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(54) Title: LARGE VOLUME EX VIVO ELECTROPORATION METHOD
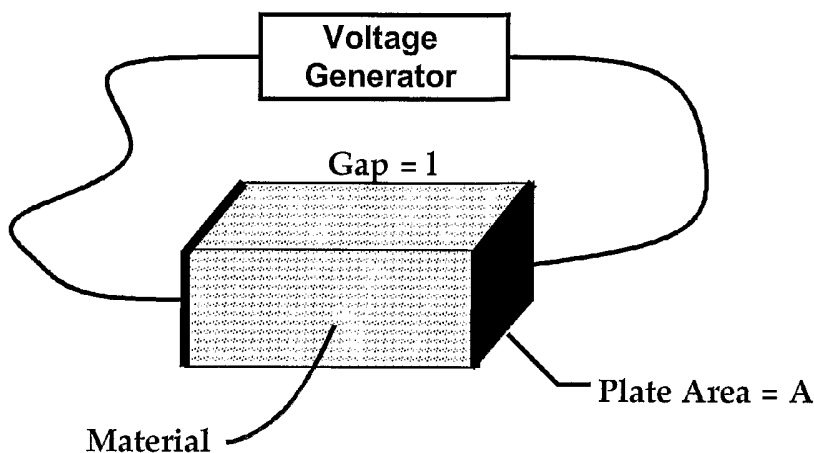
(57) Abstract: An object of the invention is to provide an electroporation method for treating vesicles with exogenous material for insertion of the exogenous material into the vesicles which includes the steps of: a. retaining a suspension of the vesicles and the exogenous material in a treatment volume in a chamber which includes electrodes, wherein the chamber has a geometric factor (cm⁻¹) defined by the quotient of the electrode gap squared (cm²) divided by the chamber volume (cm³), wherein the geometric factor is less than or equal to 0.1 cm⁻¹ , wherein the suspension of the vesicles and the exogenous material is in a medium which is adjusted such that the medium has conductivity in a range spanning 0.01 to 1.0 milliSiemens, wherein the suspension is enclosed in the chamber during treatment, and b. treating the suspension enclosed in the chamber with one or more pulsed electric fields. With the method, the treatment volume of the suspension is scalable, and the time of treatment of the vesicles in the chamber is substantially uniform.

LARGE VOLUME EX VIVO ELECTROPORATION METHOD

BACKGROUND OF THE INVENTION

5

Cross-Reference to Related Application

This application claims priority based upon
copending United States Patent Application, of Walters
and King, Serial Number 60/454,360, Filing Date 14 March
10   2003, for LARGE VOLUME EX VIVO ELECTROPORATION METHOD.

Technical Field

The present invention relates generally to ex vivo
electroporation methods, and, more particularly, to
15   electroporation methods especially adapted for clinical
and industrial applications.

Background Art

Delivering large molecules into living cells for
20   therapeutic purposes, using ex vivo or in vitro
electroporation, has been described in the literature for
many years.   The purpose of electroporation is to enhance
the movement of molecules into and out of living cells or
non-living vesicles.   The practical uses are many and
25   vary according to the complexity of material delivered,
the site of delivery and the purpose for delivery.

Complexity ranges from small drug molecules that are
otherwise difficult to get into cells to complex mixtures
of polynucleotides.

30   The site of delivery is broadly divided into in vivo
and ex-vivo delivery.   The choice of an in-vivo site is
based upon the location of the tissue to be treated and
whether or not local or systemic treatment is desired.

Clinical and industrial applications of this process
35   are possible.   Often, in clinical and industrial
applications, it is desirable to insert large molecules
into large numbers of cells and to insure that all cells

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have been processed equally. To do that, it is desirable
to process all cells simultaneously to guarantee that all
cells are subjected to the same process conditions.

   Therapeutic purposes for delivery are many. Some
5   examples are gene replacement therapy, therapeutic
genetic medicine for acquired diseases, polynucleotide
vaccines, immunotherapy, enhanced chemotherapy and many
others. Industrial and agricultural applications are
equally varied. Some examples of industrial uses are
10 extraction of material from cells produced in a
fermenter, large scale transfection for production of
recombinant protein, modification of cells for industrial
use, sterilization of liquids or vaccine production.
Some examples of agricultural uses are vaccines for
15 livestock (to include ungulates, avian species and
aquatic animals) and modification of genes for
improvement of selected traits.

   For standard in vitro electroporation, cuvettes are
usually used. These are chambers that consist of
20 parallel plate electrodes encased in plastic and have
limited capacity. Volumes used in these cuvettes are
under one milliliter. The limited volume limits the
total capacity for treating cells.

   Typical cell densities used are in the range of 1
25 million to 10 million cells per milliliter. The cells
are typically placed in a physiological medium with high
ionic content such as phosphate buffered saline, which
has a conductivity of 0.017 Siemens/cm (17,000 mS/cm) per
centimeter.

30   In electroporation, cell density is an important
parameter. If the cells are not dense enough,
therapeutic or other material is wasted. If the cells
are too dense the electric field in the proximity of each
cell is not uniform in direction or in intensity. To
35 produce consistent results that are required for clinical
applications the electric fields close to the cells must
be both uniform in direction and intensity. According to

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Fomekong et al in "Passive electrical properties of RBC
suspensions: changes due to distribution of relaxation
times in dependence on the cell volume fraction and
medium conductivity", in Bioelectrochemistry and
5   Bioenergenetics, 1998, Vol 47: 81-88), the effect of
cells on the electrical properties of cell suspensions is
dependent upon the packed cell volume of the cells. For
packed cell volumes less than 10% the distance between
cells increases rapidly and therefore the interfering
10   effect of one cell to another in the electric field
decreases rapidly below a packed cell volume of 10%. A
typical cell of 15 microns in diameter would be at 10%
packed cell volume at approximately 60 million cells/ml
(calculated using a mean cell volume of 0.000001767
15   mm3/cell). Thus cell densities under 60 million cells/ml
should be used and normally cell densities under 30
million cells/ ml are used.

TABLE 1

20

Electrode Chamber Volume milliliters

Number Out	Number In	Cell Density	
Million/ml	Million/ml	20 million/ml	40 million/ml
10	20	1	0.5
100	200	20	5
1000	2000	100	50

25   Clinical application generally requires 10 million
to 500 million cells in which the large molecules have
been properly inserted. If a treatment requires 10
million cells per dose (treatment) and 5 doses are
required, at least 50 million therapeutic cells must be
30   prepared. If the efficiency of the electroporation
process is assumed to be 50% and cells are treated at a
concentration of 20 million cells/ml then a 5 ml capacity
electrode would be required (50 million X 2 / 20

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million). If 100 million therapeutic cells are required,
a 10 ml capacity electrode would be needed.

Simply increasing the size of the electrode to
achieve the desired capacity is not practical because
this causes a proportionate increase in amperage due to a
decrease in resistance in the electrode. As the size of
the electrode increases, the resistance of the electrode
decreases as long as the conductivity of the medium used
remains constant.

If a 100 million therapeutic cells are required and
the input cell density is 20 million cells per milliliter
then a 20 ml electrode is required.

In this case just scaling the size of the electrode
up to 20 milliliters does not work. As the volume of the
electrode increases the resistance of the electrode due
to the conductivity of the media decreases. The
resistance of the media in the electrode is calculated as
follows:

Formula 1:

$$R = \frac{1}{\sigma} \frac{gap}{area} \; ohms$$

where σ = conductivity in Siemens/cm, gap is in cm and
plate area is in cm^2. In addition:

Formula 2:
$$volume = gap * area \; cm^3$$

and

Formula 3:

$$R = \frac{1}{\sigma} \frac{gap^2}{volume} \; ohms$$

FORMULAS 1, 2, and 3 are taken from Electroporation
and Electrofusion in Cell Biology, edited by Eberhard
Neumann, Arthur Sowers, and Carol Jordan, Plenum Press,

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1989, mentioned hereinabove.

The TABLE 2 below shows the electrode chamber
resistance as a function of volume for a 4-millimeter gap
5   and media conductivity of 0.017 Siemens/cm.

TABLE 2

Electrode Volume ml	Media Resistance ohms
0.5	19.2
1	9.6
5	1.92
10	0.96
50	0.19

10

When the electrode chamber volume is above 1 ml the
15   resistance of the ionic solution becomes impractically
small; significant solution heating will occur due to the
high pulse current destroying the cells.
      To address this problem a flow though technique was
developed. In this process the large volume of media
20   flows through a small treatment chamber, and the voltage
pulse waveform is applied to the parallel plates in the
chamber. The problems with this process are:
      1. Not all the cells are exposed to the same
electric field intensity and direction.
25   2. There is no guarantee that the density of the
material to be inserted and the cell density are
constant.
      3. Only uniform pulse voltages may be applied.
Variable rectangular pulse waveforms such as disclosed in
30   U. S. patent 6,010, 613 cannot be used.

In a flow through process there is no guarantee that
all cells will be subjected to the same electric field

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intensity and

direction. In this respect, because of the properties of
laminar and turbulent flow, not all of the cells will be
treated for the same period of time in a flow through

5   process. Lamina proximal to walls of flow through
conduits travel slower than lamina distal to the walls.
Flow through processes are used in both food processing
where the electric field intensity is over 20,000
volts/cm and in inserting molecules into cells for

10   therapeutic purposes.

A large body of prior art exists in the field of
electroporation, and a number of aspects of this body of
art are of particular interest herein. For example, of
particular interest herein are disclosures of the

15   electroporation medium, with special attention directed
to medium parameters. In this respect, TABLE 3 herein
sets forth a number of references relating to
electroporation medium parameters such as cations,
anions, osmolarity, and buffering.

20

TABLE 3

The following table summarizes the current state of the
25   art:

Publication	Conductivity	Cations		Anions	Osmolarity	Buffer
	(μs/cm)	High Conc.	Low Conc.			
Invention	Low (50-150)	None	Ca, Mg	Organic	L-N	Histidine
5,124,259	High	K	Ca, Mg	Organic	N	
6,040,184	Very low	None	None	None	L-N	None
6,338,965	Very low	None	None	None	L-N	None
6,368,784	High	K	Ca, Mg	Cl	N	Phos, HEPES
Djuzenova 1996	Moderate to high (800-14000)	Na, K	Ca	Cl, Sulfate	N	Phos.
Kinosita 1977	High	Na		Cl		Phos.
Dimitrov 1990	Low to Moderate		Na	Phos., Cl		Phos.
Rols 1989	Low and high	Na		Cl		Phos.
Pucilar 2001	Low and high	Na, K (if	Mg	Cl, Sulfate	N	Phos

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		used)				

More particularly with respect to TABLE 3, United
States Patent: 5,124,259 describes an electroporation
medium that provides high transfection efficiency. The
medium has potassium ions (35-105 milligram
equivalents/Liter) and organic anions and is essentially
devoid of chloride ions. The medium is highly conductive
as a result of the potassium ions. The use of low
conductive medium to allow the use of large
electroporation electrodes is not discussed.

United States Patents 6,040,184 and 6,338,965
describe an electroporation medium with essentially no
ions. The medium is made non-ionic through the use of
sugars and no inorganic ions. The patent describes
increased transfection efficiency in bacteria with the
non-ionic medium. The patent does not mention the
addition of a small amount of organic ions to provide
some conductivity and therefore some current to maintain
an electric field during electroporation.

United States Patent: 6,368,784 describes an
electroporation buffer that is also a cryoprotectant. It
also describes the use of this material for freezing
cells prior to transfection. The medium used has a high
concentration of potassium ions similar to that in
intracellular cytoplasm and similar to that described in
patent 5,124,259. The patent does not describe the use
of electroporation medium with lower conductivity to
allow the use of larger capacity electrodes.

Conductivity of the medium affects the movement of
material into cells. Djuezenova (Djuzenova et al,
Biochemica et Biophysica Acta V 1284, 1996, p 143-152)
showed that the uptake of small molecules is increased in
lower conductivity medium down to 1 mS/cm, the lowest
conductivity used in the study. Others have concurred

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that lower conductivity increases the permeability of
cells to small molecules during electroporation.
(Kinosita, K, Tsong, TY, Proc. Natl. Acad Sci, USA, 1977
V74:1923-1927) (Kinosita, K, Tsong, TY Nature, 1977
5   V268:438-440) (Dimitrov, DS, Sowers, AE, Biochem.
Biophys. Acta. 1990, V 1022:381-392).
        Kinosita found that with a given electric field,
media of high conductivity allowed leakage of small ions
(sodium and potassium) and medium of lower conductivity
10   allowed passage of larger molecules (sucrose but not
proteins) through red blood cell membranes. More
specifically, Kinosita et al disclose hemolysis of human
erythrocytes employing an electroporation step. With
respect to the cell used for electroporation, there is no
15   disclosure of electrode surface area. Therefore, and of
key importance, cell chamber volume is indeterminable. A
broad range of medium conductivities is stated. A broad
range of electrode gaps is stated. Yet, there is no
teaching provided for choosing any particular set of
20   medium conductivity and electrode gap.
        Dimitrov showed that leakage of a fluorescent dye
from electroporated red blood cells was less in medium
with a moderate conductivity compared to medium with a
low conductivity. Using a sensitive assay for
25   permeability of small molecules one group (Pucihar, G et
al, Bioelectrochemistry 2001, V 54: 107-115) showed that
lowering the conductivity of an electroporation buffer
resulted in no change of permeability at given electric
fields but an increase in viable cells. The assay used,
30   electroporation using bleomycin, detects small amounts of
uptake of small molecules and would not be sensitive to
differences in amount of electroporation in a given cell.
        Others have found just the opposite effect, such as
disclosed in "Better permeability of cells to small
35   molecules was seen during electroporation using media of
higher conductivity" (Rols, MP, Tiessie, Eur. J Biochem
1989 V 179:109-115). Rols and Tiessie showed that

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permeability to a small molecule, Trypan Blue, was
greater in high sodium medium at equal field strength and
equal number of pulses.   Others (vnd den Hoff, MJ,
Christoffels, VM, Labruyere, WT, Moorman, AF, Lamers, WH,
5   Electrotransfection with "intracellular" buffer, 1995,
Methods Mol. Biol. V48:185-197) used high levels of
potassium to mimic intracellular ionic content in an
effort to preserve cell viability. A more recent
study(Baron, S et al, J. Immunol. Meth., 2000 V 242:
10   115-126) used commercially available medium with a high
potassium content (VisSpan, Belzer UW cold-storage
solution, DuPont Pharmaceuticals) to increase
electroporation efficacy. The material delivered during
this study was macromolecules such as proteins and DNA.
15   None of the above references discussed the use of
medium with lower conductivity to achieve the movement of
macromolecules into mammalian cells. None of the
references discussed the use of medium with lower
conductivity to allow the use of larger capacity
20   electrodes.
        Other components of the medium contribute both to
transfection and to cell viability. One component that
has been used is potassium. Potassium in physiological
levels equal to intracellular amounts tends to increase
25   viability in electroporated cells. This was shown by van
den Hoff (van den Hoff et al., Nucleic Acids Res., vol.
20, No. 11, 1992, p. 2902) and others. The addition of
potassium to electroporation medium increases the
conductivity of the medium and makes the medium less
30   desirable for use in larger electrodes.
        Calcium ions also are reported to increase viability
of cells following electroporation. The reason for the
increase in viability is reported to be a contribution by
calcium in the resealing process after electroporation.
35   The increase in viability due to calcium is slightly
offset by decreased uptake of small molecules, presumably
by the same mechanism of increased pore closure due to

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calcium. The increase in viability due to small amounts
of calcium (0.1 mM), is obtained at a low cost in terms
of increased conductivity because of the small amount
used. Therefore, the addition of calcium to
5   electroporation medium is desirable.
     Osmolarity of the medium affects cell viability and
the efficiency of movement of large molecules through
cell membranes. Most electroporation is done using media
with normal osmolarity. However, the use of hypoosmolar
10   media can increase the efficiency of DNA transfection.
(van den Hoff et al, Nucleic Acids Res., vol. 18, No. 21,
1990, p. 6464) (Golzio et al., Biophys. J., vol. 74,
1998, pp. 3015-3022). Osmolarity can be adjusted in
electroporation media using non-ionic compounds such as
15   sugars, sugar alcohols, aminosugars of other non-toxic
organic compounds. These materials do not add to the
conductivity. Conductivity can be precisely controlled
using inorganic anions with inorganic or organic cations.
     The use of non-ionic organic material to adjust
20   osmolarity without affecting conductivity is desirable.
     Other references include:
Melkonyan et al., "Electroporation efficiency in
mammalian cells is increased by dimethyl sulfoxide
(DMSO)", Nucleic Acids Res., vol. 24, No. 21, 1996, pp.
25   4356-4357 and Rols et al., "Control by ATP and ADP of
voltage-induced mammalian-cell-membrane permeabilization,
gene transfer and resulting expression", Eur. J.
Biochem., vol. 254, 1998, pp. 382-388.

30   Other parameters are of interest herein with respect
to electroporation methods and apparatus disclosed in the
prior art. Of particular interest are the parameters of
capacity, environment for cell treatment (static or
flow), treated material, whether clinical use is provided
35   for, and media or buffer used. TABLE 4 sets forth a
number of U. S. patents with respect to these parameters.

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TABLE 4

Patent	Capacity	Static or flow	Treated material	Clinical use	Media or buffer used
4,695,472	Large	Flow	Food	N	Food
4,695,547	Small	Static	Cells	N	Any
4,838,154	Large	Flow	Food	N	Food
4,849,089	Small	Static	Cells	N	Any
4,882,281	Small	Static	Cells	N	Any
5,048,404	Large	Flow	Food	N	Food
5,098,843	Large	Flow*	Cells	Possibly	Non-Ionic
5,128,257	Small	Static	Adherent cells	N	Ionic
5,134,070	Small	Static	Adherent cells	N	Ionic
5,137,817	Small **	Static	Cells	Y	Any
5,173,158	Small	Static (on filter)	Cells	Possibly	Any
5,186,800	Small	Static	Bacteria	N	Low ionic
5,232,856	Small	Static	Adherent cells	N	Any
5,235,905	Large	Flow	Food	N	Food
5,283,194	Small	Static	Cells	Possibly	Any
5,545,130	Large	Flow	Blood	Y	Ionic
5,676,646	Large	Flow	Blood	Y	Ionic
5,720,921	Large	Flow	Blood	Y	Ionic
5,776,529	Large	Flow	Food	N	Ionic
5,874,268	Small	Static	Adherent cells	N	Any
6,001,617	Small	Static	Adherent cells	N	Any
6,074,605	Large	Flow	Blood	Y	Ionic

Notes for TABLE 4:

*Electric field is always on, no pulses, effective pulse
width determined by flow rate

** Electrodes plated onto surface

More specifically with respect to the patents set
forth in TABLE 2, United States Patent 4,695,472
describes the treatment of food by electroporation using
a large volume flow-through chamber. Cannot reduce
conductivity of food, has large effective capacity, no
clinical use.

United States Patent 4,695,547 describes round
electrodes for electroporation within round tissue

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culture plates. No low conductive medium, no large size,
no clinical use

United States Patent 4,838,154 describes the
treatment of food by electroporation using a large volume
5   flow-through chamber. Cannot reduce conductivity of
food, has large effective capacity, no clinical use.

United States Patent 4,849,089 describes round
electrodes for electroporation using fully enclosed
chambers. No low conductive medium, no large size, no
10   clinical use

United States Patent 4,882,281 describes round
electrodes for electroporation within round tissue
culture plates. No low conductive medium, no large size,
no clinical use.

15   United States Patent 5,048,404 describes the
treatment of food by electroporation using a large volume
flow-through chamber. Cannot reduce conductivity of
food, has large effective capacity, no clinical use.

United States Patent 5,098,843 describes a flow
20   through electroporation chamber for transfection of
cells. The pulse is always on and the effective pulse
width is determined by the time in the chamber (flow
rate). Non-ionic medium is described, large volume
capacity, possible clinical use but not described.

25   United States Patent 5,128,257 describes an
apparatus for transfecting cells grown as adherent cells.
Apparatus consists of multiple parallel plates placed on
a monolayer of cells. Only buffer described is PBS
(highly ionic), large capacity difficult due to monolayer
30   of cells. Clinical use not described.

United States Patent 5,134,070 describes a chamber
for culturing cells on an optically transparent surface
that is conductive. The chamber is for electroporation
of the adherent cells. Low-ionic medium is mentioned in
35   the claims but no specific formula is discussed. Large
capacity difficult because of adherent cells, no clinical
use mentioned.

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United States Patent 5,137,817 describes a variety
of electrodes. The example used non-ionic medium,
however it mentions that a variety of different ionic
strength media can be used. In vivo and in vitro
5   electrodes are described. The in vitro electrodes are
small capacity because they have electrodes plated onto
surfaces (not easily scalable). Low ionic medium used,
small capacity, clinical uses mentioned for in vivo
electrodes.

10   United States Patent 5,173,158 mentions the
electroporation of cells that are trapped in pores of a
non-conducting membrane. Low voltages are possible
because all current flows through the membrane pores.
Electroporation medium conductivity or ionic content is
15   not mentioned. No clinical use is mentioned. Small
capacity due to the need to trap cells in a pore.

United States Patent 5,186,800 describes the
transfection of prokaryotes (bactreria). Low ionic
medium is used. Does not describe the use of low ionic
20   medium with mammalian cells. States small capacity is
desired. No clinical use described.

United States Patent 5,232,856 describes
electroporation where one electrode is partially
conductive. A tilted electrode may be used on one of the
25   electrodes to compensate for the uneven electric fields
generated using one partially conductive electrode.
Although not clear in the claims, the partially
conductive electrode is for adherence of cells to its
surface. Ionic content of medium not mentioned.
30   Adherence would limit size. Clinical use is not
mentioned.

United States Patent 5,235,905 describes the use of
electroporation to process liquid food. Large capacity
flow through electrode is described. Ionic content of
35   food is not adjustable. Large static capacity is not
described. Clinical use is not described.

United States Patent 5,283,194 mentions the

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electroporation of cells that are trapped in pores of a
non-conducting membrane. Low voltages are possible
because all current flows through the membrane pores.
Electroporation medium conductivity or ionic content is
5   not mentioned. No clinical use is mentioned. Small
capacity due to the need to trap cells in a pore.
       United States Patent 5,514,391 describes the use of
electroporation to process liquid food. Large capacity
flow through electrode is described. Ionic content of
10   food is not adjustable. Large static capacity is not
described. Clinical use is not described.
       United States Patent 5,545,130 and United States
Patent 5,676,646 describe a flow through electroporation
device. It is designed to treat whole blood. Material
15   can be added to the blood that is not ionic but blood is
highly ionic. Large capacity is due to flow through.
Low conductivity is not mentioned for increasing
capacity. Large static capacity is not described.
Clinical use is described.
20   United States Patent 5,720,921 describes a flow
through electroporation chamber. A modification is made
to add flexible walls to buffer pressure changes. The
main example given is to treat red blood cells by
introducing material in them that increases the release
25   of oxygen from the cells. An electroporation medium is
used that is conductive. Large capacity is due to flow
through. Low conductivity is not mentioned for
increasing capacity. Large static capacity is not
described. Clinical use is described.
30   United States Patent 5,776,529 describes the use of
electroporation to process liquid food. Large capacity
flow through electrode is described. Ionic content of
food is not adjustable. Large static capacity is not
described. Clinical use is not described.
35   United States Patent 5,874,268 describes an
electroporation chamber designed to electroporated
adherent cells. The intent of the invention is to reduce

the number of cells needed. Large capacity is not
mentioned. Specific electroporation buffers are not
mentioned (just a statement about using any
electroporation buffer). Clinical use is not described.

5   United States Patent 6,001,617 describes an
optically transparent electroporation chamber fro
treatment of adherent cells. Size is limited by adherent
cells. No low ionic medium is discussed. No clinical
use is discussed.

10   United States Patent 6,074,605 describes a flow
through electroporation chamber. The main example given
is to treat red blood cells by introducing material in
them that increases the release of oxygen from the cells.
An electroporation medium is used that is conductive.

15 Large capacity is due to flow through. Low conductivity
is not mentioned for increasing capacity. Large static
capacity is not described. Clinical use is described.

        Another aspect of the prior art relates to the
20 parameters of conductivity in conjunction with electrode
dimensions (height, width, and gap), presence or absence
of a cuvette, volume, and dimension, such as shown in
TABLE 5.

25 TABLE 5
1.TABLE   5
2.Electrode Dimensions
Static, no adherent cells

Publicati on	Conductivi ty	Electrode Dimensions			Cuvett e	Volume	Dimension
	(μs/cm)	Heigh t	Width	Gap			
			mm	mm	mm	ml	
5,124,259	High (~10K)	2	87.5	4	N	0.7	0.23
6,040,184	Very low				Y		0.1-0.4
6,338,965	Very low				Y		0.1-0.4
6,368,784	High (~17K)				4		0.4
Djuzenova 1996	Moderate to high (800-14000)			6	N	1.2	0.3
*Kinosita 1977	Saline and sucrose		5-100	2-10	N, cross sectio		Not determinab le from

					n= 50-200 mm^2		publication
Reimann 1975	PBS	30	30	10			0.11
Dimitrov 1990	Low to Moderate (~100-10K)			2	N	.003	66
Pucilar 2001	0.0011 - 1.61 S/m			2		.05	0.8
Baron 2000	High (~17K)				4	.4	0.4
Schwister 1985	PBS	30	30	10	N	10	0.11
Mussauer 2001	1.5-3.5 mS/cm				2	.4	0.1
Mussauer 1999	1-8 mS/cm			6		1.1	0.33
Fomekong 1998	0.064-1.447 S/cm			5		.884	0.28
5,128,257	Saline	10-20	50-80		0.5-1.5		
5,186,800	Water				0.5 - 2.5	0.001-1	0.5-hundreds

5

        Having discussed prior art above, it is clear that
10   the foregoing body of prior art does not teach or suggest
     electroporation methods and apparatus which have the
     following combination of desirable features: (1) can be
     used for clinical and therapeutic purposes wherein all
     cells, ex vivo or in vitro, are subject to substantially
15   the same process conditions; (2) is scalable so that
     substantially large volumes of ex vivo or in vitro cells
     can be processed in a relatively short period of time;
     (3) achieves increased cell capacity without increasing
     the size of electrodes resulting in excessively large
20   amperage requirements; (4) limits heating within the
     treatment cell to low levels; (5) exposes substantially
     all ex vivo or in vitro cells to the same electric field
     intensity and direction; (6) provides that the density of
     the material to be inserted into the treatment chamber
25   can be held constant; (7) permits variable rectangular
     pulse waveforms such as disclosed in U. S. Patent No.
     6,010,613 can be employed; (8) avoids problems in flow

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through treatment cells that are due to laminar and
turbulent flow conditions; (9) permits the use of medium
with lower conductivity to achieve the movement of
macromolecules into mammalian cells and to allow the use
5   of larger capacity electrodes; and (10) is easily
scalable to large capacity without using a flow through
treatment chamber for cells to be treated.

The foregoing desired characteristics are provided by
the unique large volume ex vivo electroporation method of
10   the present invention as will be made apparent from the
following description thereof. Other advantages of the
present invention over the prior art also will be
rendered evident.

15

DISCLOSURE OF INVENTION

In view of the above, it is an object of the present
invention is to provide a large volume ex vivo
20   electroporation method which can be used for clinical and
therapeutic purposes wherein all cells, ex vivo or in
vitro, are subject to substantially the same process
conditions.

Still another object of the present invention is to
25   provide a large volume ex vivo electroporation method
that is scalable so that substantially large volumes of
ex vivo or in vitro cells can be processed in a
relatively short period of time.

Yet another object of the present invention is to
30   provide a large volume ex vivo electroporation method
which achieves increased cell capacity without increasing
the size of electrodes resulting in excessively large
amperage requirements.

Even another object of the present invention is to
35   provide a large volume ex vivo electroporation method
that limits heating within the treatment cell to low
levels.

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Still a further object of the present invention is
to provide a large volume ex vivo electroporation method
which exposes substantially all ex vivo or in vitro cells
to the same electric field intensity and direction.

5   Yet another object of the present invention is to
provide a large volume ex vivo electroporation method
that provides that the density of the material to be
inserted into the treatment chamber can be held constant.

10   Still another object of the present invention is to
provide a large volume ex vivo electroporation method
which permits variable rectangular pulse waveforms such
as disclosed in U. S. Patent No. 6,010,613 can be
employed.

15   Yet another object of the present invention is to
provide a large volume ex vivo electroporation method
that avoids problems in flow through treatment cells that
are due to laminar and turbulent flow conditions.

Still a further object of the present invention is
20   to provide a large volume ex vivo electroporation method
that permits the use of medium with lower conductivity to
achieve the movement of macromolecules into mammalian
cells and to allow the use of larger capacity electrodes.

25   Yet another object of the present invention is to
provide a large volume ex vivo electroporation method
which is easily scalable to large capacity without using
a flow through treatment chamber for cells to be treated.

30   These together with still other objects of the
invention, along with the various features of novelty
which characterize the invention, are pointed out with
particularity in the claims annexed to and forming a part
of this disclosure. For a better understanding of the
35   invention, its operating advantages and the specific
objects attained by its uses, reference should be had to
the accompanying drawings and descriptive matter in which

there are illustrated preferred embodiments of the
invention.

    To achieve the foregoing and other advantages, the
present invention, briefly described, provides a static
5   chamber with large volume to insure all cell are subject
to the same electric field intensity and direction and
the density of the cells and material are uniform. With
this invention any waveform may be used. This invention
is a voltage waveform generator connected to an electrode
10   with parallel plates with has low conductivity media, a
cell density of 20 million cells or less. The invention
uses media with conductivity between 50 μS/cm and 500
μS/cm. The invention may be used in clinical
applications and has a closed sterile chamber into which
15   the cells and large molecules are inserted and removed.

    In accordance with one aspect of the invention, a
method is provided of treating vesicles with exogenous
material for insertion of the exogenous material into the
vesicles includes the steps of:
20   a. retaining a suspension of the vesicles and the
exogenous material in a treatment volume in a chamber
which includes electrodes, wherein the chamber has a
geometric factor (cm^{-1}) defined by the quotient of the
electrode gap squared (cm^2) divided by the chamber volume
25   (cm^3), wherein the geometric factor is less than or equal
to 0.1 cm^{-1}), wherein the suspension of the vesicles and
the exogenous material is in a medium which is adjusted
such that the medium has conductivity in a range spanning
0.01 to 1.0 milliSiemens, wherein the suspension is
30   enclosed in the chamber during treatment, and
        b. treating the suspension enclosed in the chamber
with one or more pulsed electric fields,
        wherein in accordance with a. and b. above, the
treatment volume of the suspension is scalable, and
35   wherein the time of treatment of the vesicles in the
chamber is substantially uniform.

    Preferably, the chamber is a closed chamber.

- 20 -

Preferably, the chamber has at least a 2 milliliter
capacity. The chamber and the contents thereof can be
sterile. Preferably, the chamber includes entry and exit
ports for entry and removal of the suspension.
5   Preferably, the electrodes are parallel plate electrodes.

The electric fields are substantially uniform
throughout the treatment volume. The electric fields can
include a rectangular voltage pulse waveform to produce a
10 uniform pulse electric field between parallel plate
electrodes greater than 100 volts/cm and less than 5,000
volts/cm, substantially uniform throughout the treatment
volume.
The vesicles can be living cells, and the medium can
15 be a physiological medium and has a conductivity between
50 and 500 μS/cm. The number of living cells that are
treated in the chamber at one time can be more than 10
million in number. Furthermore, the number of living
cells that are treated in the chamber at one time can be
20 more than 20 million in number.
The vesicles can be autologous cells that are to be
returned to a donor after treatment with the exogenous
material. The vesicles can be syngeneic cells that are
to be given to a recipient other than the donor. The
25 vesicles can be zenogeneic cells. The vesicles can be
artificial liposomes.
The pulsed electric fields can be from electrical
pulses which are in a sequence of at least three non-
sinusoidal electrical pulses, having field strengths
30 equal to or greater than 100 V/cm, to the material. The
sequence of at least three non-sinusoidal electrical
pulses has one, two, or three of the following
characteristics (1) at least two of the at least three
pulses differ from each other in pulse amplitude, (2) at
35 least two of the at least three pulses differ from each
other in pulse width, and (3) a first pulse interval for
a first set of two of the at least three pulses is

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different from a second pulse interval for a second set
of two of the at least three pulses.

 With the method of the invention, the temperature
rise during vesicle treatment is miniscule.

5 The method of the invention is scalable in a range
spanning 2 to 10 milliliters. The method of the
invention can be carried out in sequential batches.

 The exogenous material can be a therapeutic
material. The exogenous material can be a therapeutic
10 product formed from the treatment of the vesicles with
exogenous material. The exogenous material can be
selected from the following group: a polynucleotide;
DNA; RNA; a polypeptide; a protein; and an organic
compound.

15 The exogenous material can include numerous base
pairs, for example, at least eight base pairs.

 With the invention, the chamber has a chamber
volume, the suspension has a suspension volume, and the
suspension volume is greater than the chamber volume. In
20 this respect, an initial portion of the suspension volume
is moved into the chamber, retained and treated in the
chamber, and moved out from the chamber. Then, an
additional portion of the suspension volume is moved into
the chamber, retained and treated in the chamber, and
25 moved out from the chamber.

 Still further portions of the suspension volume are
sequentially moved into the chamber, retained and treated
in the chamber, and moved out from the chamber. These
steps can be repeated until the suspension volume is
30 depleted.

 In accordance with another aspect of the invention,
an electroporation apparatus is provided which includes a
chamber which has a chamber volume of at least 2
milliliters. A pair of electroporation electrodes are
35 contained within the chamber. An electroporation medium,
carrying vesicles in suspension, is contained in the
chamber between the electroporation electrodes. The

medium has a conductivity between 50 and 500 mS/cm. A
source of pulsed voltages is electrically connected to
the electroporation electrodes, and means for adding
material to the chamber for electroporation treatment
5   therein. Also, means are provided for removing treated
material from the chamber.

Preferably, sealing means are connected to the
chamber for providing a sealed chamber. The sealing
means can include a quantity of elastomer material.

10   Preferably, the sealed chamber is sterile inside the
chamber. Preferably, the chamber includes vent means for
venting air when fluid is moved into the chamber. The
vent means can include a filter member in a wall of the
chamber. Alternatively, the vent means can include a

15   vent cell in fluid communication with the chamber.

The chamber includes a chamber inlet and a chamber
outlet.
A first reservoir can be provided in fluid communication
with the chamber inlet, for containing the vesicle-
20   bearing electroporation medium prior to introduction into
the chamber. A second reservoir can be provided in fluid
communication with the chamber inlet, for containing a
chamber flushing material for flushing treated vesicle-
bearing medium out from the chamber. A third reservoir
25   can be provided in fluid communication with the chamber
outlet, for receiving treated, vesicle-bearing medium
that is flushed out from the chamber.

The first reservoir, the second reservoir, and the
third reservoir can be comprised of flexible bags. An
30   inlet valve can be connected between the chamber inlet
and the first reservoir and the second reservoir, and an
outlet valve can be connected between the chamber outlet
and the third reservoir.

35

BRIEF DESCRIPTION OF DRAWINGS

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        The invention will be better understood and the
above objects as well as objects other than those set
forth above will become more apparent after a study of
the following detailed description thereof. Such
 5   description makes reference to the annexed drawing
wherein:
        FIG. 1 is a schematic illustration of apparatus
employed with carrying out the method of the invention.
        FIG. 2 is a graph illustrating the operating range
10   of the method of the invention, inside the triangle, and
how the operating range of the invention is outside
operating ranges of prior art electroporation methods,
indicated by small blocks outside the triangle.
        FIG. 3 is a graph illustrating the relationship
15   between charging time (in microseconds) of biological
cells and media conductivity (in microSiemens/cm) for
cells having three different diameters, namely 1
micrometer, 10 micrometers, and 100 micrometers.
        FIG. 4 is a graph showing Time Constant versus
20   Conductivity as it relates to the method of the
invention.

                   MODES FOR CARRYING OUT THE INVENTION
25

        As previously described a significant problem is the
conductivity of the media use in electroporation.   In
this process a low conductivity media is employed to keep
30   the total resistance of the media small and virtually
eliminates heating. Not just any media conductivity can
be used.   As the ionic content of the media is reduce the
number of free-ions that are available to build charge
(voltage) across the cell member is decreased.   The
35   effect is to increase the amount of time it takes to
charge the membrane.   This process is described by the
equation in Electroporation and Electrofusion in Cell

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Biology, edited by Eberhard Neumann, Arthur Sowers, and Carol Jordan, Plenum Press, 1989, on page 71. Assuming a typical cell diameter of 10 microns, the charging time is 20 microseconds at 80 μS/cm. Below 80 μS/cm the charging

5   time become too long and the pathways in cell membrane stop forming. The TABLE 6 below illustrates the resistance of the media as a function of electrode chamber volume and conductivity.

10

TABLE 6

Electrode Volume ml	Media Resistance - ohms		
	17,000 μS/cm	200 μS/cm	80 μS/cm
0.5	19.2	1600	4000
1	9.6	800	2000
5	1.92	160	400
10	0.96	80	200
50	0.19	16	40

15

Ex vivo electroporation has been demonstrated in numerous published research projects. At this point commercial applications, such as clinical transfection to produce a vaccine for the patient, requires large

20   electrodes or chambers to process millions of cells at one time. The static parallel plate chamber provides the most uniform amplitude and most uniform electric field direction of any configuration available. This uniformity is required to insure uniform treatment of the

25   target cells. It is also important not to use very high-density cell concentration such as 30 million cells/ml to insure local uniform electric fields about the cells. This invention applies to chambers larger than 1 milliliter.

30   Using larger chambers results in high current flow

- 25 -

when voltage is applied. The equations for chamber
resistance vs. conductivity of the cell and media mixture
and the chamber dimensions are as follows:

$$Volume\ of\ material = l \times A$$

$$Re sis \tan ce\ of\ Material = \rho\ \frac{l}{A} = \frac{1}{\sigma}\frac{l}{A} = \frac{1}{\sigma}\frac{l^2}{\upsilon} = \frac{GF}{\sigma}\quad ohms$$

ρ = resistivity in
ohm-cm

σ = $1/\rho$ in Siemens/cm

υ = volume of material
being treated

There is a Geometric Factor (GF), which is a
constant for any chamber dimension. As the volume of the
chamber gets larger the resistance of the material in the
chamber gets smaller thus increasing current flow.

The present invention uses an electrode with large
capacity in combination with an electroporation buffer of
defined low conductivity. This process exposes all cells
to the same treatment conditions, provides control over
the amperage required and can process large numbers of
cells. Since the cell suspension statically remains in
the chamber during application of pulsed electric fields,
complex waveforms can be used.

Another aspect of the invention further increases
capacity by alternately filling and emptying the gap
between the electrodes. In this manner, all desired
properties are met during a specific treatment and the
electrodes can be re-used for subsequent treatments in an
intermittent batch process.

This present invention specifies a range of material
conductivities, which can be used versus the chamber
dimensions, the larger the volume the smaller the

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conductivity. This invention specifies an operating area for use with the larger volume electrodes. This is illustrated in FIG. 2. Operating points of prior art published results are also presented in FIG. 2 as

5   squares. For chambers with a Geometric Factor less than 0.1 there are two limiting factors, which are related. The first is the absolute value of the chamber resistance. In this invention the chamber resistance is one ohm or greater. Operating below one ohm is view as

10 impractical. The other constraint is the conductivity of the medium in the chamber. As the conductivity decreases the charging time of the cell membrane increases because there are fewer ions external to the cell membrane.

The relationship between the Transmembrane Voltage

15 (TMV) and conductivity and cell diameter is as follows, taken from Neumann et al stated below:

Transmembrane Voltage = TMV

20 $$TMV = -1.5\, Er \left| \cos\delta \right| f(\lambda)$$

where: E = electric filed in volts/cm

r = cell radius in cm

δ = angle from electric field line in

25 degrees

f(λ) = composite conductivity

$$f(\lambda) = \frac{\lambda_o \lambda_i (2\frac{d}{r}(\ ))}{(2\lambda_o + \lambda_i)\lambda_m + (2\frac{d}{r})(\lambda_o - \lambda_m)(\lambda_i - \lambda_m)}$$

30   where: λ_o = conductivity of media external to cell milliSiemens/cm

λ_i = conductivity of cytoplasm

λ_m = conductivity of cell membrane

d = thickness of cell membrane

35

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Reference: Electroporation and Electrofusion in Cell
Biology
              Edited by Eberhard Neumann, Arthur Sowers, and
5   Carol Jordon
              Plenum Press, 1989

10   Below 1 microSiemens/cm there are so few ions that
the time to change the cell membrane is unrealistically
large.

          The preferred operating region of the present
15   invention is then:
Cell diameter   > 1 micrometer
Chamber volume   > 1 milliliter
Conductivity of Material to be treated   > 1
microSiemens/cm
20   Total resistance of material to be
          treated in chamber   > 1 ohm
Geometric Factor of Chamber   < 0.1 cm-1

          The invention uses a static chamber with large
25   volume to insure that all cells are subject to the same
electric field intensity and direction and the density of
the cells and treating material are uniform. With this
invention any waveform may be used. This invention is a
voltage waveform generator connected to an electrode with
30   parallel plates with has low conductivity medium, a cell
density of 20 million cells or less.
          A component of the invention is the use of low
conductivity medium within a defined range to limit
amperage and heat while simultaneously providing enough
35   ions to effectively electroporate cells. Typically the
medium used will have a conductivity between 50 mS/cm and
500 mS/cm.

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The invention may be used in clinical applications and with a closed sterile chamber into which the cells and large molecules are inserted and removed.

One aspect of the invention further increases
5   capacity by alternately filling and emptying the electrode. In this manner, all desired properties are met during a specific treatment and the electrode can be re-used for subsequent treatments in an intermittent batch process.

10   The conductivity of the medium used in electroporation is an important aspect of this invention. In this process, a low conductivity medium is employed to keep the total resistance of the medium small and virtually eliminate heating. There is a limit to the
15   lower conductivity medium that can be used. As the ionic content of the medium is reduced the number of free ions that are available to build charge (voltage) across the cell membrane is decreased. The effect is to increase the amount of time it takes to charge the membrane. This
20   process is described by the equation in Neumann, p71. Assuming a typical cell diameter of 10 microns, the charging time is 20 microseconds at 80 mS/cm. Below 80 mS/cm the charging time becomes too long and the pathways in cell membranes stop forming.

25   Using an electrode with a 4 mm gap, TABLE 6 illustrates the resistance of the medium as a function of electrode chamber volume and conductivity.

        In one aspect of the invention, a chamber with two electrodes is used as shown in FIG 1. An example of
30   electrode dimensions that can be used is a gap of 0.4 cm, electrode height of 2 cm and electrode length of 10 cm. The chamber can be used with a commercial electroporator such as the Cyto Pulse Sciences, Inc. PA-4000 electroporator.

35   An example of a medium that can be used with the chamber is one with the following formula:
Sorbitol 280 millimoles

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Calcium Acetate, 0.1 millimoles
Magnesium Acetate, 0.5 millimoles

    FIG. 3 is a graph illustrating the relationship
5   between charging time (in microseconds) of biological
    cells and media conductivity (in microSiemens/cm) for
    cells having three different diameters, namely 1
    micrometer, 10 micrometers, and 100 micrometers. From
    FIG. 3 it is clear that for media conductivity below 1
10 microSiemen/cm, the charging time would be so large that
    electroporation would not work.

    As to the manner of usage and operation of the
    instant invention, the same is apparent from the above
15 disclosure, and accordingly, no further discussion
    relative to the manner of usage and operation need be
    provided.
    It is apparent from the above that the present
    invention accomplishes all of the objects set forth by
20 providing a large volume ex vivo electroporation method
    which may advantageously be used for clinical and
    therapeutic purposes wherein all cells, ex vivo or in
    vitro, are subject to substantially the same process
    conditions. With the invention, a large volume ex vivo
25 electroporation method is provided which is scalable so
    that substantially large volumes of ex vivo or in vitro
    cells can be processed in a relatively short period of
    time. With the invention, a large volume ex vivo
    electroporation method is provided which achieves
30 increased cell capacity without increasing the size of
    electrodes resulting in excessively large amperage
    requirements. With the invention, a large volume ex vivo
    electroporation method is provided which limits heating
    within the treatment cell to low levels. With the
35 invention, a large volume ex vivo electroporation method
    is provided which exposes substantially all ex vivo or in
    vitro cells to the same electric field intensity and

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direction. With the invention, a large volume ex vivo
electroporation method provides that the density of the
material to be inserted into the treatment chamber can be
held constant. With the invention, a large volume ex

5   vivo electroporation method is provided which permits
variable rectangular pulse waveforms such as disclosed in
U. S. Patent No. 6,010,613 can be employed. With the
invention, a large volume ex vivo electroporation method
is provided which avoids problems in flow through

10   treatment cells that are due to laminar and turbulent
flow conditions. With the invention, a large volume ex
vivo electroporation method is provided which permits the
use of medium with lower conductivity to achieve the
movement of macromolecules into mammalian cells and to

15   allow the use of larger capacity electrodes. With the
invention, a large volume ex vivo electroporation method
is provided which is easily scalable to large capacity
without using a flow through treatment chamber for cells
to be treated.

20   With respect to the above description, it should be
realized that the optimum dimensional relationships for
the parts of the invention, to include variations in
size, form function and manner of operation, assembly and
use, are deemed readily apparent and obvious to those

25   skilled in the art, and therefore, all relationships
equivalent to those illustrated in the drawings and
described in the specification are intended to be
encompassed only by the scope of appended claims.

While the present invention has been shown in the

30   drawings and fully described above with particularity and
detail in connection with what is presently deemed to be
the most practical and preferred embodiments of the
invention, it will be apparent to those of ordinary skill
in the art that many modifications thereof may be made

35   without departing from the principles and concepts set
forth herein. Hence, the proper scope of the present
invention should be determined only by the broadest

interpretation of the appended claims so as to encompass
all such modifications and equivalents.

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CLAIMS

What is claimed is:

1. A method of treating vesicles with exogenous material for insertion of the exogenous material into the vesicles, comprising the steps of:

a. retaining a suspension of the vesicles and the exogenous material in a treatment volume in a chamber which includes electrodes, wherein the chamber has a geometric factor (cm^{-1}) defined by the quotient of the electrode gap squared (cm^2) divided by the chamber volume (cm^3),

wherein said geometric factor is less than or equal to 0.1 cm^{-1}),

wherein the suspension of the vesicles and the exogenous material is in a medium which is adjusted, such that the medium has conductivity in a range spanning 0.01 to 1.0 milliSiemens,

wherein the suspension is enclosed in the chamber during treatment, and

b. treating the suspension enclosed in the chamber with one or more pulsed electric fields,

wherein in accordance with a. and b. above, the treatment volume of the suspension is scalable, and

wherein the time of treatment of the vesicles in the chamber is substantially uniform.

2. The method of claim 1 wherein the chamber is a closed chamber.

3. The method of claim 1 wherein the chamber has at least a 2 milliliter capacity.

4. The method of claim 1 wherein the chamber and the contents thereof are sterile.

5.   The method of claim 1 wherein the chamber includes
entry and exit ports for entry and removal of the
suspension.

6.   The method of claim 1 wherein the electrodes are
parallel plate electrodes.

7.   The method of claim 1 wherein the electric fields are
substantially uniform throughout the treatment volume.

8.   The method of claim 1 wherein the electric fields
include a rectangular voltage pulse waveform to produce a
uniform pulse electric field between parallel plate
electrodes greater than 100 volts/cm and less than 5,000
volts/cm, substantially uniform throughout the treatment
volume.

9.   The method of claim 1 wherein:
        the vesicles are living cells,
        the medium is a physiological medium and has a
conductivity between 50 and 500 μS/cm.

10.   The method of claim 1 wherein the living cells being
treated are at least 10 million in number.

11.   The method of claim 1 wherein the living cells being
treated are at least 20 million in number.

12.   The method of claim 1 wherein the vesicles are
autologous cells that are to be returned to a donor after
treatment with the exogenous material.

13.   The method of claim 1 wherein the vesicles are
syngeneic cells that are to be given to a recipient other
than the donor.

14.   The method of claim 1 wherein the vesicles are

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zenogeneic cells.

15.   The method of claim 1 wherein the vesicles are
artificial liposomes.

16.   The method of claim 1 wherein the pulsed electric
fields are from electrical pulses which are in a sequence
of at least three non-sinusoidal electrical pulses,
having field strengths equal to or greater than 100 V/cm,
to the material, wherein the sequence of at least three
non-sinusoidal electrical pulses has one, two, or three
of the following characteristics: (1) at least two of
the at least three pulses differ from each other in pulse
amplitude; (2) at least two of the at least three pulses
differ from each other in pulse width; and (3) a first
pulse interval for a first set of two of the at least
three pulses is different from a second pulse interval
for a second set of two of the at least three pulses.

17.   The method of claim 1 wherein temperature rise
during treatment is miniscule.

18.   The method of claim 1 which is scalable in a range
spanning 2 to 10 milliliters.

19.   The method of claim 1 which is carried out in
sequential batches.

20.   The method of claim 1 wherein the exogenous material
is a therapeutic material.

21.   The method of claim 1 wherein a therapeutic product
is formed from the treatment of the vesicles with
exogenous material.

22.   The method of claim 1 wherein the exogenous material
is a polynucleotide.

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23. The method of claim 1 wherein the exogenous material is selected from the group consisting of DNA and RNA.

24. The method of claim 1 wherein the exogenous material is a polypeptide.

25. The method of claim 1 wherein the exogenous material is a protein.

26. The method of claim 1 wherein the exogenous material is an organic compound.

27. The method of claim 1 wherein the exogenous material includes at least eight base pairs.

28. The method of claim 1 wherein the chamber has a chamber volume, the suspension has a suspension volume, and the suspension volume is greater than the chamber volume, and wherein
       an initial portion of the suspension volume is moved into the chamber, retained and treated in the chamber, and moved out from the chamber, and
       an additional portion of the suspension volume is moved into the chamber, retained and treated in the chamber, and moved out from the chamber.

29. The method of claim 1 wherein still further portions of the suspension volume are sequentially moved into the chamber, retained and treated in the chamber, and moved out from the chamber.

30. The method of claim 1 wherein still further portions of the suspension volume are sequentially moved into the chamber, retained and treated in the chamber, and moved out from the chamber until the suspension volume is depleted.

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31. An electroporation apparatus, comprising:
      a chamber having a chamber volume of at least 2
milliliters,   a pair of electroporation electrodes
contained within said chamber,
      an electroporation medium, carrying vesicles in
suspension, contained in said chamber between said
electroporation electrodes, wherein said medium has a
conductivity between 50 and 500 mS/cm,
      a source of pulsed voltages electrically connected
to said electroporation electrodes, and
      means for adding material to said chamber for
electroporation treatment therein, and means for removing
treated material from said chamber.

32. The apparatus of claim 31, further including sealing
means connected to said chamber for providing a sealed
chamber.

33. The apparatus of claim 31 wherein said sealing means
include a quantity of elastomer material.

34. The apparatus of claim 31 wherein said sealed
chamber is sterile inside the chamber.

35. The apparatus of claim 31 wherein said chamber
includes vent means for venting air when fluid is moved
into said chamber.
36. The apparatus of claim 31 wherein said vent means
include a filter member in a wall of said chamber.

37. The apparatus of claim 31 wherein said vent means
include a vent cell in fluid communication with said
chamber.

38. The apparatus of claim 31 wherein said chamber
includes a chamber inlet and a chamber outlet.

39. The apparatus of claim 31, further including:
       a first reservoir, in fluid communication with said
chamber inlet, for containing said vesicle-bearing
electroporation medium prior to introduction into said
chamber,
       a second reservoir, in fluid communication with said
chamber inlet, for containing a chamber flushing material
for flushing treated vesicle-bearing medium out from said
chamber, and
       a third reservoir, in fluid communication with said
chamber outlet, for receiving treated, vesicle-bearing
medium that is flushed out from said chamber.

40. The apparatus of claim 39 wherein said first
reservoir, said second reservoir, and said third
reservoir are comprised of flexible bags.

41. The apparatus of claim 31 , further including:
       an inlet valve connected between said chamber inlet
and said first reservoir and said second reservoir, and
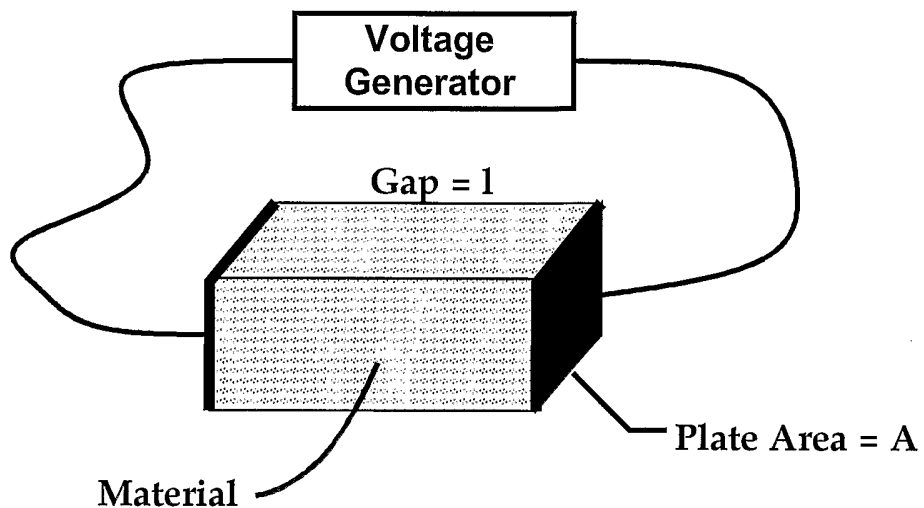   an outlet valve connected between said chamber
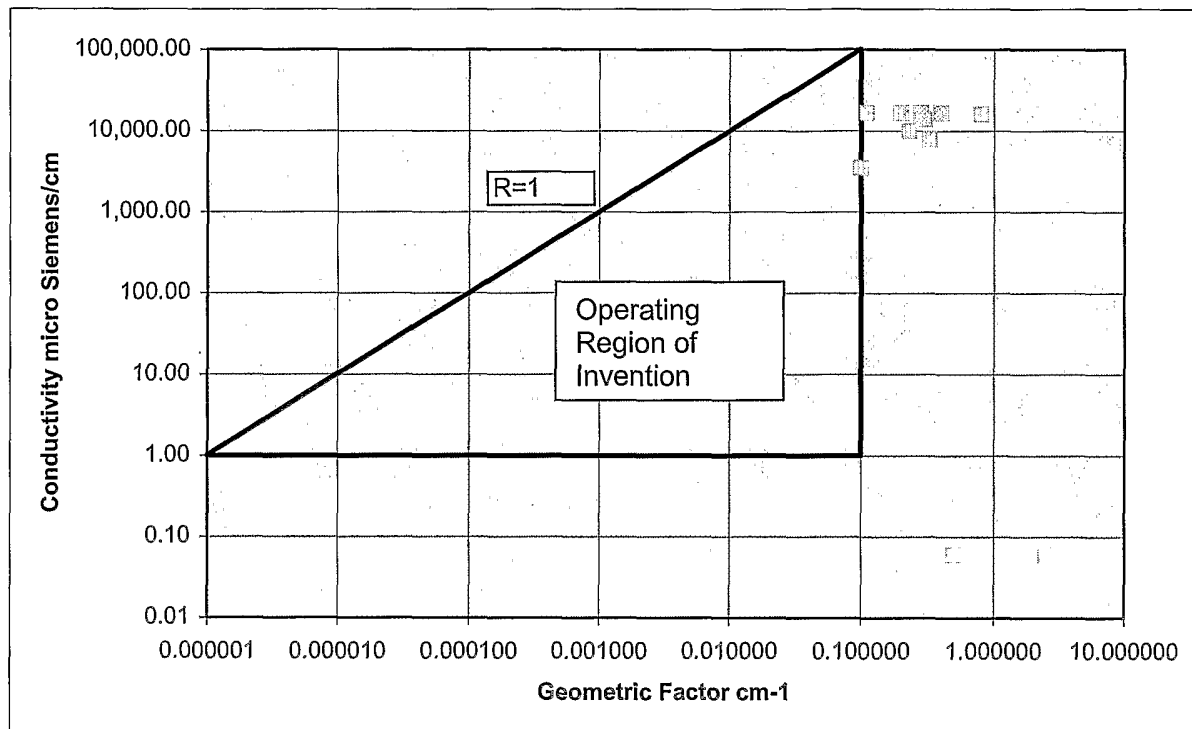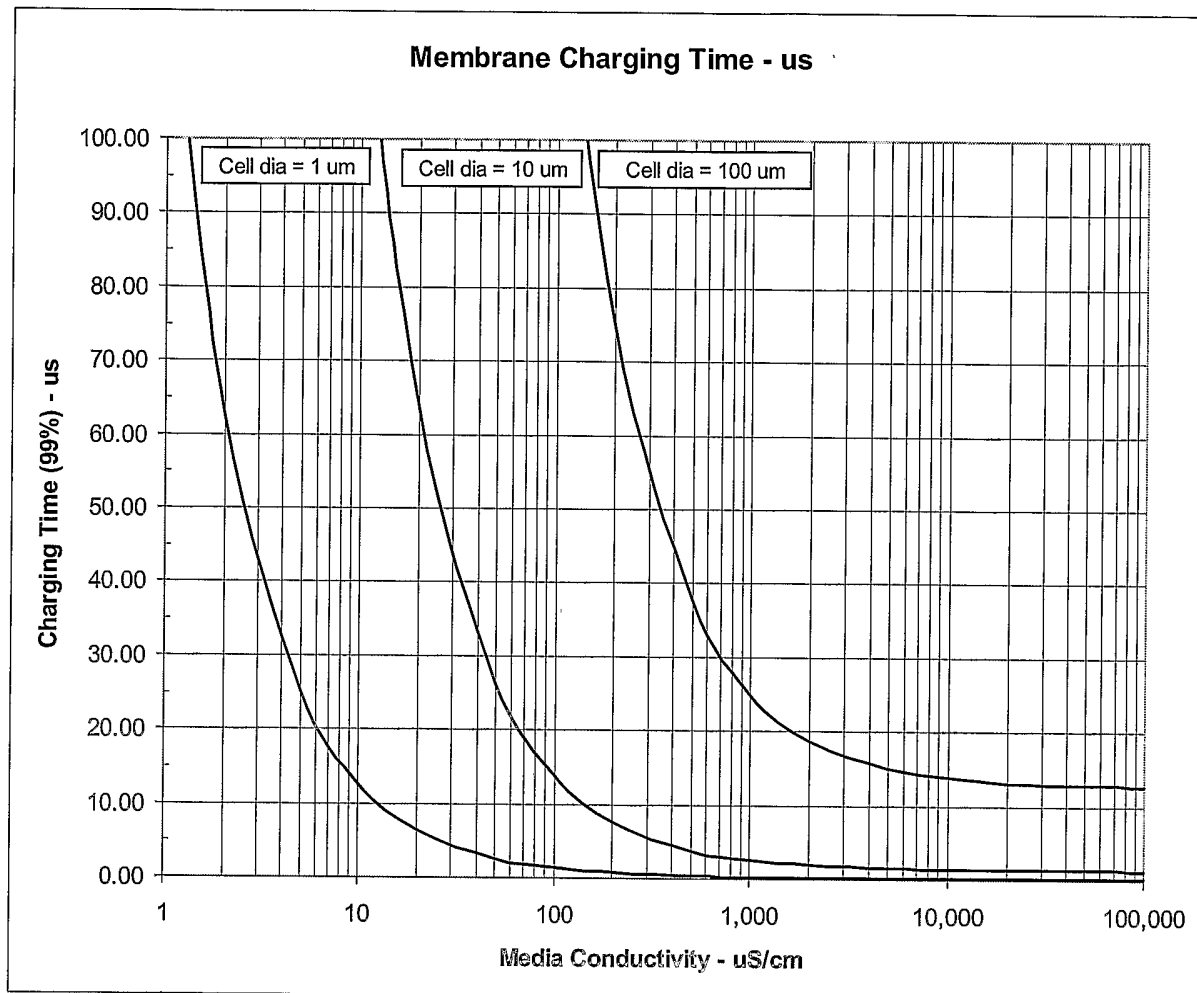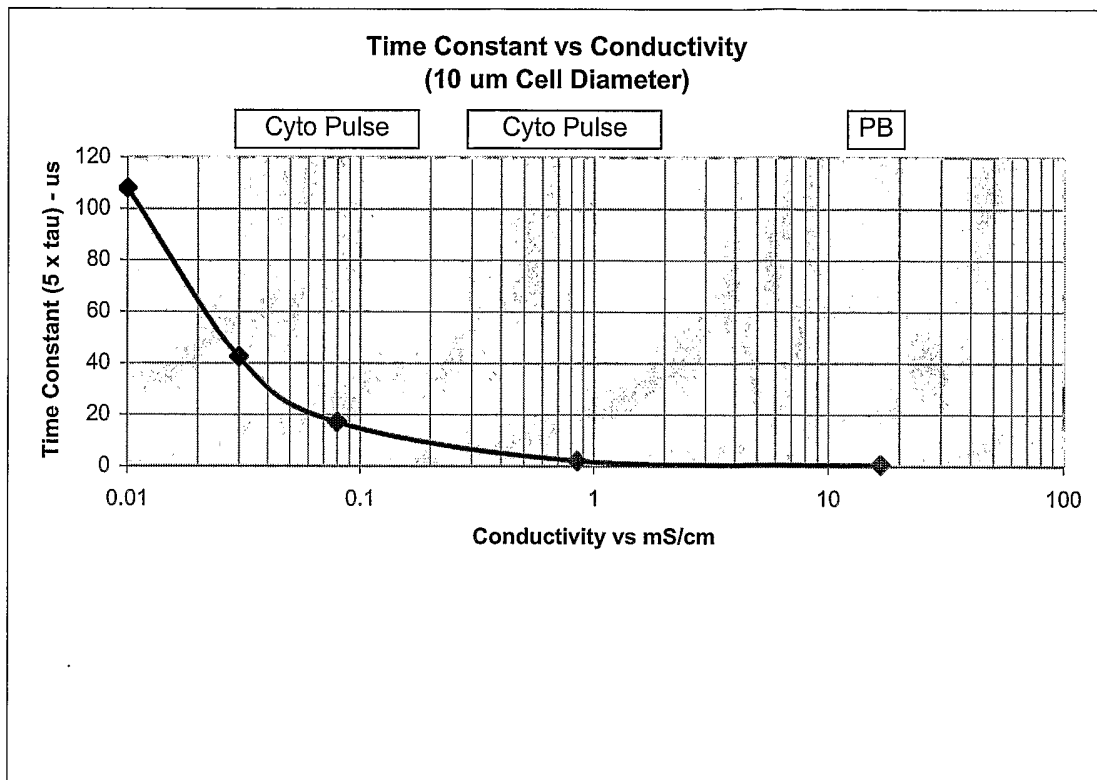outlet and said third reservoir.

FIG. 1

FIG. 2

FIG. 3

FIG. 4