



(51) International Patent Classification:

B01D 61/00 (2006.01) *B01D 69/00* (2006.01)
B01D 63/00 (2006.01) *B01D 71/00* (2006.01)
B01D 65/00 (2006.01) *B01D 71/06* (2006.01)
B01D 67/00 (2006.01) *C02F 1/44* (2006.01)

(21) International Application Number:

PCT/SG2012/000186

(22) International Filing Date:

25 May 2012 (25.05.2012)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

201103837-9 26 May 2011 (26.05.2011) SG

(71) Applicant (for all designated States except US): **NATIONAL UNIVERSITY OF SINGAPORE** [SG/SG]; 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **WANG, Honglei** [CN/SG]; c/o National University of Singapore, Faculty of Engineering, Department of Chemical and Biomolecular Engineering, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **CHUNG, Tai-Shung Neal** [US/SG]; c/o National University of Singapore, Faculty of Engineering, Department of Chemical and Biomolecular Engineering, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **TONG, Yen-Wah** [MY/SG]; c/o National University of Singapore, Faculty of Engineering, Department of Chemical and Biomolecular Engineering, 21 Lower Kent Ridge Road, Singapore 119077 (SG).

[Continued on next page]

(54) Title: PORE-SPANNING BIOMIMETIC MEMBRANES EMBEDDED WITH AQUAPORIN

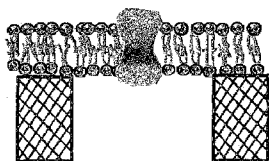


Figure 1(a)

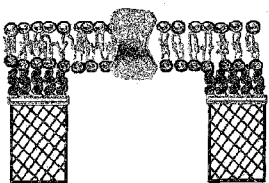


Figure 1(b)

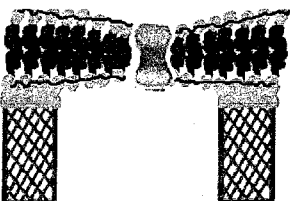


Figure 1(c)

(57) Abstract: Disclosed herein is a biomimetic membrane and its supporting substrate. The structure has a porous substrate, a metal coating on the surface of the porous substrate, an intermediary layer on the surface of the metal coating, and a thin film having a transmembrane protein is incorporated therein. Also disclosed is a method of making the membrane.





pore 119077 (SG). **HONG, Minghui** [SG/SG]; c/o National University of Singapore, Faculty of Engineering, Department of Electrical and Computer Engineering, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **CHEN, Zaichun** [CN/SG]; c/o National University of Singapore, Faculty of Engineering, Department of Electrical and Computer Engineering, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **JEYASEELAN, Kandiah** [LK/SG]; c/o National University of Singapore, Yong Loo Lin School of Medicine, Department of Biochemistry, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **ARMUGAM, Arunmozhiarasi** [MY/SG]; c/o National University of Singapore, Yong Loo Lin School of Medicine, Department of Biochemistry, 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(74) **Agent:** **AMICA LAW LLC**; 30 Raffles Place, #18-03/04 Chevron House, Singapore 048622 (SG).

(81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,

HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

- 1 -

PORE-SPANNING BIOMIMETIC MEMBRANES
EMBEDDED WITH AQUAPORIN

RELATED APPLICATION

This application claims the benefit of Singapore Provisional Application No. 201103837-9, filed on May 26, 2011. The entire teachings of the above application are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Forward osmosis (FO) has shown great potential in many water treatment applications, such as industrial waste water treatment, landfill leachate concentration, liquid food concentration, and wastewater reclamation in space. As the osmotic pressure is the only driving force in the forward osmosis process, high water permeability and water selectivity are the two most important criteria for choosing membrane materials.

Biomimetic membranes demonstrate the greatest potential for improving forward osmosis performance, and it is estimated that biomimetic membranes can have a water permeability far superior to current state-of-the-art forward osmosis polymer membranes. However, such membranes can lack the mechanical strength and stability necessary in commercial applications. The performance of a biomimetic membrane is highly dependent on the integrity of the selective layer and the density of the water channel inside the membrane. Key hurdles in fabricating planar biomimetic membranes are (1) defect formation owing to the thin and fragile self-assembled bilayer; and (2) low coverage of the porous substrate because of weak interface interaction between the selective layer and the substrate.

Thus, there is a need to incorporate aquaporin into a matrix with a desired configuration that permits water to pass through and to assemble ultrathin biomimetic membranes into useful forms with improved mechanic strength and stability.

SUMMARY OF THE INVENTION

Disclosed herein is a biomimetic membrane and its supporting substrate. The structure has a porous substrate, a metal coating on the surface of the porous substrate, an intermediary layer on the surface of the metal coating, and a thin film having a transmembrane protein is incorporated therein. The thin film is layered on top of the intermediary layer and spans one or more pores of the substrate. The transmembrane protein can be an aquaporin, such as AqpZ. The porous substrate can be alumina, polycarbonate, or other polymeric membranes prepared from phase inversion, for example cellulose acetate or sulfonated polyethersulfone or polyacrylonitrile. The metal coating on the porous substrate can be gold, silver, platinum, palladium, or any combination thereof. The intermediary layer can be polyethylene glycol, can have methacrylate groups, or can be functionalized with a photoreactive crosslinker, such as the acrylic acid derivatives methylacrylic acid, ethyl acrylate, methyl methacrylate, or combinations thereof. The thin film can be 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) or a block copolymer, such as poly(2-methyloxazoline)-*block*-poly(dimethylsiloxane)-*block*-poly(2-methyloxazoline), which is more commonly referred to as PMOXA-PDMS-PMOXA. The block copolymer can be PMOXA₁₂-PDMS₅₄-PMOXA₁₂. The block copolymer can be less than 20 nm thick. The block copolymer can have a molecular weight of the hydrophilic block from 500 to 2500 and a molecular weight of the hydrophobic block from 2500 to 10,000. The hydrophilic to hydrophobic weight ratio can be from 1:4 to 2:3. The block copolymer can be PMOXA-PDMS-PMOXA (500-2500-500), PMOXA-PDMS-PMOXA (1000-4000-1000), PMOXA-PDMS-PMOXA (1300-5000-1300), PMOXA-PDMS-PMOXA (1600-5600-1600), or higher.

Figures 1(b) and 1(c) illustrate a schematic representation of a biomimetic membrane on a porous substrate. In Figure 1(b), the biomimetic membrane is formed on a carboxylated polyethylene glycol (PEG) cushion chemisorbed on a gold layer that was deposited on a porous alumina. In Figure 1(c), the biomimetic membrane is formed on a methacrylate cushion chemisorbed on a gold layer that was deposited on polycarbonate.

Disclosed herein is a method for preparing a biomimetic membrane and its supporting substrate. The method includes incorporating a transmembrane protein into vesicles, such as by rehydrating a dry film of phospholipids or block copolymer in a suitable buffer and adding a solution containing the transmembrane protein in a detergent. After the detergent is removed, the vesicles are formed. The multilamellar vesicle solution can then be extruded using a polycarbonate membrane to control the vesicle size. The vesicles are then fused onto the surface of a porous substrate having a metal coating and an intermediary layer.

To produce a metal-coated substrate, a thin layer of metal is coated on the substrate by physical vapor deposition. The intermediary layer can be chemisorbed onto the metal-coated substrate, for example by incubating the metal-coated substrate with a solution that forms the intermediary layer. The intermediary layer can be polyethylene glycol (PEG), preferably Carboxyl-PEG-SH. Alternatively, or in combination, the substrate surface can be functionalized, such as by chemisorption of an intermediary layer having a photoreactive crosslinker.

The vesicles are fused to the thin film by incubating them together or by crosslinking them together, such as by exposing them to UV light. Optionally, the vesicles can be fused in the presence of a gentle vacuum from approximately 500 mbar to approximately 970 mbar. Optionally, the intermediary layer can be crosslinked to the thin film in order to rupture the vesicles and obtain a pore-spanning membrane.

Disclosed herein is a method of using the biomimetic membrane and supporting structure to perform osmosis. The structure is placed between two solutions of differing osmolarity.

The methods disclosed herein can be used to form structures having improved mechanical properties. Without wishing to be bound to any particular theory, it is believed that by providing an intermediate layer, a greater number of transmembrane aquaporin channels can be incorporated into the thin film. The aquaporin extends slightly beyond the lipid bilayer because the hydrophilic portions are larger than the hydrophilic heads of lipids. Without an intermediate layer, the hydrophilic portions of the aquaporin directly contact the solid support. It is

believed that the inclusion of an intermediate layer acts as a spacer to prevent the aquaporin channels from directly contacting the porous substrate.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing will be apparent from the following more particular
5 description of example embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating embodiments of the present invention.

Figure 1(a) is a schematic representation of a biomimetic membrane formed
10 directly on a porous alumina support.

Figure 1(b) is a schematic representation of a biomimetic membrane formed on a carboxylated PEG cushion chemisorbed on a gold layer that was deposited on a porous alumina support.

Figure 1(c) is a schematic representation of a biomimetic membrane formed
15 on a methacrylate cushion chemisorbed on a gold layer that was deposited on a porous polycarbonate support.

Figures 2(a) and 2(b) are graphs showing the kinetics of water permeability through vesicles in a stopped-flow apparatus.

Figure 3 is a graph showing the effect of increasing AqpZ content on the
20 permeability of DMPC vesicles.

Figures 4(a) and 4(b) are Field Emission Scanning Electron Microscope (FESEM) images of empty alumina substrates (a) before gold coating and (b) after gold coating.

Figures 4(c) and 4(d) are atomic force microscopy (AFM) images of empty
25 substrate : (c) plain porous alumina, (d) porous alumina coated with a 60nm gold layer.

Figures 4(e) and 4(f) are atomic force microscopy (AFM) images of (e) the DMPC membrane of the design of Figure 1(a) and (f) the DPMC membrane of Figure 1(b).

Figure 5 is a force indentation curve for a pore-spanning biomimetic membrane of Figure 1(a) prepared from DMPC-AqpZ vesicles having a lipid protein ratio (LPR) of 2000:1.

5 Figures 6(a) and 6(b) are histograms of (a) rupture forces and (b) indentation depths for the DMPC membrane in Figure 1(a).

Figures 6(c) and 6(d) are histograms of (c) rupture forces and (d) indentation depths for the DMPC membrane in Figure 1(b).

Figure 7(a) is an illustration of a scheme for preparing a pore-spanning membrane having an embedded aquaporin.

10 Figure 7(b) is an illustration of a scheme for modifying the surface of an intermediary layer.

Figure 7(c) is an illustration of a scheme for making an ultrathin pore-spanning membrane.

15 Figure 8 is a graph showing the kinetics of water permeability through vesicles in a stopped-flow apparatus (The curve on the top is for AqpZ incorporated ABA block copolymer vesicles and the bottom one is for the control sample without AqpZ).

Figure 9 is a graph showing the effect of increasing AqpZ content on the permeability of ABA block copolymers vesicles.

20 Figure 10(a) is an FESEM image of a top surface view of an empty polycarbonate substrate before the formation of the planar pore-spanning biomimetic membrane.

25 Figure 10(b) is an FESEM image of a cross-sectional view of an empty polycarbonate substrate before the formation of the planar pore-spanning biomimetic membrane.

Figure 10(c) is an FESEM image of a top surface view of a polycarbonate substrate after the formation of the planar pore-spanning biomimetic membrane.

Figure 10(d) is an FESEM image of a cross-sectional view of a polycarbonate substrate after the formation of the planar pore-spanning biomimetic membrane.

Figure 11 is a schematic illustrating a static test of forward osmosis performance using a biomimetic membrane (pressure retarded osmosis (PRO) mode).

Figure 12 is a graph showing the effect of AqpZ content and draw solution
5 concentration on forward osmosis water flux.

Figure 13 is a graph showing the effect of AqpZ content on forward osmosis water flux in sea water desalination.

DETAILED DESCRIPTION OF THE INVENTION

10 The membrane structure and support can be prepared as follows. The membrane is prepared on, and spans across, a porous substrate. Examples of porous substrates that can be used are alumina, track-etched polycarbonate film and polymeric membranes, such as cellulose acetate. In preferred embodiments, the substrates are fully porous, have a smooth surface, and have a pure water
15 permeability greater than $1000 \text{ L/m}^2/\text{h}/\text{bar}$. Preferably, the substrates have a uniform pore size and do not have tortuous pores. The pore size of the substrate can be between about 20 nm and about 100 nm.

Substrate surface preparation

20 A metal layer is coated onto the surface of the substrate by, for example, physical vapor deposition. The metal can be gold, silver, platinum, palladium, or any combination thereof. The thickness of the metal coating on the surface can be in the range of about 40 nm to about 60 nm. Preferably, the thickness of the metal coating can be optimized according to the pore size to avoid the pore narrowing
25 effect. For example, a gold coating of about 50 nm is recommended for a membrane support having a 50 nm pore size.

Next, the metal coated substrate is modified by adding an intermediary layer. The intermediate layer can be chemisorbed onto the metal coated porous substrate. An example of an intermediary layer is carboxylated polyethylene glycol (PEG),
30 which can be incubated on the metal coated porous substrate so that the PEG

molecules can be chemisorbed by self-assembly of thiol groups. As described more fully with respect to Example 1, the bifunctional-PEG polymer cushion significantly enhances the flexibility of thin film on the substrate and gives rise to higher atomic force microscopy (AFM) breakthrough forces when Aquaporin Z is reconstituted into the pore-suspending membrane on the bifunctional-PEG coated substrate. The PEG polymer cushion has the advantages of acting as a spacer between the membrane and the porous substrate and is believed to preserve the functional structure of the aquaporin. Although the local membrane mechanical stability can be further improved by increasing the AqpZ content, an optimal ratio of AqpZ to lipid or block copolymer is needed to achieve the highest forward osmosis (FO) performance. In some instances, the optimal molar ratio of AqpZ to lipid or block copolymer is 1:100. The block copolymer can have a molecular weight of the hydrophilic block from 500 to 2500 and a molecular weight of the hydrophobic block from 2500 to 10,000. The hydrophilic to hydrophobic weight ratio can be from 1:4 to 2:3. The block copolymer can be PMOXA-PDMS-PMOXA (500-2500-500), PMOXA-PDMS-PMOXA (1000-4000-1000), PMOXA-PDMS-PMOXA (1300-5000-1300), PMOXA-PDMS-PMOXA (1600-5600-1600), or higher. In some instances, the ABA block copolymer can be PMOXA-PDMS-PMOXA (1000-4000-1000).

Optionally, the intermediary layer can be functionalized with photoreactive crosslinkers. Any combination of chemicals that can functionalize the surface with photoreactive bonds can be applied. Examples of photoreactive crosslinkers include, but are not limited to, acrylic acid derivatives. Exemplary acrylic acid derivatives include, but are not limited to, methacrylic acid, ethyl acrylate, methyl methacrylate, and combinations thereof. Specifically, for grafting the primary amine monolayer, cystamine dihydrochloride, glutathione, thiol-polyethylene glycol-amine, peptides and proteins with thiol ends can be used. To conjugate the primary amine monolayer with methacrylate chemicals in the water phase, methacrylic acid, butyl acrylate, carboxyl- polyethylene glycol-methacrylate can be used. For conjugation occurring in the oil phase, methacrylate chemicals, such as acryloyl chloride, can be used.

Alternatively, the layer-by-layer modification can be reversed. The first monolayer is not necessarily the primary amine monolayer. For example, the first monolayer can be the carboxylated-monolayer. Chemicals such as 3-mercaptopropionic acid, lipoic acid, glutathione, thiol- polyethylene glycol- carboxyl, peptides and proteins with thiol ends can be applied for the first layer, in which case chemicals such as amine-polyethylene glycol-methacrylate, 2-isocyanatoethylmethacrylate will be needed in the sequential modification. The first method is preferred because only a few combinations of conjugating chemicals are required.

Surface modification is also not limited to layer-by-layer modification. For example, when the substrate is porous alumina, the surface can be modified directly by acryloyl chloride or 2-isocyanatoethylmethacrylate so that a thin layer of photoreactive crosslinker can be constructed.

Thin film preparation

The transmembrane water channel protein is incorporated into the thin film that will span across the pores of the substrate. The transmembrane water channel protein can be an aquaporin. Preferably, the aquaporin can be AqpZ, which is derived from *E. coli*.

The thin film can be phospholipids or block copolymers. Phospholipid or block copolymers having a hydrophilic to hydrophobic weight ratio from about 1:4 to about 2:3 and an unsaturated bond at the hydrophilic end can embed the transmembrane water channel protein and form the planar pore-spanning membrane.

The phospholipid or block copolymer can be less than 20 nm thick. The thin film can be a lipid bilayer, a single block copolymer, a mixture of different block copolymers, or a mixture of block copolymers and lipid. The thin film can preferably be dissolved in a detergent solution and form vesicles at neutral pH.

Lipids, particularly phospholipids, having a phase transition temperature lower than 37 °C can incorporate AqpZ. A preferred phospholipid is a 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC).

The block copolymer can be a diblock copolymer or a triblock copolymer, such as an ABA triblock copolymer. Preferably, the thin film is a UV-polymerizable block copolymer. A preferred block copolymer is poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline), which is abbreviated as PMOXA-PDMS-PMOXA. More preferably, the block copolymer can be PMOXA₁₂-PDMS₅₄-PMOXA₁₂. More preferably, the block copolymer is less than 20 nm thick. The block copolymer can be approximately 20% to approximately 25% hydrophilic (by molecular weight), with the remainder hydrophobic. The block copolymers can carry photoreactive crosslinkable functional groups at the hydrophilic ends, such as the acrylic acid derivatives previously described.

The transmembrane protein can be incorporated into the thin film by rehydrating the lipid or block copolymer and then mixing in a solution containing the transmembrane protein and detergent. The protein is reconstituted into the bilayer, and the detergent can be removed by dialysis or with biobeads. The vesicles are extruded with a polycarbonate membrane to control the vesicle size. Preferably, the pore size of polycarbonate membrane is larger than the pore diameter of the porous substrate

The vesicles are then fused onto the prepared substrate, which can be done by incubating the vesicles with the porous substrate. Optionally, a gentle vacuum can be applied to aid the fusion process. For example, the vacuum can be approximately 500 mbar to approximately 970 mbar. Optionally, the mixture can be gently stirred. After fusion, the vesicles are ruptured to obtain pore-spanning flat membranes. The vesicle can be ruptured by osmotic shock or strong interfacial interaction. Alternatively, if the membrane and the porous substrate contain photoreactive crosslinkable functional groups, the vesicles can be ruptured by UV crosslinking polymerization. Crosslinking also stabilizes the overall membrane structure. Preferably, the UV polymerization is carried out below 37 °C under argon protection. Optionally, the ruptured vesicles can be spread to obtain the pore suspending membrane.

The membranes thus prepared according to the methods described above can be used in water treatment applications, such as reverse osmosis and forward osmosis.

The following specific examples illustrate the disclosure herein, and are not intended to limit the scope of the invention. The teachings of all publications cited herein are incorporated by reference in their entirety.

Example 1: Preparation of a biomimetic membrane suspended by a PEG cushion

Example 1 illustrates the detailed preparation and characterization of a biomimetic membrane suspended by a PEG cushion.

Example 1.1 AqpZ incorporation and membrane permeability measurement

Aquaporin was inserted into lipid vesicles to investigate the compatibility of aquaporin and lipids. In this example, the lipid used was 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC). The vesicle suspension was prepared by the film rehydration method. The dry film of DMPC was rehydrated in 1x PBS buffer. The multilamellar vesicles solution was extruded using a polycarbonate membrane to control the vesicle size. For reconstitution experiments, 1mg/ml in 9 mM dodecyl- β -D-maltoside (DDM) was added during the formation of lipid vesicles and the mixture was stirred until the vesicles were formed. Then, the detergent was removed with biobeads. The proteoliposomes were then extruded.

The permeability of the liposome and the proteoliposome was determined using the stop-flow method. Vesicles were rapidly mixed with a hyperosmolar sucrose buffer, which causes water to permeate from the vesicle to an environmental solution and results in vesicle shrinkage. The vesicle size changes were detected by light scattering recorded at an emission wavelength of 577 nm in a stop-flow apparatus. The measuring temperature was 25 °C. As vesicles shrink, light scattering increases. Thus, an increase in the signal corresponds to a reduction in vesicle size, which can be fitted to an exponential rise equation as follows:

$$Y = A \exp(-kt) \quad (1)$$

where Y is the intensity of signal, t is the recording time (seconds), A is a constant, and k is the initial rate constant (s^{-1}). The osmotic water permeability can be
 5 calculated from Equation (2):

$$Pf = \frac{k}{(S/V_0)V_w\Delta_{osm}} \quad (2)$$

where S is the vesicle surface area (m^2), V_0 is the initial volume of vesicles (m^3), V_w is the partial molar volume of water (0.018L/mol), Δ_{osm} is the osmolarity difference
 10 that drives the shrinkage of vesicles (osmol/L), and P_f is the osmotic water permeability, in m/s.

Liposomes and proteoliposomes were rapidly mixed with a 600 mosmol/l sucrose solution as described in Borgnia et. al. *Journal of Molecular Biology* 1999; **291**: 1169-1179.

15 The initial rise of the light scattering signal was fitted by using Equation (1) and the permeability was determined from Equation (2). Figure 2 is a graph showing the kinetics of water permeability through vesicles in a stopped-flow apparatus. The data was normalized to between 0 and 1. The solid lines represent the exponential fitting curves. Figure 2(a) shows an increase in the relative light
 20 scattering signals with and without AqpZ incorporated into the DMPC vesicles at a protein to lipid molar ratio of 1:2000. The initial time rise for AqpZ-DMPC vesicles was between 20 and 30 ms, whereas the DMPC vesicles needed 2.5 s to reach equilibrium. The calculated permeabilities of DMPC and AqpZ-DMPC vesicles are 8.33×10^{-2} and 252 cm/s, respectively. Figure 2(b) is a graph showing the rise in
 25 light scattering between 0 and 10 s for the DMPC vesicles.

The incorporation of AqpZ led to a 3000-fold greater permeability compared to the pristine DMPC vesicles, thus demonstrating the reconstitution of AqpZ into the DMPC vesicles with a functional configuration. AqpZ was then reconstituted at different protein to lipid molar ratios as shown in Figure 3. The observed
 30 permeability increased remarkably and was then followed by a sharp decrease while the protein-lipid molar ratio varied from 1:6000, 1:4000, 1:2000 to 1:1000.

Example 1.2: Pore-suspending membrane preparation

Two configurations were designed as illustrated in Figures 1(a) and 1(b). In the design of Figure 1(a), vesicles were ruptured directly on a porous alumina substrate (Synkera Technologies, Inc., Longmont, Colorado, USA) by the interfacial interactions method. Without wishing to be bound by any particular theory, it is believed that vesicles are ruptured because they have electrostatic interactions with the alumina support. In the design of Figure 1(b), the alumina substrate was modified by physical vapor deposition of 40-60 nm layer of gold. Then, Carboxyl-PEG-SH (1mg/ml ethanol solution) (JenKem Technology USA Inc., Allen, Texas, USA) was incubated on the gold-coated porous alumina for more than 24 hours so that the PEG molecules were chemisorbed on gold by self-assembly of thiol groups. Liposomes and proteoliposomes were incubated with the porous alumina directly above the phase transition temperature of phospholipids for 2-8 h. Finally, the vesicles were ruptured on the PEG polymer cushion by the Interfacial interactions method. Without wishing to be bound by any particular theory, it is believed that vesicles are ruptured because they have electrostatic interactions with the carboxyl groups on the PEG cushion.

Figure 4(a) and 4(b) are Field Emission Scanning Electron Microscope (FESEM) images of empty alumina substrates (a) before gold coating and (b) after gold coating. The pore diameters before and after gold coating are estimated to be 80 ± 10 nm and 90 ± 10 nm, respectively.

Figures 4(c) and 4(d) are atomic force microscopy (AFM) images of empty substrate (c) plain porous alumina, (d) porous alumina coated with a 60nm gold layer. Figures 4(c) and (d) show the hexagonal structure obtained by AFM and the pore-to-pore distance remained at about 170 ± 10 nm before and after gold coating. The theoretical maximal penetration depth (h_{depth}), which was dependent on both the tip geometry (i.e., the radius of the tip, R_{tip}) and the pore topology (i.e., the radius of the pore, R_{pore}), was about 125nm according to this equation $h_{\text{depth}} = R_{\text{pore}}^2 / 2R_{\text{tip}}$ as used by Steltenkamp et. al. *Biophysics Journal* 2006; **91**: 217-226. In practice, the experiments showed that the penetration depth observed in the profile in Figure 4

was 45 ± 15 nm for the empty substrate and it was reduced to 40 ± 10 nm after the gold coating.

Figures 4(e) and 4(f) are atomic force microscopy (AFM) images of (e) the DMPC membrane of the design of Figure 1(a) and (f) the DPMC membrane of Figure 1(b). One can easily identify membrane-covered and -uncovered pores from the height profiles of these two designs in Figures 4(e) and 4(f). A comparison of penetration depth between Figures 4(c) and (e) as well as Figures 4(d) and (f) show that there was a 70% drop in penetration depth (*i.e.*, from 45 ± 15 nm to 15 ± 5 nm, and from 40 ± 10 nm to 15 ± 5 nm, respectively), revealing that DMPC liposomes had fused and formed the pore-spanning membrane on top of the porous alumina.

Example 1.3: Force indentation measurements of pore-suspending membrane

The local mechanical properties of the membranes were tested by conducting force indentation experiments in the center of the unsupported membrane-covered pores with a constant piezo-electric velocity of $0.5 \mu\text{m s}^{-1}$ at a temperature of 20 ± 1 °C. The force curve was recorded for more than 150 times at different pores.

The force indentation measurements were performed in the center of the pore-suspending membrane in order to obtain the elastic response of the membrane.

Figure 5 is a force indentation curve for a pore-spanning biomimetic membrane of Figure 1(a) prepared from DMPC-AqpZ vesicles having a protein to lipid ratio of 1:2000. After the initial contact between the tip and the membrane, the membrane was indented until it ruptured. Hard-wall repulsions were visible in some cases when the tip hits the pore walls at the maximal indentation depth.

25 Example 1.4: Effect of Carboxylated-PEG on the local mechanical stabilities of membranes

Figures 6(a) and 6(b) are histograms of (a) rupture forces and (b) indentation depths for the DMPC membrane in Figure 1(a). Figures 6(c) and 6(d) are histograms of (c) rupture forces and (d) indentation depths for the DMPC membrane in Figure 1(b).

Comparing Figures 6(a) and 6(c), the membrane of Figure 1(b) shows a narrower rupture force distribution. Thus, the PEG polymer layer was able to smoothen the rough alumina surface with its soft polymer chains. In other words, the PEG polymer layer could ease packing of lipids, effectively dissipating indentation stresses and improving the membrane stability. The membrane suspended on a substrate having carboxylated-PEG coating layer (*i.e.*, the membrane of Figure 1(b)) exhibited an average breakthrough force of 2.3 ± 2.1 nN, which is relatively comparable to the average breakthrough force of 3.7 ± 2.0 nN for the membrane suspended directly on porous alumina substrate (*i.e.*, the membrane of Figure 1(a)).

Comparing Figures 6(b) and 6(d), the membrane of Figure 1(b) shows a greater penetration depth. The membrane dilatation during AFM indentation could be estimated by adopting the geometry of a hemisphere to imitate the surface morphology of the indented membrane. The significant difference in penetration depths of the two designs implies that the lipid layer might flow from the edge into the pores upon indentation due to the soft PEG polymer cushion of the membrane of Figure 1(b), which increases the flexibility between the membrane and the substrate.

Example 1.5: Effect of AqpZ on the local mechanical stability of membranes

Upon indentation, a normal force was applied on the pore-suspending membrane. If the membrane was fixed on the substrate, there would be an increase in the lateral tension. The two-dimensional (2D) Young's modulus might be applicable to describe the lateral tension within the membrane between AqpZ and DMPC, as the maximum strains produced from these interactions can be represented by the Young's modulus:

$$F = 2\pi E_{2D} \frac{h^3}{r^2} \quad (3)$$

where F is the rupture force (nN), E_{2D} is the Young's modulus (N/m), h is the penetration depth (nm), and r is the hole radius (nm). The bending rigidity of the membrane can be estimated as $\sim 0.1E_{2D}d^2$, where d is the bilayer thickness of 4.5 nm, as described in Goncalves et. al. *Nature Methods* 2006; **3**: 1007-1012.

Table 1: Local mechanical properties of membrane upon AFM indentation

Membrane	Rupture force (nN)	Penetration depth (nm)	2D Young's modulus E_{2D} (N/m)*
DMPC on the membrane of Figure 1(a)	3.7 ± 2.0	26 ± 8	0.06 ± 0.05
DMPC on the membrane of Figure 1(b)	2.3 ± 2.1	47 ± 13	--
AQPz-DMPC on the membrane of Figure 1(b) (LPR 6000)	3.7 ± 2.3	24 ± 14	--
AQPz-DMPC on the membrane of Figure 1(b) (LPR 4000)	4.2 ± 3.4	32 ± 8	--
AQPz-DMPC on the membrane of Figure 1(b) (LPR 2000)	6.0 ± 3.0	31 ± 18	0.13 ± 0.09
AQPz-DMPC on the membrane of Figure 1(b) (LPR 1000)	12.0 ± 4.8	24 ± 14	0.35 ± 0.02

*The E_{2D} can only be obtained only when the membrane is relatively fixed on the pore upon indentation.

- 5 Table 1 lists the rupture forces and penetration depths of membranes having different surface functionalization and AqpZ concentration. After incorporating AqpZ, the average breakthrough force increased when compared to the DMPC layer that did include AqpZ. Even at a very high lipid-to-protein ratio (LPR=6000), the increase of the average breakthrough forces was visible. As the LPR ratio decreased
- 10 from 6000 to 1000, the breakthrough force increased drastically from 3.7 to 12.0 nN. Meanwhile, the penetration depth also decreased, indicating that the membrane became less flexible on the top of the PEG polymer cushion. The bending rigidity of the DMPC-AqpZ (LPR 2000:1) membrane ($\sim 59k_B T$) was larger than the bending modulus of a pristine DMPC bilayer ($\sim 13 k_B T$). As the LPR value decreases to
- 15 1000:1, the bending rigidity increased to $\sim 160k_B T$, indicating that the AqpZ molecules might respond to mechanical stresses less actively than DMPC molecules. Hence, the incorporation of AqpZ improved the energy barrier required for membrane rupture.

Example 2: Preparation of a biomimetic membrane suspended by a thin layer of methacrylate cushion

Example 2 illustrates the detailed preparation and characterization of a biomimetic membrane suspended by a thin layer of methacrylate cushion, as shown in Figure 1(c).

Example 2.1: Substrate surface functionalization with photoreactive crosslinker

Figure 7(a) is an illustration of a scheme for preparing a pore-spanning membrane having an embedded aquaporin. First, the surface of the porous substrate was modified with a photoreactive crosslinker. As shown in Figure 7(b), a gold coated porous polycarbonate membrane was modified by physical vapor deposition of a 40-60 nm layer of gold. Then, the gold-coated polycarbonate membrane was incubated with cysteamine solution (0.1-1.0 mg/ml in PBS solution) for more than 12 hours at 4°C so that a thin layer of cysteamine was chemisorbed on the gold by self-assembly of thiol groups. The modified polycarbonate membrane was then further activated by N-Hydroxysuccinimide / 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (NHS/EDC) and the resulting membrane was further immersed in acrylic acid (2-10% wt) PBS solution for more than 12 hours at 4°C. The membrane substrate was washed thoroughly before use.

Example 2.2: AqpZ incorporation into ABA block copolymer vesicles

AqpZ was incorporated into a thin film of the block-copolymer PMOXA₁₂-PDMS₅₄-PMOXA₁₂ (molecular weight 6000). The vesicle suspension was prepared by the film rehydration method. The dry film of PMOXA₁₂-PDMS₅₄-PMOXA₁₂ was rehydrated in 1x PBS buffer. An AQPz solution of 1mg/ml in 9 mM dodecyl-β-d-maltoside (DDM) was added during the formation of the block copolymer vesicles, and the mixture was stirred until a homogeneous mixture was formed. The detergent was removed by using biobeads. The proteopolymersomes solution was then extruded using a polycarbonate track-etch membrane to control the vesicle size. The pore diameter of the polycarbonate membrane in this study was 50 nm. The resultant concentration of proteopolymersome was 0.5-10 mg/ml.

Example 2.3: Preparation of planar pore-spanning biomimetic membranes through
2-D UV crosslinking

Figure 7(c) is an illustration of a scheme for making an ultrathin pore-
spanning membrane. The modified polycarbonate membrane was immobilized by
5 using an alumina tap on a ceramic funnel with a porous support on the top. The
proteopolymersome solution was purged with argon slowly for 10 minutes and was
dropped onto the surface of the polycarbonate membrane support upon application
of a vacuum of approximately 920 mbar. Gentle stirring was performed for 10
10 minutes. The 2-D crosslinking of AqpZ and amphiphilic material was performed by
UV irradiation on the membrane complex for 15 minutes at 4°C.

Example 2.4: AqpZ incorporation and membrane permeability measurement

The water-permeability of the PMOXA₁₂-PDMS₅₄-PMOXA₁₂ membranes
15 with and without AqpZ incorporated therein was measured similarly to Example 1.1.
Vesicles were rapidly mixed with a 1M NaCl solution (osmolar pressure = 2
osmolar/L), which causes water to permeate from the vesicle and simultaneously
results in vesicle shrinkage. The shrinkage of the vesicle size with and without
AqpZ was detected by light scattering and recorded at an emission wavelength of
20 577 nm in the stop-flow apparatus. The measuring temperature was 25°C. As
vesicles shrink, light scattering increases, and thus an increase in the signal
corresponds to a reduction in vesicle size.

The initial rise of the light scattering signal was fitted by using Equation (1)
and the permeability was determined from Equation (2). Figure 8 is a graph
25 showing the kinetics of water permeability through vesicles in a stopped-flow
apparatus, and it shows the increase in relative light scattering signals with and
without incorporation of AqpZ into the ABA block copolymer membrane at 25°C.
The initial time rise for AqpZ-ABA vesicles was between 0 and 20 ms, whereas no
obvious size change was found for the pristine ABA block copolymer vesicles
30 although the size variation was recorded for 10s. The calculated permeability of
AqpZ-ABA copolymer (molar ratio: 1:200) vesicles was $2.59 \times 10^3 \mu\text{m/s}$. The

incorporation of AqpZ led to a great improvement in the permeability of the membrane; demonstrating the reconstitution of AqpZ into the ABA block copolymer vesicles with a correct configuration.

AqpZ was then reconstituted at different protein polymer ratios as shown in Figure 9. The observed permeability increased remarkably as the ratio of AqpZ to ABA copolymer increased from 1:400 to 1:300 and then reached a plateau between a ratio of 1:200 to 1:50. Without wishing to be bound by any particular theory, one possible explanation for this phenomenon might be due to the decreasing efficiency of incorporating AqpZ.

10

Example 2.5: Morphology of the planar pore-spanning biomimetic membranes

The surface morphology and cross section of the membrane was observed by Field Emission Scanning Electron Microscopy (FESEM). Figures 10(a) and (b) showed visible pores on the substrate before polymerization. Figure 10(a) is a top-surface view, and Figure 10(b) is a cross-sectional view. Figures 10(c) and (d) showed that the pores on the top-surface were completely covered after polymerization. Figure 10(c) is a top-surface view, and Figure 10(d) is a cross-sectional view. The contrast of the pore-spanning membrane and the substrate was quite similar as the pore-spanning membrane less than 10 nm thick. The pore-spanning membrane is transparent and thin (Figure 10(c)), but it is difficult to estimate the thickness from the FESEM image.

20

Example 2.6: Performance of the planar pore-spanning biomimetic membranes

The membrane with a diameter of 3 mm was placed between the two chambers as shown Figure 11. A conductivity meter was inserted in the feed chamber to monitor the salt reverse flux. Water levels of both chambers were raised simultaneously and slowly. The water permeation through the pore-spanning biomimetic membrane was analyzed by the weight increase of the draw solution side.

25

30

The water flux of each membrane prepared from different AqpZ-ABA copolymer ratios was tested using ultrapure water as the feed and NaCl solution as

the draw solution. Figure 12 is a graph showing the effect of AqpZ content and draw solution concentration on forward osmosis water flux. The water flux of each membrane increased almost proportionally as the concentration of NaCl in the draw solution chamber increased. Table 2 shows the salt reverse flux of each test in Figure 12. Almost all the salt reverse fluxes were below 10 g/m²/h.

Table 2: Salt reverse flux of the membranes prepared using different AqpZ-ABA ratio in the forward osmosis test (PRO mode).

Concentration of drawn solution (M NaCl)	Salt leakage (g/m ² /h)			
	AqpZ-ABA (1:100)	AqpZ-ABA (1:200)	AqpZ-ABA (1:300)	AqpZ-ABA (1:400)
2.0	6.2±2.6	10.0±2.0	0.48±0.4	3.7±4.6
1.5	7.8±1.1	8.6±0.6	0.6±0.6	2.9±1.6
1.0	6.5±0.2	7.1±1.4	0.3±0.02	2.5±0.8
0.5	5.4±0.2	4.3±1.7	0.7±0.4	0.7±0.5

10

Additionally, as the ratio of AqpZ-ABA copolymer increased, the water flux also increased, which proved that the AqpZ molecules were embedded in the ABA block copolymer in a functional configuration when the vesicles were transformed to a planar membrane, as demonstrated by high water flux (see Example 2.7) and low reverse salt flux (Table 2).

15

Example 2.7: Forward osmosis performance by using seawater as feed

The water flux of each membrane prepared from different AqpZ-ABA copolymer ratios was tested using sea water (NaCl 3.5%) as the feed and 2M NaCl solution as the draw solution. The water flux of each membrane significantly increased as the ratio of AqpZ-ABA copolymer increased, as shown in Figure 13. The net driving force was equivalent to the osmotic pressure of 1.4M NaCl to pure water, but the flux of the sea water test fell to 35-45%, likely due to the internal and external concentration polarization. Thus, the data simulates the performance of the membrane for water desalination.

25

Other Embodiments

All of the features disclosed in this specification may be combined in any combination. For example, and without limitation, the PMOXA-PDMS-PMOXA copolymer of Example 2 can be used with the PEG intermediary layer of Example 1, and the DMPC thin film of Example 1 can be used with the intermediary layer of Example 1 (chemisorption of cysteamine, activated by NHS/EDC, and immersed in acrylic acid PBS solution). Similarly, the alumina substrate of Example 1 can be used with the block copolymer thin film of Example 2, and the polycarbonate substrate of Example 2 can be used with the DMPC thin film of Example 1. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other examples are also within the claims.

While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A pore-spanning biomimetic membrane structure, comprising:
a porous substrate;
5 a metal coating on the surface of the porous support;
an intermediary layer on the surface of the metal coating; and
a thin film having a transmembrane protein incorporated therein;
wherein the thin film is layered on top of the intermediary layer and spans
one or more pores of the substrate.
10
2. The structure of claim 1, wherein the transmembrane protein is an aquaporin.
3. The structure of claim 2, where the aquaporin is AqpZ.
- 15 4. The structure of claim 1, wherein the porous substrate is alumina.
5. The structure of claim 1, wherein the porous substrate is polycarbonate.
6. The structure of claim 1, wherein the metal coating is selected from the
20 group consisting of gold, silver, platinum, palladium, and combinations
thereof.
7. The structure of claim 1, wherein the metal coating is gold.
- 25 8. The structure of claim 1, wherein the intermediary layer is polyethylene
glycol.
9. The structure of claim 1, wherein the intermediary layer has methacrylate
groups.
30

10. The structure of claim 1, wherein the intermediary layer is functionalized with photoreactive crosslinkers.
11. The structure of claim 10, wherein the photoreactive crosslinkers are acrylic acid derivatives.
12. The structure of claim 11, wherein the acrylic acid derivatives are methacrylic acid, ethyl acrylate, methyl methacrylate, or combinations thereof.
13. The structure of claim 1, wherein the thin film comprises 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine.
14. The structure of claim 1, wherein the thin film comprises a block copolymer.
15. The structure of claim 14, wherein the block copolymer is poly(2-methyloxazoline)-*block*-poly(dimethylsiloxane)-*block*-poly(2-methyloxazoline).
16. The structure of claim 15, wherein the block copolymer is PMOXA₁₂-PDMS₅₄-PMOXA₁₂.
17. The structure of claim 15, wherein the block copolymer is less than 20 nm thick.
18. The structure of claim 15, wherein the molecular weight of the hydrophilic block of the block copolymer is from 500 to 2500 and the molecular weight of the hydrophobic block of the block copolymer is from 2500 to 10,000.
19. The structure of claim 15, wherein the hydrophilic to hydrophobic weight ratio of the block copolymer can be from 1:4 to 2:3.

20. The structure of claim 15, wherein the copolymer is PMOXA-PDMS-PMOXA (500-2500-500), PMOXA-PDMS-PMOXA (1000-4000-1000), PMOXA-PDMS-PMOXA (1300-5000-1300), or PMOXA-PDMS-PMOXA (1600-5600-1600).
- 5
21. The structure of claim 15, wherein the copolymer is PMOXA-PDMS-PMOXA (1000-4000-1000).
22. A method for preparing a pore-spanning biomimetic membrane structure,
10 comprising:
incorporating a transmembrane protein into a thin film;
extruding the thin film with the incorporated transmembrane protein into vesicles; and
fusing the vesicle on a porous substrate having a metal coating and an
15 intermediary layer.
23. The method of claim 22, wherein the transmembrane protein is incorporated into the thin film during rehydration of the thin film.
- 20 24. The method of claim 22, wherein vesicle fusion is performed in the presence of a vacuum.
25. The method of claim 24, wherein the vacuum is approximately 500 mbar to approximately 970 mbar.
- 25
26. The method of claim 22, further comprising incubating the vesicles with the porous substrate to fuse the vesicles to the intermediary layer.
27. The method of Claim 22, further comprising crosslinking the vesicles and the
30 intermediary layer.

28. The method of Claim 22, further comprising exposing the vesicles and the substrate to UV irradiation.
29. The method of Claim 22, further comprising chemisorbing the intermediary
5 layer onto the metal-coated substrate.
30. The method of Claim 22, further comprising forming the metal coating on the porous substrate by physical vapor deposition.
- 10 31. The method of Claim 22, further comprising functionalizing the intermediate layer with photoreactive crosslinkers.
32. The method of Claim 31, wherein the photoreactive crosslinkers are acrylic acid derivatives.
- 15 33. The method of Claim 32, wherein the acrylic acid derivatives are methylacrylic acid, ethyl acrylate, methyl methacrylate, or combinations thereof.
34. A method for performing osmosis, comprising:
20 placing the pore-spanning biomimetic membrane structure of claim 1 between two solutions of differing osmolarity.

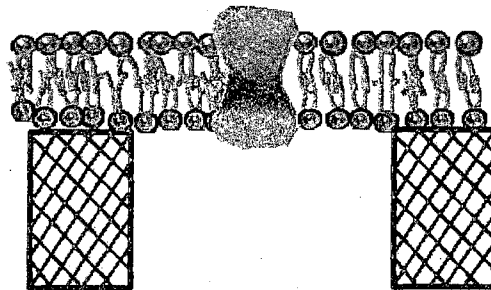


Figure 1(a)

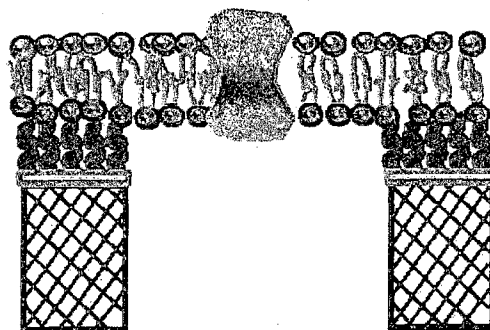


Figure 1(b)

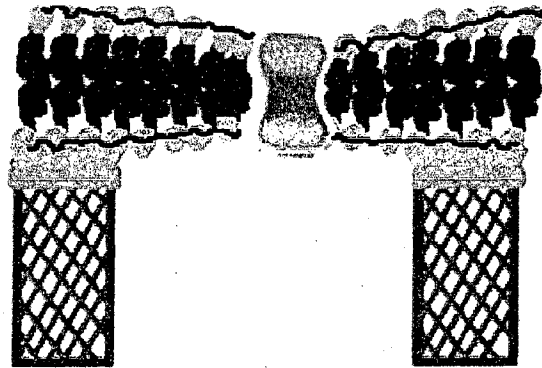


Figure 1(c)

3/14

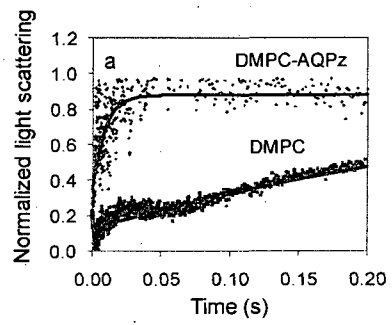


Figure 2(a)

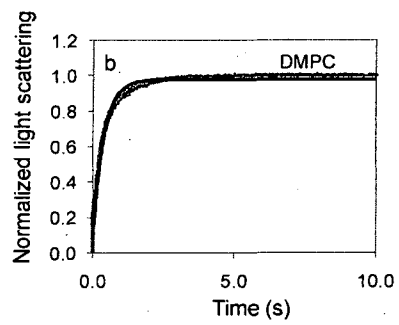


Figure 2(b)

4/14

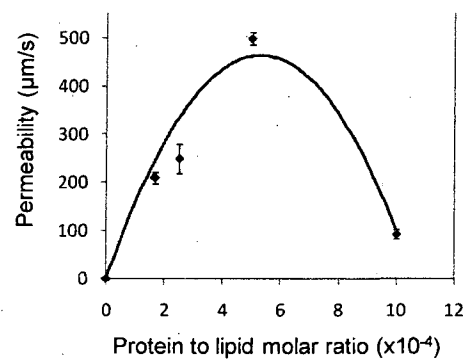


Figure 3

5/14

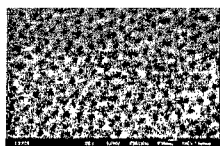


Figure 4(a)

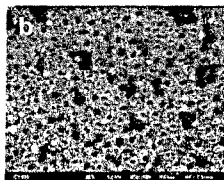


Figure 4(b)

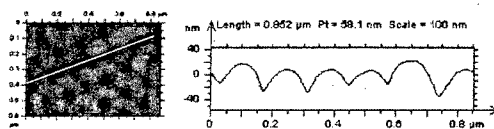


Figure 4(c)

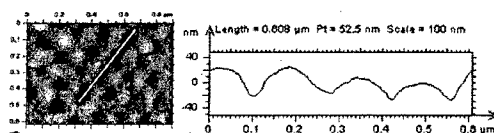


Figure 4(d)

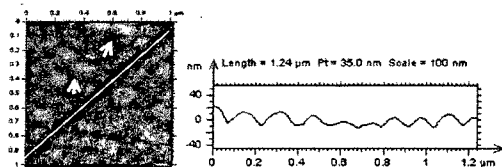


Figure 4(e)

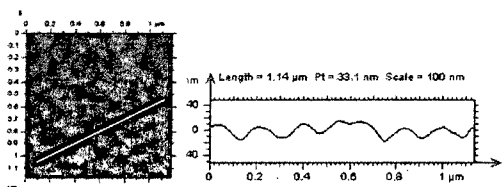


Figure 4(f)

6/14

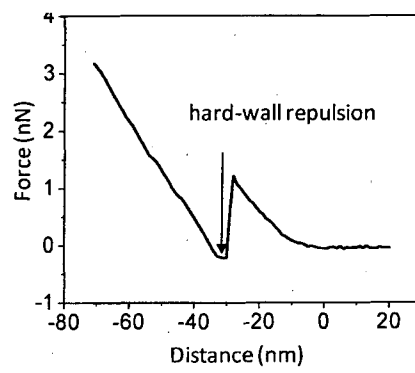


Figure 5

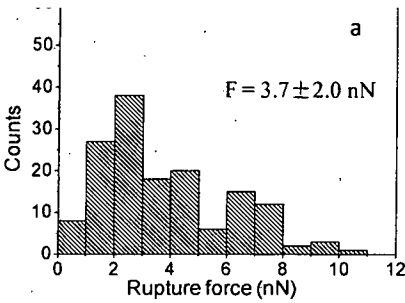


Figure 6(a)

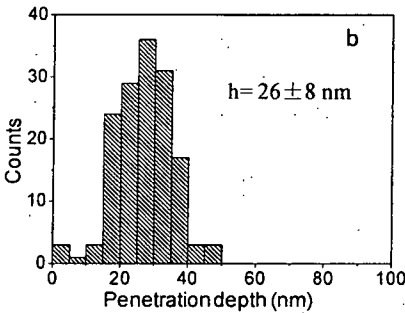


Figure 6(b)

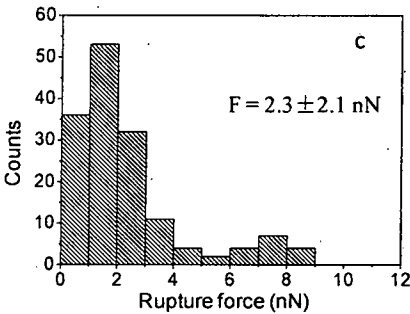


Figure 6(c)

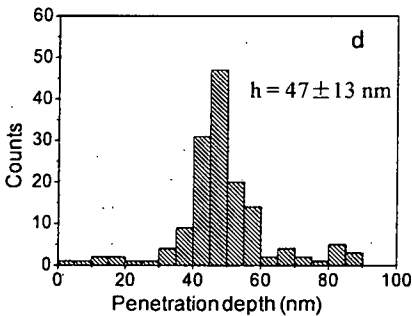


Figure 6(d)

8/14

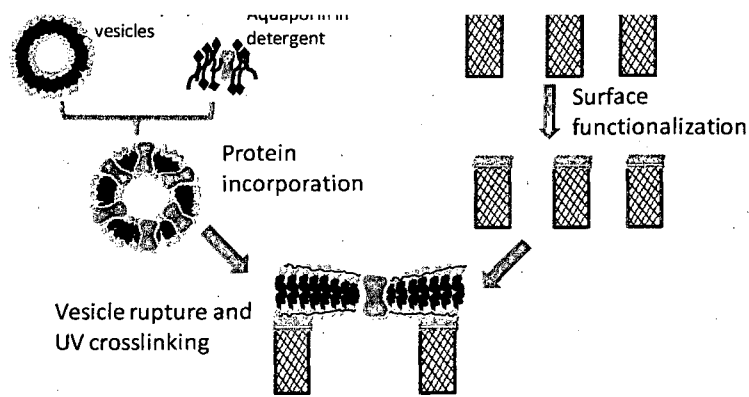


Figure 7(a)

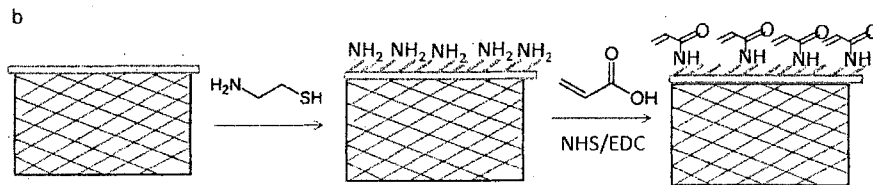


Figure 7(b)

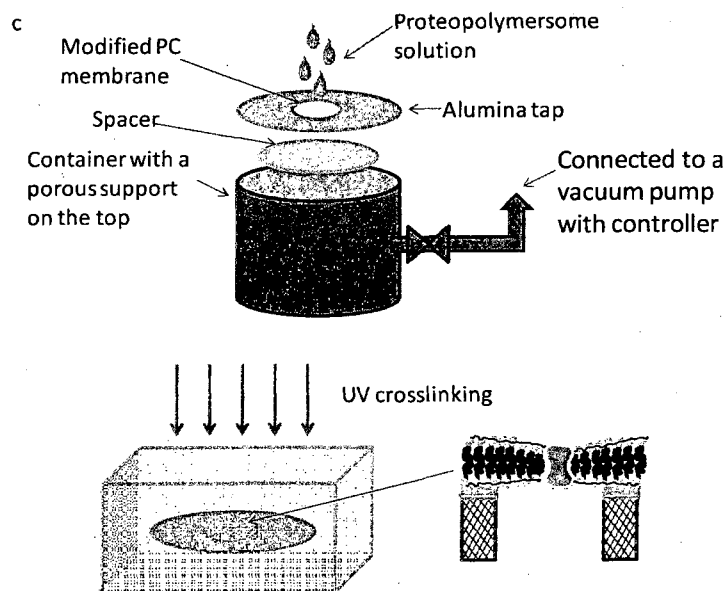


Figure 7(c)

9/14

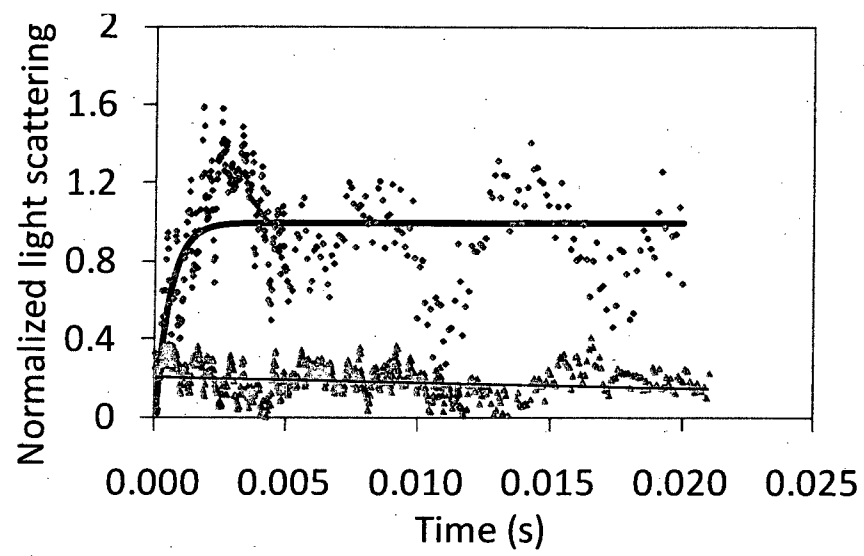


Figure 8

10/14

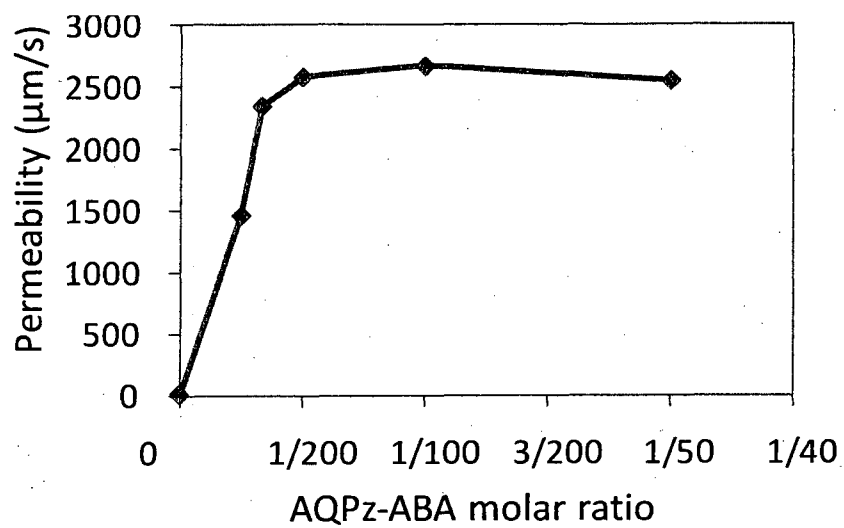


Figure 9

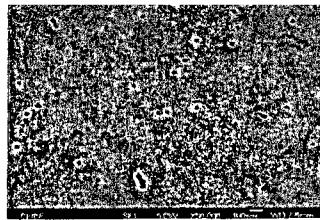


Figure 10(a)



Figure 10(b)

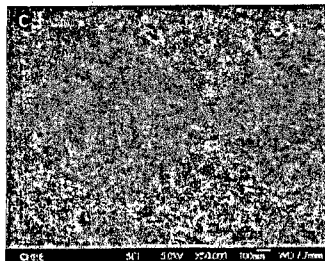


Figure 10(c)



Figure 10(d)

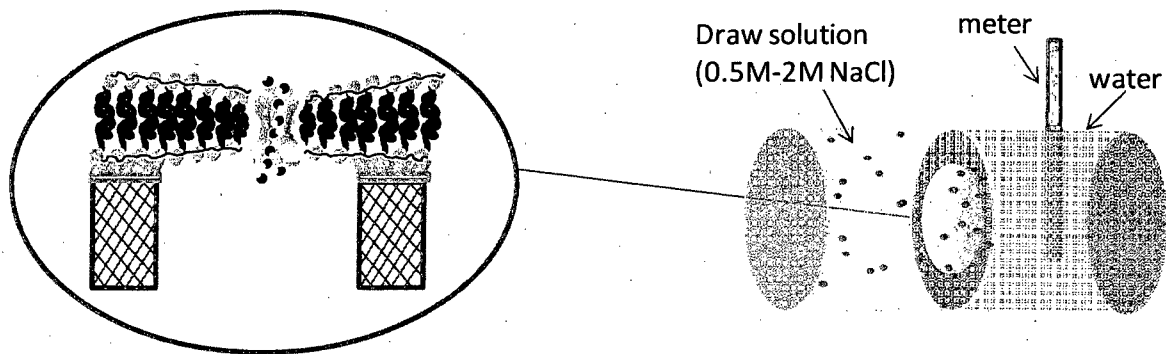


Figure 11

13/14

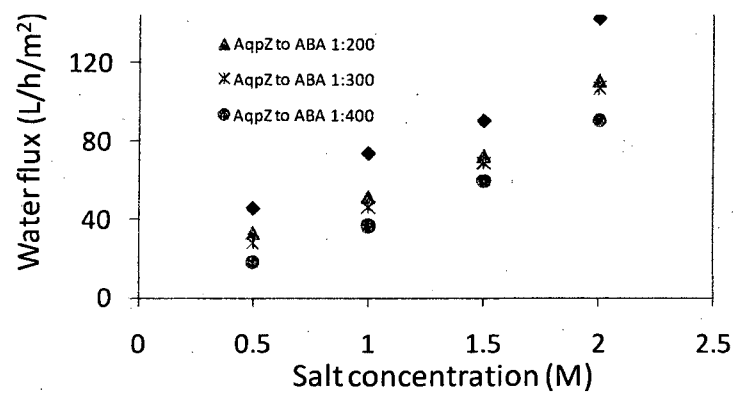


Figure 12

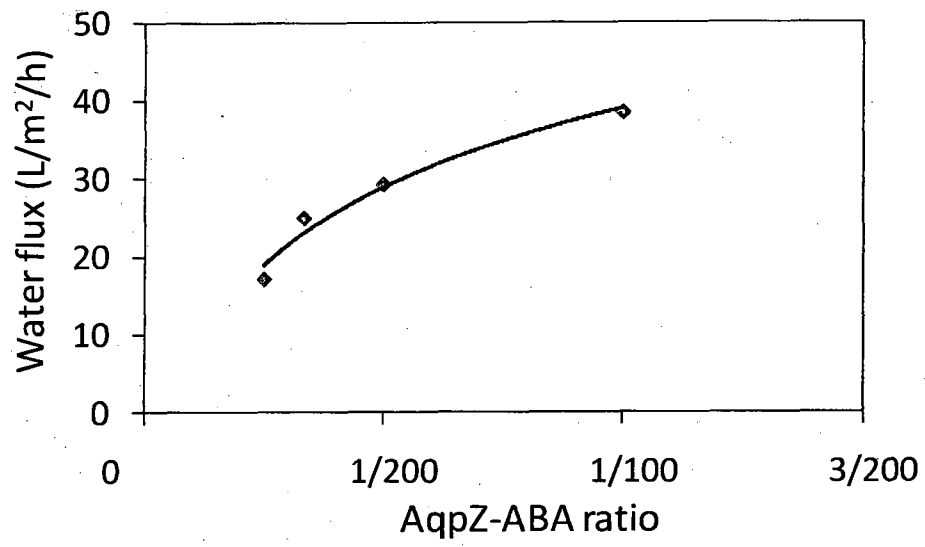


Figure 13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2012/000186

A. CLASSIFICATION OF SUBJECT MATTER

B01D 61/00 (2006.01) B01D 63/00 (2006.01) B01D 65/00 (2006.01) B01D 67/00 (2006.01) B01D 69/00 (2006.01)
 B01D 71/00 (2006.01) B01D 71/06 (2006.01) C02F 1/00 (2006.01) C02F 1/44 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC: (B01D 61/low, 63/low, 65/low, 67/low, 69/low, 71/low; C02f 1/low) and biomimetic 2w membrane+

Google patents: biomimetic membrane, metal, porous

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

☒ Further documents are listed in the continuation of Box C ☒ See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"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)	"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
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 August 2012	Date of mailing of the international search report 27 August 2012
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au Facsimile No.: +61 2 6283 7999	Authorized officer Gregory Diven AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832992

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2012/000186
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007033675 A1 (AQUAPORIN APS) 29 March 2007 Abstract; page 2 line 34- page 3 line 10; claims	1-34
A	WO 2010040353 A2 (DANFOSS AQUAZ A/S) 15 April 2010 Abstract; page 1 line 19- page 2 line 12; claims	1-34
A	US 2011084026 A1 (FREGER ET AL) 14 April 2011 Abstract; paragraphs [0011]-[0020]; examples; claims	1-34
A	US 2004049230 A1 (MONTEMAGNO ET AL) 11 March 2004 Abstract; paragraphs [0004]-[0015]; claims	1-34
A	US 2004149688 A1 (FUCHS ET AL) 05 August 2004 Abstract; paragraphs [0037]-[0120]; claims	1-34
A	CN 101301583 A (MT TECHNOLOGIES INC) 12 November 2008 Espace abstract	1-34
A	NZ 542286 A (AGRES LTD) 30 April 2008 Abstract; page 6 line 24- page 15 line 9; examples; claims	1-34

INTERNATIONAL SEARCH REPORT		International application No.			
Information on patent family members		PCT/SG2012/000186			
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.					
Patent Document/s Cited in Search Report		Patent Family Member/s			
Publication Number	Publication Date	Publication Number	Publication Date		
WO 2007033675 A1	29 Mar 2007	AU 2006246841 A1	23 Nov 2006		
		AU 2006246841 B2	23 Dec 2010		
		AU 2006294205 A1	29 Mar 2007		
		AU 2006294205 B2	16 Dec 2010		
		CA 2607371 A1	23 Nov 2006		
		CA 2621807 A1	29 Mar 2007		
		CN 101198398 A	11 Jun 2008		
		CN 101267875 A	17 Sep 2008		
		CN 102145259 A	10 Aug 2011		
		EP 1885477 A2	13 Feb 2008		
		EP 1885477 B1	17 Feb 2010		
		EP 1937395 A1	02 Jul 2008		
		EP 1937395 B1	09 Nov 2011		
		EP 2179780 A1	28 Apr 2010		
		HK 1109357 A1	28 May 2010		
		IL 187164 A	31 Jan 2011		
		JP 2008540108 A	20 Nov 2008		
		JP 2009510301 A	12 Mar 2009		
		JP 2012052552 A	15 Mar 2012		
		NO 20075674 A	12 Feb 2008		
		US 2009120874 A1	14 May 2009		
		US 7857978 B2	28 Dec 2010		
		US 2009007555 A1	08 Jan 2009		
		US 8123948 B2	28 Feb 2012		
		WO 2006122566 A2	23 Nov 2006		
		WO 2007033675 A1	29 Mar 2007		
		WO 2010040353 A2	15 Apr 2010	AU 2009301502 A1	15 Apr 2010
				CA 2739889 A1	15 Apr 2010
				CN 102238998 A	09 Nov 2011
				EP 2344269 A2	20 Jul 2011
JP 2012505167 A	01 Mar 2012				
KR 20110069839 A	23 Jun 2011				
MA 32766 B1	01 Nov 2011				
MX 2011003641 A	12 Aug 2011				
US 2011259815 A1	27 Oct 2011				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

Form PCT/ISA/210 (Family Annex)(July 2009)

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/SG2012/000186	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
		WO 2010040353 A2	15 Apr 2010
US 2011084026 A1	14 Apr 2011	US 2011084026 A1	14 Apr 2011
US 2004049230 A1	11 Mar 2004	AU 2003265250 A1	16 Feb 2004
		AU 2003265250 B2	26 Feb 2009
		CA 2497273 A1	05 Feb 2004
		CN 1703268 A	30 Nov 2005
		CN 101301583 A	12 Nov 2008
		EP 1572958 A2	14 Sep 2005
		EP 2218495 A1	18 Aug 2010
		IL 166509 A	26 Nov 2008
		JP 2006512204 A	13 Apr 2006
		JP 4444917 B2	31 Mar 2010
		KR 20050032578 A	07 Apr 2005
		KR 100803845 B1	14 Feb 2008
		KR 20070114316 A	30 Nov 2007
		US 2004049230 A1	11 Mar 2004
		US 7208089 B2	24 Apr 2007
		WO 2004011600 A2	05 Feb 2004
US 2004149688 A1	05 Aug 2004	EP 1407808 A1	14 Apr 2004
		FR 2844725 A1	26 Mar 2004
		FR 2844725 B1	07 Jan 2005
		JP 2004154769 A	03 Jun 2004
		US 2004149688 A1	05 Aug 2004
CN 101301583 A	12 Nov 2008	AU 2003265250 A1	16 Feb 2004
		AU 2003265250 B2	26 Feb 2009
		CA 2497273 A1	05 Feb 2004
		CN 1703268 A	30 Nov 2005
		CN 101301583 A	12 Nov 2008
		EP 1572958 A2	14 Sep 2005
		EP 2218495 A1	18 Aug 2010
		IL 166509 A	26 Nov 2008
		JP 2006512204 A	13 Apr 2006
		JP 4444917 B2	31 Mar 2010

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

Form PCT/ISA/210 (Family Annex)(July 2009)

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/SG2012/000186	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
NZ 542286 A	30 Apr 2008	KR 20050032578 A	07 Apr 2005
		KR 100803845 B1	14 Feb 2008
		KR 20070114316 A	30 Nov 2007
		US 2004049230 A1	11 Mar 2004
		US 7208089 B2	24 Apr 2007
		WO 2004011600 A2	05 Feb 2004
		EP 1931981 A1	18 Jun 2008
		NZ 542286 A	30 Apr 2008
		US 2008318326 A1	25 Dec 2008
		WO 2007030019 A1	15 Mar 2007
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			