(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 September 2001 (27.09.2001)

PCT

(10) International Publication Number
WO 01/70928 A1

(51) International Patent Classification[7]: C12M 3/00, C12N 15/87

(21) International Application Number: PCT/GB01/00899

(22) International Filing Date: 2 March 2001 (02.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0006655.5 21 March 2000 (21.03.2000) GB
0100583.4 10 January 2001 (10.01.2001) GB

(71) Applicant *(for all designated States except US)*: **SKWARCZUK, Vanda** [GB/GB]; 17 Co-operation Street, Failsworth, Manchester M35 9AL (GB).

(71) Applicant and
(72) Inventor: **SHIRKHANZADEH, Morteza** [CA/CA]; Apt. 312, 17 Eldon Hall Place, Kingston, Ontario K7M 7H5 (CA).

(74) Common Representative: **SKWARCZUK, Vanda**; 17 Co-operation Street, Failsworth, Manchester M35 9AL (GB).

(81) Designated States *(national)*: AT, AU, CA, CU, DE, DK, ES, FI, GB, IS, JP, LU, NO, NZ, PT, RU, SE, SG, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/70928 A1

(54) Title: METHOD AND APPARATUS FOR ELECTROPORATION OF CELLS USING ELECTRICAL PULSES OF LONG DURATION

(57) Abstract: A method and device are disclosed for introducing exogenous material into cells by electroporation using electrical pulses of long duration. The device comprises means for holding cells and a medium containing exogenous material between two electrodes, said electrodes comprising a cathode made of palladium metal and an anode made of palladium metal charged with hydrogen (Pd-H), and means for delivering an electrical pulse across said electrodes. The electrodes of the present invention have the capability of efficiently eliminating the hydrogen and oxygen gas bubbles generated at the electrodes' surface during the application of electrical pulses of long duration. Thus, unlike conventional electroporation devices, the device of the present invention allows cells to be efficiently transfected using electrical pulses of long duration without encountering the problems associated with the presence of gas bubbles during the electroporation process.

METHOD AND APPARATUS FOR ELECTROPORATION OF CELLS USING ELECTRICAL PULSES OF LONG DURATION

Field of the Invention

This invention relates to a method and apparatus for transfecting cells with exogenous materials by electroporation.

Background of the Invention

Electroporation is a technique in which cells are electrically stimulated to take up materials such as DNA and drugs from their surrounding medium. This is achieved by placing the medium containing the material and cells between two electrodes and subjecting the cells to an electrical impulse (Chang, D.C., Guide to Electroporation and Electrofusion, Academic Press, San Diego, CA, 1992). A suggested mechanism for the electroporation phenomena involves the formation of transient holes or pores in the cell membrane through which the surrounding medium containing the material may enter the cell (Neuman et.al., (1982) EMBO J., Vol.1, 841-845, Wong et.al(1982) Biochem and Biophys. Research Commun. 107, 584 - 587).

In most practical applications, cells are electroporated in suspension by placing cells between two uniformly spaced parallel electrodes. Examples of electroporation devices for the electroporation of cells in suspension are commercially available cuvettes that are fitted with parallel electrodes and electrode assemblies such as the one disclosed in the US patent 5,422,272 (Papp et.al, 1995). In recent years there has been much interest also in developing devices for the in situ electroporation of attachment-dependent cells in order to avoid removing cultured cells from their substratum. Such attachment-dependent cells are normally grown on the inner surface of a non-conductive container such as a petri dish or a tissue culture bottle. In situ electroporation of such cells can be carried out by, for example, positioning an electrode assembly close to the plated cells consisting of a pair of electrodes which serve as the "ground" (-) and "high voltage" (+) electrodes. The electrodes can be in the form of parallel plates or concentric rings. In addition to parallel plates and concentric rings, other configurations such as multiple plates and multiple concentric rings can be used. Electrode assemblies with these characteristics have been disclosed by Chang (US patent 4,822,470, 1989). Other types of electrode assembly comprising electrode array patterns that may be formed by depositing gold or

platinum on a non-conductive support have also been disclosed by Meyer (PCT application, WO 98 / 12310, March 1998). This method allows the fabrication of much smaller electrodes on the bottom of an electrode carrier for in situ electroporation of cells with smaller electrode separations in the range of 50 –100 μm.

Other devices for in situ electroporation have also been developed where the cells to be electroporated are directly cultured on optically transparent, electrically conductive electrodes or electrode arrays made of metals that are deposited on the inner surface of, for example, a tissue culture dish. Such electrodes serve both as substrates for culturing cells and as means of delivering electric pulses to the attached cells. Devices of this kind are disclosed by Baer (US patent 5,128,257, 1992) and Casnig (US patent 5,134,070, 1992). In other electroporation devices developed for the in situ electroporation of attachment dependent cells, cells are plated on a microporous membrane that is positioned between two closely spaced and parallel electrodes. Devices of this type are described by Klenchin et.al (Biophysical Journal, (1991), Vol.60, 804 – 811) and Yang et.al.(Nucleic Acids Research (1995), Vol.23, No.15, 2803 – 2810).

In addition to the in vitro applications of the electric field described above, there is also a broad range of in vivo applications, including the use of low –level direct electrical current therapy for destroying tumours (Griffin et.al., British Journal of Cancer (1995) 72, 31-34), bone repair , wound healing, nerve regeneration, and delivery of therapeutic agents using in vivo electroporation. Electroporation has been used in vivo to introduce foreign material such as DNA and drugs into living cells in body. In particular, electroporation has been used in vivo in a process termed electrochemotherapy for introducing anti-cancer agents into tumour cells (Mir et.al. , European Journal of Cancer (1991), Vol.27 , 68-72). Electrochemotherapy is carried out by infusing an anticancer drug directly into the tumour and applying an electric field to the tumour between a pair of electrodes. Electrodes are provided in various configurations such as, for example, a calliper that grips the epidermis overlying a region of cells to be treated or needle-shaped electrodes that may be inserted into the patient, to access more deeply located cells.

Plate-type electrodes aligned in parallel have been proposed to provide a uniform electric field for electrochemotherapy delivered transcutaneously (U.S. Pat.No. 5,468,223 to Mir) and U.S.Pat. No. 5,439,440 to Hofmann describes an electrode assembly for in vivo electroporation of cells that consists of parallel arrays of needle electrodes mounted on a dielectric support member. Electroporation is particularly promising for chemotherapy because some of the most effective anti-cancer drugs, such as Bleomycin, cannot successfully penetrate the membranes of certain cancer cells under normal conditions. With the application of an electric field near tumours, however, it is possible to insert the Bleomycin effectively into the cells. Electroporation in this application is especially beneficial because it can help minimize the amount of implant agent used and thus provides a means for preventing the harmful effects associated with administration of anticancer or cytotoxic agents.

In both in vitro and in vivo electroporation of cells, a high electric field is applied between electrodes to induce membrane breakdown in the targeted cells. The electric field is normally applied in a pulsed form to prevent irreversible cell damage. Waveforms, which are often used, include rectangular pulse ("square pulse") normally generated by a function generator, and the exponential decay pulse which is generated by discharging a capacitor that has been precharged at a high voltage. In the latter case, the pulse width is characterized by the decay constant τ, which depends on the capacitor selected and the ionic conductivity of the electroporation medium.

Successful electroporation is critically dependent on both biological variables and the characteristics of the applied pulse. The major electrical parameters affecting the success or efficiency of electroporation include the electric field strength, pulse wave shape, duration of the pulse (pulse length), and number of pulses applied. The most critical electrical factor for efficient electroporation is known to be the peak electric field strength generated by the electrical voltage and it is generally accepted that a threshold field intensity is required to observe the occurrence of permeabilization. It has been observed that when an electrical pulse of very short duration is used, the field strength

required for the effective poration is usually so high that the cell viability becomes unacceptably low. On the other hand, when the pulse length is large, the required field strength for successful electroporation of cells is low (Chu et. al., Nucleic Acids Res., Vol.15 (1987) 1311). Thus it appears that, to a certain extent, the field strength and the pulse length can compensate each other. In practice, the best choice of electric field strength is normally a compromise setting such that the needs for both electroporation efficiency and cell viability can be reasonably satisfied.

Besides the above parameters, the conductivity of the electroporation medium also plays an important role in the successful electroporation of cells. Thus alteration in the ionic strength of the electroporation medium can significantly affect the characteristics of the electric pulse delivered and may also change the optimal voltage required for efficient electroporation of cells. It is therefore important to monitor the shape, duration and voltage of each impulse by a storage oscilloscope to ensure that the characteristics of the delivered pulse remain unchanged from experiment to experiment.

A considerable number of studies aimed at understanding the permeabilization of biological membranes have been conducted in the past, and many parameters have been explored for optimal permeabilization (Chang et.al., Guide to Electroporation and Electrofusion, Academic Press, San Diego ,CA ,1992, Shigekawa and Dover, Biotechniques, Vol.6 (1988), 742-751). Many of the earlier studies in this field were performed by applying very short pulses (5 - 50 μ s) with high field strength (2 -10 kV/ cm). Recent studies, however, show that more efficient electroporation of cells can be achieved with rather weaker pulses (about 0.5 kV/cm) with a pulse length longer than 10ms (Teruel et.al., Biophysical Journal, Vol.73 (1997), 1785-1796). The long pulse duration apparently enhances electrophoretic movement of exogenous materials such as DNA molecules toward the cells and increases the concentration of these materials near the cell's membrane surface (Sukharev et.al., Biophysical Journal, Vol.63 (1992) 1320-1327) . This, apparently, facilitates the insertion of the exogenous materials and results in more efficient transfection of cells. At present, this " long-pulse / weak field" approach is frequently recommended, particularly when exponential pulses are used to

electroporate mammalian cells (Chang, D.C, Guide to Electroporation and Electrofusion, Academic Press, San Diego, CA ,1992).

Improved electrotransfection of cells has also been reported in cases where pulses of low voltage were applied for a relatively long periods shortly after application of an initial intense pulse of short duration (Andreason et.al., Anal. Biochem. Vol.180 (1989),269-275). Sukharev et.al. (Biophysical Journal, Vol.63 (1992) 1320-1327) have used a "two pulse" technique and have reported improved transfection of cells induced by electric pulses of long duration. The first pulse applied in this case was 6 kV / cm for 10 μs followed by the second pulse of much weaker intensity but longer duration (0.2 kV / cm for 10 ms). These studies suggest that introduction of exogenous materials such as DNA into cells by electroporation may be mechanistically complex and may require high voltage for the initial poration of cells followed by low voltage pulses of relatively long duration to maximize the transfer of exogenous materials into cells by electrophoresis. The application of low intensity pulses with long pulse duration also appears to be promising for the in vivo electroporation of cells. For example, experiments with various tumour cell lines have shown that low intensity pulses with long durations are equal or better than high intensity pulses with short pulse durations in terms of tumour cell killing (Hoffman, U.S.Pat. No. 6,055,453).

Despite the advantages associated with the use of pulses of long duration for the effective electroporation of cells, the application of this technique is limited due to the formation of gaseous hydrogen and oxygen during the course of the electroporation process. Generally, when an electric pulse of long duration (i.e., $\tau \geq 10$ ms) is applied to the electrodes in an electroporation device, the passage of a relatively large amount of charge through the electroporation medium results in the conversion of ions to considerable amounts of hydrogen and oxygen gas at the negative and positive electrodes of the electroporation device, respectively. A large amount of visible gas bubbles may be formed between the electrodes under these conditions as has been demonstrated by Chu et.al.(Nucleic Acid Res. Vol.15, No.3 (1987) 1311-1326). Oxygen bubbles are formed at the anode (positive electrode) according to the following reaction:

$$2\,H_2O \rightarrow O_2 + 4H^+ + 4e^-$$

and hydrogen bubbles are formed at the cathode (negative electrode) according to the following reaction:

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^-$$

The formation of a large amount of hydrogen and oxygen gas bubbles may severely disrupt the normal function of cells in suspension as well as cells cultured, for example, on a solid substratum (e.g. a glass plate or a micro- porous membrane) within an electroporation device. For example, attachment-dependent cells are normally grown on the inner surface of a non-conductive container such as a petri dish or a tissue culture bottle. In situ electroporation of such cells can be carried out by, for example, positioning an electrode assembly very close to the plated cells consisting of a pair of electrodes which serve as the "ground" (-) and "high voltage" (+) electrodes. The electrodes can be in the form of parallel plates or concentric rings. The distance between electrodes and the cell monolayer is normally about 1 mm or less and in some cases electrodes are placed directly on the cell monolayer(Liang et.al., BioTechniques, Vol.6, No.6, (1988), 550-558). In such cases, the gas bubbles generated can severely affect the viability of cells in close contact with the electrodes. In particular, in electroporation devices where cells are cultured on the electrode surface, such as those disclosed by Baer (US patent 5,128,257, 1992) and Casnig (US patent 5,134,070 , 1992), gas bubbles can seriously affect the attachment of the cells to the electrodes, and this may in turn adversely affect normal cellular functions and cell viability. This obviously imposes restrictions on the use of such devices in cases where pulses of long duration are required for the efficient electroporation of cells. On the other hand, the formation of a large amount of gas bubbles can interfere with the process of electroporation itself by blocking

the electrodes' surface and by altering the conductivity of the electroporation medium in the close vicinity of the electrodes. Furthermore, the random formation of gas bubbles on the electrodes' surface may disturb the uniform electric field intensity that is required for the uniform electroporation of cells.

Another major problem associated with gas bubbles during electroporation is the formation of foam in the electroporation medium in the presence of substances such as proteins that can be adsorbed at the gas-liquid interface. Electroporation of cells in the presence of antibodies, for example, has been shown to result in the formation of a significant amount of foam as a result of electrolysis (Bright et.al., Cytometry (1996) , Vol. 24, 226-233). Stabilization of foams by proteins involves adsorption of proteins at the surface of gas bubbles, surface denaturation , and finally coagulation. It is well known that proteins and other macromolecules of biological importance which may be used as implant agents for insertion into cells can be adsorbed at the surface of gas bubbles and form stable foams even at low concentrations and that their foaminess can be influenced by pH and salts (Kotsaridu et.al., Eur. J. Appl. Microbiol Biotechnol (1983), Vol.18, 60-63) . The adsorption of implant agents at the gas-liquid interface and the formation of the foam can substantially change the concentration of these substances in the electroporation medium. This would obviously lead to significant variations in the electroporation efficiency and in the transfer of exogenous material into cells since the successful transfer of the exogenous material into cells is directly related to the concentrations of these substances in the electroporation medium.

Although the mechanism of gas evolution in electroporation devices has not been previously studied in detail, the events leading to gas evolution in these devices are similar to those in other well-studied electrochemical systems equipped with gas evolving electrodes. A considerable amount of information is available in the literature concerning the mechanism of gas evolution and the effect of gas bubbles on the distribution of the electric current and voltage in electrochemical cells equipped with parallel electrodes (Janssen, L.J.J and Visser,G.J , J. Applied. Electrochemistry, Vol.21 (1991) 753 – 759 ; Vogt, H , J. Applied Electrochemistry, Vol.29 (1999) 1155 – 1159 ; Vogt, H,

Electrochimica Acta, Vol. 29 (1984) 175 –180). This information can be useful in interpreting the events that take place in electroporation devices. When pulses of short duration are applied to the electrodes in an electroporation device, the total quantity of electrical charge is usually low and thus the interfacial concentration of dissolved hydrogen and oxygen at the electrodes does not normally exceed the critical level required for the transformation of hydrogen and oxygen into a gaseous phase. Hydrogen and oxygen molecules are, therefore, transported to the bulk of the electroporation medium by molecular diffusion without the formation of gas bubbles. However, when pulses of long duration are used, a large amount of charge would normally pass through the electroporation device. Under these conditions, the interfacial concentrations of dissolved hydrogen and oxygen exceed the critical level necessary for transformation of hydrogen and oxygen into a gaseous phase. Gas bubbles, therefore, form at the nucleation sites on the electrodes in a random fashion and grow in size. Gas bubbles may interact and finally touch each other and may cover the whole or part of the electrode surface, forming an unstable gas film ("bubble curtain") which rapidly collapses and reforms. Gas bubbles attached to the electrodes' surfaces reduce the active surface area of the electrodes and result in a non-uniform distribution of current and the establishment of a non-uniform electric field during the course of the electroporation process. The coverage of the electrodes' surfaces with gas bubbles can be especially serious in cases where the dimensions of the electrodes and the spacing between them are comparable with the size of gas bubbles (i.e., when the dimensions of electrodes are in the range of 20 - 100 μm). Examples of small electrodes of this type are electrodes disclosed by Baer (US patent 5,128,257 , 1992) . Gas evolution can be particularly critical in electroporation devices such as those described by Klenchin et.al (Biophysical Journal, (1991), Vol.60, 804 – 811) and Yang et.al.(Nucleic Acids Research (1995), Vol.23, No.15, 2803 – 2810) where cells are cultured on a microporous membrane and placed between two flat electrodes during the electroporation process. In such devices gas bubbles may get entrapped between the microporous membrane and the electrodes and this may result in substantial changes in the interfacial electrical resistance during the electroporation process. This can be specially critical in electroporation devices where the microporous membrane is in close contact with one or both electrodes as in the

electroporation device described by Klenchin et.al (Biophysical Journal, (1991), Vol.60, 804 – 811).

The problem associated with gas bubbles can also be critical in devices which are designed for the in situ electroporation of cells and which are equipped with horizontal electrodes facing downward. An example of electroporation devices of this kind is the device described by Meyer (PCT application, WO 98 / 12310, March 1998) that is equipped with an interlinked set of electrodes in the range of 120 to 340 μm formed on the bottom of an electrode carrier. When pulses of long duration are applied to such electrodes, gas bubbles may accumulate under the electrodes as a result of the buoyancy forces acting on bubbles and thus, electrodes may become partially isolated from the electroporation medium. On the other hand, in electroporation devices that are equipped with vertical electrodes, gas bubbles may depart from the electrodes when they have reached a sufficient size and may remain dispersed in the electroporation medium in the interelectrode spaces and this may provide an additional local and non-steady state condition within the electroporation device. As the electric conductivity of gas bubbles is practically equal to zero, the current conducting sectional area in such cases would be restricted to the liquid phase. A bubble-liquid mixture can be considered as a random dispersion of spherical bubbles. The Bruggman equation can be used to calculate its ohmic resistance (De La Rue,R.E and Tobias, C.W, J. Electrochem. Soc. ,Vol 106 (1959)). The Bruggman equation is :

$$R = R_p (1 - \varepsilon)^{-3/2}$$

where R is the ohmic resistance of the bubble-containing solution of a void fraction, ε (volume fraction of gas in liquid) and R_p is the ohmic resistance of the bubble-free solution.

Thus, when an electric pulse of long duration is applied, the resistance of the medium may be significantly altered as the volume fraction of gas in the liquid reaches a high

level. The volume fraction of gas in the electroporation medium can especially reach to a critical level when one uses a small volume of electroporation medium. An example of a device which is especially designed for electroporation of cells in a small volume of medium is the device described by Teruel et.al.(Biophysical Journal, Vol.73 (1997), 1785-1796). The small volume of the medium allows minimizing the high cost associated with the exogenous materials such as drugs, DNA etc. However, the volume fraction of gas in such a small volume of medium can be considerably high when one uses electrical pulses of long duration. This may significantly alter the local resistance of the medium close to the electrodes, particularly when successive pulses of long duration are applied. Application of successive pulses of long duration is indeed a common procedure for the electroporation of cells in devices of this type. For example, in experiments conducted by Yokoe and Meyer (Nature Biotechnology. Vol.14 (1996) 1252 – 1256), electroporation was performed at 350 V / cm, using three voltage pulses, each 40 ms long and 40 s apart. The significant change in the local resistance of the medium as a result of gas evolution in such cases may result in an unpredictable variation in the pulse shape and pulse length from experiment to experiment, yielding variable results. The problem can be more serious when exponential-decay pulses are employed for the electroporation of cells since in such cases the pulse length, τ is directly related to the resistance of the medium in accordance with the following equation

$$\tau = RC.$$

where R is the resistance of the medium and C is the capacitance.

Thus, in order to reproduce experiments with precisely the same pulse shape and pulse length, one must ideally maintain the resistance of the medium constant by eliminating gas bubbles in an extremely short time. Even when one uses power sources that theoretically generate rectangular pulses with fixed pulse length, the unsteady state conditions associated with the formation of gas bubbles in the close vicinity of electrodes can significantly distort the output waveform during the electroporation of cells. Furthermore, the non-uniform distribution of gas bubbles in the vicinity of the electrodes

and in the inter-electrode spaces results in local heterogeneities in the electric field and, therefore, some cells may not receive adequate electric pulses with the required optimum voltage. It is well known that when long pulses in the msec range are used, small changes in field strength below or above the optimum level can result in a significant decrease in the yield of transfectants.

From the above discussion, it is obvious that fast removal of gas bubbles out of the inter-electrode spaces is an essential demand in designing electroporation devices for applications where pulses of long duration are applied for the electroporation of cells. Teruel et.al (Biophysical Journal, Vol.73 (1997), 1785-1796) have realized this problem and designed an electroporation device with vertical electrodes with an inter-electrode gap of 5 mm and equipped with especially designed slits which allow the oxygen and hydrogen gas bubbles generated at the respective electrodes to rise and exit from the device. This method, however, does not allow efficient and instantaneous removal of gas bubbles. This is because the gas bubbles formed on the electrodes' surfaces depart from the electrodes' surfaces and rise only when they have grown to a certain size. Thus, in practice gas bubbles are removed very slowly and the electrodes remain constantly covered with adherent gas bubbles during the entire electroporation process. Furthermore, it is understandable that the application of conventional methods of gas removal such as that described by Teruel et.al can be severely restricted when the gap between the electrodes is very small. For example, with "parallel –plate electrodes" which are often used for the electroporation of cells in suspension, it is desirable to have the electrodes very close to each other so that lower voltages can be employed to achieve the desired field strength. Thus, ideally, electrode assemblies are manufactured with extremely small inter-electrode gaps of less than 0.5 mm (US patent 5,422,272, Papp et.al, 1995). Such extremely small inter-electrode gaps can impose severe restrictions on the rapid removal of hydrogen and oxygen gas bubbles in a very short period during the electroporation of cells.

The fast removal of gas bubbles can be similarly restricted in devices which are normally used for the in situ electroporation of cells and which are equipped with horizontal

electrodes facing downward such as the devices described by Casnig (US patent 5,134,070 , 1992) , Meyer (PCT application, WO 98 / 12310 , March 1998), and Klenchin et.al (Biophysical Journal, (1991), Vol.60, 804 – 811). In these cases, gas bubbles may get entrapped under the electrodes and the removal of gas bubbles by conventional methods in a short time period would be extremely difficult if not impossible. In particular, in the device described by Klenchin et.al (Biophysical Journal, (1991), Vol.60, 804 – 811) in which cells are grown on a microporous membrane that is placed between two flat electrodes, gas bubbles may get entrapped between the membrane and the electrodes. Microporous membranes with a pore size of about 0.45 μm and a pore density of about $1x\ 10^{6}$ pores / cm^2 are normally used for this application since membranes with a pore size of about 0.45 μm are transparent and allow study of electroporated cells by light microscopy. However, such microporous membranes normally act as resistors since the electric discharge during the electroporation process can only occur through the pores in the membrane. Accumulation of adherent hydrogen and / or oxygen bubbles at the electrode- membrane interface can result in the blockage of a significant number of pores during the electroporation process. This is because gas bubbles normally have a relatively large diameter in the range of 20 to 100 μm and can cover a significant number of pores. This in turn results in significant local heterogeneities in the electric field during the electroporation of cells. Thus some cells may not receive the optimum field strength that is required for electropermeabilization .

Problems associated with gas bubbles may also be encountered with other downward-facing electrodes in cases where there is relative movement between the electroporation medium and the electrodes as in flow-through electroporation chambers where a suspension of cells and DNA, for example, is continuously subjected to a high electric field between two electrodes. Furthermore, as discussed before, in devices where cells are cultured on electrodes such as the device described by Casnig (US patent 5,134,070 , 1992), gas bubbles may disturb the cells attached to the electrodes and this may adversely affect the viability and normal function of the cells. Obviously, the conventional method used by Teruel et.al is entirely ineffective in alleviating the problems in such devices. Ideally, therefore, gas evolution should be prevented from the start in cases where cells

are cultured directly on electrodes or when cells are plated on a microporous membrane that is placed in the close vicinity of electrodes.

The problems associated with gas evolution may also be encountered during in vivo electroporation of cells particularly when a train of low intensity pulses with long durations are applied for the effective electroporation of cells. Under these conditions a significant amount of electrical energy would be converted into chemical energy through electrochemical reactions at the electrode-tissue interface. Substances dissolved in the tissue are consumed in the electrode reactions and new species that may have toxic effects on healthy tissue and cells are produced. These include formation of oxygen gas bubbles and toxic radicals at the anode and the evolution of hydrogen gas at the cathode. Gas evolution at the electrode-tissue interface can promote the transport and spreading of toxic species to the surrounding healthy tissues by convection. In addition, considerable variations in electrical resistance at the electrode-tissue interface can occur as a two-phase (liquid and gas) layer can be created close to the electrodes. The non-uniform distribution of gas bubbles in the vicinity of electrodes can further result in the establishment of a non-uniform current distribution and electrical field during the electroporation process.

Summary of the Invention

It is a principal object of the present invention to create an improved procedure for the electroporation of cells in which hydrogen and oxygen gas bubbles are effectively and rapidly eliminated during the electroporation process.

Thus in accomplishing this objective, there is provided in accordance with one aspect of the present invention a method for the electroporation of cells comprising the steps of placing cells and a medium containing exogenous materials between two electrodes, said electrodes comprising a cathode made of palladium (Pd) metal and an anode made of

palladium metal charged with hydrogen (Pd- H) , and applying an electrical pulse across said electrodes for electroporating cells.

Another aspect of the present invention is a device for the in vitro electroporation of cells comprising means for holding cells and a medium containing exogenous materials between two electrodes, and means for delivering an electrical pulse across said electrodes, said electrodes comprising a cathode made of palladium (Pd) metal and an anode made of palladium metal charged with hydrogen (Pd-H).

Yet another aspect of the present invention is a device for the in vivo electroporation of cells for use in electrochemotherapy and gene therapy, comprising a plurality of anodes and cathodes made of palladium (Pd) and palladium charged with hydrogen (Pd-H) respectively.

It should be noted that the materials used for constructing electrodes in prior art electroporation devices have been selected from a range of conductive materials such as aluminium, stainless steel, platinum, gold, silver, and transparent semiconductors such as indium tin oxide. In selecting electrode materials, factors such as electrical conductivity, chemical stability, mechanical stability, biological inertness and cost have been considered. Thus, it has been important that electrodes are constructed from materials that are non-toxic to cells, resistant to electrochemical attack and mechanically stable.

Commercially available disposable cuvettes equipped with aluminium electrodes (such as those supplied by BioRad, Hercules,CA and BTX, San Diego,CA) are frequently used for electroporation of cells in suspension. However, in addition to the possibility of the formation of hydrogen and oxygen gas bubbles at the electrodes, considerable solubilization of toxic cations (Al^{3+}) from aluminium electrodes has been recently reported in the literature (Loomis-Husselbee et.al., Biochem.Journal (1991),Vol.277, 883-885 ; and Friedrich et.al., Biochemistry and Bioenergetics(1998), Vol. 47, 103 – 111). More recent studies conducted by Tomov et.al. (Bioelectrochemistry (2000), Vol. 51, 207 – 209) have shown that electrodes made of stainless steel also can release toxic

ferrous ions into the electroporation medium to a concentration of several micromoles per liter when used as the anodes in an electroporation device.

The choice of anode material for electroporation devices is especially important because it determines the type of anodic reactions and the nature of the toxic species generated during the electroporation process. If the anode material is electrochemically soluble (e.g. aluminium and stainless steel), both metal dissolution and oxygen evolution can take place at the anode- electrolyte interface during the pulsing process. However, a large part of the anodic current in this case will consist of the current associated with the metal dissolution. For example, when aluminium anodes are used in an electroporation device, the following anodic reactions can occur:

Metal dissolution: $Al \rightarrow Al^{3+} + 3e^-$

Oxygen evolution: $2H_2O \rightarrow O_2 + 4H^+ + 4e^-$

The extent of each of the above reactions would primarily depend upon factors such as pulse intensity, pulse duration, number of pulses applied, and the conductivity of the electroporation medium. The metal dissolution reaction can adversely affect the electroporation process in a number of ways. For example, it has been reported that electric discharge through solution of biological macromolecules such as DNA, RNA and proteins, using aluminium anode plates, can cause precipitation of significant portions of these macromolecules. The precipitation of macromolecules is a consequence of the interaction of macromolecules with the metal ions solubilized from the anode by the electric pulse (Stapulions, Bioelectrochemistry and Bioenergetics (1999), Vol.48, 249 – 254). Precipitation of the macromolecules can result in significant errors in interpreting the results of the electroporation experiments. Toxic metal ions can further adversely affect both the properties of cell membranes (Gimmier et.al., J. Plant Physiol. (1991), Vol.138, 708 – 715) and the yield of viable permeabilized cells (Friedrich et.al., Biochemistry and Bioenergetics(1998), Vol. 47, 103 – 111). Although toxic metal ions

initially form locally at the anode- electrolyte interface, they can be transported to the entire electroporation medium by convection in a short time. This is because gas bubbles can enhance the mass transfer of toxic metal ions by agitating the solution.

Efforts have been made to minimize the amount of the released metal ions during electroporation by using platinum electrodes that can be passivated in the electroporation medium. Passivity is caused by the formation of a thin and impervious conducting oxide film on the platinum that acts as a barrier to the anodic metal dissolution reaction. However, in this case, a substantially higher portion of the anodic current would result in the formation of oxygen bubbles in accordance with the following reaction:

$$2\,H_2O \rightarrow O_2 + 4H^+ + 4e^-$$

Hydrogen bubbles are also generated at the platinum cathode with higher efficiency according to the following reaction:

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^-$$

A large amount of visible gas bubbles can be formed between platinum electrodes under these conditions as has been demonstrated by Chu et.al. (Nucleic Acid Res. Vol.15, No.3 (1987) 1311-1326). Thus, although the use of platinum electrodes eliminates the problems associated with the release of toxic metal ions, it does not eliminate problems that arise as a result of the formation of hydrogen and oxygen gas bubbles. It should be also noted that when platinum electrodes are used in electroporation devices, a possible secondary effect, in addition to oxygen and hydrogen evolution, is the anodic generation of certain intermediate species such as toxic radicals to which permeabilized cells can be sensitive. In extreme cases, there is also the possibility of the electro-oxidation of dissolved substances such as chloride ions that are often present in the electroporation medium:

$$2\,Cl^- \rightarrow Cl_2 + 2\,e^-$$

The above reaction may also occur during in vivo electroporation of tissues when platinum electrodes are used since a significant amount of chloride ions is also present in interstitial fluid in tissues.

Unlike the conventional electrodes used in the electroporation devices of prior art, the Pd cathode and Pd-H anode of the device in the present invention have the capability of effectively eliminating hydrogen and oxygen gas bubbles respectively during the electroporation process, and thus can prevent the problems associated with gas bubbles discussed above. The device of the present invention, therefore, offers an exceptional and hitherto non-existing means of subjecting cells to an electric field using electric pulses of long duration without having the disadvantages and limitations of prior art electroporation devices.

Palladium is known to have an exceptionally high capacity for absorbing hydrogen. For example, in solutions and under normal temperatures, palladium can readily absorb electrolytically generated hydrogen up to an atomic ratio of [H] / [Pd] =0.69 (Flanagan , T.B and Lewis, F.A , J. Chem. Phys., Vol. 29 , 1417 (1958). In terms of volume, it can absorb 370 times its own volume of hydrogen. Consequently one does not generally observe any evolution of gas at the beginning of the electrolysis (M Pourbaix, "Atlas of Electrochemical Equilibria in Aqueous Solutions", Pergamon Press Limited (1966). In contrast, other metals conventionally used as electrode materials such as platinum absorb only an insignificant amount of hydrogen under similar conditions and thus are ineffective in eliminating hydrogen bubbles. Another interesting property of palladium is that it is highly permeable to hydrogen and the rate of hydrogen diffusion in this metal under normal conditions is known to be extremely high (Barrer, R.M., " Diffusion in and Through Solids" , Cambridge University Press (1941)) . Thus when a palladium electrode is used as the negative electrode (cathode) for an electroporation device, it can instantaneously absorb the hydrogen atoms as they form during the electroporation process as follows:

H (adsorbed on the palladium surface) → H (absorbed in palladium)

and, therefore, the palladium electrode effectively prevents the interfacial concentration of hydrogen reaching the saturation level that is thermodynamically required for the formation of gaseous hydrogen in the form of gas bubbles.

Similarly, when a palladium electrode charged with hydrogen (Pd-H) is used as the positive electrode (anode) in an electroporation device, it can effectively prevent the formation of oxygen bubbles. In this case, as soon as an electrical charge is applied to the electrodes, the hydrogen atoms stored in the palladium metal rapidly diffuse through the solid metal to the solid / solution interface. The hydrogen atoms then consume the electrical charge and are instantaneously oxidized to hydrogen ions at the electrode surface through the following electrochemical reaction:

$$H \rightarrow H^{+} + e^{-}$$

The rapid diffusion of hydrogen atoms to the surface of the electrode ensures a rapid conversion of hydrogen atoms to hydrogen ions at the electrode surface and results in fast consumption of the electrical charge. Because of its rapid rate, the above reaction effectively becomes the predominant reaction at the electrode surface and consumes a substantial part of the electrical charge that is passed through the electroporation device. Thus, it effectively eliminates the oxygen evolution reaction that would otherwise take place at the electrode surface in the absence of the stored hydrogen in palladium. In addition, other anodic reactions such as metal dissolution reaction, anodic formation of toxic radicals, and generation of chlorine at the anode will be substantially suppressed.

Description of the Preferred Embodiment

The negative electrode of the present invention can be made substantially of palladium. However, since properties such as hydrogen permeability and hydrogen storage capacity of the electrode are of paramount importance, conventional methods known to those skilled in the art such as grain refinement, surface modifications and the addition of alloying elements can be considered as a means of improving the properties of palladium. For example, a palladium alloy composed of 77% palladium and 23% silver is known to have better properties than pure palladium in terms of the hydrogen absorption and can be used for this purpose. Similarly, palladium with nano-meter size grains is known to have improved properties concerning hydrogen permeability and, thus, can be used for this application. Surface modification techniques known to those skilled in the art such as roughening and texturing of the electrode surface can also be employed to increase the electrode surface area and to create a large surface area for hydrogen absorption. For example, a palladium electrode can be coated with palladium black to increase its surface area by electro-deposition method at 50 mA cm^{-2} in a solution of 1 g of $PdCl_2$ in 100 ml of 0.1 mol dilute aqueous HCl at room temperature.

Depending on the type of electroporation device, the negative electrode of the present invention may be made in the form of plates, flat screens, cylinders, wires and needles in an appropriate size and dimension. The negative electrode can also be made by, for example, depositing a thin film of palladium on a solid substrate made of metals, transparent glass and plastics in various forms. For example, by controlling the thickness of the palladium film, it would be possible to form an optically transparent and electrically conductive film of palladium on glass and plastics. Alternatively, the electrode can also be prepared by ion implantation of palladium atoms onto the surface of an electrically conductive substrate made of, for example, titanium, stainless steel or a glass substrate coated with gold or silver. Similarly, palladium atoms and nano-meter size palladium particles can be incorporated into thin films of oxides and ceramics that can be deposited onto glass and plastics. For example, palladium atoms and small particles of palladium can be incorporated into sol-gel derived thin film oxides such as titanium oxide

(Tour, J.M et. al., Chem.Mater, Vol.2 (1990) 647-649) and silicone dioxide. The sol-gel derived oxide films containing palladium can be deposited onto conductive glass such as a glass substrate coated with gold and silver to form transparent and conductive electrodes for in situ electroporation of cells. In a preferred embodiment of this invention, the negative electrode of the electroporation device is fabricated by depositing a thin film of palladium on a flat transparent substrate made of glass or plastics. Optically transparent Pd films with sufficient electrical conductivity can be deposited on glass and plastics by a range of conventional techniques. Cells cultured on the palladium film can be subjected to in situ electroporation while they are attached to the palladium film. In situ electroporation of cells can be carried out by, for example, positioning a Pd-H electrode or a conventional positive electrode such as a platinum electrode close to the plated cells and applying a voltage between the two electrodes. Because the palladium electrode can rapidly absorb hydrogen atoms generated during electroporation, cells can be subjected to electrical pulses of long duration without being disturbed by hydrogen bubbles. In order to improve cell attachment, the palladium film can be further covered by a microporous film of an inorganic material such as titanium oxide or indium tin oxide.

The positive electrode of the present invention can also be made substantially of palladium in various forms similar to the negative electrode as discussed above except that it is charged with hydrogen to form a Pd-H alloy such as an α- phase ([H / Pd] $\cong$ 0.33)or β- phase ([H /Pd] $\cong$ 0.66). Charging of the palladium electrode with hydrogen can be carried out by various conventional methods such as exposing palladium to a gaseous hydrogen environment or by the electrolytic method. The electrolytic method, however, is preferred since palladium and palladium alloys can be conveniently charged with hydrogen in electrolytes under normal temperatures in a very short time. A significant amount of hydrogen (up to a [H / Pd] $\cong$ 1) can be stored in palladium by this method (Krueger, F and Gehm ,G , Ann. Phys., Vol.78, P.72 ,1925). The palladium electrode is preferably charged with hydrogen shortly before it is used as a positive electrode in the electroporation process, although under normal conditions, hydrogen can be stored in palladium for a long time.

The electrodes of the electroporation device in the present invention can be used independently of each other or together in various combinations and configurations for the electroporation of cells. For example, the Pd electrode of this invention can be used as a negative electrode (cathode) in an electroporation device together with a conventional anode to absorb hydrogen atoms and to eliminate hydrogen gas bubbles during the electroporation process.

Similarly, the Pd-H electrode of the present invention can be used as a positive electrode (anode) in an electroporation device together with a conventional cathode to suppress oxygen evolution reaction. Alternatively, the Pd and Pd-H electrodes of this invention can be used together as a pair of negative and positive electrodes in an electroporation device to eliminate both hydrogen and oxygen gas bubbles. For example, the Pd and Pd-H electrodes of this invention can be used together as a pair of parallel plate electrodes for the electroporation of cells in suspension similar to the parallel electrodes described by Andreason et.al. (BioTechniques (1988), Vol.6, No.7, 650-659) and as described by Papp et al (US patent 5,422,272, 1995). In practice, both electrodes can be made of solid palladium or palladium alloys. However, prior to the electroporation of cells, one of the electrodes can be charged with hydrogen to serve as the positive electrode for the electroporation of cells. Charging of the palladium electrode with hydrogen can be performed by, for example, dipping the pair of electrodes in an electrolyte or by filling the gap between the two electrodes with an electrolyte and applying a voltage to the electrodes for a short time using, for example, a D.C power supply. The electrolyte, for example, can be a physiologically acceptable solution such as the solutions that are normally used for cell culture and for the electroporation of cells. In a preferred embodiment of the present invention, the electrolyte contains strongly reducing agents such as sodium hypophosphite. In the charging process, the Pd electrode that is connected to the negative terminal of the DC power supply would act as a cathode and would be charged with hydrogen whereas the other Pd electrode would serve as a counter electrode. The time required to charge the Pd electrode with hydrogen would primarily depend on the pH of the electrolyte and the voltage applied during the charging process.

If a high voltage is used, the charging time can be as low as a few seconds or less. In a preferred embodiment, the charging process is carried out at a relatively low voltage (eg 20 – 30 volts) for less than 300 seconds and more preferably about 60 seconds. The power supply can be, for example, a D.C power source or a function generator capable of generating an electric pulse with a predetermined amplitude and pulse length. In practice, a power generator similar to those commonly used for the electroporation of cells may be employed for the charging process. In this case, charging can be carried out using, for example, one or more exponential-decay pulses. In a preferred embodiment, charging of the Pd electrode with hydrogen is carried out using a low voltage square pulse with a pulse length of about 60 seconds. Alternatively, a train of square pulses having short pulse lengths can be employed for charging the Pd electrode with hydrogen. In addition, the electrical set-up for charging the palladium electrode may be equipped with means for controlling the charging process such that the palladium electrode can be electrolytically charged with hydrogen to a pre-determined level (e.g. charged to a pre-selected H/Pd atomic ratio).

The electrodes of the present invention can also be advantageously incorporated into a wide variety of conventional devices suitable for the in situ electroporation of attachment-dependent cells. An example of electroporation device for in situ electroporation of cells is the device described by Klenchin et.al (Biophysical Journal, (1991), Vol.60, 804 – 811) in which cells are plated on a microporous membrane that is positioned between two closely spaced and parallel electrodes. For such an application, both electrodes can be made of palladium or palladium alloys. Prior to electroporation of cells, one of the electrodes can be charged with hydrogen to serve as the positive (anode) electrode during the electroporation of cells. The charging step can be carried out conveniently and in a very short time prior to the electroporation step by placing an electrolyte between the two closely spaced Pd electrodes and applying a voltage between the two electrodes as described above. The use of the Pd and Pd-H electrodes of the present invention in this application is particularly beneficial since it results in effective elimination of hydrogen and oxygen gas bubbles that are normally generated at the interface of the microporous membranes and electrodes.

The electrodes of this invention can also be used in other types of conventional devices suitable for in situ electroporation of attachment- dependent cells such as devices described by Chang (US patent 4,822,470, 1989). For example, cells can be cultured on a solid, non-conductive substrate such as a glass slide. In situ electroporation of cells can then be carried out by positioning an electrode assembly close to the plated cells consisting of a pair of electrodes made of Pd and Pd-H that serve as the cathode and the anode respectively. The electrodes can be in the form of parallel plates or concentric rings. In addition to parallel plates and concentric rings, other configurations such as multiple cathodes and anodes can be used. Thus, Pd cathodes and Pd-H anodes can be arranged in alternating fashion into two groups. One group would be connected to the negative terminal, while the other group would be connected to the positive terminal of a high voltage power source (pulse generator) during the electroporation process. Other types of electrode assembly comprising electrode array patterns that may be formed by depositing palladium on a non-conductive support can also be used. This method allows fabrication of much smaller electrodes on the bottom of an electrode carrier for the in situ electroporation of cells with smaller electrode separations.

Similarly, the Pd and Pd-H electrodes of the present invention can be used in devices suitable for in vivo electroporation of cells for application in electrochemotherapy and gene therapy such as electrode assemblies described by Mir(U.S. Pat.No. 5,468,223) and Hofmann (U.S Pat. No. 5,439,440). For all the above applications, the electrodes can be made of palladium or palladium alloys. Prior to the electroporation of cells, one or a group of the Pd electrodes can be charged with hydrogen to serve as the positive electrode(s) for the electroporation of cells. Charging of the palladium electrode(s) with hydrogen can be carried out as described previously by, for example, dipping the electrode assembly in an electrolyte and applying a voltage between the electrodes for a very short time.

CLAIMS:

1. Apparatus for the transfection of exogenous material into cells by means of electroporation, said apparatus comprising:

a) first and second electrodes having electrically conductive surfaces substantially made of a material selected from the group consisting of palladium and palladium alloys.
b) means for electrolytically charging said first electrode with hydrogen.

c) a pulse generator having a positive pole and a negative pole.

d) means for connecting said first electrode charged with hydrogen to said positive pole and said second electrode to the negative pole of said pulse generator to generate a high electric field for transfection of exogenous material into cells.

2. Apparatus according to claim 1 wherein said means for electrolytically charging said first electrode comprises a power supply for applying a voltage between said first and second electrode and fluid retaining means for maintaining said first and second electrodes in contact with an appropriate electrolyte while said first electrode being electrolytically charged with hydrogen.

3. Apparatus according to claim 1 wherein said means for electrolytically charging said first electrode further comprises electrical means for controlling the level of the electrical charge delivered to said first electrode such that said first electrode can be charged with hydrogen to a pre- determined level.

4. Apparatus according to claim 1 wherein said first and second electrodes are each members of an electrically connected set of electrodes.

5. Apparatus according to claim 1 wherein said pulse generator is capable of generating exponential-decay pulses and square pulses of constant current and constant voltage with variable pulse lengths.

6. Apparatus according to claim 5 further comprising a selector switch connected to and arranged between said pulse generator and said electrodes, the selector switch being able to connect each electrode with either the negative pole or the positive pole of the pulse generator and thereby can allow the use of pulse generator for both electrolytic charging of said first electrode and for the electroporation of cells.

7. Apparatus according to claim 1 wherein said first and second electrodes are substantially made of a material selected from the group consisting of palladium and palladium alloys.

8. Apparatus according to claim 7 wherein said palladium alloys contain alloying elements selected from the group consisting of platinum and silver.

9. Apparatus according to claim 1 wherein said first and second electrodes are substantially made of a conductive material selected from the group consisting of palladium, palladium alloys, stainless steel and brass, and said electrically conductive surfaces are comprised of conductive coatings made of a material selected from the group consisting of palladium, palladium black and palladium alloys.

10. Apparatus according to claim 1 wherein said electrically conductive surfaces are roughened or textured.

11. Apparatus according to claim 1 wherein said electrically conductive surfaces are substantially made of a material selected from the group consisting of palladium and palladium alloys having nano-meter size grains.

12. Apparatus according to claim 1 wherein at least one of the said first and second electrodes comprises a flat, non-conductive support member having a conductive surface substantially made of a material selected from the group consisting of palladium and palladium alloys.

13. Apparatus according to claim 12 wherein said flat support member is transparent and said conductive surface is comprised of a conductive and optically transparent coating of a material selected from the group consisting of palladium and palladium alloy.

14. Apparatus according to claim 13 further comprising a micro-porous inorganic coating deposited on said conductive and optically transparent coating to improve cell adhesion thereon.

15. Apparatus according to claim 14 wherein said inorganic coating is comprised of a material selected from the group consisting of titanium oxide and indium tin oxide.

16. An improved device for electroporation of cells and tissue capable of eliminating hydrogen bubbles during said electroporation process, said device comprising a plurality of electrodes at least one of which serves as a cathode and having a conductive surface substantially made of a material selected from the group consisting of palladium and palladium alloys.

17. A method for transfection of exogenous material into cells and tissues by electroporation comprising:

Subjecting cells in contact with a solution of exogenous material to an electric field, said electric field being generated between first and second electrodes, said first electrode being an anode having a surface substantially made of palladium charged with hydrogen and said second electrode being a cathode having a surface substantially made of palladium.

18. A method according to claim 17 wherein said first and second electrodes are each members of an electrically connected set of electrodes.

19. A method according to claim 17 wherein said first electrode is being electrolytically charged with hydrogen in an electrolyte.

20. A method according to claim 19 wherein said second electrode serves as a counter electrode during said charging step.

21. A method according to claim 20 wherein said electrolyte contains at least one strongly reducing agent.

22. A method according to claim 21 wherein said reducing agent is sodium hypophosphite.

23. A method according to claim 20 wherein said electric field for transfection of cells is generated by utilizing a pulse generator capable of generating exponential-decay pulses and square pulses with varying pulse lengths.

24. A method according to claim 23 wherein the voltage required for charging said first electrode is being generated by said pulse generator.

INTERNATIONAL SEARCH REPORT

	ıtional Application No
	.../GB 01/00899

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12M3/00 C12N15/87

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12M C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, COMPENDEX, INSPEC, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TERUEL M N ET AL: "Electroporation-induced formation of individual calcium entry sites in the cell body and processes of adherent cells" BIOPHYSICAL JOURNAL, OCT. 1997, BIOPHYS. SOC, USA, vol. 73, no. 4, pages 1785-1796, XP001010253 ISSN: 0006-3495 cited in the application abstract; figure 1 --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 2001	20/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Devijver, K

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CHU G ET AL: "ELECTROPORATION FOR THE EFFICIENT TRANSFECTION OF MAMMALIAN CELLS WITH DNA" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 15, no. 3, 11 February 1987 (1987-02-11), pages 1311-1326, XP000574947 ISSN: 0305-1048 cited in the application page 1314 -page 1316 ---	1-24
A	SUKHAREV S I ET AL: "ELECTROPORATION AND ELECTROPHORETIC DNA TRANSFER INTO CELLS THE EFFECT OF DNA INTERACTION WITH ELECTROPORES" BIOPHYSICAL JOURNAL,US,NEW YORK, US, vol. 63, no. 5, 1 November 1992 (1992-11-01), pages 1320-1327, XP000646910 ISSN: 0006-3495 cited in the application the whole document ---	1-24
A	US 5 422 272 A (PAPP ANDREW A ET AL) 6 June 1995 (1995-06-06) cited in the application the whole document -----	1-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

tional Application No
PCT/GB 01/00899

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5422272 A	06-06-1995	NONE	