Universität Leipzig

Biophysics Internship

Electroporation of human erythrocytes and erythrocyte ghosts (EP)

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1 Biological membranes

The basic modules of all organisms are cells. Living cells are physically separated from the environment by a semipermeable membrane. All biological membranes consist of similar compounds. The membrane of a living cell consists of a bilayer of amphipolar lipid molecules as well as high molecular mass proteins proteins.

The essential structure of a membrane is primarily made up of a bilayer of phospholipid molecules. The polar heads of the phospholipid molecules stick out into the aqueous intracellular space and extracellular solution, because phospholipids are amphiphilic molecules and the cell membrane borders on both sides on aqueous media. The apolar tails are directed towards each other inside the bilayer. The hydrophobic tails of the lipid molecules are excluded from water in this way. The polar headgroups associate with itself and with the aqueous phase.

Different models for the arrangement of the proteins in the lipid bilayer of biological membranes have been suggested. The fluid mosaic model of lipid bilayer membranes, introduced by Singer and Nicolson, describes the essential features of biological membranes. Membranes are considered by this model as a two-dimensional oriented solution of integral proteins in the viscous phospholipid bilayer. The Singer–Nicolson fluid mosaic membrane model illustrates lateral and rotational freedom as well as random distribution of molecular components in the membrane (Fig. 1).

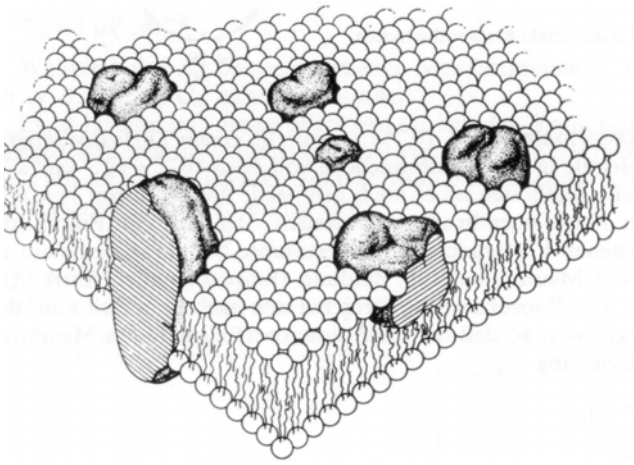


Figure 1: The fluid mosaic model of lipid bilayer membranes including membrane proteins (Adam 1995)

2 Electroporation

Even though the membrane is kept together only by hydrophilic and hydrophobic forces, it is very resistive and generally able to withstand electric fields. Transmembrane potentials of up to 120 mV are physiologic and essential for the functioning of the cell. However under the influence of strong electric fields, the lipid bilayer becomes unstable and permeable (Potter, 1988; Sowers, 1995).

Electroporation designates the use of short high-voltage pulses to overcome the barrier of the cell membrane. By applying an external electric field, which just surpasses the capacitance of the cell membrane, a transient and reversible breakdown of the membrane can be induced (Neumann et al., 1989). It is proposed that water enters the cell membrane during the dielectric breakdown and that transient hydrophilic pores are generated (Fig. 2). The formation of permeable areas occurs in the frame in less than a second whereas resealing is in the range of minutes.

This transient, permeabilized state can be used to load cells with a variety of different molecules, either through simple diffusion in the case of small molecules, or through electrophoretically driven processes allowing passage through the destabilized membrane as it is the case for DNA transfer. Initially developed for gene transfer, electroporation is now in use for delivery of a large variety of molecules: From ions to drugs, dyes, tracers, antibodies, and oligonucleotides to RNA and DNA. Electroporation has proven useful *in vitro*, *in vivo* and in patients, where drug delivery to malignant tumors has been performed (Chizmadzhev et al., 1995).

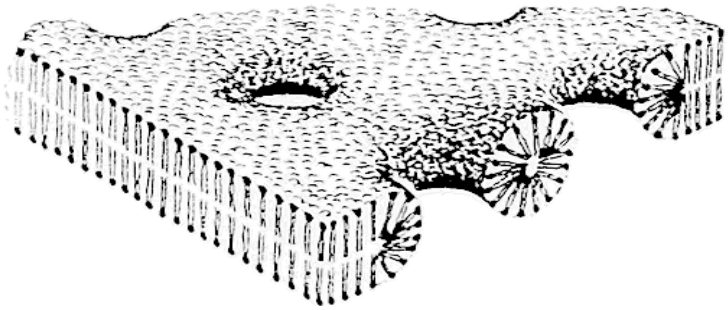


Figure 2: Pores in a lipid bilayer (Weaver and Powell, 1989)

2.1 Electroporation as an indirect field effect

Electric fields affect charges directly. Electric fields exert forces on charges, which can either move if they are free to do so, or accumulate if they are limited in their motion. The effect of electric fields is observable at free ions, dipoles and polarizable groups. If an electric field is sufficiently strong, molecules and molecular organizations, like the biological membrane, undergo structural rearrangements (Neumann, 1989).

The phenomenon of electroporation is based on the physical process of polarization of matter in electric fields. This means electroporation is an indirect field effect. The direct effect of the surface polarization precedes the structural changes of the membrane.

2.1.1 Surface polarization

The polarization of membranes results from the fundamental interaction of electric fields with charges. The primary effect of the electric field on the membrane is the induction of electric dipoles. These dipoles are oriented in the electric field.

Furthermore, the application of an external electric field leads to motions of ions in the aqueous medium along the streamlines of the field. Due to their low conductivity, membranes restrict motion of charges. This leads to the polarization of cell membranes (Fig. 3).

The accumulation of ions at the membrane surface give rise to induced surface potentials on both sides of the membrane. This results in the built-up of a potential difference across the cell membrane, the transmembrane potential $\Delta\varphi$

$$\Delta\varphi = \varphi(r) - \varphi(r-b)$$

Were r is the outer radius of the cell and b is the thickness of the membrane (Neumann, 1989).

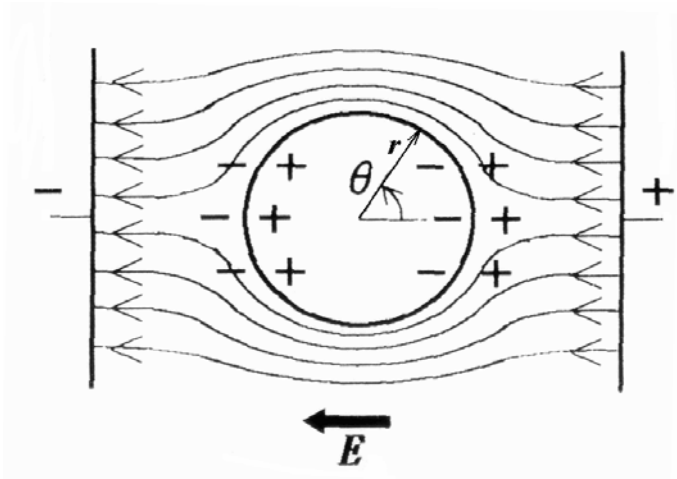


Figure 3: **Surface polarization of a spherical cell in a homogeneous electric field (Kinosita et al. 1992; Dimitrov 1995)**

The potential distribution in the region surrounding a spherical cell in an external electric field E depends on the position along the membrane. The transmembrane potential is a linear function of cosine of the angle θ between the site on the cell membrane where transmembrane potential is measured (radius vector) and the direction of the electric field. For $b << r$ is

$$\Delta\varphi = -\frac{3}{2} Er|\cos\theta| f(\lambda)$$

Where $f(\lambda)$ is an explicit function of conductivities λ_m, λ_e und λ_i of membrane, extracellular and intracellular medium.

$$f(\lambda) = \frac{1}{1+\lambda_m(2+\lambda_i/\lambda_e)/(2\lambda_i b/r)}$$

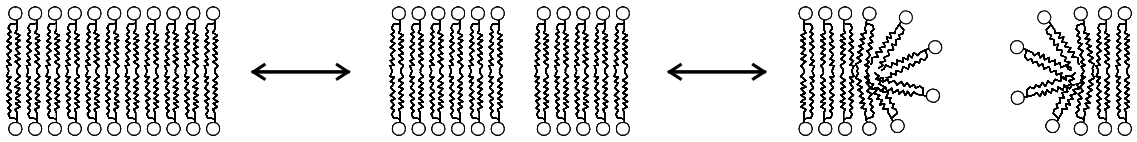
For a non-conducting membrane results with $\lambda_m = 0$ ($\lambda_m \approx 1$ nS/cm, Dimitrov 1995) $f(\lambda) = 1$.

Therefore, the maximal induced transmembrane voltage at the cell poles ($\theta = 0°$ and $\theta = 180°$) is $\Delta\varphi_{max} = -\frac{3}{2}Er$.

2.1.2 Reversible structural rearrangements

Surface polarization as a direct consequence of the electric field causes structural rearrangements in the biological membrane. This leads to pore formation, which begins with high probability on the cell poles due to the high transmembrane potential.

It was shown, that "electropores" form in the pure lipid matrix of a cell membrane, although membrane proteins might play a role in their stability (Teissie and Tsong, 1981; Needham and Hochmuth, 1989). Formation of pores occurs as a result of the rotation of lipid molecules that form the pore walls. The movement of lipid molecules in electroporation is characterized in the theoretical model by the diffusion coefficient of lipid molecules in pore radius space. The electrophoretic movement of ions and water dipoles through the spontaneous hydrophobic pores is postulated to be the first event of electroporation, after which lipid molecules rearrange to form more stable hydrophilic pores. (Fig. 4).



lipid bilayer hydrophobic pore (HO) hydrophilic pore (HI)

Figure 4: Formation of membrane pores in a lipid bilayer as indirect consequence of electric field

The formation of hydrophobic pores in the bilayer is energetically favored when the radius is very small. These hydrophobic pores are formed spontaneously by lateral thermal fluctuations of the lipid molecules. However, when their radius exceeds a critical value, a reorientation of the lipids becomes energetically favorable. This reorientation, resulting in the formation of a hydrophilic pore, can be regarded as an inversion of the initial hydrophobic pore (Fig 5). The lifetime of the hydrophobic pores is in the order of the lipid fluctuations. They are only intermediate stages in the formation of hydrophilic pores (Chernomordik, 1992).

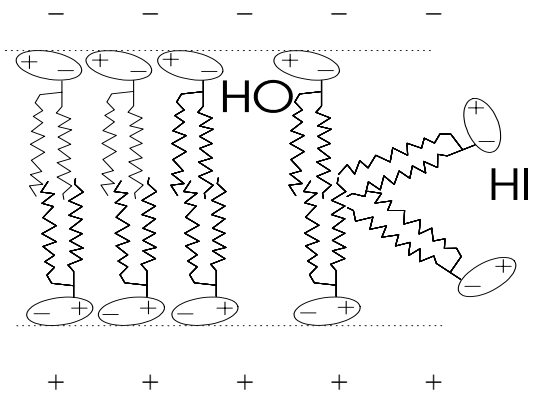


Figure 5: Molecular rearrangements of lipids due to the surface polarization in an external electric field

The diameter of hydrophobic pores d_{HO} is lower than the thickness of the membrane b. For hydrophobic electropores in lipid bilayers $d_{HO} = 0{,}7$ nm was determined (Kakorin et al., 1996). Hydrophilic pores are characterized by a considerable size. The diameter of hydrophilic pores d_{HI} is even higher than the thickness of lipid bilayers b, which is about 5 nm.

2.1.3 Irreversible secondary prozesses

All mentioned structural rearrangements are reversible. The relaxation of transmembrane current following the removal of the external pulse occurs as a result of the reorientation of the lipid molecules to close the membrane pores or shrink them.

Irreversible secondary processes, which can succeed the reversible pore formation, are the reason for the various applications of electroporation. The permeabilization due to the loss of barrier function of membrane enables electrorelease and electroincorporation, i.e. the transfer of molecules. Electrofusion of neighbor cells is a possible result of the reorganization of the membranes.

Permeation causes swelling and eventual rupture of the cell membrane The dielectric breakdown of membrane leads to the irreversible destruction of cell. Rupture can occur as consequence of large electric fields and long pulses.

The electric current leads to heating of membrane. The increase of temperature dT in time dt is given by

$$dT = \frac{U^2 dt}{R \cdot c_P \cdot \rho \cdot V}$$

Where U represents the potential, R the resistance and V the volume. For water is the spezific heat capacity $c_p \approx 4{,}18$ J/g K and the density $\rho \approx 1$ g/cm^3. This data may also be used for suspensions.

2.2 Electroporation as critical phenomenon

The onset of structural rearrangments of membrane lipids, which lead to the formation of pores in the membrane, shows a threshold behavior. It is therefore necessary to treat the electroporation as a critical phenomenon.

If the transmembrane potential achieves a critical value, the structural rearrangements of the lipid molecules begin phase transition-like. It was shown, that the critical transmembrane potential $\Delta\varphi_c$ is a function of the pulse duration Δt (Neumann, 1989; Chernomordik, 1992). For short pulses the critical value of the transmembrane potential is higher than for long pulses.

Figure 6 shows the ratio of pulse duration and critical electric field strength for cells (*Chlamydomonas reinhardtii)* found by Neumann. The correlation of the transmembrane potential $\Delta\varphi_c$ and pulse duration Δt corresponds approximately to a linear dependency between $(\Delta\varphi_c)^2$ und Δt^{-1} (Neumann, 1992).

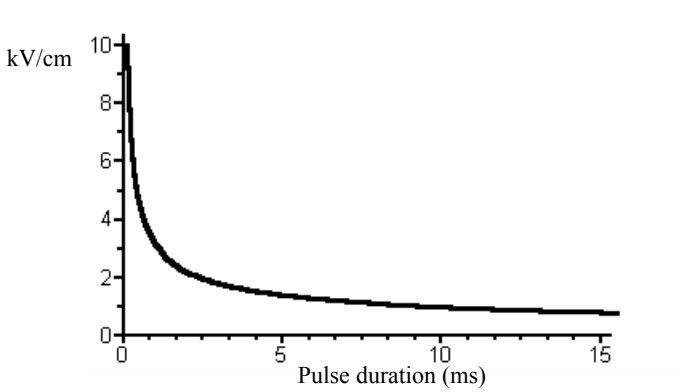


Figure 6: The critical value for field strength as function of pulse duration (Neumann, 1992)

The critical value for energy per cell W_c is given by

$$W_c = \int_o^t I_P \left|\Delta\varphi_c\right| \cdot dt$$

with polarization current $I_P = G_P\,(-\Delta\varphi_c)$ results

$$W_c = G_p(\Delta\varphi_c)^2 \cdot \Delta t\,,$$

where G_P represents the conductivity of the membrane surface.

For the critical value of electric field strength E_c results with $\Delta\varphi_c = -\frac{3}{2} E_c \cdot r$ at cell poles:

$$W_c = G_p\left(\frac{3}{2} r\right)^2 {E_c}^2 \cdot \Delta t\,.$$

2.3 Shapes of the electric pulses

Voltage pulses, used for the electroporation of biomembranes, can be differentioted by their shape, i.e. by the dependence of the voltage *U(t)* from time *t*. Due to the dependence between voltage *U* and electric field strength *E* (with the electrode distance d)

$$E(t) = U(t) / d$$

all dependencies for *U(t)* are also valid for *E(t)* as well.

There are two basic shapes of voltage pulses: the rectangle pulse and the discharge pulse. Both pulses and their associate voltages result from the discharge of the condensers. While the complete discharge of a condenser results in exponentially decaying pulses, the partial discharge of a condenser with a high capacity leads to a rectangular pulse (Fig. 7).

2.3.1 The rectangular pulse

The rectangular pulse (Fig. 2.5) is the most simple shape, because the electric voltage as well as the electric field remain constant.

Figure 7: Typilcal shape of a rectangular pulse (voltage U over time t)

2.3.2 The discharge pulse

Understanding the shape of the decaying discharge pulse (Fig. 8) is somewhat more difficult, since both, the electric voltage as well as the electric field change with time.

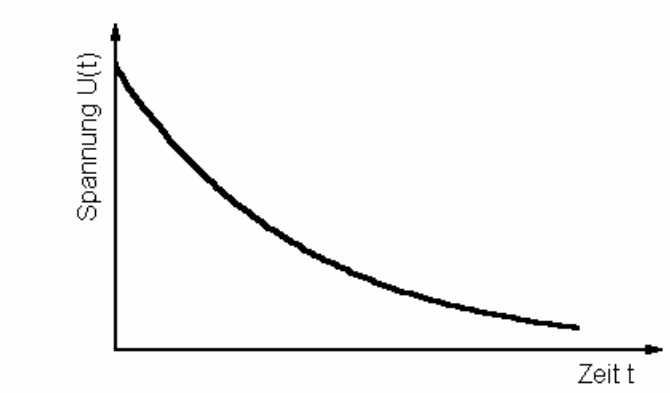


Figure 8: Typical shape of an exponentially decaying discharge pulse (voltage U over time t)

It is not possible to give suration for the complete discharge pulse. The characteristic time for the decay of the electric field E_0 is given by the time constant τ,

$\tau = RC$.

This leads to a dependence of the electric field E from time t:

$E(t) = E_0 \cdot e^{-t/\tau}$.

2.4 Progenetor II (Hoefer)

The Progenetor II (Hoefer) delivers a rectangular voltage pulse. The pulse duration varies between 10 µs and 1 s. The pulse shape derivates from the strict rectangular shape for pulses longer than 1 ms due to a discharge of the condensers.
The resistance R of the sample chamber can be estimated to be 6,7 Ω for the buffer solution used in all experiments (100 mM NaCl and 10 mM HEPES). Condensers with capacities *C* of 100 µF, 490 µF and 760 µF may be used individually as well as in combination. The time constant $\tau = RC$ for the electric discharge of the condensers into the suspension of liposomes was therefore in the range of milliseconds (table 1).

Capacity of the condensers *C*	Time constants for the discharge $\tau = RC$
100 µF	0,7 ms
490 µF	3,3 ms
760 µF	5,1 ms
(490 µF + 760 µF)	8,4 ms

Table 1: Time constants $\tau = RC$ for the discharge of the condensers avaiable for Progenetor II

The timely evolution of the condenser voltage (pulse shape) can be monitored by a storage oscilloscope (Fig. 9)

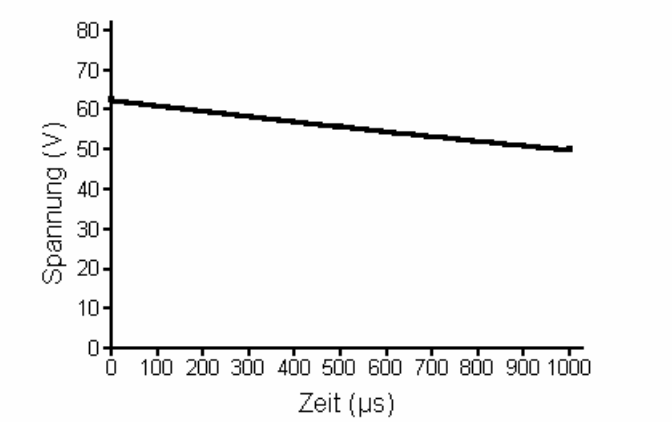


**Figure 9: Shape of the Progenetor II (Hoefer) discharge voltage pulse.
The used condenser had a capacity of 760 µF, the voltage shown by the device was 100 V. (voltage [V] vs. time [µs])**

The voltage detectable in the sample is only about 60 % of the original voltage delivered by the device (Progenetor II). This voltage can be varied between 50 and 500 V, therefore the effective sample voltage can be set between 30 and 300 V.

3 Experiment

3.1 Preparation of human erythrocytes and erythrocyte ghosts

For the preparation of erythrocytes and erythrocyte ghosts whole blood obtained from the blood donation center of the Medical Faculty of the University is used.

After careful opening of the blood bottle (with latex gloves), the blood has to be centrifuged for 5 min. at 800 g (Hereaus Labofuge) and 4 °C. The supernatant has to be removed carefully and to the cell pellet ice-cold PBS-buffer is added. After stirring, the cell suspension will be centrifuged for 10 min at 3,000g and 4°C. The supernatant is discanded once more and the whole procedure repeated twice. The results of the washing procedure are pure erythrocytes without other serum constituents or further cells (lymphocytes, leukocytes).

The cell preparation will be characterized by transmitted light microscopy, haematocrit and cell number determination (cell counting device CASY).

The preparation of erythrocyte ghosts is carried out by the use of hypotone lysis according to Schwoch and Pasow (1973). It is important that all steps are is carried out on ice and that the buffers are sufficiently precooled (4°C), since elevated temperatures cause a spontaneous closure of the erythrocyte membrane.

2ml pellet of the washed erythrocytes are suspended in 1ml PBS. Afterwards the suspension is diluted with 60ml lysis buffer (ice-cooled) and incubated for 5 min on ice. The hypotonic lysis buffer contains 1.2mM acetic acid, 4mM $MgSO_4$, 1mM $CaCl_2$ and 50µM sodium-ortho-vanadate. The presence of calcium ions causes a disruption of the lipid asymmetry in red blood cells. This means that the preferred localisation of several membrane phospholipids in the interior membrane monolayer (phosphatidylserine, phosphatidylethanolamine) and exterior membrane monolayer (phosphatidylcholine, sphingomyelin) is abolished and the phospho-lipids get scrambled. The ortho-vanadate ions inhibit in the given concentration the enzyme aminophospholipid-translocase, that is located in the erythrocyte membrane. Thus, phospholipids cannot retain the lipid asymmetry in ghost membranes.

Subsequently, the erythrocyte lysate is centifuged for 10 min at 15,000 g and 4°C in a fixed angle rotor (Jouan KR 22-i). The obtained pellet is diluted on ice with 5ml lysis buffer, which contains now additional Lucifer Yellow (1mg/ml) and 1.2mM adenosin-5-triphosphat (ATP). The ATP favors the formation of dense membranes in the closed ghosts.

After 5 min isotonic conditions are established by addition of 0.5ml 10-fold concentrated PBS that is brought to a pH of 7.4 using aqueous sodium hydroxide (0.1M NaOH). The resealing of the ghost membranes takes place by incubation of the ghost suspension 45 min at 37°C. The closed ghosts have to be washed thrice with PBS (15,000 g, 10 min, 4°C) to remove free Lucifer Yellow.

3.2 Electroporation experiments

For the electroporation experiments the Hoefer Progenerator II is used (already described above). Between the electrode plates 40 μM of erythocytes or ghosts are placed. For the several samples the aplied voltage will be gradually varied between 50 V and 500 V (in 50 V intervals).

3.3 Verification of the Electroporation

Erythrocytes

The electroporated sample between the electrodes has to be brought down with 1ml PBS and collected within a fluorescence cuvette. Afterwards the absorption at 405 nm has to be measured by a spectral photometer. As reference (100% lysis) a sample is used, which is diluted with distilled water and sonicated.

Erythrocyte ghosts

The electroporated sample has to be brought down analogously with PBS and collected within a cuvette. The excitation maximum of Lucifer Yellow fluorescence is at 427nm while the emission is at 535 nm. As reference (100% lysis) a sample is used, which is diluted with distilled water and sonicated.