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(54) Title: SELECTIVELY PERMEABLE MEMBRANES ON POROUS SUBSTRATES

(57) Abstract: A cell-free selectively permeable membrane comprising a porous substrate, a bilayer across pores of the porous substrate, and at least one membrane protein incorporated therein, and capable of mimicking desired biological functioning in the cell-free environment. Methods for forming a selectively permeable membrane comprising forming a bilayer across pores of a porous substrate, and incorporating at least one membrane protein into the bilayer. The inventive membranes are useful in, for example, the manufacture of ion-concentration devices, devices requiring selective uptake mechanisms, fuel cells, toxin sensors, and are particularly useful for structure/function studies, drug screening/selection/design, including high-throughput applications.



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SELECTIVELY PERMEABLE MEMBRANES ON POROUS SUBSTRATES**STATEMENT REGARDING FEDERALLY SPONSORED
RESEARCH OR DEVELOPMENT**

5 This invention was made, at least in part, with funds from the Federal Government, awarded through MURI grant number DAAD 19-02-1-0227 ARMY. The U.S. Government therefore has certain acknowledged rights to the invention.

FIELD OF THE INVENTION

 The present invention relates to selectively permeable membranes formed
10 across pores of a porous substrate. Both sides of the membranes are freely accessible. The invention also relates to methods for forming the selectively permeable membrane across pores of a porous substrate. Additionally, the invention relates to fuel cells, toxin detectors and protective devices comprising the selectively permeable membranes.

15 **BACKGROUND OF THE INVENTION**

 Transport systems for utilizing energy have typically relied on membranes which could not selectively uptake, concentrate or release ions and/or molecules in an organized manner. It would be advantageous to provide and use selectively permeable flexible membrane technology to incorporate membrane proteins suitable
20 for macroscopic and nanoscale preparations. Moreover, it would be advantageous to have a selectively permeable membrane which is essentially free from support material impeding access to at least one side of the membrane. Film-supported selectively permeable membranes have been previously disclosed (Cuppoletti Application No. PCT/US04/27688, the disclosure of which is fully incorporated
25 herein by reference). Thus, there exists a substantial need for an improved membrane transport system that can selectively uptake, concentrate and/or release ions and/or

molecules in an organized manner, and that can be used for macroscopic and/or nanoscale applications.

SUMMARY OF THE INVENTION

Accordingly, it is object of the invention to provide a novel selectively
5 permeable membrane having both sides of the membrane accessible. It is a further
object of the invention to provide methods for forming selectively permeable
membranes across pores of a porous substrates. It is yet a further object of the
invention to provide mechanisms and devices comprising selectively permeable
membranes including, but not limited to, fuel cells, biocides, toxin detectors and
10 protective devices against toxins.

In accordance with one aspect of the invention, a selectively permeable
membrane. The membrane comprises: a bilayer formed across the pores of a porous
substrate; and at least one membrane protein incorporated into the bilayer.

In accordance with another aspect of the invention, there are provided
15 methods for forming the inventive selectively permeable membrane. The methods
comprise forming a bilayer across pores of a porous substrate, and incorporating at
least one membrane protein into the bilayer.

In accordance with yet another aspect of the invention, a fuel cell is provided.
The fuel cell comprises a selectively permeable membrane. The membrane
20 comprises: a bilayer formed across pores of a porous substrate; and at least one
membrane protein incorporated into the bilayer. The membrane protein is capable of
establishing an electrochemical gradient of protons. The invention further provides
reduced dimension fuel cells, and fuel cells capable of variable discrete or continuous
voltage settings, and having the capability of selecting among voltages.

In accordance with yet another aspect of the invention, a toxin detector is provided. The toxin detector comprises a selectively permeable membrane according to the present invention, and means to facilitate detection of a toxin in or near the membrane.

5 In accordance with yet another aspect of the invention, a protective device against toxins is provided. The protective device comprises a selectively permeable membrane and a nonpermanent coating. The membrane is capable of establishing an acid gradient. The coating protects against diffusion of toxins that are not degraded. The protective device may be a fabric article, such as clothing intended for soldiers
10 deployed in situations which may bring them into contact with toxins.

 In accordance with yet another aspect of the invention, a high throughput assay is provided. The high throughput assay is particularly useful for rapidly screening a plurality of compounds for drug selection and design. The high throughput assay comprises a plurality of wells wherein the plurality of wells
15 comprises a selectively permeable membrane according to the present invention.

 The present invention is advantageous for producing a selectively permeable membrane that can utilize energy for the selective uptake, concentration, and/or release of ions and/or molecules in an organized manner. In addition, the selectively permeable membrane is suitable for macroscopic and/or nanoscale preparations.

20 The following detailed description will be more fully understood in view of the drawings comprising Figures 1-4. The Figures are intended to illustrate particular embodiments of the present invention, and should not be construed as limiting the scope of the invention as defined by the claims set forth herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Scanning electron micrographs (A) and functional assays (B) of polycarbonate filters with 3 different pore sizes. Microporous polycarbonate filters with 3, 5 and 8 μm pore sizes were used.

5 **(A)** Illustrates the scanning electron micrographs of the filters at 5000X magnification. The bar indicates 10 μm .

(B) Illustrates summarized resistance measurements across the filters after adding phospholipids (PL) without or with 50 ng gramicidin D (gram D) in the presence of KCl (permeant cation) or NMDGCl (impermeant cation) medium.

10 Medium is 100 mM KCl or NMDGCl with 10 mM HEPES (pH 7.4). PL used were 3:1 POPS:POPE (40 mg/ml). Data is plotted as mean $\pm$ S.E. Number of experiments is indicated in brackets. * $p=0.001$ compared with PL+KCl+gramD

Figure 2. Reconstitution of functional Kv1.5 K^+ channels in phospholipid-coated 50 μm pores in a plastic plate (A, B) and in phospholipid-coated leached PLLA membranes (C). For (A) and (B), medium on both sides of the pore was 100 mM KCl with 20 mM HEPES (pH 7.4) and phospholipids (PL) used were 3:1 POPS: POPE (40 mg/ml). Well area was 0.3 cm^2 .

15 **(A)** Sets forth typical current recordings, I - V curves and a summary of the data of experiments reconstituting membrane vesicles isolated from dexamethasone -
20 induced Kv1.5 expressing Ltk⁻ cells (Kv1.5 vesicles) into phospholipids coating 50 μm pores. Sequential current recordings at different holding potentials from -80 to +70 mV are shown: before and after addition of phospholipids (PL); after addition of Kv1.5 vesicles to the PL and finally after addition of 1 μM compound B, an inhibitor of Kv1.5 K^+ channels. Data in the I - V curves and in the summary plotted as means

$\pm$ S.E., with number of experiments in brackets. * P <0.001 vs. PL alone or PL+Kv1.5 vesicles+compd B.

(B) Illustrates control experiments using membrane vesicles isolated from Lkt⁻ cells transfected with Kv1.5 cDNA, but not induced with dexamethasone and therefore without Kv1.5⁺ channels (-Kv1.5 vesicles); boiled membrane vesicles containing Kv1.5 K⁺ channels (from dexamethasone-induced Lkt⁻ cells) and gramicidin D (gramD) reconstituted into the phospholipid bilayer. Current recordings are shown with and without vesicles or gramicidin D. Data in the summary are plotted as mean $\pm$ S.E., with number of experiments in brackets. * P <0.001 vs. PL alone, PL+vesicles (-Kv1.5 or boiled +Kv1.5).

(C) Resistance was measured of leached PLLA membranes (area 0.3cm²) after adding phospholipids (PL) and after adding Kv1.5 vesicles to the PL and finally after addition of 1 μ M compound B, an inhibitor of Kv1.5 K⁺ channels. Medium was 100 mM KCl with 10mM HEPES (pH 7.4). PL used were 3:1 POPS: POPE (40 mg/ml). Data are plotted as mean $\pm$ S.E. Number of experiments is indicated in brackets. * P <0.001 vs. PL alone, PL+Kv1.5 vesicles or PL+Kv1.5 vesicles+compound B.

Figure. 3. (A) Control experiments showing specificity of compound B as an inhibitor of Kv1.5 K⁺ currents and (B) effect of varying compound B on the Kv1.5-mediated K⁺ current.

(A) shows the effect of 1 μ M compound B on currents @ 70 mV recorded after formation of the phospholipid (PL) bilayer alone (left-hand-side) on polycarbonate filters and after reconstitution of membrane vesicles from uninduced Kv1.5-expressing Lkt⁻ cells (no Kv1.5 K⁺ channels are present) into the PL bilayers shown on the right-hand-side. Data is plotted as mean $\pm$ S.E. with number of experiments in brackets. NA, not applicable, * p <0.001 vs PL alone.

(B) shows a dose-response curve of the effect of increasing amounts of compound B on Kv1.5-mediated K⁺ currents expressed as ΔI @ 70 mV (% maximum). Data is plotted as mean $\pm$ S.E. with number of experiments in brackets. From the sigmoidal plot, IC₅₀ = 170 $\pm$ 40 nM (n=6); χ^2 = 35, p <0.001.

5 **Fig 4. Reconstitution of H⁺ transport through the gastric H/K ATPase in phospholipids coating microporous polycarbonate filters.**

(A) Representative experiment showing acidification of the medium on the *trans*-side of the phospholipid bilayer (3:1 POPS:POPE, 40 mg/ml).coating a polycarbonate filter with 0.4 μ m pores after addition of hog gastric H/K ATPase-containing
10 membrane vesicles to the bilayer followed by 5 mM MgATP and 100 μ g/ml valinomycin to the *cis* side. *Trans* medium contained 10 mM KCl and the *cis* medium contained 100 mM KCl with 10 mM HEPES, pH 7.4. The effect of 100 μ M SCH28080, a specific H/K ATPase inhibitor is also shown.

(B) Rate of acidification measured following reconstitution of hog and rabbit
15 gastric H/K ATPase-containing membrane vesicles into the phospholipid bilayer coating polycarbonate filters. Effect of 100 μ M SCH28080 is also shown. Data is plotted as mean $\pm$ S.E., with number of experiments in brackets.

Additional embodiments, objects and advantages of the invention will become more fully apparent in view of the following detailed description.

20 **DETAILED DESCRIPTION OF THE INVENTION**

Membranes found in biological systems are made of lipids capable of forming a barrier between aqueous compartments. They consist primarily of a continuous double or bilayer of lipid molecules associated with various membrane proteins. Phospholipids, sphingolipids, and glycolipids make up the three major classes of
25 membrane forming lipid molecules. These lipids are amphipathic (amphiphilic)

molecules in that they have a hydrophilic (polar) head and a hydrophobic (non-polar) tail. In the aqueous environment of cells, the polar head groups face toward the water while their hydrophobic tail groups interact with each other to create a lamellar bilayer, and to a lesser extent other aggregate structures depending on the lipid composition and conditions. For example, membrane lipids can form a variety of different shapes including spheres (vesicles), rods (tubes) and lamellae (plates) depending on lipid and water content, and temperature.

Biological membranes consisting of lipid and proteins play a crucial role in almost all cellular phenomena in living cells. The complexity of biological membranes make in vivo utilization of them prohibitive. Hence, cell-free reconstituted membranes were developed and have been known for over three decades. (See, e.g. Tien H.T., Bilayer lipid membrane (BLM) (Marcel Dekker, New York, NY), 1974). However, these are fragile structures and researches struggled to develop forms which provide capability for more rigorous and flexible manipulations. Less fragile supported membranes were developed, but these were limited to having only one side of the membrane accessible. The present invention provides a selectively permeable membrane having both sides of the membrane fully accessible, that is formed across pores of a porous membrane, making it stronger and capable of undergoing rigorous manipulations.

"Membrane protein," as used herein, is intended to include all naturally occurring and engineered proteins which are capable of associating with a lipid bilayer. Examples of membrane proteins include membrane-associated receptors, transporter proteins, enzymes, and immunogens. Proteins can associate with membranes in different ways. Integral membrane proteins contain at least one component that is embedded within the lipid bilayer. The non-polar segments of these

integral membrane proteins, which embed in the lipid bilayer perpendicular to the surface of the membrane, may consist of a hydrophobic region of the polypeptide, a covalently attached fatty acid chain or other types of lipid chains. Peripheral membrane proteins normally associate with the lipid bilayer through non-covalent interactions with these integral membrane proteins. Additionally, some peripheral membrane proteins are located entirely in the aqueous phase, associated with the membrane through a covalently attached fatty acid or lipid chain.

Glycosylphosphatidylinositol anchors, found at the C-terminus of soluble proteins, result in the attachment of these proteins to the membrane surface (Turner, A. J., Essays Biochem. (1994) 28:113-127).

Membrane proteins in biological systems are organized in various structures, leading to different functions on various surfaces and intracellular membranes of cells. Organization of membrane proteins allows for utilization of energy, uptake and concentration of ions and/or molecules across and into cells, and utilization of cell structures needed for life processes. Membrane proteins may be highly regulated. Under some physiological conditions, ion transport proteins capable of moving billions of ions per second can be reversibly silenced and again opened by intracellular regulators, thereby controlling the flow of solute. The exit and entry of ions and/or molecules across biological membranes control important life processes. Some of the most potent toxins (for example blowfish toxins that affect sodium channels, scorpion, snake and marine snail toxins and organophosphates) affect the membrane proteins and thus are highly toxic.

Membrane proteins are present in all living organisms. They are imbedded in lipid bilayer membranes that are otherwise essentially impermeable to water, to all inorganic ions, and are only permeable to small hydrophobic substances, unless the

lipid membranes also contain transport proteins. Transport processes can be primary active (using energy of light or hydrolysis of high energy phosphate compounds, e.g.), secondary active (using gradients produced by primary active transport systems, e.g.), or passive, facilitating the diffusion of substances according to the concentration or electrical gradients. Transport proteins are saturable and exhibit varying degrees of selectivity. In some cases, substrate selectivity can be broad and can be altered by changing the pore structures. Some transport proteins such as sodium channels, are very selective for a single ion, while other membrane proteins such as the multiple drug resistance protein, (MDR) which expels chemotherapeutics and other toxins from cells, are quite versatile in their transport specificity, and are capable of the transport of a number of compounds that are not structurally related. Such transporters, as they exist or after engineering, may be used to transport new materials in a specific manner.

Accordingly, the present inventor has developed a novel selectively permeable membrane having membrane proteins incorporated therein, that can utilize energy for the selective uptake, concentration, and/or release of ions and/or molecules in an organized manner. In one embodiment, the present invention is directed to a novel selectively permeable membrane. The membrane comprises a bilayer formed across pores of a porous substrate, and at least one membrane protein incorporated into the bilayer. Another embodiment provides methods for forming a selectively permeable membrane. The methods comprise forming a bilayer across pores of a porous substrate, and incorporating at least one membrane protein into the bilayer. The present invention is further directed to devices comprising the selectively permeable membrane including, but not limited to, fuel cells, toxin detectors and protective devices against toxins.

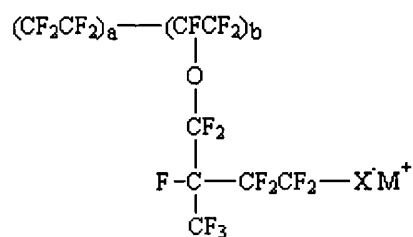
The presently inventive selectively permeable membrane comprises a bilayer formed across pores of a porous substrate. As used herein, "selectively permeable" is intended to refer to a film property which allows some ions and/or molecules to cross the film more easily than other ions and/or molecules. In one embodiment, the selectively permeable membrane is selectively permeable to protons, water, or combinations thereof. The bilayer is formed across the pores such that both sides of the bilayer are accessible. In one embodiment, the bilayer comprises a lipid bilayer.

The porous substrate may be comprised of any porous material suitable for development of a lipid bilayer and reconstitution of membrane proteins. Such materials include polycarbonate filters with manufactured pore diameter sizes of about 0.4 to about 8 μm , and laser-drilled arrays of from about 10 to about 100 μm . The ability to support lipid bilayers is dependent upon pore size. Pore diameter sizes of about 50 μm are capable of supporting stable bilayers for up to four hours. A specific embodiment provides pore sizes of less than about 20 μm . In a further specific embodiment, commercially available millipore micro porous polycarbonate filters of various pore sizes (0.4, 3, 5, and 8 μm) and a multi-well plastic plate with 10-100 μm Lenox-laser drilled holes (one per well), available from Procter & Gamble Pharmaceuticals of Cincinnati, Ohio, are used to form the bilayer.

Without intending to be limiting, other porous materials which are suitable as the porous substrate include perfluorinated polymers such as fluorinated ethylene-propylene (FEP) copolymers including TEFLON®, which is DuPont's trademark for fluoropolymer resins, and the Daikin fluoropolymer resins which are marketed in the United States of America by Sumitomo trademarked as NEOFLON®. Also suitable are FLUON® polymer resins, AGLOFON® polymer resins available from Ausimont, and KEVLAR® resins. NAFION® is a DuPont product which is a complex having

an equilibrium of ionic selectivity as well as transport properties and provides the ability to adapt to specific uses and needs, such as fuel cell operations. It is a perfluorinated polymer that contains small proportions of sulfonic or carboxylic ionic functional groups. Its general chemical structure can be seen below, where X is either

5 a sulfonic or carboxylic functional group and M is either a metal cation in the neutralized form or an H⁺ in the acid form.



NAFION[®] Perfluorinated Ionomer

A typical fluorinated polymer useful as the porous substrate according to the present invention has the following properties: thickness in the range of about 1-100 microns, weight per unit area of about 5-80 g/m²; density of about 1.3 to 2.20 g/cm³; and a break strength of about 0.2-20 kg/cm.

A bilayer is formed across the pores of the porous substrate. As used herein, "bilayer" is intended to refer to at least two layers of a lipid. The layers include, but not limited to, a biological lipid, a synthetic lipid, or combinations thereof. In one embodiment the bilayer is formed from phospholipids. The lipid bilayer is capable of forming a selectively permeable barrier between aqueous compartments.

The selectively permeable film supported membrane further comprises at least one membrane protein incorporated into the bilayer. "Membrane protein," as used herein, includes all naturally occurring and engineered proteins which are capable of associating with a lipid bilayer. Examples of membrane proteins include membrane-associated receptors, transporter proteins, enzymes, and immunogens. These

membrane proteins can utilize energy for the selective uptake, concentration, and/or release of ions and/or molecules in an organized manner. As used herein, "transport protein" is intended to refer to a membrane protein which permits the passage of certain ions and/or molecules, but not others, into the bilayer of the selectively permeable membrane. The membrane protein includes, but is not limited to, native protein, recombinant protein or combinations thereof. In one embodiment, the bilayer formed across the pores of a porous substrate may be referred to as a macroscopic membrane, while the membrane protein may be referred to as a nanostructure. A nanostructure is has domains of typically less than about 100 nanometers in size. In a further embodiment, the membrane proteins exhibit vectorial transport function.

The membrane proteins of the present invention retain at least some of their biological function after they have been incorporated into the bilayer. After formation of the selectively permeable membrane, the membrane is tested for the presence of desirable biological functioning. One means of verifying bilayer formation is by reconstituting the channel forming polypeptide, gramicidin-D, in the bilayer. This allows ion conduction through the lipid if the lipid is a single bilayer thick. Electrical resistance is monitored and gramicidin reduces the resistance of the membrane. Two non-limiting examples of functionality testing of incorporated membrane proteins include: (1) determination of functional Dv1.5 potassium channel incorporation by noting increased current across the bilayer upon treatment with vesicles containing Dv1.5 potassium channels, and a decrease in current upon addition of compound B, a specific inhibitor of Kv1.5 potassium channels; (2) determination of a functional gastric proton pump and associate protein by an assay based on the development of ATP-driven acidification across membranes containing the functional complexes, and

loss of acidification when the membranes are treated with a specific inhibitor of the proton pump, SCH28080.

In accordance with the present invention, one skilled in the art would recognize the various methods suitable for incorporating at least one membrane protein into the bilayer. In one embodiment, the membrane protein may be spontaneously inserted into the bilayer. In another embodiment, the membrane protein may be incorporated into the bilayer by a lipid vesicle, a detergent solution, or combinations thereof. The method of incorporating at least one membrane protein into the bilayer may be fully or partially conducted, or automated, by robotics.

In one embodiment, the membrane protein comprises the gastric HCl transport system which includes the gastric H/K ATPase, a K^+ channel, and a Cl^- channel protein. The K^+ Channel and Cl^- channel are regulated by changes in pH, voltage, and ion concentrations, and are further regulated by covalent modifiers. The HCl formed by this membrane protein may be used as a general biocide for killing bacteria, fungi, and viruses, a physiological function of HCl in the digestive tract. The HCl may also be used for inactivation of peptides, proteins, and acid sensitive organic compounds. "Inactivate," as used herein, includes the range from where the normal biological effect of the organic compound is diminished, to the point where it is completely eradicated, as determined from the perspective of a subject experiencing the biological effect.

Since each H^+ produced results in an equivalent of OH^- from the splitting of water, production of base on the opposite side of the membrane provides equally useful chemical secretions. HCl may be used for the concentrative uptake of weak bases (having a pH of greater than about 8 and less than about 10) such as tributylamines (a stabilizer of the neurotoxin, sarin) to high levels for the purposes of

detection of the toxin. In the case of weak bases such as tributyl amine, the un-ionized weak base passively crosses the lipid membrane, and becomes protonated. The protonated weak base accumulates, since the charged compound cannot cross the membrane. Similarly, weak acids (having a pH of greater than about 4 and less than
5 about 6) such as dinitrophenol, picric acid and trinitrotoluene may be detected with such membranes based on their ability, acting as protonophores, to collapse HCl gradients.

Moreover, chemical and electrical gradients may be inter-converted according to chemiosmotic hypothesis. Accumulation of any substance may be accomplished at
10 the expense of a gradient, as long as an appropriate membrane protein that recognizes the electrical or chemical gradient and the ion and/or molecule in question is available. Thus, HCl gradients may be used for the accumulation of another ion and/or molecule as long as another membrane protein may be identified or engineered to respond to the electrical or chemical gradients in exchange for the ion and/or
15 molecule in question.

In another embodiment of the present invention, the membrane protein may comprise ion channels and transporters involved in maintaining homeostasis. These membrane proteins include, but are not limited to, those operable to establish H, K, Na, and Ca^{2+} gradients. These gradients may be inter-converted by a variety of
20 techniques, including, but not limited to, the use of synthetic and natural exchange ionophores such as nigericin (a K/H exchanger), monensin (a Na/H exchanger), ionomycin (a Ca^{2+} /H exchanger) or a combination of FCCP (an electrogenic protonophores) with an electrogenic ionophore such as valinomycin.

When HCl transport is successfully incorporated into a selectively permeable
25 membrane, it follows that Na, K, and Ca^{2+} gradients may also be generated using

other primary active membrane proteins. The HCl transport system is not unique in producing large gradients. A close relative of the H/K ATPase is the Na/K ATPase (64% amino acid homology). The Na/K ATPase accomplishes the net electrogenic movement of 3 Na for 2 K. With other appropriate transporters, it can accomplish the production of charge and sodium gradients that can also be employed by other membrane proteins for the selective concentration (or release) of other ions and/or molecules. The Na/K ATPase is also available in large quantities and has been reconstituted into a variety of systems, including solid supported membranes. An example of sodium dependent secondary active transport proteins in nature include the sodium dependent glucose transporter that is responsible for the concentrative uptake of glucose from the intestine using the electrochemical gradient produced by the Na/K ATPase. Glucose transporters present on the basolateral membrane of the intestinal cell facilitate the downhill transport of glucose into the blood.

These and other membrane proteins, which are regulated by pH, ionic conditions, membrane voltage, and/or intracellular second messengers, may be genetically engineered to function in the environment of the presently inventive membrane when regulatory elements are missing, or when the substance would react with environmental agents, using techniques within the ability of those of ordinary skill in the art, in view of the present disclosure.

The present invention is also directed toward methods for forming a selectively permeable membrane. The methods comprise forming a bilayer across pores of a porous substrate, and incorporating at least one membrane protein into the bilayer. The membrane is accessible from both outer sides of the bilayer. One skilled in the art will appreciate the various known techniques for forming a lipid bilayer, any of which may be used herein.

One embodiment of the present invention provides a fuel cell. The permeability of substrates comprising NAFION® polymer resins to ions is well-documented and NAFION films are a common component of fuel cells. (See, for example, PCT Patent Application Serial No. WO 2005/022136 A1, the entire disclosure of which is incorporated herein by reference.) It has been previously disclosed that the application of phospholipid to one side of a NAFION® film substrate prevents back-flux and affords a superior ion concentrative means. It is also known that application of phospholipid to both surfaces of a NAFION film substrate substantially eliminates flux. Incorporation of ionophores into the phospholipid bilayer re-establishes flux. Similarly to the phospholipid-coated NAFION, a membrane according to the present invention, wherein the bilayer is a phospholipid bilayer, is substantially impermeable to ions. Upon incorporation of membrane proteins comprising ionophores into the phospholipid bilayer, ion flow is established and flux across the membrane may be measured. The present inventor surprisingly discovered that a membrane according to the present invention, comprising a phospholipid bilayer having gramicidin D incorporated into the bilayer as an ionophore, exhibits a higher flux than the NAFION® substrate alone. Gramicidin D has a unit pore size, on the average, of approximately 10 Å, and a flux of 10^8 ions per second, per gramicidin D molecule is observed across the inventive membrane. Hence, a superior ion flow may be realized across an extremely small membrane surface area by employment of the presently inventive membrane. The present invention therefore provides fuel cells capable of having significantly reduced dimensions when compared to currently available fuel cells, controlling for voltage potential.

One embodiment of the present invention provides a fuel cell comprising a selectively permeable membrane, the membrane comprising a bilayer formed across

pores of a porous substrate, and having at least one membrane protein incorporated into the bilayer. Additionally, any fuel cell source for the production of protons may be used. Uniquely, the inventive membrane serves a dual function. In addition to permitting a high rate of ion flow, it also provides a barrier to prevent the flow of ions unless a functional ion permeable membrane protein is present and activated by an appropriate driving force. The functional membrane protein permits the flow of ions with activation by an appropriate driving force. The membrane protein is capable of operating to establish an electrochemical gradient of protons. The electrochemical gradient may be continuous or discrete. In a further embodiment, the electrochemical gradient of protons is established at ambient temperatures. In a more specific embodiment, a continuous production of hydrogen ions is obtained and serves as a primary source of protons for a fuel cell.

In one specific embodiment of the fuel cell, the bilayer comprises a phospholipid bilayer, and the at least one membrane protein comprises at least one ionophore. The ionophore permits a flow of ions through the phospholipid bilayer. In a very specific embodiment, the ionophore comprises gramicidin D. The flow of ions may be controlled by a regulator of the functioning of the membrane protein, including, but not limited to, manipulation of pH, voltage, ion concentration, genetic modification, and chemical modification. Chemical modifiers may be associated with the membrane and may covalently or noncovalently modify the ionophore. The modification may silence the ionophore, or reduce/enhance ion flow through the ionophore by varying degrees. Chemical modifiers may be associated to the membrane in particular areas, ratios or other deliberate groupings, permitting selective modification and control of the ion flow and voltage. Strategically placed and triggered, such modifiers permit pre-selection of voltage across a membrane,

providing fuel cells having the capability of producing variable pre-selected discrete voltages, and/or a continuum of increasing or decreasing voltage within a pre-selected range.

A further embodiment of the present invention provides toxin detectors. A
5 toxin detector, according to the invention, comprises a selectively permeable
membrane and means to facilitate detection of a toxin in or near the membrane. One
skilled in the art will appreciate the various means for detecting a toxin in or near the
membrane, which are known in the art and suitable for use herein. In one
embodiment, the means to facilitate detection of a toxin includes, but is not limited to,
10 antibodies, peptides, enzymes, or combinations thereof that can recognize molecular
elements of an ion and/or molecule. In another embodiment, molecularly imprinted
polymers may be used as a means to facilitate detection of a toxin. In yet another
embodiment, membrane proteins may be modified in accordance with a particular ion
and/or molecule, for example, an organophosphate. Other techniques to facilitate
15 detection of a toxin include, but are not limited to, molecular imprinting, sensitized
lanthanide luminescence, and membrane bound acetyl cholinesterase.

The invention is further directed toward protective devices against toxins. The
device comprises a selectively permeable membrane according to the present
invention, and a nonpermanent coating. The membrane is capable of operating to
20 establish an acid gradient. The nonpermanent coating protects against diffusion of
toxins that are not degraded. The protective device may further comprise at least one
catalyst facilitating transport. One skilled in the art will appreciate various catalysts
which are known in the art for facilitating transport and suitable for use herein. It is
apparent to one of ordinary skill in the art that the protective device may be employed
25 to protect various materials. In one embodiment, the material may be a surface of a

living or non-living object. In a specific embodiment, the protective device comprises, at least in part, a fabric, such as clothing, and provides protection to the wearer. In a more specific embodiment, the clothing comprises a warfare related article.

5 One skilled in the art will appreciate the various known chemical agents that may be released by activation of membrane proteins that respond to electrical/chemical gradients when appropriately constituted into a selectively permeable membrane according to the present invention, in view of the present specification. In one embodiment, the chemical agent is an acid. In another
10 embodiment, the chemical agent comprises glutathione, cysteine, S-330, or combinations thereof. Specifically, these chemical agents are known to protect cells against chemical warfare agents such as mustard gas and the like. Moreover, sodium-dependent and independent cysteine transporters and glutathione transporters suggest that a native or engineered protein (such as MDR variants or engineered channels) can
15 facilitate the transport of S-330, or other substances, regardless of their chemical properties.

 Another specific embodiment of the invention provides provides a novel high throughput assay comprising a plurality of wells wherein each of the wells comprises at least one pore. A selectively permeable membrane comprising a bilayer having at
20 least one membrane protein incorporated therein is formed across the pores. The membranes mimic a desired biological function and a base level of that function is determined. Rapid screening and selection of compounds according to how they alter the base level of the biological function is possible. This assay has particular utility in drug screening and selection and the development of desired pharmaceutical agents.

Commercially available well arrays and well-known techniques in the art make this assay adaptable for automation and robotic applications.

The following example illustrates specific embodiments of the present invention and is not intended to limit the invention as defined by the claims herein.

- 5 Additional embodiments and variations within the scope of the claimed invention will be apparent to those of ordinary skill in the art in view of the present disclosure.

Example

- This example illustrates an embodiment of bilayer formation, membrane transport reconstitution and testing for functionality. The bilayer is formed over pores
10 of a polycarbonate substrate comprising naturally present pores or drilled holes. Millipore micro porous polycarbonates of various pore sizes (0.4, 3, 5, and 8 μm , e.g.) are commercially available, and a multi-well porous plastic plate with 10 - 100 μm Lenox-laser drilled holes (1/well) is available from Procter & Gamble Co. and is used for stable bilayer formation. Techniques for bilayer preparation are known by those
15 of ordinary skill in the art. In one embodiment 5 μl phospholipid is added in a 3:1 POPS:POPE 40 mg/ml in decane in plastic wells with 50 μm pores. After 30 minutes, 100 μl of 100mM KCl is added to each well. The well tray is placed into a holder filled with 100 mM DCl so that the entire bottom of the tray is covered, with no buffer or other components. The bilayer is verified according to the gramicidin-D (a
20 cation selective antibiotic) method wherein electrical resistance is monitored to observe the effect of gramicidin in reducing the resistance of the membrane. Current flow is measured by placing one electrode in the desired well and one in the surrounding liquid outside the well. The well tray is removed from the holder and placed in separate dry holder. 85 μl of the 100 mM KCl is removed from each well,
25 being careful not to disturb the bilayer. The verified bilayer is incubated with vesicles

comprising H/K ATPase or Kv1.5 potassium channels. 3 μ l of vesicles is added to each well which was determined to have a verified bilayer. Spontaneous fusion with the bilayer is allowed to proceed for 30 minutes to one hour, at which point H/K ATPase induced acidification or potassium ion currents across the bilayer membrane are measured. A desired amount of 100mM KCl is added to the well (for example, add 90 μ l of 100 mM KCl for 1000nM, add 92.5 μ l of 100 MM KCl for 750 nM). The well tray is placed back into the holder with the 100mM KCl and current flow is measured as before. Add a desired amount of a suitable 10 μ M inhibitor to achieve a final desired concentration with the final volume of 100 μ l. When the gastric proton pump H/K ATPase is reconstituted on the bilayer over the pores, ATP-dependent transport of acid across the bilayer is observed that can be inhibited by 100 μ M SCH-28080 (a specific inhibitor of the H/K ATPase). The current is measured as described earlier, after one hour. This demonstrates functional reconstitution of the gastric proton pump, H/K ATPase. When infused with vesicles comprising Kv1.5 potassium channels, the membrane exhibits potassium ion currents which are inhibited by 2-(3,4-dimethylphenyl)-3-[2-(4-methoxyphenyl)ethyl]-thiazolidin-4-one, a specific inhibitor of Kv1.5 channels. Ion currents are not present when the vesicles lack Kv1.5 or when Kv1.5 vesicles are boiled.

Claims:

1. A selectively permeable membrane comprising:
 - (a) a bilayer formed across pores of a porous substrate; and
 - (b) at least one membrane protein incorporated into the bilayer.
2. The selectively permeable membrane according to claim 1, wherein the porous substrate permits formation of a lipid bilayer and reconstitution of the membrane protein therein.
3. The selectively permeable membrane according to claim 1, wherein the pores are naturally occurring in the substrate or manufactured in the substrate, or combinations thereof.
4. The selectively permeable membrane according to claim 1, wherein the pores have average pore diameter sizes of less than about 50 μm .
5. The selectively permeable membrane according to claim 4, wherein the pores have average pore diameter sizes of less than about 20 μm .
6. The selectively permeable membrane according to claim 4, wherein the pores have pore diameter sizes of from about 0.4 μm to about 8 μm .
7. The selectively permeable membrane according to claim 4, wherein the pores comprise pore diameter sizes of 0.4 μm , 3 μm , 5 μm , 8 μm , and combinations thereof.

8. The selectively permeable membrane according to claim 1, wherein the porous substrate comprises laser-drilled arrays of from about 10 μm to about 100 μm .
9. The selectively permeable membrane according to claim 1, wherein the porous substrate comprises a multi-well plastic plate, wherein each well comprises at least one laser-drilled hole having a diameter of from about 10 μm to about 100 μm .
10. The selectively permeable membrane according to claim 1, wherein the porous substrate comprises a polycarbonate.
11. The selectively permeable membrane according to claim 1, wherein the porous substrate comprises perfluorinated polymer.
12. The selectively permeable membrane according to claim 11, wherein the perfluorinated polymer comprises fluorinated ethylene-propylene (FEP) copolymer.
13. The selectively permeable membrane according to claim 1, wherein the porous substrate comprises a polytetrafluoroethylene-based perfluorosulfonate inomer.

14. The selectively permeable membrane according to claim 1, wherein the bilayer comprises at least two lipid layers.
15. The selectively permeable membrane according to claim 14, wherein the at least two lipid layers comprise a biological lipid, a synthetic lipid, or combinations thereof.
16. The selectively permeable membrane according to claim 1, wherein the bilayer is formed from phospholipid.
17. The selectively permeable membrane according to claim 1, wherein the membrane protein comprises native protein, recombinant protein or combinations thereof, and wherein the membrane protein is capable of associating with a lipid bilayer.
18. The selectively permeable membrane according to claim 1, wherein the membrane protein comprises: receptor protein; transporter protein; ion channel protein; enzyme; immunogen; ionophore; or a combination thereof.
19. The selectively permeable membrane according to claim 1, wherein the at least one membrane protein comprises an exchange ionophore.
20. The selectively permeable membrane according to claim 19, wherein the ionophore is selected from the group consisting of nigericin, monensin, ionomycin, valinomycin, alamethicin, gramicidin D, and combinations thereof.

21. The selectively permeable membrane according to claim 20, wherein the ionophore comprises gramicidin D.
22. The selectively permeable membrane according to claim 1, wherein the membrane protein is a nanostructure and the bilayer comprises a macroscopic membrane.
23. The selectively permeable membrane according to claim 1, wherein the membrane protein is operable to exhibit a vectorial transport function.
24. The selectively permeable membrane according to claim 1, wherein the membrane protein comprises an ion channel or a transport system or a combination thereof.
25. The selectively permeable membrane according to claim 1, wherein the membrane protein is capable of establishing a gradient selected from a H^+ , K^+ , Na^+ , or Ca^{2+} gradient.
26. The selectively permeable membrane according to claim 1, wherein the membrane protein comprises a gastric HCl transport system, the system comprising a gastric H/K ATPase, a K^+ channel, and a Cl^- channel protein.
27. The selectively permeable membrane according to claim 1, wherein the membrane protein comprises a Na/K transport system, the system comprising Na/K ATPase.

28. The selectively permeable membrane according to claim 1, wherein the membrane protein is operable to generate HCl.
29. A method of inactivating an acid-sensitive organic compound, the method comprising: employing the selectively permeable membrane according to claim 28.
30. The method of inactivating an acid-sensitive organic compound according to claim 29, wherein the acid-sensitive organic compound comprises peptide and/or protein.
31. A biocide comprising the selectively permeable membrane according to claim 28.
32. A detoxin comprising the selectively permeable membrane according to claim 28.
33. A method for detecting a weak base, the method comprising: employing the selectively permeable membrane according to claim 28, wherein the HCl establishes a gradient permitting concentration of the weak base; and detecting the concentrated weak base.

34. A method for detecting a toxin comprising: detecting a weak base according to the method of claim 33, wherein the weak base is associated with presence of a toxin.
35. The method for detecting a toxin according to claim 34, wherein the toxin comprises sarin and the weak base comprises tributyl amine.
36. A method for detecting a weak acid, the method comprising: employing the selectively permeable membrane according to claim 28, wherein the HCl establishes a gradient and detecting the weak acid by observing at least a partial collapse of the gradient.
37. The method for detecting a weak acid according to claim 36, wherein the weak acid comprises dinitrophenol, picric acid or trinitrotoluene.
38. A fuel cell comprising the selectively permeable membrane according to claim 1, wherein the membrane protein is operable to establish an electrochemical gradient of protons.
39. The fuel cell according to claim 38 wherein, the electrochemical gradient of protein cells is continuous.
40. The fuel cell according to claim 39, wherein the bilayer comprises a phospholipid bilayer and the membrane protein comprises at least one

ionophore, wherein the ionophore permits a flow of ions through the phospholipid bilayer.

41. The fuel cell according to claim 40, wherein the at least one ionophore comprises gramicidin D.
42. The fuel cell according to claim 41, further comprising a regulator capable of regulating flow of ions through the phospholipid bilayer.
43. The fuel cell according to claim 42, wherein the regulator comprises a chemical modifier of the ionophore, and further wherein the chemical modifier comprises a covalent and/or a noncovalent modifier.
44. A protective device conferring protection against toxins, the device comprising the selectively permeable membrane according to claim 1, and a nonpermanent coating.
45. The protective device according to claim 44, further comprising at least one catalyst.
46. The protective device according to claim 44, wherein at least a portion of the device is formed from fabric.

47. A high throughput screening assay comprising a plurality of wells, wherein each of the wells comprises the selectively permeable membrane according to claim 1.
48. The high through put screening assay according to claim 47, wherein the assay is at least partially automated by robotics.
49. A method of forming a selectively permeable membrane, the method comprising:
- (a) forming a bilayer across pores of a porous substrate; and
 - (b) incorporating at least one membrane protein into the bilayer.
50. The method of forming a selectively permeable membrane according to claim 49, wherein the at least one membrane protein is incorporated into the bilayer by spontaneous insertion.
51. The method of forming a selectively permeable membrane according to claim 49, wherein the at least one membrane protein is incorporated into the bilayer by utilization of a lipid vesicle, a detergent solution, or combinations thereof.
52. The method of forming an selectively permeable membrane according to claim 49, wherein the method is fully or partially conducted by robotics.

53. A method of verifying formation of the selectively permeable membrane as recited in claim 1, the method comprising: reconstituting gramicidin D in the bilayer and measuring ion conduction through the bilayer.

Formation of Bilayers on Polycarbonate

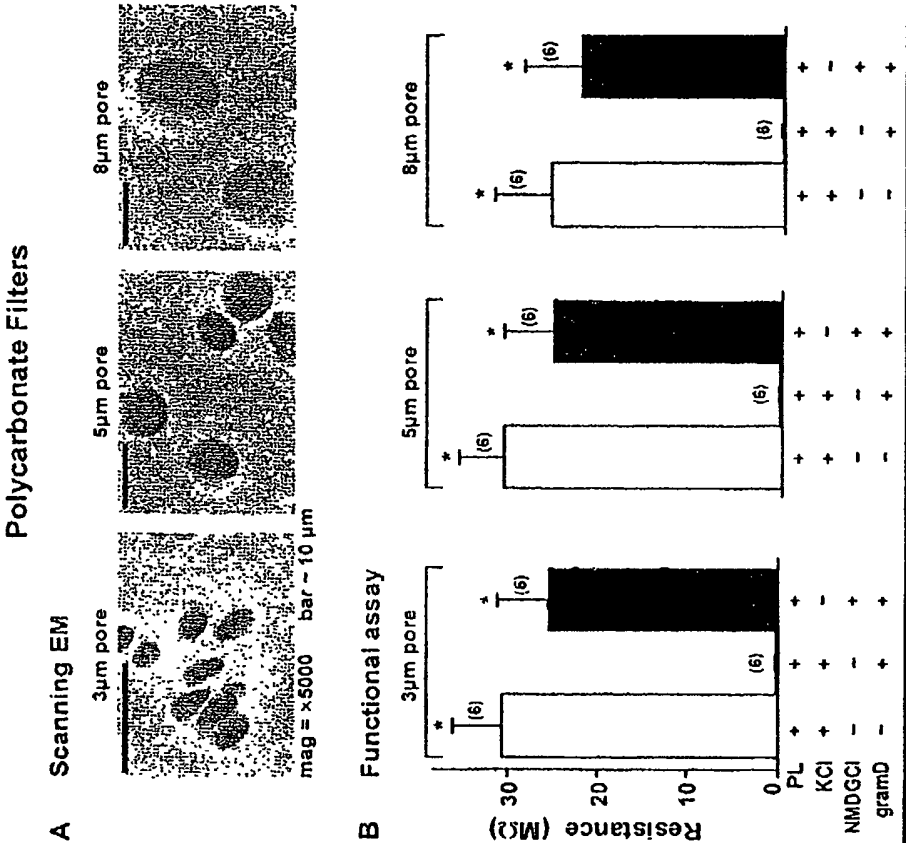


Fig. 1

Functional Reconstitution of Kv1.5 Potassium Channels

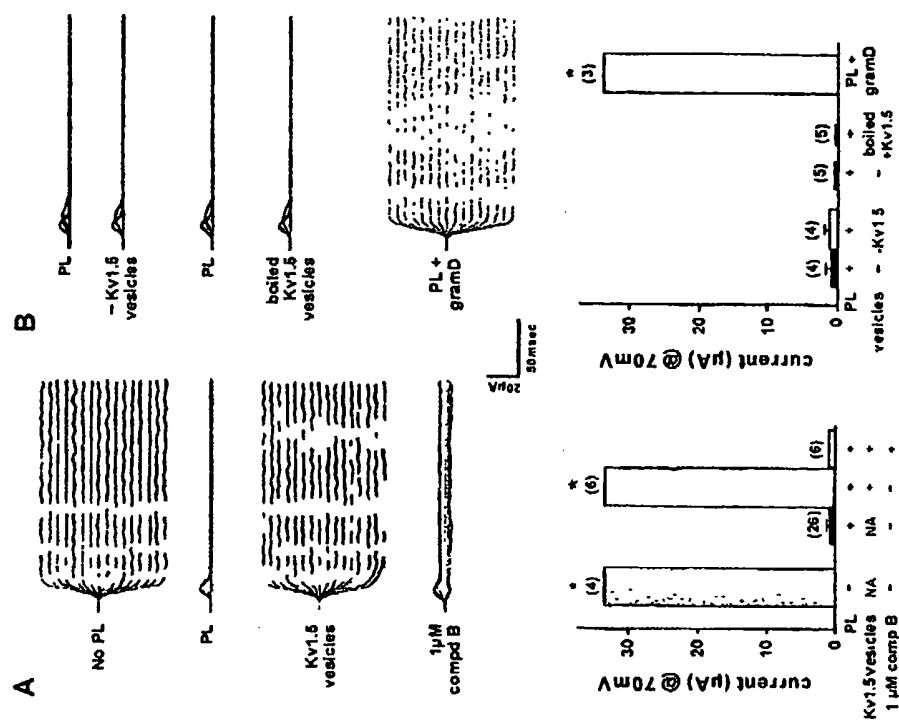


Fig. 2

Inhibition of Kv 1.5 Ion Channels By a Selective Inhibitor, Compound B

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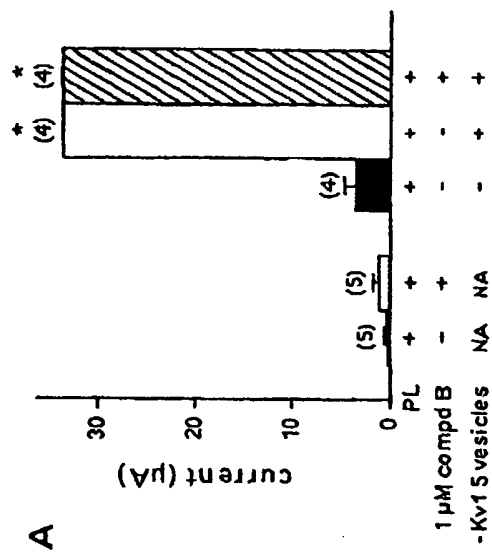


Fig. 3

Dose Dependent Inhibition of Reconstituted Kv 1.5 Ion Channels

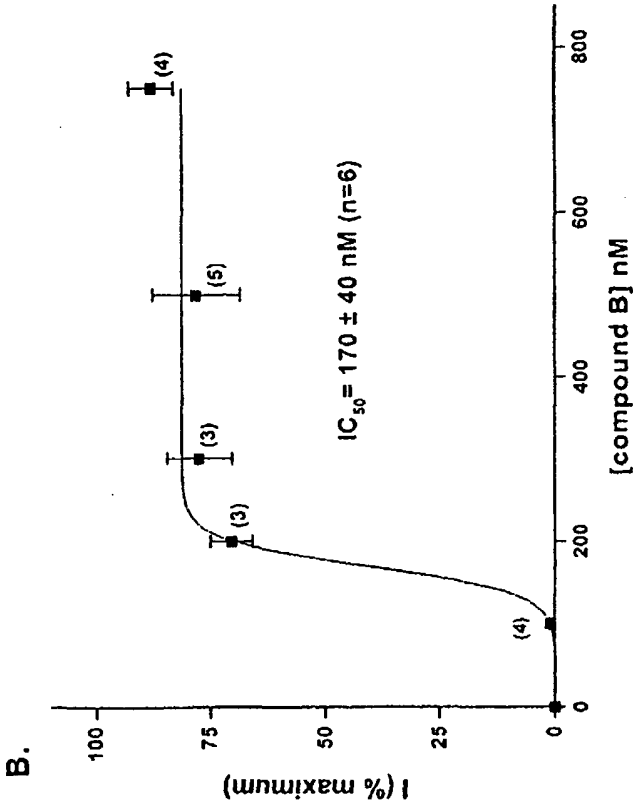


Fig. 4