells were suspended in phosphate-buffered saline with 4% v/v of fetal bovine serum to avoid attachments between the cells and glass surfaces. Micropipettes were formed from glass capillaries using a Kopf microelectrode puller and a custom-made microurograph. Micropipettes were filled by capillarity with buffer matching the cell suspension buffer, then connected via a continuous, bubble-free water pathway to a water-filled manometer. Zero pressure was set by adjusting the height of the manometer reservoir until suspended particles were motionless in the pipette lumen. Aspiration pressures were applied by adjusting the height of the reservoir using a micrometer with a resolution of 0.01 mm. Cells were observed using an inverted microscope with a 100x oil immersion objective and monochromatic illumination (480 nm). The experiments were recorded using standard video, and the length of the projection as a function of aspiration pressure was measured from the recordings. For each day of experiments, approximately eight cells were measured using the same pressure sequence, and the projection lengths at each pressure were averaged.

**RESULTS**

We first present results using a single value of resting end-to-end distances \( s_0 \) for spectrin to illustrate the general behaviors of the model and then introduce a distribution of resting end-to-end distances and demonstrate the importance of having such a distribution to better approximate actual cell behavior.

**Parameter selection and stress-strain comparisons**

An important advantage of our approach is the ability to see how changing specific characteristics of the molecular network affect the behavior of the membrane in deformation. Some examples of this are shown in Fig. 2. In the native membrane, the orientation of the network elements appears to be random. Thus, an important question to address is how increasing the number of different molecular orientations in the resting state affects the model predictions. This is shown in Fig. 2A for a simple uniaxial extension (\( \tau_2 = 0 \)). For small forces and extension, even a very small number of initial molecular orientations provides a prediction consistent with more complex expressions, but for large extensions, significant differences in the predictions emerge (Fig. 2B). This implies that the number of different molecular orientations in the resting state only affects membrane behavior when molecular extensions approach their maximum. Estimates of accuracy for different numbers of molecular orientations are summarized in Appendix D in the Supporting Materials and Methods. For example, for \( n = 3 \), errors in the calculated stress exceed 5% when extensions are greater than 70% of \( \lambda_{max} \) whereas for \( n = 6 \), extensions can be up to 90% of \( \lambda_{max} \) before errors greater than 5% are observed. Similar results were obtained for purely shear deformation (constant skeleton area). As might be expected, results for isotropic expansion of the skeleton showed no dependence on the number of molecular orientations included in the calculations (data not shown). In these examples, the maximal end-to-end distance was set to 200 nm, the persistence length was 25 nm, \( s_0 = 75 \) nm, and the molecular density was 2200/\( \mu \)m².

Dependencies of the predicted stress resultants on molecular density \( \rho_0 \), persistence length \( \rho \), and resting molecular length \( s_0 \) are straightforward because these only appear in the coefficient \( \epsilon_\beta \), which multiplies the entire expression for the stress resultant (Eqs. 5 and 6). Thus, if the ratio of \( p/s_0 \) is doubled, the stress resultants for the corresponding stretch ratios are halved, and conversely, if the density of molecules is doubled, the stress resultants are doubled. The dependence of the model predictions on the maximal stretch ratio \( \lambda_{max} \) is more complex, but, generally, the stress resultant rises more quickly with extension for smaller values of \( \lambda_{max} \) and increases asymptotically as the extension approaches \( \lambda_{max} \) (Fig. 2, C and D). Interestingly, at higher extensions, normalizing the stretch ratio to \( \lambda_{max} \) collapses the prediction to a single curve (Fig. 2D). Similar behaviors are exhibited for pure shear deformation.

It is also of interest to understand how the resistance to deformation as reflected in the material constants \( \mu \) (shear modulus) and \( K \) (area modulus) vary with membrane deformation. We have already shown that the ratio of \( K/\mu \) is 2.0 for small deformations relative to the resting state. This ratio changes substantially, however, as the membrane deforms.
Indeed, both moduli vary significantly with membrane expansion. The variation in the area modulus $K$ is shown in Fig. 2F, and the variation in $\mu$ is shown in Appendix D (Fig. S7) in the Supporting Materials and Methods. Note that when the membrane is extended (increasing $l_1/l_2$), the resistance to dilation increases more rapidly because of the nonlinear increase in resistance to stretch as the maximal stretch ratio is approached.

Comparison of the stresses (Fig. 3A) and shear modulus (Fig. 3B) obtained using the full model (Eqs. 10 and 12) and the simplified formula (Eqs. 18 and 19) show good agreement over a wide range of extensions.

Effects of natural length distributions of spectrins

A recent study using tomographic imaging of intact red cells revealed that there is a distribution of end-to-end distances for spectrin molecules in the undeformed cytoskeleton (18). The reported distribution is reproduced in Fig. 4A. Even though large values of $s_0$ occur rarely, they can have a significant impact on stress-strain behavior at larger extensions because of the singularity in molecular force as a function of extension when molecular lengths ($s$) approach $s_{\text{max}}$. This is illustrated in Fig. 4B, where the dependence of the force resultant ($\tau_1$) on extension is shown for three different but similar distributions, truncated at different values for $s_0$. The distributions with larger values for $s_0$ exhibit the greatest strain-stiffening behavior. Contributions from each individual value of $s_0$ in the distribution are shown for both small (Fig. 4C) and large (Fig. 4D) extensions.

Comparison of the prediction with micropipette aspiration experiments: analytical models

We consider three versions of the analytical models in comparison to experimental measurements. In the first version, we assume a single value for $s_0$ and fit $s_0$ and pipette adhesion energy $\sigma_{ap}$ by least-squares regression with a fixed persistence length $p$ and a fixed maximal molecular length $s_{\text{max}}$. We plotted three fits in Fig. 5A. The best-fit values (black curve in Fig. 5A) were $s_0 = 59.25$ nm and $\sigma_{ap} = 10.17$ pN/\(\mu\)m for fixed $p = 25$ nm, $s_{\text{max}} = 200$ nm, and $p_0 = 2200$ molecules/\(\mu\)m$^2$. This version of the model does not show obvious hardening unless a small $s_{\text{max}} = 140$ nm and an extremely large $p = 60$ nm is used (red curve in Fig. 5A). This is due to the high density of spectrin we...
a net compression of the skeleton in the region of the membrane outside the pipette. In keeping with this finding, we postulate an average compression of up to ~0.65% in the membrane plane outside the pipette. We considered three cases with compressions of 0.0, 0.35, and 0.65%. With fixed \( p = 25 \text{ nm} \), we obtained \( s_{\text{max}} \) and \( \sigma_{\text{ap}} \) from the fit (Fig. 5 C). When the compression of the cytoskeleton outside the pipette is larger, we obtain smaller \( s_{\text{max}} \) (and larger \( \mu_0 \)). This implies that allowing redistribution of skeletal density to the membrane portion outside the pipette reduces the appearance of strain hardening (indicated by a downward curvature to the fitted curve) such that smaller values of \( s_{\text{max}} \) are required to achieve the level of strain hardening (downward curvature) exhibited by the data.

Comparison of the prediction with micropipette aspiration experiments: ODE model

We then apply the ODE model, described in Appendix A in the Supporting Material, to solve the micropipette aspiration numerically by considering the more realistic cell shape shown in Fig. 1 B and employing the full constitutive model (Eqs. 5 and 6) instead of the simplified one. In Fig. 6, A and B, we show the effects of varying the maximal molecular length \( s_{\text{max}} \) or the persistence length \( p \) on the predictions for micropipette aspiration experiments. The initial molecular lengths \( s_0 \) were distributed as shown in Fig. 4 A. We observe that both constants affect the slope of the predicted curve, implying that it is possible to compensate for changes in one parameter with changes in the other. On the other hand, although the persistence length \( p \) only changes the slope, the maximal molecular length \( s_{\text{max}} \) changes both the slope and the curvature of the predicted curve. Introduction of the adhesive energy between the cell membrane and the micropipette, \( \sigma_{\text{ap}} \), enables fitting of the data with a range of value pairs for \( p \) and \( s_{\text{max}} \). This is illustrated in Fig. 7, in which predicted curves based on three different pairs of values show comparable agreement with the data for each of the 3 days of experiments. In Fig. 7, A and C, the pipette diameter was 1.12 \( \mu \text{m} \), and in Fig. 7 B, the diameter was 1.7 \( \mu\text{m} \). For the fitting procedure, the density of molecules on the surface was set to 2200 molecules/\( \mu \text{m}^2 \), based on proteomic measurements indicating ~300,000 tetramers per cell (12,13) and using an average cell area of ~135 \( \mu \text{m}^2 \). The distribution of \( s_0 \) values was fixed according to Fig. 4 A. The value of \( s_{\text{max}} \) was set at a constant value (140, 160, and 200 nm), and least-squares regression was performed with two free parameters: \( p \) and \( \sigma_{\text{ap}} \). Depending on the value chosen for \( s_{\text{max}} \), different values for \( p \) and \( \sigma_{\text{ap}} \) were obtained. The value of \( \sigma_{\text{ap}} \) is subject to significant measurement uncertainty because although changes in \( L_p \) can be measured very accurately, uncertainty in the location of the pipette tip can lead to errors in its absolute magnitude, and these errors contribute directly to the value of \( \sigma_{\text{ap}} \). Therefore, changes in the value of \( \sigma_{\text{ap}} \) between different experiments
do not necessarily reflect differences that have physical significance. The value of the persistence length $p$, on the other hand, is of interest because it reflects a molecular property that contributes directly to macroscopic cell behavior. The relationship between the value chosen for $s_{\text{max}}$ and the best-fit value for $p$ is given in Fig. 7D, in which the corresponding initial shear modulus is shown for each pair of $p$ and $s_{\text{max}}$. The initial shear modulus $\mu_0$ increased from 5.9 to 15.6 pN/µm with increasing $s_{\text{max}}$. The goodness of fits as reflected by the sum of squared residuals in Fig. 7 for different values of persistence length and maximal length is summarized in Appendix E in the Supporting Materials and Methods.

**DISCUSSION**

Derivation of a constitutive model based on molecular parameters facilitates an understanding of how molecular attributes affect membrane deformability. Key molecular characteristics include the density of spectrin on the membrane in the resting state $\rho_0$, the molecular persistence length $p$, the molecular lengths in the resting network $s_0$, and the maximal molecular extension $s_{\text{max}}$. In the model, the density and the persistence length appear explicitly only in the coefficient $c_p$, which is directly proportional to the magnitude of the stress for a given deformation. Consequently, the membrane modulus is expected to increase in direct proportion to spectrin density and inversely to the persistence length. In a previous study, a direct proportionality between the membrane shear modulus and the density of spectrin on the membrane has been documented (20), and this is consistent with the formulation we have developed here.

Best-fit values for the persistence length $p$ in our experiments range from 10 to 50 nm. The highest values correspond to the lowest values for $s_{\text{max}}$ (Fig. 7D), the highest degree of strain hardening, and the smallest initial shear modulus ($\sim$5.9 pN/µm). Previously reported values for persistence length fall into the lower end of this range. For single molecules or intact cytoskeletons separated from the bilayer in solution, estimates of persistence length range from 10 to 25 nm (21,22). The persistence length for molecules constrained to remain near the membrane surface might be expected to be slightly smaller or up to 25% larger, based on estimates of the effects of confinement on this characteristic (23). Although the highest values we obtain are above this range, this might be expected given the constraints on spectrin conformation because of multiple membrane attachments. For an intermediate value of $p$ around 25 nm, the fitted $s_{\text{max}}$ is around 145 nm and the corresponding initial shear modulus $\mu_0 = 9.37$ pN/µm. For the smallest value of $p$ around 8.5 nm, the fitted $s_{\text{max}}$ is around 200 nm and the corresponding initial shear modulus $\mu_0 = 15.6$ pN/µm, but this case shows very limited hardening for the size of the aspiration lengths studied here.

**Strain hardening and the distribution of resting molecular lengths**

An important characteristic of the red cell cytoskeleton is strain hardening. In addition to evidence based on determination of the shear modulus using techniques that impose different magnitudes of strain, the pipette aspiration data reported here also show evidence of this in a downward curvature of the data for larger extensions (Fig. 7). Strain hardening is expected for models based on worm-like chain theory because these molecules become asymptotically stiff as they approach their maximal extension. However, the magnitude of the stretch ratios at which this behavior occurs

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**FIGURE 4** Effect of natural length distributions of spectrin. (A) Distributions of natural length from cryo-EM measurements (18) are shown. (B) Stress resultant $\tau_1$ as a function of stretch for two distributions and a single natural length is given. (C) The contributions of each natural length to the total stress resultant $\tau_1$ are shown. (D) The same as (C) is shown, but for larger stretch. Values for the other parameters used in the calculations: $\rho_0 = 2200/\text{µm}^2$, $p = 25$ nm, $s_{\text{max}} = 200$ nm. To see this figure in color, go online.
depends critically on the ratio of $s_0$ to $s_{max}$. Values for the fully extended lengths of spectrin tetramers ($s_{max}$) are based on rotary-shadowed electron micrographs, which indicated a maximal end-to-end distance of 180–200 nm (24,25). The resting length $s_0$ is typically calculated from estimates of spectrin density and the relationship between density and $s_0$ in triangular networks: $\rho_0 = 2\sqrt{3}/s_0^2$. It is important to appreciate the implications of recent proteomic and ultrastructural findings on these values (12,13,18). Until recently, estimates of the number of spectrin molecules on the cell surface were based on density analysis of polyacrylamide gels, which put the number of spectrin tetramers on the surface of a cell at 80,000–120,000 (14). This corresponds to a density of 600–900/μm² and implies that $s_0$ ranges from 60 to 75 nm.

In relation to strain hardening, the value of $s_0 = 40$ nm implies that maximal molecular extensions in a network could approach a stretch ratio of 4.5–5.0 before significant worm-like stiffening would be observed. In this case, molecular models based on uniform networks would not predict the downward curvature that is evident in the micropipette

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**FIGURE 5** Comparison of the analytical model predictions with experimental data. (A) Fits with one global natural length without area change are shown. (B) Fits with distributed natural lengths (Fig. 4 A) without area change ($\rho_0 = 2200/μm²$) are shown. (C) Fits with distributed natural lengths (Fig. 4 A) with an assumed uniform area change $s_0$ on the flat membrane outside the pipette ($\rho_0 = 2200/μm²$) are shown. Dashed curves show the predictions beyond the experimental range. However, it should be noted that such lengths are not accessible experimentally because at longer extensions, folds begin to appear in the membrane, resulting in membrane shapes that are not amenable to analysis. Error bars for experimental data are shown as blue vertical lines. To see this figure in color, go online.

**FIGURE 6** Sensitivity of model predictions to parameter values. (A) Effect of persistence length $p$ is shown. (B) Effect of contour length $s_{max}$ is shown. Increasing either $p$ or $s_{max}$ leads to increases in the length of the projection at a given aspiration pressure.
aspiration data. This dilemma is solved by recent tomo-
graphic evidence that end-to-end distances of tetramers in
the resting state are not uniform (18). Accounting for this
distribution, our molecular-based constitutive model leads
to predictions of strain stiffening for extensions of
\(\frac{C}{2.0}\), and results in model predictions for pipette aspiration
behavior much closer to what is observed. Additionally, if
one reverses the calculation and estimates resting state den-
sity from the reported distribution of molecular lengths (us-
ing the relationship
\(r_0 = \frac{2}{\sqrt{3}}s_0\)
for triangular networks and performing a weighted sum over the values of \(s_0\)), one obtains a density of 1740/\(m^2\), a value in excellent agree-
ment with densities based on proteomic results. This last
point is strong validation for using both the distributed
values for \(s_0\) and the higher densities of spectrin indicated
by the proteomic measurements.

**Literature values for shear modulus**

Values reported for the red cell membrane shear modulus in
the literature differ significantly. For example, in micropip-
ette aspiration experiments, in which the cell experiences
moderate deformation, the shear modulus was measured
as values (6–9 pN/\(m\mu\)) (26) or 4.2 pN/\(m\mu\) (27). The shear
modulus measured using optical tweezers to stretch red
blood cells and produce small deformations was 2.5 pN/
\(m\mu\) (28,29), although some other measurements using opti-
cal tweezers gave a shear modulus of 5.7 pN/\(m\mu\) (30), 11.1–
30 pN/\(m\mu\) (31), or 200 pN/\(m\mu\) (32). In addition to direct
measurements of shear modulus, computational modeling
studies also required a small shear modulus (\(\leq 2.5\) pN/
\(m\mu\)) to predict the resting biconcave shape and the correct stoma-
tocyte-discocyte-echinocyte shape transition (4,33,34). The
ability of different constitutive laws to match experimental
measurement are compared in several studies (6,35), but
most do not account for strain-hardening behavior. Esti-
mates based on membrane fluctuation measurements
(36,37) or tank-treading phenomena (5,38,39), both of
which involve relatively small deformations, also tend to
produce smaller values. In one interpretation of thermal
fluctuation experiments, the shear modulus appears to be
very small or almost zero (36,37), whereas experiments on
tank treading give a range of shear moduli from 2.81 to
8.95 pN/\(m\mu\) depending on the deformation (5). Although
differences in experimental approaches and associated meas-
urement uncertainties as well as different approximations

**FIGURE 7** Comparison of the ODE model predictions with experimental data. Three different experiments are shown. (A) Experiment 1 with \(R_p = 0.56\) \(m\mu\). (B) experiment 2 with \(R_p = 0.85\) \(m\mu\), and (C) experiment 3 with \(R_p = 0.56\) \(m\mu\) are shown. (D) Given a value for \(s_{\text{max}}\), there is a unique value of \(p\) that
matches the slope of the data for all three experiments. The relationship between \(p\) and \(s_{\text{max}}\) is shown as the solid line:
\(p = c_1(6 - 9c_2/s_{\text{max}} + 4c_2^2/s_{\text{max}}^2)/(s_{\text{max}}/c_2 - 1)^2\), where \(c_1 = 0.0275\) and \(c_2 = 101.85\). The corresponding initial shear modulus for experiment 1 is shown at each point. Dashed
curves show the predictions beyond the experimental range. Error bars for experimental data are shown as blue vertical lines. The database can be found at:
https://datadryad.org/stash/share/6gbOC1on3URHVUT55ACqW4W3PgB4TDKerVBDk-7FWKMW. To see this figure in color, go online.
and methods of interpretation likely account for much of the differences in literature values, the strain stiffening captured using the microstructure-based model we present here may also help explain those differences. The value of the modulus in Eq. 21 approximately triples when the stretch ratio increases from 1.0 to 1.4 for \( s_{\text{max}} = 140 \text{ nm} \) (Fig. 8). Consistent with this, tank-treading experiments showed that the shear modulus can be tripled when the stretch ratio increases from 1.2 to 1.64 as shown in Fig. 8, although the absolute value of the shear modulus is at least twofold lower than those extracted from micropipette aspiration using this model.

The original analysis of micropipette aspiration, which did not consider strain hardening or cytoskeletal density changes, gave a prediction for the relationship between projection length and aspiration pressure: 
\[
\frac{R_p}{D} = \frac{\mu}{C_0} \left[ \frac{2L_p}{R_p} - 1 + \ln\left( \frac{2L_p}{R_p} \right) \right].
\]

Using this approach, the calculated shear modulus was in the range of 6–9 pN/\( \mu \text{m} \) (1,26). Our current analysis shows that failing to account for strain hardening results in an overestimate of the initial shear modulus because it reflects an “averaged” value of the increasing shear modulus with deformation. On the other hand, our analysis also shows that failing to account for local changes in skeletal density leads to underestimation of the shear modulus because the constraint of constant density leads to higher shear stresses at the same macroscopic deformation (projection length) (Fig. 5 C). Therefore, these two effects tend to compensate for each other such that the initial shear modulus values we obtain using our current model (5.9–15.6 pN/\( \mu \text{m} \)) are not much different than early estimates (6–9 pN/\( \mu \text{m} \)).

Values obtained using the simplified models

The simplified models we have derived vastly reduce the computational costs for analyzing experimental data. However, this simplicity comes with some loss of consistency for the molecular coefficients. The approximate model without cytoskeletal area change shows stronger strain stiffening and appears to match two of the three experimental data sets with high accuracy (Fig. 5). It is important to note, however, that maintaining constant cytoskeletal density introduces an important constraint on the deformation, and this accounts for the increased strain stiffening for this model. Indeed, in Fig. 5 C, increasing the assumed cytoskeletal area change for the simplified model results in less hardening. From other experimental evidence, it is clear that local changes in density do occur when the membrane is deformed.

**Dynamic remodeling, prestress of the spectrin network, and deformation mechanisms of spectrin**

Prior studies considering the molecular basis of red cell membrane elasticity have introduced important potential attributes of the network that we do not consider here, namely dynamic remodeling of the network and the possibility of prestress of the skeleton in the resting state. For example, Fai et al. built a cytoskeletal network model based on cryo-EM tomography (10,18) and demonstrated that faster cytoskeletal reorganization leads to more irreversibly broken spectrin tetramers and a smaller dimensionless tank-treading frequency for a cell undergoing tank treading in shear flow. They also found that when the cell is placed under repeated strains, cytoskeletal dynamics may play a protective role by allowing spectrin tetramers to disconnect before they would break. Zhu and Asaro showed that the unfolding of spectrin can play a significant role in strain-softening of a spectrin network (40). Although these concepts are important considerations, Discher and colleagues report that there was no change in spectrin distribution in their fluorescence-imaged microdeformation experiments over periods of 30 min (15), indicating that if remodeling does occur, it does not have a substantial effect on the distribution of strain in deformed membranes over extended times. Therefore, we believe that our decision not to consider dynamic remodeling in micropipette experiments with much smaller extensions and shorter duration is justified.

It has long been recognized that the red cell membrane is a composite structure and that both the spectrin network and the lipid bilayer contribute to its overall mechanical behavior (1). For the analysis presented here, the bilayer is not expected to make substantial contributions, except to constrain the membrane to a constant surface area, because it offers no resistance to shear deformation, and its resistance to bending is small compared with the force results generated as a result of the network deformation during pipette aspiration (1). The composite nature of the red cell membrane, coupled with the very high resistance of the bilayer to surface dilation or compression, raises...
the theoretical possibility that the skeleton itself may be slightly stretched or slightly compressed at rest, with the bilayer providing a counterbalance to make the net stress in the membrane zero at rest (41,42). The concept of a prestress in the spectrin network was introduced by Discher and colleagues to obtain agreement between their molecular-level red cell network simulations and experiments (9,43). More recently, we have identified a small adhesive energy between the membrane and the pipette surface that likely accounts for the discrepancy they observed for aspirations of biconcave cells (8), making the postulate of a skeletal prestress unnecessary for this case. (Predictions of the network density distribution in aspirated cells with long projections were also improved by inclusion of prestress, but this has not yet been addressed using the microstructure-based model we present here). The possibility of skeletal prestretch was also posited by Turlier et al. in a theory to explain how low-frequency cell fluctuations might be driven by spectrin because of changes in its phosphorylation state (44). Since then, a significant presence of myosin has been documented in red cells (45), which could provide an alternative explanation for the nonequilibrium, low-frequency fluctuations they observed. It could also be argued that long-term stress relaxation should cause network stress resultants to relax to zero over time. In summary, there is no clear evidence to confirm or refute the possibility that prestress may exist.

We consider each spectrin tetramer as a worm-like chain entropic spring in this study. Alternate models of spectrin behaving as a straight helical spring (46) or a Chinese finger trap (47), have been proposed. These models are based on electron micrographs of purified spectrin tetramers or minispectrins. Unfortunately, force-length relationships have not been derived for these cases. If such a force-length relationship existed, it would be straightforward to use the approach described here and incorporate it into our model by changing the potential in Eq. 2. Indeed, a similar approach to the one used here has been used with a model for spectrin as a helical spring to predict membrane behavior (8). That said, there are many experimental images of native spectrin in the actual RBC from quick-freeze, deep-etch, rotary replication procedure (48) or cryo-EM, in which the molecules appear to adopt random worm-like configurations (18), supporting the use of the worm-like chain potential not only here but in many theoretical and computational studies (10).

We note that the stiffening of the membrane at large extensions is reflected in both the thermodynamically defined shear modulus, \( \mu \), and the area modulus, \( K \), both of which increase substantially with membrane dilation. It is also of interest that the value of the ratio \( K/\mu \) also varies substantially from its resting value of 2.0, increasing with compression but decreasing for moderate extensions before increasing at very large extensions. Observations of the distribution of skeletal density in the projections of micropipette-aspirated cells indicated that this ratio should be \( \sim 2.0 \), but a definitive value was not provided (15). This estimate of the \( K/\mu \) ratio appears more or less consistent with the predictions of our model, but more detailed calculations would be required to draw a firm conclusion.

**CONCLUSIONS**

The work presented here makes several impactful contributions to our understanding of the physical basis of red blood cell membrane elasticity. First, we have derived a constitutive expression based on molecular properties that allows for local density changes in the deformed red cell cytoskeleton. We have generalized an expression for \( \mu_0 \), the shear modulus for a network near the undeformed shape, previously derived for a network with just three chain orientations \( n = 3 \) (7), and have shown that it is valid for arbitrary \( n \geq 3 \). Furthermore, we have developed the first, to our knowledge, molecularly based elastic model of the red blood cell membrane that accounts for both higher densities of spectrin indicated by proteomic analyses and distributed values of the resting molecular lengths obtained from electron-micrographic tomography. We have shown that for higher spectrin densities indicated by proteomics, accounting for a distribution of resting molecular lengths is essential for reproducing strain-hardening behavior exhibited by the red cell membrane in experiments. We have also obtained a relationship between the values for the maximal extended length of the spectrin tetramer and its persistence length that are consistent with membrane behavior in micropipette aspiration experiments. In addition, an analytical form of the micropipette aspiration with the simplified version of the constitutive law is derived. We find that the model exhibits hardening behavior and can help explain discrepancies found in the literature. In addition, this model can be also used to model other two-dimensional networks of flexible polymers with distributions of orientations and natural lengths, such as nuclear lamins.

**SUPPORTING MATERIAL**

Supporting Material can be found online at [https://doi.org/10.1016/j.bpj.2020.10.025](https://doi.org/10.1016/j.bpj.2020.10.025).

**AUTHOR CONTRIBUTIONS**

Z.P. and R.E.W. designed the research. Z.F., R.E.W., and Z.P. developed the mathematical models. R.E.W. performed the experiments. Z.F., R.E.W., and Z.P. analyzed the data. All authors contributed equally to writing the article.

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