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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :		(11) International Publication Number:
G01N 27/40, 33/545	A1	WO 90/02327
		(43) International Publication Date: 8 March 1990 (08.03.90)

(21) International Application Number: PCT/AU89/00352

(22) International Filing Date: 17 August 1989 (17.08.89)

(30) Priority data:
PI 9994 18 August 1988 (18.08.88) AU

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: IMPROVEMENTS IN SENSITIVITY AND SELECTIVITY OF ION CHANNEL MEMBRANE BIOSENSORS

(57) Abstract

The present invention provides a biosensor comprising at least one lipid membrane, each membrane including at least one gated ion channel. The membranes comprise a closely packed array of self-assembling amphophilic molecules and the gated ion channel has a conductance which is dependent upon an electric field applied across the membrane. The biosensor of the present invention may comprise a plurality of discrete membranes each including at least one gated ion channel. The conductance of each of these membranes is measurable independently of the conductance of the other membranes.

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IMPROVEMENTS IN SENSITIVITY AND SELECTIVITY OF ION CHANNEL MEMBRANE BIOSENSORS

Field of the Invention

The present invention relates generally to biosensors comprising membranes including at least one ion channel. In one form of the invention the conductance of the ion channels is dependent on electric field applied across the membrane. In addition, the present invention relates to biosensors comprising discrete arrays of membranes, each membrane including at least one ion channel, and the conductance of each membrane being measurable independently.

Background of the Invention

It is known that amphiphilic molecules may be caused to aggregate in solution to form two or three dimensional ordered arrays such as monolayers, micelles, black lipid membranes, and vesicles or lisosomes, which vesicles may have a single compartment or may be of the multilamellar type having a plurality of compartments.

The selectivity and flux of ions through membranes can depend on the number, size and detailed chemistry of the pores or channels that they possess. It is through these pores or channels that permeating solute molecules pass across the membrane.

It is known that membranes may incorporate a class of molecules, called ionophores, which facilitate the transport of ions across these membranes. Ion channels are a particular form of ionophore, which as the term implies are channels through which ions may pass through membranes. The measurement of current flow across membranes due to a single ion channel is known and typically yields a current of 4 pA per channel.

The use of membranes including ion channels in biosensors has been proposed. In co-pending International Patent Application No. WO89/01159 (published 9 February 1989) the production of biosensors incorporating membranes including ion channels is disclosed. The disclosure of

this application is hereby incorporated by way of cross-reference. The present invention seeks to provide biosensors of greater sensitivity.

Description of the Present Invention

The present invention consists in a biosensor comprising at least one lipid membrane each membrane including at least one gated ion channel, each of said membranes comprising a closely packed array of self-assembling amphiphilic molecules, said at least one gated ion channel having a conductance which is dependent upon an electric field applied across the membrane.

In a preferred embodiment of this aspect of the present invention, the biosensor comprises a plurality of discrete lipid membranes, the conductance of each membrane being measurable independently of the conductance of the other membranes.

In a second aspect the present invention consists in a biosensor comprising a plurality of discrete membranes, each membrane including at least one gated ion channel, each of said membranes comprising a closely packed array of self-assembling amphiphilic molecules, the conductance of each of said membranes being measurable independently of the conductance of the other membranes.

As used herein the term "gated ion channel" is defined as an ion channel the passage of ions through which is dependent on the presence of an analyte.

As used herein the term "field effect ion channel" is defined as an ion channel in which the conductance of the ion channel is dependent on an electric field applied across a membrane incorporating the ion channel.

The amphiphilic molecules are normally surfactant molecules having a hydrophilic "head" portion and one or more hydrophobic "tails". Surfactants may be any of the known types, i.e. cationic (e.g. quaternary ammonium salts), anionic (e.g. organosulfonate salts), zwitterionic

(e.g. phosphatidyl cholines, phosphatidyl ethanolamines), membrane spanning lipid, or non-ionic (e.g. polyether materials). The amphiphilic molecules are preferably such that they can be cross-linked. For this purpose it is necessary to provide the molecules with a cross-linkable moiety such as vinyl, methacrylate, diacetylene, isocyano or styrene groups either in the head group or in the hydrophobic tail. Such groups are preferably connected to the amphiphilic molecule through a spacer group such as described in Fukuda et al. J. Amer. Chem. Soc., 1986, 108 2321-2327.

Polymerisation may be performed by any of the known methods for polymerising unsaturated monomers, including heating with or without a free radical initiator, and irradiating with or without a sensitiser or initiator.

In a preferred embodiment of the present invention the amphiphilic molecules include or are decorated with at least one moiety cross-linked with at least one corresponding moiety on another of these molecules.

The ion channel used in the present invention is preferably selected from the group consisting of peptides capable of forming helices and aggregates thereof, podands, coronands and cryptands. However, it is presently preferred that the ion channel is a peptide capable of forming a helix or aggregates thereof.

Podands, cryptands and coronands have been described previously in the scientific literature (see, for example, V.F. Kragten et al., J. Chem. Soc. Chem. Commun. 1985, 1275; O.E. Sielcken et al. J. Amer. Chem. Soc. 1987, 109, 4261; J.G. Neevel et al., Tetrahedron Letters, 1984, 24, 2263).

Peptides which form α helices generally need to exist as aggregates in the membrane to form ion channels. Typically, the α helical peptides arranged to form aggregates in such a manner that an ion channel is created

through the aggregate.

It is presently preferred that the ion channel is a peptide which forms a β helix. An example of such a peptide is the polypeptide gramicidin A. This molecule has been the subject of extensive study (for further information see Cornell B. A., Biomembranes and Bioenergetics (1987), pages 655-676). The ion channel gramicidin A functions as a polar channel which traverses non-polar biological membranes. It is produced either synthetically or extracted from Bacillus brevis. In phospholipid bilayers gramicidin A is thought to exist as a helical dimer which substantially partitions into the hydrophobic region of the bilayer.

Further examples of molecules which may be used as ion channels in the present invention include gramicidin B, gramicidin C, gramicidin D, gramicidin GT, gramicidin GM, gramicidin Gm^-, gramicidin GN^-, gramicidin A' (Dubos), band three protein, bacteriorhodopsin, mellitin, alamethicin, alamethicin analogues, porin, tyrocodine, and tyrothricin.

Hereafter, the family of gramicidins will be referred to as simply gramicidin.

In the particular case of gramicidin, when the membrane is a monolayer, a monomer of gramicidin could be used as the ion channel. In a situation where the membrane is a bilayer, a synthetic analogue of dimeric gramicidin A could be used as the ion channel. In addition, where the membrane is a bilayer the ion channel may consist of two gramicidin A monomers, in which each monomer is in a different layer. In this situation the gramicidin A monomers are able to diffuse through the layers and when the two monomers come into alignment an ion channel is formed through the bilayer.

As stated above, the ion channel is gated. This may be done by a receptor moiety attached to, or associated

with, an end of the ion channel, the receptor moiety being such that it normally exists in a first state, but when bound to an analyte exists in a second state, said change of state causing a change in the ability of ions to pass through the ion channel.

The first state of the receptor moiety will normally be a state in which the passage of ions through the ion channel is prevented or hindered. Attachment of the analyte to the receptor will thus cause the receptor to enter the second state wherein ions may pass through the ion channel. In this arrangement an ion channel may be used to detect as little as a single molecule of analyte the attachment of a single molecule of analyte will cause an ion channel to open and thus cause a leak of ions across the membrane. After a brief time this ion leak may be detected as the signal for the binding of the analyte to the receptor.

As would be readily appreciated by a person skilled in the art the alternative arrangement is when the receptor moiety is in the first state ions are able to pass through the ion channel and when in the second state the passage of ions through the ion channel is prevented or hindered. The receptor moiety may be any chemical entity capable of binding to the desired analyte and capable of changing the ion channel from its first state to its second state upon binding to that analyte. The receptor moiety is any compound or composition capable of recognising another molecule. Natural receptors include antibodies, antigens, enzymes, lectins, dyes and the like. For example, the receptor for an antigen is an antibody, while the receptor for an antibody is either an anti-antibody or, preferably, the antigen recognised by that particular antibody.

More details on gating mechanisms for ion channels are provided in co-pending International Application

No. WO89/01159.

Two mechanisms are known for the field dependence of conductance. One is the electrical potential profile along the ion channel. Secondly there is the possibility of conformational change in some ion channels when an electric field is applied. Thus with application of the field; polar, dipolar and polarisable groups may change orientation and distort the ion channel or change its potential profile thus influencing its transconductance. To make an ion channel with a transconductance that can usefully be modulated by an electric field it may be necessary to incorporate or remove highly polar, dipolar or polarisable groups on the ion channel. For example substitution of residues with a very low polarisability for the highly dipolar tryptophan rings in gramicidin A renders its conductance very potential dependent. Another gross example is Alamecithin which forms a hexameric ion channel when an electric field is applied.

The ion channels of the present invention can be modified by various residues, examples of which are given in Table 1 to achieve the required results.

TABLE 1

a) DIPOLAR GROUPS:-

Suitable derivatives of virtually any non-symmetric molecule, particularly those asymmetrically substituted with electron donating groups (e.g. alkoxyartl substituents), electron withdrawing groups (e.g. alkyl or ary carboxylic acids, aldehydes, ketones, nitriles or nitro compounds or combinations of these e.g. alkoxyntroryl derivatives;

or

charged dipolar species e.g. zwitterions, ylids.

b) POLAR GROUPS:-

Species bearing positive or negative charge (e.g. ammonium salts or carboxylates).

c) POLARISABLE GROUPS:-

Species containing highly polarisable electron clouds (e.g. halides, nitriles, sulfue derivatives, phosphorous derivatives, aryl, acetylenic or olefinic derivatives).

As would be apparent from the discussion above, the gated ion channels may be cross-linked with the amphiphilic molecules. However, it is presently preferred that the gated ion channels are able to laterally diffuse through the membrane. As will become clear from the following discussion the ability for the gated ion channels to laterally diffuse through the membrane results in greater sensitivity of the biosensor.

As stated above when the biosensor of the first or second aspect of the present invention comprises a plurality of discrete lipid membranes the conductance of each membrane is measurable independently of the conductance of the other membranes. The conductance of each membrane is preferably measured by (1) providing a separate high impedance measuring line to each membrane and/or (2) by multiplexing the membranes. It is presently preferred that where a large number of discrete membranes are used that the independent measurements are made by multiplexing the membranes and more preferably by serially multiplexing the membranes. Where multiplexing is used the multiplex lines are preferably low impedance excitation (or signal source) lines (held/clamped) at the excitation value; with a single high impedance current sensing line held at ground reference to complete the circuit for each element of the array when it is switched into circuit. While it is preferred that one current sensing line is used it willbe recognised that more than one current sensing line may be provided. Either of these arrangements should result in a biosensor of optimal sensitivity.

Where the independent measurement of the conductance of the membranes is made using multiplexing it is preferred that the gated ion channels are field effect ion channels. It is also preferred that the plurality of discrete membranes including FEICs are arranged in a two dimensional array. It is presently preferred in this arrangement that the multiplex lines are driven from a complex signal such that in the two dimensional array each address line in one dimension has signal components which are cross modulated with the signals from address lines in the other dimension by the field effect ion channel.

In the biosensor of the present invention comprising a plurality of membranes including field effect ion channels, it is preferred that at least one dedicated electrode is provided on one side of each membrane which cooperates with an electrode on the other side of the membrane to enable the application of an electric potential across the membranes. It is preferred that each of these membranes is addressed by multiplexing the signal applied to the respective discrete electrodes.

As stated above biosensors made from ion channels incorporated in lipid membranes have been proposed. These typically consist of a lipid membrane containing an ion channel, which has been modified to change its ionic conductance when an analyte such as an antigen or antibody binds to it. Field effect ion channels (FEIC) can be used to improve these biosensors and their application involves the following principles:-

1. Increasing the value of "Off" to "On" resistance improves the electrical signal to noise ratio in a gated ion channel biosensor.
2. The probability that in a given period of time the molecule will react with the sensor for a given volume of analyte depends on the area of the sensor.

3. A non linear conductance can be used to improve the sensor signal to noise.

In this application the ratio of "off" to "on" resistance can be increased and shunt capacitance is reduced without increasing the time it takes for a molecule to diffuse to the sensor. Additionally field effect ion channels can be used to create a distinctive transduction signal. These techniques can be used to greatly enhance the sensitivity and selectivity of the biosensor.

The sensitivity of a biosensor, such as that described in Patent Application No. WO 89/01159 is dependent in part on the ratio of ion channel resistance to lipid membrane resistance, i.e. the "on" to "off" resistance of the ion channel incorporated in the lipid membrane. If the ratio of lipids to ion channels is too large,then the sensor's electrical impedance can be so low that impedance changes due to a sensing event are difficult to detect. Similarly if the absolute number of ion channels is too high then the sensors electrical impedance is lowered, by leakage currents through the ion channels if they are normally blocked, or by the ion channel intrinsic conductance if they are normally open.

To improve the sensitivity one can reduce the number of ion channels and reduce the sensor surface area in order to increase the signal response to the minimum number of binding events. However, a reduced surface area implies a longer time for the analyte to diffuse to the point of sensing, and for small concentrations a reduction in probability of detection. The alternative method , using flow through techniques, may not be suitable because of the small analyte volumes involved in high sensitivity tests (e.g. one droplet), and because of noise generated by the analyte flow perturbing the membrane.

A method proposed here is to set up an array of small

area sensors and to switch between them so as to move the point of sensing in the analyte. The switching can be done with a conventional electronic multiplexer, although for two dimensional arrays at least half the address lines would need to have a high impedance. Alternatively it can be done using FEIC's as part of the sensing ion channel, in which case it is possible to switch between sensing elements in a two dimensional array using low impedance lines and one common high impedance line as described in one of the following examples.

Diagnostic reliability can be improved by using a variety of functionally different tests and by measuring the statistics for sets of functionally identical tests. In both of these cases the ability to scan an array of biosensors is useful and both approaches require the availability of a mechanism for switching between biosensors.

A second method for improving sensitivity involves the use of FEIC gated ion channel biosensors which are designed with a conductance characteristic which can be readily distinguished from interfering signals such as the lipid membrane conductance and this method will also be discussed in the following examples.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following examples and accompanying Figures in which:

Figure 1 shows schematically field modulated ion channels, in which "A" shows modulated head groups; "B" shows modulated side chains; and "C" shows polymeric ion channel.

Figure 2 shows a schematic representation of a low impedance biosensor multiplexer.

Figure 3 shows a metal or glass electrode in which "A" is a side view and "B" is a view from above.

Figure 4 shows a schematic representation of an impedance bridge system.

Figure 5 shows a schematic representation of a three terminal bridge.

Figure 6 shows a schematic representation of a balanced voltage impedance bridge.

Figure 7 shows a schematic representation of a two terminal bridge.

Figure 8 shows a biosensor chip.

Figure 9 shows a cross-sectional view of the chip of Figure 8 taken along line A-A.

Figure 10 shows a cross-sectional view of the chip of Figure 8 taken along line B-B.

Example 1

ION CHANNELS WITH FIELD MODULATED TRANSCONDUCTANCE

Polar groups can be incorporated into many parts of an ion channel structure for the purpose of transconductance modulation. By way of example ion channels may be employed with polar, dipolar or polarisable residues located: at the head region of the ion channel, on the side chains of the ion channel and at the dimeric junction of an ion channel dimer.

In general the mechanisms for transconductance modulation can be direct modification of the potential profile, distortion of the channel by a conformational change or modification of the potential profile by a conformational change.

It will usually be more appropriate to measure the transconductance of such ion channels using a pulse signal or AC signal. This keeps the advantages of high signal bandwidth, avoids unwanted electrochemical effects and allows higher field strengths than a bilayer could withstand in a DC signal.

Example 2

AN ION CHANNEL WITH A FIELD MODULATED HEAD GROUP

In this case polar, dipolar or polarisable residues are attached directly or via linker groups to the mouth of the ion channel in the region of the surrounding lipid head groups (Fig. 1a). These ion channels can then be incorporated into either lipid monolayers or bilayers or can be laid down as a secondary film in series connection with a monolayer or bilayer already containing ion channels.

This form of ion channel is not as sensitive as those of Examples 3 and 4 because of the surrounding highly polar electrolyte molecules which attenuate field strength in the head group region.

If the ion channel is held in a lipid bilayer then it is also possible to use opposite polarity polar groups on each side of the bilayer to enhance sensitivity.

Example 3

AN ION CHANNEL WITH FIELD MODULATED SIDE CHAINS

In this form of ion channel polar, dipolar or polarisable residues are attached as side chains to the ion channel so that they lie within the low permittivity region of the lipid membrane (Fig. 1b). Examples are given in Table 1.

Example 4

A FIELD MODULATED POLYMERIC ION CHANNEL

This form of ion channel is used where monomers (e.g. alamethicin or gramicidin) are combined to form an ion channel. The monomers are chemically or physically linked and contain polar, dipolar or ionised groups as described previously. A field is applied which may assemble, distort or disrupt the ion channel thus modulating its ion conductance. Fig. 1(c) shows a dimer with dipolar residues attached as side chains. Distortion of the dimer by the electric field force acting on the dipolar groups

may modulate the dimer transconductance by inducing conformational changes in the region of the dimeric bond.

Example 5

AN ARRAY OF BIOMOLECULAR SWITCHES USING FIELD MODULATED ION CHANNELS

Arrays of field effect ion channels may find application wherever it is desirable to control ion flow. In particular, applications may exist in biosensors, or chemical analysis techniques such as electrophoresis.

a. A one dimensional array of field effect ion channels could be addressed using a single common high impedance signal sensing electrode and a separate low impedance signal sensing electrode for each channel.

b. A high density of ion channels could be addressed using a two dimensional array in which each side of the ion channel is addressed by separate electrodes. In this case at least half the address lines should be high impedance to reduce cross modulation. Problems with fabrication and signal bandwidth may arise because of this high impedance.

c. A high density of ion channels can be addressed by a two dimensional array in which one side of the channel is connected to an electrode which is capacitively or resistively connected to two address lines. Address lines are used as low impedance sources of signals which cross modulate when applied to a non-linear transfer point such as the non-linear conductance of the FEIC. Thus, by switching between the modulating electrodes separate elements on the array can be addressed. (Fig. 2). A single high impedance measuring electrode only is required.

Figure 2 shows schematically a low impedance biosensor multiplexer comprising an array of membranes including gated ion channels 10, an excitation source 12, a modulation source 14, a transfer function analyser 16 an

array of address lines 18, and a common sensing line 19.

Because the address lines are on the same side of the channel, and because the signal is well labelled, they can carry low impedance signals without the problems of cross modulation which would exist if they were on opposite sides. For the technique to work it is essential that the ion channel have a distinctive transconductance characteristic which can be modulated, hence the necessity to use FEIC's. The address electrodes can be AC or DC coupled.

In the fabrication of a two dimensional array of FEIC's a pattern of electrodes and resistors or capacitors is formed by etching a multilayer substrate of alternately electronically conducting and insulating materials. This substrate is then coated with a monolayer or bilayer of lipid. The lipid membrane can be formed directly on some substrate surfaces; alternatively it can be formed on a hydrogel coating over the substrate. Ideally the interconnecting resistors and conductors will be insulated from the lipid material while the electrodes are electronically coupled to the membrane either directly or by capacitive coupling. Ideally the membrane will be divided into electrically isolated array elements. This may be achieved by making wells over each element of the array.

Suitable materials for a substrate may be silicon and its oxides and nitrides, the metals (particularly palladium or platinum), the glasses, ceramics and oxides (particularly aluminium oxide and the titanates and zirconates), the conducting polymers such as nafion, and polypyrrolle, and the insulating polymers used in integrated circuit and capacitor production such as parylene, polyvinylidene fluoride, polyester and polypropylene.

Suitable materials for the lipid would be the

phospholipids, such as DMPC and DPPC, which are relatively stable. If the lipid is directly coating a metal surface such as palladium, then it would be necessary to substitute a thiol residue such as a sulfhydryl for the phospholipid headgroup.

In use the array would be placed in a liquid or hydrogel electrolyte containing a common high impedance electrode which is connected to the signal analysis equipment. If very low frequency or DC signals are being used then it may be necessary to use an additional reference electrode to balance the electrochemical potential at the signal electrodes. The signal analysis can use a variety of techniques such as: spectral analysis, cyclic voltammetry, noise analysis, dynamic impedance analysis or statistical analysis. All these methods and preferably carried out in conjunction with the decoding mechanism which is used as described below, to distinguish between interference and true signals and to distinguish between sensing elements.

Example 6

A BIOSENSOR USING AN ARRAY OF FIELD MODULATED ION CHANNELS

It is well known that arrays of biosensors would be useful for multifunctional testing. However, as described above, some forms of biosensor array can also be used to improve sensitivity, selectivity, time response and reliability.

A biosensor could be constructed, using for example an array of gated ion channel biosensors made from a field effect ion channel. An appropriate field effect ion channel is given in Example 3. Any of the switching methods described in Example 8 could be used to address the individual elements, although those described for 1 dimensional arrays would be more appropriate for small arrays and those described for 2 dimensional arrays more

appropriate for large arrays. The signal analysis methods described in Examples 5 and 8 can be combined to provide an effective addressing and detection algorithm. The reliability of detection could be further enhanced by measuring from many elements for statistical analysis.

Example 7

Ion channels with non linear conductance characteristics with electric field are known to exist.

The conductance of a lipid bilayer is known to be much less non linear with electric field than some of these ion channels.

Biosensors can be proposed based on the use of modified ion channels in lipid membranes.

Lipid membranes are known to present a significant shunt impedance to ion channels thus making it difficult to distinguish ion channel conduction acitivity from lipid conduction.

A method for increasing the sensitivity of a biosensor based on ion channels in a lipid membrane may be to use ion channels which have been modified to have an electric field dependent conductance. A complex waveform is applied to the biosensor and compared with those frequency components of the resulting signal which result from the non linear transfer function of the ion channel.

An example would be to apply an excitation voltage synthesised from two sine waves to one side of the biosensor membrane and to use a phase lock loop to measure the frequency difference component, in the current passing through the biosensor.

Let "V" represent the excitation voltage and "A" represents the current passing through the biosensor. If "f1" and "f2" represent the frequencies of the two sine waves in the excitation signal and if they are respectively the n1 and n2 sub-harmonics of a fundamental sinewave "f0" then the detected current signal can be

represented as $A\{(1/n1-1/n2) \times f0\}$. Lipid membranes can have a conductance which varies by a factor of approximately 2 over the usable range of excitation signal whereas an ion channel can be modified to act as a biosensor with a highly non linear conductance which can vary by as much as 50. Thus the ion channel would tend to have a higher level of crossmodulation of the excitation sine waves when compared to the membrane and the improvement in discrimination would be:

$$\frac{A\{(n1-n2) \times f0\} \text{ ion channel}}{A\{(n1-n2) \times f0\} \text{ membrane}}$$

If the dynamic state of biosensor impedance is being measured, for example a change in the statistics of the period of gating following a biochemical reaction, then the difference frequency of the above example should be greater than the Nyquist frequency for the shortest pulse period considered significant in the analysis.

Other signal processing strategies for biosensors based on a nonlinear ion channel are:

Spectral analysis

Cyclic voltammetry with excitation from either current or voltage sources

Noise analysis

Dynamic impedance analysis

Statistical analysis

Other modalities for discriminating ion channel from lipid membrane conductance are: optical and/or acoustic excitation of the ion channel.

Example 8

It is known that as the area of a membrane increases, the sensitivity of a system to measure ion channel activity is reduced because the membrane shunt resistance and capacitance grows while that of the ion channel remains constant.

To measure low concentrations of ion channel activity, cell areas of from 0.1 to 100 micron^2 are typical.

If the limiting sensitivity is defined as the conductance of a single channel divided by total conductance of the sensor then the dependence of limiting sensitivity on area of such a system can be expressed in terms of functions of: the area of the ion channel "f1(Ai)", the membrane area "f2(Am)" and the area of ion leakage at the membrane perimeter f3(Ae) as:

$$1 / (1 + f2(Am)/f1(Ai) + f3(Ae)/f1(Ai))$$

The functions of f1 and f2 are, to a first approximation, linear, giving admittance per unit area. However, f3 is a more indeterminate function giving leakage admittance around the biosensor cell perimeter. In a circular cell it is approximately proportional to (Rm^2-Re^2) where Rm is the radius of the biosensor and Re is the radius to the region where edge leakage occurs.

If a biosensor detects by binding analyte molecules of cross sectional area "Aan" to a few ion channels which are consequently opened or closed, then if there are N1 ion channels which can laterally diffuse through the membrane then the limiting sensitivity is given as:-

$$\frac{Am}{Aan} \times \frac{1}{1 + N1 + f2(Am)/f1(Ai) + f3(Ae)/fi(Ai)}$$

For a system in which the channels are evenly distributed but cannot laterally diffuse, the sensitivity limit is given as:

$$\frac{1}{Aan} \times \frac{N1}{1 + N1 + f2(Am)/f1(Ai) + f3(Ae)/fi(Ai)}$$

It can be seen that the advantage of a membrane which is large compared to the analyte molecule, is offset by the limiting effect of Am on electrical sensitivity. It can also be seen that simply increasing the number of ion channels overcomes this problem in systems with anchored ion channels, however, it does make detection more difficult because the ability to characterise ion channel activity by spontaneous changes in the conduction of individual channels, f1(Ai), is lost in the average conduction signal. However, if the membrane and its ion channels are divided into N2 adjacent but electrically isolated and independently measured regions, then the limiting sensitivity becomes:

"Laterally Diffusing"

$$\frac{Am}{Aan} \times \frac{1}{1 + N1/N2 + f2(Am)/(N2xf1(Ai)) + SQRT(N2)xf3(Ae)/f1(Ai)}$$

or

"Anchored"

$$\frac{Am}{Aan} \times \frac{N1}{1 + N1/N2 + f2(Am)/(N2xf1(Ai)) + SQRT(N2)xf3(Ae)/f1(Ai)}$$

By this means the electrical sensitivity can be greatly increased by reducing the limiting effect of membrane area on electrical sensitivity, and by retaining the information contained in single ion activity while allowing more ion channels to be used. The increased number of ion channels will also increase time response by reducing the lateral diffusion times. Improved sensitivity and time response in a biosensor, based on an ion channel in a lipid membrane can be achieved by

independently sensing a number of small cells distributed over the active surface area, by multiplexing or by parallel amplification or both.

Biosensors based on field effect ion channels which have been modified may also be multiplexed.

The speed of response and sensitivity of the biosensor described above are optimal when a system of parallel amplifiers is used on an array of close packed cells. A serially multiplexed system with close packed cells will be equally sensitive as the parallel system but will have a longer time response which improves with the number of parallel signal paths in the network. Spacing the sensing elements and multiplexing between them will result in an improvement in response time but a loss of sensitivity proportional to the ratio of the sensor area/sensing area.

The biosensors described below typically use a 2 or 3 terminal bridge connected to a gated ion channel modified in the membrane. Preferably multiplexing is carried out entirely by excitation electrodes with the high impedance sensing electrode(s) not being associated directly with the multiplexor.

(1) One Dimensional Array

(a) The independent measurements are set up as parallel high impedance (10^{10}ohms) amplifiers. 10,000 are required for ultimate sensitivity and time response in a 1 cm^2 sensor with close packed 100 $micron^2$ cells.

(b) The independent measurements are set up as 10,000 serially multiplexed cells. Multiplex lines are low impedance with a single current sensing line held at ground reference. Response time is typically between 20 and 200 seconds. Sensitivity is optimal.

(c) A mix of serial multiplexed and "N" parallel signal paths is used. The response time is reduced proportionally to the N amplifiers required for each

path. Note the amplifiers have to be independent and therefore isolated at high impedance from each other.

(2) Two Dimensional Array

(a) As in 3 above, however, ion channels with non linear conduction are used and the multiplexer lines are driven from a complex signal (typically "N" paired frequencies Vn(f1) and Vn(f2)) so that frequency division demultiplexing of the different frequencies corresponding to each parallel path can be carried out. Thus the time response in 2 above is reduced by "N" in a system with one high impedance line.

(b) As for 4 but where the multiplexer electrode on the membrane substrate is coupled to excitation sources via a resistor network so that two signal lines can be used to address the electrode in a two dimensional array.

(c) System as for all above biosensors but where the membranes are not close packed. This reduces the time response and/or sensitivity but for many applications this would be a useful configuration.

Example 9

1) Improved Sensitivity in a Non Linear Sensor

This example describes a device for enhancing sensitivity in a biosensor based on a gated ion channel in a lipid bilayer.

Figure 3 shows schematically metal on glass electrodes 20 from the side (a) and from above (b). The metal on glass electrodes 20 consists of a glass substrate 22, active electrodes 24, connector pads 26 and electrical connections 28 connecting connector pads 26 with electrodes 24. The electrical connections 28 and active electrodes 24 are sputtered layers.

A glass sheet 22, such as a microscope slide, is prepared by cleaning in solvent, water and chromic or nitric acid, but not detergent. Connector pads 26 are electroplated as per figure 3 and the electrode 20 is then

cleaned with distilled deionised water and by ethanol vapour degreasing or in a soxhlet extractor.

It is then quickly dried in a clean dust-free atmosphere with a jet of pure dry nitrogen obtained for example from liquid nitrogen boil off and transferred to a sputtering apparatus containing multiple targets of chromium, and either gold, palladium or platinum. The sputtering chamber should be protected from diffusion pump vapour by a liquid nitrogen cold trap. A sputter coating of 100 angstroms of chromium, followed by 200 angstroms of gold palladium or platinum, is deposited by shadow masking the pattern given in figure 3. This pattern shows two active electrodes 24, although both are not always required it is useful to have one electrode without biosensing material to act as a reference. The electrodes 24 should then be immediately coated with lipid by adsorption or Langmuir Blodgett dipping as described in the steps to prepare a biosensor given in International Patent Application No. WO 89/01159.

This form of biosensor uses a combination of bound alcohol and lipid as an insulator. The shadow mask creates a penumbral region of electrically discontinuous metal around the perimeter of the metallisation, which serves to anchor lipid support material and allow a well insulating membrane to surround and cover the electrically continuous region. Shadow masking is preferred because it avoids the chemical contamination associated with photolithography. If photolithography is used then the cleaning process described above should be repeated after the normal post photolithography cleaning procedures have been followed.

A suitable electronic system for analysis is given in figure 4. Three forms of preamplifier are shown: Fig. 5 shows a standard voltage clamp amplifier, Fig. 6 shows a balanced voltage bridge for measuring differential

impedance with a biosensor containing two active electrodes. Both elements are coated in lipid but only one includes the biosensing gated ion channels.

Figure 4 shows an example of a method to measure ion channel impedance in a membrane by using the non-linear conductance property of the ion channel. Figure 4 shows a local oscillator 31 which might typically run at 10kHz. Frequency dividers 32 and 33 derive signals of frequency F/n1 and F/n2 from the local oscillator 31. Typically n1 = 10 and n2 = 11. A summing amplifier 34 adds the two signals from frequency dividers 32 and 33, whilst buffer amplifiers 35 and 36 supply a signal to the sensing electrode. Buffer amplifier 36 also inverts the signal so that it is the opposite polarity to the signal from buffer amplifier 35, however, this inverted signal is only required where the preamplifier used is as shown in Figure 6. The system for switching (multiplexing) the signal to an array of electrodes and sensing the resultant signal with a single current sensing amplifier is shown generally as 37 and described in more detail in Figures 5, 6 and 7. The sensed signal is then further amplified by an amplifier 38 and the component of the signal with a frequency of (F/n1 - F/n2) is detected and amplified by a phase lock loop detector 39. Because this signal component results from the non-linear conductance of the ion channel it can be used to preferentially distinguish changes in the ion channel conductance from the rest of the membrane impedance which has a relatively linear conductance.

Figures 5, 6 and 7 show forms of preamplifiers suitable for use with the sensors described in the examples. Figure 5 shows a preamplifier which is more suitable for single sensors; while Figures 6 and 7 show preamplifiers which are more readily used with an array of sensors.

The preamplifier shown in Figure 5 is a standard three terminal impedance bridge comprising an amplifier 41 which supplies enough current to counter electrode 42 so that a reference electrode 43 is always held at the same potential as the command voltage. The reference electrode 43 is connected to a high impedance negative feedback input of amplifier 41 so that it accurately monitors the potential of the electrolyte solution and controls the current to the counter electrode so that the electrolyte solution is clamped to the same potential as the command voltage. The active electrode 44 is coated with the membrane and held at a zero value of potential so that current must flow into it from the counter electrode 42 dependent on the impedance of the membrane. The amplifier 45 measures this current by forcing it through a resistor 46. Thus the conductance of the membrane coating the active electrode 44 can be determined from the measured value of the potential of the electrolyte and the current passing through the membrane.

The preamplifier and electrode arrangement shown in Figure 6 comprises a balanced bridge consisting of an electrode 51 which is coated with the lipid membrane containing gated ion channels and an electrode 52 which is coated with a lipid membrane only. The two electrodes are supplied with signals which are identical but opposite in polarity so that if the electrode conductances are equal there is a zero potential in the electrolyte in which they are both immersed. A sensor electrode 53 measures imbalances in the potential of the electrolyte so that if the conductance of the electrode 51 was altered by a biosensor reaction (i.e. opening or closing of the gated ion channel) then the change in potential would be sensed by electrode 53 and amplified by a high impedance amplifier 54. Electrodes 51 and 52 can be a pair in an array of such pairs which can be addressed by switching

the excitation signal to them.

The preamplifier shown in Figure 7 represents a two terminal impedance bridge in which an amplifier 56 supplies an excitation signal to an electrode 57 which is coated with a membrane. Electrode 57 is one of an array of electrodes and the excitation signal can be switched to each electrode in the array. An electrode 58 detects the current passing through electrode 57 and amplifies it with a high impedance amplifier 59. Thus the conductance of an array of electrodes such as 57 can be measured.

2) Improved Sensitivity and Response Time in a Multiplexed Sensor

Methods are described for a biosensor and measuring system which allows multiplexing to enhance the performance of the gated ion channels in lipid membrane sensor described in International Patent Application No. WO 89/01159.

The biosensor is fabricated using a combination of silicon integrated circuit technology and lipid coating methods.

Figures 8 - 10 shows details of four mask levels necessary for fabrication with Figures 9 and 10 showing cross-sectional views taken along line A-A and B-B of Figure 8 respectively. The chip size is 7mm x 5mm with the four mask levels required to pattern the layers given as Polysilicon, silicon dioxide, Aluminium and Nitride. these are shown as Polysilicon 60, silicon dioxide 62, Aluminium 64 and Nitride 66, electrode metallisation (gold, palladium or platinum) 67. The significance of these levels is as follows:-

Polysilicon

Conducting polysilicon fingers 68 connecting each of the 10 pairs of sensing electrodes 70 to the respective aluminium bonding pads 72.

Silicon dioxide

A layer of deposited glass temporarily covering the tips of the polysilicon fingers 68 and designed to protect the pair of sensing electrodes 70. This layer is deposited after the formation of the sensing electrode metal and remains in during all subsequent operations including packaging. It is removed by hydrofluoric acid etch immediately prior to application of the lipidic biosensor film.

Nitride

A layer of deposited silicon nitride is the primary electrical insulation layer and covers the whole surface of the chip with the exception of windows over the pair of sensing electrodes 70 and bonding pads 72. Wire connecting leads 74 are provided to the bonding pads 72.

As is best shown in Figs. 9 and 10 an electrode well 78 where the biosensor membrane is positioned is provided in each one of the each pair of electrodes 70.

Summary of the Process Steps

The starting material is a 6 inch diameter wafer of 100 single crystal silicon.

1. Grow 7500 angstroms of thermal oxide
2. Deposit 4000 angstroms of phosphorous doped silicon by low pressure chemical vapour deposition.
3. Carry out ophotolithographic processes to pattern polysilicon fingers, etch in plasma.
4. Oxidise polysilicon fingers to 300 angstroms thickness
5. Deposit 600 angstroms silicon nitride by low pressure chemical vapour deposition
6. Deposit 1200 angstroms of sputtered aluminium
7. Carry out photolithographic process to pattern aluminium bond pads - plasma etch
8. Carry out photolithographic process steps to pattern windows in nitride - plasma etch
9. Deposit gold platinum or palladium

10. Pattern electrode by lift off technique
11. Deposit 8000 angstroms glass (silox) by plasma enhanced chemical vapor deposition
12. Carry out photolithographic process steps to pattern silox - plasma etch
13. Saw into chips for packaging in moulding compound and chip carrier 76.
14. The protective silox should then be removed by etching with hydrofluoric acid and coated with lipid and biosensitive ion channels as described previously.

Many configurations are possible. The pattern shown is arranged as a general test unit which shows how electrodes can be either close packed or separated and how they can be used in various bridge configurations.

In one example the two close packed elements are used to provide a cross check on each other. The 10 pairs can then be used as individual biosensing elements to scan a surface of analyte using preamplifiers such as those given in figures 6 and 7.

Another arrangement is to use them in a number of bridge circuits grouped so that some contain biosensitive ion channels, some contain ion channels which have not been modified for biosensitivity and the remainder contain only lipid material. Such grouped elements can be measured separately and compared after amplification; alternatively differential measurements can be carried out using bridges as per figures 6.

To be practical the multiplexor circuitry requires that the active elements be attached to low impedance circuitry so that conventional three terminal bridges are not appropriate. It is also desirable for cost effectiveness that the high impedance element should not be located on the sensor chip. Arrangements which achieve this are given in figure 4 and use the amplifiers outlined in Figures 6 and 7.

CLAIMS:

1. A biosensor comprising at least one lipid membrane, each membrane including at least one gated ion channel, each of said membranes comprising a closely packed array of self-assembling amphiphilic molecules, said at least one gated ion channel having a conductance which is dependent upon an electric field applied across the membrane.

2. A biosensor as claimed in claim 1 in which the ion channel is modified by incorporation or removal of polar, dipolar or polarisable groups.

3. A biosensor as claimed in claim 1 or claim 2 in which the biosensor comprises a plurality of discrete membranes, the conductance of each membrane being measurable independently of the conductance of the other membranes.

4. A biosensor as claimed in claim 3 in which at least one dedicated electrode is provided on one side of each membrane which cooperates with an electrode on the other side of the each membrane to enable the application of an electric potential across the membrane, the plurality of membranes being multiplexed by multiplexing the signal applied to the respective discrete electrodes.

5. A biosensor comprising a plurality of discrete membranes, each membrane including at least one gated ion channel, each of said membranes comprising a closely packed array of self-assembling amphiphilic molecules, the conductance of each of said membranes being measurable independently of the conductance of the other membranes.

6. A biosensor as claimed in any one of claims 1 to 5 in which the ion channel is selected from the group consisting of peptides capable of forming helices and aggregates thereof, podands, coronands and cryptands.

7. A biosensor as claimed in claim 6 in which the ion channel is a peptide capable of forming a helix or aggregates thereof.

8. A biosensor as claimed in claim 7 in which the ion channel is a peptide which forms a β helix.

9. A biosensor as claimed in claim 8 in which the ion channel is gramicidin or analogs thereof.

10. A biosensor as claimed in claim 9 in which the ion channel is gramicidin A or analogs thereof.

11. A biosensor as claimed in any one of claims 1 to 10 in which the gated ion channel can diffuse laterally within the lipid membrane.

12. A biosensor as claimed in any one of claims 1 to 11 in which the conductance of each lipid membrane is measured by means of a high impedance address lines, a separate address line being provided to each lipid membrane and/or multiplexing the membranes.

13. A biosensor as claimed in claims 12 in which the conductance of each lipid membrane is measured by multiplexing the membranes.

14. A biosensor as claimed in claim 13 in which the membranes are serially multiplexed.

15. A biosensor as claimed in claim 13 or claim 14 in which the conductance measurements are made using multiplex lines of low impedance and at least one current sensing line.

16. A biosensor as claimed in claim 15 in which there is one current sensing line.

17. A biosensor as claimed in any one of claims 1 to 11 in which the conductance of each membrane is measured by means of switching between low impedance address lines each of which supplies a signal which is measured either by a single current sensor common to all the address lines or by a number of current sensors which are electrically isolated from each other and which measure groups of address lines.

18. A biosensor as claimed in any one of claims 1 to 11 in which the conductance of each membrane is measured by

means of switching between high impedance address lines each of which supply a signal which is measured either by a single current sensor common to all the address lines or by a number of current sensors which are electrically isolated from each other and which measure groups of address lines.

19. A biosensor as claimed in any one of claims 12 to 18 which the gated ion channels are field effect ion channels.

20. A biosensor as claimed in claim 19 in which the plurality of discrete membranes are arranged in a two dimensional array.

21. A biosensor as claimed in claim 20 in which the multiplex lines are driven from a complex signal such that in the two dimensional array each address line in one dimension has signal components which are cross modulated with signals from address lines in the other dimension by the field effect ion channel.

22. A biosensor as claimed in any one of claims 1 to 11 in which the conductance of each lipid membrane is measured by means of a high impedance address line either by using a separate amplifier for each membrane or by switching one amplifier between each membrane or by switching a number of amplifiers between a number of membranes such that each membrane is measured.

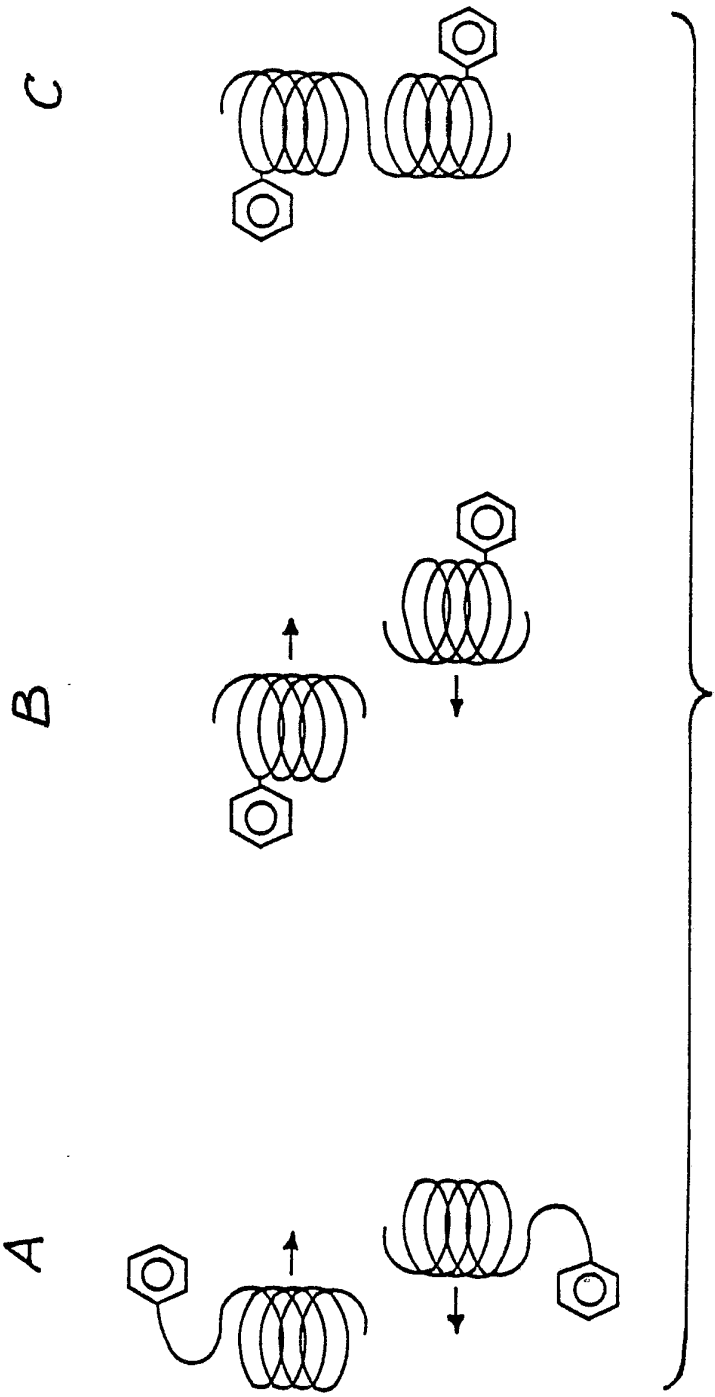

FIG. 1

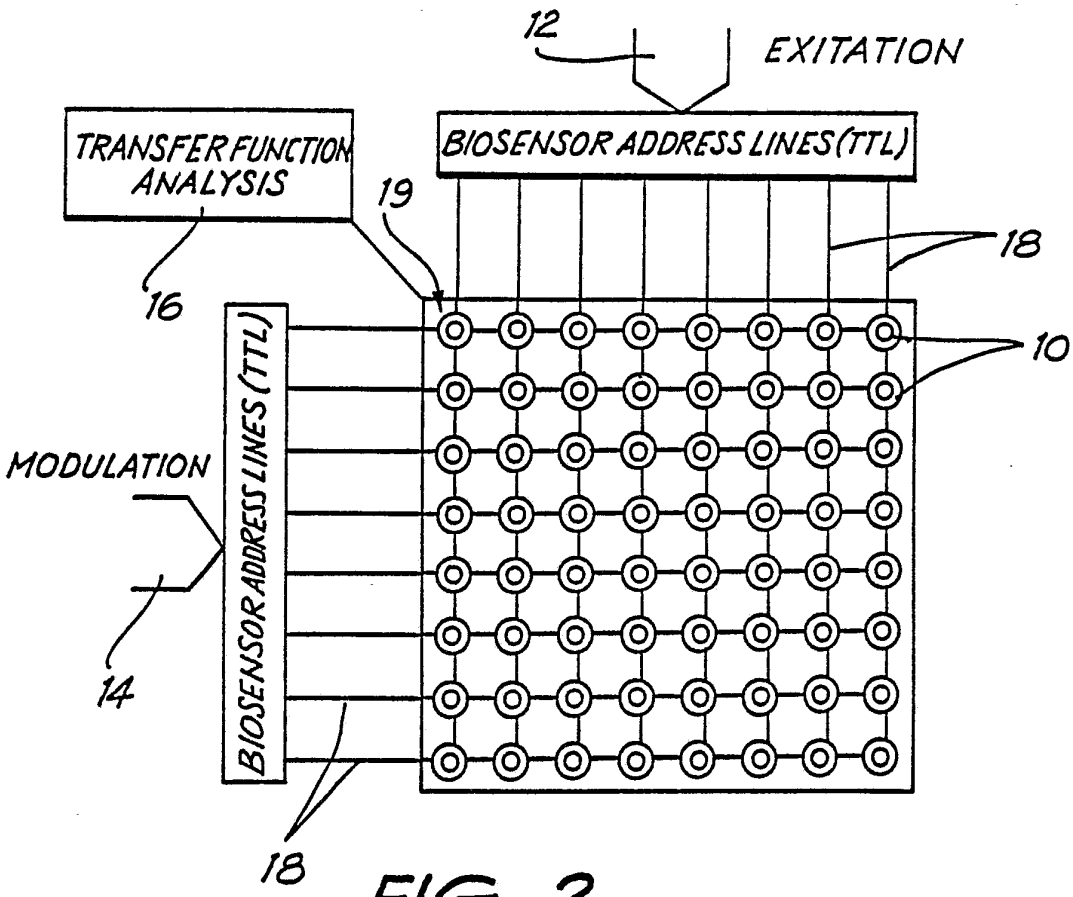
12
EXITATION
TRANSFER FUNCTION ANALYSIS
BIOSENSOR ADDRESS LINES (TTL)
19
18
16
10
MODULATION
BIOSENSOR ADDRESS LINES (TTL)
14
18

FIG. 2

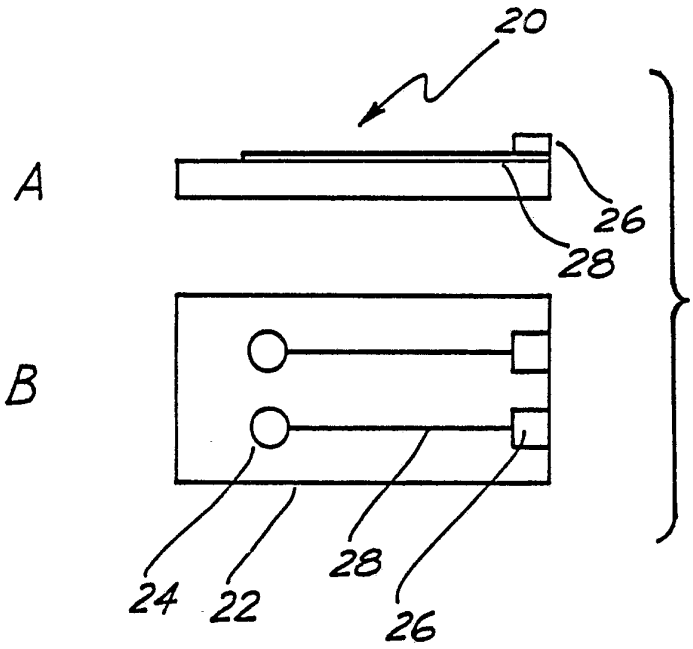

FIG. 3

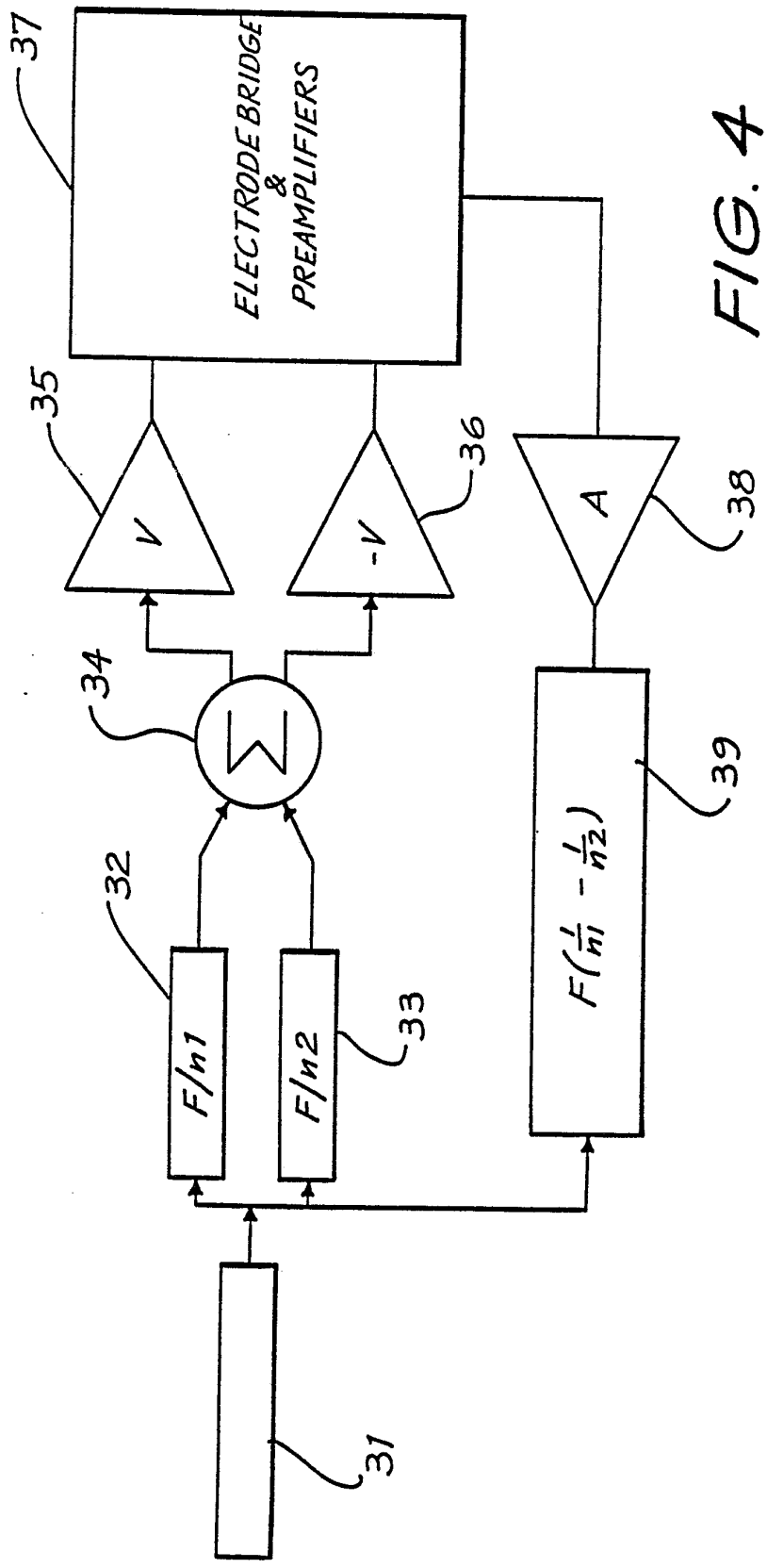

FIG. 4

SUBSTITUTE SHEET

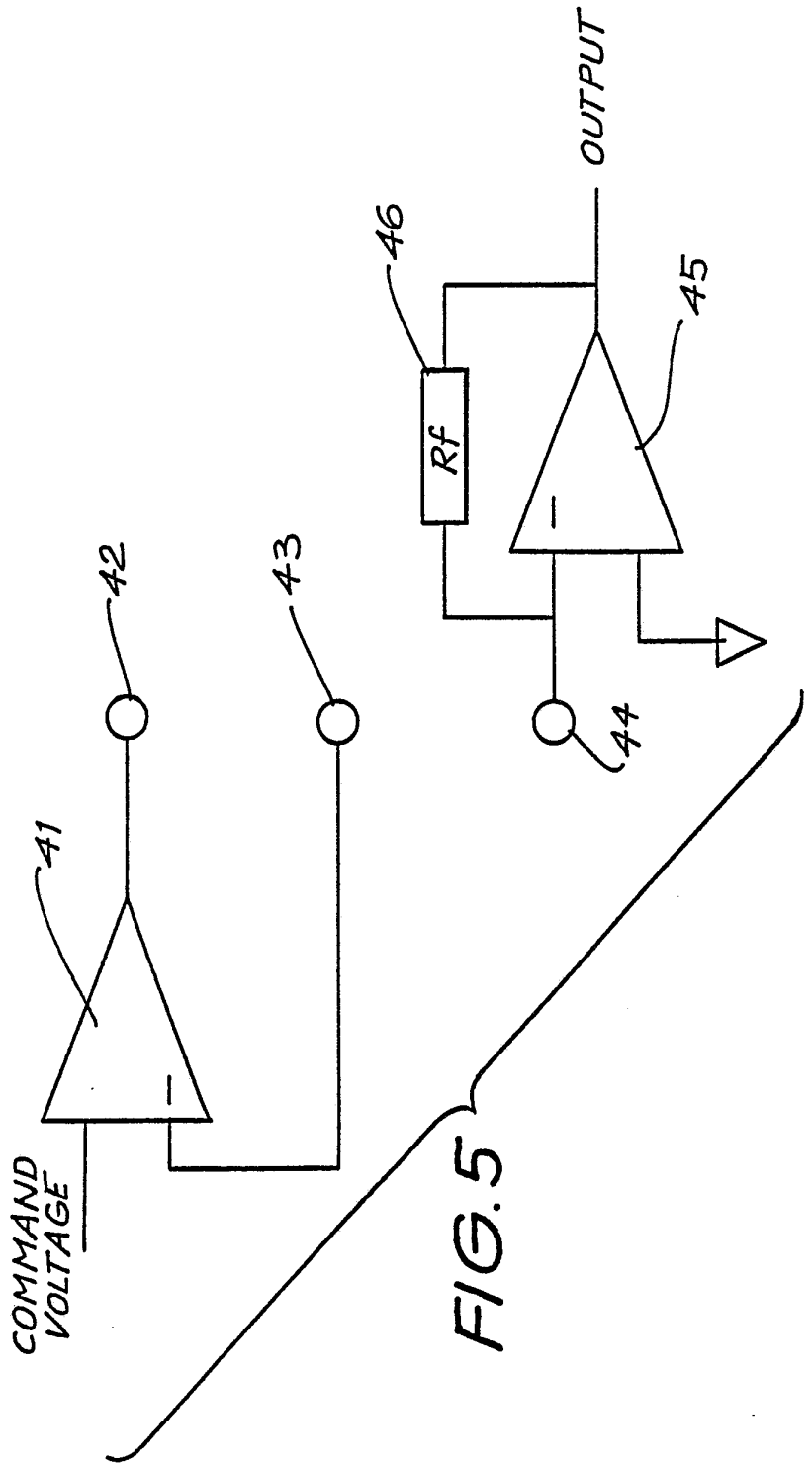

FIG. 5

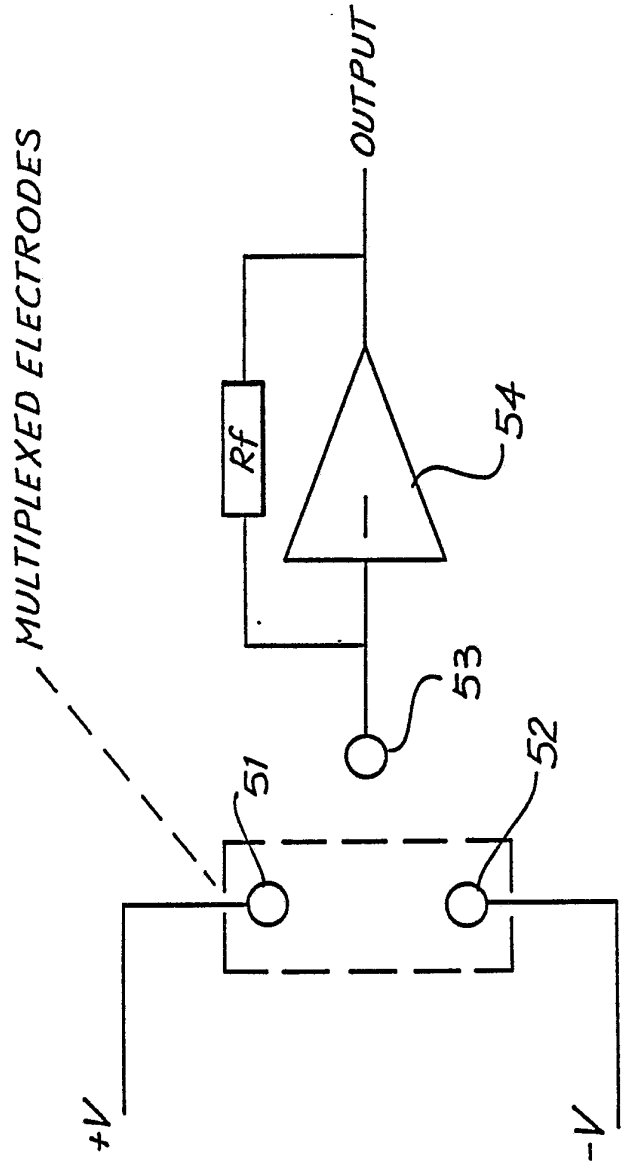

FIG. 6

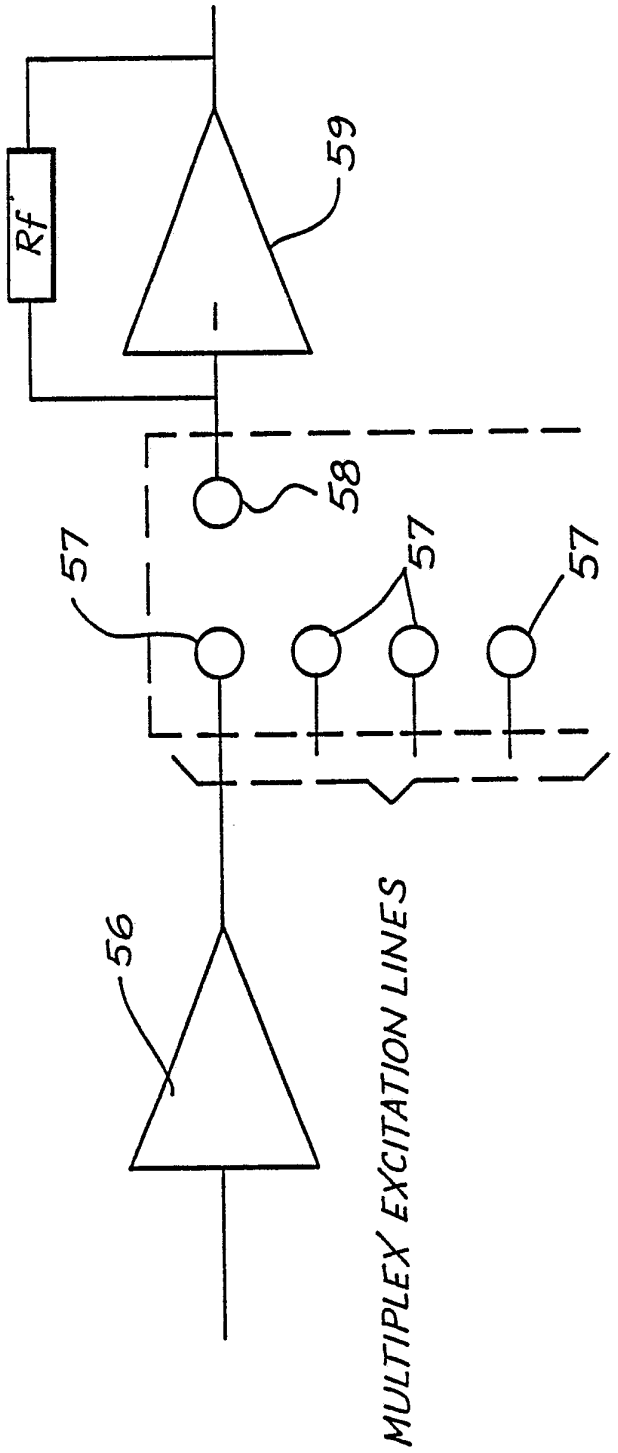

FIG. 7

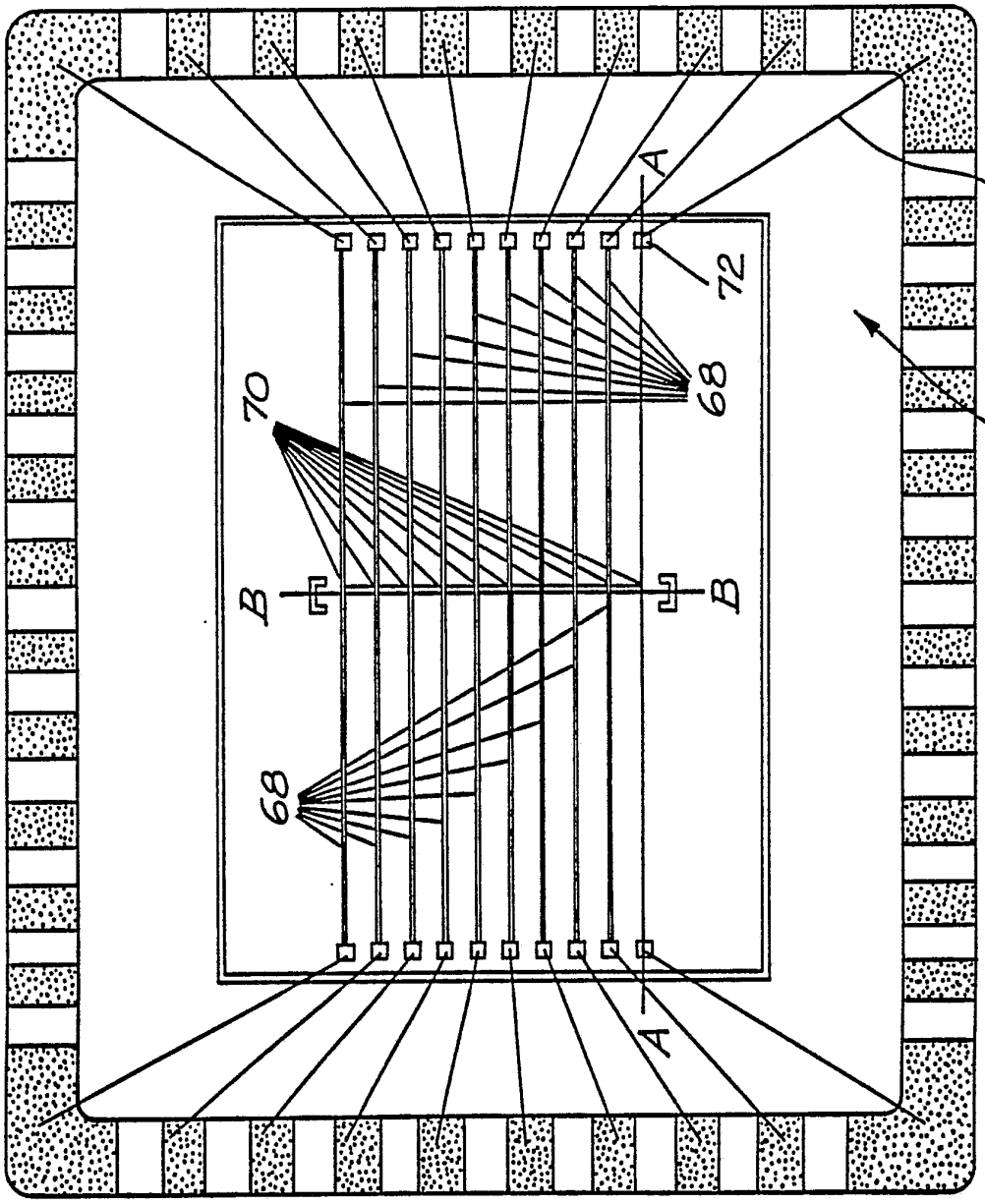

FIG. 8

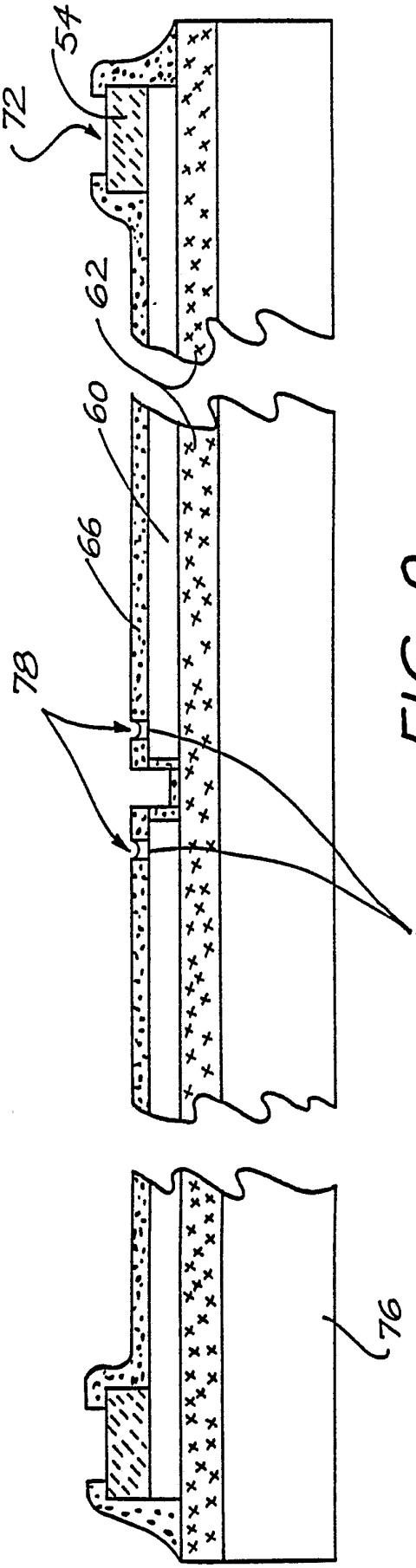

FIG. 9

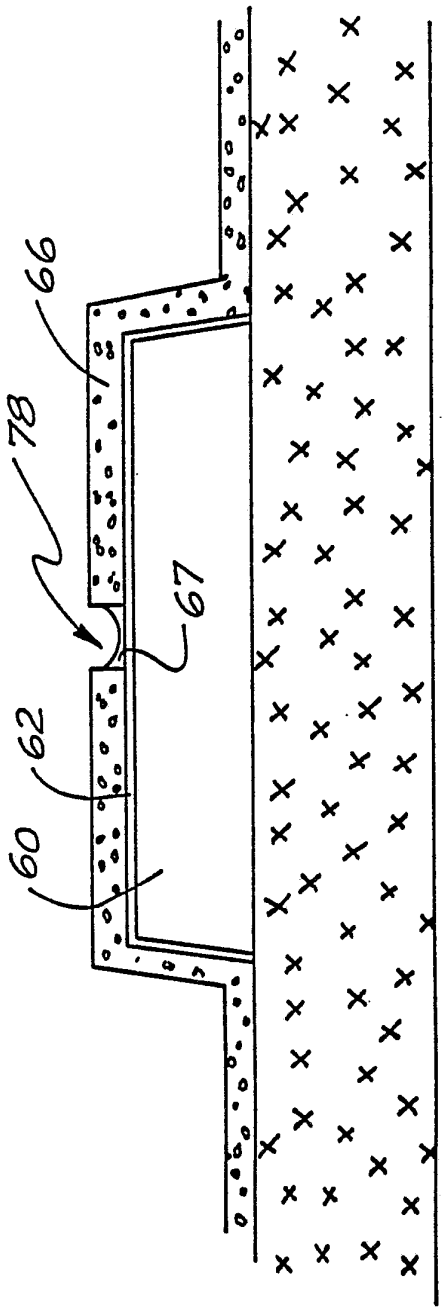

FIG. 10

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 89/00352

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

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

Int. Cl.[4] G01N 27/40, 33/545

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	WPI, WPIL, USPA G01N Keyword: "MEMBRANE" CHEM ABSTRACTS Keywords: "MEMBRANE" and "PEPTIDE" and SECTION 15 G01N 27/40

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU: G01N 27/40, 33/54, 33/545, 33/547, 33/563

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	GB,A, 2195450 (UNITED KINGDOM ATOMIC ENERGY AUTHORITY) 7 April 1988 (07.04.88)	(1-22)
X	AU,A, 40123/85 (JANATA et al) 3 october 1985 (03.10.85)	(1-22)
A	EP,A, 138150 (E.I. DU PONT DE NEMOURS AND COMPANY) 24 April 1985 (24.04.85)	

* Special categories of cited documents: 10

"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	Date of Mailing of this International Search Report
20 october 1989 (20.10.89)	26 October 1989
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	R MURRAY S. Clark RP.

Form PCT/ISA/210 (second sheet) (January 1985)