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Review

Membrane electropermeabilization effects of frequency and membrane surface order on liposome leakage

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Summary. Liposomes containing fluorescence marker were exposed to an alternating electric field of 80 V peak to peak square electric waves at different frequencies 0.01, 1, and 100 kHz to perturb the liposome permeation. The efflux of fluorescence dye after application of the electric field was measured by recording the fluorescence emission due to the complex formation reaction between the fluorescence dye and calcium ions in the bulk medium solution. Two independent sets of experiments were conducted: 1) calcium ions were present during electropulsation; and 2) they were added after electric field application. Two parameters, fluorescence emission intensity and increment of temperature of the solution in the chamber, were studied. The effect of membrane surface order on the fluorescence dye leakage from the liposomes was studied by addition of urea at threshold concentration before the liposomes sealed. The data demonstrate the existence of frequency dependency window at 1 kHz. Furthermore, the data were interpreted according to the theory of interactions of electromagnetic fields with highly polarized and deformed materials such as liposome particles. The urea caused an enhancement of the fluorescence dye leakage at frequency of 100 kHz. This effect could be explained as a decrease of the membrane binding rigidity due to the disordering effect of urea on the membrane lipid surface. Our conclusion is that the frequency and the membrane surface order are additional parameters that influence the processes of membrane electropermeabilization.

Key words: Membrane electropermeabilization, Liposome leakage, Electric field frequency, Membrane surface order

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Introduction

Recently, numerous studies have been devoted to the effects of electric field (EF) on suspensions of cells and phospholipid bilayers (Hubiniec et al., 1990; Hibino et al., 1991; Golzio et al., 1998; Rols and Teissie, 1998). It is well known that a bilayer lipid membrane is a good barrier for ions and hydrophilic molecules. Such a permeation barrier is readily modified by imposing a transmembrane electric potential exceeding the dielectric strength of the membrane. In this case the membrane-specific conductance increases dramatically, sometimes to values as high as 1 S cm^{-1} in microseconds. This effect is reversible: repetitive voltage scans did not alter the V-I characteristics of the membrane. Despite the information which has already been obtained in these studies concerning the effects of EF on membrane permeability, the process of electroporation of cell membrane as well as related phenomena remain unclear. EF intensity appears to be a crucial factor. The number of pulses and the pulse duration are two other parameters which have been shown to influence permeabilization efficiency. At a constant number of pulses and pulse duration, electropermeabilization is affected by the time between pulses. It has also been shown that the mechanism of transport across the membrane by EF treatment is a complex process. Some authors pay attention to the possibility that additional factors such as reorganization of membrane/solution interface are important in the induction of electropermeabilization (Lopez et al., 1998). It has been concluded that this is associated with a decrease in the membrane-surface organizing forces. A part of the energy required to permeabilize membranes may be used in such a case to modify the structural order of the interfacial water (Rols and Teissie, 1990; Neitchev et al., 1998). The thermodynamic implications of these conclusions have been checked by altering the membrane order. This process may be provoked at macroscopic scale by the presence of agents known to affect the structuring of water molecules at lipid/water interface (Rauch and Emmanuel, 2000).

On the other hand the EF may induce complex interactions with the membrane surface charges. The interactions of surface electric forces with dipole-dipole oriented water molecules near an interface are also of consideration. It is known that the membrane-aqueous interface may stabilize the structured bulk water by acting mainly as a momentum sink for thermal fluctuations which may otherwise disrupt the lattice stability latently present in bulk water (Hauser, 1975). The electropermeabilization of lipid suspensions deals with relaxation phenomena of liposome particles accompanying rapid Joule-heating in the solution. The latter may also cause lattice defects in the lipid structure. The lattice defects are not static structures but fluctuate with time scale of microseconds to minutes (Tsong, 1991), provoking the formation of a kink in a lipid molecule. The propagation of kink deformation occurs within 200 μs. After these rapid events there are slow, highly cooperative structure rearrangements lasting up to minutes (Genz et al., 1986). However, this process is connected with the energy transferred from the electric field to the electric charges in each infinitesimal volume of the solution. For the case of sinusoidal steady-state electromagnetic fields, accounting for the interaction on a macroscopic scale can be rather simply described as an alignment of already existing electric dipoles and movement of free charges. In liposome suspensions this process would be influenced by the frequency of the field (Durney and Christensen, 1999).

The aim of this study is to determine the effects of frequency of square wave EF and membrane surface order on the leakage of fluorescence dye from liposome suspension treated with alternating electric field.

Choice of a model and preparation of samples

Liposome suspension was used because of the wide extent of data obtained about electropermeabilization of lipid bilayers. Fluorescence dye, Quin 2 was preferred as a new calcium indicator with high selectivity against protons and other ions. Solution of urea at threshold concentration was used as a chaotropic agent perturbing the membrane surface order. For preparation of liposome suspension we used L-α-phosphatidylcholine from frozen egg yolk, commercial product of Sigma, catalog 1998 No. P-5763 without further purification. Quin 2 with molecular weight of 693.87 was purchased from Dojindo (Japan) in packing of 100 mg. Lipid, 100 mg/ml in 9:1 solution of chloroform/methanol, was dried under nitrogen gas flow in a round-bottomed tube. Then, required amounts of Quin 2 and urea in 10 ml distilled and deionized water were added to the dried lipid, and the mixture was vortexed for 60 min at room temperature until it formed a milky-white homogenous suspension. The resulted suspension was repeatedly extruded through a 0.1 μm pore-size polycarbonate filter to remove the giant liposomes, and then dialysed several times through a molecular porous dialysis membrane (Spectra/Pore MWCO:10,000) to exclude the free fluorescence dye outside the liposomes. The final liposome sample was assayed for lipid phosphorous according to a previously reported method (Kahovcova and Odavic, 1969) and stored at 5 °C in a refrigerator. It was used in one separate set of measurements. Four experimental sets of measurements were performed, two with included urea and two without urea. The final urea concentration in the stored suspension was 1M. Equal volumes of the stored suspension were taken consequently before the experiments and diluted to a volume with final lipid concentration of 2 mg/ml. The particle size distribution was monitored using a dynamic light-scattering size meter (Photal Otsuka Electronics LPA3 000, Japan).

Instruments and electric field treatment

A functional wave-forming generator, Model SG4101 (Iwatsu, Japan), and a voltage amplifier FLC-(Electronics) for creating square waves were used. The voltage applied between the electrode had an effective value of 80 V peak to peak. The chamber (3 ml capacity) was constructed from a round glass tube with diameter 1 cm and two wire Pt electrodes fixed at 1 mm distance. The experiment was performed under conditions of magnetic stirring. The enhancement of temperature due to the EF was measured before and after EF application by a sensitive miniature thermocouple type Delta (Sato, Keiryoki, Japan). EF of 30 seconds duration was applied to the sample in the chamber at frequencies of 0.01, 1 and 100 kHz. The electropermeabilization protocol was as follows: to a part (1.5 ml) of liposom suspension with concentration 2 mg/ml, 1.10^{-3} M, a solution of $CaCl_2$ was added in volume ratio 1:1. The mixture was incubated for several minutes, just prior to the EF application in the chamber. In a separate set of experiments $CaCl_2$ was added after the EF application on a sample containing only lipid suspension. Fluorescence intensities were then determined as follows.

Leakage test of fluorescence dye through liposome membrane

The EF effect on membrane permeabilization was examined by the test of leakage fluorescence dye molecules from liposomes to the dispersion medium. We used Quin 2 at a concentration of 1.10^{-2} M in water added to the dried lipid film before the liposomes sealed. (Tsien, 1980). The fluorescence intensity associated with application of electric field was an indicator of the leakage of Quin 2 molecules through the liposome membrane to the dispersion medium. The excitation wave length was 339 nm and detection was at 492 nm. Fluorescence intensities were measured in a Jasco FP-550 spectrophotometer and recorded automatically. A quartz optical cell with length of 1 cm was used. All experiments were repeated at least five times. Nontreated suspension of liposomes with added 2.10^{-3} M solution of

$CaCl_2$ in volume ratio 1:1 (total volume 3 ml) was control for absence of fluorescence dye leakage.

Liposome size and characterization

Liposome particles with embedded Quin 2 have a mean diameter of 250 nm. The particles with included urea are somewhat larger. They have a mean diameter of 330 nm as characterized by dynamic light scattering measurements. The particles were identified as large unilamellar vesicles obtained by extrusion through small pores (Hunter and Frisken, 1998). This diameter can not be significantly affected by electric field application. Measurements of liposome diameter after EF application for 30 seconds reveal a small, about 15%, increase of liposome diameter. It is not surprising, since in hyperosmotic solutions the liposome particles shrink due to the outflow of water. The shrinking reaches its maximum values within seconds after mixing the liposome suspension with a solution of $CaCl_2$ in the chamber. In the following minutes fluorescence dye leakage in the solution takes place, accompanied by liposome swelling due to the electroosmosis phenomena.

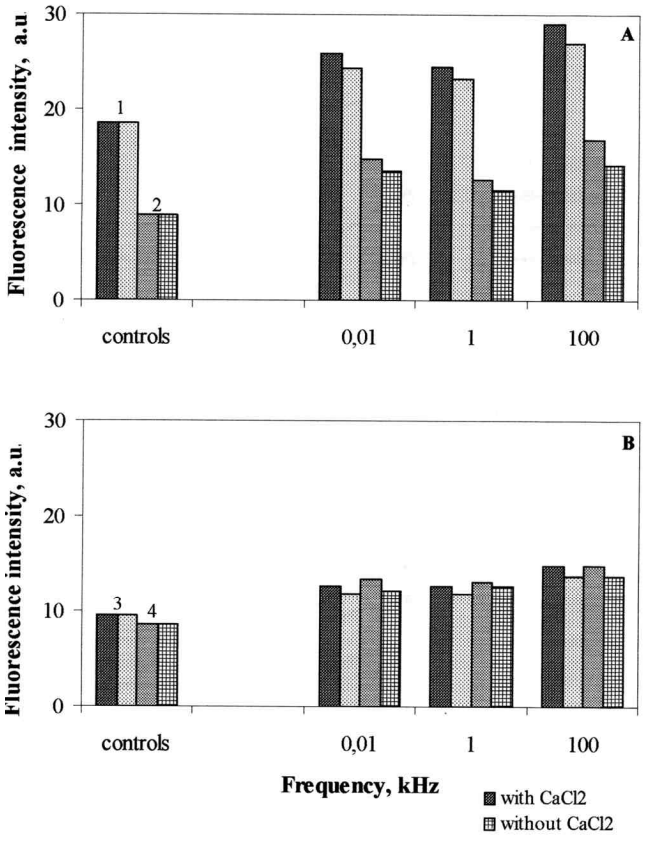

Fig. 1. Effect of electric pulse frequency on the leakage of Quin 2 from urea-treated liposomes **(A)** in two separate experiments 1 and 2, and from non-treated ones such **(B)** as in experiments 3 and 4. The control-bars in the cases with and without CaCl2 are the same. The statistical parameters are given in Table 1.

The electropulsation in hyperosmotic medium may however lead to a small increase in particle size (Tsong, 1991; Golzio et al., 1998).

Fluorescence dye leakage and effect of frequency

All data related to the leakage of Quin 2 were obtained as an increase of fluorescence dye intensity after EF treatment of the medium in the chamber at three different frequencies, in presence and absence of urea, as shown in Fig. 1A and Fig. 1B. The histograms show a permanent increase of fluorescence dye leakage in comparison to the controls. The maximal fluorescence leakage in the individual samples was achieved within 2.5-3 minutes after the wave application. The results also demonstrate higher values of Quin2 leakage in the cases when Ca^{+2} ions are present in the solution. This tendency is clearly expressed in both cases, with and without urea included in the liposomes. It is evident that Ca^{+2} ions in the solution influence the Quin 2 leakage from liposomes. The frequency dependence of Quin 2 leakage in Fig. 1A demonstrates a gradual decrease at frequencies of 0.01 and 1 kHz, and then an increase at 100 kHz. The latter is of particular significance

Table 1. Statistical parameters for data presented in Fig. 1 and Fig. 2. at significance level $p < 0.05$ for 8 experiences in presence (+) and absence (-) of $CaCl_2$ in the cell.

No. OF CONTROLS		SAMPLES FREQUENCY (kHz)		
		0.01	1	100
1.				
+ $CaCl_2$	mean: 18.6	26.0	24.6	29.0
	s.d. ±1.1	±1.3	±2.2	±2.4
	s.e. ±0.5	± 0.5	±0.7	±0.8
- $CaCl_2$	mean: 18.6	23.3	21.3	27.1
	s.d.± 1.1	± 1.6	± 1.1	± 1.9
	s.e.±0.5	± 0.6	± 0.4	± 0.5
2.				
+ $CaCl_2$	mean: 8.9	14.9	12.6	16.8
	s.d. ±0.9	±1.1	±0.8	±0.9
	s.e. ±0.4	±0.3	±0.4	±0.4
- CaCl2	mean: 8.9	13.6	11.6	14.2
	s.d. ±0.9	±1.0	±0.9	±1.3
	s.e. ±0.4	±0.4	±0.5	±0.5
3.				
+ $CaCl_2$	mean: 9.6	12.7	12.7	14.7
	s.d. ±1.2	±1.0	±0.7	±1.2
	s.e. ±0.5	±0.3	±0.4	±1.6
- $CaCl_2$	mean: 9.6	11.9	11.9	13.4
	s.d. ±1.2	±1.3	±1.6	± 0.9
	s.e. ±0.5	± 0.6	±1.7	±0.4
4.				
+ $CaCl_2$	mean: 8.7	13.4	13.2	14.8
	s.d.±1.2	±1.5	±1.4	±1.1
	s.e.±0.5	±0.5	±0.6	±0.4
- $CaCl_2$	mean: 8.7	12.2	12.4	13.9
	s.d.±1.2	±1.2	±0.8	±0.5
	s.e.±0.5	±0.5	±0.3	±0.2

considering the fact that urea is included in the liposomes. The percentage of Quin 2 leakage in this case increased by an average factor of 1.9 (with $CaCl_2$) and 1.7 (without $CaCl_2$) in comparison to the data valid for frequency of 1 kHz; 1.4 (with $CaCl_2$) and 1.3 (without $CaCl_2$), respectively.

In contrast to this finding the results in Fig. 1B obtained in absence of urea demonstrate a different tendency. There is no visible difference in fluorescence changes at frequencies of 0.01 and 1 kHz. As before, the fluorescence intensity increased at a frequency of 100 kHz in a similar manner to that in Fig. 1A. The percentage of Quin 2 leakage was higher in comparison to the results for 1 kHz by a factor of 1.6 (with $CaCl_2$) and 1.5 (without $CaCl_2$) against 1.4 and 1.3 respectively. These data confirm that the applied EF may affect the process of liposome leakage under the conditions of our experiment. They also collectively suggest a dependence of fluorescence dye leakage on the frequency of the applied electric waves. The presence of urea in the liposomes undoubtedly plays an additional role in this phenomenon.

Joule-heating of the sample solution

A rapid thermal heating from 5 to 7 °C of the medium in the chamber was recorded after the EF application. In Fig. 2A,B dependencies of temperature increments, $\Delta T = T_2 - T_1$ are given as a function of frequency in four separate experiments, where T2 is the measured temperature after EF application, and T1 is the initial temperature in the chamber. As can be seen, there is a correlation with the results found for fluorescence dye leakage at different frequencies. In Fig. 2A the values of ΔT in presence of urea decreased gradually with increasing the frequency from 0.01 to 1 kHz. The changes are negligible (about 1 °C or less). More obvious changes were registered at a frequency of 100 kHz. The values of increment in this case increased by average factors (from both experiments 1 and 2) of 1.7 (with $CaCl_2$) and 1.9 (without $CaCl_2$) in comparison to the same data for 1 kHz equal of 1. Fig. 2B shows the temperature increment changes in absence of urea in two separate experiments (3 and 4). A noticeable feature of the dependence of temperature increment on the frequency is the absence of changes at values of 0.01 and 1 kHz. A slight rise in the temperature was observed at a frequency of 100 kHz. The corresponding values at 100 kHz are higher than those at 1 kHz by a factor of 1.4 (with $CaCl_2$) and 1.3 (without $CaCl_2$) which shows a similarity with the results for fluorescence dye leakage in Fig. 1B. We could not find a correlation between the percentage of Quin 2 leakage and temperature increment. There is accumulating evidence to show that higher values of temperature increment correspond to higher values in fluorescence dye leakage as can be seen in Fig. 3. However, one would expect this effect, and it was found by other authors in cultured cells (Kinosita and Tsong, 1977).

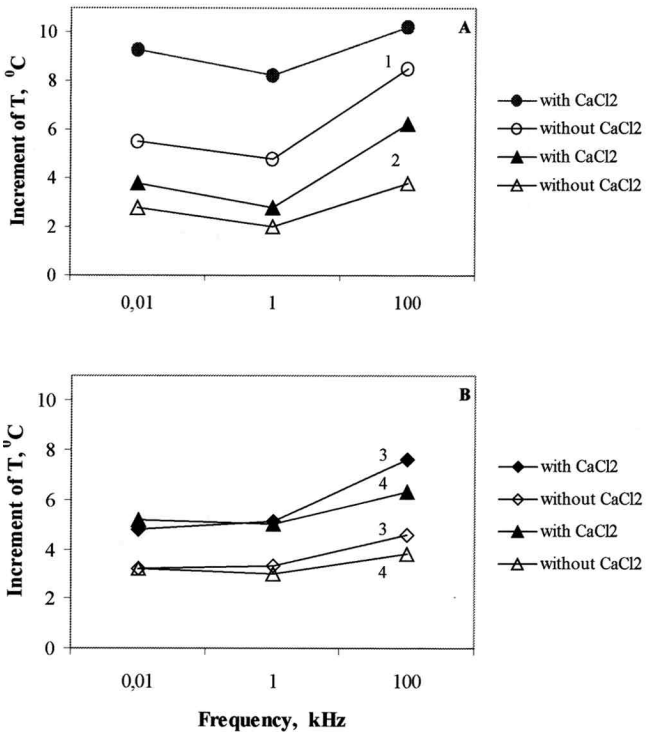

Fig. 2. Effect of electric pulse frequency on the increment of temperature in the cell. Urea-treated liposomes in experiments 1 and 2 **(A)** and non-treated liposomes in experiments 3 and 4 **(B)**. Each data point is the mean of at least five separate experiments. Error limit being 2%.

Leakage is due to the field-dependent perturbation of the liposome membrane

Membrane electropermeabilization (MEP) is an electrical technique to render lipid membranes porous and permeable, transiently and reversibly, by external voltage pulses.

A generally accepted term describing this technique is "electroporation". Recently, electroporation has gained increasing importance in cell biology, biotechnology (Neumann and Kakorin, 1996) and medicine, in particular in the new field of electroporative chemotherapy (Heller et al., 1996). MEP of small molecules depends on several physical parameters. The EF intensity is the deciding parameter inducing MEP. An increase in the number of electric waves enhances the rate of MEP. The electric wave duration is also crucial for the MEP. The number of waves and duration define EF frequency. The frequency effect on the transfer of different molecules by electric fields remains unclear. As a rule, efficiency of cell electropermeabilization to small molecule is reduced by increasing the frequency (Rols and Teissie, 1998). In our study the change of frequency means that at a constant duration (30 sec) we change the number of electric square waves. Optimum conditions

for the effect of frequency on the electropermeabilization have been obtained, mostly for incorporation of macromolecules into cells in the low frequency range 0.1 to 500 Hz (Bolognani et al., 1992; Rols and Teissie, 1998; Nikolova et al., 2000). In experiments with liposomes, the frequency range above 500 Hz is interesting because of the appearance of electroacustic waves in colloidal suspensions (Stoimenova et al., 1996). Our starting point for discussion is based on the assumption that any electric field-dependent effects are highly intensified within or on the surface of the membrane (Kinosita and Tsong, 1977; Tsong, 1991). For lipid liposomes with size above 100 nm. many authors propose an electro-deformation mechansim as a possible explanation for some anomalies in the behaviour of liposomes under conditions of electropermeabilization. As shown in this study, possible effects of the alternating electric field can be classified into those due primarily to the external applied field in the chamber, which result in a large transmembrane potential and those resulting from the gross Joule-heating of the medium. In our case the applied electric field generates a transmembrane potential lower than 1V. Synthetic phospholipid bilayers are known to break down at a transmembrane potential of a few hundred mV (Kinosita and Tsong, 1977). Our conclusion is that the effect of Quin 2 leakage from liposomes is a result of the field-induced transmembrane potential. The heating of the medium in the chamber is due to the high electroconductance of the salt aqueous solution and to electrode effects. The, membrane as a poor heat conductor, can sustain the temperature gradient generated by the Joule-heating. In such a case a thermal osmosis effect may appear, and the resultant flow of water and ions across the membrane might create a pressure difference which disrupts the liposome particle. Experimental similarity between the fluorescence intensity changes (Fig. 1) and those of increment of temperature (Fig. 2) as a function of frequency, suggests that the energy dissipation of EF by the sample is the main reason for these phenomena.

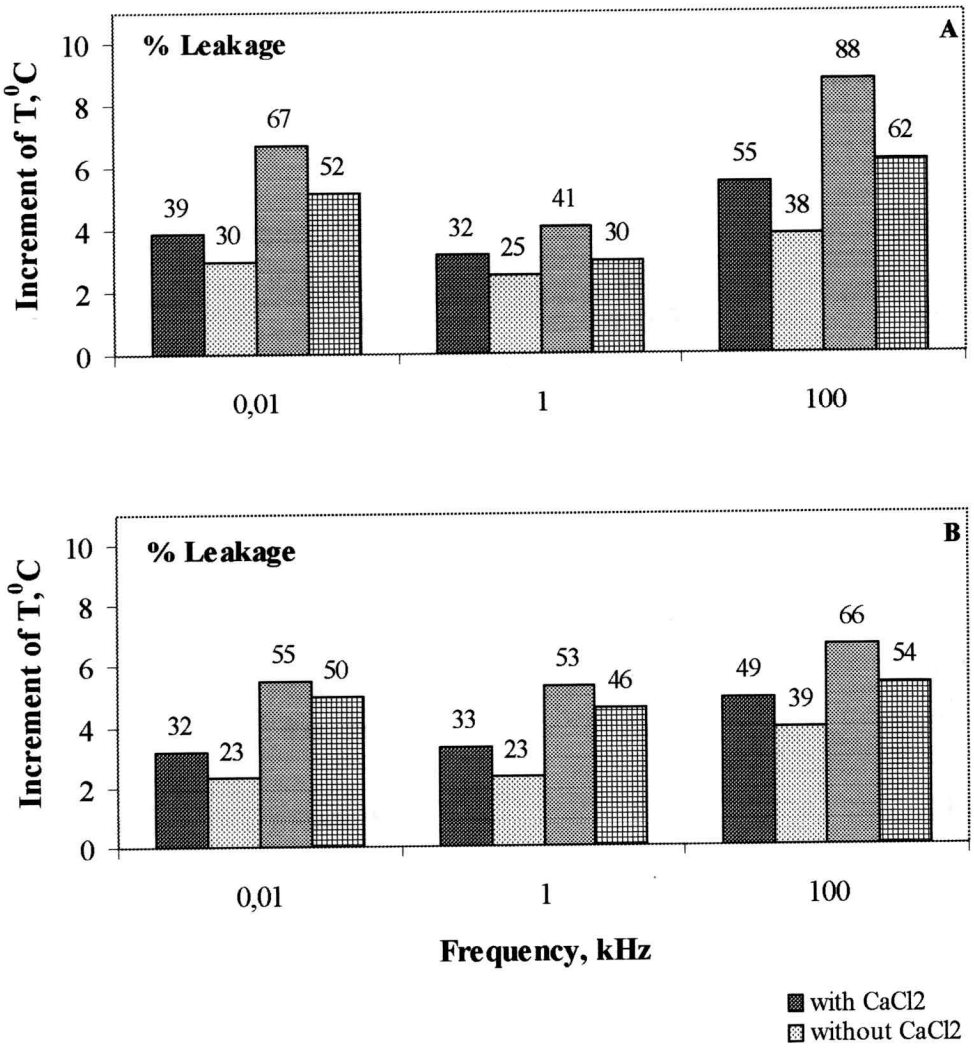

Fig. 3. Changes in temperature increment at different % leakages of Quin 2 from liposomes plotted against the pulse frequency.

However, we proceed to discuss the results obtained in view of the recent knowledge about MEP. The thermally-induced perturbation and eventual rupture of lipid membrane may have an additional effect on the fluorescence dye leakage. Firstly, will explain the differences in the data in presence and absence of $CaCl_2$ in the solution. Obviously, the presence of 1.10^{-3} M $CaCl_2$ in the chamber increases the ionic strength and osmolarity of the medium. An increase of the ionic strength may enhance significantly the electroconductivity of the medium. The data of some authors show that the field-induced change in the suspension conductivity is 10- to 100-fold larger than that due to zero-field leakage of electrolyte from the lipid vesicles (Kakorin et al., 1998). The most prominent of the electric field-induced effects is the eventual rupture of the electroporated particles. This phenomenon has been studied in great detail for erythrocyte particles by Kinosita and Tzong (Kinosita and Tsong, 1978, 1979) and recently for different model systems mainly by Neumann (Kakorin et al., 1998), and Seifert (Shillcock and Seifert, 1998). These authors showed that the Joule-heating effects and osmotic imbalance appear to be additional forces leading to membrane rupture. The rupture may influence the leakage of Quin 2 from liposomes in presence of alternating EF through the electrode processes. The free PC liposomes were electronegative. In presence of urea they are positively charged, indicating that urea molecules are attached to the liposome surface.The permeant Quin 2 molecules in all cases (absence and presence of urea) have a negative charge at neutral pH, and during application the field may give an electrodiffusive component.

From recent studies it seems that the diffusion, although facilitated through electroporation, is partially "hindered" by interaction of dye with the edge lipids of the eventual pore states in the membrane. In this study we do not mention some effects as electroaggregation and electrofusion of liposomes. The presence of both 1M solution of urea, producing a positive charge of liposome particles, and 1.10^{-3} M $CaCl_2$, should prevent any electroaggregation or electrofusion. However, there is sufficient useful information concerning these phenomena. Nowadays it is generally accepted that the EF-induced increase in the leakage of ions and larger molecules from liposome suspensions is due to the electric field. Additionally, the heating of the medium solution in the chamber may cause influx of water and ions across the membrane in an opposite direction through the thermal osmosis effect. This effect might be in some cases greater. The liposomes swell and, eventually, the membrane ruptures, thus leading to fluorescence dye leakage.

Role of EF frequency and urea

Recently some authors pay attention to the eventual role of additional factors such as electric field frequency and reorganization of membrane/liquid interface which is of importance in the processes of MEP (Rols and Teissie, 1998). In colloidal suspensions there are some threshold frequency windows responsible for formation of concentrated domains of particles. So, the factors efficient for the application of weak external field, play a role thus causing enhancement of 10^2-10^4 times. This enhancement mode could be important for electroporation. However, there is essentially no information concerning the effect of membrane surface reorganization. In our experimental conditions this is demonstrated by altering the membrane surface order using reagents known to affect the structuring of water molecules at lipid/water interface. The reagent used in this study was urea. It is known that 1M solution of urea added to liposome suspensions may have a very strong disordering effect on the interfacial structured water (Neitchev et al., 1998). The mechanism of this phenomenon is not well understood. Our data in this study demonstrate differences in the dependence of fluorescence dye leakage and increment of temperature as a function of the frequency in the cases of urea-treated and untreated liposomes. The character of these dependencies is significantly different, particularly in samples treated with urea at 100 kHz in comparison to those untreated. Certainly the presence of urea in the liposomes appears important in our explanation of the frequency effects on the leakage from liposomes. We found the existence of frequency window at 1 kHz. In particular, urea-treated liposomes in the frequency range between 0.01 and 1 kHz showed a gradual decrease in the leakage and temperature increment. At the same time the untreated liposomes showed no change in this frequency range. The maximum changes in the leakage and temperature increment were obtained with urea-treated liposomes perturbed with an electric field at 100 kHz frequency. The significance of the differences between urea-treated and untreated liposomes and electric field frequency was evaluated as reported in the theory of interactions of electromagnetic fields with high polarized and deformed materials as liposome particles (Durney and Christensen, 1999). The interaction is described microscopically in terms of three effects of the fields on the charge of the particle: induced polarization, alignment of already existing electric dipoles, and movement of "free" charges. The electric forces acting on the liposome membrane deform the particle. The efflux of fluorescence dye from the liposome to the bulk solution occurs until the curvature-elastic forces opposing the Maxwell stress electric forces on the liposome membrane are balanced. The steady-state amplitude of the deformation, corresponding to the balance of the electric and curvature-elastic forces, may depend only on the electric field strength and the membrane bending rigidity (Kakorin et al., 1998). The latter is actually known to depend on factors such as hydrophilic interactions. Such interactions can be recognised in the propensity of certain molecules and groups to be water soluble and to repel each other strongly in water. When urea is dissolved in water at

high concentration, it can be so effective in altering or disrupting the protective structured water layer around lipid bilayer, as to lead to "chaos" in water structuring at solid/liquid interface (Israelachvili and Wennerstrom, 1996). It is clear that such a situation may be favourable for the effect of the frequency of the applied field through additional redistribution or displacement of surface charges on the liposome particles. The interpretation of this phenomenon in terms of the classical theory of electromagnetic field (EM) interactions with polarized materials is given by the following equation for the specific absorbance rate, (SAR) in an infinitesimal volume (Durney and Christensen, 1999):

$$SAR = \sigma \text{ eff } E2 / \rho \text{ (W kg}^{-1}\text{)} \quad (1)$$

For the special case of EF with square waves σ eff = ($\sigma + \omega\varepsilon''$) is the effective conductivity measured in (S m^{-1}): E is the peak value of applied electric field, in Vm^{-1} and ρ is the mass density in kg m^{-3}, ω is the radian frequency in (r s^{-1}) and ε" is the dielectric constant of material. As SAR determines the energy lost by the electric field in an infinitesimal volume element ΔV of the material, from equation (1) it is clear that SAR varies both with ω and E. In our case SAR is proportional to the changes of temperature increment due to the applied electric field in the cell. Our results in Fig. 1 and Fig. 2 show good correlations between the changes of Quin 2 leakage from liposomes and changes of increment of temperature as functions of frequency. In such a case, at given E, the frequency should have an additional effect on the changes of fluorescence leakage and increment of temperature. This effect may be different and depends on the presence of urea in the liposomes, as shown in this study. The well known hypothesis regarding the possible role of frequency in MEP is that at lower values of frequency the field-dependent processes on the membrane are not so intensified within or on the surface of the membrane. In our case this is valid for the frequencies 0.01 and 1 kHz, as the electroporation has not yet reached its maximum value. At frequency above 1 kHz the field-dependent processes will perturb the cell membrane either through interaction with surface charges, dielectric forces, or local heating due to the highly promoted membrane currents. In the case of local heating a breakdown of the membrane may be observed which leads to a significant increase in liposome leakage.

Two conclusions can be drawn in this case: 1) the effect of frequency is connected with further polarization of liposome particle and 2) this effect is more pronounced in the presence of membrane surface disordering reagents. Obviously there is a relation between the frequency of the applied EF and reagents such as urea. At molecular level this dependence involves the changes in membrane bending rigidity and structural order. The interpretation of some phenomena in terms of membrane structural order leads naturally to certain implications and consequences. Thus, the modification of hydrated lipid surfaces in liposomes is related to the changes of water structure itself for example, by the adding of structure makers or breakers to the liposomes. The disruption of intrinsic structuring of water molecules at lipid surface in liposomes by urea probably contributes potentially to a decrease in membrane bending rigidity. This may be an additional and crucial factor in the membrane electroporation process.

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