coaxial electrodes **25** are attached to a nonconductive insulating holder **54** preferably made of teflon or plastic.

The gap between the inner **25***a* and outer **25***b* coaxial electrodes may vary from about 0.5 to 2.0 mm. In a preferred embodiment, the electrode **25** has a 0.7 mm gap. With this probe **20** the total volume of suspended cells to be fused or porated is about 80 μl and it is possible to do cell fusion or cell poration with as little as 20 μl of cell suspension.

The probe **20** has a handle **47** made of non-conductive material, preferably teflon. Holding means **55**, hold the outer electrode **25***a* in place.

This design has several advantages. Besides allowing the use of small volumes of cell suspension for cell fusion or cell poration, it is also simple to use and highly cost-effective. Unlike most commercial machines which require one cuvette to transfect one cell sample, this probe can serially transfect many cell samples using plates with multiple wells.

Another embodiment of the present invention for cell poration and cell fusion is shown in FIG. **14**. This figure shows the block diagram of the high power function generator which generates both the AC field for dielectrophoresis and the high power RF pulses for cell fusion and/or cell poration. The switching between the AC field and the RF field is controlled by a mechanical relay or electronic switch. The RF pulses are generated by gating the output of a radiofrequency oscillator and then passing through a MOSFET power amplifier, the power output of which may be as high as forty kilowatts.

Alternatively the AC field and the pulsed RF field can be generated by synthesizing the required electrical wave with a digital computer and amplifying these wave forms using a power amplifier. In this embodiment the protocol can be controlled entirely by the computer and thus no switching relay is needed. This computer-synthesized high power function generator has several advantages. First, very complicated wave forms can be generated to optimize the fusion and/or poration of different types of cells. Second, when the high power function generator is used in more than one protocol or by more than one user, each protocol can be stored separately in a data storage device for example, a magnetic diskette. Since the protocols can be recalled quickly, the high power function generator can be reprogrammed to generate the desired wave forms without manually readjusting all the parameters. Third, the same computer can be used as a digital oscilloscope to record the actual electrical field applied to the cells. This record can be saved in a data storage device as the permanent record of any particular cell fusion or cell poration experiment.

Excessive current is harmful to the cell because of the resulting thermal effects and pH changes. To avoid generating excessive current and the resulting effects during the application of the electric field, the suspension medium of the cells is usually a low ionic strength solution. Preferably it contains very low concentration of salts. A typical suspension medium may contain 1 mM of electrolyte including 0.4 mM Mg-acetate and 0.1 mM Ca-acetate. The medium is buffered and the pH maintained in the physiological range, for example, pH 7.5. Any buffer commonly used for biological purposes, for example, 1 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) is adequate for cell poration and/or cell fusion. Non-electrolytes are added to maintain the osmolarity of the medium at about the osmolarity of extracellular fluid. In the preferred embodiment, relatively high molecular weight, cell impermeable carbohydrates, such as sucrose and mannitol, are used to maintain the osmolarity.

For some cells, a slightly higher ionic strength in the medium seems to improve the fusion yield. For example, human erythrocytes fuse easily in 30 mM Na-phosphate. Thus, the present method of fusion can use suspension medium with an ionic strength ranging from 0.1 mM to 100 mM depending on the cell type.

The present invention for cell poration and cell fusion has a variety of uses. Many biological active substances, including DNA, RNA, organic chemicals, inorganic chemicals, drugs, antibodies, proteins, hormones, growth factors, enzymes and radioactive or fluorescent-labelled molecular probes normally cannot be readily taken up by cells. The present invention provides an effective method to transport these biological active substances into the cells. In one embodiment of the present invention, cells can be temporarily permeabilized, that is porated, by applying high-power RF pulses and the biological active substances can then enter the cells during this poration period. The porated cells can be biological cells (including, animal, human or plant cells), protoplasts, bacteria or yeasts. In another embodiment of the present invention, the biological active substances can be inserted into the cells by fusing the target cells with other biological particles which have been pre-loaded with the active substances. Such biological particles include liposomes and erythrocyte ghosts, which can be easily preloaded with desired substances using a standard osmotic shock and dialysis method. (Schlegel & Lieber *Cell Fusion* ed by A.E. Sowers Plenum Press (1987)). The target cells may be any cells which will receive the biological active substances and include isolated cells, egg cells, embroyonic cells, any primary or transformed cultured cells, or other cells in vitro.

In like manner, biological substances could be extracted from biological cells. For example, many molecules such as hormones, growth factors, enzymes, proteins and nucleic acids may not be able to cross the membrane barrier. Using the poration method of the present invention, temporary pores can be induced in the cell membrane. The non-permeable molecules can then exit the cell. This procedure could be useful in a variety of industries which use growing cells to produce biological molecules. This procedure allows the extraction of these molecules without having to kill the cells.

Example I

The ability and efficiency of the RF electroporation method to insert foreign genes into the target cell is examined using the cultured eukaryotic fibroblast cell line COS-M6 (M6). Chloramphenicol AcetylTransferase (CAT) DNA was used as a gene marker. Bacterial CAT DNA was inserted into a plasmid vector (pSV2-CAT). The CAT enzyme is not endogenously produced in mammalian cells, such as M6. Thus, the amount of CAT gene incorporated into the target cells can be assayed by monitoring the amount of CAT enzyme produced after the transfection.

The protocol was to apply 3 trains of high-power RF pulses at 10 sec intervals. Each train consists of 5 pulses (frequency 100 KHz, field strength 2.5 KV/cm, pulse width 0.5 msec).

The RF poration protocol of the present invention is a highly effective method of gene transfection. In the conventional methods of gene transfection, for example, the calcium phosphate method or the DEAE-dextran method, usually requires at least 5–10 μg of plasmid DNA for each transfection. In previous electroporation methods that used DC pulses, even larger amounts of DNA (typically 10–40 μg) were required. (Ansubel et al., Current Protocals in Molecular Biology, John Wiley & Sons, 1988). Using the RF poration method of this invention, we obtained a high level of CAT activity (76% acetylation per 25 μg of protein) when M6 cells were transfected using only 0.1 μg of CAT DNA. Furthermore, up to 10.6% acetylation per 25 μg of protein was observed when M6 cells were transfected with as little as 0.01 μg of CAT DNA. Thus, it is evident that the RF poration method has a much higher efficiency of gene transfection. The improved efficiency not only results in great savings in labor and material that is required to produce DNA, but also will allow the transfection of cells which were previously difficult to transfect.

Another advantage of the RF poration method is that it requires far less cells for gene transfection. The conventional chemical methods and the DC electroporation method typically require 2 to 10 million cells to do one transfection. With the RF method, M6 cells have been transfected with the CAT gene in high efficiency using as few as 0.1 million cells. Further experiments indicated that even lower numbers of cells (1×10^4) can be used. Currently, the minimum cell number is limited by the amount of total cell protein required to perform the CAT assay and not the ability to transfect cells. (Typically 25 micrograms of total cellular protein are needed for the CAT reaction.)

Example II

Because of the unique abilities of the RF poration method to transfect cells in small quantity and with high efficiency, the method will be particularly useful in the development of gene therapy. Many diseases are known to be caused by genetic defects. Such diseases could be treated by inserting a therapeutic gene into human cells such as bone marrow stem cells and then transplanting these cells into the human body.

For example, patients with sickle cell anemia have a defective gene which produces abnormal hemoglobin. To treat such a genetic disease, bone marrow stem cells are extracted from the patient and transfected with the normal hemoglobin gene. The transfected stem cells are transplanted back into the patient. With the appropriate vector the normal gene will be stably integrated into the genome and the patient will be able to produce normal hemoglobin.

The key step in this treatment is the transfection of the bone marrow stem cells with the normal gene. Because the number of stem cells which are extracted is relatively small, a gene transfection method of high efficiency that is suitable for extremely low cell numbers is required. The method of the present invention of poration using RF pulses uniquely has this ability. Thus this method will be highly useful for gene therapy.

The usefulness of this method for gene therapy is not limited to sickle cell anemia. This method can be applied to insert normal genes into human cells to cure many genetic diseases. Other examples include: introducing the gene for clotting factor VIII into bone marrow stem cells to cure hemophiliacs; inserting the gene for insulin into pancreatic islet cells or other human cells to treat diabetes; introducing the gene for the human LDL (low density lipoprotein) receptor into liver cells or other human cells to lower the cholesterol level in the bloodstream of hypercholesterolemia patients; and introducing the gene for human growth hormone into human cells to correct growth defects. Thus, the possibilities of using this method to insert genes into human cells to treat genetic diseases is unlimited.

Example III

Morphological Changes of the Cell Membrane during the Process of RF Field Electroporation

A fraction of a second after human red blood cells were exposed to RF pulses, they were rapidly frozen in liquid freon cooled by liquid nitrogen. The structure of the cell membranes were examined using the technique of freeze-fracture electron microscopy. In FIG. **14** the electron micrograph shows the surface structure of the red blood cell after 3 RF pulses (400 kHz, 40 μsec wide, 5 kV/cm field strength) were applied. Membrane pores with diameters of 0.1 to 0.2 micrometers were clearly seen. These pores are sufficiently large to allow a large piece of DNA to easily diffuse from the extracellular medium into the cell. Thus, there is direct evidence that the applied RF fields can induce large pores at the cell surface. The morphological evidence clearly shows that the method of the present invention is effective in inducing membrane poration to allow transfection of cells with exogeneous genes.

Example IV

An example of the advantage that the present invention has over the conventional DC (direct current) electrofusion method was seen in the fusion of human erythrocytes. The fusion events were assayed by labelling the membranes of a small number of the suspended cells with a lipophyllic fluorescent dye, for example, 1,1′,-dihexadecyl-3,3,3″,3′-tetramethylendocarbacyanine perchlorate. The cells were observed with a fluorescence microscope. Before applying the RF pulses, only the prelabelled cells give a fluorescent image and they appeared as isolated cells (see FIG. **16**A). After the cells were exposed to pulsed RF fields, unlabelled cells started to fuse with labelled cells and the dye was gradually transferred from the labelled cell to the unlabelled cell. Eventually both cells became labelled (see FIG. **16**B). This fusion process took only a few minutes following the application of the RF pulses.

Two types of cell fusion were observed in this experiment: (1) Membrane fusion, in which the fluorescent dye was transferred from the labelled cell to the unlabelled cell but the two cells did not merge their cytoplasm; and (2) cytoplasmic fusion, in which the fusing cells merged together to form one single large cell. The percentage of cells undergoing cytoplasmic fusion depends strongly on the oscillating frequency of the applied RF field The fusion yield for erythrocytes after RF pulses of different frequency are applied is shown in FIG. **17**. The highest yield of fused cells occurred when the applied RF field was oscillating at 100 KHz. The fusion yield decreased to a very low level as the frequency became too high or too low. No cytoplasmic fusion was detected when the applied field was in the form of DC pulses with the same pulse amplitude and pulse width as the RF pulses. These results clearly indicate that the RF pulse method of this invention is much

more effective in inducing cell fusion than the DC pulse method.

Another example of the advantage of the present invention over the DC electrofusion method is in the fusion of human erythrocytes with a human leukemia cultured cell line, HL-60. Fusion of these two cells types was not obtainable using the DC pulse method. The failure is probably due to the differences in cell size; erythrocytes are significantly smaller than HL-60 cells. However, using the fluorescent dye assay and the pulsed RF field of the present invention, we were able to obtain the fusion of erythrocytes with HL-60 cells.

Example V

The RF pulse method can be used to fuse cells to make hybridomas. Pigment cells from goldfish (xanthophores) were fused with a tumor cell line derived from fish skin cells. Because xanthophore cells have a built-in histochemical marker (the carotenoid droplets), it is comparatively easy to assay their fusion with non-pigmented tumor cells. FIG. **18** shows the sequential steps in the fusion of a xanthophore and a skin tumor cell. In FIG. **18**A the cells were brought into close contact by dielectrophoresis. Three pulses of RF field (40 μsec wide, frequency 400 kHz, field strength 3.3 kV/cm) were then applied. Within two minutes the cytoplasms of the two cells begun to merge (see FIG. **18**B) After 4 minutes, the cells completely coalesced into a single giant cell (see FIG. **18**C).

An important application of forming hybridomas using the RF pulse method is to make antibodies, especially human monoclonal antibodies. In this instance the biological particles to be fused can include antibody producing cells (for example, lymphocyte B cells) and continuously dividing cells (for example, cancer cells). Using a selection process, the resultant hybridoma cells can be cultured to produce specific monoclonal antibodies.

One skilled in the art will readily appreciate the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The devices, methods, procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary, and are not intended as limitations of the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

What is claimed is:

1. A device for the poration and fusion of biological particles comprising:
- a container, including a non-conducting material capable of holding liquid and said biological particles;
- electrodes positioned equidistant from each other in said container; and
- a high power function generator, attached to said electrodes for generating a high intensity electric field, including a pulsed radiofrequency having a frequency range of 1 KHz to 100 MHz, a pulse width range of 1 μsec to 100 msec, and a pulsed amplitude range of about 0.2 KV/cm to 20 KV/cm.

2. The device of claim **1**, wherein said biological particles are selected from the group consisting of human cells, animal cells, plant cells, bacteria, yeasts, protozoa, protoplasm, liposomes and erythrocyte ghosts.

3. The device of claim **1**, wherein said high intensity electric field is a pulsed radiofrequency oscillating field and includes a frequency ranges of about 0.02 MHz to 10 MHz; a pulse width range of about 20 μsec to 5 msec; and a pulse amplitude of about 0.2 KV/cm to 10 KV/cm.

4. The device of claim **1**, wherein said function generator further includes the capability of generating a continuous low power alternating current electric field for bringing said biological particles into close proximity for fusion.

5. The device of claim **4**, wherein said low power alternating current electric field includes a frequency range of about 60 Hz to the 50 MHz range and a field strength of about 20 V/cm to 900 V/cm.

6. The device of claim **4**, further comprising a mechanical relay or electronic switch for switching its output between the pulsed high intensity electric field and the continuous low power alternating current electric field.

7. The device of claim **1**, wherein:
- a wave form from the high intensity electric field is selected from the group consisting of single pulses of symmetrical oscillating fields, multiple pulses of symmetrical oscillating fields, single pulses of direct current shifted oscillating fields and multiple pulses of direct current shifted oscillating fields; and
- the shape of said oscillating fields is selected from the group consisting of sinosoidal, rectangular, square, triangular, sawtooth and combinations thereof.

8. The device of claim **1**, wherein the function generator is comprised of:
- a radiofrequency pulse generator including a gating circuit for gating the output of a radiofrequency oscillator and a power amplifier for generating the high power radiofrequency pulse from the gated output of the radiofrequency oscillator;
- an alternating current field generator; and
- a mechanical or electronic relay for switching between the radiofrequency pulse and the alternating current field.

9. The device of claim **1**, further comprising digital circuitry for synthesizing the wave forms for the high intensity electrical field and the low power AC field and a power amplifier for amplifying the synthesized electrical signals.

10. The device of claim **1** for optical microscopic observation of poration and fusion of biological particles further comprising:
- said container with a transparent bottom, wherein said container is formed by two transparent surfaces separated by spacers; and said electrodes inserted into said container, wherein said electrodes are equidistant from each other and arranged in a pattern which allows at least ten microliter of cells to closely contact the electrodes.

11. A device for cell poration and cell fusion, comprising:
- a digital microprocessor for synthesizing and generating a radiofrequency wave form and an alternating current wave form;
- a amplifier to convert the wave form generated by the digital computer to high power wave forms;
- a container, including a non-conducting material capable of holding liquid and said cells;
- electrodes positioned equidistant from each other in each container; and said electrode communicating with said amplifier for applying the electric field to said cells.

12. The device of claim **11** further including an information storage device.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,304,486

DATED : April 19, 1994

INVENTOR(S) : Donald C. Chang

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In the title, change "PORTION' to -- PORATION --, and "PULSE" to -- PULSES --.

Column 2, line 51, change "Poration" to -- poration --.

Column 6, line 65, change "η" to -- θ --.

Column 9,

line 11, place a "τ" before the equals sign at the beginnning of the equation;

line 56, change "Pair " to -- pair --;

line 68, change "Pulses" to -- pulses --.

Signed and Sealed this

Tenth Day of January, 1995

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks