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Effects of alkanols, alkanediols and glycerol on red blood cell shape and hemolysis

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Abstract

The physicochemical effects of a series of alkanols, alkanediols and glycerol on erythrocyte shape and hemolysis at 4 and 20°C were examined. We calculated the dielectric constant of the incubation medium, D_s, and the dielectric constant of the erythrocyte membrane D_m in the presence of organic solutes. The ratio $D_s/D_m = -38.48$ at 20°C defines the normal biconcave shape in a medium without hemolytic agents. A decrease in D_s/D_m favors externalization or internalization with consequent hemolysis. Alkanols and alkanediols convert biconcave erythrocytes into echinocytes, which is accompanied by an increase in the projected surface area. Glycerol converts biconcave erythrocytes into stomatocytes, which was accompanied by a marginal decrease in the projected surface area. Progressive externalization in alkanols and alkanediols or internalization in glycerol resulted in a decrease in the projected surface area and the formation of smooth spheres. The degree of shape change induced was related to the degree of hemolysis and the ratio D_s/D_m. A decrease in temperature reduced both the degree of shape change and hemolysis. Our results suggest that physicochemical toxicity may be a result of a temperature dependent hydrophobic interaction between the organic solutes and the membrane and is best interpreted by the ability of the solutes to change D_s and D_m. These results are discussed with respect to the physicochemical constants of the organic solutes.

Keywords: Erythrocyte; Hemolysis; Polyol; Cell shape; Dielectric constant; Partition coefficient; Cryoprotectant toxicity

1. Introduction

Cryoprotectant toxicity has been considered ''the central problem blocking successful cryopreservation by vitrification [1]''. Several attempts have been made to define general principles in formulating less toxic cryoprotectant solutions. Most are based on the assumption that toxicity is directly related to the cryoprotectant concentration. Complex solutions have been used in order to decrease the concentration of any one component. It has been shown that a mixture of glycerol and propylene glycol is less toxic to mouse embryos than is glycerol or propylene glycol alone [2]. Vitrification solutions designed by Sakai and co-workers for plant cells are multi-component mixtures of glycerol, ethylene glycol, propylene glycol, dimethyl sulfoxide (DMSO) and sucrose [3].

Cryoprotectant toxicity can be reduced through the addition of 'toxicity neutralizers', which appear to block the toxic effects of some cryoprotectants. Acetamide and formamide have been found to reduce the toxicity of DMSO [1]. Another approach to create less toxic vitrification solutions is to optimize the osmolality of the medium, and thereby reducing injury due to cellular dehydration. It has been experimentally shown that survival of rye protoplasts is a function of osmolality rather than the concentration of the vitrification solution [4]. In general, Fahy and co-workers [1,5] suggest that cryoprotectant toxicity at high concentrations is a result of both osmotic and biochemical injury.

The present study was designed to elucidate non-osmotic physicochemical mechanisms of the toxic action of differ-

Abbreviations: DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; ε, dielectric constant; v_m, molar volume; K, ether/water partition coefficient; D_s, dielectric constant of the solution; D_m, dielectric constant of the membrane; MI, morphological index.

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ent cryoprotectants on erythrocytes. Our interest was directed toward the shape changes involved in the process of hemolysis, and the possible role of hydrophobic forces governing them. It is well established that erythrocytes undergo shape transformations when subjected to various physical and chemical factors. ATP depletion, Ca^{2+} loading, pH changes, albumin, and a variety of amphiphilic agents have been shown to convert normal discs into echinocytes or stomatocytes [6–10]. Echinocytes are thought to be induced by forces tending to produce evaginations of the plasma membrane, while stomatocytes are thought to be a result of invagination forces. Most evidence suggests that both membrane and cytoskeleton are involved in shape regulation [11].

In the work presented here, we demonstrate the physicochemical effects of different cryoprotectants: methanol, ethanol, ethanediol, propanediol, and glycerol on erythrocyte shape and hemolysis. Using the physicochemical constants of the tested solutes, we calculate D_s and D_m in their presence. Toxicity is studied in relation to the ability of the solutes to change the ratio D_s/D_m.

2. Materials and methods

2.1. Reagents

Methanol, ethanol, 1-propanol (n-propyl alcohol), 2-propanol (iso-propyl alcohol), ethanediol (ethylene glycol), 1,2-propanediol (propylene glycol), glycerol (propanetriol), bovine serum albumin (BSA) and Tris were reagent grade or better and obtained from Sigma (St. Louis, MO).

2.2. Erythrocyte preparation and incubation procedures

Packed human erythrocytes from normal donors were obtained from the National Naval Medical Center (Bethesda, MD) within 48 h of donation and used within 7 days. Packed erythrocytes were washed in 20 mM Tris, 140 mM NaCl (pH 7.4) before use and suspended in the incubation media at a concentration of 5% hematocrit. The incubation media contained different concentrations of alkanols (methanol, ethanol, 1-propanol, 2-propanol), alkanediols (ethanediol, 1,2-propanediol), or alkanetriols (glycerol), in 20 mM Tris (pH 7.4), 140 mM NaCl. In all preparations the final concentration of NaCl (140 mM) was kept constant assuring that the measured hemolysis was not a result of osmotic swelling. Addition of all tested solutes was done at room temperature in a stepwise manner, increasing the concentration by 1 M in each step. Samples were incubated 15 min between the different steps when slowly permeating alkanediols or glycerol were added. Slow addition of the tested alkanols, alkanediols and glycerol was necessary in order to exclude osmotic injury as a possible course for the toxic action of the tested solutes. Concentrations higher than 55% (v/v) were not used in this study to ensure that osmotic injury was excluded. This concentration was determined by measuring the volume restoration following the 15 min incubation time between the different steps (data not shown). After the addition procedure was completed, samples were incubated for 30 min or longer at 20 or 4°C and processed for hemolysis measurement or microscopic examination.

2.3. Microscopy

Samples were prepared as described above with the addition of 1% (w/v) BSA to the solution. BSA was added to prevent the crenation of the erythrocytes due to the glass effect [11].

Small aliquots of the samples were examined using an Olympus VANOX-SAH-2 light microscope, equipped for Nomarski Differential Interference Contrast (DIC), using a 0.9 NA 40 × planapo objective. In order to describe the erythrocyte shape, we used the morphological classification designated by Tatsuzo et al. [12]. The transformed shape stages of externalization or internalization were graded on a scale from I to IV. They were given scores of 1 to 4 with plus or minus sign for externalization or internalization, respectively. The morphological index was calculated by multiplication of the score corresponding to the shape of transformed cells to total cells and addition of the values [12]. We also used the widely accepted terms echinocytosis and stomatocytosis for externalization or internalization, respectively. A minimum of 100 cells were scored for each sample and a normal distribution was always observed.

2.4. Image processing

A commercially available image processing and data analysis software system, Visilog, release 4.3 (Noesis Vision, Quebec, Canada) was used to analyzed the images. Computer image processing and analysis system has been described elsewhere [13]. The parameter measured was the cell's projected surface area. It should be noted that in case of invaginations the actual surface area is larger than the projected surface area.

2.5. Hemolysis

Hemolysis was measured after a 30 min incubation in the tested organic solutes at 20 or 4°C in the absence of BSA. After centrifugation (Eppendorf Microfuge at 7000 × g for 5 min) equal volumes of supernatants were mixed with Drabkin reagent and suitable dilutions were read at 540 nm using a Gilford Instruments spectrophotometer. Percent hemolysis was determined by the expression:

$$\%\text{hemolysis} = \left[\text{Abs}_{\text{sample}}/\text{Abs}_{100\%\text{lysis}}\right] \times 100, \quad (1)$$

where $\text{Abs}_{\text{sample}}$ = absorbance at 540 nm of the test sample and $\text{Abs}_{100\%\text{lysis}}$ = absorbance at 540 nm of a lysed standard sample.

2.6. Calculations

The dielectric constant of the erythrocyte membrane in the presence of organic solutes can be calculated using the Clausius-Mossotti equation for liquid mixtures as described by Orme and co-workers [14]. The first step is to calculate the membrane volume fraction of the solute of interest $f(C)$.

$$f(C) = v_a C_m = v_a KC, \quad (2)$$

where v_a is the molar volume of the solute, C_m is the solute concentration within the membrane, K is the ether/water partition coefficient, and C is the aqueous concentration of the solute. Directly measured erythrocyte membrane/water partition coefficients are not available for all solutes used in this study.

The second step is to apply the Clausius-Mossotti equation as follows:

$$Q = f(C)(\varepsilon_s - 1)/(\varepsilon_s + 2) + (1 - f(C))(\varepsilon_m - 1) /(\varepsilon_m + 2). \quad (3)$$

$$D_m(C) = (2Q + 1)/(1 - Q), \quad (4)$$

where ε_s and ε_m denote the dielectric constants of the solute and the native membrane, respectively. D_m is the dielectric constant of the membrane in the presence of the tested solute.

Replacing $f(C)$ in the above equations with the volume fraction of the solute in the incubation solution, and ε_m with the water dielectric constant, the dielectric constant of the solution D_s can be calculated. It was assumed that at the concentrations used in this study NaCl, Tris do not change the dielectric constant of the incubation solution and the membrane significantly. Molecular weight and density values of the solutes tested were taken from the CRC Handbook of Chemistry and Physics [15] and used to calculate their molar volumes. Values for the solutes and water dielectric constants were obtained from the same source and extrapolated to 20°C. The dielectric constant of the native membrane was assumed to be 2.1 [14]. The ether/water partition coefficients were obtained from Naccache and Sha'Afi [16].

3. Results

3.1. Hemolysis in the presence of alkanols, alkanediols, and glycerol

Erythrocyte hemolysis was measured after a 30 min incubation in methanol, ethanol, 1-propanol, 2-propanol, ethanediol, 1,2-propanediol and glycerol at 20 and 4°C. Our goal was to determine the solute concentration in the aqueous medium at which each of the tested solutes produces the same degree of hemolysis. Fig. 1 summarizes the results of these experiments at 20°C. For example, 1.2 M of 1-propanol was as effective as 9.9 M of methanol, 6.1 M of 1,2-propanediol, or 9.83 M of ethanediol at inducing 5% hemolysis. Higher alkanols or alkanediols resulted in equivalent hemolysis at lower concentrations. Addition of a hydroxyl group in alkanediols reduced their toxicity compared with the corresponding alcohol. Glycerol at its highest concentration used in this study (7.53 M) was not equieffective with the solutes presented, inducing only 2% hemolysis after 30 min incubation at 20°C. The same sets of experiments as presented in Fig. 1 were performed at 4°C. Solutes at concentrations sufficient to induce 5 or 50% hemolysis at 20°C were not as effective at 4°C. Concentrations that induced 100% hemolysis at 20°C induced only 5 to 10% hemolysis at 4°C. These results agree with the work of Williams and co-workers [17] on toxicity of alkanols on erythrocytes.

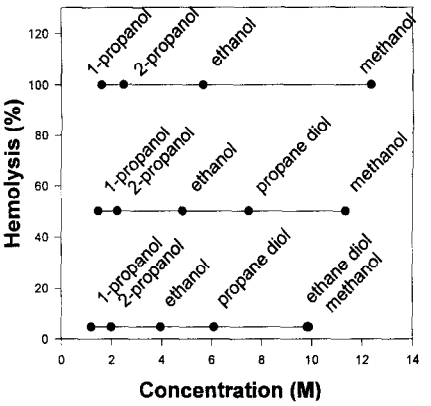

Fig. 1. Percent hemolysis at equieffective molar concentrations of different organic solutes. Equieffective concentration was assumed to be the concentration at which the same degree of hemolysis ±0.5% in different solutes is induced. Human erythrocytes were exposed to alcanols and alcandiols for 30 min at room temperature (20°C) at concentrations needed to induce 5, 50 and 100% hemolysis. Each point is the average of five independent experiments. S.D. were between 0.2 and 0.5% of the molar concentration.

Based on our results that hemolysis increases with increasing temperature and chain length of the tested organic solutes and as demonstrated by Williams et al. [17], the effect was related to the ability of the alkanols to act as surfactants. This suggests that the hydrophobic interactions are involved in membrane destabilization. We decided to take the dielectric constant of the incubation solution D_s as a measure for the solution hydrophobicity [18] and compare it to the effectiveness of different solutes. If the hydrophobicity of the solution was the only factor responsible for hemolysis, then equieffective concentrations would be expected to have the same D_s. We calculated the changes in D_s in the presence of the tested organic solutes at their equieffective concentrations from Fig. 1. The resulting values obtained are plotted against hemolysis in Fig. 2. The dielectric constant of the medium (water) without the tested solutes at 20°C is 80.2 (see Section 2.6). According to Fig. 2 hemolysis is accompanied by a decrease in D_s, compared to the dielectric constant of water.

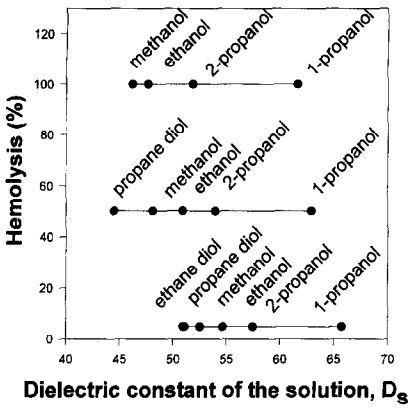

Fig. 2. Percent hemolysis at equieffective concentrations of different organic solutes is plotted against the calculated dielectric constant of the solution, D_s. The equieffective concentrations were taken from Fig. 1 and used to calculate D_s according to Eqs. 3 and 4.

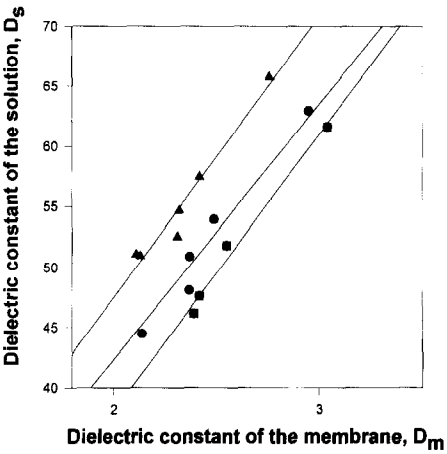

Fig. 4. Linear relationship between D_s and D_m at 5 (▲), 50 (●) and 100 (■) % hemolysis (r^2 were between 0.93 and 0.97). Data for D_s and D_m were taken from Fig. 2 and Fig. 3.

For the same solute, a lower D_s value corresponds to a higher degree of hemolysis. A decrease of D_s to a value of 65.75 in a solution of 1.2 M 1-propanol corresponds to 5% hemolysis. In the presence of equieffective concentrations of 2-propanol ($D_s = 57.47$), ethanol ($D_s = 54.66$), methanol ($D_s = 52.52$), 1,2-propanediol ($D_s = 50.94$) and ethanediol ($D_s = 51.08$), 5% hemolysis was induced at different D_s values. A comparison between the alkanediols and the corresponding alkanols showed that in presence of alkanediols the same degree of hemolysis was observed at lower D_s. The fact that different organic solutes induced the same degree of hemolysis at different D_s, suggests that D_s is not the only factor that determines the membrane destabilization in the tested solutes.

Our next step was to calculate the changes in D_m in the presence of the tested organic solutes at their equieffective concentrations (see Section 2.6). As presented in Fig. 3, hemolysis was accompanied by an increase in D_m, compared to its native value of 2.1 (see Section 2.6). For the same solute, higher values of D_m corresponded to a higher degree of hemolysis. At equieffective concentrations different solutes differed in their ability to change D_m. Alkanols induced same degree of hemolysis at a higher D_m than the corresponding alkanediols. For example, 1-propanol induced 5% hemolysis at $D_m = 2.42$, while 1,2-propanediol induced the same degree of hemolysis at $D_m = 2.13$. Ethanol was equieffective at $D_m = 2.32$, ethanediol at $D_m = 2.11$, where 5% hemolysis was observed. According to Figs. 2 and 3, alkanols were equieffective, with the corresponding alkanediols inducing the same degree of hemolysis due to a decrease in D_s, but an increase in D_m.

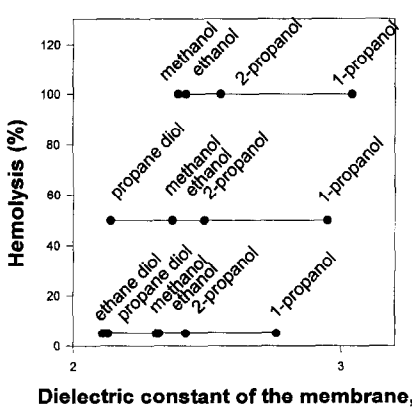

Fig. 3. Percent hemolysis at equieffective concentrations of different organic solutes is plotted against the calculated dielectric constant of the membrane, D_m. The equieffective concentrations were taken from Fig. 1 and used to calculate D_m according to Eqs. 3 and 4.

Based on these results, we propose that the ratio D_s/D_m is the parameter that best defines membrane destabilization. Fig. 4 shows a linear plot of D_s against D_m in the presence of equieffective concentrations of the tested organic solutes. A ratio $D_s/D_m = 38.48$ corresponds to the native membrane in a medium without the tested organic solutes. A decrease of this value to 23.65 ± 0.47 was accompanied with 5% hemolysis in all solutes tested. A further decrease to 21.11 ± 0.46 or 19.18 ± 0.49 corresponded to 50 or 100% hemolysis, respectively and was independent of the solute tested. In glycerol at the highest concentration used in this study (7.53 M), the ratio D_s/D_m is 26.35. This value was higher than the value needed to induce 5% hemolysis after 30 min incubation at 20°C. However, incubation of erythrocytes in glycerol at $D_s/D_m = 26.35$ for 24 h resulted in 85% hemolysis. It should be noted that the decrease in D_s/D_m in glycerol is only due to decrease in D_s without any change in D_m.

3.2. Morphology of erythrocytes in the presence of alkanols, alkanediols and glycerol

Human erythrocytes incubated with alkanols or alkanediols underwent shape changes from discocytes to echinocytes. In contrast, incubation in glycerol resulted in disc-stomatocyte transformation. The stages of erythrocyte

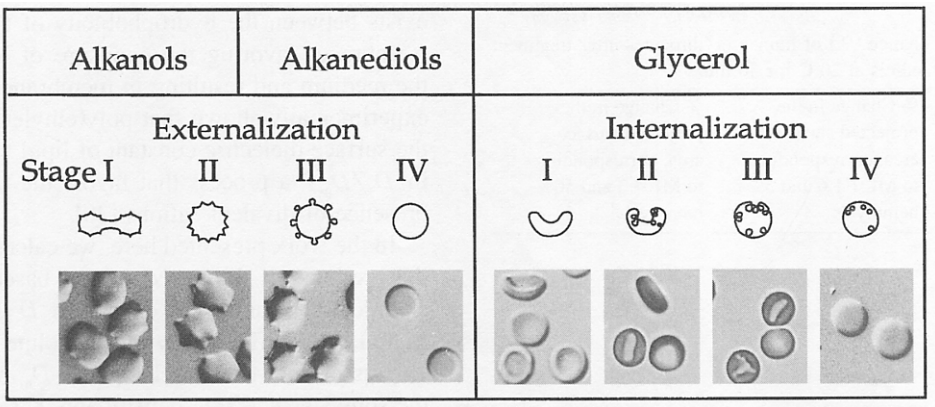

Fig. 5. Principal types of shape transformation of human erythrocytes after incubation in alkanols (methanol, ethanol, 1-propanol, 2-propanol), alkanediols (1,2-propanediol and ethanediol) and glycerol.

externalization and internalization are demonstrated in Fig. 5.

To examine the relationship between the morphological changes and hemolysis, the MI at equieffective concentrations of the tested alkanols and alkanediols was determined. Table 1 lists the results of these experiments. As seen from the table, the same degree of hemolysis was achieved at nearly the same degree of morphological change and was independent of the solute tested. An MI of 1.6 corresponded to 5% hemolysis. An increase in the MI was accompanied by an increase in hemolysis. It should be noted that changes in MI precede hemolysis. MI increases in alkanols and alkanediols from 0 (discocytes) to 1.2 without hemolysis. An agreement between morphology and hemolysis for short-chain alkanols with morphological changes preceding hemolysis was also observed by Lang-Ming Chi and co-workers [19].

In glycerol, hemolysis develops because of disc-stomatocyte transformation. Incubation for 30 min at 20°C in the highest concentration used in this study (7.53 M), glycerol induced an MI of −1.35 and 2% hemolysis. Incubation for 8 h decreased the MI to −1.8. 12 and 24 h incubation resulted in further decrease in the MI to −2.45 and −3.3, respectively, with a progressively increasing hemolysis (85% after 24 h incubation).

To test for the involvement of hydrophobic interactions in the process of erythrocyte shape transformation, we performed the same set of experiments as presented in Table 1 and in 7.53 M glycerol at 4°C. Concentrations needed to induce MI of 1.6 and 5% hemolysis at 20°C induced only a MI between 0.45 and 0.7 and no measurable hemolysis in the alkanols and alkanediols tested. In glycerol at 4°C, the MI was 0.36. The decreased temperature resulted a shift toward a lesser degree of externalization or internalization.

Changes in the projected surface area in the presence of alkanols and alkanediols at concentrations needed to induce 5 or 50% hemolysis are listed in Table 2. The initial shape change in alkanols and alkanediols (MI = +1.6) corresponding to the initiation of hemolysis was accompanied with an increase in the projected surface area. This increase was significantly higher in alkanols than in alkanediols. We explain this difference according to our findings that alkanols produce hemolysis and shape changes due mainly to change in D_m, while alkanediols produce such changes due mainly to a change in D_s. In contrast, the initial shape change in glycerol (MI = −1.35) needed to induce 2% hemolysis was accompanied with a slight decrease in the projected surface area by 3.22% (S.D. 2.5%). It should be noted that the projected surface area

Table 1
Morphological index and D_s/D_m ratio at different degrees of hemolysis in the presence of alkanols and alkanediols

	5% hemolysis		50% hemolysis		100% hemolysis	
	MI	D_s/D_m	MI	D_s/D_m	MI	D_s/D_m
Methanol	+1.52	22.71	+3.05	21.47	+3.9	19.3
Ethanol	+1.64	23.56	+3.08	20.78	+3.86	19.67
1-Propanol	+1.58	23.82	+2.89	21.33	+3.85	20.26
2-Propanol	+1.65	23.75	+2.9	21.67	+3.91	20.32
Ethanediol	+1.68	23.98	–	–	–	–
Propanediol	+1.56	24.14	+2.95	20.3	–	–

The erythrocyte concentration was 5% hematocrit and the cells were incubated with the organic solutes for 30 min at 20°C. The solute concentrations used correspond to the concentrations presented in Fig. 1 and needed to induce, 5, 50 and 100% hemolysis. Values are given as mean of three different experiments, where MI was defined based on 100 cells in each experiment. S.D. varied between 0.05 and 0.22.

Table 2
Projected surface area change (%) of human erythrocytes after treatment with alkanols and alkanediols at 20°C for 30 min

Category and name	% Change in the projected surface area, corresponding to MI +1.6 and 5% hemolysis	% Change in the projected surface area, corresponding to MI +3 and 50% hemolysis
Alkanols		
Methanol	+11.34%	−27.10%
Ethanol	+10.50%	−26.80%
1-Propanol	+10.85%	−26.67%
2-Propanol	+11.15%	−26.75%
Alkanediols		
Ethanediol	+3.38%	-
Propanediol	+5.57%	−26.62%

Values are given as mean, $N = 25$ cells. S.D. varied between 1.8 and 3.25%.

does not represent the actual surface area and in case of invaginations the actual surface area is underestimated. Assuming that the actual surface area is larger than the projected are and taking the high S.D. into account, we conclude that shape changes in glycerol take place without significant change in the surface area. This is in line with our finding that glycerol produces hemolysis without change in D_m. In all tested organic solutes including glycerol (data not shown), 50% hemolysis and the corresponding increased MI were accompanied by a significant reduction in the projected surface area. Such a decrease was a result due to exo- or endo-vesiculation. Surface area reduction after treatment with alkanols was also observed microscopically by Williams and co-workers [17].

4. Discussion

In the present study, the physicochemical effects of a series of alkanols, alkanediols and -triols on erythrocyte hemolysis and shape were examined. It was also shown that hemolysis was a result of progressive membrane externalization (in alkanols and alkanediols) or internalization (in glycerol) and was best determined by the ratio D_s/D_m. Erythrocyte shape was a result of delicate equilibrium between divergent forces [11]. The object was to describe this equilibrium in terms of the D_s and D_m. Assuming that the ratio D_s/D_m defines the erythrocyte shape, a $D_s/D_m = 38.48$ corresponded to the native discocyte in a buffer free of hemolytic agents. A decrease in this ratio due to a decrease in D_s or an increase in D_m represents a smaller difference between the hydrophobicity of the solution and the membrane. As a result a decrease in the D_s/D_m ratio favors the exposure of more membrane surface area to the medium or the membrane vesiculation (externalization or internalization as observed in this study). An increase in D_s/D_m means that a larger difference exists between the hydrophobicity of the medium and the membrane, favoring the exposure of less surface area to the medium and resulting in membrane fusion. It has been experimentally shown that poly(ethylene glycol) decreases the surface dielectric constant of lipid vesicles (an increase in D_s/D_m), a process that favors the membrane fusion in presence of divalent cations [20].

In the work presented here, we calculated D_s and D_m in the presence of the tested solutes based on their physicochemical constants. The change in D_s by a given concentration, was defined only by the solute dielectric constant, ε (Eqs. 3 and 4). The change in D_m was defined by all: the solute ε, v_m, M_w, density and K (Eqs. 3 and 4). From Eqs. 2 and 3, and Eq. 4, a solute with lower ε will induce higher decrease in D_s, while higher ε, v_m (high M_w, low density) and K will contribute to an higher increase in D_m. Comparing the toxicity action of both isomers used in this study: 1-propanol and 2-propanol, and based on their dielectric constants ($\varepsilon = 20.77$ for 1-propanol and $\varepsilon = 19.21$ for 2-propanol), densities ($D = 0.8035$ for 1-propanol and $D = 0.7855$ for 2-propanol) and v_m ($v_m = 74.78$ for 1-propanol and $v_m = 76.51$ for 2-propanol), one could expect the more hydrophobic alkanol with the larger molecular volume to be more toxic. Fig. 1 shows that the opposite of this was true. 1-propanol was more toxic compared to 2-propanol. The partition coefficient, K, for 1-propanol (1.9) was higher than the K for 2-propanol (0.64). This results in an increase in D_m in the presence of 1-propanol that contributes a critical D_s/D_m which was reached at a lower aqueous concentration. The importance of all physicochemical constants in predicting physicochemical toxicity was demonstrated by this point. Their significance was again displayed when alkanols and alkanediols are compared. Alkanediols compared to the corresponding alkanols induced the same degree of hemolysis by a decrease in D_s and an increase in D_m. In line with this finding, 1,2-alkanediols were reported to be more effective than 1-alkanols based on their membrane concentrations (D_m is a function of the solute membrane concentration see Eq. 4) in flip-flop acceleration in erythrocytes [21] and in increasing the cation permeability of lipid vesicles [22]. Schwichtenhövel and co-workers [21] also calculated D_m in the presence of alkanols and alkanediols and concluded that the calculated increases of D_m do not correlate with the increases of flip-rates. In this work we showed that it is not the change in D_m alone, but the change in the ratio D_s/D_m that best defines the effectiveness of a solute to destabilize membranes. The low K for alkanediols keeps their volume fraction within the membrane low and despite their high ε they induce a lesser increase in D_m than the corresponding alkanols. The decrease in D_s/D_m in alkanediols is achieved mostly by decrease in D_s.

Fig. 5 shows the typical shape changes in erythrocytes in the presence of alkanols, alkanediols and glycerol. As predicted by our theory, a decrease in D_s/D_m induced

internalization or externalization. Short-chain alkanols have been shown to induce echinocytosis in erythrocytes [19,23]. Our results are in agreement with the work of Lang-Ming Chi and co-workers, where they showed that the shorter the hydrocarbon chain of the alcohol, the higher the concentration needed to induce the same degree of shape change or hemolysis and an agreement between shape changes and hemolysis exists. In this work we demonstrated that the same degree of hemolysis is achieved at nearly the same MI in short chain alkanols and alkanediols.

It is tempting to seek an explanation for the difference in the shape-transforming ability between alkanols, alkanediols and glycerol. According to the bilayer couple hypothesis proposed by Sheetz and Singer [24], invagination and evagination are result of unequal expansion of the inner or outer leaflet of the bilayer. Our results showed that alkanols and alkanediols induce echinocyte formation, which was accompanied by an increase in the projected surface area, while stomatocyte formation in glycerol was accompanied with a slight decrease in the projected surface area. This suggests that alkanols, alkanediols and glycerol may govern cell shape in a manner similar to that proposed by the classical bilayer couple hypothesis contributing to an unequal surface area of the two leaflets of the bilayer [24]. It has been proposed that short-chain alcohols will concentrate in the outer leaflet of the membrane bilayer [21,25]. There is no information about the localization of alkanediols and -triols. However, as suggested by Schwichtenhövel and co-workers [21], a preferable localization of alkanediols would be the hydrophilic/hydrophobic interfacial region of the membrane because of their greater hydrophilic moiety compared to alcohols. Lang-Ming Chi and coworkers [19] demonstrated experimentally that echinocytosis and hemolysis in ethanol was accompanied by expansion of membrane lipids due to ethanol intercalation. A distribution in the outer leaflet of alkanols and alkanediols probably accounts for their echinocytosis-inducing ability. Using Eqs. 3 and 4, we calculated that, even at 100%, glycerol will cause only a marginal increase in D_m (from $D_m = 2.1$ to $D_m = 2.1024$).

Hemolysis in the presence of glycerol in our experiments was induced due to decrease in D_s/D_m by decreasing D_s. We propose that the decrease in D_s/D_m gives rise to externalization or internalization. Which one of these processes will take place depends on the way the decrease in D_s/D_m is accomplished. In the event that D_s/D_m is decreased without change in D_m, stomatocytosis will be favored. In the event that D_s/D_m is decreased with a significant change in D_m, echinocytosis will be favored. In other words, solutes that remain excluded from the membrane and reduce D_s/D_m due to reduction of D_s would be expected to be stomatogenic agents. Solutes that intercalate in the membrane and increase its D_m are expected to be echinogenic agents. This is in line with studies on distribution of cryoprotective agents into lipid interfaces, where glycerol is thought to act on the aqueous side of the monolayer [26].

Both hemolysis and shape changes were reduced at 4°C compared to 20°C. Since the ε and K are temperature-dependent, the observed reduction of toxicity with decreasing temperature was most likely the result of an increase in ε or a decrease in K, respectively. Based on this observation, we conclude that a temperature-dependent, hydrophobic mechanism is responsible for the cell shape alterations and hemolysis. These results correlate with studies performed on phospholipid bilayers [27] and microorganisms [28]. The solute-induced loss of vesicle contents was shown to involve a temperature-dependent, hydrophobic interaction between the organic solutes and the non-polar region of the membrane [27]. It has recently been shown that the hydrophobicity of a compound expressed as log K is a measure of toxicity [28].

In conclusion, we want to share some ideas that can help us in creating less toxic cryomedia. The design of less toxic cryomedia should involve the maintenance of the dielectric homeostasis of the medium and the membrane. An appropriate mixture of cryoprotectants that will keep the ratio D_s/D_m constant is desirable. Reduction in solute-induced toxicity by preserving D_s/D_m may also be achieved by adding the cryoprotective medium at lower temperatures. In line with these ideas are some already existing practical applications. In fact, it has been shown that formamide, with a dielectric constant, $\varepsilon = 109$ at 20°C, is higher than that of the water ($\varepsilon = 80.20$ at 20°C) and reduces the toxicity action of DMSO (ε of DMSO is below that of the water) [1]. This 'antitoxic' action is based on the ability of formamide to keep D_s in the presence of DMSO close to that of an aqueous medium without solutes. In addition, its low K (0.0014) will not contribute to an increase in D_m. Rall and Fahy [29] observed an increase in survival of mouse embryos if the addition of the cryomedia was done at −20°C, although this is usually performed at 0°C.

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