

Electropermeabilization of the Cell Membrane

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Abstract

Membrane electropermeabilization is the observation that the permeability of a cell membrane can be transiently increased when a micro-millisecond external electric field pulse is applied on a cell suspension or on a tissue. Applicative aspects for the transfer of foreign molecules (macromolecules) into the cytoplasm are routinely used. But only a limited knowledge about what is really occurring in the cell and its membranes at the molecular levels is available. This chapter is a critical attempt to report the present state of the art and to point out some of the still open problems. The experimental facts associated to membrane electropermeabilization are firstly reported. They are valid on biological and model systems. Secondly, soft matter approaches give access to the bioelectrochemical description of the thermodynamical constraints supporting the destabilization of simplified models of the biological membrane. It is indeed described as a thin dielectric leaflet, where a molecular transport takes place by electrophoresis and then diffusion. This naïve approach is due to the lack of details on the structural aspects affecting the living systems as shown in a third part. Membranes are part of the cell machinery. The critical property of cells as being an open system from the thermodynamical point of view is almost never present. Computer simulations are now contributing to our knowledge on electropermeabilization. The last part of this chapter is a (very) critical report of all the efforts that have been performed.

The final conclusion remains that we still do not know all the details on the reversible structural and dynamical alterations of the cell membrane (and cytoplasm) supporting its electropermeabilization. We have a long way in basic and translational researches to reach a pertinent description.

Key words Electropermeabilization, Mechanism, Membrane, Electrotransfer

1 Introduction

The permeability of a cell membrane can be transiently increased when a micro-millisecond external electric field pulse is applied on a cell suspension or on a tissue [1–3]. Under suitable conditions depending mainly on the pulse parameters (field strength, pulse duration, number of pulses), the viability of the cell can be preserved. The resulting electropermeabilization is a powerful electrochemical tool to gain access to the cytoplasm and to introduce chosen foreign molecules or to extract metabolites [4–8].

If this approach is now routinely used in cell and molecular biology for more than 20 years, one should nevertheless be aware that very few are known about what is really occurring in the cell and its membranes at the molecular levels [9, 10].

Electroporabilization is nevertheless now proposed as a very efficient way for drug, oligonucleotides, antibodies, and plasmids delivery *in vivo* for preclinical and clinical biotechnological applications [11–14]. New developments for the food and environmental industries have been proposed [15].

The clinical outcomes on melanoma are really impressive for the comfort of the patient. A safe use of this approach requires a better knowledge of the molecular processes affecting the membrane organization. Most basic investigations during the last 30 years were mostly focused on pure lipid bilayered models [16, 17]. Clearly, there are many limits in the conclusions that were obtained on these soft matter systems in order to describe what occurs in life science, i.e., cells and tissues.

This chapter is an attempt to describe the 2013 state of the art on our knowledge and the limits of the investigations and to highlight some of the still open problems.

2 Membrane Electroporabilization: The Facts

From experiments on planar bilayer membranes (BLM), it was known that lipid bilayers were not able to withstand an increase in the applied voltage above a threshold value. A conductive state followed by a rupture was observed for values of the order of 200 mV. Electropulsation induces a transmembrane potential modulation, suitable to induce a similar membrane instability. Indeed experiments on pure large lipid vesicles showed that upon the field pulse, the lipid bilayer could become leaky. This was observed online by the associated increase in conductance of a salt-filled vesicle suspension [18]. But larger molecules could leak out and be directly detected outside the vesicles as first observed with radiolabelled sucrose [19]. A very fast monitoring of the membrane leakage is obtained by electrical conductance and light scattering experiments. The process is present in less than a microsecond after (during) the onset of the pulse [20]. Similar processes were detected on biological cells [21].

Nevertheless, molecular transport of charged molecules such as propidium iodide is delayed by several tens of microseconds after the voltage onset [22]. This transport of charged molecules during the pulse is clearly mostly driven by the electric field but delayed by the membrane organization even if perturbed (a transmembrane electrophoresis). But the amazing feature is that in cells the transport remains present when the field is switched off.

Most of the transport for polar molecules up to 2 kDa occurs in this post-pulse “resealing” phase where the field is not present anymore.

Kinetic studies of electropermeabilization led to a phenomenological description in a multistep process:

1. “Charging step”—A cell was considered as a spherical shell with a dielectric membrane and with external and internal (cytoplasmic) conducting buffers. As a spherical dielectric, a position-dependent transmembrane potential (TMP) is present when the cell is submitted to an external field. This is a fast process, but it is regulated by a capacitive charging time (between 0.1 and 10 μ s). This is indeed more complicated in life science as cells are far from being spheres and that they are associated within tissues by an extracellular matrix as a highly dense population.
2. “Induction step”—The field-induced TMP increase reaches a critical value at the polar position facing the electrodes (under the simple hypothesis that cells are spheres), and this gave poorly characterized local defects in the membrane assemblies. They are called “pores” in many cases while there is still no evidence that such regular structures are present. A good definition was proposed by Neumann in 1982. They were structural transitions in terms of a hysteretic reaction model from closed to porous membrane states. These defects could be associated with water wires and were linked to contributions of lipids and proteins. A mechanical stress was present with a magnitude that depends on the buffer composition. Due to the TMP charging time and to the vectoriality of the field effect, the structural transition of the membrane affects a cap of limited size on the cell surface.
3. “Expansion step”—The density of the defects increases within the affected cap as long as the field is present at an intensity larger than a critical value. An electromechanical stress remains present. The molecular organization of the defects appears to be continuously changing during the pulse application. A self-regulation of the induced TMP is present due to the increased membrane conductance. Electropermeabilization cannot be described as an all-or-not transition.
4. “Stabilization step”—As soon as the field intensity is lower than the critical value, which is mentioned in **step 2**, a stabilization process (or several steps) is taking place within a few milliseconds, which brought the membrane to a long-lived permeabilized state for small molecules (less than 2 kDa).
5. “Resealing step”—A slow resealing in the perturbed cap is then occurring on a scale of seconds and minutes. Membrane defects are progressively annihilated. Molecular transport remains present but vanishes.

6. “Memory effect”—Some changes in the membrane properties are detected on a longer time scale (hours) while the cell behavior is back to normal.

It should be pointed out that the trigger of the field effect is very fast (down to the ns time range when very strong field pulses are delivered) [23–25]. Transport of dyes was only detected long after the ns HV pulse delivery [26]. The resulting consequences remain present several minutes after the pulse delivery before the cell could recover. Irreversible effects could be induced by a proper control of the physical parameters. Electropulsation can be used for cell eradication under the irreversible process (irreversible electroporation: IRE).

Besides the transmembrane stress due to a critical TMP, one should take into account that stretching deformations affect cells in an electric field. The cell membrane is an elastic body with a rigidity mostly due to its cytoskeleton. Hence a cell is deformed under the action of the electrodynamic forces associated with an electric field pulse (so-called the Maxwell tension). Those force field depends on pulse duration. The deformations are larger in the case of lipid systems (where the cytoskeleton network is absent) as directly observed [27, 28]. It is the result of electrostatic interactions between the induced cell dipole and the applied field E . The induced dipole moment is linked to the polarizability of the cell, as a lot of surface charges are present and can move on the cell surface. The stretch is due to the torque induced by the field pulse on the induced and permanent dipoles. The polarizability of the cell is a complex function of the membrane conductivity, the external and internal conductivities, the cell size, and the pulse duration. Its time dependence is therefore complex. After a lag of a few ns, but always in the ns second time range, the field effect reaches its maximum amplitude in that short time and is present during all the pulse. Stretching is therefore faster than the induction of the TMP. Cell membranes are mechanically stabilized by elements of the cytoskeleton, and the deformations are minute. Nevertheless, the tension experienced on the membrane can have a local effect and may affect the induction of electropermeabilization [29].

3 Membrane Potential Difference Modulation

The main source of the membrane structural modification remains supposed to be a modulation of the transmembrane potential (TMP). This is due to the dielectric character of the membrane. From a soft matter point of view, a cell can be described as an insulating shell containing a conducting solution (the cytoplasm with a conductivity λ_i , always rather high in living systems) and in suspension

in a conducting buffer (the external solution with a conductivity λ_o that can be experimentally adjusted). A cell in a field behaves as a charging spherical capacitor. The induced TMP $\Delta\Psi_E$ can be written as (when steady states are reached)

$$\Delta\Psi_E = 1.5g(\lambda)rE\cos\theta \quad (1)$$

where the vesicle shape is assumed to be a sphere, g , a complex function of the conductivities, λ , of the membrane and of the buffers; r is the radius of the sphere; E is the field strength; and θ is the angle between the normal to the membrane and the direction of the field. Being dependent on an angular parameter, the field effect is position dependent on the surface. Therefore, one side of the vesicle is going to be hyperpolarized, while the other side is depolarized. These physical predictions were checked experimentally by video microscopy on lipid vesicles by using potential sensitive fluorescent probes [30, 31]. Very large transmembrane fields result from low external applied fields due to the minute thickness of the biological membrane (average 5 nm).

This TMP alteration is reached after a very short charging time (in the microsecond time range):

$$\Delta\Psi_E(t) = 1.5g(\lambda)rE\cos\theta(1 - \exp(-t/\tau)) \quad (2)$$

The charging time τ depends on the dielectric properties of the membrane, which are more complex than for a lipid bilayer, and the conductances (i.e., ionic content) of the cytoplasm and external buffer. Under the simplifying assumptions that the membrane is a pure dielectric, then

$$\tau = rC_{memb.}(\lambda_{int.} + 2\lambda_{out}) / (2\lambda_{int.}\lambda_{out}) \quad (3)$$

It was calculated and checked to be of the order of microseconds or less for cells under physiological conditions. The consequence is that very large fields must be applied to get a significant transmembrane potential when using very short pulses (ns). These aspects are not within the scope of the present chapter. In “classical” electropulsation, as the risetime of voltage pulses in most available pulse generators is of the order of microseconds [32], it is the limiting step in the transmembrane modulation.

The external field induces a position-dependent modulation of the membrane potential difference linearly related to the intensity of the applied field. Theoretical predictions from Eq. (1) are describing the conditions that:

1. The cell shape is spherical.
2. The membrane is a dielectric.

Hypothesis 2 must then be corrected to take into account the leaks that are present in an electroporabilized membrane.

$g(\lambda)$ is associated to the electrical conductivities of the cell membrane and of the internal and of the external buffer. Its expression is

$$g(\lambda) = [\lambda_o \lambda_i (2d / r)] / [(2\lambda_o + \lambda_i) \lambda_m + (2d / r)(\lambda_o - \lambda_m)(\lambda_i - \lambda_m)] \quad (4)$$

where d is the membrane thickness.

From Eq. (4), as λ_o and λ_i are always rather high in experiments (salts are always present when working with cells except under nsPEF), it is clear that $g(\lambda)$ is under the control of λ_m . Another consequence of the membrane leakiness is that it affects the loading time of the membrane when the field is applied. Its physical definition is given by:

$$\tau = rC_m (\lambda_i + 2\lambda_o) / (2\lambda_i \lambda_o + r\lambda_m (\lambda_i + 2\lambda_o) / d) \quad (5)$$

As λ_m is dependent on the membrane leakiness, the loading time of the membrane will decrease with an increase in the membrane leakiness. $g(\lambda)$ is affected by the increase in λ_m due to electroporabilization bringing a decrease in the limiting value of the induced TMP.

Another problem must be taken into account in the description of the induced potential modulation. The vesicle shape is not a sphere for cells. An ellipsoid is a more accurate description. The effect of the field is therefore dependent on the ratio of the relative axis of the spheroid and on the orientation of the main axis relative to the field. Recent simulations predicted complex cell responses that were fairly assayed experimentally [33].

Numerical methods are used to predict the distribution of induced TMP on cells of complex shapes. Some approximations are needed due to the extreme thickness of the plasma membrane. The finite thickness, membrane with a nonzero conductivity, can be replaced by a boundary condition assigned to the interface between the cytoplasm and the external buffer. As the molecular processes inside the membrane core are not involved, the ratio λ_m/d can be treated as a single entity—the specific surface conductivity [34]. Despite the membrane as such being absent from the model, the drop of electric potential at such a discontinuity is equivalent to the transmembrane voltage induced on a membrane with a specific conductivity λ_m and thickness d . This was checked to be valid by experimental methods (fluorescence methods to measure the induced voltage). To mimic electroporabilization, this approach includes a variable membrane conductivity that is observed under experimental conditions as just mentioned [35]. This reflects the creation of conductive pathways. In the regions

exposed to a sufficiently TMP, the membrane conductivity rapidly increased with time, leading to a modified spatial distribution of TMP. Field distribution close to and within the cell was strongly different from the bulk external field [36]. This affects the cell response. Furthermore, in cell assemblies (such as spheroids mimicking tumor tissues), the field strengths were significantly lower than the external applied field under both *ex vivo* and *in vivo* conditions [37]. This brings a complicated description of the experimental system when trying to make a predictive simulation.

Another approximation in these predictions is the assumption that cell surface is smooth. Tube-like cytoplasmic projections called filopodia are present with an important role in cell–cell communication. This has not been included in the description of the field effects of cell membranes.

Due to the physiological resting potential present in living cells (an active process), $\Delta\Psi_0$, being about -40 to -60 mV, the electric field modulation of the TMP brings a resulting complex asymmetric distribution. A surface lateral gradient in TMP (and associated transmembrane field) is present during the field pulse.

Another cell deformation associated to electropermeabilization was a post-pulse effect: swelling due to the osmotic unbalance resulting from the inflow of water. The physical result was either a loss of surface ruffling or (and) an increase in surface tension [38]. The consequence is that in a train of pulses, the field is delivered on a time changing object (increase in size and in membrane conductance on localized patches).

4 Membrane Organization

Electropermeabilization is the result of structural transitions from closed to porous membrane states. The definition of the molecular components and interacting forces is a key information needed to provide an objective description of the structural transitions. Membranes are built by the self auto-assemblies of a wide diversity of amphiphilic molecules. Their stability results from the balance between a large number of weak interactions.

Permeabilization is controlled by the field strength (through the induced TMP). Permeabilization occurred only on the part of the cell surface where the TMP has been brought at its critical destructuring value. A limited cap in the part of the cell facing the electrodes is affected. The size of the cap is under the control of the applied field. Then a strong local modulation is brought by the pulse duration. Indeed nsPEF HV pulses are able to trigger a membrane conductive state when a very high voltage (field) is delivered. This was indicative that the transition of the membrane to the pre-leaky state was very fast (a few ns), but a contribution of the electromechanical stress might be present.

The field can act only on charged and dipolar groups (including interfacial water molecules). But its effects on dipoles are limited to the induction of a torque and result in a rotation to bring the dipolar moment in an antiparallel position. This is a very local movement affecting only the interfacial region and the glycerol backbone in lipids but with more stringent effects on transmembrane proteins where structural reorganization would occur [39]. The resulting shift in distribution of lateral pressures of the bilayer resulting from these very localized changes would facilitate the protein conformational transition and affect the interfacial interaction between lipid headgroups [40–42]. As a final result, the global structural organization of the membrane will be affected resulting in the exposure of non-hydrophilic regions: a metastable configuration. A repair mechanism must result.

5 Molecular Transport

The field strength controls the extent of permeabilized membrane, whereas the density of defects supporting the permeabilization is under the control of the number and the duration of electric pulses and their delay [43–45]. During each pulse, the electric field induces an electrophoretic drift of charged molecules, first towards and then across the permeabilized part of the cell surface inside the cytoplasm (but only on one side). The resulting post-pulse electro-induced long-lived permeabilization of cell membrane can be quantified in terms of the flow F_s of molecule S diffusing through the plasma membrane during the post-pulse resealing. In the case of inflow, small molecules can then diffuse freely in the cytoplasm following Fick's law across a time-dependent permeabilized surface.

The membrane slow resealing kinetic is only dependent on the cumulated pulse duration as long as irreversible (poorly characterized) damages are not induced. The total accumulation is under the control of the pulse duration that acts on the density of defects and the lifetime of resealing. The transport is dependent on the nature of the target molecule. Therefore, a larger transport is obtained for small molecules. This size effect is more complex with macromolecules (oligonucleotides, proteins) where diffusion is not present. In the case of siRNA (small interfering RNA, MW about 20 kDa), no post-pulse transport is detected [46]. Transport occurs only during the pulse supported by the induced electrophoretic drift (accumulation in the cytoplasm is therefore present only from one side of the pulsed cells), telling that a dramatic membrane structural alteration must be present to force the transmembrane transport of a 2 nm wide cylinder. The transport of plasmids is even more complex. The field-associated electric drift is just bringing an interfacial local accumulation and association of

plasmids [47]. These aggregates remain stuck for minutes at the membrane level before being transported to the cytoplasm either within actin vesicles [48] or as free molecules associated to molecular motors along the microtubules to be transferred to the nuclear envelope [49–51].

The correlation between TMP and molecular transport across the electropermeabilized part of the cell membrane was accessed for isolated cells of regular and irregular shapes, as for cells in dense suspensions. The highest TMP intensities are present in the membrane caps facing the electrodes supporting a localized induced transport [52]. At the cellular level, the local directions of electric fields, i.e., the driving force for electrophoresis-mediated transport of charged molecules during the pulse, in the extracellular domain were affected only in the vicinity of the permeabilized regions [36]. For cells in clusters, transport is limited due to the restriction of access of the molecule under study to the permeabilized membrane parts due to the close contact between cell surfaces and the extracellular matrix [53]. 3D numerical models can give access to the electric field distribution in tissues and show that along electropermeabilization a nonlinear behavior of the conductance during the field pulse is suggested to explain the experimental observations [54]. Electric field distribution inside tissue was experimentally approached *ex vivo* on liver tissue using magnetic resonance electrical impedance tomography, telling that major changes of tissue electrical conductivity were only detected in the part of the tissue where the highest electric field was present [55]. The extracellular matrix will indeed prevent the transfer of macromolecules [56, 57].

Transport associated to electropermeabilization is therefore complex and difficult to predict in tissue being indeed controlled by the tissue physiology.

6 Membrane Electropermeabilization: Structural Aspects

It is the post-pulse state that is relevant of most effects dealing with delivery. Its structural organization remains unclear. Electropermeabilization alters cell plasma membrane structure and dynamics. Very few direct experimental investigations have been performed on the structural or dynamical organization of the electropermeabilized membrane at the single cell level. Digitized video microscopy proved experimentally that the induced potential difference is indeed position dependent and is controlled by the membrane properties [58]. The increase in membrane conductance associated to electropermeabilization is therefore affecting only a limited part of the cell membrane. The cell surface organization is heterogeneous as soon as permeabilization is induced. Most observations at the single cell level under the video microscope

show that permeabilization is homogeneous on the part of the cell membrane (cap) which is altered [43, 45, 59].

Electron microscopy studies showed that very short-lived cracks were present on the red blood cell surface, but they disappeared before resealing started [60]. Osmotic swelling under hypo-osmolar conditions brings a post-pulse formation of craters on red blood cell surfaces that are illustrative of an irreversible process [61]. Freeze-fracturing analysis of pulsed melanoma illustrated defects in the dynamical assembly of lipids and proteins with a clustering of intramembrane proteins [62]. Villi and blebs were observed on the electropermeabilized cell surface in a post-pulse process [63]. ^{31}P NMR spectroscopy showed that a tilt of the orientation of the phospholipid polar head region was present in the electropermeabilized state of the membrane [64, 65].

Resealing is a complex slow process with cells where membranes remained leaky during several minutes. An interesting observation on pure lipid assemblies was that these “transient defects,” induced by the field pulse, could be stabilized for a few seconds when a hydraulic stress is applied during and after the pulse [66]. A physical strain could therefore control the resealing of the electropermeabilized cell membrane.

Drugs or physical treatments affecting the organization of the cytoskeleton were observed to alter the kinetic of resealing [67, 68]. The resealing process was strongly temperature dependent: a permeabilized state was detected during several hours on viable cells kept at 4 °C [64]. Resealing was strongly affected in starved cells with a resulting high loss in viability [69]. These last experimental observations were strong evidences, suggesting that cell membrane electropermeabilization was more than just a structural alteration of the lipid matrix but affected the cell behavior. Again one should keep in mind that a cell is an open system from a thermodynamical point of view.

Cell membrane disruption, whatever its cause, must be rapidly repaired to preserve cell viability. This was observed in most approaches to occur through an active and complex dynamical modification where endomembranes, delivered through Ca^{2+} -triggered endocytosis, are actors of the resealing process in cells [70].

This was indeed experimentally observed in prediction with a theoretical prediction [71] by approaches at the cellular and lipid bilayer levels [72–74].

Another evidence of a cellular response during the resealing process was the production of reactive oxygen species along the permeabilized part of the cell surface. This was present only during the resealing process [75, 76]. This defense mechanism plays a negative role in cell survival and can be hindered by the use of ROS scavengers [77, 78].

7 Membrane Electropermeabilization: A Thermodynamical Description

The physical chemical concepts of membrane electropermeabilization describe the chemical free energy changes of the membrane organization due to the interaction with the transmembrane field [79]. An electropermeabilized membrane was tentatively described based on the stability of a planar lipid bilayer. It was first suggested that the transient compression of the entire membrane by the electric field pulse might cause mechanical collapse leading to membrane rupture in a destructive way. A dielectric breakdown was present that could not explain why the permeabilization was reversible [80]. One model considered the membrane as a viscoelastic fluid which might rupture due to the electric stress [81]. Another description took into account that lipids were not in fixed positions in a membrane but were able to move, giving rise to the occurrence of the so-called hydrophobic pores [82]. When a voltage was applied, it increased the energy of the membrane that can increase the size of these defects up to a transition to “hydrophilic pores” where the free diffusion took place [83–86]. Reversibility should occur after the pulse due to the thermodynamical instability of such “pores.” But such “pores” had never been experimentally observed, and they could not explain the stabilizing properties of the cytoskeleton as they were proposed to be built from lipids.

One open question dealing with the electric field-induced “pores” is their physical structure. In the early model [83], they were assumed to be cylindrical and perpendicular to the membrane surface (assumed to be a neutral lipid bilayer). In the further development, they were proposed to be toroidal (large openings at the surface and a narrow neck in the middle), their edge being covered by the polar heads [86]. This geometry was associated with an energy costly packing of the fatty acid chains. Another approximation was the description of a symmetrical distribution of the polar heads while they are submitted to electric forces inducing erected configuration on one side of the membrane and a flattened one on the other. Most models are giving the conclusion that most of the “pores” are about 1 nm in diameter. This is the size of a phosphatidylcholine, the major component of the plasma membrane. It was recently proposed a channel with a more irregular cross section [87]. The conclusion is that one should take into account the high lipid dynamic and their consequences on the subsequent stages resulting from the field pulse application. The other problem is the interaction of the ions and molecules with the transmembrane pathway and their entropic cost. The use of the more relevant model with the irregular cross section proposed in [87] does not allow the use of the thermodynamical description present in the “pore” theory, where only a very simple (unrealistic) geometry is proposed.

A key feature of a membrane is that it is a fluctuating assembly where mismatches were present. Theoretical descriptions of the phase transition processes showed that such defects were prone to support the diffusion of charged species across the bilayer [88–90]. It was then suggested that electroporabilization results from the induction of local reorganizations which mismatch with native structural membrane organization would support the transmembrane flow [91, 92]. Thermal fluctuations played a role in transient defects formation [93]. If the effect of a transmembrane electric field was included under the assumption, water can be present in the defect (to explain the membrane conductance), i.e., changing the membrane capacitance, the energy term appears as dependent on the square of the defect radii and of the TMP [94]. The model suggests that under appropriate conditions, pore growth was controlled and that when the external field was removed, the membrane returned to a stable equilibrium.

Another model for electroporabilization of pure lipid assemblies based on the Smoluchowski equation predicted a rapid increase in 1 nm wide “pore” density within the first microseconds of the pulse, a saturation of TMP owing to increased conduction through the “pores,” a few pore coalescence, and a recovery time of 20 s (much longer than the experimental observations) [95]. In the theories outlined above, the field was simply a source of energy. A quite different approach considered the total stress generated in the membrane by the transmembrane field [29]. The application of the external electric field reduced the steady-state energy of the membrane and increased the area per molecule. This model offered a quite different description of the role of the TMP, where the transverse field induces a reduction of the lateral tension in the interfacial region of the membrane, i.e., weaker interactions between the phospholipid molecules at the membrane/water interface. Defect formation resulted from a competition between the external field and electrostatic structural forces at the interface.

All these approaches were obtained under the simplifying assumptions that a membrane was a lipid bilayer but with no description at the molecular level. They are more relevant of soft matter physics than from life science.

8 Electroporabilization In Silico

Atomic-scale molecular dynamics simulations were used to address ion transport through transient water pores in phospholipid membranes (but only during a few ns due to the cost in computing times). A good introduction to the basic hypothesis can be found in [96]. The predictions can be compared to the experimental data when possible. This is the case in electroporabilization with very

short strong pulses (nsPEF). But molecular dynamics simulations are computationally very time consuming. The prediction is obtained only along a few ns.

Another approach is to use coarse-grained interaction sites where four to five orders of speedup can be obtained. Coarse graining (CG) in molecular dynamics means that the details of a model are decreased by replacing them with the interaction at a coarser level. An average of four adjacent atoms is replaced by one pseudo-atom, called a coarse particle. CG model is not only fast but also considered as accurate in its predictions. Lipid area per headgroup, atom density distributions, bilayer bending modulus, and line tension can all be reproduced as fairly as with atomistic force fields. Until very recently, one limit was in the description of water where the dipolar character was lacking. This was partly corrected with a polarizable model [97]. This is a very important point in the description of field pulse effect. Water dipoles are very sensitive to the external field and its associated torque on the membrane organization and increase the probability of formation of water defects in the membrane interior and also stabilize existing defects. Therefore, it appears that the water dipoles are crucial for electroporation [98–100].

One point to take into account with all simulations is that a number of arbitrary hypothesis are needed. In the case of electric pulses effects

1. A huge bulk field is applied (not relevant to the experimental conditions as several volts are supposed to be the TMP needed for electropermeabilization, while all experimental evaluations suggest values less than 1 V). Furthermore, the field in the bulk (that is most of the time the ratio between the voltage between the electrodes divided by the distance between them) is less than 5 kV/cm under classical conditions.
2. Or an ionic unbalance is assumed to induce a capacitive voltage due to the unbalance (again the concentration values are not relevant of the experiments except it is assumed that this may be the case due to local random fluctuations). This is supposed to mimic the membrane charging due the field driven accumulation of ions along the cell membrane surface.
3. The perturbation (voltage or ionic step) is a steep step function while experimentally we got a capacitive membrane charging. A drop in the induced TMP is present after a few ns, suggesting that a conductive state is present preventing the creation of a high TMP, always assumed to be needed for the *in silico* creation of defects (so-called pores) in the lipid bilayer [23]. Only in one report a slowly increasing field (1.5 ns) was used but with CG where the polarization of water was not present [101].

4. Only a very limited part of the cell surface is taken into account (less than 100 lipids and 20 ions), and it is assumed to be flat (while on a larger scale the cell surface is highly curved).
5. The field is supposed to be applied perpendicular to the “membrane” while it is not what is expected from the description in electrical engineering as long as the membrane is a pure dielectric.

In the case of a pure fluid phospholipid (POPC), the bilayer undergoes a drastic change in terms of its molecular structure, whatever the perturbation method, i.e., when applying an electric field to MD bilayer setups [102–106] or with the charge imbalance method [96, 107–109]. The simulations showed, within a few nanoseconds, water fingers started protruding from both sides of the membrane, until a water wire connected the two baths. One should take into account that with a slower rising time of the field pulse, an asymmetric formation was obtained when using CG simulation. The sides of these water links are pathways for movement of lipid headgroups, thus forming a hydrophilic pore, but with a geometry far away from the toroidal organization. The sizes of the created hydrophilic defects (approximated as diameter of 2 nm) can be large enough to enable conduction of Na^+ and Cl^- ions from one side of the bilayer to the other, annihilating the electric stress when imposed by the ionic imbalance. As the initial charge imbalance decreases, the associated TMP decreases. When the latter reached values of a few hundred millivolts, the ionic transmembrane conductance disappears, and the water within the membrane moves back to the external baths, but bilayer alterations remain present for time spans exceeding 10 ns [110]. The reversibility simulation is even faster when an electric stress is used to induce the perturbation [102].

The formation of a water defect was observed induced by the transmembrane ionic charge imbalance, and a resulting TMP. The resulting transport of ions through the pore discharges the imbalance and makes the water file metastable, leading eventually to its sealing [107].

The likelihood of water defects formation appeared to be increased by local membrane defects involving lipid headgroups [104]. Field-directed rotation of the head group dipoles in the plane of the forced water channel, in combination with water dipole orientation (antiparallel to the field) and solvation repulsions at the aqueous–lipid interface, built the coordinated ensemble of electroporabilization events [99, 100].

Coarse-grained (CG) simulations using a polarizable coarse-grained water model, i.e., where water orientation is affected by the field, were compared to atomistically detailed models but on a longer time. The lipid bilayer reorganization could be observed with smaller TMP (1.5–3.5 V). Again water-filled pathways are formed.

The defect formation time, however, increases with decreasing field strength and may about a microsecond in the case of a system at 1.5 V. No water defect formation is observed on a microsecond time scale at 1 V. Water-filled defects disappear as soon as the TMP is less than 0.2 V.

If the charging time of the TMP is supposed to be slow (1.5 ns), defects are induced within 5–6 ns, but the formation starts from the anodic side. This asymmetry was not predicted on other MD simulations with a single lipid model.

For PC lipids in the liquid crystal state, water wires are induced with a movement of the lipids. They are short lived and disappear as soon as the field is released. Switching off the external transmembrane potential for few nanoseconds brought a complete reconstitution of the bilayer [102].

The lifetime is around 50 ns with the ionic unbalance using CG [97] where the magnitude of the TMP was decreasing more slowly. Nevertheless, they were observed to disappear when the imposed TMP was less than 0.3 V. When the MD simulation was sophisticated by adding a second step where a TMP of reduced amplitude (but nevertheless rather large) was present after the permeabilizing pulse, the water wires were observed to remain present but with no size expansion [111, 112].

Lipid in the gel state such as DPPC at 300 K was described to obey a different behavior. Indeed if it is taken into account that a local tension at the water–lipid interface is associated to the field pulse, the process is faster and may remain present when the field is decreased after the high-voltage pulse [113].

While similar behaviors were observed with pure fluid single lipid bilayers whatever the simulation methodology, these *in silico* conclusions are relevant of soft matter studies. Biological membranes are more complex assemblies where different phospholipids are mixed with proteins. Different behaviors were reported on more complex systems supposed to be closer to biological membranes.

A simple chemical modification of the fatty acid chains (oxidation of double bonds) facilitates the formation of water wires [114].

Phase behaviors of lamellar DOPE lipid membranes in water under a uniform external electric field bring different conclusions. Defects, deformation, and fusion of membranes were induced, and water/membrane interfaces are tilted to be aligned to the electric field. The membranes fuse themselves with neighbors, leading to a complex cubic structure, where three-dimensional connections are present between water and membranes [115]. Coarse-grained simulation on PE phospholipids brought the same conclusion of a phase transition from the lamellar structure to the inverted cylinder structure. The bulk applied electric field 10 ns pulse appears as a driving force for such phase transition, but the inverted cylinder phase was observed to remain present during the 50 ns following

the pulse, suggesting the occurrence of an energy barrier preventing the return to the lamellar phase [116].

Charged lipids are present in biological membranes. Heterogeneous bilayers containing mixtures of phospholipids with zwitterionic and anionic headgroups can form the water wires under MD with a strong control by the field strength. Only under very stringent conditions the charged lipid can flip across the bilayer during the simulation [117, 118]. Again the simulation conditions are far away from the experimental ones.

Phosphatidylserine is known to have a high affinity for calcium ions. The resulting effects of calcium ions on the destabilization of heterogeneous lipid bilayers were simulated by MD. Calcium ions and phosphatidylserine increase water pathway creation time and decrease pore annihilation time [119, 120].

Phospholipid distributions are known to be different in the two sides of a biological membrane.

Asymmetric assemblies (E. coli outer membrane) were simulated. Under those conditions, the water wires are again created, but they appear only on one layer (the most fluid one) [121].

Cholesterol prevents the creation of the water wires except with very strong field. This is under the control of the percentage of cholesterol [122].

On a bilayer containing a peptide nanotube channel (formed by eight cyclo[(L-Trp-D-Leu)3-L-Gln-D-Leu]), no membrane alteration is detected close to the channel, which indicates that the interactions of the peptide with the nearby lipids stabilize the bilayer. The lipid molecules located nearby the peptide are known to be strongly hydrogen bonded to the peptide. It can be therefore predicted that at high membrane protein concentrations (the case in biological membranes), the TMP necessary to rupture these hydrogen bonds should be much larger [102]. Gramicidin was found to stabilize the membrane even at large distances (up to 3 nm from the peptide) [123]. An alamethicin POPC was simulated under MD at 0.5 V/nm. High fields are predicted to affect directly the transmembrane proteins, leaving the region between the proteins unaffected [124].

Transmembrane peptides prevent the creation of water wires. They can be affected directly by the field. For example, alpha-hemolysin dramatically prevents membrane alteration, and the TMP shows a fourfold increase as compared to a pure lipid bilayer [125]. Indeed it was reported that transmembrane proteins may be directly affected by the electric pulse [126–128].

As with other parts of the basic investigations of electropemabilization, one should conclude on our present lack of knowledge to give an accurate description “in silico” of the processes taking place along biological cell membrane electropemabilization even with nsPEF. More works remain needed that should take advantage of the improvement in the computing powers.

9 Conclusion

Electropermeabilization of cell membranes appears not as punching holes in a lipid layer (the so-called “electroporation” hypothesis). Theoretical modeling and experimental data on black lipid membranes suggest the creation of aqueous pathways, a bioelectrochemical process. But this can be valid for pure single lipid model bilayer. Other descriptions suggest a more complex process where mostly the membrane–solution interface is altered. The recovery (resealing) is dependent on the cell metabolism. This biological aspect must be taken into account in its use in *in vivo* drug delivery. There is still a need of basic research for the investigations of the structural membrane alterations supporting the transport of charged molecules across the biological membranes. Most present conclusions are relevant of soft matter physics but do not take into account the complexity of living systems from a structural as well as from a thermodynamical point of view. The development of this methodology for clinical applications is very promising (electrochemotherapy, gene therapy, DNA vaccines, hybridoma production), but one cannot neglect the use for biotechnology (food processing) and environment (pathogen eradication). One open further question is the difference that may be present between classical electropermeabilization (as described in this chapter) and the new nanosecond electropulsation where very high fields are applied on a very short (ns or even ps) duration.

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Electroporation Protocols

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