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Ex vivo flow mammalian cell electropulsation

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Cell electropulsation brings a local and reversible permeabilization of cell membranes. This gives the technical possibility to introduce (load) exogenous compounds (drugs, proteins, DNA) into cells. Flow-through electropulsation allows to treat a large volume (number) of cells as requested for cell therapy. Cells are flowing through a pulsing chamber where they are submitted to a well-defined number of calibrated pulses. A proper setting of pulse frequency and flow rate controls the number of pulses. A large volume of cells can therefore be electrotreated in a small sized pulsing chamber. The viability of pulsed cells appears to be greatly preserved.

Key words: cell membrane permeability; electroporation

Introduction

Cell treatment with high intensity electric field pulses provokes a change in the membrane structure leading to a loss of its barrier function — phenomenon indicated as electropermeabilization or "electroporation".[1,2] By a proper choice of the parameters of the applied electric field, this change in the membrane permeability can be reversible or irreversible leading to leakage of cytoplasmic content and cell death.[3]

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When a short (microsecond) electric field pulse is applied to a cell, the resulting change in membrane potential difference may result in a localized long lived but reversible change in the membrane organization. This new state of the membrane is called « electropermeabilized » and can support the transfer of hydrophilic compounds into the cytoplasm and their leakage out of the cell. A key feature is that under controlled electrical conditions this membrane change is transient and the «normal» impermeable state can be recovered. The cell viability can therefore be preserved. This is obtained by a proper choice of electrical parameters (field strength, pulse duration and number of pulses) and buffers (pH, osmotic pressure and additives). This brought the technical possibility to introduce (load) exogenous compounds (drugs) into cells. A clinical development was proposed with big success (electrochimiotherapy).[4] This

transient membrane organization remains poorly characterized from a structural point of view. A peculiar associated property is fusogenicity. When cells, which are in the electropermeabilized state, are brought into contact, membrane coalescence occurs leading to the formation of viable polykaryons (electrofusion).[5] This is indicative that the repulsive hydration forces have been abolished. This was supported by the observation of an alteration of the interfacial layer of electropermeabilized cells. Another observation is that it was possible to get protein expression by electropulsing cells in a solution containing the relevant plasmid (electrotransformation, electrotransfection).[6] This again can be obtained in vivo in tumors (electrogenotherapy).[7]

Ex vivo treatment of cells appears as an interesting procedure for cell therapy. A present limit of electropulsation is that most protocols were designed for batch process. Only limited volumes can be treated due to the power limitations of most pulse generators. Furthermore safe conditions avoiding contamination (either microbial or electrochemical) need the use of rather expensive equipments (large laminar flow hood, sterilization of the cuvettes). Introduced in the 80's electric field treatment using a flow system seems nowadays a very promising technique for ex vivo cell therapy.[8-12] Our conclusions were reproduced on different cell systems by other groups [13] and very recently either on a large volume as we did in 1992 [14] or on microdevices.[15]

Recently we showed that application of series of electric pulses could provoke not only a drug loading but an important release of different cytoplasmic enzymes by a batch process. The efficiency of this process was dependent on the intensity, number and duration of pulses, on the growth phase and postpulse incubation media composition. This batch approach even very efficient was suitable only for treatment of micro volumes. Large volumes were successfully treated by the flow process.[16] A new aspect of flow-through electropermeabilization — the release of macromolecules from mammalian cells and its potential clinical application is validated in the present study.

Up-sizing of laboratory scale processes was always limited by the amount of energy to be delivered by the power generators. We presented a vast field of evidences that it is possible to utilize electropulsation using a flow system to work on a large volume of cells. Power specifications for the pulse generator are mostly driven by the required pulse frequencies. Nevertheless kHz trains can be delivered meaning that high flow rate (i.e. large volumes) can be treated (up to 1 l/min).[17,18]

All aspects of electropulsation can be obtained with the flow process: drug loading, protein and metabolite extraction, eradication, gene transfer and expression and hydrid production. A key advantage for clinical applications is that contamination can be avoided by using closed loop circuits.

The present paper described the systematic investigation of exogeneous compound loading and protein extraction from chinese hamster ovary cells by a flow electropulsation method by emphasing the good preservation of the viability of the treated cells.

Materials and methods

Cells

Chinese hamster ovary (CHO) cells were used as a model system. The WTT clone was selected for its ability to both grow in suspension and plate easily. They were grown in suspension in MEM medium as previously described.[11]

Electropermeabilization

Culture medium was removed and replaced by a pulsing buffer (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) at a cell density of 10^6 per ml. This low volume fraction of

cells was chosen to avoid any viscosity effect on the flow. Penetration of propidium iodide PI (100 μM, in pulsing buffer) was used to monitor permeabilization. Analysis was performed with a cytofluorimeter (Facscan, BD) to evaluate both the percentage of fluorescent cells (i.e. percentage of PI positive cells) and the mean level of fluorescence of the cell population.

The protein release was monitored as follows. Cells are kept 10-20 min at room temperature after the electrical treatment and then on ice. The protein concentration in the supernatant of pulsed and control cells was assayed by the Biorad kit.

Cell viability was assayed 24h after the electrical treatment by the crystal violet method by taking advantage of the selectivity of viable cells to plate on a culture dish.

Electropulsation

Electric field treatment was performed on a CNRS high power cell electropulsator generating rectangular pulses with adjustable voltage up to 1.5 kV.

A flow through pulsing chamber with a 0.3 mL volume was used. Two stainless steel flat parallel electrodes at a distance of 0.3 cm were used to apply repetitive pulses. Pulse duration (T) and frequency (Ff) were triggered by a TTL pulse generator. All pulsing parameters were monitored on line with an oscilloscope connected to a PC computer when storage was needed. An ohmic behaviour was observed. Cells were treated at room temperature with series of pulses with controlled duration and frequencies. The flow rate was in the range from 1.2 to 60 mL/min and freely adjustable with a peristaltic pump (Gilson, France).

Flow electropulsation

The basic concept was to apply calibrated pulses at a delivery frequency which was linked to the flow rate (Figure 1). The desired number of pulses was actually delivered on each cell during its residency in the pulsing chamber. The geometry of the chamber (flat parallel electrodes) was chosen to give a homogeneous field distribution on a laminar flow. Therefore, the residency time T_{res} of a given cell in the chamber was:

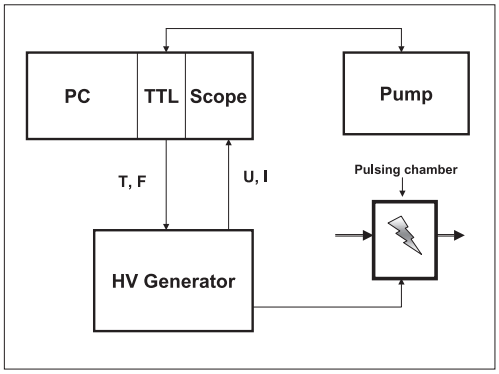

Figure 1. Configuration of a flow through electropulsator. The TTL trigger drives the delivery of the pulse with a preset duration and frequency. The voltage is set on the generator. All signals are monitored on line on the oscilloscope and the PC. The flow rate is adjusted with a peristaltic pump.

$$T_{res} = Vol / Q \quad \text{(Eq. 1)}$$

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

$$N = Vol\ F\ f / Q \quad \text{(Eq. 2)}$$

F f being the frequency of the pulses, therefore N was under experimental control. Nevertheless one should take into account that a parabolic distribution of the flow rate was present in the chamber. More pulses were applied on cells close to the walls than in the middle of the chamber

The field strength was taken as the voltage to electrode distance ratio

$$E = U/d \quad \text{(Eq. 3)}$$

d being the width between the two electrodes, U, the voltage. The field distribution

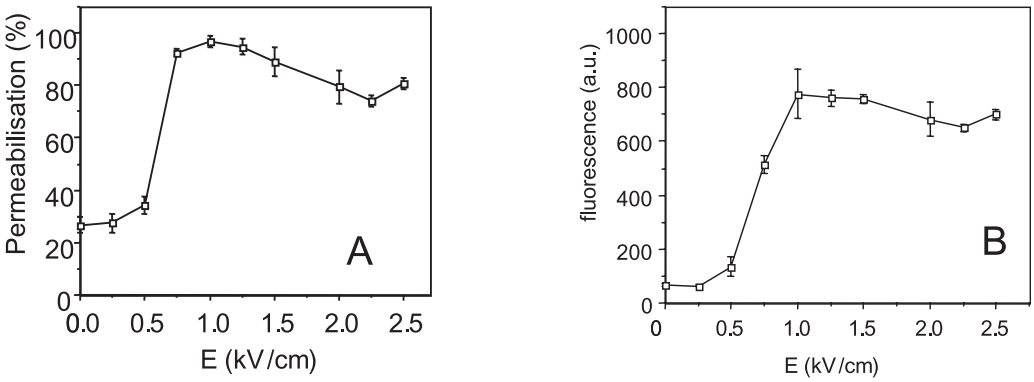

Figure 2. Control by the field on cell permeabilization. CHO cells (10^6 per ml) were treated by a train of 10 pulses lasting 1 ms at a frequency of 1 Hz with a flow rate of 1.2 ml/min. The pulsing buffer contains the hydrophilic dye PI. Permeabilization is assayed by the number of fluorescent cells (A) and by the mean fluorescence emission of the population (B) by use of a flow fluocytometer.

was homogeneous when taking into account the geometry of the chamber (parallel flat electrodes).

This average power associated to the train of pulses was:

$$\langle P \rangle = U\ I\ f\ T \qquad \text{(Eq. 4)}$$

T being the single pulse duration.
As the chamber resistance, when filled by the sample, could be approximated by

$$R = d\ /(\ \Lambda\ S) \qquad \text{(Eq. 5)}$$

where L is the conductance of the sample and S the section of the electrodes, then

$$\langle P \rangle = f\ T\ E^2\ \Lambda\ Vol \qquad \text{(Eq. 6).}$$

From Eq. 2, an increase in the flow rate Q while keeping the number of applied pulses N constant needs to increase FVolfVol, i.e. either F f or Vol (or both). Only a limited energy and current are delivered with each pulse. The power and current specifications are not requiring sophisticated designs. The only difficulty is to have a main power supply able to maintain the interpulse recharging of the internal capacitors when working at high frequencies.

Another important parameter is the nature of flow which is given by the Reynolds number Re

$$Re = (v\ d\ \rho\ )/\mu \qquad \text{(Eq. 7)}$$

Where v is the flow velocity, r, the volumic mass of the liquid, m its dynamic viscosity

In our experimental conditions, Re is always smaller than 2000, i.e. the flow is under a laminar condition. No tumbling is affecting the cell population.

Results

Effect of the field strength

A peculiar observation was that a rather high percentage of cells were PI positive just by flowing across the flow through chamber without any electrical treatment. This was much higher than the basal level in the native CHO cell population. This was associated with a decrease in the cell viability of about 30%.

A train of 10 pulses lasting each 1 ms was applied on the flow of cells at a frequency of 1 Hz (flow rate 1.2 ml/min). An increase in the number of PI positive cells and in PI stain-

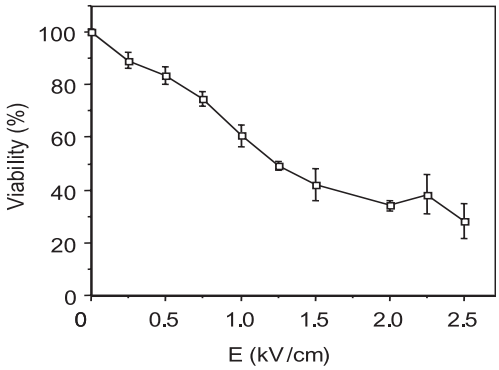

Figure 3. Viability of pulsed cells. CHO cells (10^6 per ml) were treated by a train of 10 pulses lasting 1 ms at a frequency of 1 Hz with a flow rate of 1.2 ml/min. Their viability was assayed 24 h after the treatment by the crystal violet test. The data were corrected from the effect of the flow on the control cells, which brought a 30% loss.

ing was clearly present as soon as the field strength of the applied pulses was a large as 0.5 kV/cm (Figure 2 A,B). A maximal effect was present around 1 kV/cm and a small decrease was observed for larger intensities.

The viability of the pulsed cells was affected in a field dependent way (all other parameters being kept constant). Cells in the flow process were apparently very sensitive to the field as shown by the effect of 0.25 kV/cm pulses. But cells were observed to be somehow resistant to very strong electric field (Figure 3). The loss in viability apparently leveled off above 1.5 kV/cm.

Effect of pulse duration

Cells were submitted to a train of 10 pulses at a frequency of 10 Hz and a magnitude of 1 kV/cm with a flow rate of 1.2 ml/min. These conditions were chosen by taking into account that when the pulse duration was 1 ms, all cells were permeabilized. We observed that even with a pulse duration as short as 0.1 ms the electrical treatment brought a permeabilization of all cells (Figure 4A). Increasing the pulse duration above this value did not induce any further change. PI staining was observed to increase continuously with the pulse duration (Figure 4B).

Cell viability was strongly dependent on the pulse duration. A sharp decrease was observed up to 0.2 ms followed by a slow decrease with a further increase of the pulse duration (Figure 4A).

Interestingly if the pulse duration was increased up to 5 ms, a much more limited lev-

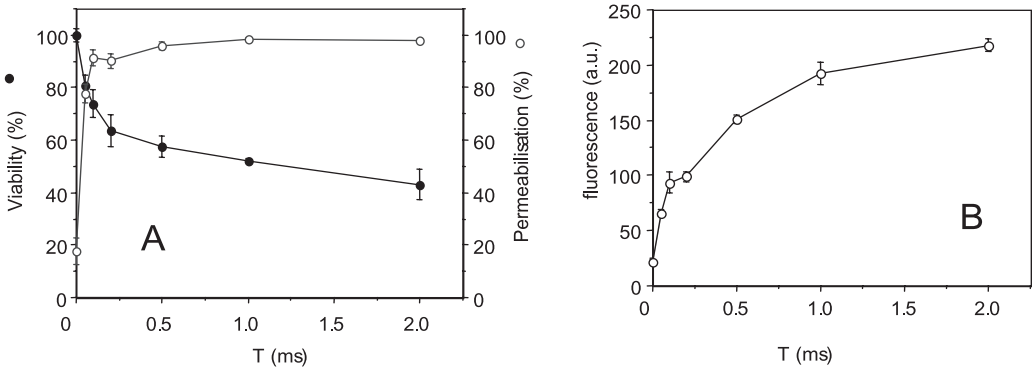

Figure 4. Effect of the pulse duration on cell permeabilization. CHO cells (10^6 per ml) were treated by a train of 10 pulses of 1 kV/cm at a frequency of 1 Hz with a flow rate of 1.2 ml/min. The pulsing buffer contains the hydrophilic dye PI. Permeabilization is assayed by the number of fluorescent cells (A) and by the mean fluorescence emission of the population (B) by use of a flow fluocytometer. Their viability was assayed 24 h after the treatment by the crystal violet test (A). The data were corrected from the effect of the flow on the control cells, which brought a 30% loss.

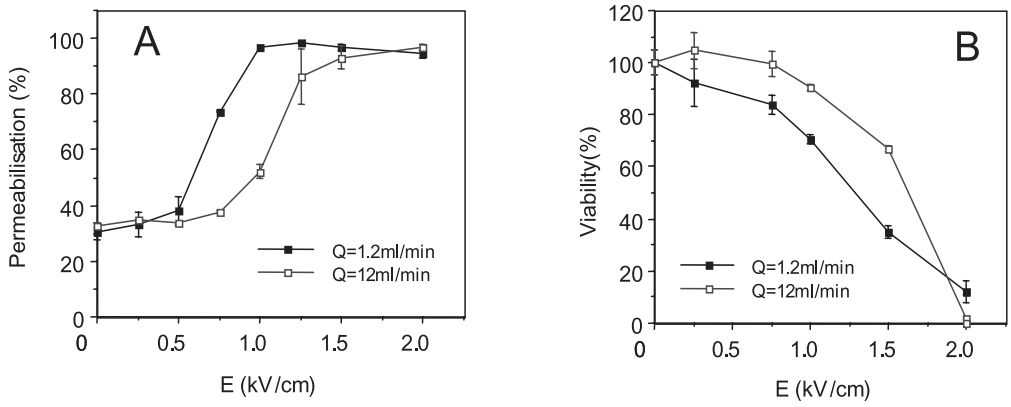

Figure 5. Effect of the flow rate on cell permeabilization. CHO cells (10^6 per ml) were treated by a train of 10 pulses of 1 kV/cm lasting 1 ms at a frequency of 1 Hz with a flow rate of 1.2 ml/min and 10 Hz with a flow rate of 12 ml/min. Permeabilization is assayed by the number of fluorescent cells (A). Their viability was assayed 24 h after the treatment by the crystal violet test (B). The data were corrected from the effect of the flow on the control cells, which brought a 30% loss.

el of permeabilization was observed and was observed to be associated to an irreversible process (cell death). No reversible permeabilization was observed.

Effect of the flow rate

Treatment of a large volume would take advantage of a high flow rate. We compared the behavior of cells when submitted to a train of 10 pulses of 1 ms. Their frequencies were adjusted to the flow rate, being 1 Hz at 1.2 ml/min and 10 Hz at 12 ml/min. The on-line monitoring of the signal on the oscilloscope showed that even under the 10 Hz procedure, the shape of the pulses remained square. The flow remained laminar under the two conditions.

Permeabilization was detected as soon as the field strength was larger than a critical value of 0.5 kV/cm. Its increase was sharper with a further increase in the field strength under the low flow rate conditions. All cells were permeabilized under the two conditions when a field as large as 1.5 kV/cm was applied (Figure 5 A).

Cell viability was less affected for a given field strength at the high flow rate (Figure 5 B). As a conclusion, reversible permeabilization was obtained under higher field conditions with the high flow rate.

Cytoplasmic protein release

We previously showed that flow-through electropulsation was an efficient approach for protein extraction from yeasts.[16] Clinical biotechnology is taking advantage of the bioproduction of proteins in mammalian cells.[19] CHO cells are one of the most successful cell factories. Results in the preceding part of this work was dealing with the loading of small (drug size like) molecules. In this part of the work we checked how effective was the flow-through electropulsation for the extraction of cytoplasmic proteins from CHO cells.

Cells were submitted to a train of 10 pulses lasting 1 ms at a frequency of 10 Hz with a flow rate of 1.2 ml/min. This pulse duration was chosen being shown in batch experiments to be needed to obtain macromolecule loading.[20] No protein release was observed with field strength up to 1 kV/mscm. High level of extraction was observed between 1.2 and 1.5 kV/cm (Figure 6). Interestingly as reported above the viability while affected by the electrical treatment remained larger than

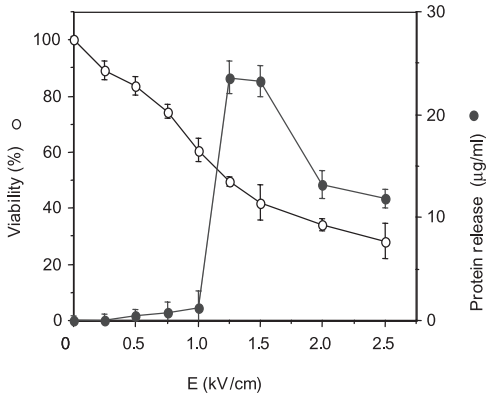

Figure 6. Electrorelease of cytoplasmic proteins. CHO cells (10^6 per ml) were treated by a train of 10 pulses of 1 kV/cm lasting 1 ms at a frequency of 1 Hz with a flow rate of 1.2 ml/min. Their viability was assayed 24 h after the treatment by the crystal violet test. The data were corrected from the effect of the flow on the control cells, which brought a 30% loss.

40% under the field conditions where the high electroextraction was obtained.

Discussion

The present study shows the flexibility and the effectiveness of the flow through electropulsation in the case of mammalian cells.

A high level of loading evaluated both in the number of PI positive cells and in the number of internalized polar molecules (as quantified by the fluorescence of PI in the present experiments) was obtained while preserving the viability of most cells. By using the 1 ms and 1 Hz conditions, we observed that up to 60% of the population could be loaded (permeabilized) and remained viable. This takes into account the fact that more than 20% of the population was killed by the flow. This clearly should be optimized for a clinical development of the method for cell therapytreatment. This can be optimized by taking into account that 0.1 ms pulses were effective to obtain a high level of loading. Short pulses may be less detrimental for cells.

Under laminar flow conditions (Re less than 2000), the maximal efficiency of loading was not affected by the flow rate up to 12 ml/min. Viability was sensitive to the flow rate. Higher flow rate can be obtained by an array of parallel chambers where all cells would be treated under the same conditions (electrical parameters, flow velocity).

The current which was delivered during the pulses under the optimized conditions (0.75 kV/cm) was only 1 Amp. The average power was 2.5 W under the high flow rate (12 ml/min, 10 Hz). This condition brought a temperature increase of only 2˚C of the cell suspension under the assumption that no heat dissipation occurred between the pulses. This conclusion further supports the safety of this approach for drug loading in cell therapy.

We observed a shift in the permeabilization /field strength plots when the flow rate was increased. This could result from the viscoelasticity of CHO cells. Their spherical shape observed under batch conditions would be altered by the drag of the flow. This drag increased with the flow rate. In simpler words, their shape turned in a more elongated one. It was well established that the sensitivity of a cell to an electric field was controlled by its shape and its orientation relative to the field lines.[21] Our observation that cell permeabilization needed higher field strength under the high flow rate where they were elongated in a direction perpendicular to the field was fully supported by the theoretical approach. This interpretation is further supported by the shift of the viability plot: elongated cells in a direction perpendicular to the field are less sensitive to the field

The protein extraction is clearly a very efficient new approach for clinical biotechnology. The observation that a large subpopulation was still viable after the train of 1 ms pulses suggested that it should be possible to recycle the pulsed population in a fermentor for a further growth of the cells. This is indicative that cell viability is not affected and

that electrochemical contamination if present is not harmful and furthermore can be decreased by a bipolar pulse. [22].

As a final conclusion, flow electropulsation offers a safe and powerful tool for the development of cell therapy

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