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Microdevice for studying the *in situ* permeabilization and characterization of *Chlamydomonas reinhardtii* in lipid accumulation phase

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ABSTRACT

Microalgae are considered as a renewable source of lipid-rich biomass feedstock for biofuels due to their high fatty acids content when cultivated in stress conditions (nitrogen starvation). Nevertheless the use of solvents in conventional extraction methods raises important environmental, health and safety issues. The application of Pulsed Electric Field (PEF) to electroporate microalgae is a promising alternative to traditional processes involved in lipid recovery, as it might permeabilize cell membrane, easing the access out of the cytoplasm, and reducing the use of solvents. In order to study the PEF effects on *Chlamydomonas reinhardtii*, we developed a microdevice that allows real time visualization during such electrical solicitation. A high number of electroporation chambers are designed on this biochip to characterize, in real-time, and in parallel, the permeabilization of cells subjected to PEF using the propidium iodide (PI). Several conditions were investigated (pulse energy, pulse duration and electrical field amplitude). Reduced energy consumption, heat effects and electrochemical reactions are obtained when applying short pulses (5 μs) of high electric field (4 to 6 $kV \cdot cm^{-1}$). Moreover, an increase is observed in cell diameter and lipid content over time in nitrogen stress conditions. The cell sensitivity to the PEF seems to be affected by the cell diameter. Finally, for the first time, lipid droplet redistribution was observed within the cytoplasm during the treatment, showing that 5 μs pulses lead to additional intracellular electroporation effects.

1. Introduction

1.1. Microalgae as a renewable source

Due to their chemical composition (proteins, pigments, starch, fatty acids) algae can be used for food, feed, medical and energy purposes [1,2]. Many algal strains are able to accumulate high amounts of fatty acids as triacylglycerols (TAGs). The accumulation reaches up to 20–50% of their dry weight in certain conditions such as high light intensity and nitrogen limitation stress [3]. Thus, algae are considered as a renewable source of lipid-rich biomass feedstock for biofuels [4]. This generation of biofuels demonstrates high productivity and no competition with food crops in comparison with the 1st and 2nd generations [5].

The overall energy consumption for each step of the production, from algae culture to downstream processes (harvesting, molecules extraction) is a decisive factor on the price of the end product [6]. The environmental impact of each step, regardless of the energetic consumption, is also a key factor [7–9]. Downstream processes, such as, classic mechanical disruption and harvesting methods, are constantly challenged because of their numerous drawbacks. Moreover, extraction methods are generally ineffective when applied on wet intact cells. New technologies have been studied to weaken algal cells prior to wet extraction, including: microwaves [10,11], ultrasounds [10,12] and electrical fields [13]. All these technologies show very low energy consumptions. However, before their use in the algae industry, their improvement at laboratory scale is mandatory. Thus, studies concerning pulsed electric fields (PEF) applied to algae cells are increasing considerably, as this process weakens cell membranes and improves the extraction of soluble compounds, such as, proteins and carbohydrates [14], or large hydrophobic molecules [15].

PEF is broadly used for other applications in biology, *e.g.*, DNA transfection [16], drug delivery into tissue cells [17,18]; and in food

Abbreviations: a.u., arbitrary units; Cm ($\mu F \cdot cm^{-2}$), membrane capacitance; DMSO, dimethyl sulfoxide; E ($kV \cdot cm^{-1}$), Electric field; E_{50} (kV/cm), electric field corresponding to 50% of permeabilization; FDA, fluorescein diacetate; IPA, isopropyl alcohol; Np, number of pulses delivered during a burst of PEF treatment; nPI_0, number of cells stained by PI in an electroporation chamber before treatment; nPI_1, number of cells stained by PI in an electroporation chamber after treatment; $ntot_1$, number of cells counted in an electroporation chamber after treatment; PI, propidium iodide; PEF, pulse electric field; r (m), cell radius; TAGs, triacylglycerols; TAP, medium tris-acetate-phosphate; TAPN-, medium tris-acetate-phosphate depleted in nitrogen; W ($kJ \cdot m^{-3}$), energy delivered; Δt_{pu} (μs), pulse width; ΔT_{pu} (°C), heating resulting from on pulse; ΔT_{burst} (°C), heating resulting from on burst; $\Delta\Psi_i$ (V), induced trans-membrane voltage; σ ($S \cdot m^{-1}$), conductivity; θ (°), angular position on the cellular membrane facing the electrodes.

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processes: treatment of fruit juices [19,20], pasteurization [21,22], sugar extraction from beets [16,23] or pigments extraction from potatoes [24]. The use of PEF on microalgae aims several targets: extraction of lipids [15,25–27], pigments [14,28] and proteins [14,29], or lysis of toxic algae [30].

1.2. Electropermeabilization of microalgae

The application of PEF raises the transmembrane potential up to a critical value and in turn induces membrane permeabilization [31] due to creation of pores, their size and reversibility of which depend on the treatment intensity and duration [32]. Thus, depending on the shape, amplitude (E), duration (Δt_{pu}) and number (N_p) of electric field pulses, different effects on algae can be modulated. Parameter values found in the literature are shown in Table 1.

Conditions found in the literature vary considerably (Table 1). Usual pulse duration ranges from several μs to several ms, while the pulse amplitude varies from 1 to 50 $kV \cdot cm^{-1}$ [33]. The achievement of a non-lethal level of poration is dependent on both parameters [18].

1.3. Electroporation parameters

The level of electroporation can be estimated from the Schwan equation (Eq. (1)) [34], which gives the potential (ΔΨi) induced on cells submitted to PEF.

$$\Delta\Psi i = -\frac{3}{2}\, r\, E\, cos(\theta) \cdot \left(1 - e^{\frac{-\Delta tpu}{\tau}}\right) \quad (1)$$

where r is the cell radius, Δt_{pu} the duration of an electric field pulse, τ the charging time of the cellular membrane and θ the angular position on the cellular membrane facing the electrodes.

The induced potential (ΔΨi) required to trigger permeabilization is known to be in the range 0.2–1.5 V for mammalian cells [33]. Algae strains differ in cell radius (r) and cell properties (affecting τ — Eqs. (2) and (3)). These features are paramount for the selection of the electric field intensity E inducing permeabilization (Eq. (1)) [29].

Furthermore, conventional electroporation uses pulses with a longer duration (Δt_{pu}) than the plasma membrane charging time (τ). The membrane charging time (τ), estimated from 0.4 to 1 μs for mammalian cells [35], depends on the membrane specific capacitance (Cm), the cell radius (r), and the medium (σ_{med}), cell wall (σ_{cw}), cell membrane (σ_m) and cytoplasm (σ_{cyto}) conductivities, respectively, as shown in Eq. (2) (deduced from the single shell model [36]) or in Eq. (3) (deduced from the double shell model where the cell wall is considered [37]).

$$\tau = r\, Cm \left(\frac{1}{\sigma cyt} + \frac{1}{2\,\sigma med}\right) \quad (2)$$

$$\tau = r\, Cm \left(\frac{1}{\sigma cyt} + \frac{\sigma med + \sigma cw}{2\,\sigma med\,\sigma cw}\right). \quad (3)$$

The full charge of the membrane requires a duration of the electric field application longer than several times the charging time τ ($\Delta t_{pu} > 5\,\tau$ to reach 95% of the final charge, when considering Eq. (1)).

For this reason, very short pulses ($\Delta t_{pu} < 1$ μs) may lead to cell apoptosis by affecting internal organelles, while the membrane charge does not reach the permeabilization level [38]. It has been shown that longer pulse durations ($\Delta t_{pu} > 1$ μs) can lead to increased pore radius and resealing time [32,39] (time needed for the membrane to recover, if the permeabilization is reversible). Besides, millisecond pulses can be applied in order to weaken the mechanical resistance of the cells, such as, cytoskeleton [40] or cell wall [29]. As shown in Eq. (1), the induced transmembrane potential $\Delta\Psi_i$ is non-homogenous on the cell surface, as it depends on the angle θ of the cell with the applied field direction. This may focus the effect of PEF to a small region of the membrane [41].

1.4. Electroporation side effects

Applying an electric field in a liquid medium may result in undesirable side effects, such as, Joule heating, water electrolysis and redox reactions at the electrodes [42]. The thermal aspect should indeed be considered when applying PEF, as the Joule effect occurs in the conductive medium. The energy delivered during the PEF treatment W (expressed in $J \cdot m^{-3}$), can be expressed thanks to Eq. (4):

$$W = |E|^2\, \Delta t_{pu}\, \sigma \quad (4)$$

where σ is the medium conductivity in $S \cdot m^{-1}$.

By neglecting thermal external exchanges (diffusion, convection), the temperature elevation (ΔT_{pu}) induced by a pulse, due to the Joule effect, can be over-estimated as shown in Eq. (5) [33].

$$\Delta Tpu = \frac{W}{C\,\rho} \quad (5)$$

where $C \cdot (J \cdot m^{-3} \cdot K^{-1})$ is the heat capacity of the medium, and ρ its density.

During the treatment, if the frequency of pulse delivery is too high and does not enable the temperature to decrease between pulses [43], temperature may increase by several dozens of degrees Celsius, affecting cell viability and degrading valuable compounds, such as, lipids, pigments or proteins. To prevent this heating, some studies on microalgae are performed in a low conductivity buffer [28,29] or with a cooling system [15,25]. However, it is well known that the conductivity of the medium increases during PEF treatment because of the leakage of ions out of the cells [26,39], which might enhance the temperature increase.

Water electrolysis may also occur on electrodes. This leads to gas production at the cathode (hydrogen) and at the anode (oxygen). These mechanisms may have many consequences during PEF application, including: interference with the electric field distribution, change in the medium conductivity, generation of reactive oxygen species and evolution of the medium pH close to the electrodes. Finally, both temperature [44] and reactive oxygen species [45] can affect the permeabilization threshold (the electric field needed to open pores in the membrane) of cell membranes.

In this paper, the effects of PEF application on algae will be observed in real time, using a dedicated microdevice [46]. This microdevice allows real time observation of the cell wall behavior and the distribution of internal lipid droplets during and after pulses application [47,48]. Moreover, the efficiency of the treatment is discussed with respect to the energetic cost of the treatment and heating aspects.

Table 1
PEF parameters (pulse duration, electric field, pulse shape) used on several algae strains.

Pulse duration (μs)	Electric field ($kV \cdot cm^{-1}$)	Pulse shape	Pulse number	Strain	Cells diameter (μm)	Study
1	23–43	Square	20–110	*Auxenochlorella protothecoides*	5–8	[26]
6–150	10–25	Square	50	*Chlorella vulgaris*	2–4	[28]
10	20	Exponential decay	1–600	*Nannochloropsis*	2–3	[14]
100	2.7	Square	21	*Chlorella vulgaris*	2–4	[27]
2 000	3	Square bipolar	30	*Chlorella vulgaris*	2–4	[29]