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Membrane bending elasticity of human erythrocyte ghosts

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An experimental study of the membrane bending elasticity of human erythrocyte ghosts is presented. The bending modulus was determined by analysing the shape fluctuations of quasi-spherical ghosts. The obtained results show that: i) the bending elasticity of erythrocyte ghosts is equal to several times the Boltzmann factor k_BT and ii) it belongs to the lower part of the existing interval of experimental values, reported in the literature for the bending elasticity of red blood cell membranes.

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1. Introduction

The human erythrocyte is one of the widely studied objects in biophysics, biology and medicine. The reason for this is the relative simplicity of the red blood cell (RBC), as well as its unambiguous importance for the functioning of the living organisms. The red cell membrane is known to be a highly-deformable, two-dimensional shell with very low area compressibility [1, 2], comprising a viscoelastic network of proteins (mainly spectrin), attached to the lipid matrix at its cytoplasmic surface. The measured stretching elasticity of the RBC membrane is in the range 300÷500 mN/m, its shear modulus in the range 2.5÷10 μN/m [2, 5-7] and its membrane viscosity equal to 3.4×10^{-7} Ns/m [6]. The elastic response of the spectrin network controls the RBC membrane deformations under a shear and stretching stress [3]. In contrast, the spectrin network is not expected to contribute to the bending stiffness, which is determined mainly by the lipid bilayer [4, 5].

Despite the numerous investigations of the RBCs, there is a relatively small quantity of data concerning the bending elasticity of their membranes. The values of this quantity, already published in the literature, belong to a quite wide interval: $k_c = (0.13 \div 4) \times 10^{-19}$ J [5, 8-10].

The bending elasticity modulus k_c of RBC membranes, obtained by analysing the thermal excitations of flickering erythrocytes, is $k_c = (0.13 \div 0.3) \times 10^{-19}$ J [8, 11]. By measuring the critical micropipette aspiration pressure, at which erythrocytes buckled, Evans [9] estimated an upper limit for the bending modulus $k_c = 1.8 \times 10^{-19}$ J. Using another technique (dynamic reflection interference contrast microscopy) Fricke et al. [12] obtained for the membrane bending stiffness of RBC a value of $k_c = 0.5 \times 10^{-19}$ J. These authors have not excluded the possibility that the surface undulations may also be determined by the lateral fluctuations of the quasi-two-dimensional spectrin/actin network. Another value of $k_c = 4 \times 10^{-19}$ J for the bending modulus of RBC membranes was published more recently [5], much higher than the other published results for the curvature modulus of RBC membranes.

The aim of the present work is to determine experimentally the value of the membrane bending elasticity modulus of quasispherical erythrocyte ghosts via analysis of their thermally induced shape fluctuations. The erythrocyte ghosts represent resealed erythrocytes of which the haemoglobin interior was discarded [13].

2. Material and methods

2.1. Ghost preparation

The concentrated erythrocyte suspension from healthy adult donors was supplied by the National Centre of Haematology and Blood Transfusion in Sofia (Bulgaria). Erythrocytes were centrifuged in a MiniSpin (Eppendorf, Hamburg) microcentrifuge at 1200 × g for 5 min at 4°C and the buffy coat was removed. The erythrocytes were washed five times with phosphate-buffered saline (10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 136.9 mM NaCl) with pH 7.4. After centrifuging the blood samples, erythrocytes ghosts (EG) were prepared by the method of Burton et al. [14] with slight modifications. Half a millilitre of the suspension, containing the washed erythrocytes with hematocrit (the proportion of blood volume that is occupied by red blood cells) Ht 30% was added to 15 ml of 5 mM phosphate buffer (pH 8.0) and centrifuged at 22 000 × g for 30 min. After the supernatant had been discarded, the pellet was re-suspended in 15 ml of 2.5 mM phosphate buffer (pH 7.8) and then centrifuged at 22 000 × g for 30 min. Finally, 15 ml of 1.25 mM phosphate buffer (pH 7.6) was added to the pellet, and EG were obtained in the pellet after final centrifuging at 22 000 × g for 30 min. The procedures of membrane isolation were performed on ice and in a centrifuge at 0-2°C. The radii of the obtained ghosts were of the order 2÷3 µm.

2.2. Observation and registration of the fluctuating ghosts

In our experiment, ghost suspensions diluted in 1/15 M phosphate buffer with pH 7.4 (66.7 mM Na_2HPO_4, 66.7 mM NaH_2PO_4) were studied under an inverted Axiovert 100 (Zeiss, Germany) microscope in phase contrast, using a long-working distance objective 63× (NA 0.75) and a home-made magnifier (3.36×), mounted before the C2400-60 (Hamamatsu, Japan) CCD camera control unit. Thereby, the pixel size became of the order of 50 nm. The video signal from the camera was fed to a frame grabber board (DT3155, Datatranslation, USA) mounted in a computer for proper digitization (768×576 8-bit pixels). Once per second, an image was acquired and recorded on the PC to obtain an image sequence of at least 200 frames for each studied ghost.

2.3. Bending elasticity measurement

The bending elasticity modulus of the erythrocyte ghost membrane was measured by the analysis of the thermally induced membrane fluctuations, described in detail elsewhere [15].

Let a frame of reference XYZ be introduced with origin O, placed in the centre of the ghost and with axis Z, parallel to the optical axis of the microscope. The radius $\rho(\vartheta,\varphi,t)$ of the fluctuating ghost in a direction determined by the polar angles (ϑ,φ) at time t is expressed as:

$$\rho(\vartheta,\varphi,t)=R_0\left[1+\sum_{n=0}^{n_{\max}}\sum_{|m|\le n}U_n^m(t)Y_n^m(\vartheta,\varphi)\right], \qquad (1)$$

where R_0 is the radius of a sphere with volume equal to that of the ghost, $n_{\max}$ is of the order of R_0/λ, where λ is the distance between the nearest neighbour molecules in the membrane, and $Y_n^m(\vartheta,\varphi)$ are the normalised spherical harmonics. For $n\ge 2$ and $|m|\le n$, the mean squared amplitude $\left\langle \left|U_n^m(t)\right|^2\right\rangle$ (later on, with $\langle\ \rangle$ we will denote the time average of the quantity in angular parentheses) of the (n,m) mode in the decomposition of the fluctuations is related to the bending elasticity modulus of the membrane k_c via the well-known expression [16]:

$$\left\langle \left|U_n^m(t)\right|^2\right\rangle=\frac{k_BT}{k_c}\frac{1}{(n-1)(n+2)\left[\bar{\sigma}+n(n+1)\right]}. \qquad (2)$$

In the above equation k_BT is the Boltzmann factor and $\bar{\sigma}=\sigma(R_0)^2/k_c+2c_0R_0+(c_0R_0)^2/2$ (where c_0 denotes the spontaneous curvature of the membrane and σ - its tension) is the dimensionless membrane tension.

The normalized instantaneous angular autocorrelation function of the ghost $\xi(\gamma,t)$, defined in its equatorial cross-section (the cross-section of the ghost membrane with the XY plane of the introduced above frame of reference) is [17]:

$$\xi(\gamma,t)=\frac{1}{(R_0)^2}\times$$
$$\times\left[\frac{1}{2\pi}\int_0^{2\pi}\rho\left(\frac{\pi}{2},\varphi+\gamma,t\right)\rho\left(\frac{\pi}{2},\varphi,t\right)d\varphi-\rho^2(t)\right], \qquad (3)$$

where:

$$\rho(t)=\frac{1}{2\pi}\int_0^{2\pi}\rho\left(\frac{\pi}{2},\varphi,t\right)d\varphi. \qquad (4)$$

The time of acquisition of each image was finite because of the finite integration time t_s of the CCD camera used ($t_s=40$ ms, equal to the European TV standard). The time t at the start of the acquisition of each image was considered as its temporal characteristic. The experimentally measured quantity was $B_n(k_c,\bar{\sigma},t_s)$, representing the amplitude of the n-th mode of the development in Legendre polynomials of the time-averaged angular autocorrelation function $\xi(\gamma)=\langle\xi(\gamma,t)\rangle$. The quantity $B_n(k_c,\bar{\sigma},t_s)$ can be expressed as [18]:

$$B_n(k_c,\bar{\sigma},t_s)=f_n^{corr}\,\frac{2n+1}{4\pi}\left\langle \left|U_n^m(t)\right|^2\right\rangle. \qquad (5)$$

The correction factor f_n^{corr} appears because of the finite value of t_s. It has been calculated to be [15]:

$$f_n^{corr}=2\left(\frac{\tau_n^m}{t_s}\right)^2\left[\exp\left(-\frac{t_s}{\tau_n^m}\right)-\left(1-\frac{t_s}{\tau_n^m}\right)\right], \qquad (6)$$

where τ_n^m is the correlation time calculated by Milner and Safran [16] for the amplitude $U_n^m(t)$:

$$\tau_n^m = \frac{\eta {R_0}^3}{k_c} \frac{2n+1}{(n-1)(n+2)[\bar{\sigma} + n(n+1)]} \times \left(2 - \frac{1}{n(n+1)} \right) . \tag{7}$$

In the last expression, η is the viscosity of the medium surrounding the membrane.

To find the value of the bending elastic modulus k_c, for each registered image of the fluctuating ghost (see Fig. 1), the contour representing the equatorial cross-section of the ghost membrane was extracted using a procedure described elsewhere [15]. The instantaneous value $B_n'(t)$ of the quantity B_n (see Eq. 5) was calculated for each of the extracted contours of the fluctuating ghost as the amplitude of the n-th mode of the development in Legendre polynomials of the instantaneous angular autocorrelation function $\xi(\gamma, t)$. Evidently, $B_n = \langle B_n'(t) \rangle$. The root mean squared $\Delta B_n' = \sqrt{\left\langle \left(B_n'(t) \right)^2 \right\rangle - \left(\left\langle B_n'(t) \right\rangle \right)^2}$ was also calculated.

For each of the studied ghosts, the function $\chi^2(k_c, \bar{\sigma}, t_s)$, defined in a standard way, was used:

$$\chi^2(k_c, \bar{\sigma}, t_s) = \sum_{n=2}^{N} \frac{\left[\langle B_n'(t) \rangle - B_n(k_c, \bar{\sigma}, t_s) \right]^2}{\left(\Delta B_n' \right)^2} , \tag{8}$$

where N was the highest number of the index n used for the fitting.

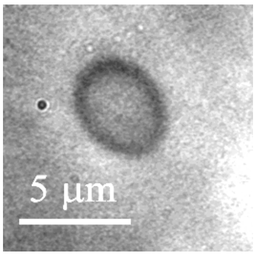

Fig. 1. Equatorial cross-sections of an erythrocyte ghost as observed by phase contrast microscopy. The diameter of the ghost is 4.3 µm.

The values of k_c and $\bar{\sigma}$ minimising the function $\chi^2(k_c, \bar{\sigma}, t_s = 40\,\text{ms})$ were determined numerically, as well as their confidence intervals. Evidently, $\bar{\sigma}$ can vary from ghost to ghost, while k_c is expected to be the same for all the studied ghosts.

3. Results and discussion

For each frame from the image sequence (1 frame per second), acquired for every studied ghost, its contour was represented by 128 radius-vectors in the plane of the contour with polar coordinates $\frac{2\pi}{128}k$, $k = 0,1,\ldots,127$. The value of N, applied in the fitting procedure, was chosen to be 7, assuring the best compromise between the number of the used amplitudes and the influence of the white noise on them.

The thermally induced fluctuations of thirty five ghosts have been observed, recorded and analysed in order to calculate the bending elasticity modulus of their membranes. Nine of them have been chosen, applying two criteria for selection.

Firstly, we required the goodness of fit for each of the accepted ghosts to be more than 0.05. For N=7 this is equivalent to the condition that the minimal value $\chi^2_{\min}(k_c, \bar{\sigma}, t_s = 40\,\text{ms})$ of the function, defined in Eq. 8, should be less than 9.5.

Formally, the quantity t_s can also be considered as an adjustable parameter (besides k_c and $\bar{\sigma}$). The value of this quantity, obtained from the minimisation of $\chi^2(k_c, \bar{\sigma}, t_s)$ (see Eq. 8) is expected to be close to the real one (40 ms). Our second criterion is based on this expectation and demands the value of 40 ms to be inside the confidence interval of t_s, obtained from the minimisation of $\chi^2(k_c, \bar{\sigma}, t_s)$ with respect to the three variables k_c, $\bar{\sigma}$, and t_s. This is equivalent to the requirement that the minimal value $\chi^2_{\min}(k_c, \bar{\sigma}, t_s)$ of χ^2, obtained with t_s as the third adjustable parameter, should be higher than $\frac{1}{2}\chi^2_{\min}(k_c, \bar{\sigma}, t_s = 40\,\text{ms})$.

The reason for a given ghost not to satisfy any of the two criteria could be that its membrane is not in a homogeneous liquid-crystalline state, which is a necessary condition for the application of the theoretical background presented above.

Assuming that the deviations Δk_c have a statistical origin, we calculated the value of the membrane bending elasticity modulus of human erythrocyte ghosts to be equal to $k_c = (0.17 \pm 0.02) \times 10^{-19}$ J. The bending elasticity moduli, measured for the nine selected ghosts, were fitted with one value of k_c and the goodness of fit was 0.7. Consequently, the hypothesis that k_c is the same for these ghosts appeared to be statistically true.

The method used by us for the measurement of k_c has the advantage of being non-invasive with respect to the studied object (floating freely in the surrounding medium), which distinguishes our study from the investigations of the bending elasticity of erythrocyte membranes (adhered to the substrate of the experimental cell), published in the literature so far (see the Introduction).

The use of red cell ghosts for studying thermally induced fluctuations also has the advantage that the ghost enables us to probe directly the physical properties of the membrane system, independently of the effect of the viscosity of intracellular haemoglobin.

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