ELSEVIER

Biochimica et Biophysica Acta 1372 (1998) 198–204

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Red blood cell shape as a function of medium's ionic strength and pH

Marta Rasia *, Adriana Bollini

Cátedra de Biofísica, Facultad de Ciencias Médicas, Santa Fe 3100, 2000 Rosario, Argentina

Received 3 November 1997; revised 17 March 1998; accepted 26 March 1998

Abstract

Glycocalyx, the characteristic first line of interaction between membrane and environment, can be visualized as a polyelectrolyte anchored to a bending-resistant matrix. This structure has an amazing resemblance with the ionized monolayers, in which, the cohesion among hydrocarbon chains is counteracted by the repulsion among similarly charged ionic heads, and thus the balance determines the curvature of the membrane. Likewise, it could be assumed that in biological membranes, repulsion among similarly charged groups in the glycocalyx could generate different curving trends. Hence, the factors directly influencing the electrostatic interaction among surface charged groups were studied, assessing the effect of the medium's ionic strength (μ) and pH, in an extensive range of values around the physiological one. The results point out μ variations inducing different shapes, depending on whether μ values were lower or higher than the physiological ones; which could be explained by the polyelectrolyte theory. The occurrence of more invaginated shapes as the medium's pH decreases, and the opposite event, when the pH increases, could be attributed to the coupling between the dissociation of the glycocalyx ionic groups and the H^+ concentration. The behavior of the cells with reduced surface charges (by neuraminidase degradation) supports the hypothesis that the observed μ and the pH effect on erythrocyte shape could be mediated by glycocalyx charged groups.

Keywords: Erythrocyte shape; Glycocalyx; Surface charge; Ionic strength; pH effect

1. Introduction

It has already been demonstrated that a wide variety of chemical agents, as well as environmental conditions, are able to transform the normal biconcave discoid shape of the red blood cells (RBC) into cup or spiculated forms [1–4]. Several papers deal with these mechanisms of action, as well as with the membrane structures that mediate these effects. The membrane cytoskeleton was consistently considered as the structure controlling the cell shape. Consequently, the shape transformation brought about by amphiphilic molecules led to the hypothesis that the lipid bilayer could be the primary determinant of membrane curvature [5]. It is also postulated [6] that the reticulum is able to preserve, but not to impose, the membrane contour.

In 1983, Schmid-Schönbein et al. [7] proposed a new hypothesis that takes account of the electrostatic repulsion among charged groups on both the cytoplasmic and the outer membrane surface. Based on this, Lerche et al. [8] conceived a biophysical model, in which the membrane curvature is a consequence of the balance between the elastic energy density – due to bending of the lipid bilayer – and the electrostatic energy density of charges in the glycocalyx and cytoskeleton. Grebe et al. [9], working on their own,

* Corresponding author. Fax: +54 (41) 484761.

0005-2736/98/$19.00
PII: S0005-2736(98)00057-1

created a computer model for the electrostatic properties of differently shaped membranes, and concluded that the changes in the distribution of charged molecules could be a reason for cell shape changes. Similarly, electric field variations in the medium, which are capable to induce immediate shape changes, were observed. This suggests a direct action on the glycocalyx, namely, the characteristic first line of interaction between the membrane and the environment.

The glycocalyx is an extracellular layer consisting of glycolipids and glycoproteins that protrude from the membrane surface. Thus, the former could be simply visualized as a polyelectrolyte anchored to an electrically neutral and bending-resistant matrix [10]. Besides, the glycocalix has an and amazing resemblance with the ionized monolayers, in which the cohesion among hydrocarbon chains is counteracted by the repulsion among similarly charged ionic heads. By analogy, we could assume that in biological membranes the repulsion among similarly charged groups, immersed in the glycocalyx could be able to generate different curving trends of the membrane.

The proposed theory has been experimentally verified modifying the medium ionic composition. Assuming a membrane ionic impermeability [11], and a brief cell medium contact, the shape modifications observed were considered as a result taking origin in electrostatic interactions in the glycocalyx.

It was amazing to observe that the cell shape did not change monotonically with μ variations as expected from membrane models already accepted. On the contrary, for μ values lower than the physiological one, the observed trend was opposite to the predicted one. This shape behavior was also observed by Deuticke in 1968 [12], Glaser in 1982 [13], and Bifano et al. in 1984 [14]; however, it has not yet been unequivocally explained.

On the other hand, regarding the influence of pH on the cell shape, stomatocytosis at low pH and echinocytosis at high pH were observed. Moreover, it was found that the response of the membrane curvature regarding the medium ionic strength is conditioned by the pH. Spiculation at low pH, and cupping at high pH, which are postulated by the gel ionic theory, were reported in the literature in isolated membranes (ghosts). Conversely, the whole RBC behavior was opposed to the one expected according to the theory [4], and in agreement with our results. Nevertheless, considering the glycocalyx anchored to the membrane matrix, in which the repulsion among charged groups develops a lateral expansive pressure, this behavior could be a consequence of the coupling between the degree of dissociation of the polyelectrolyte, and the ionic composition of the medium, as in monolayers.

2. Materials and methods

2.1. Erythrocyte preparation

Adult human blood was drawn by venipuncture, poured into heparinized tubes and immediately centrifuged at $5000 \times g$ for 10 min. The buffy coat was removed and the cells were washed three times by centrifugation, resuspended in an isotonic medium, and kept cold until use on the same day.

2.2. Incubation media

Isosmotic (290 mosM) phosphate-buffered saline (PBS) was employed. In case of pH 10, the buffer was replaced by Tris buffer. The incubation media contained a polyvalent ionic solute (Na-citrate, $MgSO_4$, $MgCl_2$ or Na_2SO_4), a 1:1 electrolyte (NaCl) and a non-electrolyte (mannitol) in variable concentrations, in order to obtain different μ values. In no case were the three solutes used under 5% of the total osmolality. Osmolality and pH of the media were controlled before use.

2.3. Erythrocyte shape

The shape of the RBC was estimated by direct microscopic observation. A drop of the suspension (Hct: 1%) was placed on a vinyl plastic slide and the cell shape was observed with an inverted microscope. Nine shapes were considered according to the following classification [2]: biconcave disc (shape index 0), three types of stomatocytes (indexes −1, −2 and −3) to spherostomatocyte (index −4); and three degrees of echinocytes (indexes 1, 2 and 3) to spheroechinocyte (index 4). The microscopic observations were made after a 1-min contact of cells with the solution

and within the next 2 min. In each preparation, an average of 150 cells were classified according to the above mentioned pattern, and a shape index value was obtained adding each shape percent fraction multiplied by the corresponding shape index.

The addition of glutaraldehyde, to preserve their shape was avoided, since irregular forms were observed with this agent [15].

2.4. Neuraminidase-treated cells

The washed cells were suspended at a 20% v/v concentration, in PBS containing 5 mM Ca^{2+} and 0.2 U of neuraminidase (*Clostridium perfringens*, Sigma) per ml of packed cells. The suspension was incubated at 37°C for an hour with gentle shaking, and the erythrocytes were removed by centrifugation at $2000 \times g$ for 5 min and washed twice.

The estimation of the superficial charges of the erythrocytes total sialic acid charge was assessed photometrically in samples of the first supernatant [16], yielding a reduction of about 90%.

3. Results

To confirm the hypothesis, the attention was focused on those factors directly influencing the electrostatic interaction among surface charged groups. Therefore, the effect of the medium's ionic strength (μ) and pH was examined, in an extensive range of values around the physiological one. In order to extend the range of μ beyond the physiological 0.145 mol/l value, while keeping normal osmolality, it was necessary to replace the 1:1 electrolyte (NaCl) with another polyvalent salt.

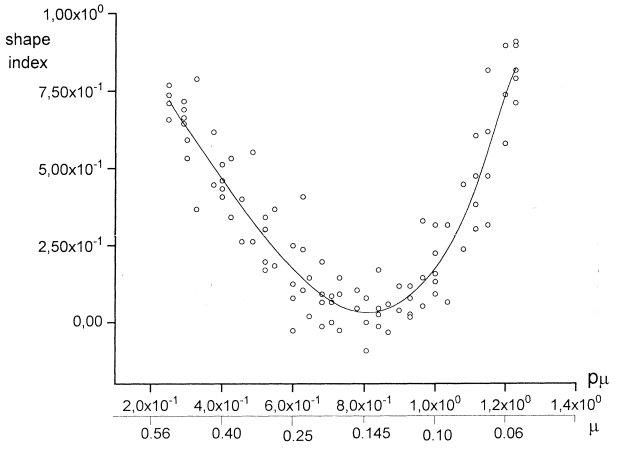

Fig. 1. Erythrocyte shape dependence on the medium's μ. The incubation media contained a polyvalent ionic solute (Na-citrate, $MgSO_4$, $MgCl_2$ or Na_2SO_4), a 1:1 electrolyte (NaCl) and a non-electrolyte (mannitol) in variable concentrations, in order to obtain different μ values. Shape indices were calculated as described in Section 2.

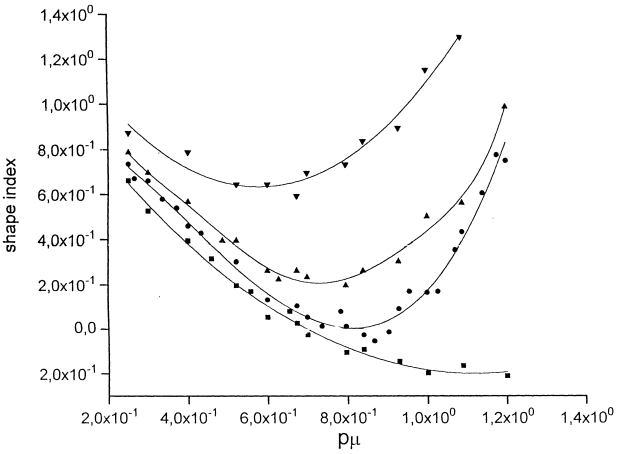

Fig. 2. Influence of pH medium on the relation erythrocyte shape/μ: Erythrocyte suspensions of different ionic strength (μ) were prepared at different pH values using phosphate buffered saline. At pH 10 Tris buffer was used. pH 4, black triangles; pH 6, black inverted triangles; pH 7.4, black circles; pH 10, black squares.

To discriminate between the μ effect and the specific solute influences: (a) the experimental media employed contained the following three kinds of solute, (1) a polyvalent salt (Na-citrate, $MgSO_4$, $MgCl_2$ or Na_2SO_4), (2) 1:1 electrolyte (NaCl), and (3) non-electrolyte (mannitol), each at 5% of the total osmolality or more, in agreement with particular experimental conditions; and (b) each μ was prepared with different solute proportions.

Fig. 1 shows the erythrocyte shape dependence on the medium's μ. It can be seen that the discocyte shape (index 0) at physiological μ is a minimum value, from which the curve attains higher values of stomatocytosis, either for lower or higher values of μ, i.e. that μ's increments yield opposite trends within the ranges of 0–0.145 mol/l, and 0.145–0.600 mol/l This non-monotonical response has also been observed by other authors. Nevertheless, the theories explaining the effect of μ above the physiological

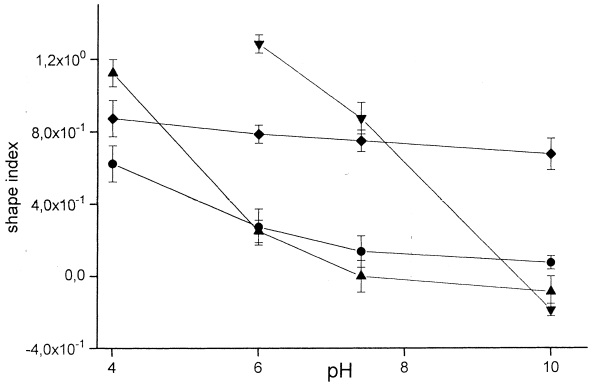

Fig. 3. pH influence on the erythrocyte shape strongly depends on the salt content of the solution. $\mu = 0.063$ mol/l, black triangles; $\mu = 0.14$ mol/l, black circles; $\mu = 0.25$ mol/l, black squares; $\mu = 0.562$ mol/l, black inverted triangles.

one are invalid in the case of μ values below the physiological one.

Fig. 2 shows that the response of the membrane curvature regarding the medium ionic strength is conditioned by the pH. Thus, the lower portion of the curve is shifted to smaller μ values, parallel to the alkalinization of the pH, and at higher pH values, the minimum ones tend to dissipate.

The influence of H^+ concentration upon the shape index, for a fixed salt concentration, is represented by the transition from one curve to another along a vertical line parallel to the index axis.

Fig. 3 shows that pH influence strongly depends on the salt content of the solution. At low μ values (0.06 mol/l) the erythrocyte shape is very sensitive to pH variations, as observed by Glaser et al. [16]. At high μ values (0.56 mol/l) the screening effect of nonprotonic ions makes the RBC shape almost independent of pH variations.

4. Discussion

The classical Sheetz and Singer's hypothesis postulates that variations of the relative areas of the hemilayers could modulate membrane curvature given origin to conformational changes. Lange et al. [6], and later Grebe et al. [17], assume that in a similar way, the electrostatic repulsion between charged residues in membrane surface is an expansive force that could change the membrane curvature. Likewise, μ decrement, either inside or outside the cell, could expand the respective hemilayer surface changing the equilibrium configuration.

Since small ions of the saline medium are able to penetrate into the glycocalyx network and screen partially the electric charges (thus diminishing their repulsion) any μ increase is expected to cause a shrinkage of the surface and a tendency to stomatocytosis. On the contrary, any μ decrease would cause echinocytic forms.

The data depicted in Fig. 1 are in agreement with the theoretical models, that relate the surface electrostatic charge to the curvature of the membrane [6,17], in the range of 0.145–0.600 mol/l. Therefore, the rise in the stomatocytic index, observed when μ decreases below the physiological value, could be considered as an anomalous behavior. Deuticke [12] observed this same type of response, i.e. the stomatogenic effect either by adding Na-citrate to the incubation media ($\mu > 0.145$), or by diminishing μ below the physiological value. Herrmann et al., in 1985 [18] working at $\mu < 0.150$ mol/l found that at higher outer NaCl concentration stomatocytes shifted to echinocytes (the outer surface is expanded instead of shrunk), and the increase of the inner ionic concentration promotes the shift to stomatocyte, in striking contrast to Lange and Grebe's hypothesis.

Deuticke attributed the stomatocytic effect at low μ values to a higher interface tension, namely, the result of pH differences between the membrane and the medium. Glaser and others [13–19] observed the same effect, and proposed that it was a consequence of a direct correlation between transmembrane potential and cell shape. Bifano et al. [14] repeated Glaser's experiments but, although their observations agreed with those reported by Glaser, additional measurements led them to the conclusion that the shape of the cells is independent of the membrane potential. Herrmann et al. [18] could not explain the shape of the charged surfaces in relation to the inner and outer NaCl concentration. They found a good correlation between ghost shape and the membrane potential, but could not find a causal relation between them. This shows that the stomatogenic effect at low μ values was repeatedly observed, but not yet unequivocally interpreted.

Our hypothesis proposes to consider the glycocalyx electrically charged structures as polyelectrolytes anchored to the membrane. Hence, it could be able

to explain the stomatogenic effect caused by a decrease of the medium's μ at low μ values, according to the polyelectrolyte dissociation theory.

In human RBC, the glycocalyx is the outer layer and the bearer of surface charged groups. The strong interaction between its charged residues was confirmed by Donath and Pastushenko [20] through the electrophoretic mobility behavior of erythrocytes, and by Herrmann et al. [21] through spin labeling studies. Hence, the latter also constitutes the highly characteristic first line of interaction between the cell and its environment. Therefore, assuming a membrane ionic impermeability [6], and a brief cell medium contact, it can be postulated that, due to the modulation of the electrostatic repulsion among the polyelectrolyte segments of the glycocalyx, the μ and H^+ concentration of the external solution could control the lateral packing density and the area of the glycocalyx. Applying Le Chatelier–Braun's principle, it could be hypothesized that the variation in the medium μ and pH could trigger immediately conformational changes, and induce a deformation on the membrane (bilayer couple hypothesis).

A similar behavior is observed in charged lipid monolayers, where the cohesion among hydrocarbon chains is counteracted by the superficial pressure caused by the electrostatic repulsion among the similarly charged ionic heads. It is already known that in these membranes conformational variations can be triggered by local μ and pH variations [22].

Considering the similarity between these membranes and the biological ones, we can envisage a lateral expansive pressure π in the glycocalyx,due to the coulomb repulsion among the polyelectrolytic segments [22,23]. Therefore, an increase in this pressure will lead to evaginated or echinocytic forms, and a decrease to invaginated or stomatocytic ones.

The double layer Gouy–Chapman's theory allows one to propose the following equation that relates π to the electrical surface potential ψ_o of the membrane [22]:

$$\Delta\pi = C_1\sqrt{\mu}\cosh\left(\frac{e\psi_o}{2kT}-1\right) \quad (1)$$

where $\Delta\pi = \pi-\pi_0$ (π_0, superficial pressure of the undissociated state); C_1 is a constant value, e is the electron charge; k is the Boltzman constant; and T is the absolute temperature.

An important effect induced by charges is that ψ_o is a function of the dissociation degree α of the acidic groups [22]:

$$\psi_o = \frac{2kT}{e}\text{arcsinh}\frac{C_2\,\alpha}{\sqrt{\mu}A} \quad (2)$$

where A is the superficial area per charged group and C_2 summarizes the constant values.

With Eq. 2 we can now relate π and ψ_o directly, either to the medium's μ, or considering the coupling between the dissociation degree and H^+ concentration to the medium's pH.

These equations show that an increase in the medium's μ leads to a decrease in ψ_o and in Δπ, as a consequence of the enhanced screening of the charges. In other words, any μ increase causes a rise in the shape index as experimentally demonstrated for values higher than the physiological one.

However, the equations do not explain the opposite tendency; for μ values lower than the physiological one, hence, they are unable to predict the change of response displayed in Fig. 1.

Finally, considering the glycocalyx as a polyelectrolyte anchored to the membrane with 2107 charges uniformly spread over an area of 137 μm^2 and 5.5 nm depth (charge density -3.5×10^6 A s/m^3), the calculated distance among charges are of the order of the Debye–Hückel's value [20], and even shorter for low μ values. Thus it was possible to define, with the aid of the polyelectrolyte theory [24], the dissociation constant of the glycocalyx as a whole:

$$K_{\text{glycocalyx}} = K_{\text{sialic}}\times\exp\left(\frac{e\psi_o}{kT}\right) \quad (3)$$

where $e\psi_o$ is the electrical work needed to draw a H^+ out of a negatively charged surface.

Being the sign of e and ψ_o opposite, the exponent becomes negative; consequently, the equation predicts that the polyelectrolyte's dissociation increases when ψ_o decreases. The penetration of positive ions into the glycocalyx leads to a decrease in ψ_o and to an impediment to the access of H^+. This could induce an increase in the dissociation degree and, through its coupling with H^+ concentration we get:

$$\frac{\alpha}{1-\alpha} = K_{\text{glycocalyx}}\times(H^+)^{-1} \quad (4)$$

The above discussion, could explain why the me-

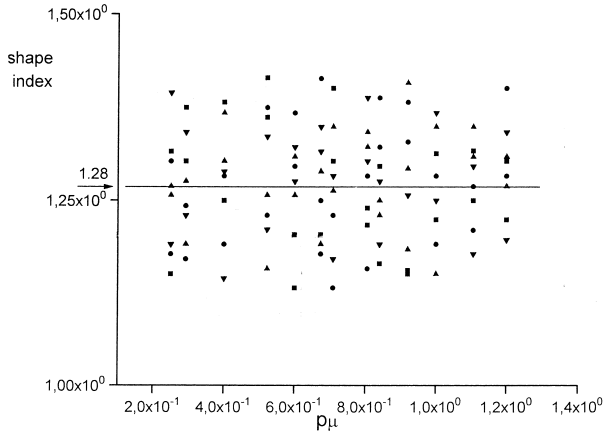

Fig. 4. Neuraminidase treated cells: washed cells were suspended in PBS containing 5 mM Ca^{2+} and 0.2 U of neuraminidase (*Clostridium perfringens*, Sigma) per ml of packed cells. The suspension was incubated at 37°C for an hour, the erythrocytes were removed by centrifugation and washed twice. Desialated cells show a mild stomatocyte shape (index 1.28), indicating that surface charges are needed to maintain the normal discocytic shape. The shape of these cells do not show a defined trend dependent on μ. Data from four separate experiments are shown.

dium's μ does not lead to the same trend under different conditions. An increase in μ always causes a lowering of ψ_o, due to the enhanced screening of the glycocalyx charges, but the decrease in ψ_o can be accompanied by a higher dissociation degree of the polyelectrolyte. These new surface charges could originate an extra electric repulsion that opposes to a potential diminution. Thus, for low μ values, i.e. when the number of positive ions in between the polyelectrolyte segment is small, the increase in the dissociation degree prevails over the screening effect. Therefore, an increase in μ causes an increase in π, the surface stretches and the stomatocyte index declines. On the other hand, for high μ values, when there is a large number of positive ions in between the polyelectrolyte segments, the dissociation does not provide enough new charges to compensate the enhanced screening effect, so the surface shrinks and the stomatocytic index rises.

This model also explains that, when the medium's pH increases, the minimum value moves towards lower μ ones (see Fig. 2), due to a higher dissociation degree. Moreover, for pH values ≥10, when the dissociation is almost complete, the model foresees that the minimum value is liable to disappear.

Furthermore, the cell shape response to pH changes through the combined effect of the pH, either on the cytoskeleton or on the membrane lipids, has also been attempted to explain. Hence, the pH variation might induce membrane lipid changes affecting RBC shape by disturbing the area balance between hemilayers, thus changing either the lipid distribution, or the lipid heads charged groups. Nevertheless, this hypothesis has not been proven [4].

The shape dependence on pH can be seen as a consequence of the coupling between the dissociation degree of the acidic groups and the H^+ concentration. The lower the pH, the fewer the charged groups, consequently, the weaker the repulsion. So medium acidification causes surface shrinkage and cell invagination.

Likewise, desialated cells show a mild stomatocyte shape (index −1.28) [17] indicating that surface charges are needed to maintain the normal discocytic shape. Moreover, the shape of these cells does not show a defined trend dependent on μ and pH (Fig. 4).

These experimental observations provide additional support to the hypothesis that μ and pH variations affect the cell shape because they affect the charged groups, at least in the present experimental conditions.

In conclusion, our results point out the role of the glycocalyx in the erythrocyte shape. According to our theory the charges in the glycocalyx thickness can trigger membrane curvature changes in response to medium μ and pH variations. This response is determined by two factors: the screening effect of the medium ions and the charged groups degree of dissociation. At higher medium μ, an increase in salt concentration reduces the electrostatic repulsion, as a result of the enhanced screening, and the surface shrinks. At lower medium μ, the increase in salt concentration produces an increase in the dissociation of the charged groups originating fresh charges; the new electrostatic repulsion overcompensates the screening effect and the surface expands. However, when the dissociation is sufficiently advanced, the screening effect is no longer compensated and the surface shrinks. The medium pH affects the membrane curvature and the μ effect, because it modulates the groups dissociation. A similar behavior may be postulated for the cytoskeleton charged groups when the cytoplasmic μ and pH are changed.

Acknowledgements

We are very grateful to Marta Bravo Luna for help in preparing the manuscript.

References

[1] L. Backman, Shape control in human red cell, J. Cell. Sci. 80 (1968) 281–298.
[2] M. Bessis, Red cell shapes: an illustrated classification and its rationale, in: M. Bessis, R.I. Wed, P.F. LeBlond (Eds.), Red Cell Shapes, Springer, New York, 1973, pp. 1–23.
[3] A. Elgsaeter, A. Mikkelsen, Shapes and shape changes in vitro in normal red blood cell, Biochem. Biophys. Acta 1071 (1991) 272–290.
[4] M. Gedde, E. Yang, W. Huestis, Shape response of human erythrocytes to altered cell pH, Blood 86, (4) (1995) 1595–1599.
[5] M.P. Sheets, S.J. Singer, Biological membranes as bailer couple. A molecular mechanism of drug-erythrocyte interaction, Proc. Natl. Acad. Sci. USA 71 (1974) 4457–4461.
[6] Y. Lange, A. Gough, T. Steck, Role of the bilayer in the shape of the isolated erythrocyte membrane, J. Membr. Biol. 69 (1982) 113–123.
[7] H. Schmid-Schönbein, R. Grebe, H. Heidtmann, A new membrane concept for viscous RBC deformation in shear. Spectrin oligomeres complexes as a Binghan fluid in shear as a dense periodic colloidal system in bending, Ann. New York Acad. Sci. 416 (1983) 225–254.
[8] D. Lerche, M.M. Kozlov, V.S. Markin, Electrostatic free energy and spontaneous curvature of spherical charged layered membrane, Biorheology 24 (1987) 23–34.
[9] R. Grebe, G. Peterhänsel, H. Schmid-Schönbein, Change of local charge density by change of local mean curvature in biological bilayers membranes, Mol. Cryst. Liq. Cryst. 152 (1987) 205–212.
[10] S. Levine, M. Levine, K.A. Sharp, D. Brooks, Theory of the electrokinetic behavior of human erythrocytes, Biophys. J. 42 (1983) 127–135.
[11] Y. Lange, R. Hadesman, T. Steck, Role of the reticulum in the stability and shape of the isolated human erythrocyte membrane, J. Cell Biol. 92 (1982) 714–721.
[12] B. Deuticke, Transformation and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment, Biochim. Biophys. Acta 163 (1968) 494–500.
[13] R. Glaser, Echinocyte formation induced by potential changes of human red blood cells, J. Membr. Biol. 66 (1982) 79–85.
[14] E.M. Bifano, T.S. Novak, J.C. Freedman, Relationship between the shape and the membrane potential of human red blood cells, J. Membr. Biol. 82 (1984) 1–13.
[15] R. Grebe, H. Schmid-Schönbein, Tangent counting for objective assessment of erythrocyte shape changes, Biorheology 22 (1985) 455–469.
[16] R. Glaser, J. Donath, Temperature and transmembrane potential dependence of shape transformations of human erythrocytes, Biochem. Bioenerg. 27 (1992) 429–440.
[17] R. Grebe, H. Wolff, H. Schmid-Schönbein, Influence of red cell surface charge on red cell curvature, Pflügers Arch. 413 (1988) 77–82.
[18] A. Herrmann, P. Müller, R. Glaser, Shape transformation of erythrocyte ghosts depends on ion concentrations, Biosci. Rep. 5 (1985) 417–423.
[19] R. Glaser, The shape of red blood cells as a function of membrane potential and temperature, J. Membr. Biol. 51 (1979) 217–228.
[20] E. Donath, V. Pastushenko, Electrophoretical studies of cell surface properties. The influence of the surface coat on the electric potential distribution and on general electrokinetic properties of animals cells, Bioelectrochem. Bioenerg. 6 (1979) 543–554.
[21] A. Herrmann, G. Lassmann, T. Groth, E. Donath, B. Hillebrecht, Conformational alterations within the glycocalyx of erythrocytes membranes studied by spin labelling, Biochim. Biophys. Acta 861 (1986) 111–121.
[22] F. Jähnig, Electrostatic free energy and shift of the phase transition for charged lipid membranes, Biophys. Chem. 3 (1976) 309–318.
[23] H. Träuble, M. Teubner, P. Woolley, H. Eibl, Electrostatic interactions at charged lipid membranes. Effects of pH and univalent cations on membrane structure, Biophys. Chem. 4 (1976) 319–342.
[24] A. Katchalsky, Theory of dissociation of polyelectrolytes, J. Polym. Sci. 7 (1951) 393–412.