PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12N 5/00	A1	(11) International Publication Number: WO 86/ 02377
		(43) International Publication Date: 24 April 1986 (24.04.86)

(21) International Application Number: PCT/US85/02029

(22) International Filing Date: 11 October 1985 (11.10.85)

(31) Priority Application Number: P 34 37 470.1

(32) Priority Date: 12 October 1984 (12.10.84)

(33) Priority Country: DE

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(81) Designated States: GB, JP, US.

Published
With international search report.

(54) Title: MEDIUM FOR THE PRODUCTION OF VIABLE FUSED CELLS

(57) Abstract

In order for a medium for the production of viable, fused cells by means of field-induced electrical fusion, particularly for the production of hybridoma cells, whereby the medium comprises an approximately isotonic, aqueous solution of electrolytes and non-electrolytes with a maximum ionic strength of 0.1, said solution containing calcium and magnesium salts, to be improved to such an extent that it adapts the fusion conditions of differently sized cells to each other during the field pulse and additionally allows closer cellular membrane contact through alteration in the structure of the medium in the region of the cellular membranes, so, that fusion can be performed with a high yield and under mild conditions and at the same time the survival rate of the cells is very high, the ratio of concentration of calcium and magnesium ions is in the range between 1:2 and 1:10 and the ionic strength of the solution is adjusted for optimum membrane contact through variation of the electrolyte concentration, whereby corresponding to a change of the electrolyte concentration is a complementary change of the non-electrolyte concentration, such that the isotonic property of the solution is constantly guaranteed by the overall osmolarity of the electrolyte and non-electrolyte parts.

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MEDIUM FOR THE PRODUCTION OF VIABLE FUSED CELLS

BACKGROUND OF THE INVENTION

The invention relates to a medium for the production of viable, fused cells by means of field-induced electrical fusion, particularly for the production of hybridoma cells, whereby the medium comprises an approximately isotonic, aqueous solution of electrolytes and non-electrolytes with a maximum ionic strength of 0.1, said solution containing calcium and magnesium salts.

The production of fused cells can be broken down into three phases; an orientation phase, the actual fusion process and a healing phase of the fused cells.

In the orientation phase, the cells which are to be fused are oriented, for example, along lines of force. Particularly suitable for this purpose is the dielectrophoretic effect which is based on polarization phenomena in the cells in the electric field and brings the cells to a certain contact distance to each other. The electric field is built up by a high-frequency alternating voltage in the range of approximately 1 MHz in order to minimize electrolysis phenomena in the medium. The prevention of electrolysis phenomena in the stage prior to fusion is extremely important because the products of electroylsis have a toxic effect on the cells, particularly when the cellular membranes are in a state of increased permeability.

The actual fusion process is initiated by a field pulse which incerases the permeability of the cellular membranes in the

contact area between two cells to such an extent that pores or penetrations occur in the membranes through which an exchange of the cytoplasm of the two cells can take place. The amplitude of the field pulse of the required field strength is inversely proportional to the radius of the cells under treatment according to the Laplace equation (see Biochimica et Biophysica Acta 692, 227-277; equation (5)) so that for small cells high field strengths and for large cells low field strengths are necessary in order to produce the penetrations or pores in the membranes. Directly after the field pulse, the field conditions orientating the cells are maintained for a short time in order to facilitate the fusion of the cellular membranes. Also of importance in this regard is the conductivity of the fusion solution which must be so limited that there is no disturbing generation of heat, as a result of which there would be convection currents which would adversely affect the orientation of the fusing cells in relation to each other.

After the alternating field has been switched off, there follows a period lasting up to 30 minutes and longer in which the healing of the cellular membranes is normally supported by temperature increase. During this time, the newly fused cell is still rather sensistive to ambient influences since the permeability of the cellular membranes is still at an increased level and, therefore, the selection mechanism of the membranes is partially inoperative.

In order to improve the fusion yields, it has been proposed hitherto to treat the cells with the enzyme pronase which, according to latest findings, reduces the mobility of the cells (FEBS Letters 163, 54 to 56 (1983)) so that, once produced,

pores in the two cells have a constant orientation in relation to each other, as a result of which the fusion of the membranes of the two cells becomes more probable. Treatment of the surface of the fusion chamber or the application of a coating of a higher-molecular substance, for example of a protein, may influence the fusion process, for which reason this coating is likewise to be considered as a part of the fusion medium. The use of the enzyme pronase leads to significant problems as regards the survival of the fused cells because the enzymatic activity of pronase lies in the decomposition of proteins and this decomposition also affects the proteins of the membrane. For the survival of the fused cells, however, it is important to conserve the proteins of the membranes, with the result that the enzyme pronase must be washed out under difficult conditions. If, during fusion, there are pronase molecules in the contact zone between the cells, and the molecules get into the interior of the cell, the dying off of the new cell is inevitable because the activity of pronase, of course, also extends to proteins inside the cell. For this reason in the above-quoted literature the proposal was made to replace the enzyme pronase by other proteins which are capable of fulfilling the same function as regards to mobility of the cells, but which do not exhibit any disadvantageous enzymatic activity as regards the viability of the cells. Even with good fusion yields, however, the number of viable cells remained very low.

Even greater problems with respect to the viability of the fused cells are encountered with the fusion of differently sized cells since, in this case, it is necessary to employ field pulses which are so great that they also create penetrations in the membrane of the small cell. However, these high field

strengths lead in the large cell also to penetrations outside the contact zone between the cells, with the result that the latter may possibly be so seriously damaged that either no fused cell whatsoever is produced, or the newly produced cell is likewise seriously damaged and, consequently, has a very small chance of survival.

Currently, fusion technology is at a level at which cells can be fused with high yields (e.g. 40% and above). However, the viability of the newly produced cells is extremely low with all hitherto known processes and media or solutions, i.e. the fused cells are usually not capable of division, so that although a fusion process provides a large number of fused cells, only a few individual cells are capable of division.

It is therefore a pricipal of the present invention to create a medium which adapts the fusion conditions of differently sized cells to each other during the field pulse and additionally allows a closer cellular membrane contact through intervention in the structure of the medium in the region of the cellular membrane, so that fusion can be performed with a high yield and under mild conditions and at the same time the survival rate of the cells is very high.

A further object of the invention is the application of a medium according to the invention for the production of viable, fused cells in a process for the non-damaging collection and orientation of the cells before and after the application of the field pulses.

SUMMARY OF THE INVENTION

A medium for the production of viable, fused cells by means of field-induced electrical fusion is provided. The medium comprises an approximately isotonic, aqueous solution of electrolytes and non-electrolytes with a maximum ionic strength of 0.1. The solution includes calcium and magnesium salts with the ratio of concentration of calcium and magnesium ions in a range between 1:2 and 1:10. The ionic strength of the solution is adjusted for optimum membrane contact through variation of the electrolyte concentration. Corresponding to a change of the electrolyte concentration is a complimentary change of the non-electrolyte concentration so that the isotonic property of the solution is constantly guaranteed by the overall osmolarity of the electrolyte and non-electrolyte parts. An electric alternating field is applied to the the medium to achieve the non-damaging collection and orientation of cells, the electric alternating field having a frequency below 100 kHz.

These and other objects and features of the present invention will be more fully understood from the following detailed description.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

According to the present invention a medium of the initially described kind is provided in which the ratio of concentration of calcium and magnesium ions is in the range between 1:2 and 1:10 with the ionic strength of the solution being adjusted for optimum membrane contact through variation of the electrolyte concentration. Corresponding to a change of the

electrolyte concentration is a complementary change of the non-electrolyte concentration, such that the isotonic property of the solution is constantly guaranteed by the overall osmolarity of the electrolyte and non-ekectrolyte parts.

By using the medium according to the invention in the production of fused cells, it has become possible with high and reproducible yields to obtain, for example, hybridoma cells whose survival rate, i.e. ability to divide, is several times higher than according to the prior art.

Particularly good results can be obtained with a medium whose calcium/magnesium ratio is in the range between 1:4 and 1:6. A particularly preferred calcium/magnesium ratio is approximately 1:5.

With relatively low ionic strengths of the medium surrounding the cells, the cell membranes possess special adhesion properties (J. Cell Sci. 63, 113 to 124 (1983)). This effect is used here selectively for the first time in the fusion of cells in order to promote a reduction of the contact distance between the membranes. When employing this effect to improve the overall yield of living, fused cells, it is particularly suitable to use calcium and magnesium salts which also perform important functions inside the cell in controlling energy conversion and metabolic processes in the cell. It is practical to use the corresponding chlorides; however, it is particularly advantages to use calcium and magnesium acetates since this prevents the toxic products of electrolysis which may occur during dielectrophoresis if chlorides are present.

A particularly preferred concentration range for the calcium ions comprises concentrations between 0.05 mM and 0.2 mM. As regards the magnesium concentrations, the range between 0.05 mM and 0.6 mM and, in particular, between 0.1 mM and 0.5 mM has proven favorable.

Buffering of the fusion medium in the case of a physiological pH value is assured in a practical manner by a phosphate buffer whereby the ionic strength of the latter must be taken into account when adjusting the overall ionic strength of the fusion medium. Particularly advantageous buffer concentrations of the phosphate buffer are in the range between 1 mM and 30mM.

The results of fusion are positively influenced by the exchange of physiological cations for biocompatible cations which at least partially compensate for the negative surface charges of the membranes. In particular, biocompatible cations exhibiting a low change density and an electrostatically weakly bound hydrate shell contribute toward improved bringing together of the two cells in that the hydrate structure at the surface of the cellular membranes is less heavily pronounced and more weakly bound, thus causing a reduced steric hindrance as the cells approach each other.

Particularly preferred media contain oligo- and/or polycationic oligo- and/or polypeptides which due to the fact that they are multiple-charged, compensate for a greater proportion of the negative surface charge of the membranes, yet still exhibit an electrostatically weakly bound hydrate shell. Particularly preferred are oligo- and/or polypeptides which have

an isoelectric point above pH=7 since, in the case of the physiological pH value, they carry an excess positive charge. It is favorable to use proteins, particularly cytochrome and/or histones as polypeptides. It is also advantageous to use oligo- and/or polylysine as biocompatible cations.

The physiological cations can also be advantageously replaced by single-charged large organic cations, particularly those of the $\{NR_xH_y\}^+$, whereby there applies x + y = 4 where x = 1 to 4 and R = alkyl residue, since, after fusion has taken place, these componds can - if necessary - be washed out of the area of the membrane surfaces with relative ease by means of an increased electrolyte concentration.

The non-electrolyte part is formed advantageously by inositol and/or glucosamine.

If the dielectrophoretic process is used for the orientation or collection of the cells, the applied alternating voltage may result in problems with products of electrolysis, even if use is made of an alternating field of relatively high frequency (for example in the MHz range). Therefore, to trap products of electrolysis, it is advisable to add further non-electrolytes to the solution. Catalase is particularly suitable in a small quantities for decomposing H_2O_2. It is advantageous to add radical scavengers to the medium individually or in combination, particularly gluthathione, albumin, cysteine or tocopherol, whereby the individual concentrations are approximately 1 mM and 1 mg/ml for catalase and albumin.

The radical scavengers are particularly practical if the calcium and magnesium salts are used in the form of chlorides. Some of the problems of electrolysis can be eliminated from the outset through the use of the corresponding acetates.

Although increasing the frequency of the electric alternating field can drastically restrain the problems of electrolysis phenomena, this is itself limited by the fact that at higher frequencies of the electric alternating field there is a reduction of the shielding effect of the cellular membranes for the cell interior with the result that the alternating field acts on the cell interior where it may cause changes which are detrimental to the viability of the cell.

Therefore, according to the present invention an electric alternating field with a frequency below 100 kHz is applied to the medium described above. In the frequency range below 100 kHz (frequencies down into the Hz range are possible) the shielding of the cellular membrane for the cell interior is virtually complete. The products of electrolysis which occur at this relatively low frequency of the electric alternating field are trapped and decomposed by the radical scavengers which are added to the medium.

It is particularly advantageous to select the frequency of the electric alternating field such that it is below the Maxwell-Wagner rotation frequency and outside other rotation frequency ranges of the cells which are to be fused.

Consequently, firstly, the cells are brought in a particularly non-damaging manner to the contact distance required

for fusion - furthermore, thanks to the now possible lengthening of the residence time of the cells in the electric field it is possible to obtain better orientation of the cells - and, secondly, there are none of the rotational motions of the cells which occur in low frequency ranges and which may partially cancel out the orientating effect of dielectrophoresis on the cells.

For each cell type there are one or more frequency ranges in which a maximum rotational motion or spinning of the cell in the suspension medium is caused (Biochemica et Biophysica Acta 694, 22 7-227 (1982), p. 239 ff). The position of these frequency ranges is mainly dependent on the type of cell, the size of cell and on the conductivity of the suspension medium.

The advantages arising through the use of this new process according to the invention with regard to apparatus and the fusion process in conjunction with the media thereby used are so great that this process as well as the associated media both independently satisfy all requiremennts for patentability.

These and further aspects and advantages of the invention are explained in greater detail below in the following non-limiting examples:

Example 1:

FUSION OF SP 2/0 MYELOMA CELLS WITH MURING LYMPHOCYTES

A cell suspension of myeloma cells (SP 2/0) and lymphocytes in the ratio of 1:10 and a total suspension density of 1.1×10^7 cells/ml are transferred into a fusion chamber in a

medium of the composition described further below. In the fusion chamber the electrodes (platinum wires with a diameter of 0.2 mm) are disposed at a distance of 0.2 mm from each other. In this example, the myeloma cells are the large cells for which a lower field strength than the one used would be sufficient for producing the penetrations or pores. In order to prevent the fusion of two or more myeloma cells, the lymphocyte cells are present in a 10-fold excess. This ratio guarantees that, basically every myeloma cell has contact with a lymphocyte cell.

Fusion is performed at a temperature of 20 C.

The medium according to the invention, in which the cells are suspended during fusion, contains 0.28 M inositol which, as a non-electrolyte basically causes the isotonic property of the fusion medium. Consequently, the electrolyte part can be kept very small so that the conductivity of the solution remains very low. The low conductivity of the solution has the advantage that, during the use of the electric alternating field for dielectrophoresis, there is only very slight heating of the solution, as a result of which problems with thermal convection currents in the fusion chamber, which would adversely affect the joining of the cell chains, are prevented.

A physiological pH value (approximately 7) of the fusion medium is assured is assured with the aid of a phosphate buffer (concentration 1mM; KH_2PO_4/K_2HPO_4)

An optimum ionic strength of the solution is obtained with the additional components of 0.1 mM calcium acetate and 0.5 mM magnesium acetate.

The electrical fusion conditions are:

Sinusoidal alternating field for 30 s with a field strength of 250 Vcm^{-1} and a frequency of 1.5 MHz.

Three direct voltage pulses with a flield strength of 3.5 $kVcm^{-1}$ and 20 s duration are supplied at intervals of 1 s.

Subsequently, a sinusoidal alternating field is again applied to the electrodes for 30 s with a frequency of 1.5 MHz and a field strength of 250 Vcm^{-1}. Directly after this, the cells are kept in the fusion medium for about 10 minutes at 37°C.

Subsequently, the fused cells - which at this point in time exhibit increased permeability of the cellular membrane - are, until the membrane penetrations have completely healed, transferred into an aftertreatment medium with a temperature of 37°C and are left there for approx. 30 minutes.

The aftertreatment medium is tailored to the fusion medium and consists basically of an aqueous solution of the following components:

80 mM	NaCl
60 mM	KCl
8 mM	Na_2HPO_4
1.5 mM	KH_2PO_4
0.5 mM	Mg acetate
0.1 mM	Ca acetate

With the aid of the fusion medium according to the invention as well as the aftertreatment of the fused cells, it has been possible to increase the yield of a few individual viable cells (prior art, e.g. FEBS Letters 137, 11 to 13 (1982)) to 140 viable, i.e. division-capable, hybridoma cells.

The good reproducibility of the yields of division-capable cells is to be particularly emphasized in this connection. Hitherto, it was only possible to fuse cells with good yields, but without being able to obtain satisfactory and reproducible results as regards their survival rate.

Example 2:

FUSION OF SP 2/0 MYELOMA CELLS WITH MURINE LYMPHOCYTES

Conditions as in example 1, but the concentration of inositol in the fusion medium is replaced by a 0.28 M concentration of glucosamine.

This fusion medium thus also possesses the properties of a fusion medium according to the invention (calcium/magnesium ratio 1:5, as well a low electrolyte concentration tailored to the adhesion properties of the cells). With 128 viable hybridoma cells, the result of fusion is similarly satisfactory to the solution containing inositol.

Example 3:

FUSION OF SP 2/0 MYELOMA CELLS WITH MURINE LYMPHOCYTES

Conditions as in example 1, but in this case the following components were additionally added to the fusion medium

to allow the low-frequency collection and orientation of the cells:

1 mM glutathione
1 mg/ml high-purity albumin
0.1 mg/ml catalase (purified by means of dialysis)

The frequency of the electric alternating field was able to be lowered to 20 kHz and the field strength of the alternating field to 180 Vcm^{-1}.

As compared to the process with a collection frequency of 1.5 MHz (example 1), it was possible to obtain a further drastic increase in the yield to 280 division-capable hybridoma cells.

Example 4:

FUSION OF TUMOR CELLS OF STRAIN K 562 AND MYELOMA CELLS SP 2/0

By exchanging physiological cations for biocompatible cations according to the invention with an electrostatically weakly bound hydrate shell, it is possible even to improve mainly the fusion yields.

The conditions of fusion (medium and field conditions) are the same as those in example 1. Table 1 compiles the results for these two cell types as functions of the addition of cytochrome.

Table 1

Cell strain	Fusion yields without cytochrome	with cytochrome
K 562	35%	90%
SP 2/0	62%	87%

It should be emphasized in this respect that the fusion yields refer to the fusion of identical cells. The addition of cytochrome to the fusion medium is 5µ g/ml.

These examples shown that through the addition of cytochrome even in systems in which a high fusion yield is obtainable without cytochrome, there is a further increase in the number of fused cells. At this point, however, it should be emphasized once again that the fusion yields must not be equated with the yields to viable cell hybrids of the kind stated in examples 1 to 3.

Example 5:

PRODUCTION OF YEAST CELL HYBRIDS

In this example, cells of the strain Saccharomyces cerevisiae AH22pADH are fused with cells of the strain Saccharomyces cereviwsiae AH215 under identical field conditions in so-called helix chambers (see FEMS Microbiol. Letters 24, 81 to 85 (1984)).

The mixture ratio of the two cell types was 1:1, the total cell syspension density was 1.5×10^9 to 2×10^9 cells/ml.

The field conditions for fusion were: alternating field with a field strength of between 250 and 275 Vcm^{-1} and a frequency of 2 MHz for 1 to 2 min both before and after the application of two square-wave direct voltage pulses (field strength 10 kVcm, pules duration 10μ s) at an interval of 0.5 s.

The compositions of the aqueous solutions in which the cells were suspended for experiments 1 to 4 are given in Table 2.

In control experiment 4 the cell suspension was not exposed to an electric field. The two observed cell hybrids are attributable to spontaneous fusion.

Experiments 1 to 3 show, firstly, the great significance of the addition of calcium and magnesium ions. The conditions in experiment 3 are, with the exception of the relatively non-critical alternating field frequency, identical with those described in the above-quoted literature. The yield was 119 division-capable hybrids; on repeating the experiment, the result was merely 42. In the quoted literature, the yield is given as between 50 and 60. The range of scatter when using a pure sorbitol solution is, therefore, very great.

Reproducible and considerably higher yields of division-capable hybrids are obtained only with the calcium and magnesium ion concentrations and concentration ratios according to the invention. The yields of experiments 1 and 2 are reproducible to $\pm$ 20%.

A comparison of the results of experiment 1 and experiment 2 shows, secondly, the improvement of the fusion

medium by exchanging the chlorides for acetates, as the result of which problems of electrolysis, which are still present even with alternating fields in the MHz range, can be drastically retrained.

Table 2

Experiment	1	2	3	Control 4
Calcium acetate	0.1 mM	0	0	0.1 mM
Magnesium acetate	0.5 mM	0	0	0.5 mM
$CaCl_2$	0	0.1 mM	0	0
$MgCl_2$	0	0.5 mM	0	0
Inositol	0.28 M	0.28 M	0	0.28 M
Sorbitol	0.92 M	0.92 M	1.2 M	0.98 M
No. of division-capable hybrids	990	584	119	2
Relative yields in %	100	59	12	0.2

While the foregoing invention has been described with reference to its preferred embodiments, various alterations and modifications will occur to those skilled in the art. All such variations and modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. Medium for the production of viable, fused cells by means of field-induced electrical fusion, particularly for the production of hybridoma cells, the medium comprising an approximately isotonic, aqueous solution of electrolytes and non-electrolytes with a maximum ionic strength of 0.1, said solution including calcium and magnesium salts with the ratio of concentration of calcium and magnesium ions in a range between 1:2 and 1:10, the ionic strength of the solution being adjusted for optimum membrane contact through variation of the electrolyte concentration, and corresponding to a change of the electrolyte concentration is a complementary change of the non-electrolyte concentration such that the isotonic property of the solution is constantly guaranteed by the overall osmolarity of the electrolyte and non-electrolyte parts.

2. Medium as defined in claim 1 wherein the ratio of concentration of the calcium and magnesium ic ıs is in the range between 1:4 and 1:6.

3. Medium as defined in claim 2 wherein the ratio of concentration of the calcium and magnesium ions is approximately 1:5.

4. Medium as defined in claim 1 wherein the calcium and magnesium ions are added to the solution in the form of chlorides.

5. Medium as defined in claim 1 wherein the calcium and magnesium ions are added to the solution in the form of acetates.

6. Medium as defined in claim 1 wherein the concentration of the calcium ions is in the range between 0.05 mM and 0.2 mM.

7. Medium as defined in claim 1 wherein the concentration of the magnesium ions is between 0.05 mM and 0.6 mM.

8. Medium as defined in claim 7 wherein the magnesium concentration is between 0.1 mM and 0.5 mM.

9. Medium as defined in claim 1 wherein the ionic strength is additionally adjusted by a phosphate buffer.

10. Medium as defined in claim 9 wherein the concentration of the phosphate buffer is in the range between 1 mM and 30 mM.

11. Medium as defined in claim 1 wherein the electrolyte part comprises biocompatible cations which replace physiological cations on negatively charged membrane surfaces and change the water structure in the region of the membrane.

12. Medium as defined in claim 11 wherein the biocompatible cations exhibit a low charge density and an electrostatically weakly bound hydrate shell.

13. Medium as defined in claim 11 wherein the biocompatible cations are oligo- and/or polycationic oligo- and/or polypeptides, respectively.

14. Medium as defined in claim 13 wherein the oligo- and/or polypeptides exhibit an isoelectric point above pH = 7.

15. Fusion medium as defined in claim 13 wherein the polypeptides are proteins.

16. Medium as defined in claim 15 wherein cytochrome and/or histones are contained as proteins.

17. Fusion medium as defined in claim 13 wherein oligo- and/or polylysine act as biocompatible cations.

18. Medium as defined in claim 11 wherein the physiological cations are replaced by single-charged large organic cations.

19. Medium as defined in claim 18 wherein compounds of type $\{NR_xH_y\}^+$ are contained as single-charged large organic cations, whereby there applies $x+y = 4$ where $x = 1$ to 4 and R = alkyl residues.

20. Medium as defined in claim 1 wherein the non-electrolyte part is constituted basically by inositol and/or glucosamine.

21. Medium as defined in claim 1 wherein said medium comprises, as a further non-electrolyte, pure catalase in small quantities for the decomposition of H_2O_2.

22. Medium as defined in claim 1 wherein said medium comprises radical scavengers as additional non-electrolytes.

23. Medium as defined in claim 22 wherein glutathione and/or albumin are contained as radical scavengers.

24. Medium as defined in claim 23 wherein glutathione and/or albumin are contained with a concentration of approximately 1 mM and/or with a concentration of approximately 1 mg/ml, respectively.

25. Medium as defined in claim 22 wherein cysteine and/or tocopherol are additionally contained as radical scavengers.

26. Medium as defined in claim 21 wherein an electric alternating field for the orientation and collection of cells in the production of viable, fused cells is applied to the medium, said electric alternating field having a frequency below 100 kHz.

27. Medium as defined in claim 26 wherein the frequency of the electric alternating field is below the Maxwell-Wagner rotation frequency and outside other rotation frequency ranges of the cells which are to be fused.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/02029

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. Cl. 435/240.
INT. Cl.[4] C12N 05/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]	
Classification System	Classification Symbols
U.S.	435/ 172.2, 240, 241, 948 935/ 52, 54, 93, 94

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Chemical Abstracts, Biological Abstracts, Index Medicus 1977-date, Cell and Electro or Electrical Fusion

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X,Y,A	N, Biochemical and Biophysical Research Communications 120 (1), 16 April 1984, Ohno -Shosaku et al, "Facilitation of Electrofusion of Mouse Lymphoma Cells by the Proteolytic Action of Proteases" p. 138-43.	1-27
Y,A	N, FEBS Letters 163(1), October 1983, Vienken et al, "Electrofusion of Myeloma Cells on the Single Cell Level" p. 54-56	1-27
A	N, Biochemical and Biophysical Research Communications 114 (2), 29 JULY 1983, Claude et al, "Homokaryon Production by Electrofusion A Convenient Way to Produce a Large Number of Viable Mammalian Fused Cells" p. 663-9	1-27
A	N, FEBS Letters 137 (1), January 1982, Vienken et al, "Electric Field Induced Fusion: Electro-Hydraulic Procedure for Production of Heterokaryon Cells in High Yield" p. 11-13	1-27

* Special categories of cited documents: [15]

"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

"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

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
09 JANUARY 1986	17 JAN 1986
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	JOHN E. TARCZA John E. Tarcza

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	N, FEBS Letters 147 (1), October 1982, Bischoff et al, "Human Hybridoma Cells Produced by Electro-Fusion" p. 64-8	1-27

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.