Pore lifetimes in cell electroporation: Complex dark pores?

James C. Weaver\textsuperscript{1,*} and P. Thomas Vernier\textsuperscript{2}

\textsuperscript{1}Harvard-MIT Division of Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA; \textsuperscript{2}Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA, 23508, USA

*Corresponding author

We review some of the basic concepts and the possible pore structures associated with electroporation (EP) for times after electrical pulsing. We purposefully give only a short description of pore creation and subsequent evolution of pore populations, as these are adequately discussed in both reviews and original research reports. In contrast, post-pulse pore concepts have changed dramatically. For perspective we note that pores are not directly observed. Instead understanding of pores is based on inference from experiments and, increasingly, molecular dynamics (MD) simulations.

In the past decade concepts for post-pulse pores have changed significantly: The idea of pure lipidic transient pores (TPs) that exist for milliseconds or longer post-pulse has become inconsistent with MD results, which support TP lifetimes of only $\sim 100$ ns. A typical large TP number during cell EP pulsing is of order $10^6$. In twenty MD-based TP lifetimes ($2 \mu s$ total), the TP number plummets to $\sim 0.001$. In short, TPs vanish $2 \mu s$ after a pulse ends, and cannot account for post-pulse behavior such as large and relatively non-specific ionic and molecular transport. Instead, an early conjecture of complex pores (CPs) with both lipidic and other molecule should be taken seriously.

Indeed, in the past decade several experiments provide partial support for complex pores (CPs). Presently, CPs are “dark”, in the sense that while some CP functions are known, little is known about their structure(s). There may be a wide range of lifetimes and permeabilities, not yet revealed by experiments. Like cosmology’s dark matter, these unseen pores present us with an outstanding problem.

Early models for lipidic pores and their lifetimes

There are three major stages of electroporation (EP): (1) pore creation during pulsing, with a highly non-linear dependence on transmembrane voltage, $\Delta \phi_m$, (2) pore expansion/contraction, also affected by $\Delta \phi_m$, and (3) pore persistence and eventual destruction, usually considered for $\Delta \phi_m = 0$ (full depolarization), with post-pulse membrane resealing attributed to pore lifetimes.

Of these creation is reasonably well understood, including agreement between molecular dynamics (MD) and continuum models for transmembrane voltages and associated membrane fields where these very different methods overlap\textsuperscript{11}. Behavior during pulsing is only occasionally measured\textsuperscript{2,4} due to significant technical difficulties, so it receives little attention.
Most experiments focus on post-pulse conditions.

Post-pulse observations and measurements are common, typically carried out a short time after pulsing stops. Often membrane recovery is described in terms of a resealing process, and the associated times interpreted as lipidic pore lifetimes. These are often given as a single number for planar lipid bilayers, vesicles and for cells, with the latter our focus. In this case there are relatively few reported values, and these are in the range 0.01 to 100 s. Measured quantities include both electrical (resistance/conductance or whole cell clamp voltage) and chemical (e.g. transported amount of Ca$^{++}$, propidium$^{++}$ or calcein$^{-4}$). However, pores themselves are not directly observed.

Molecular dynamics results relevant to TP lifetimes

Behavior of lipidic pores within an aqueous environment (water or water plus some ions) is now relatively well studied. The impact of MD on understanding pore behavior, from creation to destruction, is a major accomplishment. MD has already changed what we think when EP and pores are considered in a predominantly lipid/aqueous environment. At the same time support for the traditional view of long-lived lipidic pores is weakening due to:
Figure 2: A MD pore energy landscape, based on Wohlert et al. 2006. This pore energy landscape has no barrier to pore destruction. This suggests that delays in pore destruction are kinetic. Once expanded, a pore must contract to reach the size at which it vanishes. Instead of a “cusp” that defines a barrier in the free energy plots (Fig. 1), the pore energy decreases monotonically with size, with no barrier as a point of “no return” is reached (the sudden change in downward slope as pore destruction commences). MD thus predicts a rapid pore destruction rate, and a correspondingly short TP lifetime, of order $\sim 100$ ns.

1. MD results often suggest/show that the interior interface of a TP is not fully covered with phospholipid head groups, so the probability of close opposing head groups that repel is becoming small. This repulsion is essential to the traditional model. Without significant repulsion the hypothetical destruction barrier for lipidic pores has lost its foundation.

2. MD results also show that once created, TPs vanish rapidly, typically within $\sim 100$ ns. This rapid loss is reported in many publications, consistent with a $\sim 100$ ns lifetime. Wohlert et al. explicitly shows that the pore energy landscape does not have a significant barrier to pore destruction, which suggests that delays in pore vanishing are essentially kinetic (once expanded, a pore must contract to reach the size at which it vanished). Instead of a “cusp” that defines a barrier in the free energy plots, the pore energy is sloping down slightly, with no barrier to surmount before pores vanish. This essentially guarantees a rapid pore destruction rate, and does not depend on...
how many time-dependent MD simulations are used.

Recent results from many MD papers show that pure lipid pores have $\sim 100$ ns lifetimes. This has dramatic consequences: If there are $10^6$ pores during a pulse, within about $2 \mu s$ the probable number of pores is $\sim 0.001$ (essentially zero). Important examples are the sequence of events that show these short lifetimes, an example of landscape with no barrier at all to pore destruction and an explicit demonstration that $\sim 100$ ns lifetimes are expected.

**Experimental support for long-lived pores**

Our argument that traditional TPs vanish within $\sim 2 \mu s$ after a well defined pulse end is only a beginning. The obvious question it raises is: What accounts for post-pulse behavior for times well beyond about $2 \mu s$? Our reply is that in addition to early qualitative conjectures, there are now several experiments that together strongly suggest that one or more pore-like entities exist. That there characteristics are not yet fully established is expected: This topic has received relatively little or prolonged attention. In recognition of the supporting evidence we briefly note some, with only a short comment regarding what the evidence is for each supporting experiment. This can range from qualitative to quantitative, with varying degrees of specificity. Below we briefly describe, in chronological order, publications that provide support for existence of one or more long-lived pores.

**Concepts, theory and experiments supporting complex pore-structures**

A qualitative conjecture in the early 1990s suggested that entry of non-lipidic molecules should increase the lifetime of a structure based on both the traditional lipidic TP and a partially-occluding inserted molecule.

Just about a decade ago an important experiment described the post-pulse recovery of the PM, reporting that the conductance returned to (within experimental noise) its initial value in $\sim 15 \text{ m}(900 \text{ s})$. We conservatively assume that 5 exponential lifetimes corresponds to essentially full recovery, and this yields a recovery (resealing) lifetime of $\sim 180 \text{ s}$. This is a very long lifetime compared to MD.

Subsequently experimental results for delayed development of plasma mammalian permeability were reported with measurement times running out to $1,800 \text{ s}$. The delayed permeability increases occurred at post-pulse times of order $100$ to multiples of $100 \text{ s}$ for a single $40 \mu s$ at various electric field strengths. Similar experiments with very different pulse conditions ($60 \text{ ns}$ pulse trains) reported similar delayed permeability increases.

Another pulse train experiment reports that more pores can be created following an inter-pulse spacing of $0.1 \text{ s}$ ($10 \text{ Hz}$ pulse rate). This means that the $0.1 \text{ s}$ interval allows significant recovery of pores, and most importantly, the corresponding decreased PM conductance. A decrease of pore number and total membrane conductance, $G_m$, means that new pores can be created by the next pulse, for the same strength-duration pulse. A specific example of this general expectation was recently published. Other types of experiments also suggest more than one type of pore exists.

Finally, a rather different type of experiment reports the important result that post-pulse there is evidence for a contribution of active transport. The conventional view is that the PM is fully
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depolarized, so that any transport is purely diffusion. This report shows otherwise, and provides further support for one or more types of long-lived permeability states that exist long after TPs have vanished. Provocative experiments describe results that explicitly suggest “long-lived nanopores”). This observation and associated characterization is important, even if not yet fully established in details. Even more recently an experimental study based on multiple pulses (“pulse trains”) provides evidence that some long-lived pores are involved, with their longevity related to the capability to create more pores after the inter-pulse interval of $\sim 10^{-2}$ s new pores are created.

Very recent experiments with single pulses of very large strengths and correspondingly short duration show that some type of pore-like structure persists, and is able to interact with well-known resting potential source to indicate that both diffusion and electrophoresis are involved in transporting test solutes through what must be some type of long-lived pores, well into post-pulse times. What do not survive are traditional, purely lipidic pores, and this realization suggests that we direct attention to complex pores, some of which have extremely long lifetimes.

Acknowledgments

This work was supported by AFOSR MURI grant FA9550-15-1-0517 on Nanoelectropulse-Induced Electromechanical Signaling and Control of Biological Systems, administered through Old Dominion University.

We thank J. V. Stern, E. Sözer, R. S. Son, K. C. Smith, O. N. Pakhomova, A. G. Pakhomov, T. R. Gowrishankar and A. E. Esser for stimulating discussions, and K. G. Weaver for computer support.

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