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Biomedical applications of electric pulses with special emphasis on antitumor electrochemotherapy

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Abstract

Short and intense electric pulses (EP) are regularly used in almost all molecular and cellular biology laboratories to introduce foreign DNA, as well as other exogeneous molecules, into living cells. Besides these in vitro applications, some in vivo applications have recently emerged. Biomedical application of EP is thus a new interdisciplinary field at the frontier of physics, chemistry and biology. This article intends to give an informative background and an overview of several presentations from the XIIth Symposium on Bioelectrochemistry and Bioenergetics that dealt with this subject, as well as from the two round tables organized by the authors [1]. Two procedures have already entered clinical trials: the electroinsertion of CD4 molecules on red blood cell membranes, which uses EP delivered ex vivo, and antitumor electrochemotherapy, which uses EP delivered in vivo. An overview of current research on the latter is given in more detail.

Keywords: Electrochemotherapy; Antitumor treatment; Electropermeabilization; Electroloading

1. Cell electropermeabilization

The delivery of appropriate short and intense electric pulses (EP) to living cells, either in suspension or in tissue, results in a transient and reversible alteration of their cell membrane [1]. Consequently, the plasma membrane becomes permeable to a large variety of hydrophilic molecules that are otherwise unable to diffuse through the plasma membrane. Thus these molecules, such as dyes, drugs, oligonucleotides, proteins and nucleic acids [2], can enter the cells. The use of EP to introduce DNA (electrotransfection) is now the most widespread technique used to genetically modify any kind of bacterial, plant, fungal or animal cells [3–5].

At the cellular level, cell electropermeabilization is understood and can be manipulated and controlled. The cell membrane insulates the cytoplasm from the external medium, and when a uniform and quasi-stationary electric field (E_{ext}) is applied to a suspension of cells, a transmembrane potential ($\Delta\phi_m$) is induced within a few microseconds [6]. Using a simplified hypothesis (spherical cells of radius r, membrane thickness and conductivity negligible), the transmembrane potential induced at the point of the plasma membrane defined by the polar angle with respect to the E_{ext} direction is given by the relationship:

$$\Delta\phi_m = 1.5 \times E_{ext} \times r \times \cos\Theta$$

Cell electropermeabilization is a threshold phenomenon: permeabilization of the membrane occurs only above a threshold value of the transmembrane potential $\Delta\phi_s$, fairly constant among biological membranes. $\Delta\phi_s$ corresponds to an applied electric field E_s (so that $\Delta\phi_s = 1.5 \times E_s \times r$) which depends on the radius of the cells.

At the molecular level, the phenomenon is not yet completely understood, in spite of intense fundamental research on molecular modifications occurring in membranes submitted to EP. During the EP, large areas of the cell membrane can be perturbed, but the perturbed area

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rapidly decreases after the end of the EP. However, long-lasting permeable structures remain present for long periods with respect to the EP length. The lifetime of permeable structures depends on the number of pulses applied, their duration and the temperature [7–9]. Several reports favor the idea of EP-induced lipid rearrangements that lead to the appearance of hydrophilic pores, termed electropores [10,11]. Consequently, the result of cell exposure to EP was termed electroporation. However electropores have never been observed using electron microscopy, except under artifactual conditions that do not prove their existence [12]. Another possible explanation of the phenomenon is the appearance of large hydrophobic pores resulting from an increase of lipid fluctuations in the lipid bilayer. These fluctuations are associated with changes in phospholipid polar head group orientation and with concomitant alteration of the structured water in the vicinity of the membrane polar groups [13]. Such changes could result in the observed rise of membrane permeability [14]. Since the exact mechanisms of the phenomenon at the molecular level are not yet available, the term electropermeabilization seems more appropriate than electroporation.

The increase in membrane permeability achieved by EP allows low molecular weight hydrophilic molecules (such as nonpermeant dyes, drugs, oligonucleotides, low molecular weight dextrans) to diffuse easily across the membrane [15–18]. Uptake can be detected even if these molecules are added after the EP delivery. The main requirement is obtaining permeabilization in the vast majority of cells with a concomitant good conservation of cell viability. Short (100 μs) square-wave pulses of an electric field intensity adjusted to the manipulated cell type (a determination that can be easily performed using the Lucifer Yellow assay [16] or similar tests) are adequate for the electroloading of low molecular weight hydrophilic molecules.

High molecular weight hydrophilic molecules (such as antibodies, enzymes, nucleic acids, high molecular weight dextrans) can also be introduced into the cells by means of EP ([19–22] and M. P. Rols et al., abstract P II-11 of the XIIth BES Symposium). In that case, the presence of the exogeneous molecule is required at the time of the EP delivery to the cells. Moreover, long pulses (several milliseconds long) give better results. It is possible that these molecules do not diffuse freely through the transient permeable structures and that an interaction between them and the membrane during the length of the electric pulse is required, in a sort of foot-in-the-door situation. Then, macromolecules would be completely taken up by the cells after the end of the EP.

Electroloading of exogeneous hydrophilic molecules is not the only possible utilization of cell electropermeabilization. The membrane structural perturbations of a cell in an electropermeabilized state allow proteins possessing a transmembrane hydrophobic domain to be inserted in the plane of the lipid bilayer. This phenomenon was named electroinsertion [23,24]. The electrically-induced modifications of the plasma membranes also promote a fusogenic state of the cells ([25,26] and M. Jaroszeski et al., abstract P II-5 of the XIIth BES Symposium). Electrofusion can occur between cells of the same or of different types, between cells and tissues [27] and between cells and vesicular structures such as liposomes [28]. Electrofusion can also allow the introduction of nonpermeant molecules into cells through their fusion with preloaded erythrocyte ghosts [29].

Electric pulses can be delivered (i) in vitro, on cultured cells in suspension [30] or attached to a substratum [31], (ii) ex vivo, on tissues [32,33] or on cells (e.g. blood cells) removed from living organisms and then reinoculated [34,35], or (iii) in vivo, i.e. in situ, on the living organisms [36,37]. Cell electropermeabilization in tissues treated ex vivo or in vivo has recently been demonstrated [38,39]. The diversity of the consequences of cell electropermeabilization and the variety of conditions under which cell electropermeabilization can be performed have opened the way for many biomedical applications of short and intense electric pulses.

2. Biomedical applications of electric pulses

Table 1 gives an overview of the feasible direct biomedical applications that are either in use or about to become reality.

The ex vivo electroloading of red blood cells was first described in 1978 [40], but was never used for clinical purposes. Recently, platelets and white blood cells have also been electroloaded ex vivo [34,35]. The basis and the feasibility of the continuous electroloading of pharmacological substances in white blood cells have been demonstrated [41]. An interesting feature of this approach is that a careful adjustment of the EP conditions allow the electroloading without a previous isolation of the white blood cells: the whole blood can be submitted to the EP and only the larger cells are electropermeabilized, with no modifications to the red blood cells. The electroloading of white

Table 1
Biomedical applications of electric pulses

In vivo delivery of short and intense electric pulses
Electropermeabilization
Transdermal drug delivery
Electrochemotherapy
Electrofusion
Electrofusion of cells to tissues
Electrofusion of liposomes to tissues
Ex vivo delivery of short and intense electric pulses
Electroinsertion of proteins in red blood cell membrane
Electroloading of active molecules into leukocytes

blood cells can be achieved during the continuous flow of the complete blood through a tube-type electrode. This biomedical application of EP was shown to be effective in achieving targeting of antibiotics in inflammatory foci. The white blood cells electroloaded with antibiotics were reinjected into rats, reaching inflammatory areas in vivo [42].

Electric pulse-enhanced transdermal drug delivery is the subject of several recent reports describing in vitro investigations using skin of various origins. Hitherto only preliminary preclinical results, obtained with non-active low molecular weight dyes, have been available ([43] and U. Pliquett and J. C. Weaver, abstracts P II-9 and O III-1 of the XIIth BES Symposium). The future of EP-enhanced transdermal drug delivery is still linked to results that have to be obtained with pharmacologically active substances. This application suffers the absence of a complete theory of the changes occurring at the level of the main barrier of the skin (the substratum corneum) as well as in the underlying cutaneous tissue. The suggested "electropermeabilization" of the lipid layers of the substratrum corneum or of the membranes of the corneocytes had, however, not yet been demonstrated. In addition to this, an alternative hypothesis is being developed: a Joule heating mediated phase transition of the lipids constituting the substratum corneum (J. Teissié, abstract O III-2 of the XIIth BES Symposium).

Another application of EP was presented by Dr. G. Hofmann during the symposium (abstract OII-5). The data concerning the electrointroduction of liposomes through the substratum corneum could be promising but need to be confirmed both experimentally and theoretically. In particular, it is necessary to determine whether during EP delivery electropermeabilization or electrofusion of liposomes with skin occurs. Indeed, cell to cell electrofusion as well as electrofusion of cells to tissues can be obtained. Cell to cell electrofusion was used in the production of monoclonal antibodies, by fusing antibody-producing cell lines with myeloma cells [44]. Cell to tissue electrofusion was well demonstrated in the case of human cells fused in vivo, i.e. in situ, to the cornea of rabbits [27]. This method possesses interesting potential for the development of new investigational tools. Human cells containing receptors to human-specific bacteria, such as the pathogen *Neisseria gonorrhoeae*, can be fused to naturally resistant tissues. Then, after the fusion of human cells to rabbit corneas, it is possible to study a human-restricted infection and to analyze the influence of potential antiinfectious treatments using an animal model ([45] and R. Gilbert et al., abstract P II-4 of the XIIth BES Symposium).

Transfer of relevant membrane molecules can be achieved not only from a cell membrane to another cell membrane, as in the case of cell electrofusion, but also by electroinsertion of proteins. Electroinsertion allows the transfer of purified membrane proteins directly from the external medium to a cell membrane ([23] and J. Teissié *et al.*, abstract P II-13 of the XIIth BES Symposium). Even if membrane proteins possessing only one transmembrane domain are electroinserted with high efficiency, there are several potential clinical applications. Promising preclinical data have been obtained after electroinsertion of the full-length recombinant CD4 molecule (the receptor of the AIDS agent, the Human Immunodeficiency Virus) in red blood cells [46]. Preliminary clinical evaluation presented at this symposium by Dr. C. Nicolau (abstract S VII-3) has shown that such $CD4^+$ tagged red blood cells were able to reduce the load of HIV in seropositive patients. The basis of this application, as well as that of electrochemotherapy, described below, is fairly well understood and clinical trials are soundly based on extensive in vitro and preclinical studies.

Table 2
Antitumor electrochemotherapy

Transplanted subcutaneous tumors			
B 16 melanoma	in C57B1/6 mice	Villejuif (F)	Mir et al., 1991
B 16 melanoma	in C57B1/6 mice	Tampa (FL)	Heller et al., 1994
B 16 melanoma	in C57B1/6 mice	Ljubljana (SI)	Serša et al., 1994
LPB sarcoma	in C57B1/6 mice	Villejuif (F)	Mir et al., 1991
KB carcinoma	in nude mice	Villejuif (F)	Mir et al., 1991
SA 1 fibrosarcoma	in A/J mice	Ljubljana (SI)	Serša et al., 1994
Ehrlich tumor ascites	in CBA mice	Ljubljana (SI)	Serša et al., 1994
MBT-2 tumor cells	in C3H mice	Fukushima (J)	Yamaguchi et al., 1994
Spontaneous subcutaneous tumors			
Breast adenocarcinoma	in C3H/Bi mice	Villejuif (F)	Belehradek et al., 1991
Fibrosarcoma	in cats	Alfort (F)	Mir et al., submitted
Transplanted internal tumors			
RG2 glioma	in Fisher rat brain	Lund (S)	Salford et al., 1993
VX2 carcinoma	in rabbit liver	Villejuif (F)	Ramirez et al., submitted

Preclinical trials performed using bleomycin and runs of 4 or 8 square-wave electric pulses of 100 μs and 1000–1500 V cm^{-1} (subcutaneous tumors) or 800 V cm^{-1} (internal tumors) delivered at the frequency of 1 Hz.

Table 3
Antitumor electrochemotherapy: situation of the clinical trials (December 1994)

Trial	M.D. responsible for the trial	Ph.D. colloborating with the medical team	Type of tumors treated	Number of patients
Villejuif (F)	C. Domenge	L.M. Mir/S. Orlowski	Head and neck C.	14
			Breast A.	1
Toulouse (F)	J.M. Bachaud	J. Teissié/M.P. Rols	Head and neck C.	2
			Melanoma	4
			Kaposi's sarcoma	1
Tampa (FL)	D. Reintgen	R. Heller/R. Gilbert	Melanoma	3
			Basal cell C.	2
			Breast A.	1
Ljubljana (SI)	Z. Rudolf	G. Serša/D. Miklavčič	Melanoma	6
Reims (F)	P. Coninx	L.M. Mir/A. Penet	Head and neck C.	3

3. Antitumor electrochemotherapy

Chemotherapy is hampered by a poor targeting of the existing drugs to the tumor cells. This is also the reason for secondary effects that limit the amounts of drug that can be administered. Bleomycin (BLM) is a nonpermeant cytotoxic drug, unable to diffuse through the plasma membrane, and is only carried in low amounts into the cells by specific receptors [47]. This mechanism of uptake limits BLM cytotoxicity, even when cells are exposed to huge extracellular concentrations. Cell electropermeabilization enables efficient BLM electroloading and thus allows the drug to be highly cytotoxic at concentrations several orders of magnitude lower than those necessary to kill unpermeabilized cells [30,48]. In vivo, antitumor efficacy of systemically (intramuscularly or intravenously) administered BLM is also highly potentiated by the delivery of short and intense EP at the level of the tumor. This biomedical application of EP, a new antitumor treatment, was termed electrochemotherapy ([49,50] and L. M. Mir, abstract S VII-2 of the XIIth BES Symposium). Runs of 4 or 8 square-wave EP of 100 μs were delivered at a frequency of 1 Hz [36,51]. The electric field intensities applied were 1000–1500 V cm^{-1} for the treatment of subcutaneous tumors using transcutaneously delivered EP and 800 V cm^{-1} for the treatment of internal tumors using EP directly delivered to the tissues through inserted electrodes ([52] and L. H. Ramirez et al., abstract P II-8 of the XIIth BES Symposium). Table 2 summarizes the preclinical data obtained using such EP on different animal tumors in Villejuif and in other laboratories [36,51–55].

Exponentially decaying pulses, as well as single square-wave EP, both of higher amplitudes and durations, were used by Okino et al. [56–58]. However, they observed a limited antitumor efficacy and more side effects than those obtained under our treatment conditions. Other attempts to find new ways for bleomycin delivery, such as intratumoral drug injection (R. Heller et al., abstract O III-3 of the XIIth BES Symposium), or to evaluate the efficacy of other cytotoxic drugs such as cisplatinum (G. Serša et al., abstract O III-5 of the XIIth BES Symposium), are under evaluation and can provide further extensions to electrochemotherapy. Moreover, an immunological component was evident in the electrochemotherapy antitumoral effects, manifesting in the destruction of residual tumor cells [59,60]. After appropriate local immunostimulation using Interleukin-2-secreting cells injected into in the electrochemotherapy treated tumors, systemic antitumor effects were obtained [61].

The first clinical phase I-II trial was initiated at the Institut Gustave-Roussy. Patients with small permeation nodules of head and neck squamous cell carcinomas entered this study and 50% of the treated nodules disappeared. Moreover, treatment was conveniently tolerated and no major side-effects were in evidence [62]. At present, several groups (Table 3) have begun to treat various types of subcutaneous tumor in patients with encouraging results (to be reported). These trials, as well as future trials on internal tumors made possible by the current preclinical data (Table 2), will allow the evaluation of electrochemotherapy. However, the absence of side-effects noticed up to now and the rapid detection of antitumor effects in these early studies add to the confidence in the future of this method.

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