Digression on Membrane Electroporation for Drug and Gene Delivery

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Membrane electroporation (ME) defines an electrical technique to render lipid membranes porous and permeable, transiently and reversibly, by external voltage pulses. Although there are numerous applications of ME to manipulate cells, organelles and tissues in cell biology, biotechnology and medicine, yet the molecular mechanism of ME is only slowly being understood. A general chemical-thermodynamical approach for the quantitative description of cell membrane electroporation has been developed to provide the framework to quantitatively rationalize electroporative cell transformation and electroporative uptake of drug-like dyes into cells, as well as electrolyte efflux from salt-filled electroporated vesicles.

Mechanistically, the electroporative transfer of gene and drug-like dyes involves the coupling between an interactive contact formation of the permeates with the cell surface membrane and the structural electroporation-reseling cycle

\[ C \Rightarrow (P) \]

where C is the closed and (P) represents a number of different porated membrane states, respectively. The experimentally accessible concentration fraction \( f_p = [(P)] / [(C) + [(P)]] \) of porous states is related to thermodynamic and electro-mechanic parameters such as temperature and the electric field strength, membrane rigidity or curvature. The results of the theoretical approach, mainly based on electrophical data of lipid vesicles, have been successfully used to analyze single cells and to specify conditions for the practical purpose of direct electroporative gene transfer and drug delivery, in particular in the new medical disciplines of electroporative chemotherapy and electroporative gene vaccination.

Key words: electro transformation; dye uptake; conductometry; electrophysics of lipid vesicles

Introduction

One of the fundamental features of life is the separation of the internal and external compartments of living organisms. In medicine, the separation barriers of organs can be artificially opened and closed by conventional scalpel surgery. Recently, a novel micro-surgery on the level of cells has been introduced. The mini scalps of this cellular surgery are electric voltage pulses. The new electrophysics of electrochemotherapy of tumors and of electro-gene therapy (see, e.g., (1-4)) are based on the concept and method of membrane electroporation (ME).

Methodologically, the applied voltage pulses make the cell membranes porous and transiently permeable to otherwise impermeable substances (5-6). Functionally the structural changes induced by ME, conditions the membrane for a variety of secondary processes such as transport and fusion, which are coupled to the electric pore formation and reseling cycle. Historically, the first direct functional electro-
transfer of naked genes (electrotransformation) into suspend- 
ed cells has been achieved by Neumann et al. (1982), (6).

Besides electroperorative transfer of drugs and genes, the pheno- 
mena of electofusion of single cells to large syncitia (7) and 
electroinsertion of foreign proteins into (electroperorated) 
membranes (8) are structurally also based on ME. The tech-
tique of ME is not only used to manipulate cells, organelles 
and tissue in cell biology but has also been applied to 
ehance isolotophoric drug transport through skin by ME 
(see, e.g., (9-12)). Of great medical importance is certainly 
the electrotransfer of chemotherapeutics into cancer tissue, 
see, e.g., (13-17). Despite these impressive medical applica-
tions of ME, the techniques still have to be improved and 
the practical procedures have to be further optimized. For this 
purpose it is necessary to understand the mechanism of elec-
roperorative drug and gene transfer into cells in more details.

Lipid Bilayers and Electroporation

No doubt, the elaborate theories for the complex molecular 
transport through the electroporated skin (18) and single cell 
membranes (19) use the same concepts as those developed 
for the mechanism of electric pore formation in pure lipid 
membranes (20, 21). The specific properties of real cell 
membranes must, however, be included into lipid electropo-
ration models as additional parameters. See, e.g., (22, 23).

The first theories of ME are based on data of planar lipid 
bilayers (20, 24-27). Natural membranes are usually curved. 
Therefore lipid vesicles are appropriate model systems for 
specific, lipid-related details of ME in cells (21, 28-31). For 
instance, electrooptic data show that the area difference 
elasticity (ADE) energy originating from the different packing 
density of the lipid molecules in the two leaflets of the curved 
vesicle membranes can dramatically increase both extent and 
rate of ME (32). Interestingly, the adsorption of the protein 
anxin V on lipid membrane surfaces decreases the extent 
and rate of ME by changing the ADE-energy of the curved 
vesicle. On the other hand, a concentration difference of elec-
trolyte between the two sides of the membrane increases the 
s spontaneous curvature due to the difference in the Debye 
screening length (33). Curvature and concentration difference 
between the outside and the cell interior rationalize the observa-
tion that vesicles made of phosphatidlycholine-Na-phos-
phatidylglycerol and of radius 50 nm can be electroporated at 
the surprisingly low transmembrane potential $|\Delta \phi_{m}| = 37.5$ 
mV and the pulse duration 100 ms (29, 30), whereas for plan-
lar membranes and 100 $\mu$s pulse length $|\Delta \phi_{m}| = 500$ mV (27).

The Electropore Concept

The concept of ME has originally been derived from electri-
cally-induced permeability increases (electropermeabiliza-
tion) as judged from the release of intracellular material (5) or 
from the electrodiffusive uptake of macromolecules such as 
DNA (6). In planar membranes, the dramatic increase of the 
specific membrane conductance from about 10 nS cm$^{-2}$ up to 
1 S cm$^{-2}$ has been analyzed in terms of electropores; see, e.g., 
Glaser et al. (1988), (20). Direct evidence for electropores 
can be derived from imaging data of 0.1 $\mu$m-diameter pore-
like structures in electroporated red blood cells (34). These 
larger pores must develop from yet not visible smaller pores. 
Voltage-sensitive fluorescence microscopy at the membrane 
level shows dramatic decreases in the transmembrane potent-
tial at the pore caps of see urchin eggs concomitant with an 
increase in the ionic conductivity of the membrane (35).

Membrane electroporemeabilization requires pores in order to 
permit ions and molecules to cross the electrically modified 
membrane. For instance, a small ion of diameter 0.4 nm and 
of charge $e = 1.6 \times 10^{-19}$ C, passing through the membrane of 
d = 5 nm thickness, the Born energy barrier is $\Delta W_{\text{Born}} = 65 \times 
kT$, where at $T = 298$ K (25°C) $kT = 4.11 \times 10^{-21}$ J. Thus the 
required transmembrane potential $|\Delta \phi_{m}| = \Delta W_{\text{Born}}/e$ to 
overcome the barrier is $|\Delta \phi_{m}| = 1.7$ V. An even larger value would 
be needed for two-valent ions (3.5 V). Nevertheless, the trans-
membrane potential required to electroporate the cell mem-
brane does usually not exceed 0.5 V (27). It is obvious that 
a reduction of the barrier can be achieved by aqueous pores in 
the membrane phase. In such pores or channels the value of the 
barrier energy is lowered by a factor of 3 to 4 (36). The diame-
ter of electropores is usually very small: $\phi_{p} \leq 1$ nm (21, 27), 
yet there seems to be no alternative for pores in order to ration-
alize ion transport through the electroporated membranes (37).

Materials and Methods

Preparation of Lipid Vesicles

As already stated, lipid vesicles are suited to study funda-
mentals of electroporation related to cellular systems. 
Unilamellar phospholipid vesicles can be readily prepared by 
the extrusion method (see, e.g., (37)), using the commercial 
chloroflour: methanol lipid extract of 20% (weight) lecithin 
(also called Soy 20 or Avanti 20) kept deep-frozen at $-80^\circ$ C. 
The purified extract contains 20% (weight) phosphatidyli-
choline (PC), 10% phosphatidic acid (PA), 30% phosphatidyl-
ethanolamine (PE), 20% phosphatidinositol (PI) and 20% 
other, not specified lipids from Avanti Polar Lipids.

The vesicular lipid phase of the total concentration $[L_{t}] = 1$ 
mM is suspended in 0.2 M NaCl solution and freeze-thawed 
five times in liquid nitrogen to obtain solute equilibration 
between trapped and bulk solution. In order to remove 
external NaCl, the vesicle suspension is dialyzed against degassed 
0.33 M sucrose solution of the same osmolarity. The final 
NaCl concentration in the bulk is $c_{\text{ex}} = 0.2$ mM and in the
References and Footnotes


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where $\Omega = 4 \cdot \alpha^2 \cdot RT/(F \cdot \sqrt{3} \cdot \alpha \cdot a \cdot E \cdot h)$, $n = b/d$ (see Fig. 7a) and $\alpha = (1 - RT/(F \cdot v_m))$ are the dimensionless factors, and $\lambda_{ex}$ is the conductivity of electrolyte in the bulk, $\lambda_0 + F^2 \cdot D \cdot (c(0) + c(d))/RT$ and LambertW is a special function (30).

**Electroportative Cell Transformation**

It is recalled that direct transfer of naked DNA into cells and microorganisms by membrane electroportation has become the method of choice in cell biology and medicine. The model calculations for the experimental data have shown that the electrodiffusion of DNA is about 10 times more effective than simple diffusion. In quantitative terms, the flow coefficient is $k_p^0 = 7 \times 10^{-3} \text{s}^{-1}$ and the fraction of the maximum electroporated area, conditioning the electrodiffusive DNA flow in yeast cells, is $f_p^* = 0.023\%$ (44). The mean radius of the pore in the DNA-permeable patches is estimated to be $r_p = 0.39 (\pm 0.05) \text{nm}$. The mean number of pores in the membrane permeable to DNA in yeast cells is given by $N_p = S_p/r_p \cdot r_p^2 = 2.2 (\pm 0.2) \times 10^4$ per cell of radius $a = 2.7 \text{nm}$. The mean pore radius $r_p$ is in the same order of magnitude as $r_p = 0.35 (\pm 0.05) \text{nm}$ of electroporated lipid vesicles (28, 41) and $r_p \approx 1 \text{nm}$ of planar membranes (see, for instance, (20)). These pore sizes appear to be too small for free diffusion of DNA molecules. The pore size is not sufficient for the entrance of a free end of a linear DNA molecule (diameter of the double helix $\Phi = 3 \text{nm}$) and not at all for the entrance of the more bulky circular DNA. Up to now, the theoretical proposals of Pastuchenko and Chizmadzhev (1992), (49), have not been checked experimentally. The experimental data suggest that the transport of DNA, as said before, is a diffusive migration enhanced by electrophoresis across the porous membrane patches.

**Electroportative Uptake of Drug-like Dyes**

The results of the analysis of the electroportative dye transport into B-lymphosoma cells may serve as a quantitative basis for optimization strategies to improve the conditions for drug delivery by membrane electroporation, such as in electrochemotherapy. In the field strength range $0 \leq E/kV \text{cm}^{-1} \leq 2.1$, the equilibrium distribution constant $K_p$ of the electroporation reaction increases enormously because of exponential dependence of $f_p$ on $E$ (19, 23). The saturation value of the induced transmembrane potential difference of the electroporated B cell membrane is calculated to be $\Delta \phi_m = -0.7 \pm 0.1 \text{V}$. The value $\Delta \phi_m = -0.7 \text{V}$ is typical of that estimated for most cell membranes by Weaver and Chizmadzhev (1996), (27). It does not suggest any extraordinary high local transmembrane conductivity. The mean pore radius of the pore state permeable to the dye SERVA blue was estimated to be $r_p = 1.2 \text{nm}$, in line with previous estimates (27), yet this value seems to be rather large. An open pore of this size should lead to a locally significant transmembrane conductivity $\lambda_{m}$, reducing the local transmembrane voltage (28), eventually causing leakage of cell components and finally cell death. Note, however, that the detection of the dye permeable pore state is only possible, when the SERVA blue molecules are interactively passing through the pore. Therefore, the pore is temporarily occluded by the dye molecule, preserving the original low conductivity. The fraction of the area occupied by the dye-permeable pores is $f_p^* = 3.5 \times 10^{-4}$, or $0.035 \pm 0.003\%$ at $E = 2.1 \text{kV cm}^{-1}$ at the pulse duration $t_p = 200 \mu\text{s}$, which is rather small. This corresponds to $N_p = (1.5 \pm 0.1) \times 10^5$ pores per average cell of radius $a = 12.5 \mu\text{m}$. The small numbers rationalize the rather slow transmembrane flow of the dye molecules leading to cell coloring. The fraction $f_p^* = 3.5 \times 10^{-4}$ compares well with the range $2 \times 10^{-4} < f_p^* < 2 \times 10^{-3}$ of conductive electrolytes in sea urchin eggs at approximately the same experimental conditions, derived from fluorescence imaging data by Habino et al. (1993), (35). One important aspect is the longevity of the porous membrane states, such that the main drug delivery occurs in the long-lasting resealing phase. Additionally, local cooling should speed up the effective drug influx because low temperatures slow down the resealing processes.

**Conclusion**

In summary, the external field does not only make the cell membranes structurally permeable but also greatly enhances the transport of ionic molecules like DNA or drug-like dyes by electrophoretic migration.

Remarkably, the pore radii are relatively small. The density of the electropores is very small: $f_p \leq 10^{-2}$. It is noted that the pore fraction increases continuously with the electric field strength in a strongly nonlinear fashion. The electroporation-induced massive permeability changes (to small ions, dye molecules, proteins and nucleic acids such as DNA in electroporative gene transfer), however, require an interactive transport formalism, where $f_p$ is a part of the transport coefficient. The data base suggests that the cross-membrane transport of macromolecules transiently involves cooperative adsorption complexes, with several membrane pores progressing progressively coalescing in contact with the (electro-)diffusing polymer and annealing after the passage of the permeates. The experimental conditions for procedural optimization strategies must be adjusted to these fundamental results.

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Figure 6: (a) Relative conductivity increase ($\Delta \lambda / \lambda_0$) of the suspension of the salt filled vesicles of radius $a = 90$ nm as a function of the field strength $E$. (b) The fraction of the electroproated membrane area ($S_f/S_0$) as a function of the field strength $E$. $S_f/S_0$ is calculated from the electroporophotic relaxations, yielding characteristic rate parameters of the electroporation - rescaling cycle in its coupling to ion transport. Data from reference (37): $\lambda_0 = 2.85$ mS m$^{-1}$, $T = 293$ K (20$^\circ$).

Figure 7: (a) The electric potential $\varphi_{ex}$ of an external transmembrane field and $\varphi_{im}$ the image potential of an ion along the pore $x$-axis. (b) Forces from the induced image charge in the pore walls on the moving ion (arrows).

Actually, if a point charge $g$ is situated near the boundary of two dielectrics, say between water and the lipid phase of the membrane, $g$ in water induces in the neighboring membrane an image charge $g'$. Since $\varepsilon_w = \varepsilon_m$, $g'$ has the same sign as $g$. Therefore, $g$ experiences a repulsive force from $g'$ tending to repel the ion from the lipid phase. Even at constant pore radius, the height of the energy barrier can be reduced by an external electric field. For the case of a trapezium form of the electrostatic barrier in the pore, the membrane conductivity can be specified as an explicit function of the field strength (36):

$$\lambda_m = \Omega \cdot \lambda_{\alpha} \text{Lambert} \left( \frac{f_p \cdot \lambda_0}{\Omega \cdot \lambda_{\alpha}} \exp \left( \frac{F \cdot (\sqrt{3} \cdot \alpha \cdot a \cdot E \cdot n/2 - \varphi_{im}^0)}{RT} \right) \right)$$

[15]
undulations and membrane electroporation. Fortunately, the different time scales of these processes permit to separate ME from membrane smoothing and stretching (see Fig. 4). The characteristic time of membrane stretching and smoothing can be calculated with the help of Eq. [12]:

$$\tau = \frac{3\eta \cdot a^3}{\frac{48 \cdot \kappa}{5} + \frac{3}{2} \sigma_0 \cdot a^2 \left(1 + \frac{K}{\sigma_0 + K/L}\right)}$$

where $\eta$ is the viscosity of water, $K$ is the membrane compression module, $\sigma_0$ is the initial lateral membrane tension and $L = 8 \cdot \pi \cdot \kappa/(kT)$ is a dimensionless parameter. Here $\tau \leq 1\mu s$. The relative increase in the membrane surface area by stretching and smoothing can not exceed the value given by:

$$\frac{\Delta S}{S_0} = \frac{72 \cdot \varepsilon_0^2 \cdot \varepsilon_w^2 \cdot E^4 \cdot a^6}{45 \left(62 \cdot \kappa + 10 \cdot \sigma_0 \cdot a^2 \left(1 + \frac{K}{\sigma_0 + K/L}\right)\right)^2}$$

The characteristic time constant of electroporative increase in the membrane area is usually much larger than 1 $\mu s$. For example, at $\Delta \phi_m = -0.3 V$, $\tau_p = 5\mu s$, (37).

Since the extent of ME is always small, $f_p \ll 1$, the equilibrium constant can be approximated by $K_p = f_p/(1-f_p) \approx f_p$. Hence we may specify:

$$f_p = K_p = K_p(a^1 = 0) \cdot \exp\left[\beta \cdot a^{-1}/kT\right]$$

where the constant $K_p(a^1 = 0)$ covers the remaining thermodynamical, mechanical and electrical contributions to pore formation such as the pore edge energy and the chemical and electric polarization terms at $\Delta \phi^N = -0.3 V$. For the term $\beta$ see Eq. (4). In accordance with Eq. (7), the fraction of the electroporated membrane $f_p = S_p/S_0$ at the nominal transmembrane potential $\Delta \phi^N = -1.5 \cdot E \cdot a = -0.3 V$, referring to the vesicles pole caps at zero membrane conductivity, steeply decreases with increasing $a$ (Fig. 5). This suggests that the degree $f_p$ of pore formation is strongly affected by the membrane curvature $H = 2/a$. The analysis of the data in Figure 5 in terms of Eq. (14) yields $K_p(a^1 = 0) = 1.2 \pm 0.1 \cdot 10^{-3}$ and $\beta = 81 \pm 5 kT \cdot nm$. The value of $K_p(a^1 = 0) = 1.2 \cdot 10^{-3}$ suggests that, at $\Delta \phi^N = -0.3 V$ and $a^1 = 0$ (planar membrane), only 0.12% of the membrane area is covered by pores. Applying the usual values of the parameters $K = 2.95 \cdot 10^{-20} J$ and $\alpha = 2.0$, the value of $\beta = 81 \pm 5 kT \cdot nm$ indicates that the formation of one conical average pore of $f_p = 0.35 nm$ in a vesicle of $a = 25 nm$ leads to the dissipation of the Gibbs free energy by $\Delta G_{ADE}/N_A = -\beta \cdot a^1 = 3.2 \cdot kT$. At the larger vesicle radius $a = 170 nm$, the energy release is smaller: $\beta \cdot a^1 = 0.5 kT$.

Another approach to investigate ME and shape deformation of small vesicles is to load the vesicles with highly concentrated electrolyte and to measure the time course of the increase in the suspension conductivity caused by electrolyte efflux through the pores.

The analysis of turbidity and conductometric relaxations in salt-filled vesicles, exposed to a rectangular field pulse in the field strength range $0 \leq E/MVm^{-1} \leq 7.5$ and the pulse duration $t_p = 10 \mu s$ (37), shows that the surface fraction $f_p$ of membrane electropores increases with $E$ and levels off at about $E = 2 MVm^{-1}$ (Fig. 6 b). The suspension conductivity $\lambda$, however, non-linearly increases with $E$ without level-

![Figure 4: The relative increase $f_p = S_p/S_0$ in the vesicle surface area due to formation of membrane electropores as a function of time (a) at different pairs of vesicle radius and field strength: a (nm)/E (MV/m) = 25/8; 38/5.3; 80/2.5, respectively, for the constant nominal transmembrane potential $\Delta \phi^N = -1.5 \cdot E \cdot a = -0.3 V$.](image)

![Figure 5: The surface fraction of membrane electropores $f_p = S_p/S_0$ at (constant) $\Delta \phi^N = -1.5 \cdot a \cdot E = -0.3 V$. The solid curve represents the data fit with Eq. (14).](image)
If the total amount \( n^\text{in} = N_c^\text{in} \cdot N_c \) of transported molecules inside all the \( N_c \) cells is small compared to \( n^\text{out} \), i.e. \( n^\text{in} \ll n^\text{out} \), integration of Eq. [8] within the boundaries \( t = 0 \) and \( t = t_E \) yields:

\[
n^\text{in}(t_E) = k_f^0 \cdot c^0 \cdot N_c \cdot V_c \cdot \int_0^{t_E} S_p(t) \, dt,
\]

where \( c^0 = n^\text{out}/N_c \) and \( S_p(t) = S_0 \cdot f_p(t) \) is the time course of the build up of the electrophoretic surface, connecting transport with \( f_p \).

The conventional flow coefficient of non-equilibrium thermodynamics is given by \( k_f^0 = P_p \cdot S_0 \cdot V_c \), where \( P_p = \gamma_p \cdot D/d \) is the pore permeability coefficient, \( D \) is the diffusion coefficient of the molecules passing through the electropores, and \( \gamma_p \) is the pore partition coefficient, given by \( \gamma_p = [n_p]/[n^\text{in}] \), where \( [n_p] \) and \( [n^\text{in}] \) are the concentrations of molecules in the pore water and within the cell, respectively. The (electro)diffusion coefficient \( D \) involves the diffusion coefficient \( D_0 \) and the electrical part, given in Eq. [8]. Hence, \( D = D_0 \cdot \{(1 + 3\lambda_0)^{-1} \cdot \cdot \cdot f(\gamma_m) / (E(\cdot \cdot \cdot kT))\} \). The dependence of the degree \( f(t_E) \) of electroporation / coloring of the cell ensemble on the pulse time \( t_E \) is then given by (43, 44):

\[
f(t_E) = \frac{n^\text{in}(t_E) \cdot [1 - y(t_E)]}{n^0_s - n^\text{in}(t_E) \cdot [1 - y(t_E)]},
\]

where \( n^0_s \) is the initial amount of cell components which bind the permeated, now intracellular DNA or dye molecules. The term \( y(t_E) \) is specified as:

\[
y(t_E) = \exp(k_b \tau_T \cdot [n^\text{in}(t_E) - n^0_b])
\]

where \( \tau_T \) is the characteristic time constant of cell transformation / coloring and \( k_b \) is the bimolecular rate constant of the binding of the transported molecules to intracellular sites.

As in the case of planar membranes (45), the transport of DNA or drug molecules appears to be a thermally - diffusive migration of the molecules which transiently interact with many small pores of an electroporated membrane patch. Adsorbed DNA appears to be located into the surface, thereby pushing lipid molecules transiently aside. The inserted DNA / drug-like dye is at least partially pulled through the electroporated bilayer patches by the electric field (electrodiffusion). The longevity of the electroporated membrane state facilitates after-field diffusion of DNA and drug-like dyes into the cell (6). For small charged molecules, the electric drug meets a smaller hydrodynamic resistance that the larger DNA molecules. The data base suggests (43, 44), that the DNA adsorption is coupled to (at least two) electroporated membrane states (\( P_1 \) and \( P_2 \)), and the adsorption of dye molecules SERVA blue is coupled to a minimum of three states (\( P_1, P_2, \) and \( P_3 \)), differing in the pore radius. Once the molecule is a part of the membrane, the actual cross-membrane transport does not require the presence of an external field. For ionic molecules, transport is greatly accelerated by electrophoresis in the membrane field (23, 46, 47).

**Results and Discussion**

**Optical Detection of Rapid (\( \mu \)s) Membrane Electroporation**

The observed electrooptic relaxations of vesicle suspensions reflect very rapid (\( \mu \)s) structural changes in the lipid membrane (21, 28, 31) and deformation of the vesicle shape in an electric field (37). However, the primary optical signals (Fig. 3) must at first be correctly decoded. Here a Mie-type numerical code (48) has been applied to recalculate the turbidity minus and plus modes to obtain the fractional increase \( f_p = S_p/S_0 \) of the membrane surface area.

**Figure 3:** Turbidity relaxations. The plus mode \( \Delta T^+ / T_0 = (\Delta T^2 + 2\Delta T \Delta T^+)/(\Delta T^2) \), reflecting the entrance into the membrane and the decrease in the vesicle volume, and the minus mode \( \Delta T^- / T_0 = (\Delta T^- \Delta T^-)/(\Delta T^-) \) of the turbidity changes \( \Delta T \) at the parallel (\( \sigma = \| \)) and perpendicular (\( \sigma = \perp \)) polarization modes (\( \lambda = 355 \) nm), respectively, relative to the zero field turbidity \( T_0 \), as functions of the time \( \tau \), at the two (extreme) field strengths \( E = 1.0 \) MV m\(^{-1}\) and \( E = 7.5 \) MV m\(^{-1}\), respectively. Rectangular electric pulse of the field strength \( E \) and the pulse duration \( t_p = 9.8 \mu s \) at \( T = 293 \) K (20\(^\circ\) C). Salt-filled unilamellar lipid vesicles, mean radii \( s = 90 \pm 10 \) nm, internal [NaCl] = \( c_\text{in} = 0.2 \) M, total lipid concentration \( [L_1] = 1.0 \) mM, suspended in isotonic 0.33 M sucrose and 0.2 mM NaCl solution; vesicle number density \( \rho_v = 2.4 \cdot 10^{14} \) ml.

The increase in the membrane surface area of lipid vesicles in an electric field is generally a complex process comprising membrane stretching, smoothing of membrane thermal...
is the conventional Gibbs reaction energy (without tension, curvature and electric terms) where \( \nu \) and \( \mu \) are the stoichiometric coefficients (with sign) and chemical potentials of the molecules, respectively, constituting the phase \( \alpha \). It is further remarked that \( \Delta M_{m} \) is a function of the applied external field \( E \) and is dependent on geometry and conductivities.

**Effect of Membrane Curvature and Electrolyte Gradient on Electroporation**

For small vesicles, the curvature term is very important for the membrane electroporability; the surface pressure term is negligibly small and is therefore not considered. The curvature energy is related to the deviation \( \Delta(S) = S - S_{0} \) of the area difference \( \Delta S \) between the two monolayer leaflets from its equilibrium value \( S_{0} \). At approximately equal number of lipid molecules within the outer and the inner leaflets and neglecting the alterations in the interfacial energy with changing curvature (21), the curvature term of the \( C \rightarrow (P) \) state transition is given by (34):

\[
\int_{0}^{H} \Delta_{\alpha} \beta dH = N_{A} \cdot \int_{0}^{H} (\beta_{p} - \beta_{c}) dH = \frac{64 \mu^{2} - \alpha \cdot \kappa \cdot \frac{r_{p}^{2}}{d} \cdot \frac{N_{A}}{a} \cdot \left( \frac{1}{a} + \frac{H_{0}^{el}}{2 \pi \alpha} \right)}{d} \tag{4}
\]

where \( N_{A} \) is Avogadro's constant, \( \beta_{p} = 0, \beta = \beta_{c}, \kappa \) is the elastic module and \( \alpha = 1 \) is the material parameter (42). The electrical part of the spontaneous curvature \( H_{0}^{el} \) for the case of thin membrane \( d \ll a \) and for low surface charge density or high salt concentration, is explicitly given elsewhere (34).

Different to cylindrical pores of volume \( V = \pi \cdot r_{p}^{2} \cdot d \), the conus-shaped \( P \) pores in the vesicle membrane reduce the lipid packing density difference and, consequently, the free energy of the electroporated vesicle system. Eq. [4] indicates that the smaller the vesicle radius \( a \) and the larger the electrical spontaneous curvature \( H_{0}^{el} \), the larger is the Gibbs energy released by the pore formation. The mean pore radius \( r_{p} \) of conus-shaped pores is defined by \( r_{p} = (r_{out} + r_{in})/2 \) where \( r_{out} \) and \( r_{in} \) are the pore radii in the outer and inner leaflets of the bilayer, respectively. The factor \( \xi \) is \( (r_{out} - r_{in})/(r_{out} + r_{in}) \) characterizes the pore conicity. For instance, at \( r_{p} = 0.35 \) nm, there are 7 lipid molecules in the outer pore edge and 5 lipid molecules in the inner pore edge, hence \( \xi = 0.352 \).

The electric polarization term at the positional angle \( \theta \) is expressed as (Kakorin et al. 1996):

\[
\varepsilon_{p} \Delta M_{m} \cdot \int_{0}^{L} \frac{k^{2} \lambda_{m}(\theta) \cdot \cos^{2} \theta \cdot E^{2}}{8 \cdot d} \int_{0}^{L} \frac{N_{A} \cdot (1 + \frac{H_{0}^{el}}{2 \pi \alpha})}{d} \cdot \frac{r_{p}^{2}}{d} \cdot \frac{N_{A}}{a} \tag{5}
\]

where \( \varepsilon_{0} \) is the vacuum permittivity and \( f(\lambda_{m}) \) the conductivity factor. The difference \( (\varepsilon_{w} - \varepsilon_{L}) \) refers to the replacement of lipids by water during the \( C \rightarrow (P) \) transitions. The line tension term is characterized by:

\[
\int_{0}^{L} \gamma(\gamma_{H} - \gamma_{C}) dL = N_{A} \cdot (1 + \frac{H_{0}^{el}}{2 \pi \alpha}) \cdot \frac{r_{p}^{2}}{d} \cdot \frac{N_{A}}{a} \cdot \frac{\gamma}{2 \pi r_{p}} \tag{6}
\]

where \( \gamma = \gamma_{H} - \gamma_{C} \) is the pore line tension, \( L = 2 \pi r_{p} \) is the circumference and \( r_{p} \) is the radius of the pore; note that \( \gamma_{C} = 0 \). Applying Eq. [2] - Eq. [6], the equilibrium constant of the global reaction scheme in Eq. [1] is given by:

\[
K = \sum_{i} \exp \left( -\frac{\gamma_{i} r_{p}}{2 \pi} \right) \cdot \exp \left( -\frac{\gamma_{j} r_{p}}{2 \pi} \right) \cdot \frac{N_{A}}{a} \cdot \frac{\gamma}{2 \pi r_{p}} \int \Delta_{m} \cdot \Delta \gamma \cdot \Delta \theta \cdot \Delta \phi \cdot \Delta \tau \tag{7}
\]

where

\[
K = \exp (-\Delta_{R} G^0 / RT)
\]

is the distribution constant at \( E_{m} = 0, \gamma = 0 \) and \( \beta = 0 \).

Since usually \( K_{p} = 1 \), we see from Eq. [2] that \( f_{p} = K_{p} / (1 + K_{p}) = K_{p} \). Hence the degree of poration \( f_{p} \) and poration energetics are explicitly connected by

\[
f_{p} = \exp (-\Delta_{R} G^0 / RT)
\]

**Transport of Drug-like Dyes and DNA Through the Electropores**

The electrodissipative transport of DNA or charged drug-like dye molecules into and across the electroporated membrane of the average cell is described by the Nerst-Planck equation for the mole flow density vector of the amount \( n^{in} \) of DNA or dye molecule in the direction of the external field \( E \).

The DNA-influx is given by (41,43):

\[
\frac{dn_{in}^{in}}{dt} = -\frac{D_{0}}{d} \cdot \frac{n_{in}^{in}}{V_{c}} \cdot \frac{n_{out}}{V_{c}} \cdot \frac{1 - \frac{1}{kT}}{kT} \cdot \Delta \phi_{m} \tag{8}
\]

where \( S_{p} \) is the electroporated surface area and \( V_{c} \) the volume of the average cell, \( D_{0} \) is the zero-field effective diffusion coefficient of DNA or dye molecule across the electroporated surface patches (in interactive contact with the molecules), \( \Delta \phi_{m} \) is the absolute value of the effective charge number (with sign) of the transported molecule, \( \Delta \phi_{m} \), the \( \theta \) - average electrical potential difference contribution induce by the applied field. Here

\[
\Delta \phi_{m} = 0.5 \int_{0}^{\pi/2} \Delta \phi_{m} \cdot \sin \theta \cdot \sin \theta = -\left( \frac{3}{8} \right) \cdot a \cdot E \cdot f(\lambda_{m}),
\]

where \( n_{out} \) is the amount of DNA or dye molecules and \( v \) the volume of the bulk solution, respectively (21).
conductivity \( \lambda_L(t) \), without pulsing, is due to the leakage of salt ions through the membrane of the salt-filled vesicles. In order to account for the conductivity contributions of Joule heating and electrode effects, the conductivity \( \lambda_R(t) \) of the blank solution of sucrose and NaCl of identical initial conductivity as for the vesicle suspension has been recorded using the same electrical pulse parameters. The field-induced relative change in the suspension conductivity \( \Delta \lambda_R(E)/\lambda_0 \) due to membrane electroporation and salt release through electropores is given by: \( \Delta \lambda_R(E)/\lambda_0 = (\Delta \lambda_L(E) - \Delta \lambda_R(E) - \Delta \lambda_L(E))/\lambda_0 \), where \( \Delta \lambda_L(E) = \lambda_L(E) - \lambda_0 \), \( \Delta \lambda_R(E) = \lambda_R(E) - \lambda_0 \), and \( \Delta \lambda_L(E) = \lambda_L(E) - \lambda_0 \) (29).

Theory

Chemical and Physical Pore Properties

In the electric field the charge redistribution during the ionic interfacial polarization on both sides of the membrane dielectrics is equivalent to building up electric condenser plates. It is recalled that the membrane is a highly dynamic phase of mobile lipid molecules in contact with water and hydrophobically held together by the aqueous environment. Such a charged condenser with both mobile interior and mobile environment favors the entrance of water molecules to produce aqueous pores (P) with higher dielectric constant \( \varepsilon_w = 80 \) compared with \( \varepsilon_P = 2 \) of the replaced lipids (state C). In this sense the lipid membrane is an open system with respect to \( H_2O \) molecules and ions charging the condenser to yield the total charge g.

The purely electrical aspect of ME may be described in terms of a change \( dG_{el} \) of the electrical part (\( G_{el} \)) of the total Gibbs energy of the source (\( |\Delta \phi_{ml}|d\gamma \)) by \( dG_{el} = |\Delta \phi_{ml}|d\gamma - F \cdot dx \), where \( |\Delta \phi_{ml}| \) is the induced transmembrane potential (difference), \( F \) is a generalized force acting on the dielectrics and tending to change the water content by \( dx \), replacing lipids thereby forming pores, and \( g \) is the charge. At zero membrane conductivity (\( \lambda_m = 0 \)), the external field maintains a constant potential difference \( |\Delta \phi_{ml}| \). Therefore ME proceeds at constant \( |\Delta \phi_{ml}| \) and the replacement of lipids by water means \( dG_{el} = |\Delta \phi_{ml}|d\gamma/2 > 0 \). This physically rigorous picture apparently contradicts to the spontaneous pore formation requiring a decrease of the free Gibbs energy (G). Therefore, the chemical-thermodynamical description of ME requires the transformed Gibbs energy \( \tilde{G} = G - E_m \cdot M \), covering also the electric source of the membrane field \( E_m \) (Fig. 2). Thus the total energy change of ME is given by \(-|\Delta \phi_{ml}|d\gamma/2 \) (41). Here, \( E_m = -|\Delta \phi_{ml}|d\gamma \) is the transmembrane field strength, \( M \) is the (induced) electric dipole moment of the pore regions and \( G \) is the ordinary Gibbs energy, containing the chemical potentials of the \( H_2O \) and lipid molecules constituting a pore. Note that at \( E_m = 0 \), we have \( \tilde{G} = G \). At constant pressure and temperature, a field-induced state transition is characterized by \( \Delta R \tilde{G} = \tilde{G}(P) - \tilde{G}(C) \leq 0 \); see Eq. [1], in line with spontaneity and stability criteria of equilibrium thermodynamics.

In the simplest case, pore formation and closure is chemically modeled as an overall structural change

\[
C \rightleftharpoons (P) \tag{1}
\]

between closed (C) and pore (P) states, respectively. The analytical theory connects the fractional concentration change or extent of pore formation \( f_p = [(P)]/[(P) + [C]] \) with the energetics of the process. In particular, the dependence of \( f_p \) on the applied external field \( E \) is explicitly specified. Here the square brackets refer to concentration or surface density, and the round brackets symbolize that \( (P) \) globally represents several coupled pore states, \( (P) = P_0, P_1, P_2, \ldots \) and \( [(P)] = [P_0] + [P_1] + [P_2] + \ldots \).

The overall equilibrium constant for the process in Eq. [1] is defined by:

\[
K_p = \frac{[(P)]}{[C]} = \frac{f_p}{1 - f_p} = \exp \left[ \frac{-\Delta R \tilde{G}}{RT} \right] \tag{2}
\]

where \( \Delta R \tilde{G} = \tilde{G}(P) - \tilde{G}(C) \) is the standard value of the transformed Gibbs reaction energy, \( R \) the gas constant and \( T \) the absolute temperature. Note that \( \Delta R \tilde{G} \) covers the various energetic contributions in the form of:

\[
\Delta R \tilde{G} = \Delta R \tilde{G} = \int_0^L \Delta R \Gamma dL + \int_0^A \Delta R \Gamma dS_p + \int_0^H \Delta R \beta dH - \int_0^\beta \Delta R Mg E_m \quad \tag{3}
\]

where \( \gamma \) is the line tension (or pore edge energy density) and \( L \) is the edge length, \( \Gamma \) is the surface energy density and \( S_p \) is the pore surface in the surface plane of the membrane, \( \beta \) is the curvature energy term and \( H \) is the vesicle curvature, including the spontaneous curvature \( H_0 \). In the case of the spherical vesicle and \( H_0 = 0 \), the curvature is given by the radius \( a \) of the midsurface of the bilayer: \( H = 2a^{-} \). Note that \( \Delta R = d/d\xi \), where \( \xi \) is the molar advancement of the two state process. The term \( \Delta R \tilde{G} = \sum_j \sum_i v_{ij} \cdot \mu_j^+ \)
vesicle interior $c_{in} = 0.2$ M, respectively. The vesicle mean diameters $\varnothing = 50, 76, 160$ and 340 nm have been determined by dynamic light scattering measurements. The final total lipid concentration used for the electro-optical measurements ($[\text{L-}] = 1$ mM) corresponds to a vesicle number density of $p_v = 10^{14} - 10^{16}$ dm$^{-3}$. At these conditions, the average distance between the surfaces of single vesicles in all cases is larger than the diameter, qualifying the suspension as diluted with practically no vesicle-vesicle contacts, also during the field pulse of 10 µs duration.

**Cells and Plasmid DNA**

Yeast cells of Saccharomyces cerevisiae AH 215 strain (MATa, *leu* 2-3, 2-112, *his* 3-11, 3-15) are used. The strain has been cultivated in YEP (1% yeast extract Difco, 2% peptone Difco, 2% glucose) media up to an optical density of $OD = 1.3-1.4$ at the wavelength $\lambda = 650$ nm, corresponding to a cell density of $p_c = 10^8$ cells/ml. The reversion rate of *leu 2* is less than $10^{-9}$, which is extremely low. The plasmid YEP 351 (5.6 kbp, relative molecular mass $M_r = 3.6 \times 10^6$) compensates *leu 2* mutation of *S. cerevisiae* (38). The isolation and purification of the plasmid DNA in the supercoiled form has been performed using the method of Maniatis *et al.* (39). The DNA/Ca$^{2+}$ titrations and the electropulsing experiments are performed with cell suspensions of 1 M sorbitol, 1 mM Tris·HCl at pH 7.2. The condition [Ca] = 0 has been achieved by 1 mM EDTA, pH 7.2. The conductivity of the medium at [Ca] = 1 mM is $\lambda_0 = 0.076$ S m$^{-1}$. The temperature of all experiments is $T = 293$ K (20º C).

**Cell Coloring**

The FcγR$^+$ mouse B cell line IIA1.6 has been cultivated as described in reference (40). The cells are washed twice with Click’s medium (Biochrom KG, Berlin/Germany) without fetal calf serum and resuspended. The cell density is $p_c = 6 \times 10^6$ ml$^{-1}$. SERVA Blue G (SBG, $M_r = 854$) has been obtained as a powder from SERVA Feinbiochemica, Heidelberg/Germany. Fresh solutions of 1.7 mM SBG in serum-free Click’s medium are prepared just before each experiment. SBG is an established protein-dye which does not penetrate intact cell membranes (Bradford, 1976). For all electroporation experiments the final protein-dye concentration is $c_p = 1.17$ mM and $p_c = 6 \times 10^6$ ml$^{-1}$. Hence the linear cell density is $182$ cells cm$^{-1}$ and the mean distance between the cell centers is $d = 55$ µm. The IIA1.6 cells are about 25 µm in diameter, thus the average cell separation is about a cell diameter. No cell clustering is observed.

**Electro-Optical Relaxations**

Rectangular pulses of field strengths up to $E = 8$ MV m$^{-1}$ and of duration $t_E = 10$ µs are applied by cable discharge to the sample cell equipped with parallel planar graphite electrodes, thermostated at $T = 293.0 \pm 0.1$ K (20º C). The field induced changes in the transmittance of plane-polarized light are measured at the wavelength $\lambda = 365$ nm (Hg-line; highest accuracy).

The light intensity change $\Delta I^\sigma$, caused by the electric pulse and measured at the polarization angle $\sigma$ relative to the direction of the applied external field vector $E$, is related to the optical density change by $\Delta OD^\sigma = OD^\sigma(E) - OD^\sigma_0 = - \log (1 + \Delta I^\sigma/\Delta I^0)$, where $\Delta I^0 = I^0(E) - I^0$ is the light intensity change from $I^0$ (at $E = 0$) to $I^0(E)$ in the presence of E. OD$^\sigma(E)$ and OD$^\sigma_0$ are the optical densities at E and at E = 0, respectively (Fig. 1). Generally, OD = A + T, comprising both absorbance (A) and turbidity (T) along the light path length $t = 1$ cm. In the case $A << T$, we have $\Delta OD^\sigma = \Delta T^\sigma$.

The field induced changes $\Delta T^\parallel$ and $\Delta T^\perp$ at the two light polarization modes $\sigma = 0^\circ$ (||, parallel to the external field vector $E^\parallel$) and $\sigma = 90^\circ$ (⊥, perpendicular to $E^\perp$) are given by $\Delta T^\parallel = T^\parallel - T_0$ and $\Delta T^\perp = T^\perp - T_0$, respectively.

Generally, the difference (or minus) mode $\Delta T^- = \Delta T^\parallel - \Delta T^\perp$ is not the classical dichroism $\Delta T$. There are usually contributions from non-orientational processes, characterized by the turbidity plus term $\Delta T^+ = (\Delta T^\parallel + 2\Delta T^\perp)/3$, covering changes in the scattering cross section due to entrance of water and ions into the electroporated lipid membrane as well as changes of the vesicle volume. Here we have $\Delta T^- = \Delta T$ and the scattering minus term $\Delta T^-$ thus reflects dominantly the elongation of vesicles in the direction of the electric field.

![Figure 1: Scheme for electrophoretic measurements of nanometer-sized vesicles. $I_0^\sigma$ is the intensity of the light entering the vesicle suspension at $x = 0$, $t$ that after the passage of the sample at $x = t$, at the light polarization $\sigma$; $t$ is the optical path length.](image)

**Conductivity Measurements**

The conductivity measurements during a rectangular electric pulse of the duration $t_E = 10$ µs and in the field strength range $1 \leq E/MV m^{-1} \leq 7.5$ have been carried out in sample cells equipped with planar stainless steel or graphite electrodes; electrode distance $t = 0.5$ or $t = 0.9$ mm; thermostated at 293 K (20º C). The initial conductivity $\lambda_0$ of the vesicle suspension before pulsing ($\lambda_0 = 28.5 \mu$Sm cm$^{-1}$) and the total conductivity $\lambda_T(t)$ after pulse application are recorded with a KNICK digital conductometer (Krüss, Hamburg). The leak


