Cellular Engineering

Stability of spiculated red blood cells induced by intercalation of amphiphiles in cell membrane

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Abstract—*The stability of speculated red blood cells, induced by intercalation of amphiphilic molecules into the cell membrane, is studied. It is assumed that the stable red blood cell shape corresponds to the minimum of its membrane elastic energy, which consists of the local and non-local bilayer bending energies and of the skeleton shear elastic energy. The cell volume and the membrane area are kept constant. It is calculated that the number of spicules of the stable echinocytic shape is larger when the amphiphile concentration is higher, which is in agreement with experimental observations. Also, it is established that, in explaining the stability of the echinocytic shape of the red blood cell, it is necessary to include the membrane skeleton shear elasticity.*

Keywords—*Red blood cell, Cell shape stability, Echinocyte, Membrane skeleton, Membrane elastic energy*

Med. Biol. Eng. Comput., 1998, **36**, 251–255

1 Introduction

AMPHIPHILIC MOLECULES intercalate readily into the red blood cell (RBC) membrane. At sublytic concentrations, they induce changes in the normally discocytic RBCs to either spiculated (echinocytic) shapes (Fig. 1) or invaginated (stomatocytic) shapes (DEUTICKE, 1968; BRETCHER and BESSIS, 1972; SHEETZ and SINGER, 1974; ISOMAA *et al.*, 1987).

To explain the RBC shape changes induced by the amphiphiles, the bilayer couple model was formulated (SHEETZ and SINGER, 1974; EVANS, 1974). According to this model, the RBC shape changes arise from a selective incorporation of nphiphilic molecules into the inner or outer layer of the RBC plasma membrane bilayer. In terms of the bilayer couple model, the RBC shape changes continuously at constant membrane area A and constant cell volume V, owing to the continuous change in the conditions, which causes the continuous change in the difference between the outer and the inner monolayer areas of the bilayer ΔA (SHEETZ and SINGER, 1974; EVANS, 1974; SVETINA and ŽEKŠ, 1996). In other words, the experiments (SHEETZ and SINGER, 1974; ISOMAA *et al.*, 1987) strongly indicate that, when the area difference is lowered, the discocytic RBC shape is changed towards the stomatocytic shape, whereas, when the area difference is increased, the discocytic RBC shape is changed towards the echinocytic shape.

Shape changes connected to the change in the area difference ΔA were also observed in giant phospholipid vesicles. The disocytic shape of the vesicles transformed into the stomatocytic shape as ΔA was lowered (KÄS and SACKMANN, 1991). However, the giant lipid vesicles have never been reported as having attained true echinocytic shapes, i.e. shapes with many spicules. As the RBCs contain the cytoskeleton underlying the membrane that can withstand shear deformations over long periods (MOHANDAS and EVANS, 1994), whereas the giant lipid vesicles are without the cytoskeleton, it is indicated that the skeleton of the RBC membrane may be responsible for the formation and stability of the echinocytic shapes (IGLIČ, 1997).

The scope of this work is the theoretical description of the echinocytic RBC shape changes induced by intercalation of amphiphilic molecules in the outer layer of the membrane bilayer. By introducing a mathematical description of the RBC shape that is based on the bilayer couple model, the role of the membrane skeleton is stressed, and it is explained why the experimentally observed number of echinocyte spicules is larger when the concentration of the echinocytogenic amphiphile molecules in the erthythrocyte suspension is higher (SHEETZ and SINGER, 1974; ISOMAA *et al.*, 1987).

2 Methods

2.1 *Model*

The stable RBC shape is defined as the shape corresponding to the minimum of the membrane elastic energy under given geometrical constraints. In this work, membrane area A and cell volume V are taken to be constrained. Therefore, of all possible shapes of the RBC, the shape of the minimum membrane elastic energy at given A and V is sought. To

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First received 25 July 1997

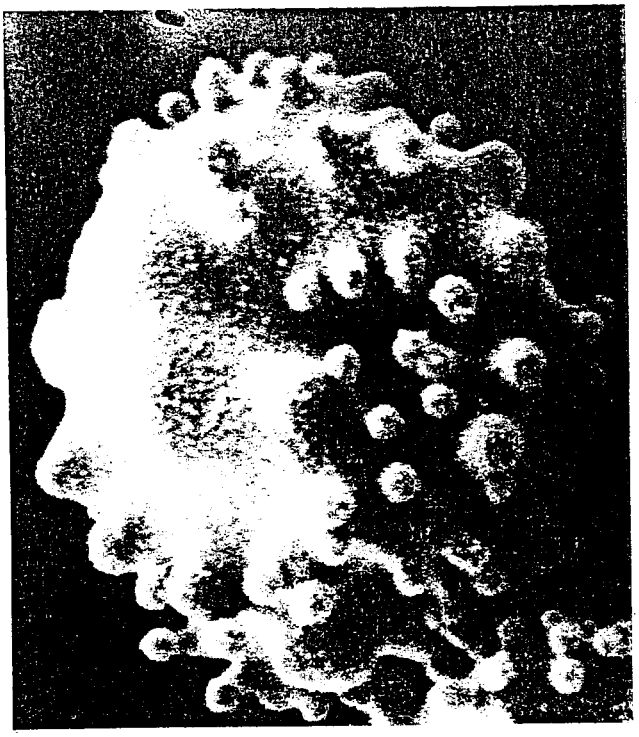

Fig. 1 *Scanning electron micrograph of RBC treated with dodecyltrimethylammonium bromide*

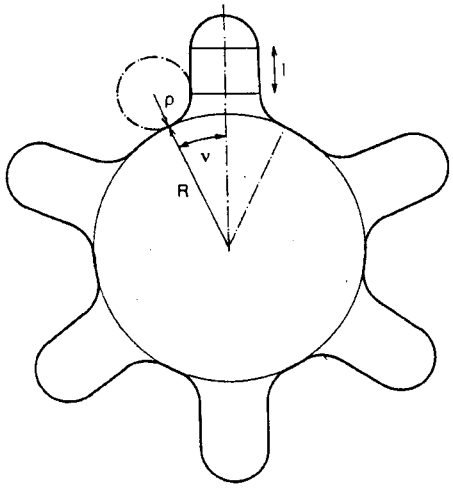

Fig. 2 *Cross-section of model echinocyte red blood cell shape characterised by five geometrical parameters (IGLIČ, 1997)*

achieve the variation of the shape, a parametrical model of the echinocyte involving five geometrical parameters is used (IGLIČ, 1997) (Fig. 2). It is taken that there are n spicules distributed on the cell body, and that the spicules are axisymmetric with respect to the normal to the membrane surface. The model parameters are: the radius of the cell body R; the number of spicules distributed on the cell body n; the length of the spicule cylinder l; the radius determining the base of the spicule ρ; and the angle v (Fig. 2). The corresponding mathematical expressions for the cell volume $V(\rho, n, v, l, R)$, the plasma membrane area $A(\rho, n, v, l, R)$, and the area difference $\Delta A(\rho, n, v, l, R)$ for the model echinocyte shape are described in detail elsewhere (IGLIČ, 1997).

2.2 *Membrane energy*

The membrane elastic energy W consists of the local bending energy W_b, the non-local bending energy W_r and a membrane skeleton shear elastic energy W_s (EVANS and SKALAK, 1980; SVETINA and ŽEKŠ, 1996) as follows:

$$W = W_b + W_r + W_s \tag{1}$$

where it is taken that the skeleton is attached to the bilayer over the entire inner surface of the membrane (IGLIČ *et al.*, 1995; SIKORSKI and BIALKOWSKA, 1996). The local bending energy of the bilayer is given by (HELFRICH, 1973)

$$W_b = \frac{1}{2} k_c \int (C_1 + C_2)^2 dA \tag{2}$$

where the integration is performed over the membrane area A, k_c is the local membrane bending modulus, and C_1 and C_2 are the principal curvatures.

The non-local bending energy due to net compression and expansion of both lipid layers resulting from the curvature change can be expressed as (SVETINA and ŽEKŠ, 1996)

$$W_r = \frac{1}{2} k_r (\Delta A - \Delta A_o)^2 / (A\delta^2) \tag{3}$$

where

$$\Delta A = \delta \int (C_1 + C_2) dA \tag{4}$$

k_r is the non-local membrane bending modulus, δ is the distance between the neutral surfaces of both layers of the bilayer, and ΔA_o is an RBC membrane parameter. It is emphasised that the parameter ΔA_o is an increasing function of the number of amphiphilic molecules intercalated in the outer layer of the membrane bilayer (SVETINA and ŽEKŠ, 1996). The difference between the areas of the two bilayer layers ΔA is, in general, different from ΔA_o.

Although it was shown recently that the membrane skeleton is locally compressible (MOHANDAS and EVANS, 1994), the essential characteristic of the shear elasticity can be satisfactorily described by assuming, for simplicity, that the bilayer and the skeleton are laterally incompressible (WAUGH, 1996). Therefore, in this work, the shear energy of the skeleton is calculated using an approximate expression (EVANS and SKALAK, 1980)

$$W_s = \mu \int \left(\frac{1}{2} (\lambda_m^2 + \lambda_m^{-2}) - 1 \right) dA \tag{5}$$

where λ_m is the dimensionless principal extension ratio along the meridional direction, and μ is the shear modulus of the membrane. The mathematical expressions for the local bending energy $W_b(\rho, n, v, L, R)$ and the shear energy $W_s(\rho, n, v, L, R)$ for the model echinocyte shape (Fig. 2) are described in detail elsewhere (IGLIČ, 1997).

In the following, cell volume V, membrane area A, area element dA, principal curvatures C_1 and C_2 and area differences ΔA and ΔA_o are normalised relative to the corresponding values for the spherical cell of radius $R_o = (A/4\pi)^{1/2}$. Therefore the relative cell volume is $v = 3V/4\pi R_o^3$; the relative membrane area is $a = A/4\pi R_o^2 = 1$; the relative area element is $da/4\pi R_o^2$; the relative principal curvatures are $c_1 = C_1 R_o$ and $c_2 = C_2 R_o$; and the relative area differences are $\Delta a = \Delta A/8\pi\delta R_o$ and $\Delta a_o = \Delta A_o/8\pi\delta R_o$. All the contributions to the membrane elastic energy (eqns. 2, 3 and 5) are normalised relative to the local bending energy of the sphere $8\pi k_c$, to yield

$$w_b = 1/4 \int (c_1 + c_2)^2 da \tag{8}$$

$$w_r = (k_r/k_c)(\Delta a - \Delta a_o)^2 \tag{9}$$

$$w_s = (\mu/4k_c) \int ((\lambda_m^2 + \lambda_m^{-2}) - 2) R_o^2 da \tag{10}$$

It can be seen from eqns. 8–10 that the relative membrane elastic energy $w = w_b + w_r + w_s$ and, consequently, the stable RBC shape is completely determined by the RBC parameters v and Δa_o and the ratios of the membrane constants k_r/k_c and μ/k_c. It was recently measured that $k_r/k_c \cong 5$ (WAUGH *et al.*, 1992), and the estimation of μ/k_c from experimental determination of μ and k_c (EVANS, 1974; WAUGH and EVANS, 1979) gives $\mu/k_c \cong 10^{13}$ m^{-2}. The value of R_o is 3.3 μm.

The stable RBC shape is obtained by minimisation of the membrane elastic energy w at given membrane parameter Δa_o and the ratios k_r/k_c and μ/k_c when the constraint of fixed v is taken into account. First, the shape of a chosen Δa is calculated. As the non-local bending energy w_r does not influence the RBC shape at given Δa (SVETINA and ŽEKŠ, 1996), the cell shape at given Δa can be determined by minimisation of the energy $w_b + w_s$ (IGLIČ, 1997). In this procedure, three of the five geometrical parameters of the parametrical model (the angle ν, the length of the spicule cylinder l and the radius of the cell body R) are determined from the two constraints for the membrane area and the cell volume and from the requirement for the chosen area difference. The remaining two parameters (the radius determining the base of the spicule ρ and the number of spicules n) are determined by minimisation of $w_b + w_s$. Then, the stable RBC shape (IGLIČ *et al.*, 1996) is calculated by finding the shape corresponding to the absolute minimum of the total relative membrane elastic energy w over all possible Δa values.

3 Results

First, the role of the membrane skeleton in determination of the echinocytic RBC shapes is studied. A model echinocyte of a chosen relative volume v and a chosen relative area difference Δa is considered. Fig. 3 shows the calculated number of spicules of such a model echinocyte as a function of the ratio μ/k_c. The calculated shapes are obtained by minimisation of the energy $w_b + w_s$. It can be seen that for $\mu/k_c \neq 0$ the calculated echinocyte shapes have more than one spicule, whereas, for $\mu/k_c = 0$, the calculated echinocyte shapes with minimum energy $w_b + w_s$ have only one spicule (IGLIČ, 1997). As the observed echinocyte shapes have many spicules (Fig. 1) (BRETCHER and BESSIS, 1972; ISOMAA *et al.*, 1987), it can be concluded that, when explaining the echinocytic RBC shapes with many spicules, it is necessary to include the membrane skeleton shear elasticity.

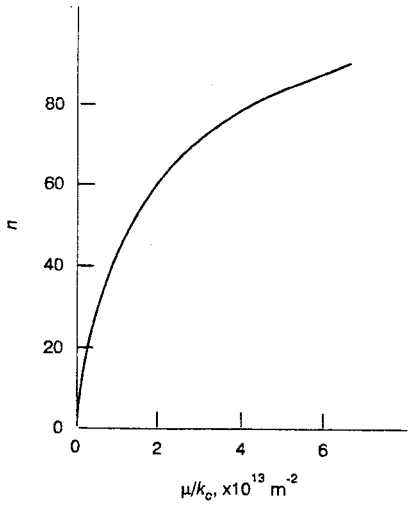

Fig. 3 *Calculated number of echinocyte spicules determined by minimisation of energy $w_b + w_s$ at given Δa, against ratio μ/k_c. Relative cell volume is $v = 0.6$; ratio $k_r/k_c = 4$; and relative area difference is $\Delta a = 2.4$*

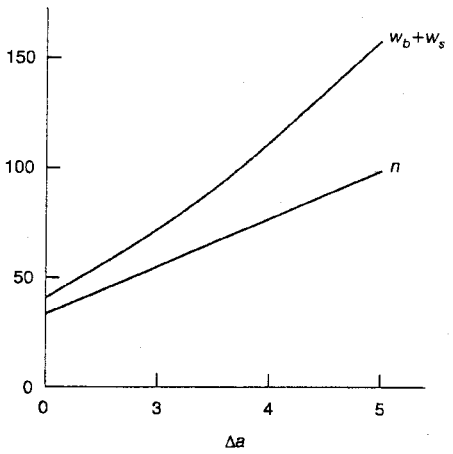

Fig. 4 *Dependence of energy $w_b + w_s$ and corresponding number of spicules n on relative area difference Δa for relative cell volume $v = 0.6$, $k_r/k_c = 4$, and $\mu/k_c = 10^{13}$ m^{-2}*

Next, it is explained how the stable RBC shapes are obtained. Fig. 4 shows the dependence of the energy $w_b + w_s$, minimised at chosen relative area difference Δa, for different values of Δa. It can be seen that the minimised energy $w_b + w_s$ monotonously increases with increasing Δa. Also, Fig. 4 shows the number of echinocyte spicules n corresponding to the minima of $w_b + w_s$ for different Δa. It can be seen that the number of spicules n also monotonously increases with increasing Δa.

As the stable echinocyte shape is defined to be the shape corresponding to the absolute minimum of the relative membrane elastic energy w, as well as the terms w_b and w_s considered in Fig. 4, the non-local bending term w_r should also be included and minimised with respect to Δa. The non-local bending term depends quadratically on Δa (eqn. 9), so that adding this term to the monotonously increasing $w_b(\Delta a) + w_s(\Delta a)$ (Fig. 4) gives a minimum of the total relative membrane elastic energy w at some finite value of $\Delta a = \Delta a_{stable}$, this value being significantly influenced by the RBC membrane parameter Δa_o (Fig. 5*b*).

In explaining the effect of the intercalation of the amphiphilic molecules into the RBC membrane on the stable echinocyte shapes, it is assumed, in accordance with the bilayer couple model, that the change in the shape is caused by the change in Δa_o. It is considered that the RBC membrane parameter Δa_o is larger if the outer area of the RBC plasma membrane is increased owing to the intercalation of the amphiphilic molecules (SVETINA and ŽEKŠ, 1996).

To show the effect of increasing Δa_o (i.e. the increase in the number of intercalated amphiphiles in the outer layer of the bilayer) on the stable echinocyte shape, Fig. 5*a* shows the dependence of Δa_{stable} on Δa_o. For the sake of clarity, the total relative energy of the RBC membrane $w = w_b + s_s + w_r$ as a function of the relative area difference Δa for three different values of Δa_o is depicted in Fig. 5*b*. The stable RBC shapes corresponding to the three minima are marked and are also indicated in Fig. 5*a*. It can be seen that Δa_{stable} increases with increasing Δa_o.

Finally, Fig. 6 shows the number of echinocyte spicules of the stable shapes (n_{stable}) on the amount of intercalated amphiphiles represented by Δa_o. It can be seen that n_{stable} increases with increasing Δa_o, as can be expected from the dependence of n on Δa (Fig. 4). The increase in n_{stable} with increasing Δa_o is in agreement with experimental observations (SHEETZ and SINGER, 1974; ISOMAA *et al.*, 1987).

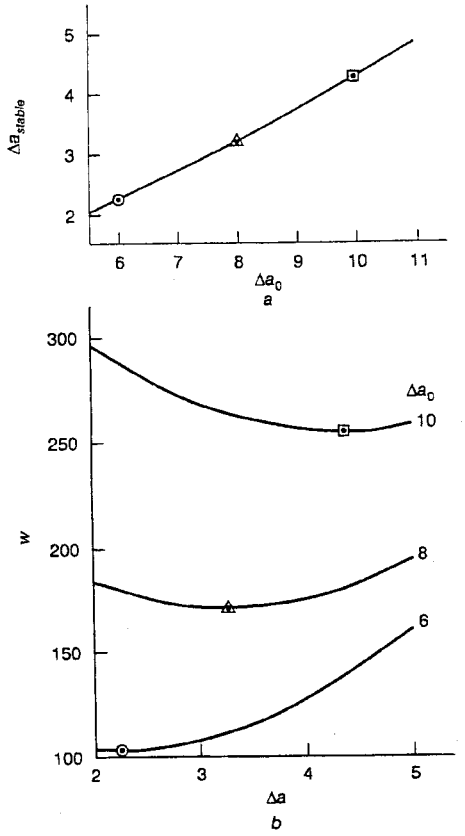

Fig. 5 *(a) Dependence of relative area difference of stable echinocyte shapes Δa_{stable} on RBC membrane parameter Δa_0 representing number of amphiphilic molecules intercalated in outer membrane layer (A). (b) Stable echinocyte shapes are obtained by minimisation of total relative elastic energy of RBC membrane $w = w_b + w_s + w_r$ over relative area difference Δa. Stable RBC shapes corresponding to three different choices of RBC membrane parameter Δa_0 are marked. Values of relative volume and ratios of membrane constants k_r/k_c and μ/k_c are given below Fig. 4*

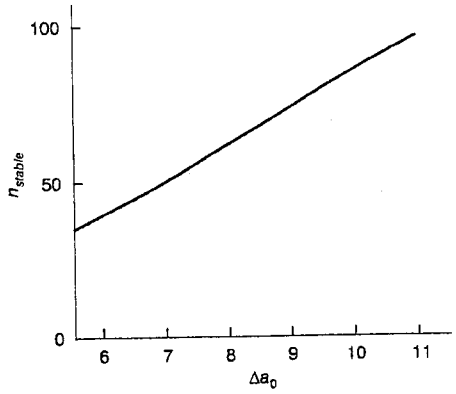

Fig. 6 *Dependence of number of echinocyte spicules of stable shapes n_{stable} on RBC membrane parameter Δa_0 representing the amount of amphiphilic molecules intercalated in outer membrane layer. Values of relative volume and ratios of membrane constants k_r/k_c and μ/k_c are given below Fig. 4*

4 Discussion

The bilayer couple model and the requirement of the minimum RBC membrane bending energy can explain the stability of numerous observed RBC shapes (BERNDL *et al.*, 1990; SVETINA and ŽEKŠ, 1996). However, these studies of RBC shapes were limited to the range of area differences ΔA where the spiculated RBC cells, i.e. echinocytes, cannot exist. Namely, echinocyte RBC shapes have much higher ΔA than other RBC shapes. Therefore, in this work, the analysis of the RBC shape stability is extended to the range of higher ΔA where echinocytes can exist.

The membrane skeleton appeared to have a secondary role in most of the RBC shape changes (ZARDA *et al.*, 1977; SHEETZ, 1983; GEDDE *et al.*, 1995). This can be at least partially explained by the results of experiments by MOHANDAS and EVANS (1994) indicating that the area expansivity modulus and the bending constant of the skeleton are, in normal conditions, a few orders of magnitude smaller than the corresponding constants of the bilayer.

However, it is shown in this work that the membrane skeleton is important in stabilisation of echinocytic RBC shapes. It is shown that, by neglecting the membrane shear elasticity, the calculated stable echinocyte shapes have only one spicule, whereas, by taking into account also the membrane skeleton shear elasticity, the calculated stable echinocyte shapes have many spicula, which is in agreement with experimental observations. This is also supported by our recent preliminary observations showing that lamprey erythrocytes, in which the membrane skeleton appears to be deficient (OHNISHI and ASAI, 1985), do not form well-defined spicules upon treatment with echinocytogenic amphiphiles.

In this work, the spontaneous curvature of the membrane bilayer C_o (HELFRICH, 1973) was not explicitly considered in the local bending energy term (eqn. 1). However, the effects described by the spontaneous curvature are effectively described by being contained in the RBC membrane parameter ΔA_o (MIAO *et al.*, 1994). In other words, the presented continuum description of the membrane elastic energy cannot account for all the features of the molecular origin and therefore cannot clearly reveal the differences between the C_o and the ΔA_o RBC membrane parameters.

The range of ΔA_o where echinocytic RBC shapes can be expected is estimated. SHEETZ and SINGER (1974) report values of $\Delta A_o/A$ between 0.005 and 0.019 for different types of amphiphile. This range of ΔA_o values corresponds to the interval of Δa_o values between 3 and 10.5 for $\delta = 3$ nm and $R_o \cong 3.3$ μm ($A = 138\ \mu m^2$), which is in the range of the values of Δa_o considered in this work (Figs. 5 and 6).

Previous studies of echinocyte shapes involved situations where only short spicules were considered (LANDMAN, 1984), a spicule alone was considered (STOKKE *et al.*, 1986), or the volume was not constrained and the number of the spicules of the stable RBC shape was assumed in advance (WAUGH, 1996). As, in our model, the whole cell is considered, whereas the number of spicules of the stable shape is determined in the minimisation of the membrane elastic energy, the presented work provides an extension of the mathematical description of the stable echinocyte shape.

5 Conclusions

It is calculated that the number of echinocyte spicules is larger when the number of amphiphiles intercalated in the outer membrane layer is larger. Also, it is stressed that the membrane skeleton shear elasticity is essential to stabilise the echinocyte shape. These conclusions agree well with the experimental data (SHEETZ and SINGER, 1974; ISOMAA *et al.*, 1987).

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