Effects of cell orientation and electric field frequency on the transmembrane potential induced in ellipsoidal cells

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A B S T R A C T

The transmembrane potential (Δϕ) induced by external electric fields is important both in biotech applications and in new medical therapies. We analyzed the effects of AC field frequency and cell orientation for cells of a general ellipsoidal shape. Simplified equations were derived for the membrane surface points where the maximum Δϕ is induced. The theoretical results were confirmed in experiments with three-axial chicken red blood cells (a:b:c = 6.66 μm:4.17 μm:1.43 μm). Propidium iodide (PI) staining and cell lysis were detected after an AC electroporation (EP) pulse. The critical field strength for both effects increased when the shorter axis of a cell was parallel to the field, as well as at higher field frequency and for shorter pulse durations. Nevertheless, data analysis based on our theoretical description revealed that the Δϕ required is lower for the shorter axis, i.e. for smaller membrane curvatures. The critical Δϕ was independent of the field frequency for a given axis, i.e. the field strength had to be increased with frequency to compensate for the membrane dispersion effect. Comparison of the critical field strengths of PI staining in a linear field aligned along semi-axis a (142 kV m⁻¹) and a field rotating in the a–b plane (115 kV m⁻¹) revealed the higher EP efficiency of rotating fields.

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1. Introduction

Membrane electropermeabilization (EP, also referred to as electroporation or dielectric breakdown) is probably the most important technique for the introduction of extracellular molecules, which do not penetrate cell membranes under normal conditions. It is generally assumed that membrane re-conformation and the formation of aqueous pores play a decisive role in the process of penetration [1]. EP is observed when the transmembrane potential (Δϕ) induced by the external field reaches a “critical voltage” of approximately 1 V [2–4]. Its magnitude depends on the experimental conditions [5,6]. Nevertheless, thorough investigations have shown that the experimental observation of a “critical voltage” results from the strongly nonlinear auto-regulation processes involved in pore formation [7–9]. One important effect is the voltage drop in the external medium for the current flowing through the pore. This voltage drop increases with pore size, leading to a down-regulation of Δϕ. Nevertheless, the assumption of a “critical voltage” is feasible for practical reasons and we will use the term in the following.

EP of cells depends on several electric parameters, e.g. field strength, pulse duration, number of pulses [6,10–17] and pulse shape [18–20]. Cell size, shape and orientation, medium conductivity and temperature are also important [6,21–23]. Δϕ is proportional to the length of the axis oriented in the field direction and a shape factor that is related to the depolarizing factors [24–25]. Chang et al. [26] described how AC-fields are more effective in EP than DC fields, and result in an increased cell viability. The frequency dependence of Δϕ in AC-fields depends on medium conductivity [22]. Capacitive membrane bridging causes the Δϕ to decrease with increasing field frequency [4,22,27–34]. Clearly, rotating AC-fields generated by the superposition of two perpendicularly oriented fields are even more effective than the linear AC-fields [28,35,36].

New developments focus on EP in microfluidic devices with an EP process controlled at the single cell level [37,38]. The aims are to achieve high effectiveness in the genetic manipulation of cells [39–43] and effective cell lysis prior to subcellular analysis [44].

Gimsa and Wachner [25,45] have derived analytical expressions of Δϕ for both orientations of the symmetry axis of spheroidal cells, assuming a low conductive membrane and a highly polarizable cytoplasm. The same ansatz could be extended to arbitrarily oriented cells of the general ellipsoidal shape, including all electrical parameters [30]. Recently, we derived simplified expressions avoiding the complex depolarizing factors for spheroidal cells [46]. In this work, we analyze the Δϕ induced by AC field pulses in arbitrarily oriented ellipsoidal cells. The theoretical description is compared to experimental results obtained from the EP of the roughly ellipsoidal chicken red blood cells (CRBCs). Experiments were conducted...
in a chip chamber with two comb-shaped electrodes. Each comb had three interdigitating fingers, allowing for five different inter-electrode distances. The EP of the cells was determined from the two criteria, propidium iodide (PI) permeabilization detected by fluorescence-staining of the cell nuclei and cell lysis, respectively. We considered the effects of field strength, pulse field frequency, pulse duration and cell orientation.

2. Theory

2.1. Δp for general orientation of ellipsoidal cells

2.1.1. A finite element ansatz for Δp of the oriented single shell model

Gimsa and Wachner [30] have presented a simplified finite element ansatz for the Δp of a single shell ellipsoidal model. The model consists of the internal, membrane and external media, designated by the indices i, m and e, respectively. Each medium is presented by a prismatic element [see [30]]. The impedance \( Z^e \) of each element is given by the geometry (cross-sectional area \( A \) and length \( l \)):

\[
Z^e = \frac{1}{\sigma^e} \quad \text{with} \quad \sigma^e = \sigma + j \omega \varepsilon \varepsilon_0
\]

(1)

\( \varepsilon^e, \varepsilon, \varepsilon_0 \) and \( j \) stand for the complex specific conductivity of the considered medium, relative permittivity, permittivity of vacuum and \( \sqrt{-1} \), respectively. Eq. (1) is equivalent to a resistor-capacitor (RC) pair (see lump model in [30]). The length \( l \) in Eq. (1) is given by the dimensions. The cross-sectional areas \( A \) of each element are assumed to be equal and infinitely small. They are oriented perpendicular to the field. Starting from this “finite element model”, for an ellipsoidal cell with semiaxis a oriented in parallel to the field direction \( \Delta p \) at pole a can be expressed by the voltage divider properties of the lump model [see [30] for details]:

\[
\Delta p_a = -a_i E_{m,e} \Delta E_a = -\left( \frac{Z^e_{ia} + Z^m_{ia}}{Z^e_{ia} + Z^m_{ia} + Z^{m,\infty}_{ia}} \right) Z^{m,\infty}_{ia} \Delta E_a
\]

(2)

\( E_{m,e} \) and \( E_{m,i} \) stand for the external field in x-direction, the effective internal local field of the body and the cytoplasmic field, respectively. Please note that \( \Delta p_a, E_{m,e} \) and \( E_{m,i} \) are complex terms. Nevertheless, for simplicity we only mark the impedances \( Z^e \) of the prismatic elements by asterisks. \( Z^{m,\infty}_{ia} \) stands for the maximum of \( \Delta p_a \), determined by the influence radius \( a_{infl} \) along axis a. Index a refers to the geometry along semiaxis a. No axis index is required for the membrane impedance because the membrane elements are assumed to possess the same geometry along each principal axis. Please note that the model is largely consistent with (or even superior over) the Laplace-description for reasonable cell properties [30].

2.1.2. Δp for arbitrarily oriented cells of the general ellipsoidal shape

The general ellipsoidal model is described by three principal semiaxes \( a, b, \) and \( c \) of different length. Special cases are spheroidal models with \( a=b=c \) and spherical ones with \( a=b=c \). The semi-axes can be used to determine a Cartesian, orthonormal coordinate system \( x, y, \) and \( z \) where \( x, y, \) and \( z \) are parallel to \( a, b, \) and \( c \), respectively (Fig. 1). The homogeneous external field \( E \) is oriented arbitrarily within this coordinate system. Its orientation is determined by the angles \( \alpha \) and \( \gamma \). The local vector \( P \) of the membrane point under consideration is determined by the angles \( \alpha \) and \( \gamma \). The induced transmembrane potential \( \Delta p_a \) at a membrane point (index p) is:

\[
\Delta p_a = \left( \frac{Z^m_{ia} + Z^{m,\infty}_{ia}}{Z^m_{ia} + Z^{m,\infty}_{ia} + Z^{m,\infty}_{ia}} \right) Z^{m,\infty}_{ia} \Delta E_a
\]

(3)

for an arbitrary orientation of the inducing field [see [30] for details]. \( a_{infl}, b_{infl} \) and \( c_{infl} \) stand for the influence radii of the ellipsoidal cell along the three semiaxes. Again, all \( \Delta p_a \) terms are complex. The influence radius along each semiaxis refers to a certain distance from the center of the cell [45]. In the center, a symmetry plane can be defined for each semiaxis that is oriented perpendicular to the semiaxis. Field components \( E_x, E_y, E_z \) parallel to a semiaxis will not change the potential in the respective symmetry plane for symmetry reasons. The \( \Delta p_a \)-components at the three poles \( a, b, \) and \( c \) are solely induced by the respective field components \( E_x, E_y, E_z \) along the semiaxes. \( d_a, d_b, \) and \( d_c \) denote the distance of the membrane point to the three symmetry planes. At the poles \( d_a = a, d_b = b, \) and \( d_c = c \). For a cytoplasmic conductivity much higher than the membrane conductivity and a very thin membrane, \( \Delta p_a \) is given by the sum of the vector components of Eq. (3) [see [30] for a detailed consideration]. Neglecting the permittivities of the cytoplasm and the external medium, from Eq. (3) we obtain [30]:

\[
\Delta p_a = \frac{-a_{infl} d_a}{(1 + a_{infl}^2 (1/\sigma_m + (a_{infl} - a)/\sigma_m)) \sqrt{1 + f^2}} \frac{E_x}{f_{a,m}} + \frac{-b_{infl} d_b}{(1 + b_{infl}^2 (1/\sigma_m + (b_{infl} - b)/\sigma_m)) \sqrt{1 + f^2}} \frac{E_y}{f_{a,m}} + \frac{-c_{infl} d_c}{(1 + c_{infl}^2 (1/\sigma_m + (c_{infl} - c)/\sigma_m)) \sqrt{1 + f^2}} \frac{E_z}{f_{a,m}}
\]

(4)

with

\[
f_{a,m} = \frac{1}{2 \pi f_m \sigma_m} \frac{1}{\sigma_m (1/\sigma_m + (a_{infl} - a)/\sigma_m)} + \frac{1}{\sigma_m (1/\sigma_m + (b_{infl} - b)/\sigma_m)} + \frac{1}{\sigma_m (1/\sigma_m + (c_{infl} - c)/\sigma_m)}
\]

(5)

\( C_m, g_m \) stand for the area-specific membrane capacitance in \( \text{Fm}^{-2} \) and the area-specific membrane conductance in \( \text{Sm}^{-2} \), respectively. They are given by \( C_m = \varepsilon_0 e_m d/m \) and \( g_m = \varepsilon_m d/m \) with \( d \) being the membrane thickness. \( f, f_{a,m}, \sigma_m, \alpha_m \) and \( \sigma_m \) stand for the external field frequency, the characteristic frequency of membrane polarization along semiaxis \( a \), and the internal and external conductivities, respectively. Expressions analogous to Eq. (5) hold along the other two semiaxes.

2.1.3. Maximum of \( \Delta p_a \) for semiaxis b being oriented perpendicular to the external field

The field vector is parallel to the \( x-z \) plane for semiaxis b being orientated perpendicular to the external field (\( \alpha=0^\circ \), Fig. 2A). A new analytical expression can be derived from Eq. (3) for the membrane surface point reaching the maximum \( \Delta p_a \) if only peripheral points in the \( a-c \) plane (\( \beta=0^\circ \)) are considered. The angle \( \alpha \) determining this point can be obtained considering the \( \alpha \)-dependence of the sum of the \( x \)- and \( z \)-components of Eq. (3) (compare to Eq. (4)). The \( \alpha \)-dependence of Eq. (3) is obtained after introduction of angular coordinates for the distances \( d_a, d_b, \) and \( d_c \) [see [30] and compare to
Eq. (9)). Assuming the first derivative of the obtained function to be zero for \( |E_x|=|E_y|\) and \( |E_z|=0 \), i.e. \( \gamma=45^\circ \) and \( \delta=0^\circ \) we obtain:

\[
\alpha_{(\Delta \phi_{\text{max})}} = \arctan \left( \frac{|\Delta \phi_{\text{c}}|}{|\Delta \phi_{\text{a}}|} \tan \gamma \right)
\]

Introducing \( \alpha_{(\Delta \phi_{\text{max})}} \) into Eq. (3) leads to the maximum of \( \Delta \phi \):

\[
\Delta \phi_{\text{max}} = \sqrt{|\Delta \phi_{\text{c}}|^2 \cos^2 \gamma + |\Delta \phi_{\text{a}}|^2 \sin^2 \gamma}
\]

Analogous expressions hold for fields oriented in the \( x-y \) and \( y-z \) planes. In these cases, the field orientation and the membrane point under consideration will be defined by the angles \( \delta \) and \( \beta \), respectively (Fig. 2B). Similarly, also other field orientations can be considered. An interesting consideration is the frequency dependence of the \( \Delta \phi_{\text{max}} \) point for \( \gamma=\delta=45^\circ \). In this case, the site of \( \Delta \phi_{\text{max}} \) will exhibit a complex frequency-dependent trajectory at the membrane surface.

2.2. DC-limit of \( \Delta \phi_p \) for oriented ellipsoidal cells with zero membrane conductance

2.2.1. General orientation of a cell of the general ellipsoidal shape

For zero membrane conductance and at very low frequency or DC fields, Eq. (3) can be reduced to:

\[
\Delta \phi_p = \begin{vmatrix}
\frac{d}{a} \\
\frac{d}{b} \\
\frac{d}{c}
\end{vmatrix} \Delta \phi_{\text{c}} = \begin{vmatrix}
\frac{d}{a} \\
\frac{d}{b} \\
\frac{d}{c}
\end{vmatrix} \Delta \phi_{\text{b}}
\]

After introduction of angular coordinates for the distances \( d_x, d_y, \) and \( d_z \) (see [30]) into Eq. (3) we obtain:

\[
\Delta \phi_p = -\frac{E}{a b c} \cos \gamma \cos \alpha \cos \beta + \sin \alpha \sin \beta + \sin \alpha \cos \beta + \cos \gamma \sin \delta
\]

2.2.2. \( \Delta \phi_p \) for a perpendicular orientation of semiaxis \( b \) to the external field

For semiaxis \( b \) being oriented perpendicular to the external field, Eq. (9) can be reduced to:

\[
\Delta \phi_p = \frac{E(d_{\text{infl}} \cos \gamma \cos \alpha + d_{\text{infl}} \sin \gamma \sin \alpha)}{\sqrt{a^2 \sin^2 \alpha + c^2 \cos^2 \alpha}}
\]

considering only peripheral points in the \( a-c \) plane (Fig. 2A). The angle \( \alpha_{(\Delta \phi_{\text{max})}} \) determining \( \Delta \phi_{\text{max}} \) is given by (compare to Eq. (6)):

\[
\alpha_{(\Delta \phi_{\text{max})}} = \arctan \left( \frac{d_{\text{infl}} \tan \gamma}{d_{\text{infl}} \tan \gamma} \right)
\]

and after introducing \( \alpha_{(\Delta \phi_{\text{max})}} \) into Eq. (10) we obtain (compare to Eq. (7)):

\[
\Delta \phi_{\text{max}} = -E \sqrt{a^2 \sin^2 \gamma + c^2 \sin^2 \gamma}
\]

Eqs. (11) and (12) indicate that the \( \Delta \phi_{\text{max}} \) of ellipsoidal cells for the above simplifications depends on field orientation \( (\gamma) \) and cell shape, i.e. the influential radii along semiaxes \( a \) and \( c \) (Fig. 2A). Analogous expressions hold for the peripheral points of the \( a-b \) (Fig. 2B) and \( b-c \) planes.

2.3. DC-limit of \( \Delta \phi_p \) for spheroidal cells with zero membrane conductance

2.3.1. General orientation of a spheroidal cell

Spheroidal models are oblate and prolate when their symmetry axes are shorter \( (c<a=b) \) and longer \( (c>a=b) \) than the semiaxes \( a \) and \( b \), respectively. For arbitrarily oriented spheroidal cells \( \Delta \phi_p \) is given by [45]:

\[
\Delta \phi_p = -\frac{d_{\text{infl}}}{a} (E_x d_x + E_y d_y) - \frac{c_{\text{infl}}}{c} E_z d_z
\]

From Eq. (9) we obtain:

\[
\Delta \phi_p = \frac{-E a c}{\sqrt{(a^2 \sin^2 \alpha + c^2 \cos^2 \alpha)}}
\]

\[
\frac{d_{\text{infl}} \cos \alpha \cos \beta}{a} \left( \frac{\cos \gamma \cos \delta}{\cos \gamma \sin \alpha} \right) \left( \frac{\cos \gamma \sin \delta}{\sin \gamma} \right)
\]

2.3.2. \( \Delta \phi_p \) at peripheral membrane points with a perpendicular orientation of semiaxis \( b \) to the external field for a spheroidal cell

For \( \delta=0^\circ \) the field vector lies in \( x-z \) plane. If we further assume \( \beta=0^\circ \), \( \Delta \phi_p \) in Eq. (9) can be reduced to Eq. (10). Nevertheless, \( d_{\text{infl}} \) and \( c_{\text{infl}} \) are based on complex expressions for the depolarizing factors [45]. Recently, we derived simple approximating equations for \( d_{\text{infl}} \) and \( c_{\text{infl}} \) of spheroids avoiding complex expressions (see Appendix A). Introducing Eqs. (A.1) and (A.2) into Eq. (10) leads to:

\[
\Delta \phi_p = -\frac{a (E+a+c) \cos \alpha + (a+c) \sin \alpha}{2 (a+c) \sqrt{a^2 \sin^2 \alpha + c^2 \cos^2 \alpha}}
\]

The same expressions can be introduced into Eqs. (11) and (12) to determine the \( \Delta \phi_{\text{max}} \) and the angle \( \alpha_{(\Delta \phi_{\text{max})}} \) under which it occurs. Furthermore, limiting cases of the shape can be considered. For a thin disk \((c<<a)\) Eq. (15) reduces to:

\[
\Delta \phi_p = -\frac{a E (2 \cos \gamma \cos \alpha + \sin \gamma \sin \alpha)}{2 \sqrt{a^2 \sin^2 \alpha + c^2 \cos^2 \alpha}}
\]
3.2.1. EP chip chamber and instrumentation

A glass chip with two comb-shaped platinum electrodes with three fingers was used for EP experiments. Our new design allowed for five different inter-electrode gaps of 80 µm, 100 µm, 150 µm, 300 µm and 450 µm generating five electric field strengths from the same supply voltage (Fig. 3). The chips were fabricated by GeSiM GmbH, Grosserkmannsdorf, Germany (www.gesim.de). Only the three short-voltage fields were used in the CRBC experiments. The AC fields were generated by two 180°-phase shifted square-topped signals with a key ratio of 1:1 from a function generator HP 81130A (Hewlett Packard GmbH, Germany). The resistances of the on-chip temperature sensors were calibrated for their temperature dependencies and measured before and after pulse application by a multimeter (model 2000, Keithley Instruments Inc., USA).

After the EP pulse, a short temperature increase has been observed with a maximum of 0.9 degrees for the longest pulse duration of 200 ms. The temperature peak was observed approx. 50 s after the pulse. The main reason for this behavior was the heat dissipation by the terminating resistors. The temperature approached room temperature approx. one minute after the peak.

3.2.2. Electric field in the chamber

The electric field strength \( E \) is given by a Fourier series for a square-wave AC-pulse with a key ratio of 1:1 [36]:

\[
E = \frac{4V_{ss}}{\pi d} \exp(j\omega t) - \exp(-j3\omega t)/3 + \exp(j5\omega t)/5 + \ldots + \ldots
\]

\( d, V_{ss}, \omega \) and \( t \) stand for the distance of a pair of plane-parallel electrodes, the voltage difference between the electrodes, \( \sqrt{-1} \), the circular frequency, and time, respectively. The Fourier series contains only odd harmonics. While the effective field of the full series is \( V_{ss}/d \), the effective field generated by the sinusoidal first harmonic is \( 2\sqrt{2}V_{ss}/(\pi d) \) leaving a missing 9.94% contribution for all other harmonics of the series. The missing contribution decreases to 5.05% and 3.36% when the third and fifth harmonics are included in the series. This consideration might be interesting in the frequency range of membrane dispersion when capacitive membrane bridging reduces the induced transmembrane potential with increasing frequency, i.e. membrane dispersion leads to a relatively stronger decrease in the contributions of the higher harmonics.

3.2.3. Cells and sample preparations

CRBCs were chosen because of their 3-axial ellipsoidal shape. Fig. 4 presents micrographs of the cells (Scanning Electron Microscope, DSM 960A, Carl Zeiss, Oberkochen, Germany). The cells were obtained from the BfR (Bundesinsitut für Risikobewertung, Berlin, Germany). Fresh blood samples taken from the wing vein of Italian cocks were preserved at a 1:1 dilution in Alsever’s solution (18.66 g dextrose, 4.18 g NaCl and 8.0 g tri-Na-citrate-2-hydrate dissolved in 1 liter distilled water, pH 6.1) as an anticoagulant. This suspension had a cell concentration of approx. 19%. It was stored no longer than three days. Cells were suspended in a mixture of a sucrose and a NaCl solution for experiments. Both solutions had an osmolarity of 300 mOsm and contained 1 mM phosphate buffer solution, pH 6.8. The mixture was adjusted to a medium conductivity of 0.12 S/m. The signal for the HP 8130A function generator. Waveform and amplitude of the AC-fields were monitored with an oscilloscope HP 54610B (Hewlett Packard GmbH, Germany). The resistances of the on-chip temperature sensors were calibrated for their temperature dependencies and measured before and after pulse application by a multimeter (model 2000, Keithley Instruments Inc., USA).

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conductivity was controlled by a conductometer (inoLab, WTW GmbH, Weilheim, Germany) in the temperature compensation mode (20 °C).

3.2.4. EP experiments
PI (Fluka, Switzerland, molecular weight 668.39) binds to DNA and is used as a fluorescence marker of cell nuclei [48]. Cells were suspended in 5 ml measuring solution containing 10 µM PI [36]. 7 µl of this suspension with a cell concentration of 0.03% v/v were transferred to the measuring chamber that was sealed by a cover slip. EP experiments were conducted with sedimented cells a few minutes after transfer. The uptake of PI by single cells was observed with a fluorescence microscope (Olympus IX71, Japan). The images were recorded on hard disk and monitored by a computer interface using the Cell-P program of our imaging system (Soft Imaging System GmbH, Germany). Cells already stained before pulse application were excluded from interpretation. EP was judged 3 min after pulse application. Non-lysed cells did not lose their hemoglobin content. PI permeabilized cells detected by fluorescence-staining of their nuclei and non-permeabilized cells were distinguished amongst the non-lysed cells. Data were obtained for 10–15 cells collected in 4–5 repeats for each experimental condition.

4. Results and discussions

4.1. Theoretical analysis

4.1.1. DC-limit of Δϕp for cells of the general ellipsoidal shape with zero membrane conductance
The following analysis is based on the theoretical descriptions introduced above. Table 1 presents the CRBC parameters used. In the experiments, cells were oriented with semiaxes b or c perpendicular to the external field (Fig. 2A and B). For the first orientation, δ=0° and β=0° for peripheral points in the a–c plane (see Figs. 1 and 2A). The Δϕp of any point depends on the field angle γ. Maxima at the poles are special cases of Eq. (3) and given for a parallel orientation of the field and semiaxes a (|Δϕa,| = a|infl| E) and c (|Δϕc,| = c|infl| E), respectively. At a field angle of γ=45° (Fig. 2A) Eq. (12) becomes:

\[ |Δϕ_{a,c,}^{\text{max}}| = \left(\frac{\sqrt{2}}{2}\right) E\sqrt{a_{\text{infl}}^2 + c_{\text{infl}}^2} \]

Analogous expressions are obtained for cell orientations in the a–b and b–c planes. It can be shown that the equation holds for cell models with a very thin membrane, zero membrane conductance, and a highly conductive cytoplasm at low frequencies. Under these conditions, the membrane point of Δϕp is solely determined by the object geometry.

Table 2
Shape parameters of a spheroidal model for theoretical considerations

<table>
<thead>
<tr>
<th>Semiaxes</th>
<th>Influential radii calculated from depolarizing factors</th>
<th>Simplified equations (Appendix A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spherical model</td>
<td>a = b = c = 3.5 µm</td>
<td>5.25 µm</td>
</tr>
<tr>
<td>Spheroidal model</td>
<td>c/a = 1:3.5 (oblate)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a_{infl} = b_{infl}</td>
<td>4.18 µm</td>
</tr>
<tr>
<td></td>
<td>c_{infl}</td>
<td>3.06 µm</td>
</tr>
<tr>
<td></td>
<td>c/a = 2:1 (prolate)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a_{infl} = b_{infl}</td>
<td>5.96 µm</td>
</tr>
<tr>
<td></td>
<td>c_{infl}</td>
<td>8.47 µm</td>
</tr>
</tbody>
</table>
We therefore designated the angle of this membrane point “geometry-determined limiting angle \( \alpha \) \( \Delta \phi_{a,c_{\max}} \) is 7.2° obtained for the geometric parameters of Table 1 (Fig. 5A). \( \Delta \phi_{a,b_{\max}} \) is 23.78° in the \( a-b \) plane (Fig. 5B).

4.1.2. DC-limit of \( \Delta \phi_{p} \) of oriented spheroidal cells with zero membrane conductance

We consider the spheroidal shape in the following for the sake of completeness. Semiaxes \( a \) and \( b \) were fixed to a typical cell value of 3.5 \( \mu \)m. Semiaxis \( c \) was varied. Three axes ratios were considered, \( c:a=1:1 \) (spherical), \( c:a=1:3.5 \) (oblate), and \( c:a=2:1 \) (prolate). A field orientation perpendicular to semiaxis \( b \) \( (\delta=0^\circ) \) can be assumed without limitation in generality. \( \Delta \phi_{p} \) of the peripheral membrane points \( (\beta=0^\circ) \) was calculated either by the full model (Eq. (10)) using exact influential radii (Table 2) or by the simplified Eq. (15) (Fig. 6). The \( \Delta \phi_{p_{\max}} \) values were found at the poles of semiaxis \( a \) \( (\alpha=0^\circ) \) and \( c \) \( (\alpha=90^\circ) \) for field orientations in parallel \( (\gamma=0^\circ, \text{Fig. 6A}) \) and perpendicular \( (\gamma=90^\circ, \text{Fig. 6C}) \) to semiaxis \( a \). For \( \gamma=45^\circ \), \( \Delta \phi_{p_{\max}} \) can be expressed in analogy to

Fig. 6. Comparison of the DC-limits of \( \Delta \phi_{p} \) for the full (Eq. (10), solid lines) and simplified (Eq. (15), dashed lines) spheroidal models. Zero membrane conductance was assumed. (A–C): \( \Delta \phi_{p} \) dependence on \( \alpha \) for peripheral membrane points in \( a-c \) plane \( (\beta=0^\circ) \) for semiaxis \( b \) perpendicular to the field \( (\delta=0^\circ) \). The field orientations is \( \gamma=0^\circ \) (A), \( \gamma=45^\circ \) (B) and \( \gamma=90^\circ \) (C). (D): trivial case of a field orientation perpendicular to the symmetry axis \( c \) \( (\gamma=0^\circ, \delta=0^\circ) \). The \( \Delta \phi_{p} \) dependence on \( \beta \) is a cosine function for peripheral membrane points in the \( a-b \) plane \( (\alpha=0^\circ) \). The \( \Delta \phi_{p} \) magnitude is higher for prolate than for oblate cells.

Fig. 7. Frequency dependence of the angle \( \alpha \) of the \( \Delta \phi_{p_{\max}} \) membrane point on cell parameters for a field orientation of \( \gamma=45^\circ \) in the \( a-c \) plane. (A) internal conductivity \( \sigma_{i} \); (B) external conductivity \( \sigma_{e} \); (C) membrane conductance \( g_{m} \); (D) area specific membrane capacitance \( C_{m} \).
Eq. (20) (Fig. 6B). For a field orientation perpendicular to semiaxis c (∈=0°), Δϕ_{max} = -a_{m}E for all angles in the a–b plane (Fig. 6D). The errors of the simplified equations for prolate and oblate shapes are usually much smaller than 10% compared to the full model (Fig. 6).

4.1.3. Dependence of the angle of Δϕ_{max} on cell parameters and frequency for tilted cells of the general ellipsoidal shape

The site of Δϕ_{max} depends on cell and medium parameters when the object is exposed to the field under a certain angle. For a qualitative consideration we used two different field orientations δ=45° (γ=0°: field in a–b plane), as well as γ=45° (δ=0°: field in a–c plane). The effect of cell parameters on the location of Δϕ_{max} is considered a function of frequency in Figs. 7 and 8. The angles robustly approaches the geometry-determined limiting angles of 7.2° (a–c plane, Fig. 7) and 23.78° (a–b plane, Fig. 8) at low frequencies for a low membrane conductance.

Generally, three frequency plateaus are obtained for the angles. The first and second plateaus occur at frequencies from 0–10^4 Hz and 10^5–10^7 Hz, respectively. While deviations of the first plateau from the geometry-determined limiting angle depend on membrane conductance, especially at low external conductivities, the third (permittivity) plateau is largely independent from the cell parameter variations considered. Only membrane permittivity variations have a slight effect (Figs. 7D and 8D). In contrast, the second plateaus of α and β are strongly affected by the cell parameters. It is interesting, that α and β may be higher or lower than the geometry-determined limiting angle. The reason is that the polarizability balance along the two semiaxes in the field plane may be deflected either to the shorter or the longer semiaxis, i.e. the shorter or the longer semiaxis may be higher polarizable [49]. The plateau levels of α and β are independent of C_m variations. Nevertheless, higher C_m values shift the transition frequency from the first to the second plateaus toward lower frequencies (Figs. 7D and 8D). Please note that the assumption of cell properties varying along the three axis, e.g. of different cytoplasmic permittivities, results in a more complex behavior of the angle of Δϕ_{max}. Additional transitions and frequency plateaus are obtained for such assumptions (data not shown). Nevertheless, such a situation is realistic for biological cells with non-confocal membranes [47].

The Δϕ balance along the three semiaxes is plotted in Fig. 9 to consider the physical background of the angle transitions in Figs. 7 and 8. Fig. 9A shows that the frequency dependence of Δϕ is qualitatively very similar along the three semiaxis. At low frequency (< 10^5 Hz) the amplitude of Δϕ is frequency independent. The amplitude of Δϕ is highest for the longest

Fig. 8. Frequency dependence of the angle β of the Δϕ_{max} membrane point on cell parameters for a field orientation of δ=45° in the a–b plane. (A) internal conductivity, α_i; (B) external conductivity, α_e; (C) membrane conductance, g_m; (D) area specific membrane capacitance, C_m.

Fig. 9. Frequency dependence of the absolute values of Δϕ according to the full model (Eq. (2), Table 1). (A) Frequency dependence of Δϕ at the poles for a field orientation along the semiaxes a, b and c, respectively. Insert: curves cross one another at around 100 MHz. (B) amplitudes of Δϕ at the poles relative to Δϕ_{a}. Please compare to the transitions of Δϕ_{a,b,c} and Δϕ_{a,b,c} in Figs. 7 and 8, respectively.
semiaxis \((\Delta \phi_a > \Delta \phi_b > \Delta \phi_c, \text{Fig. 9A})\). Transitions of the relative values occur at frequencies above 100 kHz (Fig. 9A). Above 100 MHz the sequence of the magnitudes even reverses \((\Delta \phi_c > \Delta \phi_b > \Delta \phi_a; \text{Fig. 9A insert, Fig. 9B})\). The transitions in the relative magnitudes are identical to the transitions of \(\alpha\) (compare dashed lines in Fig. 9B to dashed line in Fig. 7) and \(\beta\) (compare dotted lines in Fig. 9B to dashed line in Fig. 8).

4.1.4. Electric field distribution in the EP chamber

We used COMSOL 3.3A Multiphysics program (Comsol AB, Stockholm, Sweden) to consider the three-dimensional electric field distribution in the inter-electrode space of the chip chamber for an electrode height of 100 nm, i.e. the thickness of the platinum layer (Fig. 3). Aqueous solution was assumed to cover both electrodes by 5 µm, the approximate filling height in the EP experiments. The constant field strength found in the center between the electrodes is altered at distances lower than 10 µm to the electrodes. While the field strength is increased close to the chamber bottom \((z < 1 \mu m)\) it is decreased for heights of \(z = 3 \mu m\) and \(z = 5 \mu m\) at short distances to the electrodes. This is the reason for an increased number of lysed cells in the immediate vicinity of the electrode edges (see cell number 6 in Fig. 10E).

4.2. EP results

4.2.1. Dependence of PI permeabilization and cell lysis of oriented CRBCs on electric field strength, frequency and pulse duration

CRBCs are nucleated and have a three-axial ellipsoidal shape. The nuclei are clearly visible (Figs. 4 and 10). The semiaxes measures obtained from 60 cells were \(a:b:c = 6.66 \pm 0.19 \mu m; 4.17 \pm 0.26 \mu m; 1.43 \pm 0.08 \mu m\). Experimental considerations were restricted to the case of semiaxis \(c\) being perpendicularly oriented to the external field \(E\) \((\gamma = \alpha = 0^\circ\); see also Fig. 2B). Fig. 10 presents examples of phase-contrast and fluorescence micrographs of CRBCs immediately and 3 min after the pulse. Three

![Fig. 10. EP of oriented CRBCs suspended in 10 µM PI solution (σₑ = 0.12 S m⁻¹). Left column (A, C, E and G): phase contrast micrographs immediately after the pulse. Right column (B, D, F and H): fluorescence micrographs 3 min after pulses of \(E = 200 kV m^{-1}\) (16 Vpp, electrode distance 80 µm). Different pulse durations of 1 ms (E), 10 ms (A and C) and 100 ms (G) as well as different field frequencies of 1 kHz (A, E and G) and 150 kHz (C) were used. Cell numbers and the orientation angles of their semiaxes \(a\) with respect to the field are indicated. PI permeabilized (small fluorescent spots, normal nucleus size) and lysed (E: number 6, G: numbers 3 and 4, swollen nucleus) cells were distinguished from the appearance of their nuclei.](image-url)
angle-classes of cell orientation were considered as indicated in Fig. 10 ($\delta=0^\circ, 45^\circ$ and $90^\circ$).

**Frequency dependence of EP:** the pulse duration was kept constant at 10 ms to consider the frequency dependence. Examples are shown in Fig. 10A–D. Cells of all orientations were permeabilized for PI at 1 kHz (Fig. 10A and B). Only cells oriented parallel to the field (cells 1 and 2) were permeabilized for PI at 150 kHz, while cells oriented at $45^\circ$ and $90^\circ$ (cells 5 and 6) were not permeabilized (Fig. 10C and D). Detailed results on the frequency dependence are given in Fig. 11A–C.

**Pulse duration dependence of EP:** the frequency was kept constant at 1 kHz to consider the pulse duration dependence. Examples are shown in Fig. 10E–H. Four out of six cells were permeabilized for PI at 1 ms (Fig. 10E and F). All cells were permeabilized for PI by the longer 100 ms pulse. Two cells even lyed (cells 3 and 4 in Fig. 10G and H). Lysed cells lost their hemoglobin content after pulse application, their nuclei swelled. An example is cell number 6 in Fig. 10E. For its location close to the electrode, it is the only lysed cell, even though it is oriented at $72^\circ$. Detailed results of the pulse duration dependence are given in Fig. 11D–F.

The percentages of PI permeabilized and non-lysed cells were plotted over field strength for different pulse durations and field frequencies to analyze the electroporation properties of CRBCs. Sigmoidal curves were fitted to the experimental data (Fig. 11):

$$y = \frac{a}{1 + \exp((Es0_5 - E)/b)}$$

Where $y$, $a$, and $b$ denote the percentages of cells complying with one of the two criteria (PI permeabilization or non-lysis), the final ($b$ negative) or starting ($b$ positive) percentage of cells for zero-field strength and the slope of the sigmoidal curve. $E$ is the electric field strength of a data point and $E_{50}$ determines the electric field strength corresponding to the 50% criterion [13,50]. All curves were fitted using a nonlinear least-square regression program (SigmaPlot 9.5).

Fig. 11A–C present selected results for the frequency dependence for a constant pulse duration of 10 ms. The figure shows a higher EP efficiency at lower frequencies (compare to Gimsa and Wachner [29]).

Fig. 11D–F present selected results for the effect of pulse duration for a constant frequency of 1 kHz. This frequency is low enough to be still in the plateau range of the DC-limit (Fig. 9) and high enough to avoid electrode effects. Higher field strengths are required for an effective EP at shorter pulse durations and higher numbers of lysed cells are found at longer pulse durations [14,15]. Clearly, EP efficiency is higher at a cell orientation of $\delta=0^\circ$ than at $\delta=45^\circ$ and $90^\circ$ [23].

### 4.2.2. Critical field strength for oriented cells in dependence of field strength and pulse duration

We defined critical field strengths $E_{crit}$ for PI permeabilization and cell lysis from the 50%-values of the fitted curves in Fig. 11, i.e. 50% of the cells comply with one of the criteria at $E_{crit}$ [1]. The $E_{crit}$ for PI permeabilization was always lower than for cell lysis independent of cell orientation.

Fig. 12 presents the frequency and pulse duration dependence of the two $E_{crit}$ parameters. The parameters increase for higher frequencies (Fig. 12A) and shorter pulses (Fig. 12B). Below 100 kHz...
Parameter Cell orientations permeabilization for different orientations of semiaxis increases linearly with frequency also below 100 kHz (Fig. 12A). The stronger than those resulting in PI permeabilization\[7]
large aqueous pores, membrane rupture, and cell lysis are much
reason may be that the nonlinear processes related to the formation of
the optimal frequency for differentiation of PI permeabilization and non-lysis.

Please note that $\Delta \phi$ for PI permeabilization was independent of frequency, whereas it is increasing at higher frequencies [22]. This behavior is in accordance with Eqs. (4) and (5). Nevertheless, $E_{\text{c}}$ for cell non-lysis increases linearly with frequency also below 100 kHz (Fig. 12A). The reason may be that the nonlinear processes related to the formation of large aqueous pores, membrane rupture, and cell lysis are much stronger than those resulting in PI permeabilization [7-9]. Further, the different relations of the $E_{\text{c}}$ values for PI permeabilization and cell non-lysis (Table 3) at the different angles of cell orientation are most probably resulting from these properties. Our model does not describe complex changes of the membrane properties during the process of pore formation and membrane rupture. For these reasons, no $\Delta \phi_{\text{c}}$ values were derived from the cell lysis data.

A field frequency of 10 kHz was chosen to search for the optimal pulse duration. This frequency is high enough to exclude electrode

The obtained $\Delta \phi_{\text{c}}$ values for PI permeabilization are given in Table 3. Theoretical $\Delta \phi_{\text{c}}$ values were only calculated for PI permeabilization for the reasons discussed above.

Fig. 13 presents the frequency dependence of PI permeabilization. The low deviation of the curves from the data points indicates that the cell parameters of Table 1 are reasonable assumptions for CRBCs. The assumption of a specific membrane capacitance even higher than 0.025 F m$^{-2}$ (Table 1) would probably result in a theoretical $E_{\text{c}}$ increase already at lower frequencies and improved fits at higher frequencies (see also [47]). Nevertheless, other probe molecules may yield different values.

Our results confirm that the membrane sensitivity toward the field depends on membrane curvature. Three factors may be important for this effect: i) the larger the area oriented perpendicularly to the field (low curvature) the larger the area experiencing a high $\Delta \phi$ and the higher the probability of pore formation. As a result the membrane sensitivity for the induced $\Delta \phi$ will be higher along the two shorter semiaxes; ii) the surface tension generates forces attracting membrane molecules from other membrane areas. These forces are stronger in areas of higher curvature around the poles of the longer axis, i.e. when molecules are becoming available by the growth of pores they are faster collected in areas of high curvature leading to a facilitated pore growth in areas of low curvature. Nevertheless, such a global effect may require a membrane property that provides argument iii): areas of higher curvature may be stabilized by molecular structures near the poles of the longer semiaxis. As a result, the membrane will be less sensitive toward field-induced distortions in these areas. In practice, a combination of the above and

![Fig. 12. Comparison of $E_{\text{c}}$ for PI permeabilization and non-lysis for various field frequencies (A, const. pulse duration of 10 ms) and pulse durations (B, const. field frequency of 1 kHz). Three cell orientations are considered. Dashed lines in (B) denote the average of the orientations for the two $E_{\text{c}}$ criteria. The dotted vertical line marks the optimal frequency for differentiation of PI permeabilization and non-lysis.](image1)

![Fig. 13. Fitted frequency dependencies of $E_{\text{c}}$ of PI permeabilization for three cell orientations over frequency (data of Fig. 12A), Eqs. (4) and (5) and the parameters of Table 1 were used. The fits yielded the $\Delta \phi_{\text{c}}$-values of Table 3.](image2)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cell orientations</th>
<th>$E_{\text{c}}$/kV m$^{-1}$</th>
<th>$\Delta \phi$/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{c}}$/kV m$^{-1}$ (PI permeabilization, 1 kHz, 10 ms)</td>
<td>$\delta$=0°</td>
<td>141.89±7.53</td>
<td>0.99</td>
</tr>
<tr>
<td>$E_{\text{c}}$/kV m$^{-1}$ (non-lysed cells, 1 kHz, 10 ms)</td>
<td>$\delta$=0°</td>
<td>208.29±3.93</td>
<td>0.99</td>
</tr>
<tr>
<td>$E_{\text{c}}$/kV m$^{-1}$ relative to $E_{\text{c}}$ at 0°</td>
<td>$\delta$=0°</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$\Delta \phi_{\text{c}}$/V (theoretical analysis of data in Fig. 13)</td>
<td>$\delta$=0°</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Please note that $E_{\text{c}}$ for PI permeabilization for a field rotating in the $a$-$b$ plane was approx. 115 kV m$^{-1}$ corresponding to a $\Delta \phi_{\text{c}}$ of 0.79 V at 0°.
other unknown effects may explain our findings. Please also note that
the actual curvatures in CRBCs are different from those of a general ellipsoid of the same axis-lengths (Fig. 4).

5. Conclusions

We developed a model for the Δϕ induced in three-axial ellipsoidal and spheroidal cells with an arbitrary orientation within the field in order to analyze the effects of cell orientation, field strength and frequency. The model was tested in EP experiments on ellipsoidal CRBCs of different orientations in a glass chip chamber. The chamber provided homogeneous fields in large areas between the electrodes. The permeabilization and lysis rates were only increased for cells sedimented in the immediate vicinity of the electrodes due to the strongly inhomogeneous fields at these sites. Integrated temperature sensors allowed us to prove that the EP pulse did not induce a strong temperature increase in the chamber.

The percentages of PI permeabilized and lysed cells increased with pulse duration and decreased with field frequency. The optimal pulse duration and field frequency, i.e. a high percentage of PI permeabilization at a low lysis rate, were approx. 10 ms and 10 kHz for CRBCs at an external conductivity of 0.12 S m⁻¹. Theoretical analysis showed that Δϕ is highest when the longest semiaxis is oriented in parallel to the external conductivity of 0.12 S m⁻¹. The plot of the relative influence radius c(Ω) vs. the symmetry axis c._

The general ellipsoid is always unity [51,52] if it follows that a + b + c = 2 c(Ω) + c along the symmetry axis c._  

\[ \frac{a + b + c}{c} = 2c(Ω) + c \]  

(A1)

\[ \frac{a + 2c}{c} = 1 + \frac{1}{\sqrt{c(a)}} \]  

(A2)

References


Appendix A

Simplified equations of the influential radius for spheroidal cells

The plot of the relative influential radius Ω/Ω compared to the logarithm of the axis ratio exhibits a sigmoidal shape for spheroids (see [46] for more details). This behavior can be approximated by:

\[ \frac{Δϕ_{max}}{Δϕ} = \frac{a + 2c}{c} = 2 - \frac{1}{\sqrt{c(a)}} \]  

(A1)

The limiting values for infinitely thin disks, spheres and infinitely long cylinders 1, 1.5, and 2 are correctly reflected by Eq. (A1). Since the sum of the depolarizing factors (n₁ + n₂ + n₃) along the three principal axes of a general ellipsoid is always unity [51,52] it follows that a + b + c = 2 c(Ω) + c along the symmetry axis c._

\[ \frac{a + 2c}{c} = 1 + \frac{1}{\sqrt{c(a)}} \]  

(A2)


