Cytoskeleton influence on normal and tangent fluctuation modes
in the red blood cells

S.B. Rochal†‡ and V.L. Lorman‡

†Laboratoire de Physique Theorique et Astroparticules, CNRS - Universite Montpellier 2, Place
Eugene Bataillon, 34095 Montpellier, France

‡Physical Faculty, Rostov State University, 5 Zorge Street, 344090 Rostov-on-Don, Russia

(March 31, 2022)

Abstract

We argue that the paradoxal softness of the red blood cells (RBC) in fluctuation spectra experiments is apparent. We show that the effective surface shear modulus $\mu_s$ of the RBC obtained from fluctuation data and that measured in static deformation experiments have the same order of magnitude. A simple micromechanical model of the RBC developed for this purpose accounts for the influence of a finite-thickness cytoskeleton on the fluctuations of the composite membrane-cytoskeleton system. The spectrin network cytoskeleton with the bulk shear modulus estimated as $\mu \approx 105 \div 165$ Pa contributes to both normal and tangent fluctuations of the system and confines the fluctuations of the lipid membrane. The ratio of mean square amplitudes of the RBC normal and tangent fluctuations $\langle X_n^2 \rangle / \langle X_t^2 \rangle$ calculated in the frame of the model is 2-3 orders of magnitude smaller than it is in the free membrane with the same bending and shear moduli.

87.68.+z, 83.60.-a, 87.16.-b, 87.17.-d
The mechanical properties and fluctuation spectrum of the living cell depend strongly on the coupling between its fluid phospholipid membrane (ME) and stiff cytoskeleton (CS) composed of the cross-linked biopolymer networks [1-4]. In red blood cells (RBC), investigated intensively by physicists since many years [5-7], the coupling between the lipid bilayer and the spectrin filaments network provides the ME with viscoelastic properties. In addition to its bending modulus $K \approx 10^{-19} - 10^{-20}$ J, the RBC ME acquires two dimensional (2D) static shear modulus revealed by micropipette suction [5] or by other types of static deformation response. Measurements of thermally activated shape fluctuations [8] refine the picture and give the access to the dynamic properties of RBC. The basic theoretical model [9] explaining both statics and dynamics of RBC considered the ME as practically independent on the CS influence, but with strongly variable 2D shear modulus. Namely, thermally excited surface undulations of RBC (the cell flickering) [8] was described as a motion of a fluid ME with $\mu_s \approx 0$. Thus the free ME model [9] has established the opinion that RBC combine paradoxally liquid-like and solid-like mechanical properties, with solid-like behavior being manifested only during large-scale shape changes [9-10]. However, the modern microrheological technique [e.g. 11-12] has shown that mechanical behavior of RBC satisfies Hook’s law in a broad region of deformation [13]. This result is incompatible with both deformation scale separation principle and non-linear shear modulus behavior resulting from the model [9].

As it will be shown later apparent softness of RBC obtained from the fit of thermal fluctuation spectra by the free ME model is related to the simplified form of its elastic energy. In [9] it is reduced to the ME bending and shear terms only. In recent theoretical works [14-17] it was proposed to take into account the influence of viscoelastic CS properties on the coarse-grained mechanics of the composite ME-CS system. Energy of surface tension and especially the term responsible for the ME confinement were introduced in the system’s Hamiltonian [16-17]. Resulting model clarified the effective bending modulus behavior during normal fluctuations. Besides, it demonstrated the physical factors which limit normal fluctuations of the ME. By contrast, model [16-17] could not explain the main RBC paradox.
the apparent change of its shear properties from the solid-like to the liquid-like ones. In
approach [16-17] the CS was treated as an infinitely rigid shell (i.e. with an infinite shear
modulus) making impossible the calculation of the effective shear modulus for the ME-CS
structure. Infinite rigidity of the CS prevents any tangent fluctuation in the model, and
thus does not allow to compare its predictions with the results of the free ME model [9].
Note that the conclusion about liquid-like behavior of RBC was obtained in [9] from the
calculation of the ratio $<X_n^2> / <X_t^2>$ of mean-square amplitudes of normal and tangent
fluctuations (see Fig. 1). To solve the problem authors of Ref. [18] proposed to consider the
dependence of $\mu_s$ value on the wave vector. In their model $\mu_s$ modulus tends to zero only
for the fluctuations with wavelengths smaller than the mesh size of the spectrin network.
Nevertheless, the $<X_n^2> / <X_t^2>$ ratio has not been calculated.

In the present work we propose more realistic continuum model of the composite ME-
CS system which brings a solution to the paradoxal problem of the RBC apparent softness
in thermal fluctuation measurements. We show that the 2D shear modulus of the RBC
obtained from the fluctuation data and that measured in static deformation experiments
have the same order of magnitude $\mu_s \approx 6 \times 10^{-6}$ N/m. For that purpose, in addition to the
ME confinement, we take into account the contribution of normal and tangent fluctuation
modes of a finite-thickness CS to the surface free energy of the ME-CS system. These
modes, determined in the simplest case of incompressible CS by its bulk shear modulus $\mu$
only, are rapidly attenuated in the cell interior. Normal CS fluctuations modify the ME
bending properties and tangent fluctuations supply the composite system with a finite 2D
shear modulus $\mu_s$. Mean-square amplitudes of normal and tangent fluctuations of the system
are calculated in the framework of the proposed model. They are perfectly consistent with
the RBC flicker spectroscopy data [8,19] but their ratio $<X_n^2> / <X_t^2>$ is 2-3 orders
of magnitude smaller than that attributed in [9] to the solid-like ME. This result shows
that the RBC fluctuational softness is apparent and its shear properties in both static and
dynamic experiments are the same.

The material constants for these estimations were obtained by a double-step numerical
fit of the effective bending rigidity dependence on the wave vector $q$ [19]. The same type of experimental data is frequently used in the theoretical estimations of the RBC mechanical properties [16,17,20]. The data being obtained using classical Fourier transform, the models are usually formulated in the framework of plane geometry. We also follow this approach but limit the region of its applicability. A model which does not take into account the curvature of the system is valid [14-17] only for a treatment of fluctuations with the wavelengths $\lambda << R$, where $R \approx 4 \mu m$ is the effective RBC radius. The description of the ME-CS system motion is performed then in terms of continuum mechanics. This approximation is favored by the fact that the attachment points between the spectrin network and the bilayer are rather rare and their area is negligible (Fig. 1). Besides the actin nodes there exist additional ME-CS connections through ankryn complexes distributed randomly. Together with defects and dynamic structural rearrangements in the spectrin network they contribute to the entropic elasticity of the RBC CS and justify the continuum description. Finally, we consider the fluctuation wavelength region for which the influence of active processes is reduced to the increase in the effective temperature [21-22].

The free energy of normal thermal fluctuations of a ME-CS system contains the fluctuation energy of a flat ME and the CS contribution $W$:

$$F = \int dS \left( \frac{1}{2} K (\Delta u^m)^2 + \frac{1}{2} \sigma (\nabla u^m)^2 + \frac{1}{2} \gamma (u^m)^2 + W(u^c) \right).$$  \hspace{1cm} (1)

Here $u^m$ is a normal displacement of the ME, $K$, $\sigma$ and $\gamma$ are the coefficients of bending, surface tension and pinning of the ME, respectively; $u^c$ is a displacement field of the CS.

The ME fluctuation energy (first three terms in Eq. (1)) is limited to the sum of bending, surface tension and ME confinement energy contributions. In a plane geometry adopted in the present work bending fluctuations are independent on stretching and the energy due to the bending-stretching coupling can be omitted, though in a general system with curvature it cannot be neglected [15,23,24]. The CS is considered as an incompressible viscoelastic plate (Fig. 1) with the thickness $h \approx 0.03 \mu m$ and with a finite 3D shear modulus $\mu$. The last term in (1) expresses the surface density of the CS fluctuation energy:
\[ W = \int_0^h dz \mu \epsilon_{ij} \epsilon_{ij}^* \]  

(2)

where \( \epsilon_{ij} = \frac{1}{2} \left( \frac{\partial u_{ij}^e}{\partial x_j} + \frac{\partial u_{ij}^e}{\partial x_i} \right) \) is the CS strain tensor, \( u_{ij}^e = u_x^e, u_y^e, u_z^e \) and \( x_i = x, y, z \); and \( z \) is directed perpendicular to the ME-CS interface. Due to the small Reynolds number of the system the motion of both ME and CS is overdamped. Thus the state of CS is determined from the motion equation of an incompressible medium with neglected inertia terms:

\[ \nabla p = \mu \Delta u^e + \eta \Delta \dot{u}^e \]  

(3)

with \( p \) standing for pressure, \( \eta \) for the CS viscosity and \( \dot{u}^e \) for the CS velocity field. In a more general case of a compressible isotropic medium the energy (2) would have two terms related to two independent elastic Lame coefficients \( \lambda \) and \( \mu \). The equation of motion of a compressible isotropic medium would contain the term \( \lambda \ \text{grad div } u \), where \( u \) is a displacement field. This term stands for the density of forces of isotropic deformation. In the incompressible medium \( \text{div } u = 0 \) and \( \lambda \) tends to infinity. As it is shown in [25] the limit value of the term \( \lambda \ \text{grad div } u \) in the incompressible medium is equal to \(-\nabla p\). This leads to the motion equation of the incompressible medium in the form given by Eq. (3).

Thermally excited CS fluctuation modes are rapidly attenuated in the direction perpendicular to the ME plane. The solution of Eq. (2) compatible with bending fluctuations of the ME and satisfying \( \text{div } u^e = 0 \) condition describes the mode of this type:

\[ u_x^e = -i U_q qz \exp(-qz + iqx); \quad u_y^e = U_q(1 + qz) \exp(-qz + iqx); \quad u_z^e = 0; \]  

(4)

\[ p = -2iq U_q (\eta \omega + i \mu) \exp(-qz + iqx); \]  

(5)

\[ \dot{u}^e = -i \omega u^e. \]  

(6)

The wave vector of fluctuations in (4-6) is chosen along the \( x \) axis, \( U_q \) expresses the amplitude of normal displacements at the ME-CS interface \( z = 0 \) (see Fig. 2). All tangent displacements in the mode of this type vanish at the interface.

In the present work we consider the physical situation, where at the interface the CS velocities \( \dot{u}^e \) and displacements \( u^e \) coincide with those of the ME: \( \dot{u}^e|_{z=0} = \frac{\partial u^m}{\partial t} \) and
$u^c|_{z=0} = u^m$. In a more complicated case of ME-CS mutual gliding, only the normal components of both velocity and displacement fields are preserved at the interface and a viscous drag influences tangent fluctuation modes. However, in the plane geometry approximation this does not affect the normal fluctuation modes (which are of primary interest in the RBC model) and maintains the relation $u^m = U$, where $u^m$ and $U$ are the normal displacement of ME and CS, respectively.

Finally, the contribution $W_q$ of the CS to surface fluctuation energy (1) is obtained by substitution of bending displacement field (4) into elastic energy (2) and following integration over the CS thickness:

$$W_q = \mu q |U_q|^2 \left(1 - \exp(-2qh)(2q^2 h^2 + 2qh + 1)\right).$$  \hfill (7)

After development of free energy functional (1) into Fourier series we get the total energy of normal ME-CS fluctuations in the form:

$$E = S \sum_q E(q)|U_q|^2$$  \hfill (8)

suitable for analysis of the RBC fluctuation spectrum [8,19]. Function $E(q)$ is expressed as

$$E(q) = K q^4 + \sigma q^2 + 2W_q/|U_q|^2 + \gamma.$$  \hfill (9)

The sum in Eq. (8) runs over all possible $q$ in a square plate with the side equal to $\pi R$; the interface area $S$ is taken to be $(\pi R)^2$. Note that in the limit $qh \to 0$ CS fluctuations simply renormalize the ME bending rigidity: $W_q \approx 4\mu h^3 |U_q|^2 q^4/3$; and in the limit $qh \to \infty$ CS fluctuation wave transforms into the wave of normal fluctuations on the surface of a semi-infinite medium: $W_q \approx \mu q |U_q|^2$. Due to a strong exponential attenuation of solution (4-6) with the depth $z$ the last approximation is suitable in a wider region $q \geq h$.

The authors of [19] give the fluctuation spectra of RBC as the dependence of $-\log < |U_q|^2 > q^4$ on the wave vector $q$ in the wavelength region from 0.25 $\mu m$ to 2 $\mu m$. They stress also that the region of fidelity of their results does not exceed 0.5 $\mu m \leq \lambda \leq 1 \mu m$ limits. Long-wavelength limit is determined by the validity of the plane geometry approximation
(used in [19] for the data processing). Lack of fidelity in the short-wavelength region is related to the experimental technique limitations. Therefore, we performed the fit of the RBC fluctuation spectrum in the fidelity region only. Using equipartition \( E(q) \propto |U_q|^2 \) one can fit the expression \( q^4/E(q) \) proportional to the experimental value \( q^4 < |U_q|^2 > \), and usually related to the effective bending rigidity [16,17,20]. Double-step least-square fit (Fig. 3) of relative values of \( q^4 < |U_q|^2 > \) leads first to the expressions of the RBC material constants in function of bending modulus \( K \). The inset in Fig. 3 shows the relative contributions of the bending, surface tension, CS fluctuation and confinement energies to the total mode energy \( E(q) \) for one of the three cells studied in [19]. Similar relation between different contributions exists for two other cells. Then, taking into account the average value \( K = 3.4 \times 10^{-20} \) J estimated in [19] we obtain the absolute values of the constants: \( K^{(1)} \approx 4.9 \times 10^{-20} \) J; \( \sigma^{(1)} \approx -5.9 \times 10^{-6} \) N/m; \( \gamma^{(1)} \approx 3.1 \times 10^8 \) N/m\(^3\); \( \mu^{(1)} \approx 148 \) N/m\(^2\); \( K^{(2)} \approx 2.2 \times 10^{-20} \) J; \( \sigma^{(2)} \approx -2.3 \times 10^{-6} \) N/m; \( \gamma^{(2)} \approx 1.8 \times 10^8 \) N/m\(^3\); \( \mu^{(2)} \approx 105 \) N/m\(^2\). The fit of experimental data [19] for the third cell (not shown in Fig. 3) results in \( \mu^{(3)} \approx 165 \) N/m\(^2\). The found value of the CS 3D shear modulus \( \mu \) is in a good agreement with an effective spring constant of spectrin filament \( \approx 4 \times 10^{-6} \) N/m in 30 nm thick CS. The effective value of the 2D shear modulus \( \mu_s \) can be estimated in the simplest way as \( \mu_s \approx \mu h = (3.2 \div 5) \times 10^{-6} N/m \).

Let us now show that to explain the RBC behavior in thermal fluctuation experiments we do not need to suppose vanishing of its effective 2D shear modulus \( (\mu_s \approx 0) \). To do this we reexamine two main arguments of the free ME model [9] which have lead to the conclusion about negligible \( \mu_s \) value and, then reanalyze the same experimental data in the framework of the composite ME-CS model developed in the present work.

The arguments developed in [9] can be resumed qualitatively in the following way: 1) The ratio of mean-square amplitudes of normal and tangent fluctuations \( < X_n^2 > / < X_t^2 > \) in the model [9] has the order of magnitude \( \sim \mu_s R^2 / K \). 2) Thermal fluctuation experiments show that the RBC thickness fluctuation profile has the peak near the rim of the cell [8]. Due to the RBC shape the main contribution to the thickness fluctuations near the rim comes
from tangent fluctuations (see Fig. 1). Thus, in a system with $\mu_s \approx 0$ and, consequently, with strong tangent fluctuations, the peak should find itself near the rim. With the value of $\mu_s \approx 6 \times 10^{-6} \text{N/m}$ known from static experiments the model [9] gives the $< X_n^2 > / < X_t^2 >$ ratio 2-3 orders of magnitude greater than the experimental one. The discrepancy is then attributed to the high value of $\mu_s$ and to preserve the model the effective 2D shear modulus is considered to be vanishing.

Estimation of the $< X_n^2 > / < X_t^2 >$ ratio in the framework of the present model gives however quite different result which is consistent with the static $\mu_s$ value. The main difference with respect to the model [9] is determined by the contribution to total energy (8) from the CS, both from its fluctuations and from the ME confinement. Mean-square amplitudes of normal and tangent fluctuations of the composite ME-CS system can be, in principle, calculated using equipartition theorem. To avoid however, a cumbersome discussion of different mode polarizations for tangent fluctuations we prefer to illustrate the results in the way proposed in [26] and based on the fluctuation-dissipation theorem. Mean-square amplitudes of normal and tangent fluctuations are then determined by static normal and tangent response functions, respectively [26,27]:

$$< X_i(0)X_i(0) > = K_B T \alpha_i(0),$$

(10)

where response $\alpha_i(\omega)$ defines amplitude $A_i(\omega)$ of ME particle motion under periodic external force $F_i = F_i^0 \exp(-i \omega t)$ application: $\alpha_i = A_i/F_i^0$. The values of responses at zero frequency, and consequently, the amplitude of mean-square fluctuations are independent on the system dissipative properties. The responses of a flat ME have been discussed in detail in [14,15]. Using the same formalism we obtain normal response $\alpha_n(0)$ of the composite ME-CS system in the form:

$$\alpha_n(0) = \int_{q_{\min}}^{q_{\max}} \frac{q dq}{2\pi E(q)},$$

(11)

where $E(q)$ is defined by Eq. (9). The minimal wave vector $q_{\min}$ of fluctuation which contributes to response (11) is determined by the RBC finite size: $q_{\min} = 1/R$. Upper cut-off $q_{\max}$ is defined [27] by the fact that the number of fluctuation modes in the system is finite.
In the lipid ME it is determined by the total number $N$ of lipid molecules: $q_{\text{max}} = \sqrt{N} q_{\text{min}}$. In such a case the number of normal fluctuation modes is equal to $N$.

The expression for the tangent response $\alpha_t(0)$ is obtained along the same line. In the general case the function $\alpha_t(\omega)$ can be presented as $\alpha_t(\omega) = \alpha_{t}^{sh}(\omega) + \alpha_{t}^{st}(\omega)$, where $\alpha_{t}^{sh}(\omega)$ and $\alpha_{t}^{st}(\omega)$ are contributions of the shear and stretching fluctuation modes, respectively [14]. However, the lipid ME is commonly considered as incompressible. In such a case $\alpha_{t}^{st}(\omega) = 0$ and $\alpha_t(\omega) = \alpha_{t}^{sh}(\omega)$. Note, that shear fluctuations in the proposed model depend mainly on the CS properties. To obtain the energy of shear modes we use the solution of Eq. (3) compatible with shear fluctuations of the ME:

$$u_c^x = 0; \quad u_c^x = U_q \exp(-qz + iqx); \quad u_c^z = 0; \quad p = 0; \quad \dot{u}^c = -i\omega u^c.$$  \hspace{1cm} (12)

Similar to mode (4-6), thermally excited shear CS fluctuations are rapidly attenuated in the $z$-direction (see Fig. 2). Substitution of displacement field (12) into elastic energy (2) and integration over the CS thickness results in the shear fluctuation energy

$$E^{sh} = S \sum_q \mu q |U_q|^2 (1 - \exp(-2qh)).$$  \hspace{1cm} (13)

Corresponding shear contribution to the tangent response of the composite system is then expressed as:

$$\alpha_{t}^{sh}(0) = \int_{q_{\text{min}}}^{q_{\text{max}}} \frac{dq}{4\pi\mu(1 - \exp(-2qh))}. \hspace{1cm} (14)$$

To calculate responses (11) and (14) we use the RBC material constants $K$, $\sigma$, $\gamma$ and $\mu$ fitted above and take $N \approx 4.4 \times 10^7$ molecules [28]. The normal response $\alpha_n(0)$ is estimated to be $\sim (6 \div 9) \times 10^4$ m/N. This value is 2-3 orders of magnitude smaller than the response function of the free ME model with the same bending rigidity. For the tangent response function $\alpha_t(0)$ we obtain $\alpha_t(0) \sim (8 \div 11) \times 10^5$ m/N. Thus, final $< X_n^2 > / < X_t^2 >$ ratio is also 2-3 orders of magnitude smaller than that in the free ME model and has no contradiction with the thickness fluctuation profile experiment. Let us stress, that the value obtained in the approximation of an incompressible ME-CS system is an upper limit for
the $< X_n^2 > / < X_t^2 >$ ratio. The system compressibility increases the $< X_t^2 >$ value and preserves the $< X_n^2 >$ one. It means that for a compressible system our approach works even better.

Knowledge of responses (11) and (14) allows us to make a little better estimation of $\mu_s$ modulus than that given by the relation $\mu_s \approx \mu h$. The following estimation is more appropriate for the static microrheological experiments using the probe particles technique (see, for example [13]). In such experiments the cell is usually replaced for data processing by a 2D solid-like ME. The static shear properties of the both systems are roughly equivalent provided $\alpha_{sh}^s(0) = \beta_{st}^s(0)$, where $\beta_{st}^s(0)$ denotes the shear contributions to the tangent response of the 2D solid-like ME. The response $\beta_{st}^s(\omega)$ of the 2D ME in connection with the PP microrheology was studied in [14,15]. Its value

$$\beta_{st}^s(0) = \int_{q_{min}}^{q_{PP}} \frac{dq}{4\pi q\mu_s}$$

(15)

depends on the wave-vector cut-off $q_{PP} = 2\pi/R_{PP}$ [14,15], where $R_{PP}$ is the radius of the contact area between the RBC and the PP (i.e. silica bead). The equality between $\alpha_{sh}^s(0)$ (see Eq. (14)) and $\beta_{st}^s(0)$ results in the following effective 2D shear modulus $\mu_s$:

$$\mu_s \approx \mu ln \left( \frac{2\pi R}{R_{PP}} \right) / \left( \frac{2\pi}{R_{PP}} + \frac{1}{2h} ln \left( \frac{1 - exp \left( -4\pi h/R_{PP} \right) R}{2h} \right) \right).$$

(16)

Taking $R_{PP} \approx R/20$ we obtain that the CS with the bulk shear modulus $\mu \approx 105 \div 165$ Pa and $h \approx 30$ nm thickness induces in the composite ME-CS system the 2D shear modulus $\mu_s \approx (5.1 \div 8.1) \times 10^{-6}$ N/m. This value is very close to that obtained in static deformation experiments [5-7].

In addition to the resolution of the RBC apparent softness paradox, the composite ME-CS model has a striking feature: negative $\sigma$ value obtained from the fit of fluctuation spectra [19]. Negative osmotic pressure difference $\Delta P = 2\sigma/R \approx 1 \div 1.5$ Pa insures a good contact between the ME and the CS. It makes the mechanical interaction between the parts of the composite system much less dependent on the attachment points. In such a case the CS induces (through the controlled tension mechanism) additional ME fluctuations which can
even result in an elastic instability of the cell [29]. A high negative surface tension would break the symmetry from the normal biconcave RBC to the echinocyte shape. On the other hand, a high positive surface tension corresponding to a sufficiently big positive osmotic pressure difference would make a normal cell unstable with respect to the spherical shape. These points attract considerable attention in the cell biology field [30, 31].

More detailed physical discussion of the surface tension could be done in the frame of a more rigorous model taking into account both finite shear modulus $\mu_s$ and the real RBC shape. In such a model surface tension becomes a tensor characteristic $\sigma_{ij}$ of the ME that satisfies the Laplace equilibrium equation:

\[ \sigma_{\theta\theta}/R_\theta + \sigma_{\phi\phi}/R_\phi = \Delta P. \]

Here $R_\theta$ and $R_\phi$ are the RBC curvature radii along $\theta$ and $\phi$ spherical coordinates, respectively. Due to the rotational symmetry of the non-pathological RBC shape $\sigma_{\theta\phi} = 0$. The values of the $\sigma_{\theta\theta}$ and $\sigma_{\phi\phi}$ components are dependent on the $\theta$ coordinate of the ME point. Thus, change in signs of principal curvatures $1/R_\theta$ and $1/R_\phi$ can alternate compressed and stretched regions at the cell surface. If $\Delta P$ is negative (as it is obtained in the present model) then the ME region near the rim is compressed: positive curvatures near the rim lead to $\sigma_{\theta\theta} < 0$ and $\sigma_{\phi\phi} < 0$. This fact favors normal fluctuations and increases their contribution to $< \delta d^2 >$ (see Fig. 1). Near the cell center the RBC shape is locally concave and both curvatures are negative. The ME is stretched in this region ($\sigma_{\theta\theta} > 0$ and $\sigma_{\phi\phi} > 0$) and its normal fluctuations are reduced. Consequently, the amplitude of normal fluctuations increases with the distance from the cell center. Remarkably, it puts negative osmotic pressure value in a good agreement with the location of the thickness fluctuation profile peak.

In conclusion, we showed that the RBC paradox, consisting in solid-like elastic behavior in static suction and liquid-like behavior in thermal fluctuations experiments is apparent. The influence of the finite-thickness CS which confines the ME and contributes to the fluctuation energy of the composite ME-CS system reduces considerably normal fluctuations of the RBC. Contrary to the predictions of the free ME model, the amplitude of normal fluctuations in the composite system is smaller than the amplitude of tangent fluctuations. Resulting effective 2D shear modulus of the RBC shows in thermal fluctuation spectra the value typical for the
solid-like elastic behavior.

The authors thank G. Mennessier for helpful discussions. S.B.R. is grateful to the Biannual Program of the French Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la Recherche for financial support.

**Figure captions**

Fig. 1. Equilibrium shape and fluctuations of the RBC. Mean-square amplitude of the RBC thickness fluctuations $< \delta d^2 >$ depends on the mean-square amplitudes of normal $< X_n^2 >$ and tangent $< X_t^2 >$ fluctuations and on the cell surface point: $< \delta d^2 > = < X_n^2 > \cos^2 \beta + < X_t^2 > \sin^2 \beta$; here $\beta$ is the angle between the vertical direction and the RBC surface normal. Near the rim of the cell the main contribution to $< \delta d^2 >$ comes from tangent fluctuations. Inset: Schematic representation of the RBC membrane and finite-thickness cytoskeleton.

Fig. 2. Displacement fields in the cytoskeleton. 2D sections by the plane containing $z$ and $x$ axes are shown. The three different modes presented are characterized by the same wave vector $q$. The membrane is located in $(x, y)$ plane at $z = 0$ level. According to the boundary condition (see in the text), the displacement of the membrane surface is equal to that of the cytoskeleton at $z = 0$ level, therefore the membrane is not shown. (a) Bending mode. (b) Shear mode. The displacement field is perpendicular to the plane of the figure. Two opposite directions of the displacement field are shown by crosses and full circles. Their size is proportional to the displacement value.

Fig. 3. Wavelength dependence of $q^4/E(q)$ ($E(q)$ is given by Eq. (9)) related to the RBC effective bending rigidity in the flicker spectroscopy experiments [19]. Fits (solid lines) of the data for two different cells (rhombuses and squares) are presented. Inset: Calculated wavelength dependence of the terms in the normal fluctuation mode energy $E(q)$: bending contribution $E_1 = Kq^4$, surface tension contribution $E_2 = \sigma q^2$, CS fluctuations contribution $E_3 = 2W_q/|U_q|^2$ and confinement $E_4 = \gamma$. Though the surface tension term is negative, the
globally positive mode energy $E(q)$ insures stability of the system with respect to fluctuations.
REFERENCES

[1] B. Alberts et al., *Molecular Biology of the Cell* (Garland, New York, 1994).

[2] F. Brochard and J.F. Lennon, J. Phys (Paris), **36**, 1035 (1975).

[3] E.A. Evans, Biophys. J., **13**, 941 (1973); E.A. Evans, Biophys. J., **16**, 597 (1976).

[4] R.E. Waugh, Biophys. J., **70**, 1027 (1996).

[5] D.E. Discher, N. Mohands, E.A. Evans, Science, **266** 1032 (1994).

[6] V. Heinrich, K. Ritchie, N. Mohandas, and E. Evans, Biophys. J., **81**, 1452 (2001).

[7] J.C.M. Lee and D. E. Disher, Biophys. J., **81**, 3178 (2001).

[8] K. Zeman, H. Engelhardt, and E. Sackman, Eur. Biophys. J., **18**, 203 (1990).

[9] M.A. Peterson, Phys. Rev. A, **45**, 4116, (1992).

[10] M. Peterson, H. Strey, and E. Sackmann, J. Phys. II (France) **2**, 1273 (1992).

[11] E. Helfer, S. Harlepp, L. Bourdieu, J. Robert, F. C. MacKintosh, and D. Chatenay, Phys. Rev. Lett., **85**, 457 (2000); E. Helfer, S. Harlepp, L. Bourdieu, J. Robert, F. C. MacKintosh, and D. Chatenay, Phys. Rev. Lett., **87**, 088103 (2001).

[12] E. Helfer, S. Harlepp, L. Bourdieu1, J. Robert, F. C. MacKintosh, and D. Chatenay, Phys. Rev. E, **63**, 021904 (2001).

[13] G. Lenormand, S. Henon, A. Richert, J. Simeon, F. Gallet, Biophys. J., **81**, 43 (2001).

[14] A.J. Levine and F.C. MacKintosh, Phys. Rev. E, **66**, 061606 (2002).

[15] S.B. Rochal, V.L. Lorman, and G. Mennessier, Phys. Rev. E, **71**, 021905 (2005).

[16] N. Gov, A.G. Zilman, and S. Safran, Phys. Rev. Lett., **90**, 228101 (2003).

[17] N. Gov and S. Safran, Phys. Rev. E, **69**, 011101 (2004).

[18] R. Lipowsky, and M. Girardet, Phys. Rev. Lett., **65**, 2893 (1990).
[19] A. Zilker, H. Engelhardt, and E. Sackmann, J. Phys. (Paris) 48, 2139 (1987).

[20] J.B. Fournier, D. Lacoste, and E. Raphael, Phys. Rev. Lett., 92, 018102 (2004).

[21] J. Prost, J.B. Manneville, and R. Bruinsma, Eur. Phys. J. B, 1, 465 (1998).

[22] J.B. Manneville, P. Bassereau, D. Levy, and J. Prost, Phys. Rev. Lett., 82, 4356 (1999).

[23] Z. Zang, H.T. Davis, and D. M. Kroll, Phys. Rev. E, 48, R651 (1993).

[24] H. Yoon and J.M. Deutsch, Phys. Rev. E, 56, 3412 (1997).

[25] L. Landau and E. Lifchitz, Theory of elasticity (Mir, Moscow, 1983); L. D. Landau and E. M. Lifchitz, Hydrodynamics, (Pergamon, New York, 1981).

[26] P.M. Chaikin and T.C. Lubensky, Principles of Condensed Matter Physics (Cambridge University Press, Cambridge, UK, 2000).

[27] L.D. Landau and E.M. Lifshitz, Statistical Physics (Pergamon, London, 1958).

[28] D. Marsh, CRC Handbook of Lipid Bilayers (CRC Press, Boca Raton, FL, 1990).

[29] G. Lim, M. Wortis, and R. Mukhopadhyay, Proc. Natl. Acad. Sci. U.S.A. 99, 16766 (2002).

[30] S.K. Boey, D.H. Boal, and D.E. Discher, Biophys. J., 75, 1573 (1998).

[31] D.H. Boal, Biol. Bull., 194, 331 (1998).
Fig. 1
Fig. 2(a)
Fig. 2(b)
$\frac{q^4}{E(q)}$, a. u.

Wavelength $\lambda$ (µm)

$E_1$, $E_2$, $E_3$, $E_4$