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Recent biotechnological developments of electropulsation. A prospective review

J. Teissié [a,*], N. Eynard [a], M.C. Vernhes [a], A. Bénichou [a], V. Ganeva [b], B. Galutzov [b], P.A. Cabanes [c]

[a] *IPBS CNRS (UMR 5089), 205 Route de Narbonne, 31077 Toulouse Cedex, France*
[b] *University of Sofia, Sofia, Bulgaria*
[c] *SEM EDF, Paris, France*

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Abstract

During the last 25 years, basic research has improved our knowledge on the molecular mechanisms triggered at the membrane level by electric pulses. Applied aspects may now be used under safe conditions.

Electropulsation is known as a very efficient tool for obtaining gene transfer in many species to produce genetically modified organisms (GMO). This is routinely used for industrial purposes to transfer exogenous activities in bacteria, yeasts and plants. The method is simple and of a low cost.

But electropulsation is not limited to this application for biotechnological purposes. It is known that the field-associated membrane alterations can be irreversible. The pulsed species cannot recover after the treatment. Their viability is strongly affected. This appears as a very promising technology for the eradication of pathogenic microorganisms. Recent developments are proposed for sterilization purposes. New flow technologies of field generation allow the treatment of large volumes of solution. When high flow rates are used, microorganisms are submitted both to a hydromechanical and to an electrical stress. The synergy of the two effects may be present when suitable pulsing conditions are chosen. Several examples for the treatment of domestic water and in the food industry are described.

Walled microorganisms are affected not only at the membrane level. We observed that alterations are present on the cell wall. A very promising technology is the associated controlled leakage of the cytoplasmic soluble proteins. Large dimeric proteins such as β-galactosidases can be extracted at a high yield. High volumes can be treated by using a flow process. Extraction of proteins is obtained with many systems including mammalian cells.

Keywords: Electropulsation; Biotechnology; Microorganisms; Yeast; Amoebae; Legionella

1. Introduction

When during the Szeged BES meeting in 1987, the principal investigator was asked to give a survey of the effects of electric fields and currents on living cells and their potential use in biotechnology, two main emerging fields were present [1]. Taking benefit of the basic research results of the early 1980s, electrofusion and electrotransformation were starting to be used not only in academic laboratories while cell electroactivation was expected to be a fruitful technology. More than 10 years later, while electrofusion is used for the formation of plant hybrids rather routinely, its use in other biotechnological fields is somewhat limited with the exception of its key function in animal cloning [2]. Electrotransformation (electrically mediated gene transfer) is routinely used at the bench to obtain genetically modified organisms (GMO) [3–5].

But no significant progress at the industrial scale has been made to obtain by such technologies:

(a) an increase in the growth of the microorganism
(b) an increase in the metabolic activity of micro-organisms

while this was expected in 1987.

In fact biotechnological applications remain focused on small-scale experiments. Getting a limited number of transformed microorganisms is enough to prepare the availability

* Corresponding author. Tel.: +33-5-6117-5812; fax: +33-5-6117-5994.
E-mail address: justin@ipbs.fr (J. Teissié).

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of GMO for the market. The selected microorganisms can be grown and expanded under selective pressure.

New developments of electropulsation in biotechnology are obtained when large volumes can be treated. Metabolites can be extracted or introduced as a result of electropermeabilization. They can be small sized but cytoplasmic proteins can be the target by using suitable electrical parameters [6]. Microorganisms can be eradicated when stringent pulse conditions are used, which bring an irreversible electropermeabilization [7].

2. Theory

When applied on a cell suspension, an external field induces a time- and position-dependent membrane potential difference modification ΔV

$$\Delta V(M) = fgrE\cos\theta(M)(1-\exp(-t/\tau)) \quad (1)$$

where f is dependent on the cell shape, g on the conductance of the outer and inner buffers and of the cell membrane, $2r$ is the length of the cell in the direction of the field, E is the field strength, θ is the angle between the direction of the field and the normal to the membrane plane at the point of interest M and τ is the membrane capacitance loading time. The field pulse is supposed to be a square wave.

The loading time is described by

$$\tau = rC_{\mathrm{m}}g^* \quad (2)$$

where C_{m} is the membrane unit capacitance and g^* a complex function of the conductances. For microorganisms, τ is in the microsecond range. It increases with a decrease in the conductance of the external buffer and with the cell size.

The resulting membrane potential difference is the sum of the resting membrane potential difference (assumed to be independent of the external field) and of the field-dependent modulation.

Electropermeabilization is triggered as soon as locally the resulting membrane potential difference reaches a critical value (between 200 and 300 mV). This means that for a (spherical) given cell, this is obtained when

$$E\cos\theta(M)(1-\exp(-t/\tau)) = E_{\mathrm{p}} \quad (3)$$

The conclusion is that for long pulses (duration larger than several τ) for a field intensity E ($E > E_{\mathrm{p}}$), a cap on the cell surface is in the permeabilized state and its surface is

$$A\ \mathrm{perm} = 2\pi r^2(1-E_{\mathrm{p}}/E) \quad (4)$$

but clearly its size will depend on the pulse duration when this duration is in the order of τ.

The density of local defects supporting the permeabilization is increased with pulse duration and number of successive pulses but not with the delay between pulses if delay is larger than 1 ms and shorter than 10 s.

These conclusions on spherical cells can be used with ellipsoidal cells (rod-like bacteria) but one must take into account the orientation of their long axis relative to the field [8].

3. Technological problems linked to large volume treatment

Working on large volumes can be obtained by an up-sizing of the present laboratory-scale processes. Batch technology is always limited by the amount of energy that can be delivered by the power generators. The volume Vol that can be treated with a pulse of duration T at a field E in a buffer with a conductance Λ requires an available energy:

$$W = E^2\Lambda\mathrm{Vol}\ T \quad (5)$$

that is, 15 kJ is needed to pulse 1 l of phosphate buffer saline (PBS) at 1 kV/cm during 1 ms. This is clearly a technical limit in the design because with a width between the electrodes of 1 cm (to limit the voltage to 1 kV), the current would reach 15 kA!

Other methodologies are clearly needed. Flow processes appear to be a suitable approach. We introduced the technology in the mid-1980s [9] for an up-scaling of electrofusion [10,11] and showed some years later that it can be used for electrotransformation but the plasmid cost was high [12]. The possibility to treat blood samples was proposed for clinical applications [13].

4. Flow electropulsation

The basic concept is to apply calibrated pulses as in batch process but at a delivery frequency that is linked to the flow rate (Fig. 1). The relationship between frequency and flow is such that the desired number of pulses is actually delivered on each cell during its residency in the pulsing chamber. The geometry of the chamber is chosen to give a homogeneous field distribution and a uniform flow rate (turbulent flow conditions may be advantageous). Therefore, the residency time T_{res} of a given cell in the chamber is:

$$T_{\mathrm{res}} = \mathrm{Vol}/Q \quad (6)$$

where Vol is the volume of the pulsing flow chamber and Q is the flow rate. The number of pulses delivered per cell is:

$$N = T_{\mathrm{res}}F \quad (7)$$

F being the frequency of the pulses, which is set by the pulse generator, which also controls the pulse duration T and the voltage U.

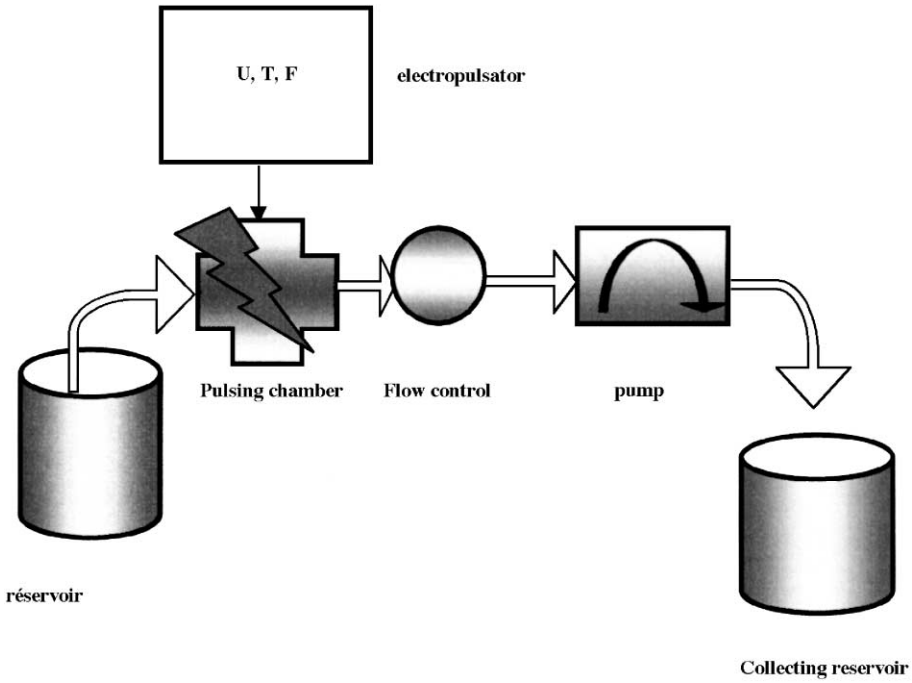

Fig. 1. Flow electropulsation. (A) Cells are taken from the reservoir. (B) They flow through the pulsing chamber where a controlled number of calibrated pulses is applied. The pulsing chamber is connected to the high-power pulse electropulsator where the voltage U, the pulse duration T and the pulse frequency F are under control. (C) The flow Q is obtained by a pump and controlled. (D) Pulsed cells are collected and processed in a collecting reservoir.

The field strength is

$$E = U/d \tag{8}$$

d being the width between the two electrodes that are flat and parallel.

The description of the flow electropulsation device is cartooned in Fig. 1.

The electrical requirements are reasonable. To treat 1 l of PBS per minute with 10 pulses of 1 ms requests a power of 2.5 kW from Eq. (5).

5. Protein extraction

Yeasts (*Saccharomyces*, *Kluyveromyces*, *Picha*) are a well-established cell factory for the production of endogenous proteins (alcohol dehydrogenase, aldehyde dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, 3-phosphoglyceric phosphokinase, β-galactosidase, alcohol oxidase). Their electrotransformation to produce exogenous proteins follows an easy-to-perform protocol on intact systems [14].

A technological bottle neck is the extraction of proteins from the cytoplasm under conditions where the protein integrity (i.e., activity) is preserved. Many approaches are proposed and already used in the biotech industry. As the cell wall must be degradated, drastic mechanical (bead mill homogenization, rotor stator homogenization, high-pressure homogenization, US disintegration), chemical (autolysis by solvent, membrane disintegration, heat, pH and osmotic shock) or enzymatic (zymolase, lyticase) methods are used. A critical drawback is presently due to the nonspecificity of these methods: the vacuoles are destroyed allowing the proteases to have a free access to the cytoplasmic enzymes. Another limit is that these methods are energy consuming because the treatment is most of the time operated at high temperature.

Taking into account our recent observations with batch processes [15,16], flow processes were developed to bring electroextraction at an industrial scale [17].

A simple procedure is applied. Yeasts cells are washed and suspended in pure water, a low-conductance medium. A limited number of pulses is applied with pulse duration in the millisecond time range. Field intensities are less than 4 kV/cm. Pulsed cells are then incubated in 0.105 M salt solution (PBS and glycerol as osmotic protector) at room temperature. A slow release of cytoplasmic proteins is obtained, but up to 90% of the cell content can be recovered within 6–8 h (100% being assumed to be obtained by the bead mill process or the enzyme lysis procedures). A key feature is that the specific activity of the recovered proteins is higher by a factor of 1.5–2 than with the mechanical extraction. Electrophoretic characterization of the extracted proteins does not indicate a size limit in the recovered proteins. Indeed dimeric β-galactosidase (250 kDa) is extracted.

The electric conditions that are requested are easily obtained due to the low current intensity that is needed as the experiments are run on a suspension in pure water.

Optimization of the extraction procedure can be obtained by playing on the electrical parameters (field intensity, pulse duration, number of pulses) in such a way as to obtain a high flow rate. The cellular load can be high (up to 20% dry w/v).

The present results are mostly focused on the yeast system, but we were able to obtain analogous results with mammalian cells [18] and other walled systems may be targets (plant cells, molds) [19]. While proteins are products with a high added value, the electro-assisted extraction is valid for small metabolites as illustrated with the use of ATP leakage to quantitatively assay cell electropermeabilization in batch experiments.

6. Pathogen eradication

Electropulsation is known for many years to cause irreversible membrane permeabilization when drastic electrical conditions are used (Fig. 2). This offers a new physical approach for the elimination of microorganisms.

6.1. Food industry

Cold sterilization is supposed to eliminate the microorganisms in food (milk, fruit juices) while preserving the ''real'' taste of the product [7]. The idea is that the field is able to disrupt the cell envelope but is too weak to inactivate enzymes [19]. Among the two technologies presently under development: high pressure (HP) and high-intensity electric field pulses (HELP), the electropulsation approach is already on the market with a prototype of pilot scale equipment able to work a flow rate of 300 l/h. Electrical parameters are always using strong electric pulses (more than 20 kV/cm) with microsecond pulse duration with a capacitor discharge technology.

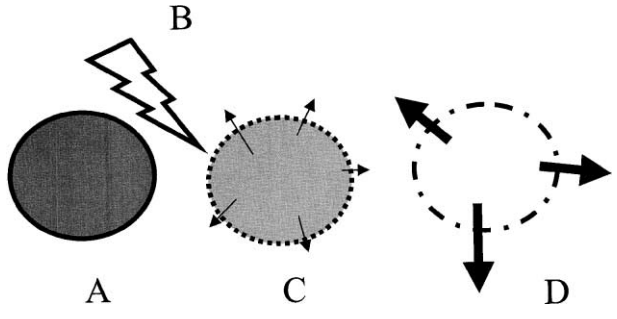

Fig. 2. Irreversible electropermeabilization. (A) Intact cells. Their cytoplasm content is pictured in dark grey. (B) Electropulsation. (C) Cell membranes are permeabilized. The cytoplasm content leaks out as shown by the light grey colour and the small arrows. (D) The cell membrane is irreversibly permeabilized and cannot be repaired. All the cytoplasmic content leaks out.

6.2. Legionella in domestic water

A health problem is present in developed countries: how to get rid of pathogens with a technology preserving the environment. Since the epidemic in 1976, Legionella are known to be present in domestic water and its inhalation through aerosol is deleterious for human beings. Legionella are present in many systems: water heater, shower, central heating, air cooler. Its growth in domestic water must be eliminated. Several methodologies have been proposed: chlorine treatment or heat shock but only an online treatment will bring a safety level.

Electropulsation has been shown in Toulouse to be a suitable approach when used in a batch approach. A low-field long-duration square wave repetitive pulse approach (20 times 10 ms at 550 V/cm) was designed to cause irreversible electropermeabilization. The electrical parameters (50 Hz frequency) were suitable to develop a flow technology able to treat suitable volumes of domestic water (which conductivity is low, less than 1 mS/cm). The cost in energy is limited as the current to be delivered is with a low intensity and that moderate field intensities (applied voltage) are needed. A 6 log efficiency in eradication has been obtained with a lab pilot system at a flow rate of 24 ml/min. A scaled-up system is under development.

6.3. Amoeba downstream of power plants

The presence of pathogenic amoebae (*Naegleria fowleri*) is detected at increasing level in the closed looped cooling systems of power plants that use water for cooling. This is due to the facilitated growth of protozoa above 40 °C. A continuous treatment system of the cooling water at the system drain appears necessary. Different methods have been proposed when chlorine treatment of the water [20] was forbidden by councils for Public Health. Pilot studies are under evaluation using different physical methods such as UV light, ultrasound treatment or electropulsation.

Electropulsation can be applied for batch eradication either alone or with a concomitant chemical or UV treatment [21,22]. The results show that eradication can be obtained under low-field long-pulse duration conditions by inducing an irreversible permeabilization. Interestingly, permeabilization was obtained under conditions that were more stringent than for smaller mammalian cells such as Chinese ovary cells (CHO) while it is assumed that the field effects are larger on larger cells. Another interesting result was, that as already observed on CHO cells [23], the eradicating effect of the field pulses was not directly linked to the energy that was delivered to the cell. This observation is very important for industrial developments to reduce the cost of the treatment. Short pulses with a high field intensity (microseconds, more than 10 kV/cm) were the most cost-effective for eradication. Death was then not due to a classical process of irreversible permeabilization.

Electropulsation was then used on a flow process. In a lab pilot system, it was observed than the direction of the pulse field versus the flow was very important. Eradication was not possible at high flow rate when the perpendicular orientation was used while it was obtained with a parallel orientation [18]. A pilot set up was recently tested on a power plant [24]. Results are encouraging. A 2 log eradication was obtained for less than 1 MW when treating 1 m^3/s.

7. Consequences for basic research

Most of the results are explained by an irreversible permeabilization. Nevertheless in the case of protein extraction, leakages of species with molecular weights larger than 200 kDa are evidences that defects are present in the yeast wall. The outflow was slow (several hours) suggesting that no large defects were present. This was confirmed by electron microscopy studies [7]. The creation of these defects as a result of membrane electropermeabilization remains unexplained.

Electropermeabilization results from low-field long-lasting pulses and is easily monitored by the associated leakage of small compounds. But optimized eradication in the food industry and in the case of amoebae is obtained under different electrical conditions. Indeed the effect of short pulses is not supported by such a model.

Conditions for permeabilization are not energy optimal. The membrane charging time is always shorter than the pulse duration. As a consequence, the field-induced membrane potential difference does not reach its steady-state value. As the membrane-permeabilized part is under the control of the induced potential difference, a smaller fraction is brought to the permeabilized state with a lower density of defects than obtained with long-lasting pulses. This conclusion opens the question of the origin of the microorganism death under these pulsing conditions. Other physical factors are present when a field pulse is applied on a vesicle [25,26]. Electrical fields induce mechanical forces. As it is a field effect on a field induced dipole, the general expression of the force F is given by

$$F = UE^2 \quad (9)$$

where the U parameter is dependent on the frequency of the field and on the membrane state [27]. As due to electropermeabilization, which is triggered within less than 1 μs, the membrane state is affected and as U is time dependent, F changes during the pulse in a complex way. But the final result is that a time-dependent strain is applied on a cell with a time-dependent membrane organization.

One should consider that a cell is a viscoelastic body and that a change in its geometry would result. This is clearly observed on liposomes [28,29]. Elongation is predicted to be present in the early steps of the field pulse [25,27]. A change in the shape of the vesicle results. In a solution with a low conductivity, this is associated to its elongation in the direction of the field, that is, for small values of θ, would result in lower critical values of the damaging field intensities. The mechanical stress on the membrane organization should have direct damaging effects and clearly facilitate the outflow of cell content. This would explain the observations on microorganism lysis obtained in the reported biotech applications. Basic study investigations are needed to confirm these predictions. Therefore, observations at the single-cell level are strongly needed.

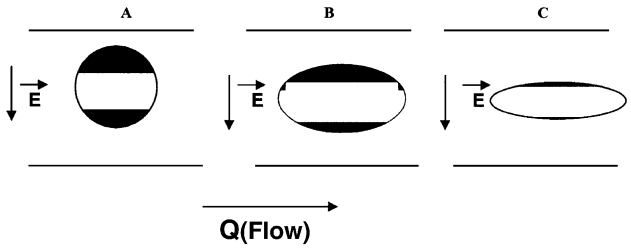

Fig. 3. The hydrodynamic drag affects the cell shape in the flow process. The field is applied in the perpendicular orientation to the flow. (A) There is no flow; the cell is spherical. For a given field intensity, two large caps on the cell surface are brought to the electropermeabilized state. (B) A flow is present. Being a viscoelastic body, the cell shape changes and becomes an ellipsoid with a decrease of its size in the direction of the field. The electropermeabilized cap size is decreased for the same applied field intensity. (C) A high flow is present. The hydrodynamic cell deformation is even higher. Almost no electropermeabilization can be induced by the electric field pulse.

The dependence of eradication on the flow rate and on the orientation of the field relative to the flow suggests that the hydrodynamic drag may:

1. prevent the reorientation of rod-like bacteria during the field pulse, a step which facilitates electropermeabilization [30]
2. induce a deformation of cells in the direction of the flow, resulting in a decrease of its size in the direction of the field and of its sensitivity to the field (Fig. 3)

The physical conditions present in the flow conditions cannot therefore be described by taking into account exclusively the electrical stress associated to the external field but the mechanical tensions associated to the flow are other parameters affecting the stability of the membrane.

8. Conclusions

Electropulsation can be in the coming years a powerful tool for the biotech industry. But such a development, as already observed during the last 20 years for the obtainment of GMOs, needs an improvement of our basic knowledge on

the molecular processes affecting the cell organization during the field pulses.

Acknowledgements

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