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Raguse et al.

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[54] SENSOR MEMBRANES

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[22] Filed: Apr. 9, 1997

Related U.S. Application Data

[62] Division of Ser. No. 406,853, filed as PCT/AU93/00509 Oct. 1, 1993 published as WO94/07593 Apr. 14, 1994, Pat. No. 5,637,201.

[30] Foreign Application Priority Data

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Jul. 8, 1993 [AU] Australia PL9863

[51] Int. Cl.⁶ G01N 27/26

[52] U.S. Cl. 204/418; 204/403; 435/4; 435/7.1; 435/289.1; 435/817

[58] Field of Search 427/331, 337, 427/384, 388.1, 430.1; 204/403, 418, 282, 296; 435/4, 7.1, 11, 289.1, 817

[56] References Cited

U.S. PATENT DOCUMENTS

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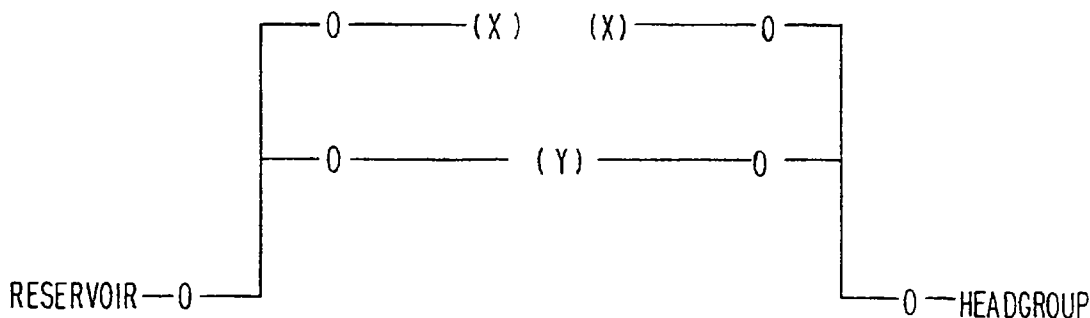
Primary Examiner—Bruce F. Bell

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[57] ABSTRACT

The present invention relates to a novel method for producing an electrode membrane combination. This novel method employs compounds including a linker lipid for use in attaching a membrane including a plurality of ionophores to an electrode and providing a space between the membrane and the electrode, the electrode being either in part or totally made up of the linker lipid. The linker lipid comprises within the same molecule a hydrophobic region capable of spanning the membrane, an attachment group used to attach the molecule to an electrode surface, a hydrophilic region intermediate said hydrophobic region and the attachment group and a polar head group region attached to the hydrophobic region at a site remote from the hydrophilic region.

24 Claims, 10 Drawing Sheets



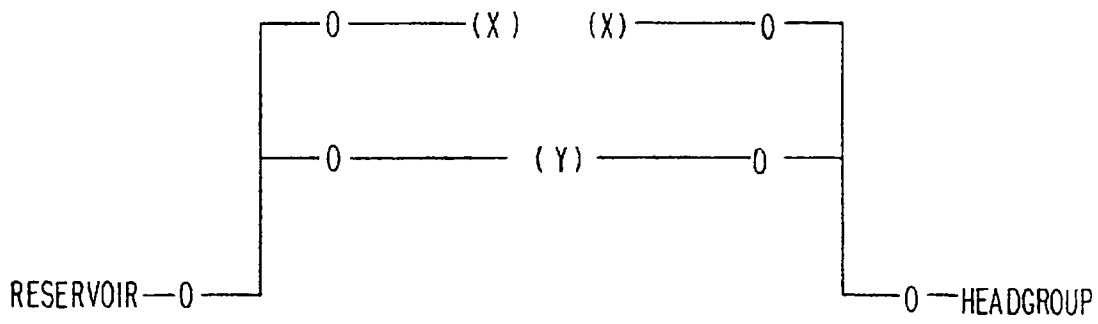


FIG. 1

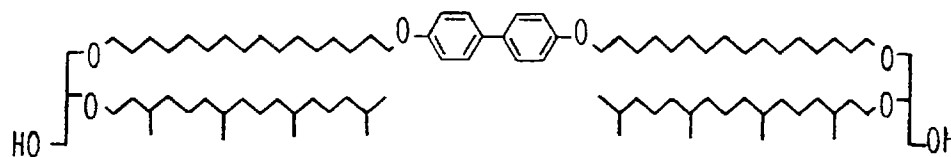


FIG. 2

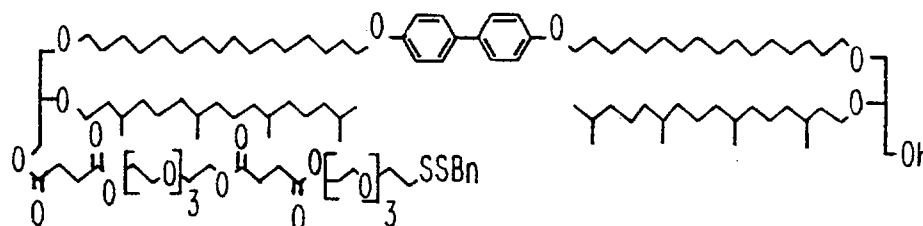


FIG. 3

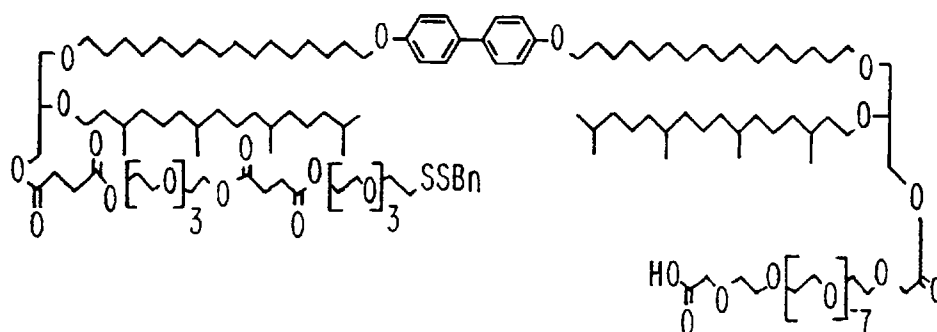


FIG. 4

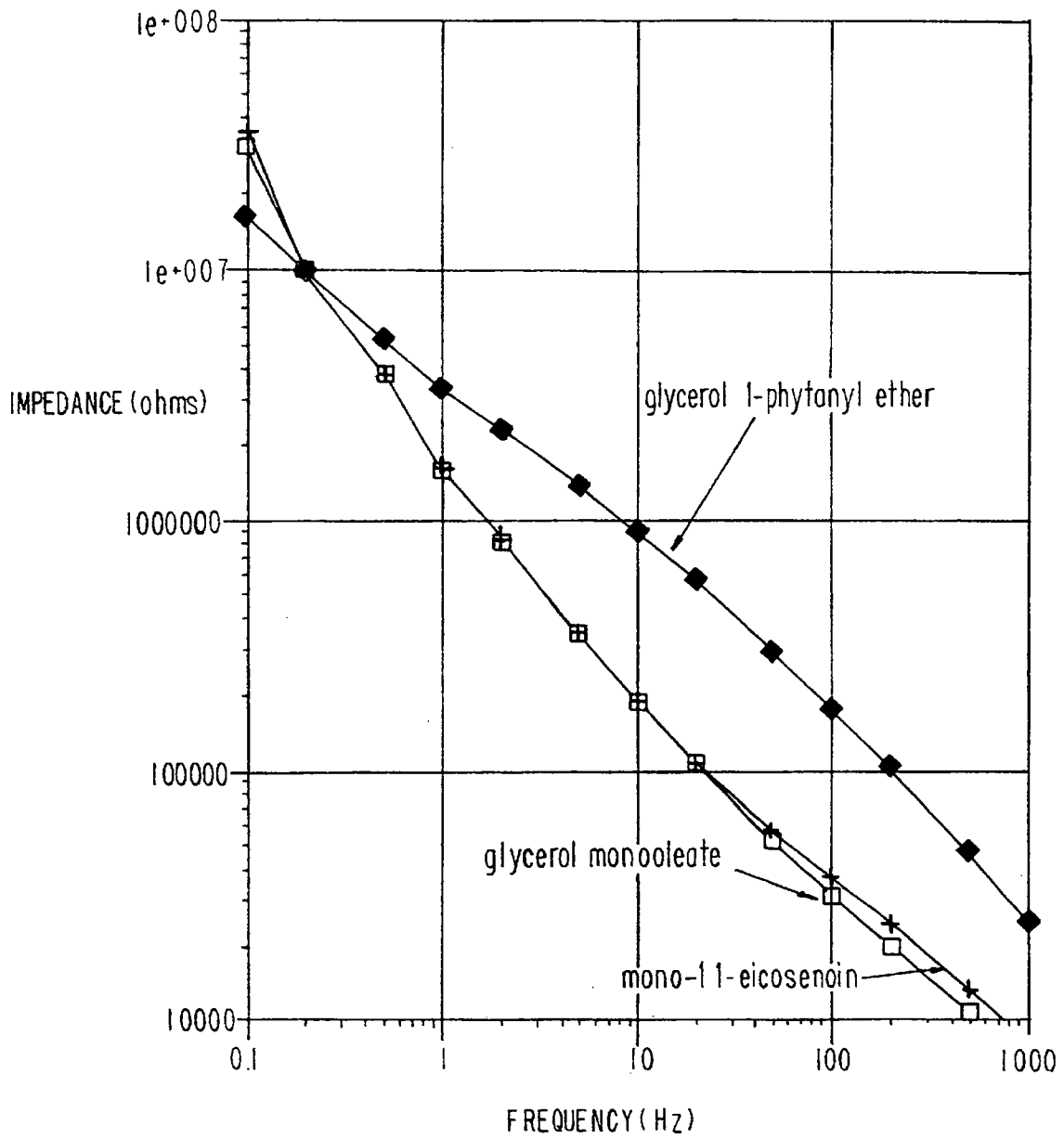


FIG. 6

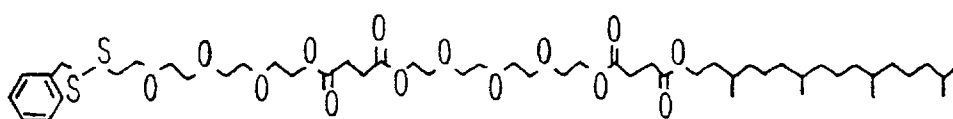


FIG. 7

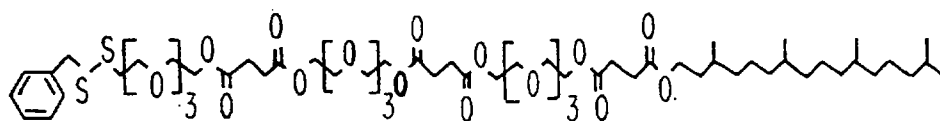


FIG. 8

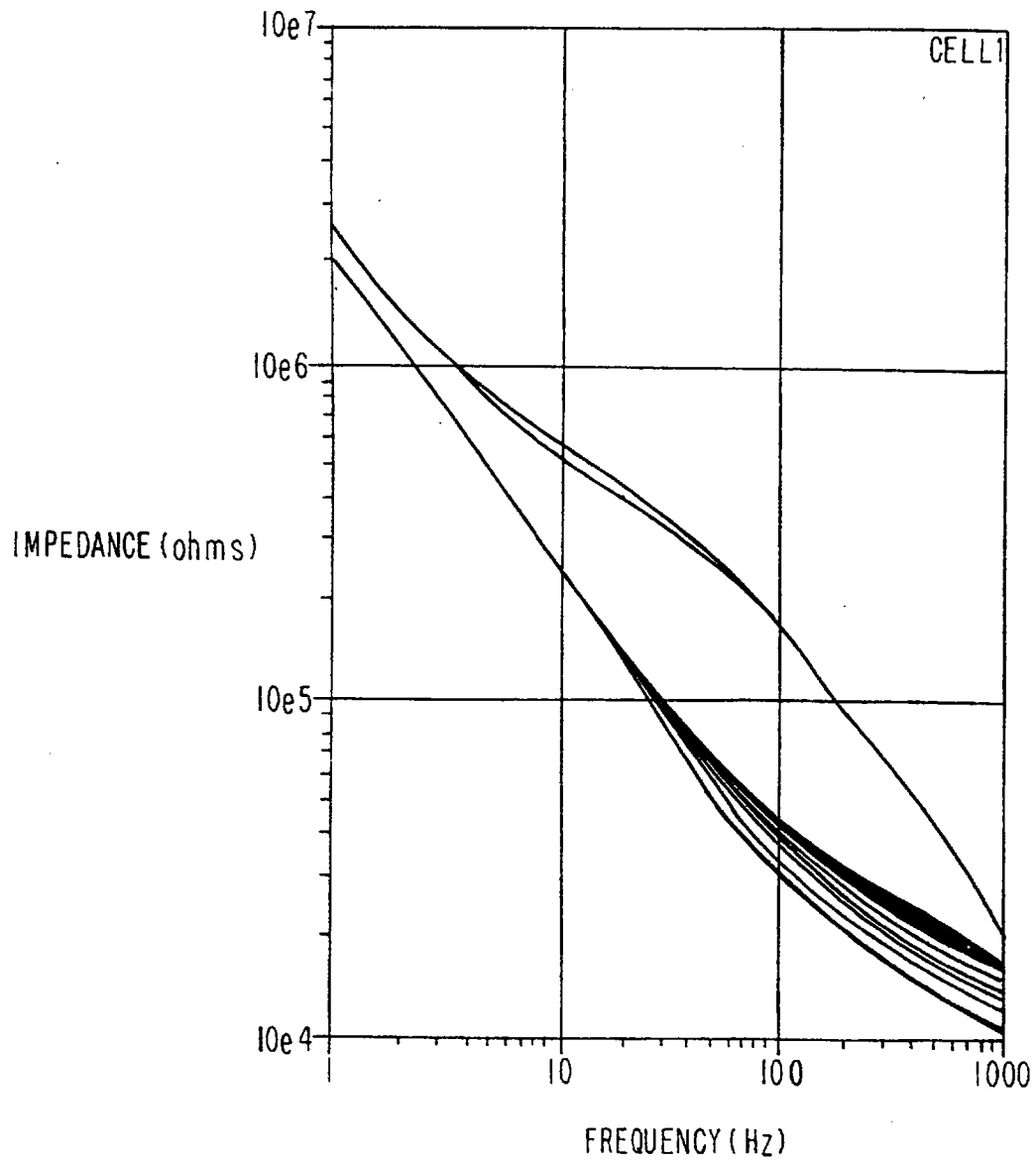


FIG. 9

FIG. 10

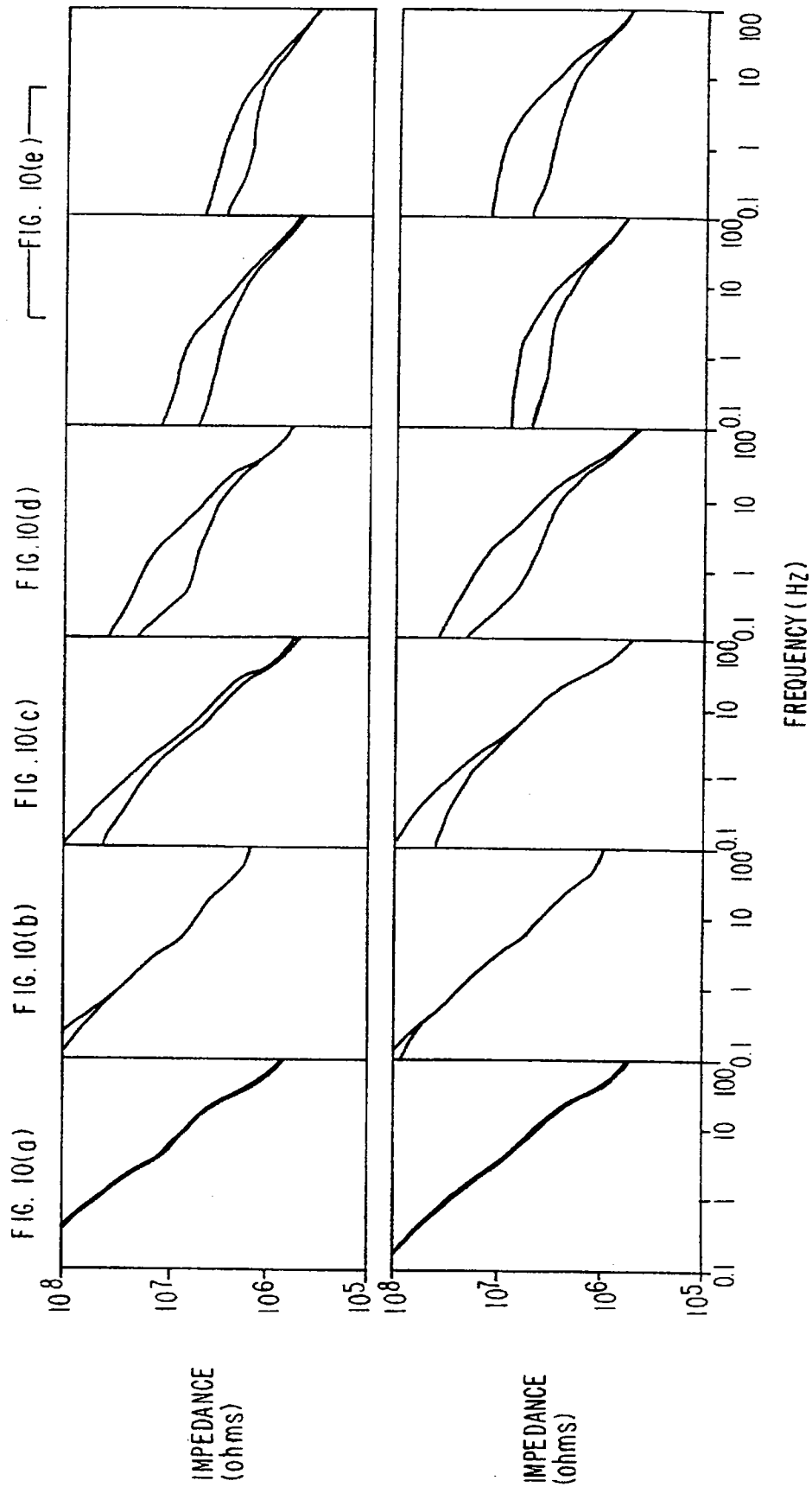


FIG. 11

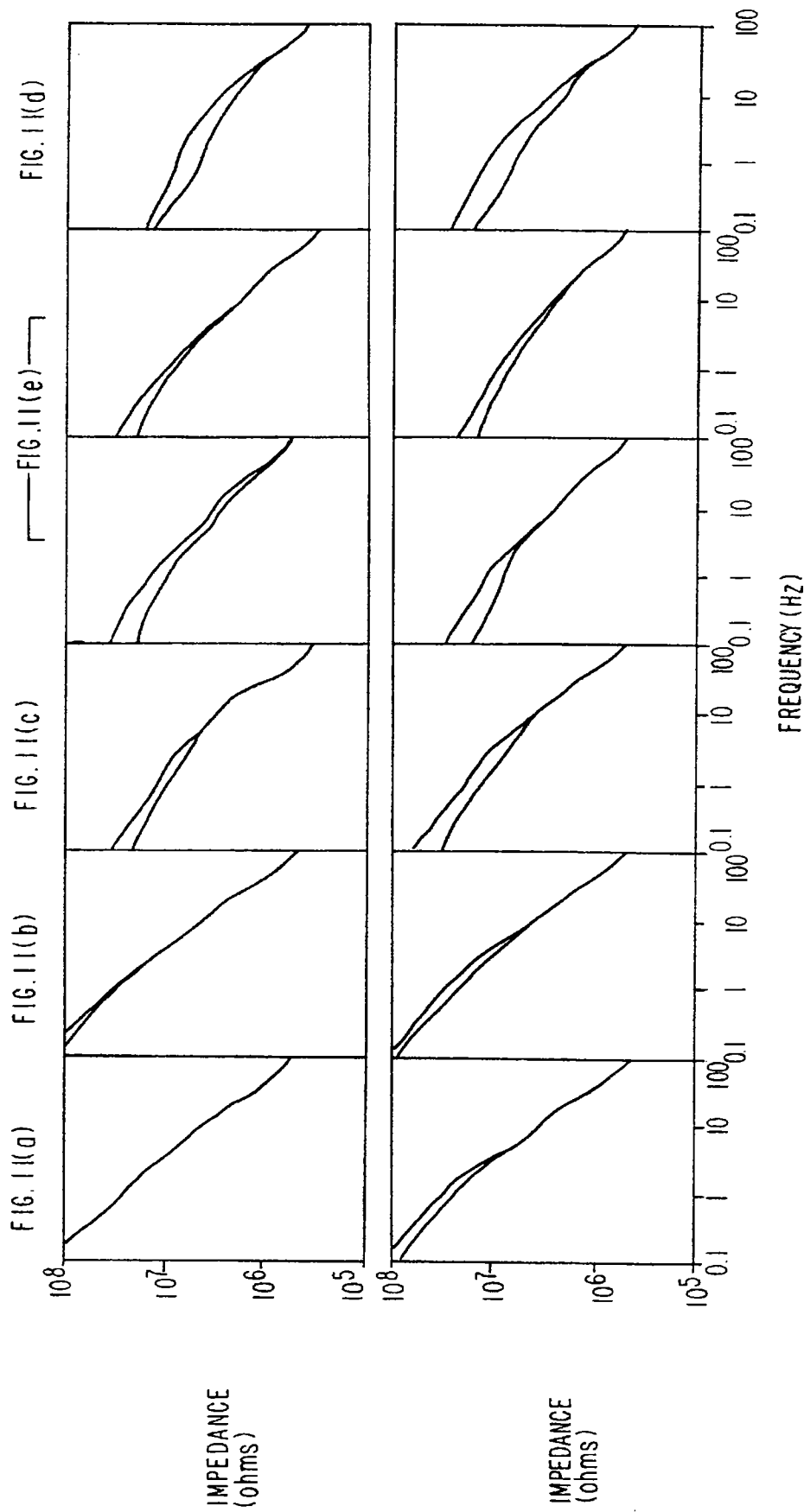
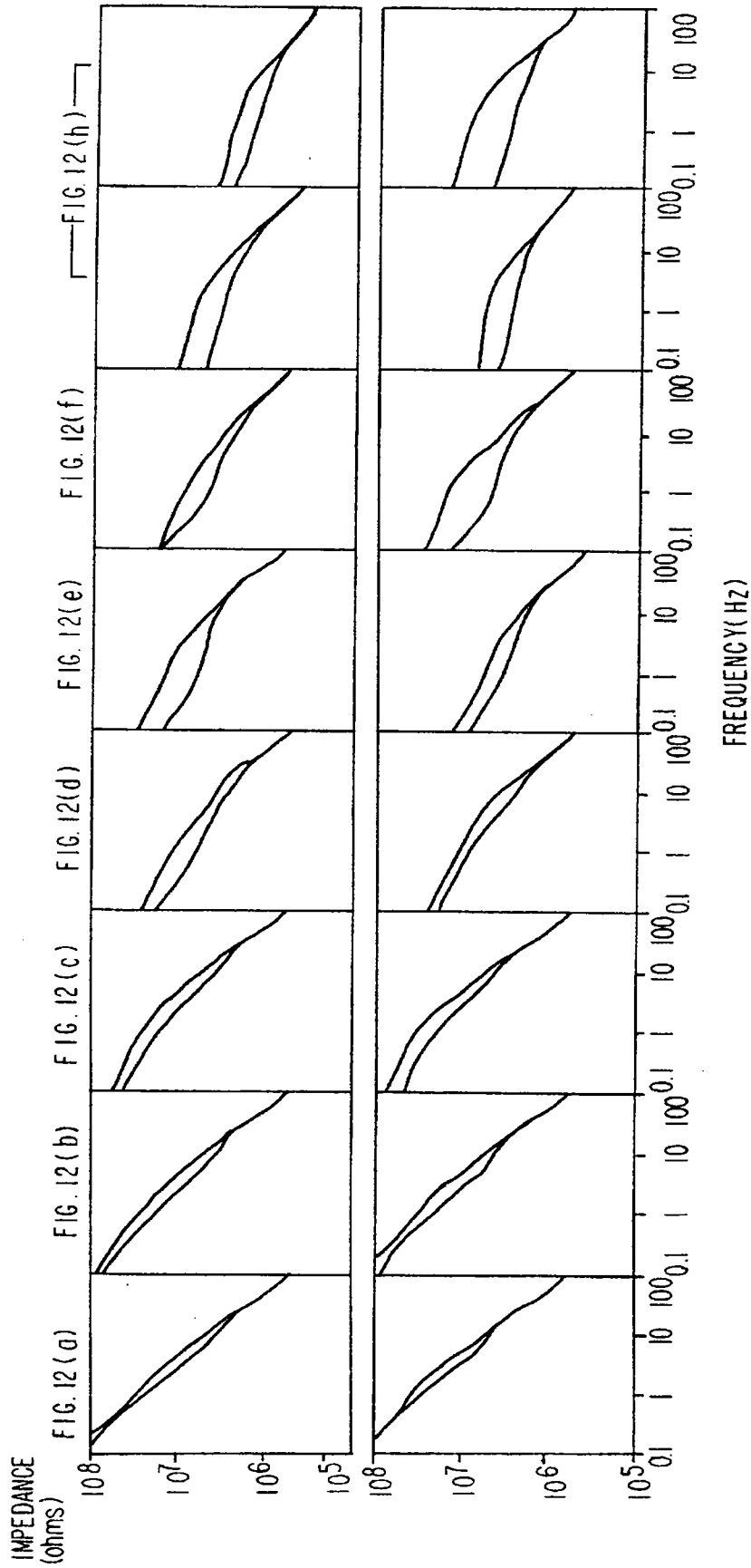


FIG. 12



SENSOR MEMBRANES

This is a division of application Ser. No. 08/406,853, filed as PCT/AU93/00509, Oct. 1, 1993, published as WO94/07593, Apr. 14, 1994, now U.S. Pat. No. 5,637,201.

FIELD OF THE INVENTION

The present invention relates to electrode membrane combinations for use in ion selective electrodes and biosensors. In addition, the present invention relates to methods for the production of such electrode membrane combinations and the use of ion selective electrodes and biosensors incorporating such electrode membrane combinations in the detection of analytes. The present invention also relates to novel compounds used in the electrode membrane combinations.

BACKGROUND OF THE INVENTION

Lipid bilayer membranes (also known as black lipid membranes—BLM's) are well known in the biological and chemical fields. The ability of ionophores to modulate the ion flux through these membranes is also well known. Modulation of the ion flux of the membrane in response to specific molecules is also known, especially in the biochemical fields. The lipid bilayer membranes are however extremely fragile and sensitive to non-specific physical and chemical interference. The preparation and properties of the BLM's are fully described in textbooks and literature articles.

It has been known since 1967 that ionophores incorporate into lipid bilayers (P. Mueller et al, *Biochem. Biophys. Res Commun.*, 26 (1967) 298; A. A. Lev et al *Tsitologiya*, 9 (1967) 102;) in BLM's and that the selective ion flux through the membrane could thus be monitored. Possibility of producing a lipid bilayer containing ionophores on an ionic hydrogel reservoir and using such as an ion selective electrode has also been suggested (U. J. Krull et al, U.S. Pat. No. 4,661,235, Apr. 28, 1987), however no means of obtaining reproducible and stable bilayer membranes have been taught in the art. Using a Langmuir-Blogett bilayer and multilayer approach (T. L. Fare et al *Powder Technology*, 3, (1991), 51-62; A. Gilardoni et al, *Colloids and Surfaces*, 68, (1992), 235-242) has been attempted however the ion selectivity was inadequate and the response time was too slow for practical purposes, stability was not adequate and the LB technique is generally considered to be too difficult for industrial applications.

Ionophores in the context of the present invention are any of the naturally occurring lipophilic bilayer membrane compatible ion carriers such as valinomycin, nonactin, methyl monensin or other naturally occurring ion carriers, or synthetic ionophores such as lipophilic coronands, cryptands or podands, or low molecular weight (<5000 g/mol) naturally occurring or synthetic ion channels such as gramicidin, alamethicin, melittin or their derivatives. Additionally tri-alkylated amines or carboxylic acids such as phytic acid may serve as proton ionophores.

Ion channels may also include large, lipid membrane compatible, protein ion channels, especially where their function and stability is enhanced through their incorporation into lipid bilayers that are essentially free of extraneous alkane material.

In the broad context of the present invention lipids are deemed to be any amphiphilic molecules, either naturally occurring or synthetic, containing a hydrophobic hydrocarbon group and a hydrophilic head group.

Biosensors and ion selective electrodes incorporating gated ionophores in lipid membrane combinations have been disclosed in International Patent Application Nos PCT/AU88/00273, PCT/AU89/00352, PCT/AU90/00025 and PCT/AU92/00132. The disclosure of each of these references is incorporated herein by reference.

As is disclosed in these applications, suitably modified receptor molecules may be caused to co-disperse with amphiphilic molecules and produce membranes with altered surface binding properties, which are useful in the production of biosensor receptor surfaces of high binding ability and high binding specificities. It is also disclosed that ionophores such as polypeptide ionophores may be co-dispersed with amphiphilic molecules, thereby forming membranes with altered properties in relation to the permeability of ions. There is also disclosure of various methods of gating these ion channels such that in response to the binding of an analyte the conductivity of the membrane is altered. The applications also disclose methods of producing membranes with improved stability and ion flux using chemisorbed arrays of amphiphilic molecules attached to an electrode surface and means of producing lipid membranes incorporating ionophores on said chemisorbed amphiphilic molecules. Additionally, means of co-dispersing ion selective ionophores with amphiphilic molecules thereby producing ion selective membrane combinations are disclosed.

The present inventors have now determined improved means of increasing the stability and ion flux properties of the lipid membranes through the use of novel synthetic lipids and lipid combinations, and novel means of membrane assembly.

In various embodiments the present invention consists in the use of novel bilayer membrane spanning lipids and bilayer lipids and methods of assembly thereof, in order to modulate the properties of the lipid sensor membrane so as to control the ion transport properties of the ionophore, the thickness and fluidity of the membrane, the stability of the membrane, the response to serum, plasma or blood, and the non-specific absorption of proteins to the membrane.

SUMMARY OF THE INVENTION

In a first aspect, the present invention consists in a linker lipid for use in attaching a membrane including a plurality of ionophores to an electrode and providing a space between the membrane and the electrode in which the membrane is either in part or totally made up of the linker lipid, the linker lipid comprising within the same molecule a hydrophobic region capable of spanning the membrane, an attachment group used to attach the molecule to an electrode surface, a hydrophilic region intermediate said hydrophobic region and the attachment group, and a polar head group region attached to the hydrophobic region at a site remote from the hydrophilic region.

In a preferred embodiment of the present invention, the head group region is selected from the group consisting of groups normally associated with naturally occurring or synthetic lipids such as glycerol, phosphatidyl choline, phosphatidyl ethanolamine, mono-, di- or tri-methylated phosphatidyl ethanolamine, phosphatidic acid, phosphatidyl serine, phosphatidyl glycerol, phosphatidyl inositol, disubstituted head groups as found in cardiolipins, ganglioside head groups, sphingomyelin head groups, plasmalogen head groups, glycosyl, galactosyl, digalactosyl, sulfosugar, phosphosugar, N-acetyl neuramic acid, sialic acid, amino-sugar head groups, carbohydrate head groups, gal(beta1-3) galNAc(beta1-4) [NAcNeu(alpha2-3)gal(beta1-4)glc-

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ceramide, oligomers of ethylene glycol, ethylene glycol, oligomers of propylene glycol, propylene glycol, amino acids, oligomers of amino acids, combinations of oligomers of ethylene glycol or propylene glycol functionalised with amino acids or other ionic species or any combination or derivative of the above.

It is generally preferred that the head group is a naturally occurring or synthetic head group that can be used to minimise the non-specific binding of proteins onto the surface of the membrane.

In a further preferred embodiment of the present invention, in order to provide surface characteristics that minimise the non-specific binding of proteins, it is preferred that the head group is a polyethylene glycol ranging in molecular weight of between 600–6000 g/mol.

In a further preferred embodiment, the head group is a phosphatidyl choline group.

In a further preferred embodiment, the head group is a glycerol head group.

In a further preferred embodiment, the head group is a biotin or a biotinylated 6-aminocaproic acid group or an N-biotinylated oligomer of 6-aminocaproic acid.

In a further preferred embodiment, the head group is a Gal(beta1-3)galNAc(beta1-4)[NAcNeu(alpha2-3)gal(beta1-4-Glc-ceramide head group.

In a further preferred embodiment, of the present invention it is preferred that the head group is a group capable of being used to covalently link a protein molecule onto the linker lipid. The protein molecule may be either a receptor such as an antibody or an antibody fragment or may be an enzyme or may be a protein molecule chosen in order to impart biocompatible properties to the membrane.

It is further preferred that the head group is terminated in a carboxylic acid group capable of being used to conjugate the linker lipid with a protein molecule via the amine groups on the protein.

It is further preferred that the head group is a polyethylene glycol in the molecular weight range 400–1000 g/mol terminated in a carboxylic acid group.

In a further preferred embodiment, the head group is a group capable of being covalently linked with a protein molecule via the aldehyde groups generated from the oxidation of carbohydrate groups on the protein molecule.

In a further preferred embodiment, the head group is a hydrazide derivative.

In a further preferred embodiment, the head group is a polyethylene glycol terminated in a carboxy hydrazide derivative.

In a further preferred embodiment, the head group is a group capable of being covalently linked to a protein molecule via free thiol groups on the protein molecule.

It is further preferred that the head group is a maleimide derivative.

In a further preferred embodiment, the head group is a group capable of being covalently coupled to a carboxylic acid group on a protein molecule.

It is preferred that the hydrophobic group has the general structure as shown in FIG. 1 where the group (X) is a hydrocarbon chain that is approximately half the length of the group (Y).

It is preferred that the group (X) will generally be between 10–22 carbons in length and may be a saturated, unsaturated or polyunsaturated hydrocarbon, or may be an alkyl substituted hydrocarbon such as the phytanyl group or other mono- or permethylated hydrocarbon chain.

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It is further preferred that the group (X) is a phytanyl group.

In a preferred embodiment of the present invention, the group (Y) in FIG. 1 is a single chain hydrocarbon group of length between 20–60 Å long.

In a further preferred embodiment the group (Y) consists in a single chain group that is between 20–60 Å long and contains within the chain a rigid spacer group such as biphenyl ether or biphenylamine or other biphenyl compound. The rigid spacer group serves the function of making the synthesis of the group simpler as it easily enables the coupling of two smaller alkyl chains onto the rigid spacer group, enabling long sections of the group (Y) to be synthesised readily. The rigid spacer group also enhances the ability of the linker lipid to assume the membrane spanning conformation of the linker lipid as opposed to an U-shaped conformation within the membrane.

In a further preferred embodiment, the group (Y) is a single chain group that is between 30–50 Å long and contains within the chain a N,N'-alkyl substituted 4,4'-biphenyl amine group.

In a further preferred embodiment, the group (Y) is a single chain group that is between 30–50 Å long and contains within the chain a 4,4'-biphenyl ether group.

In a further preferred embodiment, the group (Y) is a bis-hexadecyl 4,4'-biphenyl ether.

In a further preferred embodiment, the group (Y) is a bis-tetradecyl 4,4'-biphenyl ether.

In a further preferred embodiment, the group (Y) is a bis-dodecyl 4,4'-biphenyl ether.

In a further preferred embodiment, the group (Y) is a single chain group that is between 20–60 Å long and contains within the chain an alkyl substituted amine.

In a further preferred embodiment, the group (Y) consist in a single chain group that is between 20–60 Å long and contains within the chain a bis-alkylated pentaerythritol group.

In a further preferred embodiment of the present invention, the membrane spanning lipid is a single chain lipid in which the group (X) in FIG. 1 is absent.

In a further preferred embodiment, the group (Y) contains groups that can alter their conformation in response to an external stimulus such as light, pH, redox chemistry or electric field. The change in conformation within the group (Y) will allow the properties of the membrane such as thickness to be controlled through such external stimulus. This can in turn be used to modulate the conduction of ion channels through modulation of the on/off times of the channels and the diffusion of the channels.

In a preferred embodiment, where the group (Y) alters its conformation in response to light stimulus, the group (Y) contains a 4,4'- or 3,3'-disubstituted azobenzene.

In a further preferred embodiment the group (Y) contains a group that undergoes a spiropran-merocyanine equilibrium in response to light stimulus.

In a further preferred embodiment of the present invention, the hydrophobic region of the linker lipid consists of oligomers of long chain amino acids, such as 11-aminoundecanoic acid, 16-aminohexadecanoic acid or other amino acid where the carbon chain is preferably between 6–20 carbons long, and where the amino acids are linked via amide linkages.

It is further preferred that the amide groups are tertiary, alkyl substituted amide groups, where the alkyl groups are

phytanyl groups or saturated or unsaturated alkyl groups between 1-18 carbons in length.

The nature of the hydrophilic group, the attachment group and the electrode are as described in PCT/AU92/00132.

As is set out in this earlier application it is preferred that the attachment region of the linker lipid is attached to the electrode surface by chemisorption. In a situation where the electrode is formed of a transition metal such as gold, platinum, palladium or silver, it is preferred that the attachment region includes thiol, disulphide, sulphide, thione, xanthate, phosphine or isonitrile groups.

In further preferred embodiment the electrode is formed of gold, silver, platinum or palladium and the attachment region includes either a thiol or a disulfide group, the linker lipid being attached to the electrode by chemisorption.

In an alternate embodiment where the electrode is formed such that a hydroxylated surface is formed on the electrode, it is preferred that the attachment region includes silyl groups such as silyl-alkoxy or silyl chloride groups. The hydroxylated electrode surface may be prepared by a number of techniques known to someone skilled in the art and may consist of oxidised silicon or oxidised metals such as tin, platinum, iridium.

In yet a further preferred embodiment the electrode is formed of oxidized silicon, tin, platinum or iridium and the attachment region includes silyl groups, the linker lipid being attached to the electrode by covalent attachment.

The hydrophilic region of the linker lipid is preferably a long chain hydrophilic compound. The hydrophilic region of the linker lipid may be composed of oligo/poly ethers, oligo/poly peptides, oligo/poly amides, oligo/poly amines, oligo/poly esters, oligo/poly saccharides, polyols, multiple charged groups (positive and/or negative), electroactive species or combinations thereof. The main requirement of the hydrophilic region of the linker lipid is that it allows the diffusion of ions through the ionophores provided in the membrane. This is achieved by the placement of suitable ion and/or water binding sites along or within the length of the long chain that makes up the reservoir region.

In a preferred embodiment of the invention the hydrophilic region consists of an oligoethylene oxide group. The oligoethylene oxide group may consist of four to twenty ethylene oxide units.

In a further preferred embodiment the hydrophilic region consists of a subunit of tetraethylene glycol attached to succinic acid. This tetraethylene glycol/succinic acid subunit may be repeated 1-4 times.

In a further preferred embodiment the hydrophobic region of a proportion of the linker lipids have covalently attached thereto an ionophore via a hydrophobic spacer.

As set out above the molecule having a hydrophobic region as shown in FIG. 1 incorporating a rigid spacer group provides a number of advantages. This hydrophobic region can, of course, be synthesized separately from the hydrophilic region, attachment region, and polar head group region. This hydrophobic region, or synthetic lipid, is believed to be new in its own right and can be included in bilayer membranes as a membrane spanning lipid to improve various characteristics of the membrane, such as stability.

Accordingly, in a second aspect the present invention consists in a synthetic lipid for use in bilayer membranes, the synthetic lipid having a structure as shown in FIG. 1 in which Y is a single chain group that is between 20 and 60 Å long and contains a rigid spacer group and X are hydrocarbon chains approximately half the length of Y or are absent.

It is preferred that the group (X) will generally be between 10-22 carbons in length and may be a saturated, unsaturated or polyunsaturated hydrocarbon, or may be an alkyl substituted hydrocarbon such as the phytanyl group or other mono- or permethylated hydrocarbon chain.

It is further preferred that the group (X) is a phytanyl group.

In a further preferred embodiment the rigid spacer group is a biphenyl ether or biphenylamine or other biphenyl compound.

In a further preferred embodiment, the group (Y) is a single chain group that is between 30-50 Å long and contains within the chain a N,N'-alkyl substituted 4,4'-biphenyl amine group.

In a further preferred embodiment, the group (Y) is a single chain group that is between 30-50 Å long and contains within the chain a 4,4'-biphenyl ether group.

In a further preferred embodiment, the group (Y) is a bis-hexadecyl 4,4'-biphenyl ether.

In a further preferred embodiment, the group (Y) is a bis-tetradecyl 4,4'-biphenyl ether.

In a further preferred embodiment, the group (Y) is a bis-dodecyl 4,4'-biphenyl ether.

In a further preferred embodiment, the group (Y) is a single chain group that is between 20-60 Å long and contains within the chain an alkyl substituted amine.

In a further preferred embodiment, the group (Y) consist in a single chain group that is between 20-60 Å long and contains within the chain a bis-alkylated pentaerythritol group.

In a further preferred embodiment of the present invention X in FIG. 1 is absent.

In a further preferred embodiment the synthetic lipid includes a head group. Preferred head groups are those listed in the first aspect of the present invention.

The present inventors have determined that the linker lipids described in the first aspect of the present invention as well as the linker molecules described in PCT/AU92/00132, where the attachment group is a thiol and the hydrophobic region is a single hydrocarbon chain, or where the attachment group is a thiol or disulfide group and the hydrophobic region is made up of two hydrocarbon chains, then, when these linker lipids are adsorbed onto a freshly prepared noble metal electrode a close packed monolayer membrane is formed that does not permit the ionophore to easily penetrate into the membrane, hence restricting the ion flux through the membrane. If electrode surfaces are used that are contaminated then the adventitious introduction of defect sites, where the chemisorption of the sulfur containing groups does not occur, will allow ionophores to penetrate into the monolayer. Contamination of the surface of a gold electrode can occur by adsorption of contaminants from air over a period of minutes to hours and results in electrode surfaces that can suffer from poor reproducibility and stability. The present inventors have devised a more controlled method of producing membranes with the required spacing between the linker molecules, while still retaining the efficient and reproducible attachment of the hydrophilic molecules onto the electrode surface during the deposition of the first layer.

In the prior art it has always been believed necessary that on forming a second lipid layer onto coated electrodes an apolar containment vessel is required in order to obtain sealed bilayer membranes on solid substrates. Additionally, the prior art teaches that an alkane co-solvent such as decane, dodecane, tetradecane or hexadecane is beneficial in

