Chapter 4
Cell Membrane Electropulsation: Chemical Analysis of Cell Membrane Modifications and Associated Transport Mechanisms

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Abstract The transport of substances across the cell membrane is complex because the main physiological role of the membrane is the control of the substances that would enter or exit the cells. Life would not be possible without this control. Cell electropulsation corresponds to the delivery of electric pulses to the cells and comprises cell electroporation and cell electropermeabilization. Cell electropulsation allows for the transport of non-permeant molecules across the membrane, bypassing the physiological limitations. In this chapter we discuss the changes occurring in the cell membrane during electroporation or electropermeabilization as they allow to understand which molecules can be transported as well as when and how their transport can occur. Electrophoretic or diffusive transports across the cell membrane can be distinguished. This understanding has a clear impact on the choice of the electrical parameters to be applied to the cells as well as on other aspects of the experimental protocols that have to be set to load the cells with non-permeant molecules.

4.1 Introduction

The cell membrane, also termed plasma membrane, highly regulates all the exchanges between the outside and the inside of the cell. In particular, the cell membrane is a barrier that prevents the unregulated penetration/leakage of molecules of vital importance for the cell physiology, such as all the substrates/products of the cell metabolism, ions, sugars, or amino acids. None of these molecules can cross the

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J. Kulbacka, S. Satkauskas (eds.), *Transport Across Natural and Modified Biological Membranes and its Implications in Physiology and Therapy*,
Advances in Anatomy, Embryology and Cell Biology 227,
DOI 10.1007/978-3-319-56895-9_4

cell membrane by free diffusion. Other large hydrophilic molecules are also unable to cross the membrane by diffusion. These molecules and the small hydrophilic molecules that are not transported through channels or pumps are termed "non-permeant" molecules (Silve et al. 2012a); they can only reach certain cell compartments (but neither the cytosol nor the nucleosol) by endocytosis/exocytosis.

To allow the uptake of non-permeant molecules, cell membrane perturbations must be initiated that will transiently rupture the membrane impermeability. One of the most popular and practical way to reversibly permeabilize the cell membrane consists in the application of adequate electric pulses. The term electroporation was first introduced by Neumann and Katchalsky in 1972. The modelization of the phenomenon began almost as early and started with 1D approximations of the membrane (Litster 1975). The initial basis of analytical models was the Schwan equation:

$$\Delta\Psi = 3/2.E.R.\cos(\theta)$$

(the transmembrane potential $\Delta\Psi$ at a given point on the membrane is equal to the electric field amplitude E times the radius of the cell R times the cosine of the angular coordinate θ).

This equation implies that polarization in the cells occurs at the poles according to the electric field orientation and thus will in priority induce an electrical breakdown of the membrane in these regions.

There are two main mathematical approaches to model the pore formation: either static or dynamic. Older models focused on the static approach as in Pastushenko and Chizmadzhev (1982). The other approach, the dynamic one, considers the temporal evolution of the pore density (Krassowska and Filev 2007). This model links the number of pores to the voltage applied and then evaluates the evolution of the pore radii. The flaw of this model is that it is highly dependent on the mesh size. Using a fine mesh gives different results from a coarser one. Indeed, with the Krassowska model, small changes in initial non-measurable parameters can result in very different outcomes (Poignard et al. 2016). This can be a consequence of the fact that many initial variables are only approximations, because measuring them, for example, the conductivity and permittivity of the membrane or the cytosol, is possible (Wang et al. 2017) but difficult. Indeed, the cytosol is not just a solution of proteins and other biological compounds since the cytoskeleton and the vesicles occupy a large fraction of the cytoplasmic volume. To address this problem, a new model (the Leguèbe model) has been developed which relies on a lower number of variables. Moreover it considers two steps in the pulsing process: the generation of defects (possibly pores) at the poles of the cell membrane, followed by the diffusion of these defects in the cell membrane after the pulses delivery (Leguèbe et al. 2014). The Leguèbe model suggests a two-step approach of electropulsation. The membrane conductance fluctuates between the conductance at rest, the conductance during the pulse for a fully permeabilized membrane, and the conductance after the pulse which reflects the long-term effect of electroporation, also termed electropermeabilization.

Molecular dynamics (MD) have proven to be helpful to understand the interaction of cells with electric pulses. As far as the electroporation is concerned, MD allow for the study of the membrane structure at a nanoscale level. This computational method enables the simulation of the evolution of the atoms composing the membrane and its surroundings during up to tens of nanoseconds. If the force corresponding to the simulated electric field is high enough, pore generation can occur during the computational time. The process of pore generation is stochastic and simulations often require a high field amplitude. Since each individual molecule is considered in the calculations of the simulation, the region of interest is limited by the number of elements that the computer is able to handle simultaneously. Therefore, there are constraints in both the size of the sample considered and the duration of the simulation in order to avoid that running MD simulations are too computationally heavy. In particular, the nanosecond time range of this modeling is limited compared to the time during which the cell remains permeabilized. Nevertheless, in the case of nanosecond pulses, the predictions of the MD have been experimentally validated (Breton et al. 2012). More details of the results provided by these numerical approaches are reported in Chap. 1 of this volume.

Another relevant issue concerns the fact that the cell membrane is not the only membrane in the cell that may undergo modifications when the cell is submitted to an electric pulse. If nanosecond pulses are used, the membrane of internal organelles can also be porated (Schoenbach et al. 2001; de Menorval et al. 2016). Such an electroporation of the internal organelles of the cell may allow exchanges of molecules between different internal compartments of the cell, namely, between the cytosol and the endoplasmic reticulum or between the cytosol and the mitochondria. Therefore, after exposure to electric pulses, the complexity of the possible transports of molecules must be taken into account.

A further issue is that there is no consensus up to now about the term "electroporation." Electroporation refers to the generation of pores via pulsed electric fields (PEFs), while the term "electropermeabilization" refers to the generation of defects by PEFs. It is sometimes considered a more suitable term because even after the pore resealing, the cell can remain in a permeabilized state for periods of time that are orders of magnitude longer than the pulse duration. Recently the term "electropulsation" has been proposed to describe the action of submitting cells to electric fields.

In this chapter, we present recent findings that have improved the understanding of the interaction of electric pulses with cell membranes, and we address the mechanisms that may underlie the transport of molecules either during or after the pulse delivery.

4.2 Recent Experimental Data on Chemical Modifications of Membranes Submitted to Electric Pulses

It has been known for a long time that the application of electric pulses to cells induced the appearance of reactive oxygen species (ROS) in the cell medium (Bonnafous et al. 1999). It has also been reported that the addition of antioxidants

could reduce the death rate of cells provoked by pulses (Gabriel and Teissié 1994). Finally, some groups used indirect measurements of phospholipid oxidation by absorbance studies to show that pulses could peroxidize membrane phospholipids (Benov et al. 1994). More recently, mass spectrometry has been used to directly investigate the chemical processes taking place during or after electropulsation at the level of membranes. The study was conducted on a well-known membrane model, the giant unilamellar vesicles (GUVs). These vesicles were constituted of either saturated or unsaturated phospholipids since these lipids are both present in all cell membranes. The mass spectra obtained clearly showed that electric pulses can induce the oxidation of the unsaturated membrane phospholipids. Whatever the duration of the pulses (millisecond, microsecond, or nanosecond pulses), unsaturated lipids were oxidized, while saturated lipids remained intact. The mass spectra clearly presented a classical oxidation pattern showing peaks corresponding to the addition of oxygen atoms as well as peaks of low masses corresponding to lipids that underwent chain breaks after oxidation. Mechanistic studies conducted on the same GUV model showed that the presence of light or dioxygen could be cofactors in the oxidation (M. Breton et al., submitted). More importantly, the concentration of ROS in the solution increased after pulsing only if unsaturated vesicles were present in the solution. Therefore, the electric pulses should not directly cause ROS generation and lipid peroxidation. It seems more likely that the main effect of the electric pulses is that they facilitate the peroxidation induced by the ROS already present in the solution before pulsing. Finally, the effects of the pulse parameters on the level of lipid oxidation follow the same trend as the effects of the pulse parameters on the level of cell permeability. Indeed, if the duration, the number, or the voltage of the pulses is increased, the level of oxidation is increased. This is the case for all kinds of pulses (millipulses, micropulses, nanopulses).

4.3 Cell Electropulsation Model

Our group proposed a new model which describes both the immediate effects of the pulses during their application to the cells as well as the chemical and physical consequences of the pulses on the cell membranes. One of the bases of this model is that when a very high electric field is applied on a membrane, non-permeant molecules such as water or ROS can enter the membrane first as water fingers and later as water pores (Tarek 2005). This model contains two steps: electroporation followed by electropermeabilization. Electroporation happens during and shortly after the application of the pulses. During this step, pores form, and therefore water and ROS can enter the lipophilic area of the membrane where the oxidable lipid chains are. The oxidation of the membrane phospholipids can take place. The membrane is then highly conductive and completely permeable. When the pulse is finished, the pores rapidly close in tens of nanoseconds. The electropermeabilization step initiates. The conductivity of the membrane sharply decreases, while the decrease of the membrane permeability is more progressive. However, since some oxidized lipids remain

in the membrane until they are removed by membrane repair mechanisms, the conductivity and permeability of the membrane remain slightly higher than their basal level. During the electropermeabilization step, the membrane permeability will depend on the density of the oxidized lipids in the membrane. Right after the end of the pulse, the oxidized lipids are concentrated in the porated area. The density of oxidized lipids is high and the membrane is very permeable. Gradually, the oxidized lipids will diffuse laterally in the membrane. The density of oxidized lipids will decrease, and therefore the permeability of the membrane will decrease. A low level of membrane conductivity and permeability will remain until the complete removal of oxidized lipids from the membrane. Cells can renew their membrane lipids by the process of endocytosis/exocytosis which can last for several hours (Ullery et al. 2015). The membrane will hence remain slightly permeable and conductive for a few hours. This model is valid for all kinds of pulses ranging from nanosecond to millisecond duration. It presents different kinetics which are consistent with all the previous experimental studies that did not fit with the theories of electroporation or electropermeabilization. According to this model, it seems important to take into account both the physical and the chemical consequences of cell pulsation when studying the transport of molecules inside the cells by electrotransfer.

4.4 Study of the Interfacial Water Around Biological Samples Submitted to Electric Pulses

Many groups have studied the water/lipid interface based on vibrational spectroscopy techniques (Bonn et al. 2010; Gruenbaum and Skinner 2011; Nagata and Mukamel 2010; Nihonyanagi et al. 2013). It has been reported that the interfacial water molecules are highly organized due to strong hydrogen bonds close to lipid head groups (Lopez et al. 2004) and to preferential water orientation pointing toward the lipids tails (Chen et al. 2010). CARS microscopy has been used to investigate the orientation of water in the lipid/water interfaces of multilamellar vesicles (Cheng et al. 2003). Teissie and colleagues suggested that this interfacial water could be considered as the first barrier to overcome in order to allow molecules to cross the plasma membrane (Teissie 2007). Molecular dynamics simulations have revealed that the application of an intense electric field on a lipid membrane disorganizes the interfacial water by creating aqueous pores into the membrane (Tarek 2005). Water molecules play a key role in the initiation and the stabilization of these aqueous pores (Tieleman 2004; Ziegler and Vernier 2008). Due to the dipole moment of water, the water molecules are mainly oriented along the electric field, which contributes to stabilization of these aqueous pores (Tokman et al. 2013). The lifetime of these aqueous pores is still under debate in the community (Dehez et al. 2014; Pavlin and Miklavčič 2008; Ziegler and Vernier 2008). Nevertheless, it is commonly admitted that the aqueous pores are initiated and collapse within nanoseconds or tens of nanoseconds, respectively, after the beginning and the end of the pulsed electric fields (Delemotte and Tarek 2012). Due to the nanosecond time scale

and the nanometer size scale of the electropores, obvious experimental proofs of the presence of the electropores created by the pulsed electric fields are still missing. In an experimental study, we focused on an indirect consequence of the delivery of pulsed electric fields on biological membranes by monitoring the interfacial water thanks to a unique Coherent anti-Stokes Raman scattering (CARS) microscope (Silve et al. 2012b). The illumination geometry specifically enhances the CARS signal at the interfaces. Thus, CARS spectra of water close to biological samples (DC-3F cells and GUVs) exposed or not to PEFs have been acquired. Two families of water, coined "interfacial water" and "interstitial water," are associated to two different vibrational wavenumber spectra bands. The 3000–3230 cm^{-1} and 3300–3450 cm^{-1} bands are known to be associated, respectively, to the interfacial water and the interstitial water (Gruenbaum and Skinner 2011). Our results show that the CARS intensity ratio between these two families is highly affected by the pulsed electric field delivery. The interstitial water becomes predominant in the water vibrational spectrum after the PEFs treatment. The differences in the spectra of GUVs before and after the pulsed electric field treatments are larger than those of the spectra of DC-3F cells. We associate this result with the absence of proteins in the membrane of GUVs. Indeed, the presence of proteins contributes to the reduction of the interfacial water/lipid signal. This study reports the first experimental proof of the effect of pulsed electric fields on the water and especially the water/lipid interface of biological samples. More experiments still have to be performed in order to improve the understanding of the underlying mechanisms of the electropermeabilization process such as the determination of a dose-effect relationship or the application of different types of electric pulses.

4.5 Biochemical Characterization of Live Cells Exposed to Pulsed Electric Fields

As reported in the previous section, mass spectrometry studies have demonstrated the chemical modification of phospholipids induced by the delivery of PEFs on GUVs. In order to assess the effect of pulsed electric fields on the biochemical composition of cells, confocal Raman microspectroscopy was used to acquire the Raman signature of live cells exposed or not to classical electric pulses parameters: 8 pulses, 100 μs duration, 1000 V/cm field magnitude, and 1 Hz repetition rate. Confocal Raman microspectroscopy is a label-free and noninvasive optical technique which provides detailed information about the molecular composition of the samples, especially about the proteins, lipids, and DNA contents of the cells. This instrumentation technique has reached a mature state since the discovery in 1928 of the physical underlying mechanism by Sir Raman (Raman 1928). It has been commonly used to characterize cells (Downes et al. 2011) and drug delivery systems (Smith et al. 2015) or to perform biomedical diagnoses (Kong et al. 2015). Major modifications in the Raman signatures of live cells were noticed when comparing the pulsed group to the control group when performing the acquisition in a cytoplasmic area (Fig. 4.1).

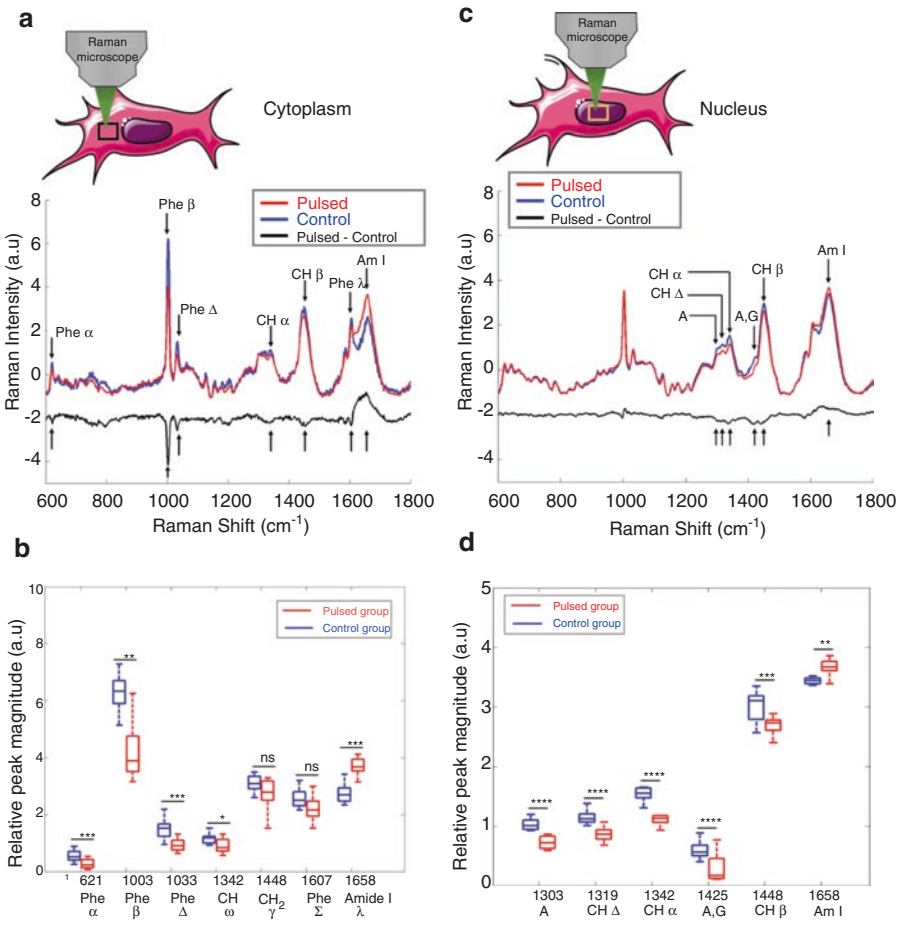

Fig. 4.1 Effect of the delivery of electric pulses on the Raman signatures of live cells at the cytoplasm (**a**, **b**) and the nucleus (**c**, **d**) locations. (**a**) Mean Raman signatures of control and pulsed cells acquired at the cytoplasm. The differential spectrum (pulsed minus control) is displayed with a vertical offset for more clarity. (**b**) Statistical analysis of the magnitude of seven critical bands. (**c**) Mean Raman signatures of control and pulsed cells acquired at the nucleus. The differential spectrum (pulsed minus control) is displayed with a vertical offset for more clarity. (**d**) Statistical analysis of the magnitude of six critical bands. The electric pulses parameters were fixed to eight pulses, 100 µs duration, 1000 V/cm field magnitude, and 1 Hz repetition rate. Statistics were conducted with a Student's t-test (*NS* non-statistically significant, *: p-value < 5%, **: p-value < 1%, ***: p-value < 0.1%, ****: p-value < 0.01%) (Adapted from Azan et al. (2017))

Especially, the vibrational modes of phenylalanine (621, 1003, 1033, and 1607 cm^{-1}), amide I (1658 cm^{-1}), and lipids (1448 cm^{-1}) were highly impacted by the delivery of pulsed electric fields (Fig. 4.1a, b) (Azan et al. 2017). Phenylalanine is a nonpolar and hydrophobic amino acid present in many transmembrane domains (Unterreitmeier et al. 2007). It has been demonstrated that the 1658 cm^{-1} Raman band is a biomarker of the secondary structure of proteins (Maiti et al. 2004). Our results experimentally

confirm for the first time the effects of electric pulses on proteins (Azan et al. 2017) that were predicted by a numerical model showing the unfolding of proteins under an intense electric field (Cournia et al. 2015). When the Raman signatures of pulsed and non-pulsed cells were acquired at the nucleus location, vibrational models associated to DNA were also impacted by the pulsed electric fields (Fig. 4.1c, d). This result is in agreement with previous studies reporting that DNA is sensitive to the ROS generated in the culture medium by the delivery of electric pulses (Gabriel and Teissié 1994; Pakhomova et al. 2012; Wiseman and Halliwell 1996).

4.6 Transport Phenomena

Under the context of the theories and facts developed in the previous sections of this chapter, it is essential to distinguish the various transport possibilities that may occur across the membrane at the various steps of the electropulsation process. Here we will consider transport mechanisms under electropulsation conditions that preserve cell viability. Indeed, in the case of irreversible electroporation, when the membrane cannot fully recover its initial impermeability (one of the definitions of the "irreversible electroporation"), all molecules, sooner or later, will be able to freely cross the cell membrane.

Under normal conditions, in the absence of any physical or chemical perturbation, the membrane is impermeable to the non-permeant molecules, that is, to all the molecules that are not actively transported across the membrane and that cannot diffuse through the membrane (mainly through the lipid bilayer) because of their size and physicochemical characteristics. All the metabolically important small molecules (like sugars, amino acids, dipeptides, iron, osmotically important ions such as sodium or potassium, second messengers like calcium ions) do not freely cross the membrane: their transport is highly regulated. No large molecule can cross the plasma membrane, except the molecules that can generate pores in the membrane or affect the membrane structure (like the chains of the vegetal toxins responsible for the internalization of the catalytic chains of the same toxins or like the cell-penetrating peptides such as those derived from TAT or melittin) (Salomone et al. 2014).

When an electric pulse is delivered to cells, and as soon as the cell enters the first step of the membrane destabilization caused by the electric pulse, that is, the "electroporation" step, non-permeant molecules can start crossing the membrane (Fig. 4.2). They can cross either by diffusion through the aqueous pores created in the membrane or by electrophoresis. Indeed, because of the presence of the electric field, charged molecules will be efficiently pulled through the membrane. For highly charged molecules or, better to say, for molecules possessing a high ratio of charges per mass, this transport can be very efficient even for ultrashort pulses as demonstrated by Breton et al. (2012). In 10 ns, one pulse of 10 ns and 5.8 kV/cm was sufficient to permeabilize the membrane of GUVs and to introduce siRNAs inside the GUVs. Limited diffusive transport also occurs, but at a low level, for several reasons.

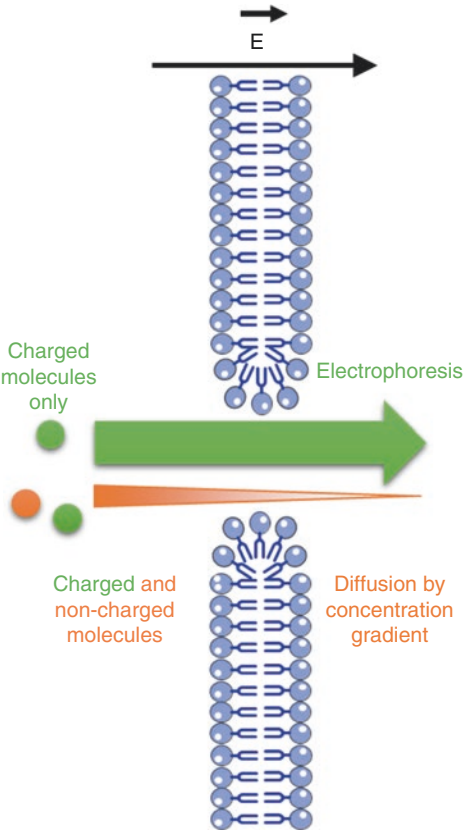

Fig. 4.2 Scheme presenting the transport of molecules during the electroporation step. The electric field is present. The duration of this step is directly related to the duration of the electric pulse, and so it ranges from a few nanoseconds to milliseconds. Charged molecules are mostly pulled through the membrane by electrophoresis. Limited diffusive transport also occurs for both charged and non-charged molecules

The first one is that the duration of the pulses, and therefore the half-life of the electropores, is always very short to preserve the cell viability. The second one is that this diffusion will only occur through the electropores, that is, through a small area of the cell surface, which will restrict the flux of molecules across the membrane. The third one is that unless there is a huge concentration gradient between the outside and the inside of the cell, the number of molecules transported through a small area during an extremely short time (Fick's law) will be very small and thus not easily detectable. Nevertheless, this transport exists and could be shown using GUVs prepared with a non-oxidable lipid, for example, the DSPC that is a fully saturated phospholipid. Because GUVs are just a phantom of a cell, they can be pulsed many times without the restrictions imposed by viability preservation. Because the lipids of these DSPC GUVs cannot be oxidized, even if pores form during the pulse delivery, the membrane recovers its full integrity when the pores close. DSPC GUVs prepared in 240 mM sucrose (sucrose inside the GUVs) and diluted in 260 mM glucose (glucose outside the GUVs) show a strong optical contrast. Two minutes after one single pulse, or a very limited number of pulses, no change in this contrast is observed (while GUVs prepared with an unsaturated oxidable lipid will already lose this

contrast, see next paragraph). However, after the delivery of a high number of long pulses (e.g., of 5 ms duration), a loss in contrast is detected: it reflects the exchange of glucose and sucrose across the electropores generated during the pulse delivery. The cumulated duration of the pulse and the large concentration gradients of the two sugars allow for a sufficient mass transport of the sugar molecules across the membrane, and therefore the contrast loss, even if sugars are neutral, non-charged molecules. Thus, not only the electrophoretic transport can occur through the pores during the pulses delivery but also the diffusive transport.

After the pulse, and after the rapid closure of the electropores, the cells remain permeabilized for some time, in the seconds to minutes range (Fig. 4.3). No electrophoretic transport driven by an external electric field can occur since the electric pulses have ended. The contribution of a transmembrane potential difference across the electropermeabilized membrane has been evoked by Dr. T. Vernier and his colleagues and is under study: the electric field across the membrane could indeed favor or not favor the passage of charged species, as a function of the sign of the charge(s) of these molecules. However, with respect to the "electroporation" step, the "electropermeabilized" state is characterized by the massive transport of charged or non-charged non-permeant molecules across the membrane. One of the main reasons is the duration of the "electropermeabilized" state: seconds or minutes, as already mentioned. These durations are several orders of magnitude longer than the

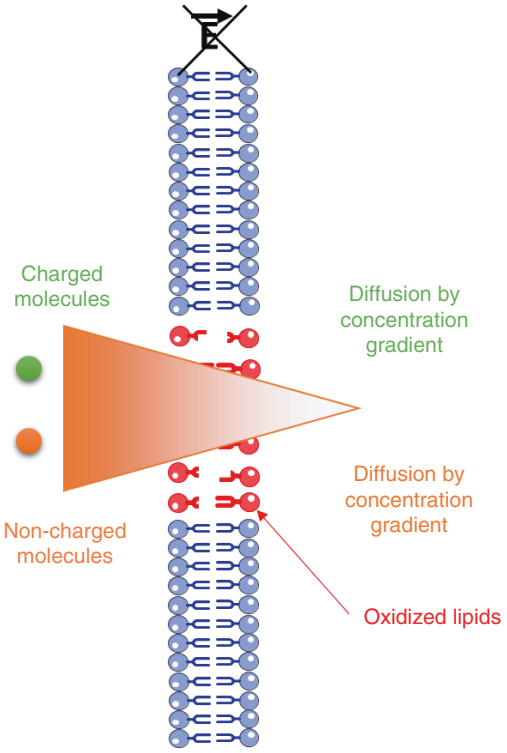

Fig. 4.3 Scheme presenting the transport of molecules during the electropermeabilization step. There is no electric field. This step has a long duration ranging from seconds to hours. The membrane is leaky because it contains oxidized phospholipids. Charged and non-charged molecules can be massively transported by diffusion if a concentration gradient is present

pulse duration, even in the case of the longest pulses, the msPEF. The duration of the permeabilized state is the consequence of the lipid oxidation, since it is well known that a membrane containing oxidized lipids (Wong-Ekkabut et al. 2007; Vernier et al. 2009; Rems et al. 2016) is a "leaky" membrane. The coefficient of diffusion through an oxidized membrane can be orders of magnitude larger than the coefficient of diffusion through a native, nonoxidized membrane (Rems et al. 2016). This explains why the optical contrast of a GUV prepared using an unsaturated lipid is lost 2 min after the delivery of a single pulse or very small number of pulses: sugars could cross the membrane during the 2 min, a time extremely long with respect to the pulse duration. This diffusive transport is, of course, not constant during the duration of the electropermeabilized state. There are probably two reasons why very large molecules have to be present in the pulsing medium at the time of the pulse delivery: (1) in the case of highly charged molecules, such as long nucleic acid that are too large to move through diffusive processes, to benefit from the electrophoretic transport and to at least initiate the contact with the membrane; (2) in the case of large non-charged molecules such as large proteins, to find regions with a very large density of oxidized lipids, therefore, to find regions with very large diffusive capacities. It is important to recall that DNA fragments of up to 150 kbp or proteins of 150 kDa, which were complete antibodies (Bobinnec et al. 1998), have been transported inside the cells by electropulsation. According to our model, and in particular according to the lateral diffusion of the lipids that will "dilute" the oxidized lipids generated in the areas where electropores were formed, the density of the modified lipids and hence the diffusion capabilities will progressively decrease, which will progressively restrict the diffusion of the molecules as a function of their size. The kinetics of such a progressive restriction in the molecule transport have been analyzed in the case of the anticancer drug bleomycin (Silve et al. 2012a). In another respect, the total number of molecules transported (independently of the external concentration and thus of the gradient of the concentration across the membrane) is directly related to the size of the molecules transported, under identical pulsation conditions and using the same cells to evaluate these transports (Mir 2008).

In conclusion, the properties of the molecules transport across the electropulsed membranes are in agreement with the membrane impermeability rupture model. During the electroporation phase, the electrophoretic transport of non-permeant molecules across the electropulsed membrane is more effective than the diffusive transport, while in the electropermeabilization phase, the diffusive transport will be the most efficient one.

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