Effect of pulsed electric fields on biological cells: 
adding some pieces to the large puzzle

Aude Silve
Thanks to ...

Institute for Pulsed Power and Microwave Technology (IHM)
Who am I?

PhD
Effects of nanosecond pulsed electric field on living cells and tissues.

Post-doc
Measurement of TMV induced by nanosecond pulses by means of fluorescence probe
Introduction

Effect of External Electric Pulses on Biological Cells

*Cell membrane*

**Resting state**
*Impermeable membrane with very low conductivity*

**Anode**

**Cathode**

**Membrane Charging**
*=> high transmembrane voltage*

**Modification of membrane properties**

*Electroporation or Electropermeabilisation?*
**MilliPulse / MicroPulse**

- **Magnitude « A »**: 10 to 300 V/mm
- **Duration « t »**: 10 μs to 20 ms
- **Rising edge « τ »**: 1 to 20 μs

**Parameters**

**NanoPulse**

- **Magnitude « A »**: 1 to 30 kV/mm
- **Duration « t »**: 3 ns to 300 ns
- **Rising edge « τ »**: 500 ps to 10 ns

**Classical versus Nano**
Impact of Pulse parameters | 

Other determining parameters: 
- Number of pulses 
- Shape of pulses 
- Repetition rate 
- Type of cells (shape, size, ...) 
- Buffer 
- Temperature 
- etc. ...

Reversible and Irreversible

Field magnitude

E

100 V/mm

20 V/mm

No effect

Reversible permeabilisation

Irreversible permeabilisation

Thermal effects

100 μs 20 ms

Pulse duration

E

T
**Detecting changes of membrane’s permeability**

- Usually by studying diffusion of normally not permeant ions or molecules
  - Release of intracellular metabolites (e.g., ATP)
  - Release or uptake of fluorescent markers (e.g., PI, Lucifer Yellow, Calcein)
  - Uptake of biologically active molecules (e.g., cytotoxic drugs, DNA, antibodies)
Detecting changes of membrane’s permeability

- Usually by studying diffusion of normally not permeant ions or molecules
  - Release of intracellular metabolites (e.g., ATP)
  - Release or uptake of fluorescent markers (e.g., PI, Lucifer Yellow, Calcein)
  - Uptake of biologically active molecules (e.g., cytotoxic drugs, DNA, antibodies)

Detecting changes of membrane’s conductivity

- With electrical or opto-electrical measurements
  - Voltage clamp on lipid bilayers
  - Patch-clamp approach on single cells
  - Bio impedance methods in vivo or in biological tissues
  - Voltage sensitive dyes
Detecting changes of membrane’s permeability

-> Usually by studying diffusion of normally not permeant ions or molecules
- Release of intracellular metabolites (eg: ATP)
- Release or uptake of fluorescent markers (eg: PI, Lucifer Yellow, Calcein)
- Uptake of biologically active molecules (eg: cytotoxic drugs, DNA, antibodies)

Detecting changes of membrane’s conductivity

-> With electrical or opto-electrical measurements
- Voltage clamp on lipid bilayers
- Patch-clamp approach on single cells
- Bio impedance methods in vivo or in biological tissues
- Voltage sensitive dyes

Detecting the following physiological consequences

- Cell death, in vitro
- Tumor regression in vivo
Changes of membrane’s permeability
An example: bleomycin to detect reversible permeabilisation.

Detection of permeability

- Cell survival
- Mitotic death

Bleomycin
Detection of permeability

Even a single 10ns Pulse enables penetration of Bleomycin

Silve, Leray, Mir, Bioelectrochemistry, 2012
Injection: 1 to 2 M LPB cells (LPB: murine fibrosarcoma)

T0: treatment:
- Average tumor volume: 30 – 50 mm³
- Retro-orbital injection of Bleomycin (100 µg in 100 µl) or Physiological serum
- Application of electric pulses

Image: D-dot sensor output

Graph: Applied pulses computed from D-dot output
What is the life time of permeability?

- Pulse applied at Time t=0.
- Survival rates are normalized to the control submitted to the bleomycin only
- Bleomycin concentration of 30 nM.
- Viability assessed by cloning efficiency test
- DC3F cells

With this diagnostic: resealing seams to happen in a couple of minutes
Is there a maximum size of molecules that can be transported?

Many experiments such as studies of cells osmotic swelling after PEF suggest that membranes are permeable mostly to very small molecules. However, no size limit has been detected:

Larger molecules still penetrates

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Weight (Da)</th>
<th>( C_{\text{int}} ) (% of ( C_{\text{ext}} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lucifer Yellow</td>
<td>457</td>
<td>100</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>1 500</td>
<td>33</td>
</tr>
<tr>
<td>Oligonucleotide</td>
<td>12 000</td>
<td>10</td>
</tr>
<tr>
<td>Pokeweed Antiviral Toxin</td>
<td>30 000</td>
<td>1</td>
</tr>
<tr>
<td>Antibody</td>
<td>150 000</td>
<td>not quantified</td>
</tr>
</tbody>
</table>

Cells: DC3F 8 pulses: 100 µs, 140 V/mm
- PEF induce an increase of membranes permeability to normally non permeant molecules

- Small water soluble molecules can cross the permeable membrane easily but no absolute size limit of the molecule that can be transported could be detected

- After the pulse, a resealing can be observed

  Resealing time are in the order of seconds to minutes

  Resealing time depends on the pulses parameters

  Resealing time depends on temperature and on biological factors

  Recovery of membrane’s integrity is at least partially a biologically active process
Changes of membrane’s conductivity
Bioimpedance

Potato: a powerful biological sample

Typical Impedance Measurement

Extra-cellular medium (Re)
Membrane capacitive behaviour (Cm)
Intra-cellular medium (Ri)
**Normalized Impedance Drop**

\[
NID = \frac{R_{\text{real}}(Z_{100\text{Hz}}^{\text{perm}}) - R_{\text{real}}(Z_{400\text{kHz}}^{\text{perm}})}{R_{\text{real}}(Z_{100\text{Hz}}) - R_{\text{real}}(Z_{400\text{kHz}})}
\]

*NID* = 1 : no or very low increase of membrane’s conductivity

*NID* → 0 : high increase of membrane’s conductivity
Micropulses: impact of the repetition rate

Duration between two pulses

Repetition rate = \frac{1}{T}

Pulses: 100 µs, 80 V/mm

Silve, Guimerà Brunet, Al-Sakere, Ivorra, Mir, BBA 2014
The high conductivity increase observed during the pulse cannot be detected a few seconds after the pulse. The conductivity increase detected after the pulses is much lower but persistent.
Persistent Low Conductivity Increase

In vivo conductivity of rat liver

- Measurement of the conductivity of the complete liver, reveals high increase of the conductivity on the membranes

- Relaxation of high conductivity state
  \( \tau \sim 100 \text{ ms} \)

- Additionally to the high conductivity increase, a persistent low conductivity increase can be detected

Fig. 5. Conductivity evolution during the electroporation sequence in an example from the irreversible group (8 pulses of 100 \( \mu \text{s} \) and 1500 V/cm). Points marked with circles and joined with a dashed line represent the quasi-DC conductivity obtained at the end of each electroporation pulse.

Ivorra and Rubinsky, Bioelectrochemistry, 2007
Detecting conductivity changes

Whole-cell + Patch-clamp

100 μm

2 μm
Benefit of patch-clamp in whole cell configuration

- TMV is directly imposed and can be measured
- Current through the membrane can be recorded
- Homogeneous changes over the whole membrane surface
  => averaging possible
Current-voltage relation

Patch-clamp

Physiological Response

Supraphysiological Response

V_{\text{CLAMP}}

Threshold for conductivity Increase Patch-clamp

**Current-voltage relations - 10 ms pulses - DC3F cell**

<table>
<thead>
<tr>
<th>Threshold potential (mV)</th>
<th>DC-3F</th>
<th>BY-2 protoplast*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depolarisation</td>
<td>+201 ± 7 (n=18)</td>
<td>+205 ± 7 (n=9)</td>
</tr>
<tr>
<td>Hyperpolarisation</td>
<td>-231 ± 8 (n=18)</td>
<td>+273 ± 7 (n=9)</td>
</tr>
</tbody>
</table>

*data from Wegner et al. (2011)*

Relaxation of the high conductivity state

\[ \tau = 16.8 \pm 2.8 \text{ ms} \]
Two phenomenon involved?

Patch-clamp | 1st sweep
---|---
2nd sweep
3rd sweep
4th sweep

-300
-200
-100
0
100
200
300

Current (nA)

-2
0
2

Trans-membrane voltage difference (mV)

3.4 nS

1.7 nS

-0.6
-0.3
0.0
0.3
0.6

Current (pA)

-300
-200
-100
0
100
200

Trans-membrane voltage difference (mV)
Correlation with PI uptake

PI uptake

Voltage pulse

Current response

100 mV
2 ms
20 nA
2 ms

6000 7000 8000

-100 0 100
-1500
-1000
-500
0
500
1000

Current (pA)
Trans-membrane voltage (mV)
prepulse
postpulse 0.775 nS/pF
0.042 nS/pF

-1.28 + 0.57 + 1.41 + 4.56 + 7.29 + 8.53

Patch-clamp |
- PEF induce an increase of membranes conductivity

- This conductivity increase can be detected on lipid bilayer, on single cells and on biological tissue

- Relaxation of the high conductivity states is fast (10-100 ms). Much faster than the resealing of the permeable state

- Some experimental data show after relaxation of the high conductivity state, a persistent low conductivity increase.
Pores or not?
Basis of the models
- Pores described using the free-energy of membrane
- Pore density obtained using Smoluchowski’s equation

\[ \frac{\partial N_{ep}(t, V)}{\partial t} = \alpha e^{(V/V_{ep})^2} \left( 1 - \frac{N_{ep}}{N_0} \exp\left(-q(V/V_{ep})^2\right) \right) \]

Some aspects still under discussion:
- Size of the predicted pores vs size of molecules transported
- Predicted Life time of pores vs observed life time of permeabilization
- Cumulative effects of pulses and effect of repetition rate
Experimental evidences

Pores or not ?

Sengel and Wallace, PNAS, 2016

Tieleman et al, J.AM. CHEM. SOC. 2003

go to : S10-1 and PA-51

BUT ... those defects reseal immediately when the voltage on the membrane is removed ...
Traditional models based of description of pores

Pore density ↔ Conductivity $S_m$ ↔ Permeability
Two variables to describe the state of the membrane

Traditional models based of description of pores

Pore density ↔ Conductivity $S_m$ ↔ Permeability

New modelling approach

Two variables to describe the state of the membrane

$S_m$ / Conductivity
$P_m$ / Permeability

Two variables to describe the state of the membrane

\[ S_m, \quad P_m \]

Conductivity \( S_m \)  Permeability \( P_m \)

Two phenomenon induced by external electric field

\[ X_1, \quad X_2 \]

Something fast (short dynamics) \( X_1 \)  Something else (?) \( X_2 \)

Mathematical approach

A two step model

\[ \forall t > 0, \quad s \in \Gamma \]

\[ S_m(t, s) = S_0 + X_1(t, s)S_1 + X_2(t, s)S_2 \]

\[ P_m(t, s) = P_0 + X_1(t, s)P_1 + X_2(t, s)P_2 \]

A two step model

Mathematical approach

$S_m(t, s) = S_0 + X_1(t, s)S_1 + X_2(t, s)S_2$

Something ???
- Only during the application of pulse
- High conductivity increase

Something else ????
- Long lasting phenomenon
- Little contribution to conductivity

$S_m$

$S_0$

$t$

Pulse
A two step model

Mathematical approach

\[ S_m(t, s) = S_0 + X_1(t, s)S_1 + X_2(t, s)S_2 \]

Contribution of pores ???
- Only during the application of pulse
- High conductivity increase

Something else ????
- Long lasting phenomenon
- Little contribution to conductivity
Lipid oxidation?

S07-5 (Gailliegue) S09-5 (Mir)

Need for additional proofs

- Need for calibration (E, t, N etc. ...)

Breton, Silve and Mir, under revision
Voltage Sensitive Dyes
Voltage Sensitive Dyes

The molecule

Annine 6

- di-4-ANEPPS (Gross 1986, Ehrenberg 1987, Lejewska 1989)
- ANNINE-6 (Frey 2006, Flickinger 2010, Berghoefer 2012)
Voltage Sensitive Dyes

Principle of experiments

- The fluorescence change $F/F_0$ give you information on transmembrane voltage value
- Temporal resolution : $T_{\text{laser}} \sim 5 \text{ ns}$
- To obtain images at different time during an electric pulse, $\Delta t$ can be modified
Voltage Sensitive Dyes

Analysis, using the «Local equivalent electric field»

\[ C_m \frac{\partial V}{\partial t} + \left( \frac{2\sigma_e \sigma_i}{r_c (\sigma_i + 2\sigma_e)} + S_m \right) V = \frac{3\sigma_e \sigma_i}{\sigma_i + 2\sigma_e} E_{\text{ext}} \sin(\theta) \]
Voltage Sensitive Dyes

Analysis, using the «Local equivalent electric field»

\[ E_{\text{ext}} \sin \theta \]

\[ \frac{F}{F_0} \text{ vs. } \theta [^\circ] \]

\[ \frac{F}{F_0} \text{ vs. } E_{\text{ext}} \sin \theta [\text{kV.m}^{-1}] \]
Voltage Sensitive Dyes

What about surface conductance?

**Quasi-static approximation**

\[ V_1 = \frac{2\sigma_e \sigma_i}{S_m r_c (2\sigma_e + \sigma_i) + 2\sigma_e \sigma_i} V_0 \]

\[ V_0 = \frac{3}{2} r_c E \sin \theta \]
Voltage Sensitive Dyes

What about surface conductance?

Pulse parameters that generate the same conductivity increase of the membrane

\[ V_1N_0 = \frac{2\nu}{\nu_i + 2\nu_e} \]

= Pulse parameters inducing the same pore density?
The applications
What kind of applications?

Medical applications

Industrial applications

Lluis Mir

Wolfgang Frey
Benefits of the method:
- Efficient
- No side effect
- Local treatment
- Treatment specific to cancerous cells
- Cheap
- No hospitalization is necessary
- Simple procedure
- Structures like vessels or nerves are preserved

Response after a single treatment (total 171 nodules)

CR : Complete Response, PR : Partial response (↓ volume >50%),
NC : No significant change, PD : Progression
From ESOPE European clinical trial

CR : 73.7%, PR : 11.1%, NC : 10.5%, PD : 4.7%
Medical Application / Electrochemotherapy

Design and Realization of the “Cliniporator” (IGEA – 2004)

**Electrodes for medical application**

ECT Centers in Europe

<table>
<thead>
<tr>
<th>Countries</th>
<th>Centers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italy</td>
<td>41</td>
</tr>
<tr>
<td>Germany</td>
<td>46</td>
</tr>
<tr>
<td>Great Britain</td>
<td>16</td>
</tr>
<tr>
<td>France</td>
<td>10</td>
</tr>
<tr>
<td>Spain</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total EUROPA</strong></td>
<td><strong>139</strong></td>
</tr>
</tbody>
</table>

**Treated Patient**

- 3 000 in 2013
- ~11 000 by end of 2014

Pulse voltage: 100 bis 1000 Volts
Number of pulses: 1 à 20
Duration of pulses: 100 μs
Repetition frequency: 1 à 5000 Hz
Applications in the food industry

Sugar industry
2 µs - 500 kV/m - 5 kJ/kg

Throughput: ~ 10 t / h

Applications in the food industry

Sugar industry

Applications in the food industry

2 µs - 500 kV/m - 5 kJ/kg

Throughput: ~ 10 t/h

Advantages:

- Better extraction of juice enables a decrease of the extraction temperature
- Better water extraction in the pulp press saves evaporation energy for drying
- Over all up to 30% less energy required

Dr. Martin SACK (KIT, IHM)
Applications in the food industry

PEF treatment of crushed grapes

Advantages:

- Improved extraction
- Fast processing
- No use of enzymes

**Future applications... Bioeconomy**

**Microalgae, a concentrate of valuable components**

- Colorants, antioxidants, vitamins:
  - astaxanthin,
  - ß-carotene

**proteins**

**lipids, fatty acids:**
- primary target for energetic use

**lipids**

**pigments**

- O$_2$

**oligo/polysaccharides**
- alginate
- agar
- carrageenan
- glucans

**Biomass**

**light**

H$_2$O CO$_2$

**nutrients**

**Many potential Market**
- Food industry
- Feed
- Pharmaceutical industry
- Biofuel

**Two main challenges:**
- Reduction of cultivation costs
- Extraction

Adapted from I. Hariskos, C. Posten: Biotechnology Journal, 2014, 9 (with permission)
Thanks to ...

BioEM 2017
5-9 June West Lake Villa
Hangzhou, China

EBEA
european bioelectromagnetics association

Institute for Pulsed Power and Microwave Technology (IHM)