Mechanism for the conductivity changes caused by membrane electroporation of CHO cell-pellets†‡

Marco Schneere, Thomas Seipp, Uwe Pliquett, Sergej Kakorin and Eberhard Neumann*

Physical and Biophysical Chemistry, Faculty of Chemistry, University of Bielefeld, P.O. Box 100 131, D-33501 Bielefeld, Germany. E-mail: eberhard.neumann@uni-bielefeld.de; Fax: +49 521 106 29 81; Tel: +49 521 106 20 53

Received 19th July 2004, Accepted 29th October 2004
First published as an Advance Article on the web 23rd November 2004

Electric field pulses, applied to densely packed pellets of Chinese hamster ovary (CHO) cells of mean radius \( a_c = 7.5 \pm 0.7 \) μm, cause major electric conductivity changes, described by three kinetic normal modes. The first mode reflects Wien effects of ionic atmosphere perturbations and ion pair dissociations (cell surfaces). Using Maxwell’s conductivity equation, the second and third mode are converted to the respective membrane conductivity modes. Electrothermodynamic analysis in terms of structural transitions from closed (C) to porated (P) membrane states of very different lifetimes, according to the scheme (C \( \rightleftharpoons \) C1 \( \rightleftharpoons \) (P2 \( \rightleftharpoons \) P3), yields the mean zero-field pore conductivities \( \kappa_{f,2} = 1.00 \pm 0.05 \) nm (P2) pores and \( \kappa_{f,1} = 1.5 \pm 0.1 \) nm (P3) at \( T = 293 \) K (20 °C). The relaxation time \( \tau_2 \) (P2-formation) reflects the rate limiting step (C \( \rightleftharpoons \) C1), associated with the activation dipole moment of \( \Delta m_1 = 63 \times 10^{-30} \) m or (19 Debye units), suggesting orientation changes of dipolar lipid head groups, in the solution membrane interfaces preceding the actual pore formations. Besides: the field-dependencies of the pore fractions \( f_2 \) and \( f_3 \) (order of \( 10^{-1} \)), the field reduction factors \( f_{j,\leq 1} \) and the membrane voltage, we obtain the zero-field pore conductivities \( \kappa_{f,2} = 1.7 \times 10^{-7} \) mS cm\(^{-1}\) (P2) and \( \kappa_{f,3} = 0.10 \) mS cm\(^{-1}\) (P3) and the membrane conductivity \( \kappa_{m} = 3.2 \) mS cm\(^{-1}\). The post-field conductivity changes, due to the long-lived P2- pores, are analyzed in terms of time- and field-dependent efflux coefficients. The characteristic post-field pore resealing time \( \tau_m = \tau_2^p = 45 \pm 3 \) s is independent of the field strength of the causative pulse and independent of the distance between the two electrodes. These results are an essential part for the optimization of the electrical pulse parameters, also for the clinical electrotansfer of bioactive substances into aggregated biological cells (tissue).

1. Introduction

Membrane electroporation (MEP) is a concept and technique to render cell membranes transiently porous and permeable to otherwise impermeable substances. The various applications of MEP include the direct functional transfer of genes (electrotransfection), release of proteins, and the electrotransfer of ionic dyes and drugs into cells. MEP of cell tissue gains increasing importance especially in clinical applications such as electrochemotherapy (ECT) of skin tumors and for gene therapy. In brief, when electric field-pulses are applied to densely packed pellets of Chinese hamster ovary (CHO) cells may serve as model systems for tissue. Traditionally, electric field-induced permeable pores in cell membranes have been identified by the transfer of ionic dyes  and by the small-ion transport reflected in conductivity changes.

Abidor et al. (1994) have pioneered the electroporation of cell aggregates using a particular set-up with a small cell pellet near to one of the two electrodes and the supernatant. Here, we use a configuration where only the cell aggregate is in contact with the electrodes. The pellet fills the total chamber space with only minimal amounts of solvent in the interstitial volume between the densely packed cells and no supernatant. Previously, Kinosita and Tsong (1979) have shown that electric field pulses (1.5 \( \leq E/kV cm^{-1} \leq 6 \) and 80 μs pulse duration) cause drastic increases in the conductivity of sucrose suspensions of erythrocytes. The conductance relaxations during and after the pulse have been discussed in terms of membrane processes: pore formation and pore expansion, but also other non-pore contributions. In the mean time, salt-filled lipid vesicles and mouse skin tumors have been shown to exhibit kinetic conductivity curves similar to those of erythrocytes and to be analysable in terms of kinetic normal modes. Here we show that the kinetics of the pellet conductance changes reflects the rate-limiting steps of electric pore formations in the membranes of the pellet cells.

† Electronic supplementary information (ESI) available: Appendices 2–5 with derivations of the main equations. See http://www.rsc.org/suppdata/cp/b4/b411037d/
2. Materials and methods

2.1 Preparation of the cells and cell pellets for electroporation

Chinese hamster ovary cells (CHO-K1) are cultivated in cell culture flasks (NUNC, Wiesbaden, Germany) in a DMEM/F12-medium containing fetal calf serum, in a CO2-incubator at 37 °C. For the electroporation experiments the cells are first trypsinized for 3 min at 20 °C. The trypsinized cells are washed twice and then resuspended in 290 mM sucrose solution to preserve isosmolarity. The cell density of the starting suspension is \( \rho = 4 \times 10^7 \) cell ml\(^{-1}\). The cell suspension is placed between the two planar stainless steel electrodes, mounted in a single-use polycarbonate cuvette (PLASTIBRAND, BRAND, Wertheim, Germany) and then centrifuged in specially modified cup holders at various centrifugation speeds for 5 min (Kontron Hermle ZK 365, HERMLE Labortechnik, Weihingen, Germany). In order to avoid shunting pathways due to supernatant medium, a Teflon slide is moved into the space between the electrodes on top of the cell pellet, to remove the remaining medium.

The four electrode system (used for some experiments) consists of the two conventional parallel stainless steel electrodes with an electrode distance \( l = 2 \text{ mm} \). Into these electrodes is inserted a gilded conductor board slide with the two small measuring electrodes of the electrode distance \( l = 1 \text{ mm} \). Every experiment has been performed at \( T = 293 \text{ K (20 °C)} \). The cells have been visualized by a phase contrast microscope (BH-2, OLYMPUS, Hamburg, Germany).

2.2 Electric field pulses and conductance relaxations

Single rectangular field pulses have been applied with a modified ELEKTROPORATOR II® pulse generator (DIALOG, Düsseldorf, Germany). The pulse generator is particularly suited for biophysical studies of membrane electroporation. The time courses of voltage \( U(t) \) and current \( I(t) \), respectively, during field-pulse application are monitored on the screen of a TEKTRONIX TDS 720A (Beaverton, Oregon, USA) and then computationally processed. The conductance of the sample chamber before and after the field pulse is recorded by a conventional conductometer (KNICK 600, Berlin, Germany) as a function of time, respectively, to monitor ion leak and resealing of electroporated cells.

In the four electrode system, the conventional outer electrodes are connected to the pulse generator ELEKTROPORATOR I (Dialog, Düsseldorf, Germany) and the inner ones to an oscilloscope (Tektonix TDS 540 D, TEKTRONIX, Beaverton, USA), respectively; the inner, measuring electrodes are connected via a differential high voltage probe (SI 9000, Scheid AG, Switzerland), yielding the actual voltage \( U \) across \( l = 1 \text{ mm} \) of the cell sample.16 Hence the applied field strength is given by \( E = U/l \). The changes \( \Delta \Lambda(t) \) with time \( t \) of the conductance \( \Lambda = I/U \) are expressed in terms of relative conductance and conductivity changes, respectively, by:

\[
Y(t) = \Delta \Lambda(t)/\Lambda_0 = \Delta \lambda(t)/\lambda_0, \tag{1}
\]

where \( \Delta \Lambda = \Lambda - \Lambda_0 \) and \( \Delta \lambda = \lambda - \lambda_0 \) are the field-induced changes in the conductance \( \Lambda = \lambda(A/l) \) and conductivity \( \lambda \), calculated from the cell constant \( A/l \) of the measuring chamber of electrode area \( A \) and distance \( l \) between the electrodes. \( \Lambda_0 \) and thus \( \lambda_0 \) refer to the zero field-pulse solution before pulsing, respectively.

3. Results

3.1 Packing density and transmembrane transport of small ions

CHO cells in sucrose solutions of various packing densities \( \rho \) have first been subjected to a single, rectangular electric pulse of field intensity \( 1 \text{ kV cm}^{-1} \) and duration \( t_E = 1 \text{ ms} \). The effect of \( \rho \) is most dramatically seen in the after-pulse conductivity relaxations \( Y_{\text{off}}(t) \) of the suspension (pellet plus supernatant) heading at respective stationary values, clearly indicating resealing of electropores, formed previously during the pulse (Fig. 1). It is also seen that the increase in the packing density, caused by the increased centrifugation velocity (RCF) before the pulse, leads to smaller values of the conductivity relaxations \( Y_{\text{off}}(t) \) of all samples is within \( 0.09 \pm 0.01 \text{ mS cm}^{-1} \). The data suggest that the decrease of the stationary values \( Y_{\text{off}}(t = 300 \text{ s}) \) results from the decrease in the portion of conductive solution in the interstitial space of the cells in the pellet with increasing packing density. On the other hand, the \( \lambda_0 \) of the cell pellet depends on the volume fraction \( f_c \) (Fig. 2).

3.2 Conductivity changes during and after field-pulse application

In-field conductivity changes. In Fig. 3, it is seen that the densely packed cells in the remaining low-conductive medium of 290 mM sucrose, exhibit relative conductivity increases during, \( Y(t) \), and also after field-pulse application, \( Y_{\text{off}}(t) \), respectively, that are particularly large. The conductivity values \( Y(t_E) \) at the end of the pulse at \( t_E = 1 \text{ ms} \), increase with increasing field strength. The in-field conductivity changes \( Y(t) \) show several kinetic modes. The curves (2) and (3) in Fig. 3 display a very rapid, not time-resolved increase (only observed...
for $E \geq 0.8 \text{kV cm}^{-1}$ followed by a slower increase with at least two, kinetically very different, contributions. As compared to the results of the field-induced conductivity changes of salt-filled vesicles, the rapid phase indicates external cell surface effects whereas the two slow relaxations are suggestive for electric field-induced formation of membrane pores.

Post-field conductivity changes. After pulse termination at $t_0$, there is a dramatic decrease of $Y$ from the in-field value $Y(t_0)$ to the initial value $Y^{off}(t = 0)$, measured shortly after pulse termination on the time scale of seconds. The rapid conductivity decrease is followed by a slower increase due to continuing electrolyte efflux through the resealing membrane pores, finally termination on the time scale of seconds. The rapid conductivity decrease is followed by a slower increase due to continuing electrolyte efflux through the resealing membrane pores, finally reaching a stationary value, indicating no further release of electrolyte (see also Fig. 1). Fig. 4 shows an example of conductivity changes observed for a sequence of three electric field pulses applied to one cell-pellet. In line with the data represented in Fig. 3, the in-field conductivity changes suggest formation of membrane pores, which partially reseal in the after-field pulse period. The respective overall conductivity increase after the pulse in each case suggesting continuing electrolyte efflux into the small intercellular (interstitial) space.

Flow analysis. The uptake of dye molecules by electroporated cells (within 30 min) is stronger very near to the electrode surfaces [M. Schmeer, PhD thesis, University of Bielefeld, 2004], indicating that the field-induced permeabilization is stronger in the high local fields of, although polished, yet not quite smooth, metal surfaces. If the surface-adherent CHO-cells are more strongly affected than those of the bulk pellet and release large amounts of electrolyte, the released cations ($K^+$, $Mg^{2+}$, etc.) and anions ($HCO_3^-$, $Cl^-$, $H_2PO_4^-$, etc.) will cross the pellet of thickness $l$ along the field lines of the weak measuring voltage between the electrodes. This one-dimensional (electro-) diffusion is characterized by the time constant $t_0 = l^2/2D$, where $D = 2 \times 10^{-5} \text{cm}^2 \text{s}^{-1}$ is a mean diffusion coefficient for small cations and anions (at $T = 293 \text{ K, } 20 \text{ °C}$). For two different electrode distances, here $l_2 = 2l_1$, the ratio of the diffusion time constants is $t_{02}/t_{01} = (l_2/l_1)^2 = 4$. The resealing time $t_R$ is a kind of characteristic time derived from the $Y^{off}(t)$ data at $t \geq t_R$. According to Kakorin et al. (1998), the field-off relaxations are described by:

$$Y^{off}(t) = Y^{off}(1 - \exp[-k(t-t_0)/t_R] - \exp[-t/t_R]),$$

where the stationary value is given by $Y^{off} = 1/(1-\exp[-k(t-t_0)/t_R])$.

Data evaluation yields the flow coefficient $k_{p}(t_0)$ at time $t_0$ and the value of $t_R$; see Fig. 5. In eqn. (2), $Y^{off}$ is the amplitude and $k(t_0) = 3f_s(t_0)\eta_3/\mu_0$, where $f_s(t_0)$ is the pore fraction of the $P_3$ pore state at $t = t_0$, $\eta_3 = \eta_2/\eta_0$ is the permeability coefficient for pore type $P_3$, $\gamma = \rho_3/\rho_0$, the distribution coefficient of ions in the pore ($\gamma_0$) of length $d_R$ relative to the cell interior ($c_{w0}$) and $d_0$ the cell radius. As expected, $t_R$ is independent of $E$.

If the $Y^{off}(t)$ curve were due to the diffusion of electrolyte from irreversibly electroporated CHO cells touching the electrode surface, the relation $t_{R0}(l_2)/t_{R0}(l_1) = t_{F0}(l_2)/t_{F0}(l_1) = 4$ should hold. Experimentally it is found that $t_{R0}(l_2) = t_{R0}(l_1)$ holds true in the range $0 \leq E/kV \text{ cm}^{-1} \leq 1.5$ (Fig. 6), suggesting no diffusion of formerly intracellular ions from the electrode surface. This result is consistent with $t_R = \sqrt{t}$.
i.e., \(Y_{\text{MF}}(t)\) is caused by the resealing of the long-lived pores, underlying the kinetic in-field normal mode (3); see below.

### 3.3 Size distribution of CHO-cells, packing density and conductivity

Due to different growth states, the cell radius of the suspended CHO-cells (phase contrast microscope) is different for every cell. The size distribution of the cells can be represented by a Gauss-like function \(P(a_i) = n_i/N\), where \(n_i\) is the number of cells with the particular radius \(a_i\) relative to a total amount of \(N\) cells; data not shown. The mean cell radius (standard deviation) is \(\bar{a}_c = 7.5 \pm 0.7 \mu m\). The conductivity \(\sigma_0\) of a CHO-cell suspension or pellet in 290 mM sucrose without application of an electric field pulse decreases with increasing cell density \(\rho = N/V\), represented in terms of the volume fraction \(f_c\) of the cells (Fig. 2). If the packing of the cells in a pellet (aggregate) is, for instance, viewed as a face-centered cubic order, this cubic cell configuration comprises \(N = (1/2) \times 6 + (1/8) \times 8 = 4\) cells per cubic unit; see below. The volume fraction of the maximum cubic packing is given by \(f_c = 0.67\). Experimentally, the packing density of the CHO cells in the pelleted used here, corresponds to a volume fraction \(f_c = 0.67\), being smaller than the value \(f_c = 0.74\) for actual cell–cell contact. The mean distance between the cell surfaces is \(L = 2\bar{a}_c = 1 \mu m\), where \(L = 16 \mu m\). Since \(2\bar{a}_c = 15 \mu m\), on average, there is no tight cell–cell contact.

### 4. Data analysis and discussion

#### 4.1 Packing density affects field-induced transmembrane potential

For spherical cells of radius \(a_i\) in a diluted suspension, the stationary value of the field-induced potential difference \(\Delta \varphi_{\text{ind}}\) is given by the Fricke equation \(^{18}\) in the form:\(^{19}\)

\[
\Delta \varphi_{\text{ind}}(\theta) = -1.5f_j a_i E \cos \theta, \tag{3}
\]

as the electric potential drop in the direction of the external field (vector) \(E\), where \(\theta\) is the angle between the membrane site considered and the direction of \(E\) through the midpoint of the cell. Because for bi-anisotropic cells \(a_i\) is a function of the thickness of the lipid membrane is usually taken as \(a_i = 5 \text{ nm}\), the inequality \(d_{\text{ex}} \ll a_i\) holds true and the conductivity term \(f_j\) is approximated by \(f_j = 1 - \lambda_{\text{md}}(a_i/d_{\text{ex}})(2 + \lambda_{\text{md}}/\lambda_{\text{ax}}))/(2\lambda_{\text{ex}})^2\) where \(\lambda_{\text{md}}\) and \(\lambda_{\text{ax}}\) are the conductivities of the interior and exterior medium, respectively, and \(\lambda_{\text{ex}} = d_{\text{ex}} G_{\text{m}}\) is the membrane conductivity. The specific membrane conductance at zero applied field \(G_{n0}\) of cellular membranes is in the range \(1 \leq G_{n0} / \text{mS cm}^{-2} \leq 100\). \(^{20}\)

Because here \(\lambda_{\text{md}} \approx 20 \lambda_{\text{ex}}\), the conductivity factor \(f_j\) reduces to \(f_j = 1 - \lambda_{\text{md}}(a_i/d_{\text{ex}})^2/\lambda_{\text{ex}}^2\). In any case, in this notation \(\lambda_{\text{md}}\) refers to a homogeneous membrane and eventually to a cosine \(\theta\)-average, if an external field is applied. For the pole caps facing the plane electrodes (\(\cos \theta = 1\)), eqn. (3) is reduced to the cap average value \(\Delta \varphi_{\text{ind}}(\theta) = -1.5 E a_i f_j\) of the suspended (s) cells.

According to Abidor et al., densely packed cells in a pellet are modeled as parallel rows of resistances, along the electrode distance \(l^2\). Every resistance represents one membrane perpendicular to the direction of the external electric field vector, such that each cell contributes with two membranes, i.e., two resistances. In this configuration the induced transmembrane potential difference \(\Delta \varphi_{\text{ind}}\) for densely packed cells (p) is given by \(\Delta \varphi_{\text{ind}}(p) = -U/(2n),\) where \(n = l/(2\bar{a}_c)\) is the number of cells in one cell layer between the electrodes. Substitution yields:

\[
\Delta \varphi_{\text{ind}}(p) = -E a_i f_j, \tag{4}
\]

where \(E = U/l\). We use eqn. (4) throughout as a practical approximation. The induced voltage has been calculated for various theoretical cell configurations and orientations;\(^{21}\) for a simple cubic lattice, at the pole caps, eqn. (4) applies.

Comparison with the expression for \(\Delta \varphi_{\text{ind}}(s)\) yields \(\Delta \varphi_{\text{ind}}(s) = 1.5 \Delta \varphi_{\text{ind}}(p)\). Therefore, at the same external voltage, dense packing of the cells reduces the transmembrane potential at the pole caps by a factor of 2.3. With increasing packing density, the geometrical shape factor \(F\) in \(\Delta \varphi_{\text{ind}}(p) = -F E a_i E \cos \theta\) formally decreases in the range of \(1.5 \geq F \geq 1.0\).

### 4.2 In-field conductivity relaxations

The actual relative conductivity changes \(\gamma(t)\) during the field pulse are first analyzed in terms of kinetic normal modes according to the general ansatz \(\gamma(t) = \sum Y_i(t) = \sum Y_i(1 - \exp[-(t/t_i)])\) for \(i = 1,2,3,\ldots\) modes,\(^{3}\) where \(Y_i\) is the amplitude and \(t_i\) the relaxation time of mode \(i\), respectively. If applied to the data set in Fig. 3, the general expression can be specified as

\[
Y_i(t) = Y_i + Y_2(1 - \exp[-(t/t_2)]) + Y_3(1 - \exp[-(t/t_3)]), \tag{5a}
\]

where \(Y_2\) and \(Y_3\) are the amplitudes and \(t_2\) and \(t_3\) the relaxation times of mode (2) and mode (3), respectively. The formation of \(P_3\) pores, depending on the built up of the \(P_2\) pores, is at the expense of \(P_3\) pores is according to eqn. (5b).

Since the data suggest that \(t_2 \gg t_3\), it is practical to define the slope parameter \(m = Y_2/(t_2 - t_3)\) for the time range \(t > t_2\) with the practical, artificial setting \(Y_2(t = t_3) = 0\). Generally, we have, of course, \(Y_3(t = 0) = 0\). For the short-time range \(0 \leq t < t_3\), where the approximations \(Y_3(1 - \exp[-(t/t_3)]) = Y_3/t_3 = m t_3\) and \(Y_2(1 - \exp[-(t/t_3)]) = -t_3(1 - \exp[-(t/t_3)])\) apply, eqn. (5b) reduces to:

\[
Y_3(t) = m t_3(1 - \exp[-(t/t_3)]), \tag{5c}
\]

as the practical approximation for the in-field part of \(Y(t)\), see Fig. 7, curve (d). It is shown below that the ratio \(Y_3(t)/Y_3\) equals the respective ratio \((f_2(t) - f_2(0))/(f_2 - f_2(0))\) of the fractions of \(P_3\) pores. Note, that eqns. (5) qualify the contributions of the modes of type \(i\) in terms of parallel electric circuit elements. In Fig. 7, the separate parallel contributions to the experimental in-field curve \(Y(t)\), denoted by (a), are visualized for \(E = 1.5\text{ kV cm}^{-1}\) in terms of eqns. (5).

---

**Fig. 7** Visualization of the kinetic normal mode analysis of the measured relative conductivity changes \(\gamma(t) = \lambda(t)/\lambda_a\). Curves (a) and (e), during and after application, respectively, are typical for a field-pulse of \(E = 1.5\text{ kV cm}^{-1}\) and duration \(t = 1\text{ ms}\), applied to a CHO-cell pellet prepared at RCF = 90 g in 290 mM sucrose; \(\lambda_a = 0.09\) mS cm\(^{-1}\) at \(T = 293\text{ K}\). The curve (b) refers to \(Y(t) - Y_1\) and curve (c) refers to the electroporation phase (2) according to \(Y_2(t) = Y_3(1 - \exp[-(t/t_2)])\). Curve (d) represents the electroporation phase (3) according to eqn. (18) of the text. The time constants \(t_2\) and \(t_3\) refer to of the kinetic normal modes (2) and (3), respectively.
Within the field strength range $0 \leq E \leq 4$ kV cm$^{-1}$, the conductivity amplitudes $Y_1$ and $Y_2$, respectively, of the electroporative phases (2) and (3), in terms of the relative changes $l_{iP}$, in the membrane conductivity $\lambda_{m}$ (Fig. 10c) with increasing field strength $E$. Iterative data fit (dashed lines) according to eqns. (33) and (34) of the text with (a) $\Delta \lambda_{2,0} = 2.4 \times 10^{-13}$ S m$^{-1}$ and (b) $\Delta \lambda_{3,2} = 1.0 \times 10^{-13}$ S m$^{-1}$ yields $\tau_{2,0} = 29$ cm$^2$ kV$^{-1}$ and $\tau_{3,2} = 40$ cm$^2$ kV$^{-1}$, respectively. The resulting mean pore radii are $r_2 = 1.0 \pm 0.1$ nm and $r_3 = 1.5 \pm 0.1$ nm, respectively, for $P_2$ and $P_3$. The solid lines represent the data fit in the entire range of the field strengths with the conductivity factor $f_{2,0} = f_{2,2} = f_{3,3}$ in (a) and (b) with $f_{2,0}$ in (c), respectively, see Fig. 10(a).

It is recalled that the documentation of the measured conductivity relaxations according to $\Delta(l) = \lambda_0 + \sum_i \Delta \lambda_i = \lambda_0 + \sum_i Y_i$ for $i = 1, 2, 3$, in terms of the relative changes $Y_i = \Delta \lambda_i / \lambda_0$, where $\Delta \lambda_i = \lambda_i - \lambda_{i-1}$, is appropriate for the data logistics and permits instructive comparisons between the various pellet aliquots. The aim, however, is to calculate the respective changes $\Delta \lambda_i$ in the membrane conductivity $\lambda_{m}$ of the cells in the pellet. Since the kinetic phase $\Delta \lambda_1 = \lambda_0 (1 + Y_1)$ does not

---

**Fig. 8** The amplitudes $Y_1$ and $Y_2$, the slope $m_1$ and the time constant $\tau_2$, respectively, of the kinetic normal modes, of the pellet conductivity changes during field-pulse application ($t_0 = 1$ ms) to CHO-pellets prepared at RCF = 90 g in 290 mM sucrose, all as a function of the applied field strength $E$. There are apparent threshold values (arrows): $E_{th}(1) = 0.90 \pm 0.05$ kV cm$^{-1}$ for phase (1), $E_{th}(2) = 0.70 \pm 0.05$ kV cm$^{-1}$ for the electroporative phases (2) and (3), respectively.

**Fig. 9** The field-induced changes in the membrane conductivity (a) $\Delta \lambda_{m2,0}$ due to $\Delta \lambda_2 = \lambda_0 (1 + Y_2)$ of the electroporative phase (2), (b) $\Delta \lambda_{m3,2}$ due to $\Delta \lambda_3 = \lambda_0 (1 + Y_3)$ of the electroporative phase (3) at the time $t_0$ and (c) the relaxation rate $\tau_2$ with increasing field strength, too, see below.
...and several porous states (P) [or clusters rally, electric pore formation appears to involve several closed membrane states (C) and several porous states (P) that contribute to \( \lambda_{\text{m}} \), we rearrange \( \lambda(t) \) according to \( \lambda(t) = \lambda(t) - \Delta \lambda(t) \) and obtain the terms \( \lambda_i(t) = \lambda_0 + \sum \Delta \lambda_i \) for \( i = 2, 3 \), for further analysis. Thus, respectively:

\[
\lambda_2^{(t)} = \lambda_0 + \Delta \lambda_2(t), \quad \lambda_3^{(t)} = \lambda_0 + \Delta \lambda_2 + \Delta \lambda_3. \tag{6}
\]

It is now \( \lambda_2^{(t)} \) which enters the calculation procedure to yield the respective membrane conductivity \( \lambda_{\text{m},\text{t}} \), see below. Finally, the separate field-induced changes \( \Delta \lambda_i \) constitute the kinetic normal modes for \( i = 2, 3 \), according to \( \Delta \lambda_i(t) = \lambda_i(t) - \lambda_i \). Specifically,

\[
\Delta \lambda_2(t) = \lambda_2^{(t)} - \lambda_0, \quad \Delta \lambda_3(t) = \lambda_3^{(t)} - \lambda_2^{(t)}, \tag{7}
\]

where \( \lambda_2^{(t)} \) is the (time-independent) maximum value of \( \lambda_2^{(t)} \) and, obviously, \( \lambda_1 \) holds true.

### 4.3 General chemical electrothermodynamics of MEP

Physical-chemically, the dynamics of pore formation and resealing is viewed as a local, cooperative structural transition of \( n \) neighbouring lipid molecules (L) and water (W) to form a lipid pore cluster \( L_n(W) \). If, for instance, one lipid in one monolayer is replaced by water molecules, \( n \geq 12 \) lipids form a membrane spanning aqueous pore through the two monolayers according to the scheme \( nL + W \rightleftharpoons L_n(W) \). Structurally, electric pore formation appears to involve several closed membrane states (C) and several porous states (P) [or clusters

\[ L_n(W) \] and has been previously modelled by the structural overall scheme (C) \( \rightleftharpoons (P) \).

In appendix 1, it is derived how the overall distribution constant \( K = \left[ \left( C \right) \right]/\left( \left( P \right) \right) = f(1 - f) \) and the overall degree of poration \( f = \left[ \left( P \right) \right]/\left( \left( C \right) \right) + \left[ \left( C \right) \right] = K(1 - K) \) are connected to the reaction energetics by \( K = \exp[\Delta G/RT] \), where \( \Delta G \) is the overall standard value of the transformed Gibbs reaction energy; \( R = k_B N_A \), where \( k_B \) is the Boltzmann constant, \( N_A \) the Avogadro constant and \( T \) the absolute (Kelvin) temperature. Note that the required Legendre transformation \( G = G - E \mu_{\text{m}} M \), where \( M \) is the electric (dipole) moment relates the appropriate Gibbs reaction energy to \( K \) through \( \Delta G = \Delta G_{\mu} = \Delta G_{\mu} - \Delta G M \mu_{\text{m}} \) for \( E \), where \( E \) is the (Maxwell) membrane field.

The conventional Gibbs reaction energy term \( \Delta G \) covers the various non-electrical contributions such as the chemical transitions, the line tension, the surface tension, the curvature, etc. The electric polarization energy \( \Delta G_{\mu} = \exp[t \varepsilon C_0^2/\varepsilon_{\text{in}}] \) enters the calculation procedure to yield the overall standard reaction dipole moment \( \Delta G M \mu_{\text{m}} = \Delta G M \mu_{\text{m}} \), which is the difference between the overall molar moments \( M \) (P) and \( M \) (C) of the porous and the closed membrane states, respectively.

#### 4.4 Electrothermodynamics of coupled poration steps

**MEP reaction scheme.** It is recalled that the two relaxation modes \( \Delta \lambda_2(t) \) and \( \Delta \lambda_3(t) \), documented as \( \gamma_2(t) \) and \( \gamma_3(t) \) in Fig. 7, have very different normal mode relaxation times: \( \tau_2 \ll \tau_3 \). However, the apparent threshold field strengths are approximately the same. These features are consistent with at least two types of porous states, \( P_2 \) and \( P_3 \), which are reactively coupled. For this case, the previous global scheme (C) \( \rightleftharpoons (P) \) is first specified as the two-step reaction sequence (C) \( \rightleftharpoons P_2 \rightleftharpoons P_3 \), where obviously the (C) \( \rightleftharpoons P_2 \) transition is much more rapid than the slower poration transition \( P_2 \rightleftharpoons P_3 \). The physical rationalization of the rapid relaxation mode, however, requires the assumption of at least one intermediate closed state \( C_1 \) besides C. It is shown below that the minimum reaction scheme for a consistent physical-chemical data description is the three-step reaction cascade

\[
(C \rightleftharpoons C_1) \rightleftharpoons P_2 (\rightleftharpoons P_3) \tag{8}
\]

In this scheme, the rapid step \( C \rightleftharpoons C_1 \) is coupled to the slow poration transition \( P_2 \rightleftharpoons P_3 \) via a very rapid intermediate step \( C_1 \rightleftharpoons P_2 \). The intrinsic steps of type \( i \) are characterized by the intrinsic equilibrium constants \( K_i' = K_i/k_{\text{c},i} \) as the respective concentration ratios, and the intrinsic relaxation rates \( 1/\tau_i = k_i' + k_i'' \), where \( k_i' \) and \( k_i'' \) are the intrinsic rate coefficients for the forward and backward directions, respectively. Explicitly, we have:

\[
K_i' = [C_1][C] = k_i' k_{i,1} \quad \text{and} \quad 1/\tau_i' = k_i' + k_i''
\]

\[
K_i'' = [P_2][C_1] = k_i'' k_{i,2} \quad \text{and} \quad 1/\tau_i'' = k_i'' + k_i''
\]

\[
K_i = [P_3][P_2] = k_i k_{i,3} \quad \text{and} \quad 1/\tau_i = k_i + k_i''
\]

**Normal relaxation mode (2).** Obviously, the rapid relaxation mode (2) refers to the sequence

\[
(C \rightleftharpoons C_1) \rightleftharpoons P_2 \tag{9}
\]

The strong inequality \( \tau_2^0 \ll \tau_3^0 \) justifies to consider the rapid reaction sequence in the first part of the conductivity relaxation \( \Delta \lambda_2(t) \) as partially not affected by the very small change \( \Delta \lambda_3(t) \) in the slow step \( P_2 \rightleftharpoons P_3 \). See the appendix 2 in the supplementary information (ESI). The electric field dependence of the normal mode relaxation rate \( 1/\tau_2 \) suggests that the mode (2) comprises the rate limiting rapid step \( C \rightleftharpoons C_1 \) coupled to the very rapid step \( C_1 \rightleftharpoons P_2 \). The overall equilibrium constant for
the coupled steps of the mode (2) in eqn. (9) is given by

$$K_2 = \frac{[P_2]}{[C] + [C_1]} = \frac{f_2}{1 - f_2}$$ (10)

It is shown below that the field-induced changes $\Delta \lambda_{on}$ in the membrane conductivity $\lambda_{on}$ refer to the changes $\Delta f_i$ of the overall pore fractions $f_i$. Because $\lambda_{on}$ refers to all membrane states, the concentration sum $c_0 = ([C] + [C_1]) + ([P_2] + [P_3])$ comprises the specified ion-conductive part $([P_2] + [P_3])$ and the not yet specifiable, leak-conductive part $[C] + [C_1]$. The overall pore fractions, respectively, are given by:

$$f_2 = [P_2]/c_0, \quad f_3 = [P_3]/c_0$$ (11)

Substitution shows that $f_2 = K_{21}(1 + K_{22}(1 + K_2))$ and $K_2 = K_2'K_1'(1 + K_3)$.

Field dependence of the amplitude of the pore fraction $f_2$. The analytical concept views the conductivity relaxation $\Delta \sigma(t)$ as reflecting the membrane conductivity mode $\Delta \lambda_{on}(t)$, being rate-limited by the structural mode:

$$f_2(t) = f_2^0(1 - \exp[-t/\tau_2]),$$ (12)

where $f_2$ is the amplitude of the $f_2(t)$-relaxation and $f_2^0$ the pore fraction at zero applied field. The data evaluation shows that $f_2$ is in the order of $10^{-5}$, such that eqn. (A.1.8) of Appendix 1 applies in the form:

$$f_2 - f_2^0 = \frac{K_2}{K_2'} = \exp(b_2/f_2^0E^2)$$ (13)

Analogous to eqn. (A.1.7), the field term $b_{2,0}^0 = b_{2,0}/f_2^0$ contains $V_{2,0} = V_2(P_2) - V_2(C_0,C_1)$ as the reaction volume of the overall state transition (C, C_1) → P_2, from which the mean pore radius $r_2$ is obtained; see eqn. (A.1.5).

Relaxation rate $1/\tau_2$. If the structural relaxation according to eqn. (9) involves the very rapid equilibration step $C_1 \rightleftharpoons P_2$ coupled to the slower C $\rightleftharpoons C_1$ transition, the relaxation rate $1/\tau_2$ in eqn. (12) is given by

$$1/\tau_2 = k_2 + k_{-2} = k_1' + k_{-1}'(1 + K_3),$$ (14)

where the overall rate coefficients are given by $k_2 = k_1$ and $k_{-2} = k_{-1}'(1 + K_3)$. Compared to the intrinsic rate $1/k_1' = K_1'(1 + K_3)$, the coupling is covered by the term $1/(1 + K_3)$. Since $k_2$ is in the order of $10^{-3}$, i.e. $\tau_2 \approx 1$, the P2 relaxation-mode (2) is kinetically controlled by the step C $\rightleftharpoons C_1$. If the step C $\rightleftharpoons C_1$ comprises field-induced reorientations of the dipolar head groups of the lipids in the solution/membrane interfaces, as suggested previously and indicated by the results of molecular dynamic studies, the relaxation rate reflects a permanent dipole moment mechanism. In the surface area affected by the field, the distribution $K_1' = [C]/[C] = k_{-1}'/k_1'$ is then biased towards $K_1' \gg 1$. Therefore the inequality $k_1' \gg k_{-1}'$ holds true and the approximation $1/\tau_2 = k_1'$ is justified.

In the Arrhenius-like expression for the field-dependence of the rate coefficient $k_1' = (k_1')^0\exp[x_1]$, the field exponent is specified by $x_1 = \Delta m/L_d(k_B T)$, where $\Delta m$ is the average transition dipole moment of the rotational change of the interfacial head group dipole and $L_d$ is the Onsager directing field. Here, $E_d$ is $E_d = x_1E$. Substitution of eqn. (4) yields $E_d = \epsilon_0\langle \lambda_{on}/\Delta \lambda_{on}\rangle f_2^0 E$. The rate-limiting transition C $\rightleftharpoons C_1$ involves states which are characterized by the (leak) conductivity term $\lambda_{on}$ (see below), corresponding to a (leak) field factor $f_2^0$ (see eqn. (4)). The field dependence of $1/\tau_2 = k_1'$ is then given by:

$$\frac{1}{\tau_2} = \frac{1}{\tau_2^0} \exp(b_1' f_2^0 E)$$ (15)

where $\tau_2^0$ refers to $E = 0$. In the range $0 \leq E/kV cm^{-1} \leq 1$, we use the approximation $f_{2,1} = 1$ (as done with $f_{2,2}$ and $f_{3,3}$, see below). Because of $1/\tau_2 = k_1'$, we see that $x_1 = b_1' f_{2,1}E$, where $b_1' = \epsilon_0\Delta m/L_d(k_B T)d_{av}/[\langle \lambda_{on}/\Delta \lambda_{on}\rangle]$. The characteristic activation volume is $\Delta m = 63 \times 10^{-30}$ C m (or 19 Debye units). It is remarked that, formally, the simpler scheme $C \rightleftharpoons P_2$ is characterized by $1/\tau_2 = k_{-2}' + k_{-2}'(1 + K_3)$. Since, however, $K_3 < 1$, the expression $1/\tau_2 = k_{-2}'$ would imply that a backward step promotes a field driven forward process. Because this is contrary to physical experience, the field dependence of $1/\tau_2$ necessitates the assumption of the intermediate state $C_1$ to describe the measured conductivity mode (2).

Slow normal mode (3). During the field-induced formation of the long-lived pore state $P_3$ from the state $P_2$, the formation of $P_3$ pores from the state reaction $C \rightleftharpoons C_1$ can be considered as rapidly equilibrating during the slow transition step $P_2 \rightleftharpoons P_3$. The slow mode (3) is therefore described by the overall process

$$(C = C_1 = P_2) = P_3$$ (16)

The (overall) equilibrium constant of this mode is defined as

$$K_3 = \frac{[P_3]}{[P_2] + [C] + [C_1]} = \frac{f_3}{1 - f_3}$$ (17)

where the relation $c_0 - [P_3] = [P_2] + [C] + [C_1]$ has been applied. Recalling eqns. (11) and (10), we see that

$$f_3 = \frac{[P_3]}{c_0} = \frac{K_3'K_3''}{1 + K_3'(1 + K_3''(1 + K_3'))}$$

Data analysis suggests that $K_3' \gg 1$ and $K_3'' < 1$; therefore the approximations $K_3 = K_3'K_3''$ and $f_3 \approx K_3'K_3''$ as well as the inequality $K_3' > 1$ hold true. See Appendix 2 in the electronic supplementary information (ESI).†

The $f_2(t)$-relaxation and the field dependence of the amplitude $f_3$. The kinetics of the relaxation mode $\Delta \sigma(t)$, reflecting the membrane conductivity mode $\Delta \lambda_{on}$, is analyzed in terms eqns. (5). The structural $f_2(t)$-relaxations in the small time range $0 \leq t < \tau_3$ are given by

$$\frac{f_2(t) - f_2^0}{(f_2 - f_2^0)} = \frac{Y_2(t)}{Y_3} = \frac{t - \tau_2(1 - \exp[-t/\tau_2])}{\tau_3}$$ (18)

where $f_2$ is the amplitude of $f_2(t)$ and $f_2^0$ the fraction of pore state $P_3$ at zero applied field. Here, too, the field dependence of $f_3$ and $K_3$ are connected by:

$$\frac{f_3}{K_3} = \frac{K_3}{K_3'} = \exp(b_3' f_3' E^2)$$ (19)

where $b_3' = \epsilon_0\langle \lambda_{on}/\Delta \lambda_{on}\rangle$ and $V_3 - V_2$. See eqn. (A.1.7). Since the mode (3) refers to the transition $P_2 \rightleftharpoons P_3$, the respective reaction moment is $\Delta m = \Delta m_{P_3} = \Delta m_{P_2}$. The mean pore radius $r_3$ is calculated from $V_3 = V_2 + \Delta V_{r_3}$ according to $r_3 = (V_3/4\pi)^{1/3}$ and $\Delta V_{r_3}$ is calculated according to $r_3 = (V_3/4\pi)^{1/3}$. It is remarked that the inequality $\Delta V_{r_3} > V_2$ indicates a stronger field dependence of $K_3$ as compared to that of $K_2$. This feature is suggestive for a stronger field-dependence of the relaxation rate $1/\tau_3$, compared to that of $1/\tau_2$.

Relaxation rate $1/\tau_3$. Analogous to mode (2), the mode (3) relaxation rate is given by:

$$1/\tau_3 = k_{-2}' + k_3'K_3''(1 + K_2),$$ (20)
where the coupling of the intrinsic transition $P_2 \cong P_3$ (with $1/\tau_3 = k_3 + k_{-3}$) to the more rapid steps $C \cong C_1 \cong P_2$ (considered as being equilibrated during the pore transition $P_2 \rightarrow P_3$) by the coupling factor $K_2/(1 + K_2) \approx K_2$. In the field strength range $0 \leq E/kV \text{ cm}^{-1} \leq 0.9$, the weak dependence of $K_5 = f_{32}$ on $E$ permits the rough approximation $K_5 \approx K_5^0$. The terms $K_1$ and $1/\tau_1$ exhibit similar, strongly non-linear dependences on $E$, as the terms $K_2$ and $1/\tau_2$, see eqns. (14) and (15) as well as eqn. (20). Analogous to $K_5 \approx K_5^0$, we may now safely assume, that in the low-field strength range $0 \leq E/kV \text{ cm}^{-1} \leq 0.9$, the approximation $\tau_3 = \tau_3^0$ is applicable in practice. Experimentally, the data of mode (3) only refer to the time range $0 \leq t \leq t_0$ (Fig. 7), the amplitudes $Y_3 = m_3 t_5$ are not directly accessible. Applying eqn. (18) to the low field strength range we obtain:

$$f_i(t_i) - f_i^0 = \frac{t_i - \tau_i}{\tau_i^0} \left(1 - \exp\left[-t_i/\tau_i^0\right]\right)$$

(21)

describing the sigmoid onset and the linear part of mode (3).

### 4.5 Parallel MEP reaction scheme

If, alternatively to the model presented by eqn. (8), the pore formation processes are independent structural transitions, occurring in parallel according to $C \cong P_2$ and $C \cong P_3$, respectively, where $C = \{[C] + [P_2]\} + \{[C] + [P_3]\}$, the total membrane conductivity change $\Delta \lambda_m = \Delta \lambda_m(I) + \Delta \lambda_m(II) + \Delta \lambda_m(III)$ is simply the sum of the contributions $\Delta \lambda_m(I) = \Delta \lambda_{m,1}$ and $\Delta \lambda_m(II) = \Delta \lambda_{m,2}$ and $\Delta \lambda_m(III) = \Delta \lambda_{m,3}$. The individual pore fractions are, respectively, defined as $f_{1}^0 = \{[P_2]\}/\{[C] + [P_2]\} = f_{2}(1 - f_{1}^0)$ and $f_{1}^{\text{relax}} = \{[P_3]\}/\{[C] + [P_3]\} = f_{3}(1 - f_{1}^{\text{relax}})$. Since $f_{1}^0$ contains and vice versa, the normal mode data fit to be iterative, starting with the well-justified approximation $f_{1}^0 = f_{3}(1 - f_{1}^{\text{relax}})$, it is found that indeed, within the accuracy of data, $f_{1}^0 = f_{3}$ holds and also $f_{1}^{\text{relax}} = f_{3}$ hold true. As a consequence, the parallel model leads to the same numerical values for $r_{23}, r_{3}, r_{1}^*, b_{2,0}^*, b_{3,0}^*$, respectively, as the consecutive model above.

### 4.6 Membrane conductivity $\lambda_m$ of cells in a pellet

**Conductivity and pore states.** Analogous to pure lipid membranes, the plasma membrane conductivity of a cell in a pellet is expressed as parallel contributions according to:

$$\lambda_m = \sum_i \lambda_{p,i} + \lambda_{m,1}$$

(22)

In view of the total state sum: $c_0 = \{[P_2]\} + \{[C] + [P_2]\} + \{[C] + [P_3]\}$, the terms $\lambda_{p,i}$ are the (field-dependent) conductivities of the pores of type $i; i = 2, 3$ correspond to the pore states $P_2$ and $P_3$. The states $C$ and $C_1$ may contain ion channel proteins and other ion leaks associated with a parallel conductivity $\lambda_{m,1}$, corresponding here to $i = 1$.

Obviously, at zero applied field, eqn. (22) takes the form:

$$\lambda_{m,0} = \sum_i \lambda_{p,i} + \lambda_{m,1}$$

(23)

Note that the measured zero-field conductivity $\lambda_m$ of the pellet refers to the zero-applied-field membrane conductivity $\lambda_{m,0}$. In line with eqn. (23) and analogous to eqns. (6), the field-induced relaxations of the membrane conductivities $\lambda_m$ are given by:

$$\lambda_m = \sum_i \Delta \lambda_{p,i} + \lambda_{m,0}$$

(24)

comprising the changes $\Delta \lambda_{p,i} = \sum_i (f_{i}^{\text{relax}} - f_{i}^{0})$, where $f_{i}^{0}$ and $f_{i}^{\text{relax}}$ are the zero-field pore fraction and pore conductivity of pore type $i$, respectively. Now, analogous to the conductivity normal modes $\Delta \lambda_i = \lambda_i - \lambda_{m,1}$ in eqn. (7), the membrane conductivity modes are generally given by:

$$\Delta \lambda_{m,i} = \lambda_{m,i} - \lambda_{m,i-1} = \sum_i \Delta \lambda_{p,i}$$

(25)

where it is obvious that, formally, $\lambda_{m,1} = \lambda_{m,0}^0$. Therefore the modes $\Delta \lambda_2 = \lambda_2 - \lambda_{m,1}$ and $\Delta \lambda_3 = \lambda_3 - \lambda_{m,1}$ reflect, respectively, the two membrane modes:

$$\Delta \lambda_{m,2} = \lambda_{m,2} - \lambda_{m,1} = f_{2}^{\text{relax}} - f_{2}^{0}$$

$$\Delta \lambda_{m,3} = \lambda_{m,3} - \lambda_{m,2} = f_{3}^{\text{relax}} - f_{3}^{0}$$

(26)

Substitution of the expression for $\lambda_{m,i}$, eqn. (23), into eqn. (24) for $i = 2$ and $i = 3$, respectively, leads to the integral membrane conductivities:

$$\Delta \lambda_{m,2} = f_{2}^{\text{relax}} + f_{3}^{\text{relax}} + \lambda_{m,1}$$

$$\Delta \lambda_{m,3} = f_{2}^{\text{relax}} + f_{3}^{\text{relax}} + \lambda_{m,1}$$

(27)

thus specifying the general expression in eqn. (24). Note that eqns. (28) correspond to the experimental terms $\lambda_m$ of eqns. (6).

**Maxwell equation for $\lambda_{m,i}$** According to Maxwell, the experimental conductivities $\lambda_m$ referred to the zero-applied-field membrane conductivity $\lambda_{m,1}$, of spherical objects in a suspension can be expressed in terms of equivalent conductivities for cells $\lambda_{m,i}$, respectively, and the volume fraction $v_a$ of the cells in the suspension. Here, we apply this approach to the case of pellets of densely-packed cells and specify: 19

$$\frac{\lambda_{m,i} - \lambda_{m,1}}{2\lambda_{m,i} - \lambda_{m,0}} = f_{i}^{\text{relax}} + f_{i}^{0}$$

(29)

where $f_{i}$ is defined in the context of the data in Fig. 2. The equivalent conductivity for a coated sphere, with the coat conductivity $\lambda_{m,1}$ and the core conductivity $\lambda_{m,0}$ has been given by Danzer et al. 27 and Fricke. 18 Applied to the CHO-cells of the pellet we have:

$$\lambda_{m,1} = \lambda_{m,1} - \lambda_{m,0}^0 = \frac{2 \times (1 - v) \lambda_{m,1} + (1 + 2v) \lambda_{m,0}}{2 \lambda_{m,1} - \lambda_{m,0}}$$

(30)

As documented previously, 26 the field-dependence of $\lambda_{p,i}$ is described in terms of an electrostatic barrier caused by the permeating ions, when passing the low dielectric pore walls. In Appendix 3 of the ES1, it is derived that:

$$\lambda_{p,i} = \lambda_{p,i}^{0} \exp\left[\psi_{i}^{0}\right]$$

(32)

where $\psi_{i}^{0} = \psi_{i}^{0} \exp\left[\psi_{i}^{0}\right]$ and $\lambda_{p,i}^{0}$ is a geometrical parameter of the trapezium model for the energy barrier. 26 F the Faraday constant and $\lambda_{p,i}^{0}$ is the intrinsic barrier potential of the image forces in the volume $V_i$ of the mean pore radius $r_i$. See Appendix 4 of the ES1. Substitution of eqns. (31) and (32) into eqn. (25) yields the general expression for the membrane conductivity modes $\Delta \lambda_{m,i}$ (corresponding to the measured modes $\Delta \lambda_i$) according to:

$$\Delta \lambda_{m,i} = f_{i}^{\text{relax}} \exp\left[\psi_{i}^{0}\right] E^2 + \psi_{i}^{0} E - 1$$

(33)
The data analysis proper starts with the data fit to the simplified relation
\[ \Delta \lambda_{\text{am}, \text{f}} = f_0 \lambda_0 (\exp \beta_0 E_0^2) - 1 \]  
for the field strength range \( 0 \leq E \text{ kV cm}^{-1} \leq 0.8 \), where the approximation \( f_2 = 1 \) and the inequality \( b_0 E_0^2 > \lambda_0 E \) is applicable. The first fit yields preliminary values for \( f_0 \lambda_0 \) and \( b_0 \), respectively, thus preliminary values for the polarization volume \( \lambda_0 \) and \( E_0 \). This \( \lambda_0 \) value is used to calculate the field parameter \( c_0 \) (for \( f_2 = 1 \)) in eqn. (32). Further on, \( c_0 \) is inserted into eqn. (33) and the iterative data fit yields the final values \( b_0 \), \( c_0 \), \( f_2 \), \( \lambda_0 \), \( V_f \) and \( \lambda_0 \).

**Characteristics parameters of mode (2).** The data set \( \Delta \lambda_{\text{am}, \text{f}}/E \) (Fig. 9a) is used to get the field dependence of the amplitude \( \Delta \lambda_{\text{am}, \text{f}}(E) \). Data fit using the eqns. (33) and (34) yields the parameters: \( f_0 \lambda_0 \), \( b_0 \), \( c_0 \), \( c_0/2 \), respectively, for the field strength range \( 0 \leq E \leq 1 \text{ kV cm}^{-1} \). \( b_0 = 29 \text{ cm}^{-2} \text{ kV}^{-2} \), \( c_0/2 = 15.7 \text{ nm}^2 \), \( c_0/2 = 1.0 \pm 0.1 \text{ nm} \), \( c_0/2 = 2.89 \text{ cm}^{-2} \text{ kV}^{-2} \), and the field dependence of \( f_0 \lambda_0(E) \) represented in Fig. 9(a). In Appendix 3 of the ESI it is derived that, for \( f_0 \), \( \lambda_0 \), \( c_0/2 \) amounts to \( 0.2 \times 10^{-5} \text{ S cm}^{-2} \), leading to the zero-field pore fraction \( f_2 = 1.4 \times 10^{-14} \). On applying eqn. (13), the field dependence of the amplitude \( f_0 \) is obtained (Fig. 10b).

**Characteristics parameters of mode (3).** The data evaluation of the normal mode (3) refers to the limited time range \( 0 \leq t \leq t_E \) = 1 ms. It is recalled that the slow post-field conductivity relaxation (at \( E = 0 \)) is consistent with the rescaling of the long-lived pores of type \( P_3 \), characterized by the time constant \( \tau_R \). We therefore set: \( \tau_R = \tau_E \). Now, the data set \( \Delta \lambda_{\text{am}, \text{f}}(t) \) is rearranged to yield the relaxation mode \( \Delta \lambda_{\text{am}, \text{f}}(t) \). Data fit using eqns. (33) and (34) and \( f_0 \lambda_0 \) from eqn. (30) \( f_0 \lambda_0 = 1.0 \times 10^{-12} \text{ S m}^{-1} \), \( f_0 \lambda_0 = 40 \text{ cm}^{-2} \text{ kV}^{-2} \) and \( \lambda_0 = 1 \times 10^{-13} \text{ S m}^{-1} \). The mean radius of \( P_3 \) pores, resulting from \( \lambda_0 = 2 \) cm, \( \lambda_0 = 69 \text{ cm}^{-2} \text{ kV}^{-2} \), amounts to \( \lambda_0 = 1.5 \pm 0.1 \text{ nm} (\Delta \lambda_{\text{am}, \text{f}} = 33.5 \text{ nm}) \). According to Appendix 3 of the ESI\( ^\dagger \) we calculate \( \lambda_0 = 0.3 \text{ mS cm}^{-2} \) and \( f_0 \lambda_0 = 10^{-14} \). The pore fraction \( f_0 \lambda_0(E) \) at the end of the applied pulse is calculated from eqn. (21) and is represented in Fig. 10(b). Substitution of \( \sum \lambda_0 \) \( \sum \lambda_0 = \sum \lambda_0 \) \( \lambda_0 \) \( \lambda_0 \) gives \( \sum \lambda_0 \) in practice by the leak \( \sum \lambda_0 \) of electrolyte from the cell during the field pulse phase (3) and after the pulse (10off(t)). However, the majority of \( P_3 \) pores appear to close to the cell surface within a few milliseconds after pulse termination, i.e. at \( t = 1 \text{ ms} \). It is remarked that, on a time scale of a few seconds, the increase of \( \lambda_0 (t) \) starts at exactly the in-field end value \( \lambda_0 (t) \), see Fig. 7. If this value is due to ion efflux through one large pore, this pore state must be fully established within the time interval of the relaxation time \( \tau_2 \) of the formation of pore state \( P_3 \) (Fig. 8c) to permit continuous efflux under Maxwell stress to cause the delayed linear increase in \( \lambda_0 (t) \). The zero-field time constant \( \tau_E \) = 45 s could provide the efflux of electrolyte from the cell during the field pulse phase (3) and after the pulse (10off(t)). However, the majority of \( P_3 \) pores appear to close to the cell surface within a few milliseconds after pulse termination, i.e. at \( t = 1 \text{ ms} \). It is remarked that, on a time scale of a few seconds, the increase of \( \lambda_0 (t) \) starts at exactly the in-field end value \( \lambda_0 (t) \), see Fig. 7. If this value is due to ion efflux through one large pore, this pore state must be fully established within the time interval of the relaxation time \( \tau_2 \) of the formation of pore state \( P_3 \) (Fig. 8c) to permit continuous efflux under Maxwell stress to cause the delayed linear increase in \( \lambda_0 (t) \). The zero-field time constant \( \tau_E \) = 45 s, which now would be the closing time constant of the large pore formed in the field. Because of this large difference, it is unlikely that one large pore is responsible for the long-lived pore state \( P_3 \).

**5. Conclusions**

Applying Maxwell’s conductivity equation to the electric-field induced changes of densely packed cell pellets, the membrane conductivities at zero-field and the pore conductivities at least two types of largely different pore life times have been calculated. The kinetic normal mode analysis, in terms of parallel circuit contributions, yields the polarization volumes, thus the pore radii of the two pore states, and the activation dipole moment of the structural transition preceding the actual pore formations. Further on, the concept of MEP in terms of membrane state transitions also provides numerical values of the very small zero-field pore fractions and the conductivity factors limiting the membrane voltage through the transmembrane ion flows. The intrinsic pore conductivities have been calculated using the trapezium barrier model for the image forces caused by the permeating ions. Due to dense (3), as specified be eqn. (18) of the consecutive model, is negligibly small.
packing, the field-induced membrane voltage is smaller in the pellet by a factor of 2/3 compared to that of cells in diluted suspension. In summary, the study has provided a general scheme for the evaluation of the conveniently measurable conductivity changes in cell pellets. The procedure is suggestive for applications to cells in contact as in tissue, in order to optimize the biotechnological protocols for the electro-transfer of bioactive substances like drugs and genes in modern electrotherapies, as well as in cell fusion technology, based on the method of membrane electroporation.

6. Glossary

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Conductance ($A = I/U$)</td>
</tr>
<tr>
<td>φ</td>
<td>Electric potential</td>
</tr>
<tr>
<td>$f_{i,1}$</td>
<td>Field reduction factor for pore type $i$</td>
</tr>
<tr>
<td>$τ_i$</td>
<td>Intrinsic relaxation time of reaction step $i$</td>
</tr>
<tr>
<td>$\bar{a}_c$</td>
<td>Mean cell radius</td>
</tr>
<tr>
<td>$\bar{r}_i$</td>
<td>Mean pore radius of pore type $i$</td>
</tr>
<tr>
<td>$\bar{t}_i$</td>
<td>Normal mode relaxation time of mode $i$</td>
</tr>
<tr>
<td>$f_c$</td>
<td>Volume fraction of cells</td>
</tr>
<tr>
<td>$k_d(t)$</td>
<td>Fick’s law flow coefficient (time-dependent)</td>
</tr>
<tr>
<td>$\lambda_c (λ_0)$</td>
<td>Pellet conductivity, $(E = 0)$</td>
</tr>
<tr>
<td>$\lambda_c (λ_0)$</td>
<td>Pore fraction as surface area fraction, $(E = 0)$</td>
</tr>
<tr>
<td>$\lambda_{in} (λ_{in})$</td>
<td>Cell internal (external) conductivity</td>
</tr>
<tr>
<td>$\lambda_{in} (λ_{in})$</td>
<td>Cell membrane conductivity, $(E = 0)$</td>
</tr>
<tr>
<td>$\lambda_{in} (λ_{in})$</td>
<td>Rate coefficient for forward (backward) direction of step $i$</td>
</tr>
<tr>
<td>$Y_i$</td>
<td>Relative conductivity term for mode $i$ $(y_i = Δ\lambda_i/λ_0)$</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>$d_m$</td>
<td>Membrane thickness</td>
</tr>
<tr>
<td>$ρ$</td>
<td>Cell number density</td>
</tr>
<tr>
<td>$E$</td>
<td>Applied (Maxwell) field strength</td>
</tr>
<tr>
<td>$E_m$</td>
<td>Membrane field strength</td>
</tr>
<tr>
<td>MEP</td>
<td>Membrane electroporation</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugation velocity (in units of g)</td>
</tr>
</tbody>
</table>

Appendix 1
Electrothermodynamically, the isothermal–isobaric field dependence of $K$ on the (Maxwell) membrane field $E_m$ is given by the van’t Hoff relationship:

$$\left( \frac{∂ \ln K}{∂ E_m} \right)_{ρ,T} = \frac{ΔK M^\circ}{RT}$$  

(A1.1)

Integration for $E_m = 0$, referring to $K_0 = f^0(1 - f^0) = \exp[-ΔR G/RT]$, and $E_m$ yields the electric field exponent:

$$X^\circ = \ln \frac{K}{K_0} = \frac{1}{E_m} \frac{ΔK M^\circ}{RT} = \frac{ΔE_m}{RT}$$  

(A1.2)

According to Abidor et al. (1979), pore formation is viewed as the entrance of water of dielectric constant $\bar{v}_w$, replacing an equivalent overall volume $V$ of the lipid phase of dielectric constant $\bar{v}_d$, specifying the polarization energy as proportional to the difference $(\bar{v}_w - \bar{v}_d)$. Applying this concept to the scheme (C) $\cong (P)$, the overall reaction moment is $ΔP M^\circ = V_{P,C} ΔR p^\circ$, where $V_{P,C}$ is the molar volume increase due to the formation of pore state P from the closed membrane state C. The reaction polarization, as a molar quantity, is given by $ΔP M^\circ = N_A η_0 (v_w - v_d) E_m$, where $η_0$ is the (dielectric) permittivity of the vacuum. Substitution and explicit integration in eqn. (A1.2) yields

$$X^\circ = \frac{V_{P,C} η_0 (v_w - v_d)}{2 k_B T} E_m^2$$  

(A1.3)

We now use the pellet approximation and specify eqn. (4) of the text as

$$E_m = - \frac{Δ\rho_m(P)}{d_m} = \frac{\bar{a}_f E}{d_m}$$  

(A1.4)

Assuming cylindrical pores, $V_{P,C} = πd_m^2$, a mean pore radius is defined by

$$\bar{r} = \left( \frac{π}{2} \right)^{1/2} (V_{P,C}/πd_m)^{1/2}$$  

(A1.5)

Substitution of eqn. (A1.4) in eqn. (A1.3) yields $X^\circ = b_p M^\circ E^2$ and eqn. (A1.2) yields

$$K = K_0 \exp[(b_p M^\circ E^2)]$$  

(A1.6)

where the field term $b_p M^\circ = b_p M^\circ E^2$ is given by

$$b_p M^\circ = \frac{V_{P,C} η_0 (v_w - v_d) E_m^2}{2 k_B T d_m^2}$$  

(A1.7)

In all cases of MEP of cells and lipid vesicles, the overall surface fraction of pores does not exceed the order of magnitude of $10^{-3}$ to $10^{-2}$. Hence, for each homogenous phase $(e)$, $P = η_d (e - 1) E$ holds, where $E$ is the Maxwell field of the phase. A change of the phase material within the volume $V$ leads to the moment change $ΔM = VAP = V\bar{v}_d ΔE$, where $ΔE = η_w - η_d$.

Acknowledgements
We gratefully acknowledge the instructive discussions with Prof. Schultz, Düsseldorf with respect to electrode design. Prof. Mir, Villejuif and Prof. Miklavcic, Ljubljana concerning the electrochemical analysis of cell pellets. We thank R. Gerke for carefully processing the figures. We thank the Deutsche Forschungsgemeinschaft for grant Ne 227/9-3, -94, the ministry MSWF of the land NRW for grant Elminos, the Fonds Chemie and the European Union, Brussels, for grant QLK-3-CT-1999-00484 to E. Neumann.

References