

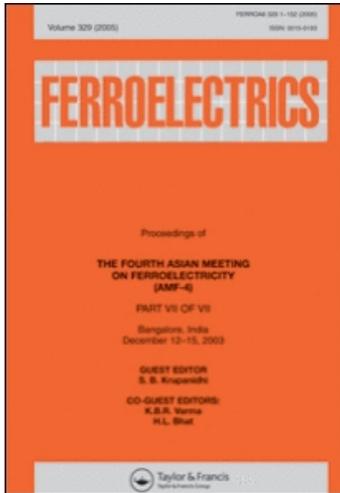
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THE ELECTROPORATION HYSTERESIS

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Electroporation and electrofusion have developed to powerful methods in cell biology and gene technology. However, the molecular mechanisms of the electroporative gene transfer and of the electric membrane fusion are still not known. Electroporation renders the membrane transiently porous and at the same time fusiogenic. In the present digression electroporation is discussed in terms of structural rearrangements in the membrane. The onset of electric pore formation is viewed as a critical phenomenon. In order to cover the reversible as well as the irreversible processes of electroporation and electrofusion, the concept of the relaxation hysteresis of membrane electroporation is introduced.

1. INTRODUCTION

In physical chemistry and biophysics electric field methods have traditionally been applied to probe the ionic-electric properties and reactivities of molecules and molecular organizations such as biological membranes.^{1,2} Nowadays electric field pulse techniques also gain increasing importance in cellular and molecular biology, in gene technology and in medicine. In particular, the methods of electroporation³ and electrofusion⁴⁻⁷ have developed to powerful tools for cell manipulations (for reviews see references⁸⁻¹⁰) and for the physical chemical study of electrically induced structural rearrangements in membranes.²

Until now there is only indirect evidence that the applied electric pulses cause structural reorganizations in the cell membranes; for early reference see Sale and Hamilton,¹¹ Neumann and Rosenheck,¹² Lindner *et al.*¹³ Although there are a couple of model approaches toward theories for electropermeabilization and electrofusion^{9,10,11-12} it is fair to say that the detailed mechanisms of field-induced restructuring of membranes in electroporation and electrofusion processes are not known. In addition, in this new field there is still a need to classify the observations in terms of physical concepts and to establish an unequivocal terminology based on physical chemical principles. In the present account, electroporation and electrofusion are discussed in terms of field-induced structural rearrangements in the membranes. In particular, electroporation is viewed as a critical phenomenon and the concept of the relaxation hysteresis is introduced to elucidate the reversible and the irreversible aspects of electroporation and electrofusion.

2. THRESHOLD AND STRENGTH-DURATION PARAMETERS

There are several experimental parallels between electroporation and electrofusion. Both field effect phenomena show threshold behavior. For a given cell the

numerical values of the threshold field strengths E_c are (almost) the same and depend inversely on the cell diameter. No doubt, both cell fusion and electroporative material exchange are clearly induced by the external field pulse. But, because of the longevity of the field-induced structural changes, the actual fusion events and the main part of the material exchange are, by and large, after-field effects.^{3,13,23-25}

In summary the data suggest that it is one and the same primary field effect on the membrane structure: leading to cell fusion if cell membranes are brought into contact before or after pulsing,²³ or causing DNA uptake if the DNA is adsorbed to the cell surface before (or after) pulsing.

Threshold Parameters

The threshold field strength E_c for electroporation (electropermeabilization and electrofusion) is a kind of "point of no return".¹⁸ If the electric field $E (\geq E_c)$ is maintained, the electropores induced by the supercritical field increase in number and size^{14,15,21} until, at a supercritical number density and pore size, the membrane ruptures (dielectric breakdown). If electric pulses of short duration Δt are applied, the field is already switched off before rupture can occur and the previous low-permeability state is apparently restored completely.

It is therefore pertinent to view membrane electroporation as a critical phenomenon, characterized by critical values for the extent ξ_c of structural rearrangement, for the field strength E_c and for the pulse duration Δt_c . In our structural model the primary requirement for the onset of electroporation is that the threshold ξ_c has to be reached; see Figure 1. In this context the subcritical

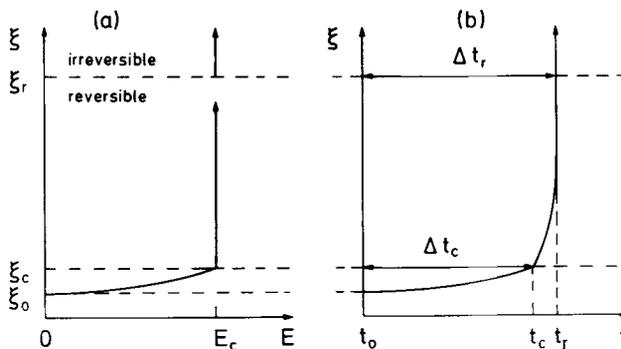


FIGURE 1 Electroporation viewed as a critical membrane phenomenon associated with critical values for the field strength E_c , the pulse duration Δt_c and the degree of structural rearrangement ξ_c . (a) electroporation starts when the threshold ξ_c is attained; the minimum field strength to reach ξ_c is E_c . At $E \geq E_c$ the electroporation process is unidirectional, i.e. irreversible, until the rupture threshold ξ_r is reached where the membrane breaks down irreversibly. If however, the field is switched off before ξ_r is attained, the electroporated membrane reseals (reversible electroporation cycle). (b) The critical pulse duration Δt_c to trigger the electroporation process at ξ_c is the smaller the larger the field strength $E (> E_c)$. The reversible electroporation cycle requires that the pulse length Δt is smaller than the rupture time Δt_r . The changes of ξ from ξ_0 to ξ_c represent reversible subcritical rearrangements such as, e.g., the increase of size and number of hydrophobic defect sites.

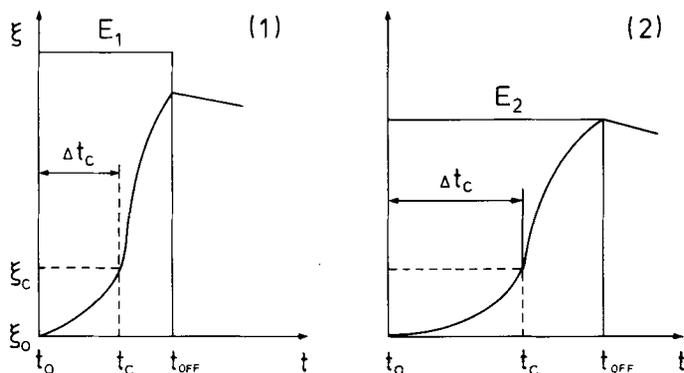


FIGURE 2 Strength/duration ($E/\Delta t_c$) relationship for the onset of the electroporation process at $\xi = \xi_c$. The critical pulse length Δt_c is the smaller the larger the field strength: $\Delta t_c(E_1) < \Delta t_c(E_2)$, if $E_1 > E_2$.

changes from ξ_0 to ξ_c represent reversible structural rearrangements such as e.g., the increase in number and size of hydrophobic defect sites and micropores in the bilayer. The minimum field strength to attain the critical value ξ_c is the critical field E_c . Once the threshold ξ_c is reached ($E \geq E_c$) the actual electroporation starts and proceeds unidirectionally (no return) until the rupture threshold ξ_r is attained where the membrane disintegrates. If the field is reduced below E_c or switched off before ξ_r is reached, the electropores or electrocracks²² reseal or anneal such that the original membrane state appears to be completely restored (reversible electroporation).¹⁸

Strength-Duration Relationship

Similar to other electric membrane phenomena such as nerve excitation, the onset of electroporation is associated with a strength-duration relationship. Since the threshold ξ_c is attained faster at a higher field strength (Figure 1), the minimum pulse duration Δt_c that is required for the onset of the electroporation process is the smaller the larger the applied external field (Figure 2).

If indeed the value E_c decreases with the independently chosen pulse length Δt ,²⁶ this feature may result from the stochastic nature of electropore formation.^{18,22} The larger Δt the larger is the probability of nucleation of the electropores at a smaller field strength.

The organization of biological membranes is highly complex: uneven surface distribution and uneven membrane thickness; structural coupling to external matrix and to intracellular cytoskeletal elements. It is therefore not possible to exactly calculate the strength-duration parameter set $E_c/\Delta t_c$ from first principles.

The usual expression given for E_c in terms of the cell radius a and the critical transmembrane voltage ($V_{m,c}$) is only an approximation. The approximation for the maximum value at the pole cap regions (where $\cos \delta = +1, -1$, respectively; see also Figure 3) is given by

$$E_c \approx 2 |V_{m,c}|/3a \quad (1)$$

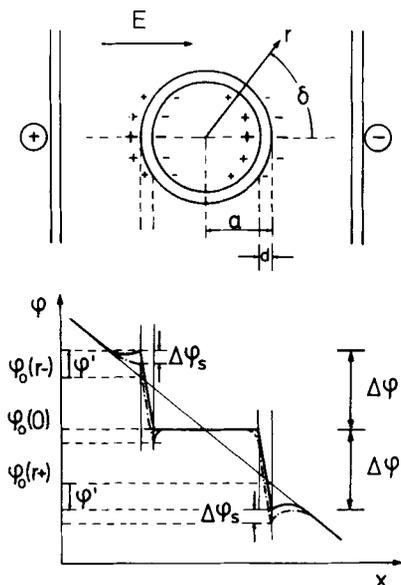


FIGURE 3 Interfacial polarization of a spherical nonconducting shell of thickness d and outer radius $r = a$ in a constant external field \mathbf{E} . The stationary electric potentials are given in polar coordinates of the radius vector \mathbf{r} and the angle δ , such that the conducting interior of the cell has the constant reference potential $\varphi_0 = 0$ for $0 \leq r \leq (a-d)$. For $r > a$, $\varphi_0 = -\mathbf{E} \cdot \mathbf{r} = -E \cdot r \cos \delta$. The total potential $\varphi(r)$, relative to $\varphi_0(0) = 0$, is given by $\varphi(r, \delta) = \varphi_0(r, \delta) + \varphi'(r, \delta)$, where $\varphi'(r, \delta)$ is the contribution of the interfacial polarization. The $\Delta\varphi$ terms are the interfacially induced, cross-membrane potential differences in the absence of fixed ionic groups and adsorbed ions, ($\Delta\varphi_s = 0$). The dash/point line models schematically the potential profile in the presence of fixed surface charges (here negative).

Equation (1) is practically very useful, even for the estimate of the threshold of the initial field strength of exponentially decaying pulses (CD-pulses); $V_{m,c} \approx 0.5$ to 1 V for short duration pulses ($\Delta t \approx 10 \mu\text{s}$) and $V_{m,c} \approx 0.2\text{--}0.5$ V for longer pulse duration ($\Delta t \geq 0.1$ ms).

3. FIELD AMPLIFICATION BY INTERFACIAL POLARIZATION

The magnitude of the applied field strength and the dependence of the threshold E_c on the cell radius indicate that the field effect on the membrane structure is indirect. The data suggest that interfacial polarization precedes the structural transitions.

The actual membrane field affecting the lipids and proteins is strongly amplified by the interfacial polarization.^{27,24} The time constant (τ_p) of the build-up of the interfacial polarization $\Delta\varphi$ is dependent on a and the conductivities (λ) of the cell interior, the cell membrane and of the external medium.²⁷ In brief, the electroporation and electrofusion data indicate the sequence of events:²⁸

$$E \rightarrow \Delta\varphi \rightarrow \Delta\xi \quad (2)$$

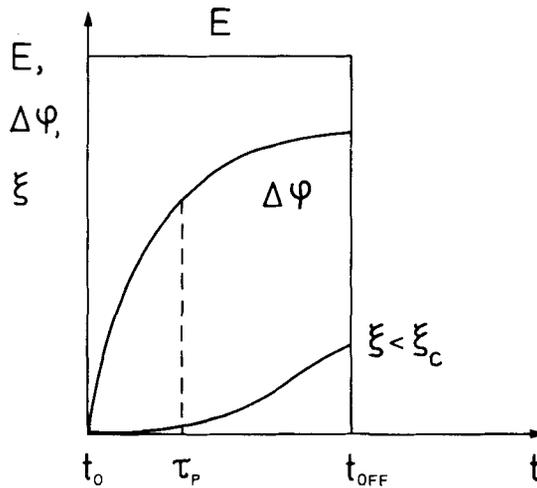


FIGURE 4 Amplified field effect. The external field pulse ($E, \Delta t$) causes the interfacial potential difference $\Delta\varphi$. The electric field, equivalent to $\Delta\varphi$, in turn induces structural rearrangements $\xi(t)$ in the membrane. Thus $\xi(t)$ is delayed with respect to the application of E at t_0 . The time course $\Delta\varphi(t)$ is represented as a simple exponential process (time constant τ_p). If the pulse duration at E is $\Delta t < \Delta t_c$, the time course $\xi(t) < \xi_c$ models a subcritical change.

where E causes the change $\Delta\varphi$, and $\Delta\varphi$ in turn causes the change in the extent $\Delta\xi$ of membrane rearrangements. In this sense the $\xi(t)$ function is delayed with respect to the application of the field pulse (Figure 4).

It is well known that all cell membranes have a natural electric potential difference, $\Delta\varphi_m$; typically, $\Delta\varphi_m = -70$ to -100 mV relative to the outside potential (zero).

The stationary value of the actual transmembrane voltage V_m , relative to the direction of the (constant) external field vector \mathbf{E} , results from contributions from the (diffusion) potential $\Delta\varphi_m$, from asymmetric surface charges ($\Delta\varphi_s$) and the interfacial polarization. The voltage drop V_m in the direction of E is given by:²⁸

$$V_m \approx -\Delta\varphi_m \frac{|\cos \delta|}{\cos \delta} - \frac{3}{2} f(\lambda) E a |\cos \delta| \quad (3)$$

where E is the amount of \mathbf{E} and the conductivity factor $f(\lambda) \leq 1$ may be approximated by $f(\lambda) = 1$ for a nonconducting membrane. Equation (3) correctly covers the signs and the angular position dependence of V_m relative to \mathbf{E} . At the pole caps in the \mathbf{E} direction, $|\cos \delta| = 1$, yielding the maximum values of V_m .

An average value for the stationary transmembrane electric field strength E_m relative to the external field vector may be estimated from:

$$E_m \approx -V_m/d \quad (4)$$

where d is the membrane thickness.

4. THE RELAXATION HYSTERESIS OF ELECTROPORATION

Without doubt, the electroporation and electrofusion data indicate that on the one hand reversible primary processes and on the other hand irreversible secondary events are involved; see Table I.

Ionic-dielectric polarization of the membrane/solution interfaces and structural rearrangements in the membrane are essentially reversible processes. Material exchange and fusion processes are passive, unidirectionally occurring relaxation phenomena of irreversible nature. Furtheron, the data indicate that direct external field effects on the membrane structure are of minor extent. Rather, interfacial polarization at first leads to the strong (amplified) transmembrane field which in turn induces the major structural rearrangements.²⁸

Obviously, a transient permeability increase¹² indicates transient membrane

TABLE I
Fundamental processes of the electroporation hysteresis of membranes

Physical-chemical processes	Electric terms
I. Reversible primary processes	
1. <i>Primary electric events</i>	
1.1 Electric dipole induction and dipole orientation	Dielectric polarization
1.2 Redistribution of mobile ions at phase boundaries membrane/solution, including (a) ionic atmosphere shifts (b) local activity changes of effectors, e.g., H ⁺ -(pH-changes) or Ca ²⁺ -ions.	Ionic-dielectric interfacial polarization (Maxwell-Wagner, β -dispersion)
2. <i>Structural rearrangements</i>	Electro-restructuring
2.1 Conformational changes in protein and lipid molecules	
2.2 Phase transitions in lipid domains, resulting in pores, cracks (via pore coalescence) and percolation	Electroporation Electropores, electrocracks Electropercolation
2.3 Annealing and resealing processes	
II. Irreversible secondary processes	
1. <i>Transient material exchange</i>	Electropermeabilization
1.1 Release of internal compounds, e. g. hemolysis	Electrorelease
1.2 Uptake of external material, e.g. drugs, antibodies	Electroincorporation, Electrosequencing
1.3 Transfer of genetic material, e.g. DNA, mRNA, viroids, . . . with stable cell transformation	Electrotransfection Electrotransformation, Electroporative gene transfer
2. <i>Membrane reorganizations</i>	
2.1 Cell fusion (if membrane contact)	Electrofusion
2.2 Vesicle formation (budding)	Electrovesiculation Electrobudding
2.3 Electromechanical rupture	Dielectric breakdown
3. <i>Tertiary effects</i>	
3.1 Temperature increase due to dissipative processes	Joule heating, dielectric losses
3.2 Metal ion release from metal electrodes	Electroinjection
3.3 Electrode surface H and O in statu nascendi	Electrolysis

“openings”: pores or cracks which reseal after pulsing. When the cycle of permeability increase and decrease is modelled on the level of electropores,^{14,18,22} the formation-resealing cycle of a pore is represented as a cyclic local change of the membrane structure. In addition, local deformations of the pore edges may lead to crater-like pore structures.²⁸

Metastable States and Unidirectional Transitions

As outlined previously, the apparent dependence of the threshold field strength E_c on the pulse length²⁶ indicates a membrane specific critical threshold ξ_c at which the electroporation process is triggered. Once initiated, the process is unidirectional, i.e., irreversibly running at constant E ($>E_c$). Therefore, the membrane state just before the onset of the electroporation process must be metastable. The minor structural rearrangements before electroporation ($\xi_0 < \xi < \xi_c$) are apparently reversible but occur on a metastable level.²⁹

At short pulse durations ($\Delta t < \Delta t_r$) such that the rupture threshold ξ_r was not reached, the electroporated (and fusiogenic) membrane is again metastable with respect to less porous structures. The pore resealing process (at $0 < E < E_c$) is also unidirectional, i.e. irreversibly running until intact bilayer structures are restored.

In this sense membrane electroporation represents a cycle of structural rearrangements (Figure 5), where the intermediate states of the annealing process are probably different from those of the electroporation process in the presence of the external field.

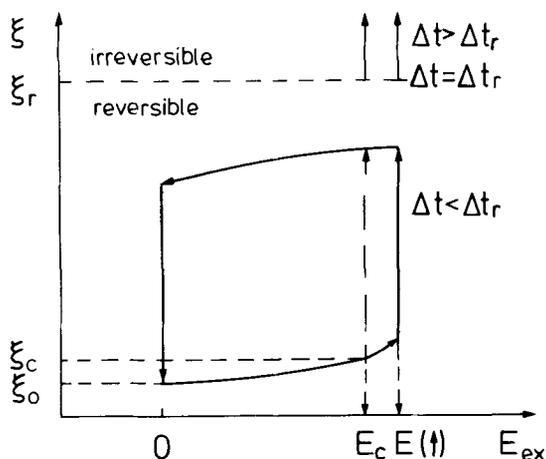


FIGURE 5 The relaxation hysteresis of the membrane electroporation. The cyclic change in the extent of structural rearrangements ξ (at $E > E_c$ and $\Delta t(E) < \Delta t_r$) are displayed as a function of the external field (E_{ex}). The cycle comprises reversible as well as irreversible elements. The subcritical changes of ξ between ξ_0 and ξ_c ($E < E_c$) are reversible. At the supercritical field strength E ($>E_c$) the structural rearrangements associated with the electroporation process are unidirectional, i.e. irreversible. If the field duration is larger than the rupture threshold (Δt_r), the membrane ruptures. If however the field pulse is switched off at $\Delta t < \Delta t_r$, we remain in the reversible electroporation domain. The (slow) return at $E = 0$ from the upper branch to $\xi = \xi_0$ is unidirectional; the resealing or annealing processes are irreversibly occurring relaxations to the initial state ξ_0 .

The physical conception that comprises both (reversible) metastable states and (irreversible) unidirectional transitions in a cyclic manner is called hysteresis.²⁹ Therefore the electroporation/resealing cycle may be analyzed and understood in terms of a structural relaxation hysteresis.

Energetics of the Electroporation Hysteresis

The energetics of the field-induced structural rearrangements in the electroporation/resealing cycle can be thermodynamically treated independent of any special mechanism.

The characteristic reaction free enthalpy $\Delta_r G(E)$ for the transition from the intact bilayer state to pore configurations is given by

$$\Delta_r G(E) = \Delta_r G - \int_0^{E_m} \Delta_r M dE_m \quad (5)$$

where E_m is given by Equations (3) and (4) and $\Delta_r G$ is the reaction free enthalpy in the absence of E .^{30,2} Obviously, pore formation is energetically unfavorable at $E = 0$; hence $\Delta_r G > 0$.

The reaction dipole moment $\Delta_r M$ is related to the difference between the moments M_w of the water filled pore and M_m of the bilayer having the same size as the aqueous pore.

Applying the dielectric continuum model the polarization moments (relative to vacuum) are given by

$$M = \epsilon_0(\epsilon - 1)vE_m \quad (6)$$

where ϵ_0 is the dielectric permittivity of the vacuum, ϵ the dielectric constant of the medium and v is the volume of the electroporated membrane.

The polarization of the water ($\epsilon_w \approx 80$) near the pore edge in the pore wall¹⁸ is energetically more favorable (by a factor of about 40) than the polarization of an equal volume of lipid bilayer ($\epsilon_m \approx 2$).¹⁴ With $\Delta_r M = N_A (M_w - M_m)$, where N_A is the Avogadro constant, we can specify

$$\Delta_r M = N_A \epsilon_0 (\epsilon_w - \epsilon_m) v(\delta) E_m \quad (7)$$

If $d \ll a$, the electroporated membrane volume $v(\delta)$ is given by $v(\delta) \approx 4\pi a^2 d(1 - |\cos \delta|)$ where the fraction of spherical membrane shell affected by a supercritical field is $(1 - |\cos \delta|) = 1 - E_c/E$ (Figure 3).

It is seen from Equation (7) that, because of $\epsilon_w > \epsilon_m$, we obtain $\Delta_r M > 0$. Therefore, the unidirectional electroporation process is associated with $\Delta_r G(E) < 0$; obviously because $\int \Delta_r M dE_m > \Delta_r G$; see Equation (5). On the same line, the initial driving force for the unidirectional pore resealing process is given by $\Delta_r G_{res.} = -|\Delta_r G(E)|$.

In the case of unidirectional transitions, starting from metastable states, the rate equation for the extent ξ_i of the i -th mode of the structural rearrangements,

$$d\xi_i/dt = k_{ij}\xi_i - k'_{ij}(1 - \xi_i) \quad (8)$$

is reduced to the simple form

$$d\xi_i/dt = k_{ij}\xi_i \quad (9)$$

Because of the irreversibility the reverse process can be neglected. The general

rate coefficient k_{ij} represents a combination of the rate constants k_j of all elementary steps j contributing to the mode i .

Finally, the field dependence of the rate constant k_j is described by

$$k_j(E) = k_j(0)e^{f\Delta_r M_j^* dE/RT} \quad (10)$$

where $\Delta_r M_j^*$ is the transition dipole moment of the activated state, R the gas constant, T the absolute temperature and $k_j(0)$ the value at $E = 0$.

The general remarks and the explicit expressions given in this programmatic study may provide a general framework for the thermodynamic and kinetic analysis of the electroporation and electrofusion processes in terms of a hysteresis formalism.

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