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- (73) Patenthaver: **Applied Biomimetic ApS, Østager 2, Vollerup, 6400 Sønderborg, Danmark**
- (72) Opfinder: **GRZELAKOWSKI, Mariusz Piotr, c/o Applied Biomimetic, Inc., 50 West Watkins Mill Road, Suite S101, Gaithersburg, MD 20878, USA**
- (74) Fuldmægtig i Danmark: **Zacco Denmark A/S, Arne Jacobsens Allé 15, 2300 København S, Danmark**
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**HONG LEI WANG ET AL: "Mechanically robust and highly permeable AquaporinZ biomimetic membranes", JOURNAL OF MEMBRANE SCIENCE, vol. 434, 1 May 2013 (2013-05-01), pages 130-136, XP055199453, ISSN: 0376-7388, DOI: 10.1016/j.memsci.2013.01.031**  
**WENYUAN XIE ET AL: "An aquaporin-based vesicle-embedded polymeric membrane for low energy water filtration", JOURNAL OF MATERIALS CHEMISTRY A, vol. 1, no. 26, 1 January 2013 (2013-01-01), page 7592, XP055198883, ISSN: 2050-7488, DOI: 10.1039/c3ta10731k cited in the application**  
**Roxana Stoenescu ET AL: "Asymmetric ABC-Triblock Copolymer Membranes Induce a Directed Insertion of Membrane Proteins", Macromolecular Bioscience, vol. 4, no. 10, 20 October 2004 (2004-10-20), pages 930-935, XP055309699, DE ISSN: 1616-5187, DOI: 10.1002/mabi.200400065**  
**Katarzyna Kita-Tokarczyk ET AL: "Block copolymer vesicles-using concepts from polymer chemistry to mimic biomembranes", Polymer, vol. 46, no. 11, 1 May 2005 (2005-05-01), pages 3540-3563, XP055075604, ISSN: 0032-**

Fortsættes ...



# DESCRIPTION

## Field of the invention

[0001] The present invention relates to a process for making membranes. Specifically, it relates to a process for making water filtration membranes.

## Background of the invention

[0002] Conventional nanofiltration or reverse osmosis water filtration membranes have been known for many decades. Typically, they are made by casting a support membrane (often polysulfone or polyethersulfone); immersing the resulting cast in an aqueous solution of a diamine; removing excess from the surface of the membrane; immersing the membrane in an organic solution of a trifunctional acyl halide; and curing the resulting product to produce a polyamide layer. Washing and secondary coating are then carried out as necessary.

[0003] It is known from WO 01/32146 that membrane proteins may be incorporated into the walls of vesicles made from amphiphilic ABA block copolymers. This document includes extensive discussion of the nature of the polymers, and discloses that the polymers may have polymerizable groups at both chain ends. These polymerizable groups can be polymerized after the formation of the self-assembled vesicles, the polymerisation occurring exclusively intravesicularly. WO 2004/011600 discloses that aquaporins may be incorporated into triblock co-polymers to form a membrane which will only pass water, excluding all contaminants. Since these disclosures, much work has been carried out to develop commercially viable membranes incorporating transmembrane proteins, and particularly water filtration membranes based on aquaporins. The challenge is to produce a working membrane, which is physically sufficiently robust to withstand the necessary conditions.

[0004] WO 2009/076174 describes a method of preparing substantially flat membranes based on block copolymers and aquaporins. Dong et al, J. Mem. Sci. 2012, 409-410, 34-43, creates block-copolymer vesicles incorporating an aquaporin, but then breaks the vesicles using a vesicle rupturing method to deposit a planar monolayer of polymer on the surface of a support. WO 2010/146365 describes vesicles which may have aquaporins embedded in them, suspended in an oil phase to form a liquid membrane. According to Zhao et al, J. Membrane Sci. 2012, 422-428, various proposed methods of producing aquaporin membranes include polymer tethered bio-layers, biomembrane aperture partition arrays, membrane supported lipid bilayer via vesicle fusion, and vesicles suspended over membrane pores, but most of these are not able to withstand the high hydrostatic pressure that is required. Zhao's own solution to the problem is in effect to use a conventional membrane preparation as described above, modified by addition of aquaporin-loaded lipid vesicles (i.e. liposomes) to the aqueous solution of diamine. The result provides liposomes embedded in a polyamide layer. Although Zhao reports

the results obtained positively, it is clear from the data provided that although a small increase of water flux is obtained (Fig. 4(a)) no enhancement of the ability of the membrane to reject solute is found compared with conventional membranes (Fig. 5). It is believed that this is because the aquaporin-loaded liposomes become completely surrounded by polyamide, and thus the primary water flux through the membrane is via the polyamide (i.e. via the conventional path of the base membrane), and only partially through the aquaporin channels. WO 2013/043118, also from Zhao et al, describes the same technology and also discloses that block copolymers can be used to form vesicles, either containing or not containing aquaporins, and embedded in a polyamide layer. Again, the results plainly show that water flux via the polyamide layer and not exclusively via the aquaporin channels is obtained.

**[0005]** Xie et al, J. Mater. Chem A, 2013, 1, 7592, and WO 2013/180659, describe a process comprising (i) incorporating aquaporin into self-assembled polymer vesicles based on a polymer primarily (95%) having methacrylate end groups but also containing some (3%) carboxylic acid end groups; (ii) cross-linking the methacrylate end groups using UV light; (iii) depositing and covalently immobilizing the cross-linked vesicles on a support in such a concentration that isolated vesicles are disposed separately from each other on the surface of the support; and (iv) creating a thin polymer layer between the individual vesicles by the process known as "surface imprinting". In this process, it is important that the size of the immobilized vesicles is such that they are larger than the thickness of the imprinted polymer layer to prevent blockage of the aquaporin water channels. The process is said to exhibit high mechanical strength and stability during water filtration, but it is also stated that the most critical issue is that the imprinted polymer layer was not sufficiently dense to prevent all of the solute and water molecules from permeating. Further, only very limited flow rates are obtainable by such a system Duong et al, J. Memb. Sc., 409-410 (2012) 34-43 describes the formation of flat membranes by fusing vesicles comprising transmembrane proteins and in particular Aquaporin by Langmuir Blodgett methods, thus opening the vesicles to form planar structures. Wang et al, J. Memb. Sc. 434 (2013) 130-136 describes vesicular biomimetic membranes formed by cross-linking AqpZ-embedded block copolymer vesicles, followed by immobilizing vesicles on the membrane support via covalent binding.

**[0006]** Accordingly, there still remains a need for a process which leads to a physically robust membrane incorporating transmembrane proteins, particularly a membrane which uses aquaporins acting effectively for water filtration.

### **Summary of the invention**

**[0007]** The present disclosure provides a filtration membrane which comprises a porous support and, covalently bonded to a surface thereof, a layer comprising a plurality of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from an amphiphilic block copolymer; characterised in that within said layer, vesicles are covalently linked together to form a coherent mass. The thickness of the layer will be greater than the average diameter of the vesicles. In absolute terms, the thickness of the layer is suitably at

least 0.04 microns.

**[0008]** The present disclosure further provides a process for the preparation of a filtration membrane according to the invention, which comprises providing an aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from an amphiphilic block copolymer having reactive end groups; depositing said suspension of vesicles on a surface of a porous support; and providing reaction conditions such that covalent bonds are formed between different vesicles and between vesicles and said surface.

**[0009]** The invention provides a filtration membrane which comprises a porous support and, covalently bonded to a surface thereof, a continuous layer comprising a plurality of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from an amphiphilic block copolymer, and said transmembrane proteins being selected from aquaporins and glyceroporins; beta-barrel transmembrane proteins; and alpha-helical transmembrane proteins; characterised in that within said layer, vesicles are covalently linked together to form a coherent mass in which said layer of vesicles has a thickness greater than the average diameter of the vesicles, wherein said amphiphilic block copolymer is a diblock copolymer AB having a hydrophilic and a hydrophobic block or a triblock copolymer ABA having hydrophilic end blocks and a hydrophobic inner block, wherein the filtration membrane may be prepared by a process that comprises providing an aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from an amphiphilic block copolymer having reactive end groups; depositing said suspension of vesicles on a surface of a porous support; and providing reaction conditions such that covalent bonds are formed between different vesicles and between vesicles and said surface, wherein the process comprises:

1. (i)

1. (a) providing a first aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups X;
2. (b) providing a second aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups Y which are reactive with polymer end groups X;
3. (c) depositing said suspensions of vesicles on a support having a surface which is reactive with either polymer end groups X or Y; and
4. (d) causing reaction of end groups X with end groups Y, and either end groups X or end groups Y with the surface of the support;

or wherein the process comprises:

2. (ii)

1. (a) providing an aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups X;
2. (b) providing an multifunctional linking agent having at least two reactive groups Y

which are reactive with polymer end groups X;

3. (c) depositing said suspension of vesicles and said multifunctional linker on a support having a surface which is reactive with either polymer end groups X or reactive groups Y; and
4. (d) causing reaction of end groups X with groups Y, and either end groups X or groups Y with the surface of the support.

and wherein in either (i) or (ii), groups X are amine groups and groups Y are carboxylic acid, activated carboxylic acid, and/or azide groups; or groups X are carboxylic acid, activated carboxylic acid, and/or azide groups, and groups Y are amine groups; or groups X and Y are click chemistry groups.

**[0010]** The invention further provides a process for the preparation of a filtration membrane which filtration membrane comprises a porous support and, covalently bonded to a surface thereof, a continuous layer comprising a plurality of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from an amphiphilic block copolymer, and said transmembrane proteins being selected from aquaporins and glyceroporins; beta-barrel transmembrane proteins; and alpha-helical transmembrane proteins; characterised in that within said layer, vesicles are covalently linked together to form a coherent mass in which said layer of vesicles has a thickness greater than the average diameter of the vesicles, wherein said amphiphilic block copolymer is a diblock copolymer AB having a hydrophilic and a hydrophobic block or a triblock copolymer ABA having hydrophilic end blocks and a hydrophobic inner block, which process comprises providing an aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from an amphiphilic block copolymer having reactive end groups; depositing said suspension of vesicles on a surface of a porous support; and providing reaction conditions such that covalent bonds are formed between different vesicles and between vesicles and said surface, wherein the process comprises either:

1. (i)

1. (a) providing a first aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups X;
2. (b) providing a second aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups Y which are reactive with polymer end groups X;
3. (c) depositing said suspensions of vesicles on a support having a surface which is reactive with either polymer end groups X or Y; and
4. (d) causing reaction of end groups X with end groups Y, and either end groups X or end groups Y with the surface of the support; or

2. (ii)

1. (a) providing an aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups X;

2. (b) providing an multifunctional linking agent having at least two reactive groups Y which are reactive with polymer end groups X;
3. (c) depositing said suspension of vesicles and said multifunctional linker on a support having a surface which is reactive with either polymer end groups X or reactive groups Y; and
4. (d) causing reaction of end groups X with groups Y, and either end groups X or groups Y with the surface of the support, wherein in either (i) or (ii), groups X are amine groups and groups Y are carboxylic acid, activated carboxylic acid, and/or azide groups; or groups X are carboxylic acid, activated carboxylic acid, and/or azide groups, and groups Y are amine groups; or groups X and Y are click chemistry groups.

**[0011]** Preferably, the filtration membrane is a water filtration membrane, and preferably the transmembrane protein is an aquaporin. Throughout this Specification and claims, unless the context requires otherwise, any reference to a filtration membrane should be understood to include a specific reference to a water filtration membrane, and any reference to a transmembrane protein should be understood to include a specific reference to an aquaporin.

#### **Detailed description of the invention**

**[0012]** In complete contrast to the process of Xie mentioned above, it is an essential feature of the present invention that the support carries a layer of vesicles in which multiple vesicles are close packed together. The packing in the layer may for example be hexagonal close packing. The layer of vesicles present on the support surface is thicker than the average diameter of the vesicles, i.e. it is of greater thickness than would be provided by a single layer of vesicles. It is preferred that the layer should have a thickness equivalent to at least 2, for example at least 10, preferably at least 50, more preferably at least 150, and most preferably at least 200, times the average diameter of vesicles. Preferably the layer is not more than 500 times, for example not more than 300 times, the average diameter of a vesicle. So, for example, the layer may have a thickness of from 2 to 500, for example from 50 to 300, especially from 200 to 300 times the average diameter of the vesicles. In absolute terms, the thickness of the vesicle layer is preferably at least 0.01, for example at least 0.04, for example at least 0.1, for example at least 0.2, for example at least 2, preferably at least 10, more preferably at least 30, and most preferably at least 40, microns. There is no particularly preferred maximum thickness for the layer. The layer may for example have a thickness up to 100, for example up to 60, microns. So, for example, the layer may have a thickness of from 0.01 to 100, for example from 0.04 to 100, for example from 0.2 to 100, preferably from 10 to 60, especially from 40 to 60, microns.

**[0013]** To increase robustness, the layer of vesicles in the finished membrane is preferably provided with a protective top coating layer, or a second support layer on the opposite side from the support layer. This top coating may for example provide added protection from

mechanical damage during a rolling process. It may for example comprise a hydrophilic polymer, for example polyvinylalcohol.

**[0014]** The process of the invention is carried out according to either the first or second preferred embodiments described herein. In the first preferred embodiment, there is provided a process for the preparation of a membrane according to the invention, which comprises:

1. (a) providing an aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups X;
2. (b) providing a multifunctional linking agent having at least two reactive groups Y which are reactive with polymer end groups X;
3. (c) depositing said suspension of vesicles and said multifunctional linker on a support having a surface which is reactive with either polymer end groups X or reactive groups Y; and
4. (d) causing reaction of end groups X with groups Y, and either end groups X or groups Y with the surface of the support.

**[0015]** In the second preferred embodiment, there is provided a process for the preparation of a membrane according to the invention, which comprises:

1. (a) providing a first aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups X;
2. (b) providing a second aqueous suspension of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups Y which are reactive with polymer end groups X;
3. (c) depositing said suspensions of vesicles on a support having a surface which is reactive with either polymer end groups X or Y; and
4. (d) causing reaction of end groups X with end groups Y, and either end groups X or end groups Y with the surface of the support.

**[0016]** The process of the invention results in a physically robust layer of polymer vesicles linked to each other, optionally via a linker, and also linked to the surface of the support.

**[0017]** It is not necessary that all the block copolymer molecules used in the invention should have reactive end groups. The proportion of block copolymer molecules having reactive end groups is not critical, provided that there are sufficient groups to react with reactive groups either in a second population of vesicles or in a multifunctional linker, to form a coherent mass. Generally, at least 10%, for example at least 20%, for example at least 30 %, for example at least 40%, for example up to 60%, or up to 100%, of the block copolymer molecules used to

form the vesicles will have functional end groups X or Y. Similarly, it is not required that only one type of end group X or Y is present. It may for example be desired to use blends of block copolymers, one containing one reactive end group X(1), for example an end group including an -NH<sub>2</sub> group, and the second containing a different reactive end group X(2).

**[0018]** The end groups on any particular polymer molecule may be the same as each other, or they may be different, but preferably they are the same. For example, one end group may be a reactive end group X, while the other end group may be a non-reactive group. The exact nature of the groups will of course depend on the nature of the process and also on the nature of the surface of the support.

**[0019]** Suitable reactive groups are amine groups (reactive with for example carboxylic acid, activated carboxylic acid and/or azide groups), carboxylic acid, activated carboxylic acid and/or azide groups (reactive with for example amine groups Y), and "click chemistry" groups (for example azide or alkyne groups, which are respectively reactive with alkyne and azide groups Y). The use of amine groups is particularly preferred.

**[0020]** A wide variety of amine-based end groups is available, and these may contain -NH<sub>2</sub> and/or -NH- groups. It has been found that when providing amphiphilic block copolymers with such end groups, the ability of the block copolymer to self-assemble into vesicles is enhanced: this is surprising, as generally it is expected that the properties of amphiphilic block copolymers which most influence vesicle formation are (i) the size and nature of the blocks; and (ii) the polydispersity of the polymer.

**[0021]** When using a multifunctional linking agent, the reactive groups present in that agent may be the same as each other, or they may be different. They must be such as to react with complementary reactive groups present in the vesicles and/or with the surface of the support. Suitable groups are as mentioned above. When using a multifunctional reagent, the reagent may for example contain 3 or 4 reactive groups, but preferably it contains two reactive groups, and any reference herein to a multifunctional reagent should be understood to include a specific reference to a difunctional reagent.

**[0022]** In a preferred embodiment of the invention, the vesicles contain reactive groups which include an amine group; and a complementary reactive group is provided which is an activated carboxylic acid group or an azide, for example a phenylazide, group.

**[0023]** In one embodiment of the invention, the surface of the support may be functionalised in one or more steps to introduce specific reactive groups Z capable of reacting with complementary reactive groups X and/or Y. Suitable groups include amine groups (reactive with for example carboxylic acid or activated carboxylic acid groups X and/or Y); carboxylic acid or activated carboxylic acid groups, (reactive with for example amine groups X and/or Y); and "click chemistry" groups (for example azide or alkyne groups reactive with alkyne or azide groups X and/or Y). One example of a multi-step functionalization of a surface is hydrolysis of a polyacrylonitrile surface using acid, e.g. hydrochloric acid, to introduce surface carboxylic acid

groups, which may subsequently be activated using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) followed by conversion into alkyne groups, for example using propargylamine, or into azide groups, for example using amino-triethyleneglycol-azide. However, in another embodiment of the invention, it may not be necessary to functionalise the surface of the support, because X and/or Y may be reactive with groups already present in the material forming the support. For example, Y may be an azide group: such groups are highly reactive once activated using UV light, and are capable of reacting with C-H bonds present in many polymers present in support materials. Specifically, azide, especially phenylazide, groups are capable of covalently bonding with polysulfones, which as discussed below, are a preferred support material for use in the present invention.

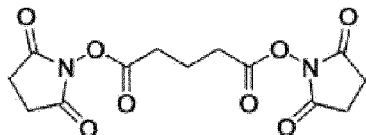
**[0024]** Where reference is made to an activated carboxylic acid group, this should be understood to include any conventional activated carboxylic acid group, for example an activated ester such as an N-hydroxysuccinimide ester, or an acid halide. Such activation techniques are well known in the art. In a preferred embodiment, activated carboxylic acid end groups are produced by the reaction of a carboxylic acid group with EDC and NHS. This is a well-known technique often used in the world of protein conjugation and immobilization. The reaction of a carboxyl group with EDC and NHS results in formation of an amine reactive NHS ester.

**[0025]** When using a multifunctional linker, its exact nature is not crucial, provided that it is capable of reacting efficiently to cause linking of the vesicles together by reaction of the X and Y groups.

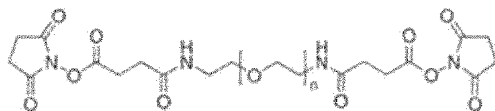
**[0026]** Suitable multifunctional linkers include homobifunctional crosslinkers, that is, crosslinkers with the same functionalities at both ends. Examples which are capable of binding to amine groups include:

1. (i) NHS esters. Typical esters include:

disuccinimidyl glutarate:

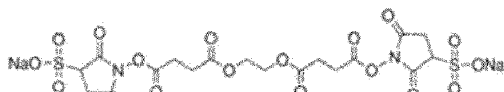


bis(succinimidyl) polyethylene glycol:

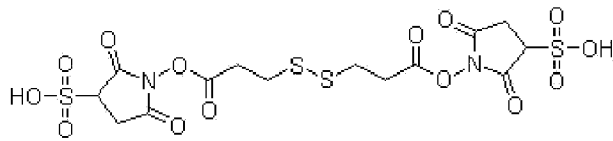


for example bis(succinimidyl penta(ethylene glycol));

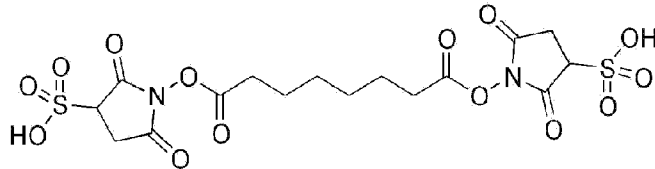
ethylene glycol bis(sulfosuccinimidylsuccinate):



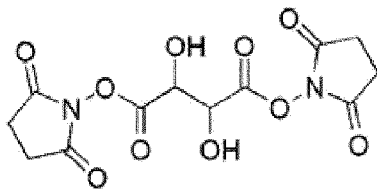
3,3'-dithiobis(sulfosuccinimidylpropionate):



bis(sulfosuccinimidyl)suberate:



disuccinimidyl tartrate:

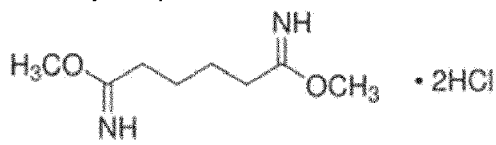


Reagents of this type react with primary amines in slightly alkaline conditions, for example at a pH of 7.2-8.5, for example 7.2-8.0, and yield stable amide bonds. Reaction temperatures are typically in the range of from 0 to 30, for example from 4 to 25°C. The reaction produces N-hydroxysuccinimide which can be removed via dialysis or desalting. The reaction may for example be carried out in PBS buffer at pH 7.2-8.0 for 0.5 to 4 hours at room temp or 4°C.

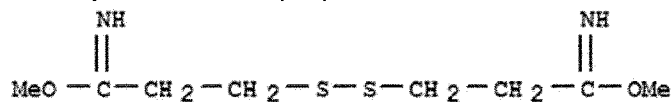
Sulfo NHS esters contain an -SO<sub>3</sub> group on the NHS ring. This has no effect on the chemistry of the reaction, but such reagents tend to have increased water solubility.

2. (ii) Imidoesters. Typical imidoesters include the following (often obtained as dihydrochloride salts):

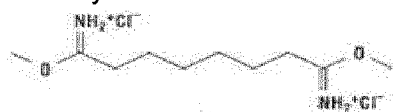
dimethyl adipimidate:



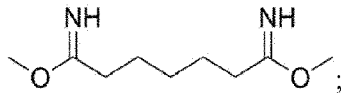
dimethyl 3,3'-dithiobispropionimidate:



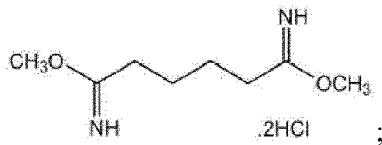
dimethyl suberimidate:



dimethyl pimelimidate:

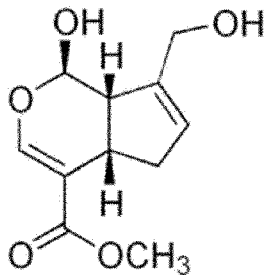


dimethyl adipimidate:

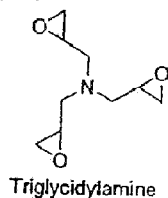


Imidoesters react with primary amines to form amidine bonds. To ensure specificity for primary amines, the reaction is typically carried out in amine-free alkaline conditions (pH 9-11, for example pH10) with borate buffer.

3. (iii) genipin, which has the formula:



4. (iv) epoxides, for example triglycidylamine:

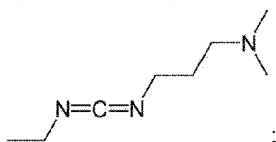


5. (v) dialdehyde compounds, for example  $\text{HOC}(\text{CH}_2)_x\text{CHO}$ , where  $x$  is 1 to 6. Typical dialdehydes include glutaraldehyde, succinaldehyde, glyoxal, malondialdehyde, and phthalaldehyde.

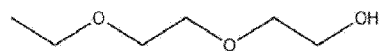
6. (vi)  $\text{COOH-PEG-COOH}$ . This reagent is water-soluble, and if desired may be activated with EDC/NHS to provide reactivity with amines.

**[0027]** Suitable multifunctional linkers also include heterobifunctional crosslinkers, that is, crosslinkers with different functionalities at both ends. Examples include:

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (usually obtained in the form of the hydrochloride):



carbital



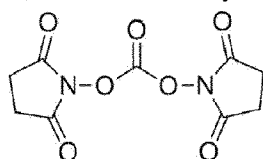
epoxides, for example triglycidalamine;



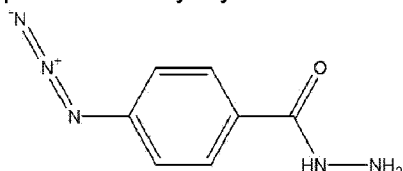
sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate;

poly(2-hydroxyethyl-co-2-methacryloxyethyl aspartamide);

N,N'-disuccinimidyl carbonate:

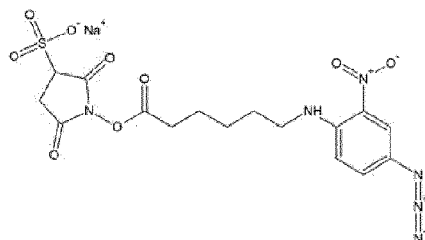


p-azidobenzoyl hydrazide:



**[0028]** The process of the invention may utilise "click chemistry", which may for example utilise the reaction of an azide with an alkyne. For example, an alkyne group may be introduced as a group X or Y by reaction of a primary amine with an NHS ester. Many azide-PEG-azide linkers are available commercially.

**[0029]** Preferably a multifunctional linker includes a  $(\text{CH}_2)_m$  chain in which m is from 2 to 20, preferably from 3 to 10, especially from 3 to 9. An especially preferred difunctional linker is the commercially available product N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate. This product has the formula:



**[0030]** The sulfosuccinimide group is a reactive group Y which is an activated carboxylic acid ester, capable of reacting spontaneously with amine groups. The phenylazide group is a group Y which is inert under light-free conditions, but becomes highly reactive when activated using

UV light, reacting readily with amine groups. In the absence of amine groups, the activated group is also capable of reacting with groups of a lower reactivity, even in some circumstances with a C-H bond; specifically, it is capable of reacting with the aromatic C-H groups in a polysulfone.

**[0031]** The conditions under which step (d) of the process of the invention described above, i.e. causing reaction of complementary reactive groups X and Y, and reaction of either X or Y with the surface of the support, is carried out, will of course depend on the nature of the various reactive groups. In some embodiments, the reactive groups will react with each other spontaneously once contacted together under suitable conditions. In other embodiments, photo-activatable groups may be present, in which case the reactants may be contacted together, and subsequently photoirradiated to initiate reaction. In a preferred embodiment of the process of the invention, both mechanisms are combined by using a multifunctional reagent having a first group Y which reacts on contact with an end group X, and a second group Y which reacts with an end group X and with the surface of the support on irradiation with UV light.

**[0032]** Thus, the steps of one embodiment of the process of the invention may be carried out as follows:

(a) providing an aqueous solution of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups X;

(b) providing an multifunctional, preferably difunctional, linking agent having at least two reactive groups Y which are reactive with polymer end groups X, including a first reactive group Y(1) being capable of reaction with polymer end groups X under a first set of reaction conditions, and a second reactive group Y(2) which is unreactive with polymer end groups X under said first set of reaction conditions but which is reactive with polymer end groups X under a second set of reaction conditions;

(b') mixing said aqueous solution of vesicles with said multifunctional linking agent under said first set of reaction conditions so that reactive group Y(1) reacts with polymer end groups X;

(c) depositing the resulting solution on a support which is reactive with second reactive group Y(2), in an amount sufficient to produce the desired layer of vesicles; and

(d) causing reaction of end groups X with said second reactive group Y(2), and second reactive end groups Y(2) with the surface of the support, by applying said second set of reaction conditions.

**[0033]** Any suitable reaction conditions which differentiate the two reaction steps may be used. For example, the first set of reaction conditions may involve groups X and Y(1) which react at a first temperature while the second set of reaction conditions may involve groups X and Y(2)

which react at a second, higher, temperature. However, in a preferred embodiment, X and Y(1) are such that they react spontaneously on contact, or with heating if necessary, while X and Y(2) are such that they react only when activated by photoirradiation. Accordingly, a particularly preferred process comprises:

- (a) providing an aqueous solution of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having reactive end groups X;
- (b) providing a multifunctional, preferably difunctional, linking agent having at least two reactive groups Y which are reactive with polymer end groups X, including a first reactive group Y(1) being capable of reaction with polymer end groups X on contact, and a second reactive group Y(2) being capable of reaction with polymer end groups X on photoirradiation;
- (b') mixing said aqueous solution of vesicles with said multifunctional linking agent under conditions such that said first reactive group Y(2) reacts with polymer end groups X;
- (c) depositing the resulting solution on a support which is reactive with second reactive group Y(2), in an amount sufficient to produce the desired layer of vesicles; and
- (d) applying photoirradiation to cause reaction of end groups X with said second reactive group Y(2), and second reactive end groups Y(2) with the surface of the support.

**[0034]** In a particularly preferred embodiment, the invention provides a process which comprises:

1. (a) providing an aqueous solution of vesicles having transmembrane proteins incorporated therein, said vesicles being formed from amphiphilic block copolymers having terminal amine groups;
2. (b) mixing said aqueous solution of vesicles with N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate under conditions such that reaction between the succinimidyl group of said hexanoate reacts with said polymer terminal amine groups;
3. (c) depositing the resulting solution on a support; and
4. (d) irradiating said deposit with UV light to cause reaction of the azide group of said hexanoate with polymer terminal amine groups and with the surface of the support. Within this embodiment, preferably the block copolymer is one of those preferred polymers mentioned below, particularly amine-terminated [(poly)2-methyl-2-oxazoline] [(poly)dimethyl siloxane] [(poly)2-methyl-2-oxazoline].

**[0035]** In all the above embodiments, the amount of suspension deposited in step (c) is sufficient to provide the surface of the support with a continuous layer of vesicles. Generally, after step (d) has been carried out, this layer will be in the form of a coherent mass which has a thickness greater than the average diameter of the vesicles; or, in absolute terms, has a

thickness of at least 0.04 microns.

**[0036]** A very wide range of reaction conditions may be used to effect the process of the invention. In one embodiment, when using a multifunctional linker, the quantity of multifunctional linker used will be such that the total quantity of reactive groups Y present is in excess of the total quantity of polymer end groups X present to ensure adequate crosslinking. Control of pH, temperature and other reaction conditions is conventional and within the normal practice of the skilled man.

**[0037]** The amphiphilic block copolymer is a diblock copolymer AB having a hydrophilic and a hydrophobic block, or, preferably, a triblock copolymer ABA having hydrophilic end blocks and a hydrophobic inner block. The use of such copolymers in the formation of vesicles is well known, and a very wide range of hydrophilic polymers and hydrophobic polymers may form the blocks A and B.

**[0038]** Hydrophobic polymers include for example polysiloxanes, for example polydimethylsiloxane or polydiphenylsiloxane, perfluoropolyether, polystyrene, polyoxypropylene, polyvinylacetate, polyoxybutylene, polyisoprene, polybutadiene, polyvinylchloride, polyalkylacrylates, polyalkylmethacrylates, polyacrylonitrile, polypropylene, polytetrahydrofuran, polymethacrylates, polyacrylates, polysulfones, polyvinylethers, and poly(propylene oxide), and copolymers thereof.

**[0039]** The hydrophobic segment preferably contains a predominant amount of hydrophobic monomers. A hydrophobic monomer is a monomer that typically gives a homopolymer that is insoluble in water and can absorb less than 10% by weight of water.

**[0040]** Suitable hydrophobic monomers are dimethylsiloxanes, C<sub>1</sub>-C<sub>18</sub> alkyl and C<sub>3</sub>-C<sub>18</sub> cycloalkyl acrylates and methacrylates, C<sub>3</sub>-C<sub>18</sub> alkylacrylamides and -methacrylamides, acrylonitrile, methacrylonitrile, vinyl C<sub>1</sub>-C<sub>18</sub> alkanoates, C<sub>2</sub>-C<sub>18</sub> alkenes, C<sub>2</sub>-C<sub>18</sub> haloalkenes, styrene, (lower alkyl)styrene, C<sub>4</sub>-C<sub>12</sub> alkyl vinyl ethers, C<sub>2</sub>-C<sub>10</sub> perfluoro-alkyl acrylates and methacrylates and correspondingly partially fluorinated acrylates and methacrylates, C<sub>3</sub>-C<sub>12</sub> perfluoroalkylethylthiocarbonylaminoethyl acrylates and methacrylates, acryloxy- and methacryloxyalkylsiloxanes, N-vinylcarbazole, C<sub>1</sub>-C<sub>12</sub> alkyl esters of maleic acid, fumaric acid, itaconic acid, mesaconic acid, vinyl acetate, vinyl propionate, vinyl butyrate, vinyl valerate, chloroprene, vinyl chloride, vinylidene chloride, vinyltoluene, vinyl ethyl ether, perfluorohexyl ethylthiocarbonylaminoethyl methacrylate, isobornyl methacrylate, trifluoroethyl methacrylate, hexa-fluoroisopropyl methacrylate, hexafluorobutyl methacrylate, tris(trimethylsilyloxy)silylpropyl methacrylate, and 3-methacryloxypropylpentamethyldisiloxane.

**[0041]** The hydrophobic polymer may include a single type of polymer or more than one type of polymer, such as two or more of those mentioned above.

**[0042]** A preferred hydrophobic polymer is a polysiloxane, especially (poly)dimethylsiloxane.

**[0043]** The mean molecular weight (g/mol) of one segment B is preferably in the range from about 500 to about 50,000, preferably in the range from about 800 to about 15,000, more preferably in the range of about 1,000 to 12,000, particularly preferably in the range from about 5,000 to about 12,000.

**[0044]** In addition to the hydrophobic segment B, the amphiphilic copolymer includes at least one, preferably two, segments A which include at least one hydrophilic polymer, for example polyoxazoline, polyethylene glycol, polyethylene oxide, polyvinyl alcohol, polyvinylpyrrolidone, polyacrylamide, poly(meth)acrylic acid, polyethylene oxide-co-polypropyleneoxide block copolymers, poly(vinylether), poly(N,N-dimethylacrylamide), polyacrylic acid, polyacyl alkylene imine, polyhydroxyalkylacrylates such as hydroxyethyl methacrylate, hydroxyethyl acrylate, hydroxypropyl acrylate, and polyols, and copolymers thereof

**[0045]** The hydrophilic segment preferably contains a predominant amount of hydrophilic monomers. A hydrophilic co-monomer is a monomer that typically gives a homo-polymer that is soluble in water or can absorb at least 10% by weight of water.

**[0046]** Suitable hydrophilic monomers include hydroxy 1-substituted lower alkyl acrylates and methacrylates, acrylamide, methacrylamide, (lower alkyl) acrylamides and methacrylamides, N,N-dialkyl-acrylamides, ethoxylated acrylates and methacrylates, polyethyleneglycol-mono methacrylates and polyethyleneglycolmonomethylether methacrylates, hydroxyl-substituted (lower alkyl)acrylamides and methacrylamides, hydroxyl-substituted lower alkyl vinyl ethers, sodium vinylsulfonate, sodium styrenesulfonate, 2-acrylamido-2-methylpropanesulfonic acid, N-vinylpyrrole, N-vinyl-2-pyrrolidone, 2-vinyloxazoline, 2-vinyl-4,4'-dialkyloxazolin-5-one, 2- and 4-vinylpyridine, vinylically unsaturated carboxylic acids having a total of 3 to 5 carbon atoms, amino(lower alkyl)- (where the term amino also includes quaternary ammonium), mono(lower alkylamino)(lower alkyl) and di(lower alkylamino)(lower alkyl) acrylates and methacrylates, allyl alcohol, 3-trimethylammonium 2-hydroxypropylmethacrylate chloride (Blemer,QA, for example from Nippon Oil), dimethylaminoethyl methacrylate (DMAEMA), dimethylaminoethylmethacrylamide, glycerol methacrylate, and N-(1,1-dimethyl-3-oxobutyl)acrylamide.

**[0047]** Specific examples of hydrophilic monomers from which such polymers can be made are cyclic imino ethers, vinyl ethers, cyclic ethers including epoxides, cyclic unsaturated ethers, N-substituted aziridines,  $\beta$ -lactones and  $\beta$ -lactams. Further suitable monomers include ketene acetals, vinyl acetals and phosphoranes. Suitable cyclic imino ethers include 2-oxazoline. If a 2-oxazoline having an alkenyl group in 2 position is used as hydrophilic monomer, a polymerizable unsaturated group is provided within segment A (in a side chain) of the amphiphilic segmented copolymer to serve as the polymerizable unsaturated group necessary for the final polymerization to obtain a polymeric product or as an additional polymerizable unsaturated group which offers the possibility of direct crosslinking in the preparation of the polymer. The most preferred cyclic imino ether is 2-C<sub>1-3</sub>alkyloxazoline, especially 2-methyloxazoline. The most preferred vinyl ethers are methyl vinyl ether, ethyl vinyl ether and

methoxy ethyl vinyl ether.

**[0048]** A preferred hydrophilic polymer block is (poly)2-C<sub>1-3</sub>alkyl-2-oxazoline, especially (poly)2-methyl-2-oxazoline.

**[0049]** The mean molecular weight (g/mol) of one segment A is suitably in the range from about 200 to about 50,000, preferably in the range from about 800 to about 15,000, more preferably in the range of about 1,000 to 12,000, particularly preferably in the range from about 5,000 to about 12,000.

**[0050]** Synthesis of block copolymers by polymerisation is well known, and the length of the one or more segments which are to be copolymerized on the starting segment can be easily controlled by controlling the amount of monomer (hydrophilic or hydrophobic) which is added for the copolymerization, and/or by the addition of suitable chain-terminating capping agents. In this way the size of the segments and their ratio can easily be controlled.

**[0051]** As is well known in the art, the absolute and relative lengths of the hydrophilic and hydrophobic blocks are important in determining the suitability of the copolymers for forming vesicles (so called polymer hydrophobic ratio). Further, the length of the blocks should be such that the thickness of the vesicle wall is broadly comparable with the length of the transmembrane protein so that the protein can be readily incorporated into the vesicle walls without the channel becoming blocked. For example the thickness of the vesicle wall may be in the range of from 1 nm to 50 nm. The length of the hydrophobic block B is particularly important, and this should preferably be no greater than 150 repeat units.

**[0052]** An especially preferred block copolymer for use in the present invention is PAOXA-a-PDMS-b-PAOXA-a, especially PMOXA-a-PDMS-b-PMOXA-a, in which PAOXA is (poly)2-C<sub>1-3</sub>alkyl-2-oxazoline, PMOXA is (poly)2-methyl-2-oxazoline, and PDMS is (poly)dimethyl siloxane. Preferably each a independently is a number between 5 and 100, preferably between 10 and 100, and b is a number between 5 and 150, preferably between 20 and 150. Various PAOXA-PDMS-PAOXA polymers are commercially available, and others can be readily synthesised by known methods.

**[0053]** Reactive end groups X may be present following initial synthesis of the copolymer, or they may be introduced following the copolymer synthesis. For example, the copolymers may already contain suitable end groups when using a particular monomer, for example a dienepolymer such as polybutadiene or polyisoprene, or if the monomer used for making a hydrophilic segment comprises an unsaturated side chain, for example 2-allyl-oxazoline. Alternatively, the polymer may already contain reactive end groups when the polymerisation has been stopped by use of a suitable capping agent. If not present following initial synthesis, it is possible to introduce reactive groups by suitable reactions at the end of the relevant block. For this purpose, the polymerization of the growing segment may be terminated after a suitable chain length is reached and the initiator group present at the chain end capped, for example, either by using specific reagents such as hydroxy styrene, allyl alcohol,

hydroxyethylmethacrylate, propargyl alcohol, allyl amines and propargyl amine, or by using KOH/EtOH or primary amines leaving -OH or -NH- groups or unsaturated groups at the end of the growing segment. Hydroxyl groups may also be introduced into the copolymers by employing suitable comonomers in the copolymerization, e.g. 2-hydroxy-alkyloxazolines. The hydroxyl or -NH- groups may then be reacted, e.g. with an isocyanate carrying a polymerizable unsaturated group. Preferred examples of such bifunctional compounds are vinyl isocyanate, allyl isocyanate, acryloyl isocyanate, styrene isocyanate, vinyl benzyl isocyanate, and propargyl isocyanate. Other reactive groups can be introduced by methods known to those skilled in the art.

**[0054]** In an especially preferred embodiment, the polymers used in the present invention have amine end groups. Most preferably the polymer is amine-terminated PAOXA-a-PDMS-b-PAOXA-a, for example one of those PAOXA-PDMS-PAOXA polymers mentioned above, carrying amine end groups.

**[0055]** An amine end group may contain an -NH<sub>2</sub> group or an -NH- group, or both. In a particularly preferred embodiment of the invention, the amphiphilic block copolymers are terminated by end groups X having the formula -NHR in which R represents an alkyl group which may be straight-chain or branched having from 1 to 6 carbon atoms substituted by at least one, for example 1, 2 or 3, -NH<sub>2</sub> groups. Preferably such an end group X has the formula -NH-CH-(NH<sub>2</sub>)<sub>2</sub> or, preferably, -NH-(CH<sub>2</sub>)<sub>n</sub>-NH<sub>2</sub>, in which n is an integer from 2 to 6, preferably 2 to 4, especially 2. Such end groups may be introduced by reacting a polymer having -OH end groups with a suitable reactive amine NH<sub>2</sub>R, for example a diamine, for example H<sub>2</sub>N-(CH<sub>2</sub>)<sub>n</sub>-NH<sub>2</sub>, especially H<sub>2</sub>N-(CH<sub>2</sub>)<sub>2</sub>-NH<sub>2</sub>, or triamine, for example N.([CH<sub>2</sub>]<sub>n</sub>NH<sub>2</sub>)<sub>3</sub> or CH.([CH<sub>2</sub>]<sub>n</sub>NH<sub>2</sub>)<sub>3</sub>, for example CH(NH<sub>2</sub>)<sub>3</sub> or tris(3-aminopropyl)amine. Branched oligomeric imines may also be used.

**[0056]** Amphiphilic block copolymers of the type PAOXA-a-PDMS-b-PAOXA-a, in which PAOXA is (poly)2- C<sub>1-3</sub>alkyl -2-oxazoline and PDMS is (poly)dimethyl siloxane, which contain end groups including both an -NH<sub>2</sub> group or an -NH- group, i.e. containing both a primary and a secondary amine group, especially -NH-CH-(NH<sub>2</sub>)<sub>2</sub> or -NH-(CH<sub>2</sub>)<sub>n</sub>-NH<sub>2</sub>, are novel and are claimed in our copending application reference no. 22883 WO. Vesicles formed from such polymers and having transmembrane proteins incorporated therein are also novel and claimed in our copending application.

**[0057]** Block copolymers can be prepared in the form of vesicles by methods well known in the art. Generally, these methods involve either solvent displacement or solvent-free rehydration. In solvent displacement methods, the block copolymer is dissolved in an organic solvent before mixing with water. After mixing, and optionally removing the organic solvent, spontaneous self-assembly of vesicles results. In solvent-free rehydration, dry block copolymer is brought into contact with an aqueous medium whereupon hydration results in the spontaneous self-assembly of vesicles. In a special case of solvent-free rehydration, the thin-film rehydration process, block copolymer is dissolved in an organic solvent which is then removed under

conditions such that a thin film is formed. This film is then hydrated by contacting with water.

**[0058]** Vesicles having a desired size and low polydispersity can be obtained by known methods, for example by extrusion of large uni- and multi-lamellar polydisperse vesicles through one or more membranes of known pore size. Track etched polycarbonate membranes, for example Isopore (Trade Mark) membranes available from Millipore, are suitable for this purpose. Suitably, the vesicles used in the present invention have an average diameter in the range of from 30 to 10,000, preferably 50 to 1000, more preferably 100 to 400, especially from 150 to 250, nm.

**[0059]** The propensity of known PAOXA-a-PDMS-b-PAOXA-a polymers to form vesicles, rather than other self-assembly structures such as micelles, depends primarily on the absolute and relative sizes of the blocks. Thus, when the polymer is terminated with -OH groups, and when the blocks are relatively high molecular weight, for example as in PAOXA<sub>14</sub>PDMS<sub>55</sub>PAOXA<sub>14</sub> or higher, micelles tend to be formed, which means that lower molecular weight polymers need to be used if vesicles are required. Surprisingly, the presence of an end group including both an -NH<sub>2</sub> and an -NH- group makes a major difference, and the use of PAMOXA-a-PDMS-b-PAOXA-a, for example PAOXA<sub>14</sub>PDMS<sub>55</sub>PAOXA<sub>14</sub> and in particular PMOXA<sub>14</sub>PDMS<sub>55</sub>PMOXA<sub>14</sub> having such end groups, for example:



particularly



has proved particularly valuable for the preparation of vesicles.

**[0060]** Overall, the use of functional group terminated polymers, particularly amine-terminated polymers, together with a complementary multifunctional linking agent gives major advantages compared with known processes for the preparation of working filtration membranes.

**[0061]** However the vesicles are formed, the vesicle formation process can be carried out in the presence of transmembrane proteins, especially aquaporins, whereby the transmembrane protein becomes incorporated into the wall of the vesicle. Generally, the process is carried out in the presence of a detergent which assists in maintaining the integrity and biological function of the protein. Thus, the above rehydration steps may be carried out using an aqueous solution of a transmembrane protein, preferably also including a detergent. The use of aquaporins is preferred, and aquaporins are robust under a wide range of process conditions.

**[0062]** Aquaporins are biological cell transmembrane proteins whose function is to selectively transport water and no other molecules; the transport channel of the protein is a two-way channel through which water can flow in either direction. They are expressed by many human

cell types, and also by bacterial and plant cells. Any of the different members of the aquaporin family of proteins can be used in the present invention. Suitable aquaporins include Aqp 4, Aqp1 and, especially, Aqp Z. Aquaporins may exist in monomeric, dimeric, tetrameric and higher oligomeric forms, as well as mutated, conjugated and truncated versions of the primary sequence. Provided that the biological function of the aquaporin, i.e. the selective transport of water, is maintained, any of these may be used in the present invention.

**[0063]** Any other transmembrane protein having desirable transport properties may be used in the present invention. Variants of such transmembrane proteins, including naturally or non-naturally occurring variants and orthologs or paralogs of such proteins may be used. Such proteins include for example:

- **Monotopic Membrane Proteins**

**[0064]**

- **Cyclooxygenases**

- Ram Prostaglandin H2 synthase-1 (cyclooxygenase-1 or COX-1): *Ovis aries*
- Ram Prostaglandin H2 synthase-1 (COX-1) R120Q/Native Heterodimer: *Ovis aries*
- Aspirin Acetylated COX-1
- Cyclooxygenase-2: *Mus Musculus*

- **Squalene-Hopene Cyclases**

- Squalene-hopene cyclase: *Alicyclobacillus acidocaldarius*

- **Monoamine Oxidases**

- Monoamine Oxidase B: Human mitochondrial outer membrane
- Monoamine Oxidase A: Rat mitochondrial outer membrane
- Monoamine Oxidase A: Human mitochondrial outer membrane
- G110A mutant

- **Hydrolases**

- Fatty acid amide hydrolase: *Rattus norvegicus*

- **Oxidoreductases (Monotopic)**

- Sulfide:quinone oxidoreductase in complex with decylubiquinone: *Aquifex aeolicus*
- Electron Transfer Flavoprotein-ubiquinone oxidoreductase (ETF-QO): *Sus scrofa*

- **Peptidoglycan Glycosyltransferases**

- Peptidoglycan Glycosyltransferase: *Staphylococcus aureus*
- Peptidoglycan Glycosyltransferase penicillin-binding protein 1a (PBP1a): *Aquifex aeolicus*
- Peptidoglycan Glycosyltransferase penicillin-binding protein 1b (PBP1b): *Escherichia coli*
- **Peptidases**
  - Signal Peptidase (SPase): *Escherichia coli*
  - Signal Peptide Peptidase (SppA), native protein: *Escherichia coli*
- **Dehydrogenases**
  - Glycerol-3-phosphate dehydrogenase (GlpD, native): *Escherichia coli*
- **Dihydroorotate Dehydrogenases (DHODH, class 2)**
  - Dihydroorotate Dehydrogenase: *Escherichia coli*
  - Dihydroorotate Dehydrogenase: *Rattus rattus*
  - Dihydroorotate Dehydrogenase, apo form: *Homo sapiens*
  - Dihydroorotate Dehydrogenase: *Plasmodium falciparum*, 3d7
- **Polymerases**
  - TagF teichoic acid polymerase: *Staphylococcus epidermidis*
- **ADP-Ribosylation Factors**
  - ADP-ribosylation factor (ARF1), myristoylated: *Saccharomyces cerevisiae*
  - ADP-ribosylation factor (ARF1\*GTP), myristoylated: *Saccharomyces cerevisiae*
- **Isomerases**
  - RPE65 visual cycle retinoid isomerase: *Bos Taurus*

- **Transmembrane Proteins: Beta-Barrel**

[0065]

- **Beta-Barrel Membrane Proteins: Multimeric**
  - Porin: *Rhodobacter capsulatus*
  - Porin: *Rhodospirillum rubrum*

- OmpK36 osmoporin: *Klebsiella pneumonia*
- Omp32 anion-selective porin: *Comamonas acidovorans*
- Omp32 anion-selective porin: *Delftia acidovorans*
- OmpF Matrix Porin: *Escherichia coli*
- OmpC Osmoporin: *Escherichia coli*
- OmpG \*monomeric\* porin: *Escherichia coli*
- PhoE: *Escherichia coli*
- LamB Maltoporin: *Salmonella typhimurium*
- LamB Maltoporin: *Escherichia coli*
- LamB Maltoporin: *Escherichia coli*
- ScrY sucrose-specific porin: *Salmonella typhimurium*
- MspA mycobacterial porin: *Mycobacterium smegmatis*
- OprP phosphate-specific transporter: *Pseudomonas aeruginosa*
- OprD basic amino acid uptake channel: *Pseudomonas aeruginosa*
- OpdK hydrocarbon transporter: *Pseudomonas aeruginosa*
- PorB outer membrane protein, native structure: *Neisseria meningitidis*
- **Beta-Barrel Membrane Proteins: Monomeric/Dimeric**
- TolC outer membrane protein: *Escherichia coli*
- TolC outer membrane protein, ligand blocked: *Escherichia coli*
- TolC outer membrane protein (Y362F, R367E): *Escherichia coli*
  - C2 Form
  - P2:2:2 form
- VceC outer membrane protein: *Vibrio cholera*
- OprM drug discharge outer membrane protein: *Pseudomonas aeruginosa*
- CusC heavy metal discharge outer membrane protein: *Escherichia coli*
- CusBA heavy-metal efflux complex outer membrane protein: *Escherichia coli*
- BenF-like Porin (putative): *Pseudomonas fluorescens*

- OprM drug discharge outer membrane protein: *Pseudomonas aeruginosa*
- apo BtuB cobalamin transporter: *Escherichia coli*
- BtuB: *Escherichia coli*
- apo BtuB by in meso crystallization: *Escherichia coli*
- Colicin I receptor: *Escherichia coli*
- OmpA: *Escherichia coli*, 2.5Å
- OmpA with four shortened loops: *Escherichia coli*
  - Called  $\beta$ -barrel platform (BBP)
- OmpT outer membrane protease: *Escherichia coli*
- Pla Plasminogen activator (native 1): *Yersinia pestis*
- OmpW outer membrane protein: *Escherichia coli*
  - Orthorhombic Form
  - Trigonal Form
- OprG outer membrane protein: *Pseudomonas aeruginosa*
- OmpX: *Escherichia coli*
- TtoA Outer Membrane Protein (OMP): *Thermus thermophilus* HB27
- OmpLA (PldA) outer membrane phospholipase A monomer: *Escherichia coli*
- OmpLA (PldA) active-site mutant (N156A): *Escherichia coli*
- OpcA adhesin protein: *Neisseria meningitidis*
- NspA surface protein: *Neisseria meningitides*
- NaIP autotransporter translocator domain: *Neisseria meningitides*
- NanC Porin, model for KdgM porin family: *Escherichia coli*
- Hia1022-1098 trimeric autotransporter: *Haemophilus influenza*
  - Hia992-1098
- EspP autotransporter, postcleavage state: *Escherichia coli*
- EstA Autotransporter, full length: *Pseudomonas aeruginosa*
- PagP outer membrane palmitoyl transferase: *Escherichia coli*

- FadL long-chain fatty acid transporter: *Escherichia coli*
- FadL long-chain fatty acid transporter A77E/S100R mutant: *Escherichia coli*
  - ΔS3 kink
  - P34A mutant
  - N33A mutant
  - ΔNPA mutant
  - G212E mutant
- FadL homologue long-chain fatty acid transporter: *Pseudomonas aeruginosa*
- FauA alcaligin outer membrane transporter: *Bordetella pertussis* TodX hydrocarbon transporter: *Pseudomonas putida*
- TbuX hydrocarbon transporter: *Ralstonia pickettii*
- Tsx nucleoside transporter (apoprotein): *Escherichia coli*
- FhuA, Ferrichrome-iron receptor: *Escherichia coli*
- FepA, Ferric enterobactin receptor: *Escherichia coli*
- FecA, siderophore transporter: *Escherichia coli*
- HasR heme-uptake receptor: *Serratia marcescens*
  - Ile671Gly mutant
- FptA, pyochelin outer membrane receptor: *Pseudomonas aeruginosa*
- FpvA, Pyoverdine receptor: *Pseudomonas aeruginosa*
- FpvA, Pyoverdine receptor (apo form): *Pseudomonas aeruginosa*
- P pilus usher translocation domain, PapC13 0-640: *Escherichia coli*
- **Beta-Barrel Membrane Proteins: Mitochondrial Outer Membrane**
- VDAC-1 voltage dependent anion channel: *Human*
- VDAC-1 voltage dependent anion channel: *Murine*
- **Omp85-TpsB Outer Membrane Transporter Superfamily**
- FhaC Filamentous Hemagglutinin Transporter: *Bordetella pertussis*
- TeOmp85-N POTRA domains: *Thermosynechococcus* anaOmp85-N *Anabaena* sp. PCC7120
- BamA: *Escherichia coli*
- BamE: *Escherichia coli*

- **Non-constitutive. Beta-sheet Pore-forming Toxins**

- Alpha-hemolysin: *Staphylococcus aureus*
- LukF: *Staphylococcus aureus*
- Perfringolysin O (PFO) protomer: *Clostridium perfringens*
- Anthrax Protective Antigen (PA) and Lethal Factor (LF) Prechannel Complex: *Bacillus anthracis*
- Lymphocyte preforin monomer: *Mus musculus*

- **Transmembrane Proteins: Alpha-Helical**

**[0066]**

- **Non-constitutive. Alpha-helical Pore-forming Toxins.**

- Cytolysin A (ClyA, aka HlyE): *Escherichia coli*
- FraC eukaryotic pore-forming toxin from sea anemone: *Actinia fragacea*

- **Outer Membrane Proteins**

- Wza translocon for capsular polysaccharides: *Escherichia coli*
- Porin B monomer: *Corynebacterium glutamicum*
- Type IV outer membrane secretion complex: *Escherichia coli*
- Bacteriorhodopsin (BR): *Halobacterium salinarium*
- Halorhodopsin (HR): *Halobacterium salinarium*
- Halorhodopsin (HR): *Natronomonas pharaonis*
- Sensory Rhodopsin I (SRI): *Anabaena (Nostoc) sp. PCC7120*
- Sensory Rhodopsin II (SRII): *Natronomonas pharaonis*
- Archaerhodopsin-1 (aR-1): *Halorubrum sp. aus-1*
- Archaerhodopsin-2 (aR-2): *Haloroubrum sp. aus-2*
- Xanthorhodopsin: *Salinibacter ruber*

- **G Protein-Coupled Receptors (GPCRs)**

- Rhodopsin: Bovine Rod Outer Segment (*Bos Taurus*)
- Rhodopsin: Squid (*Todarodes pacificus*)
- $\beta$ 1 adrenergic receptor (engineered): *Meleagris gallopavo* (turkey)
- $\beta$ 2 adrenergic receptor: *Homo sapiens*
- Methylated  $\beta$ 2 adrenergic receptor: *Homo sapiens*
- A2A adenosine receptor: *Homo sapiens*
- CXCR4 Chemokine Receptor: *Homo sapiens*
- Dopamine D3 Receptor: *Homo sapiens*

- **Autonomously Folding "Membrane Proteins" (Sec-independent)**

- Mystic membrane-integrating protein: *Bacillus subtilis*

- **Glycoproteins**

- Glycophorin A transmembrane-domain dimer: *Homo sapiens*

- **SNARE Protein Family**

- Syntaxin 1A/SNAP-25/Synaptobrevin-2 Complex: *ratus ratus*

- **Integrin Adhesion Receptors**

- Human Integrin  $\alpha$ IIb $\beta$ 3 transmembrane-cytoplasmic heterodimer: *Homo sapiens*

- **Histidine Kinase Receptors**

- ArcB (1-115) Aerobic Respiration Control sensor membrane domain: *Escherichia coli*
- QseC (1-185) Sensor protein membrane domain: *Escherichia coli*
- KdpD (397-502) Sensor protein membrane domain: *Escherichia coli*

- **Immune Receptors**

- Transmembrane  $\zeta$ - $\zeta$  dimer of the TCR-CD3 complex: *Homo sapiens*
- DAP12 dimeric: *Homo sapiens*

- **Channels: Potassium and Sodium Ion-Selective**

- KcsA Potassium channel, H<sup>+</sup> gated: *Streptomyces lividans*
- KcsA Potassium channel E71H-F103A inactivated-state mutant (closed state): *Streptomyces lividans*
- KcsA Potassium channel E71I modal-gating mutant: *Streptomyces lividans*

- KvAP Voltage-gated potassium Channel: *Aeropyrum pernix*
- Kv1.2 Voltage-gated potassium Channel: *Rattus norvegicus*
- Kv1.2/Kv2.1 Voltage-gated potassium channel chimera: *Rattus norvegicus*
  - F233W Mutant
- MthK Potassium channel, Ca<sup>++</sup> gated: *Methanothermobacter thermautotrophicus*
- Human BK Channel Ca<sup>2+</sup>-activation apparatus: *Homo sapiens*
- Kir3.1-Prokaryotic Kir Chimera: *Mus musculus* & *Burkholderia xenovornas*
- Kir2.2 Inward-Rectifier Potassium Channel: *Gallus gallus*
- KirBac1.1 Inward-Rectifier Potassium channel: *Burkholderia pseudomallei*
- MlotiK1 cyclic nucleotide-regulated K<sup>+</sup>-channel: *Mesorhizobium loti*
- mGIRK1 G-Protein Gated Inward Rectifying Potassium Channel: *Mus musculus*
- NaK channel (Na<sup>+</sup>complex): *Bacillus cereus*
  - D66/S70E Mutant
  - D66N Mutant
  - D66E Mutant
- CNG-mimicking NaK channel mutant: *Bacillus cereus*
- NaK channel; K<sup>+</sup> selective mutant: *Bacillus cereus*
- **Channels: Other Ion Channels**
- GluA2 Glutamate receptor (AMPA-subtype): *Rattus norvegicus*
- M2 proton channel: *Influenza A*
- M2 proton channel: *Influenza B*
- ASIC1 Acid-Sensing Ion Channel: *Gallus gallus*
- ATP-gated P2X4 ion channel (apo protein): *Danio rerio* (zebra fish)
- Nicotinic Acetylcholine Receptor Pore: *Torpedo marmorata*
- Prokaryotic pentameric ligand-gated ion channel (pLGIC): *Erwinia chrysanthemi*
- Prokaryotic pentameric ligand-gated ion channel (GLIC): *Gloeobacter violaceus*
  - E221A mutant

▪ Prokaryotic pentameric ligand-gated ion channel (GLIC), wildtype-TBSb complex: *Gloeobacter violaceus*

- Wildtype-TEAs complex
- E221D-TEAs complex
- Wildtype-TMAs complex
- Wildtype-bromo-lidocaine complex
- Wildtype-Cd<sup>2+</sup> complex
- Wildtype-Zn<sup>2+</sup> complex
- Wildtype-Cs<sup>+</sup> complex

▪ MscL Mechanosensitive channel: *Mycobacterium tuberculosis*

▪ MscS voltage-modulated mechanosensitive channel: *Escherichia coli*

▪ CorA Mg<sup>2+</sup> Transporter: *Thermotoga maritima*

▪ MgtE Mg<sup>2+</sup> Transporter: *Thermus thermophilus*

▪ SLAC1 anion channel, TehA homolog (wild-type): *Haemophilus influenzae*

- F262A mutant
- F262L mutant
- F262V mutant
- G15D mutant

◦ **Channels: Protein-Conducting**

▪ SecYEβ protein-conducting channel: *Methanococcus jannaschii* ◦ **Channels: Aquaporins and Glyceroporins**

▪ AQP0 aquaporin water channel: *Bovine lens*

▪ AQP1 aquaporin water channel: Human red blood cell

▪ AQP1 aquaporin water channel: Bovine red blood cell

▪ AQP4 aquaporin water channel: rat glial cells

- S180D Mutant

▪ AQP4 aquaporin water channel: Human

▪ AQP5 aquaporin water channel (HsAQP5): human

▪ AqpM aquaporin water channel: *Methanothermobacter marburgensis*

▪ AqpZ aquaporin water channel: *Escherichia coli*

▪ AqpZ aquaporin (C9S/C20S), T183C mutant: *Escherichia coli*

- L170C Mutant
- AqpZ aquaporin mutant F43W : *Escherichia coli*
  - H17G/T183F Mutant
  - F43WH174G/T183F Mutant
- SoPIP2;1 plant aquaporin: *Spinacia oleracea*
- GlpF glycerol facilitator channel: *Escherichia coli*
- GlpF glycerol facilitator channel, W84F/F200T-mutant: *Escherichia coli*
- PfAQP aquaglyceroporin: *Plasmodium falciparum*
- Aqy1 yeast aquaporin (pH 3.5): *Pischia pastoris*
- **Channels : Formate Nitrate Transporter (FNT) Family**
  - FocA, pentameric aquaporin-like formate transporter: *Escherichia coli*
  - FocA formate transporter without formate: *Vibrio cholerae*
  - FocA formate transporter: *Salmonella typhimurium*
- **Channels: Urea Transporters**
  - Urea transporter: *Desulfovibrio vulgaris*
  - Connexin 26 (Cx26; GJB2) gap junction: *Human*
- **Channels: Amt/Rh proteins**
  - AmtB ammonia channel (mutant): *Escherichia coli*
  - AmtB ammonia channel (wild-type): *Escherichia coli*
    - H168E Mutant
    - H168A Mutant
    - H168F Mutant
    - H318A Mutant
    - H318 Mutant
    - H318F Mutant
    - H168A/H318A Mutant
  - Amt-1 ammonium channel: *Archaeoglobus fulgidus*
  - Rh protein, possible ammonia or CO<sub>2</sub> channel: *Nitrosomonas europaea*
  - Human Rh C glycoprotein ammonia transporter: *Homo sapiens*

- **Intramembrane Proteases**

- GlpG rhomboid-family intramembrane protease: *Escherichia coli*
  - W136A Mutant
  - S201T Active-Site Mutant
- GlpG rhomboid-family intramembrane peptidase: *Haemophilus influenzae*
- Site-2 Protease (S2P). Intramembrane Metalloprotease: *Methanocaldococcus jannaschii*
- Signal Peptide Peptidase (SppA), native protein: *Escherichia coli*

- **Membrane-Bound Metalloproteases**

- apo-FtsH ATP-dependent metalloprotease: *Thermotoga maritima*

- **H<sup>+</sup>/Cl<sup>-</sup> Exchange Transporters**

- H<sup>+</sup>/Cl<sup>-</sup> Exchange Transporter: *Salmonella typhimurium*
- H<sup>+</sup>/Cl<sup>-</sup> Exchange Transporter: *Escherichia coli*
  - E148A Mutant
  - E148Q Mutant
  - S107A/E148Q/445A Mutant
- Monomeric H<sup>+</sup>/Cl<sup>-</sup> Exchange Transporter: *Escherichia coli*
- +/Cl<sup>-</sup> Eukaryotic Exchange Transporter: *Cyanidioschyzon merolae*
- H<sup>+</sup>/Cl<sup>-</sup> Eukaryotic Exchange Transporter: *Synechocystis sp. pcc 6803*

- **Bacterial Mercury Detoxification Proteins**

- MerF Hg(II) transporter: *Morganella morganii*

- **Multi-Drug Efflux Transporters**

- AcrB bacterial multi-drug efflux transporter: *Escherichia coli*
- AcrB bacterial multi-drug efflux transporter, apo protein, N109A mutant: *Escherichia coli*
- AcrB bacterial multi-drug efflux transporter, D407A mutant: *Escherichia coli*
- MexB bacterial multi-drug efflux transporter: *Pseudomonas aeruginosa*
- CusA metal-ion efflux pump: *Escherichia coli*
- EmrE bacterial multi-drug efflux transporter: *Escherichia coli*
- NorM Multidrug and Toxin Compound Extrusion (MATE) transporter (apo form): *Vibrio cholerae*

o **Membrane-Associated Proteins in Eicosanoid and Glutathione Metabolism (MAPEG)**

- Microsomal Prostaglandin E Synthase 1: *Human*
- 5-Lipoxygenase-Activating Protein (FLAP) with Bound MK-591 Inhibitor: *Human*
- Leukotriene LTC<sub>4</sub> Synthase: *Human*

o **Major Facilitator Superfamily (MFS) Transporters**

- LacY Lactose Permease Transporter (C154G mutant): *Escherichia coli*
- LacY Lactose Permease (wild-type) with TDG: *Escherichia coli*
- FucP Fucose Transporter in outward-facing conformation: *Escherichia coli*
  - N162A Mutant
- GlpT Glycerol-3-Phosphate Transporter: *Escherichia coli*
- EmrD Multidrug Transporter: *Escherichia coli*
- PepT<sub>So</sub> Oligopeptide-proton symporter: *Shewanella oneidensis*

o **Solute Sodium Symporter (SSS) Family**

- vSGLT Sodium Galactose Transporter: *Vibrio parahaemolyticus*
  - K294A Mutant

o **Nucleobase-Cation-Symport-1 (NCS1) Family**

- Mhp1 Benzyl-hydantoin transporter : *Microbacterium liquefaciens*

o **Betaine/Choline/Carnitine Transporter (BCCT) Family**

- BetP glycine betaine transporter: *Corynebacterium glutamicum*
- CaiT carnitine transporter: *Escherichia coli*
- CaiT carnitine transporter: *Proteus mirabilis*

o **Amino Acid/Polyamine/Organocation (APC) Superfamily**

- AdiC Arginine:Agmatine Antiporter: *Escherichia coli*
  - N22A, L123W Mutant
  - N101A Mutant
- apo ApcT Na<sup>+</sup>-independent Amino Acid Transporter: *Methanocaldococcus jannaschii*

o **Amino Acid Secondary Transporters**

- LeuTAa Leucine transporter: *Aquifex aeolicus*

- Wild-type LeuT transporter: *Aquifex aeolicus*
  - E290S Mutant
- Mutant LeuT transporter with Nitroxide Spin Label (F177R1): *Aquifex aeolicus*
  - I204R1 Mutant
- Glutamate Transporter Homologue (GltPh): *Pyrococcus horikoshii*
- Aspartate Transporter Li<sup>+</sup>-Bound State(GltPh): *Pyrococcus horikoshii*
- **Cation Diffusion Facilitator (CDF) Family**
- YiiP Zinc Transporter: *Escherichia coli*
- **Antiporters**
- NhaA Na<sup>+</sup>/H<sup>+</sup> antiporter: *Escherichia coli*
- Mitochondrial ADP/ATP Carrier: Bovine heart mitochondria
- **Energy-Coupling Factor (ECF) Transporters**
- RibU, S Component of the Riboflavin Transporter: *Staphylococcus aureus*
- **ATP Binding Cassette (ABC) Transporters**
- BtuCD Vitamin B12 Transporter: *Escherichia coli*
- Sav1866 Multidrug Transporter: *Staphylococcus aureus*
- Molybdate Transporter ModB2C2: *Archaeoglobus fulgidus*
- ModBC Molybdate ABC Transporter: *Methanosarcina acetivorans*
- HI1470/1 Putative Metal-Chelate-type ABC Transporter: *Haemophilus influenza*
- MsbA Lipid "flippase" with bound AMPPNP: *Salmonella typhimurium*
- P-Glycoprotein: *Mus musculus* (mouse)
- MalFGK2-MBP Maltose uptake transporter complex: *Escherichia coli*
- MetNI Methionine uptake transporter complex: *Escherichia coli*
- FbpC ferric iron-uptake transporter nucleotide-binding domain: *Neisseria gonorrhoeae*
- **Superfamily of K<sup>+</sup> Transporters (SKT proteins)**
- TrkH potassium ion transporter: *Vibrio parahaemolyticus*
- Calcium ATPase: Rabbit sarcoplasmic reticulum

- Na,K-ATPase: Pig Kidney
- Na,K-ATPase: Shark
- Na,K-ATPase Regulatory Protein FXYD1: *Human*
- Phospholamban homopentamer: Human sarcoplasmic reticulum
- Plasma Membrane H<sup>+</sup>-ATPase: *Arabidopsis thaliana*
- **V-type ATPase**
  - Rotor of V-type Na<sup>+</sup>-ATPase: *Enterococcus hirae*
  - V1-ATPase Complex: *Thermus thermophilus*
  - A3B3 complex of V1-ATPase: *Thermus thermophilus*
- **F-type ATPase**
  - F1-ATPase from bovine heart mitochondria: *Bos Taurus*
  - ATP synthase (F1c10): *S. cerevisiae*
  - F1 ATPase: *S. cerevisiae*
  - Rotor (c11) of Na<sup>+</sup>-dependent F-ATP Synthase: *Ilyobacter tartaricus*
  - Rotor (c14) of H<sup>+</sup>-dependent F-ATP Synthase of spinach chloroplasts: *Spinacia oleracea*
  - Rotor (c15) of H<sup>+</sup>-dependent F-ATP Synthase of an alkaliphilic cyanobacterium: *Spirulina platensis*
  - Rotor (c13) of H<sup>+</sup>-dependent F-ATP Synthase: *Bacillus pseudofirmus*
  - Peripheral stalk of H<sup>+</sup>-dependent F-ATP Synthase: *Thermus thermophilus*
- **Phosphotransferases**
  - Diacylglycerol kinase (DAGK): *Escherichia coli*
- **Hydrolases**
  - Estrone Sulfatase: *Human placenta*
- **Oxygenases**
  - Particulate methane monooxygenase (pMMO): *Methylococcus capsulatus*
  - Particulate methane monooxygenase (pMMO): *Methylosinus trichosporium* OB3b
- **Oxidoreductases**
  - Sulfide:quinone oxidoreductase: *Aquifex aeolicus*

- Electron Transfer Flavoprotein-ubiquinone oxidoreductase (ETF-QO): *Sus scrofa*
- Glycerol-3-phosphate dehydrogenase (GlpD, native): *Escherichia coli*
- NarGHI Nitrate Reductase A: *Escherichia coli*
  - K86A Mutant
  - H66Y Mutant
- NrfH Cytochrome C Quinol Dehydrogenase: *Desulfovibrio vulgaris*
- DsbB-DsbA Periplasmic Oxidase Complex: *E. coli*
- DsbB-Fab complex: *Escherichia coli*
- wtDsbB-DsbA(Cys133A)-Q8 Complex: *E. coli*
- Vitamin K epoxide reductase: *Synechococcus sp.*
- **Mo/W bis-MGD Oxidoreductases**
- Polysulfide Reductase PsrABC (native): *Thermus thermophiles*
- **Electron Transport Chain Complexes: Complex I**
- Complex I membrane domain: *Escherichia coli*
- Complex I complete: *Thermus thermophiles*
- **Electron Transport Chain Complexes: Complex II**
- Native Fumarate Reductase Complex: *Escherichia coli*
- Fumarate Reductase Complex: *Wolinella succinogenes*
- Formate dehydrogenase-N: *Escherichia coli*
- Succinate dehydrogenase (Complex II): *Escherichia coli*
- Succinate:ubiquinone oxidoreductase (SQR, Complex II): *porcine heart mitochondria*
- Succinate:ubiquinone oxidoreductase (SQR, Complex II): *chicken heart mitochondria*
- **Electron Transport Chain Complexes: Complex III (Cytochrome bcl)**
- Cytochrome bc1: *Bos Taurus*
- Cytochrome bc1: *Gallus gallus*
- Cytochrome bc1: *Sarcomyces cerevisiae*
- Cytochrome bc1: *Rhodobacter Sphaeroides*

- **Electron Transport Chain Complexes: Cytochrome b6f of Oxygenic Photosynthesis**

- Cytochrome b6f complex: *Mastigocladus laminosus*
- Cytochrome b6f complex: *Chlamydomonas reinhardtii*
- Cytochrome b6f complex: *Nostoc* sp. PCC 7120

- **Electron Transport Chain Complexes: Complex IV (Cytochrome C Oxidase)**

- Cytochrome C Oxidase, aa3: *Bos taurus* (bovine) heart mitochondria
- Cytochrome C Oxidase, aa3: *Paracoccus denitrificans*
  - N131D Variant
- Cytochrome Oxidase, cbb3: *Pseudomonas stutzeri*
- Cytochrome ba3: *Thermus thermophilus*
- Cytochrome C Oxidase wild-type: *Rhodobacter sphaeroides*
- Ubiquinol Oxidase, cytochrome bo3: *E. coli*

- **Nitric Oxide Reductases**

- Nitric Oxide Reductase: *Pseudomonas aeruginosa*

- **Photosystems**

- Photosystem I: *Thermosynechococcus elongates*
- Photosystem I (plant): *Psium sativum*
- Photosystem II: *Thermosynechococcus elongates*
- Photosystem II: *Thermocynechococcus vulcanus*

- **Light-Harvesting Complexes**

- Light-Harvesting Complex: *Rhodopseudomonas acidophila*
- Light-Harvesting Complex: *Rhodospirillum molischianum*
- Light-Harvesting Complex LHC-II, Spinach Photosystem II: *Spinacia oleracia*
- Light-Harvesting Complex CP29, Spinach Photosystem II: *Spinacia oleracia*
- Light-Harvesting Complex LHC-II, Pea Photosystem II: *Pisum sativum*

- **Photosynthetic Reaction Centers**

- Photosynthetic Reaction Center: *Blastochloris viridis*

- Photosynthetic Reaction Center: *Rhodobacter sphaeroides*
- Photosynthetic Reaction Center: *Thermochromatium tepidum*

**[0067]** The support may be made of any suitable microporous material. It may for example be based upon a conventional membrane support, as used in reverse osmosis or ultrafiltration membranes. Such supports may for example be made from a polyolefin, cellulose, regenerated cellulose, cellulose acetate, polyacrylonitrile, polyethersulfone, or polysulfone. In a preferred embodiment of the invention, the support is made from a polysulfone.

**[0068]** Chemical functionality of the support membrane may be delivered in the form of additives, which may be either low molecular weight or polymeric, to the casting dope, or functionalization of the support surface, for example by chemical treatments, graft polymerisation or plasma polymerization. By these means, the following chemical transformations of the support may for example be accomplished: conversion of amine groups into carboxylic acid groups, or vice versa; conversion of aldehydes into amines; and conversion of hydroxyl groups into carboxylic acid groups. All such reactions are well known in the art.

**[0069]** Porous ultrafiltration membranes may for example be prepared by air casting, where the dissolved polymer solution passes under a series of air flow ducts that control the evaporation of the solvents in a very slow manner; solvent or emersion casting, where the dissolved polymer is spread onto a moving belt and run through a bath of liquid, and the liquid in the bath exchanges with the solvent in the lacquer and causes the formation of the pores; thermal casting, where heat is used to drive the solubility of the polymer in a given solvent system. The lacquer is then cast out onto a moving belt that is being cooled. Quenching the heat in the lacquer causes precipitation to start and the pores to form. Materials typically used in the process include but are not limited to cellulose regenerated, cellulose nitrate, cellulose acetate, polyamide, polysulfone, poly(ether sulfone), polycarbonate, poly(ether imide), poly(2,6-dimethyl-1,4-phenylene oxide), polyimide, poly(vinylidene fluoride), polytetrafluoroethylene, polypropylene, polyacrylonitrile, poly(methyl methacrylate), polyvinyl alcohol, and polydimethylsiloxane. The morphology of the cast is regulated by the configuration of the final module. It may for example comprise a flat-sheet for spiral wound elements; hollow-fibre for hollow-fibre elements; or it may be tubular.

**[0070]** Preparation of a membrane having a layer comprising a coherent mass of vesicles, said layer having a defined thickness, may be achieved by control of the concentration of vesicles present in the solution of vesicles applied to the support and/or by the volume of solution deposited on the support.

**[0071]** Xie et al, J. Mater. Chem A, 2013, 1, 7592, discloses processes involving crosslinking during the preparation of the polymer vesicles, but this crosslinking, which did not change the structure or dimension of the polymer vesicles (col. 2 p. 7596 top paragraph) is always internal crosslinking between the crosslinkable end groups corresponding to the groups X of the

present invention. Similarly, the crosslinking disclosed in WO 01/32146 is always internal crosslinking. It is of course possible, depending on the nature of the various groups present, for internal cross-linking to occur in the vesicles of the present invention, but it is an essential feature of the present invention that external crosslinking, preferably via a multifunctional linker, also takes place. The advantage of the present invention over the methods disclosed by Xie et al, and by Zhao et al, J. Membrane Sci. 2012, 422-428 and WO 2013/043118, is that any possible pathway through the membrane other than through the transmembrane proteins embedded in the walls of the polymer vesicles, is minimised, while providing a large number of possible transmembrane proteins per unit surface area of the support membrane, thus maximising flux through the membrane. The process is technically simple, and the resulting membranes are physically robust.

**Brief description of the figures****[0072]**

Figure 1 shows the NMR spectrum of the polymer prepared in step 1 of Example 1.

Figure 2 shows the results of the molecular weight cut-off experiments of step 4 of Example 2.

Figure 3 shows the results of the flow testing experiments of step 5 of Example 1.

Figures 4 and 5 are scanning electron microscopy images of the membranes prepared in Example 1.

Figure 6 shows the results of the dynamic light scattering measurements of Example 2.

Figures 7A and 7B show LSM imaging micrographs of vesicles prepared in Example 4.

Figure 8 shows the effect of incorporating Aquaporin Z protein into vesicles as described in Example 4.

Figure 9 is a micrograph of the membrane of Example 4.

Figure 10 shows the effect of internally cross-linking the polybutadiene in the membrane of Example 4.

**[0073]** The following Examples illustrate the invention.

**Example 1.****Materials:**

[0074]

- 2-methyl-2-oxazoline, Sigma
- Triethylamine, Sigma
- Hexane, anhydrous, Sigma
- Ethylene diamine, Sigma
- Trifluoromethanesulfonic acid, Sigma
- Ethyl acetate, Sigma
- Aquaporin-Z stock solution 1mg/ml in 1% octyl glucoside and 100 mM NaMPOS buffer at pH 7.5
- 100 mM NaMPOS buffer at pH 7.5
- Chloroform (Puriss)
- Octyl glucoside (Anatrace)
- Amine functional polymer vesicles 10mg/mL in Na.MOPS
- PoPR (Polymer to Protein ratio, mass)
- N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate, sulfo-SANPAH (Pierce; Product No. 22589)
- Dextrans (American Polymer Standards Corporation)
- 365nm UV lamp (Entela UVP)
- 47mm Membrane stamp
- 25mm Membrane stamp
- Polysulfone membrane; pore size 150 nm (cut-off over 1000kDa)

### 1) Polymer preparation - Primary/Secondary-Amine terminated poly-2-methyloxazoline-poly-di-methyl-siloxane-poly-2-methyloxazoline (PMOXA-PDMS-PMOXA)

#### Step a). $\alpha,\omega$ -Hydroxy-butyl-poly-di-methyl-siloxane (PDMS) synthesis:

[0075] Targeting the molecular weight of 4000 g/mol, 93.03 g (0.34 mols) of octamethylcyclotetrasiloxane and 6.97g (0.0025 mols) 1,3-bis(hydroxybutyl)-tetramethyldisiloxane were charged into a 3-necked round bottom Pyrex reactor with an argon inlet, thermometer and condenser. Trifluoroacetic acid 6.55 g (0.05755 mols) was added. The reaction mixture was heated at 60°C for 48 hours. After this time the excess trifluoroacetic acid was extracted with distilled water until the aqueous extract was neutral. Then the reaction mixture was stripped off under high vacuum to remove the cyclic side products. Ester groups were further converted to alcohols by a weak base catalyzed hydrolysis in THF and an equal volume of 5% aqueous sodium carbonate solution at 40-45°C, for 48 hours. Organic and aqueous phases were separated out. The 83.72 grams of product were recovered by the evaporation of THF. The product was evaluated for molecular weight by proton NMR and

molecular weight distribution by GPC in chloroform.

### **Step b). Primary/Secondary-Amine terminated PMOXA-PDMS-PMOXA synthesis**

**[0076]** Hydroxyl-terminated PDMS synthesized as in step a above was used in the synthesis of poly PMOXA-PDMS-PMOXA amphiphilic block copolymer.

**[0077]** In a three-neck round bottom flask 50 grams (0.012 mols) of PDMS were kept under high vacuum for 24 h. In the next step, a reaction flask was filled with dry argon, and the polymer was dissolved in dry hexane (200 ml) and added to the three-neck flask via septum. Cooled (0-5 deg C) PDMS was then activated by drop-wise addition of 6.62 g (0.02346 mols) of trifluoromethanesulfonic anhydride in presence of 2.45 g (0.024 mols) of triethyl amine and allowed to post-react for 3 hours. The activated PDMS was further filtered under argon and hexane was removed under reduced pressure. 250 ml of dry ethyl acetate was added to redissolve the activated polymer, and ring-opening polymerization of 2-methyloxazoline was started upon addition of 23.5 g (0.27 mols) dried 2-methyl oxazoline at 40 deg C. After 12 hours reaction under argon, a 3-fold excess, 4.14 g (0.069 mols) of butyl-di-amine was added as terminating agent. Product was recovered under high vacuum and evaluated for molecular weight by proton NMR (shown in Figure 1) and molecular weight distribution by GPC in chloroform. The product was 100 % soluble in ethanol and 99.5% insoluble in hexane. The remaining 0.5% was found to be unreacted PDMS as shown by proton NMR.

### **2). Polymer vesicles/proteo-vesicles preparation:**

**[0078]** 50 mg of ABA block-co-polymer was dissolved in 2 ml of chloroform in a round bottom flask (Pyrex 100 ml). Chloroform was then removed under high vacuum to form a thin film of polymer. This film was hydrated with either 5 ml of buffer (control) or 5 ml of aqueous stock solution of Aquaporin-Z and stirred overnight. In these samples the amount of added protein was varied from 1:1 to 1:1200 polymer to protein ratio. Detergent was subsequently removed by dialysis in 30 kDa dialysis membranes in NaMOPS buffer. The resulting product was then extruded through track-etched membranes to uniform 200 nm size.

### **3). Coating**

**[0079]** In this step, the concentration of deposited vesicles was kept constant and monitored by matching the count rate (250kcps) in Dynamic Light Scattering (Malvern Zetasizer Nano) with static attenuator.

**[0080]** Sulfo-SANPAH (SS) solution (10mM in 100mM NaMOPS pH 7.5) was allowed to react with vesicles prepared as in step (1) in the absence of light (250µL of vesicle solution

combined with 50 $\mu$ L SS for 15-minutes). A series of 47mm polysulfone membranes (Nano H2O Inc, 150nm) were cut by punch press and placed into Teflon membrane holders and rinsed with deionized water. Excess water was removed by compressed air and 300 $\mu$ L (each) of SS-activated vesicles/proteo-vesicles solutions were placed onto polysulfone support membranes. The membrane holders were then placed under UV light approximately 5cm from the source and covered with foil for protection for 30 minutes. Excess reactants were then removed from the membrane surface using a 1 ml pipette without touching the membrane surface. The above steps were repeated three times, following which the membranes were removed from the holders and 25mm diameter membrane samples were cut from the coated area using a punch press. These were then rinsed in excess 100 mM NaMOPS pH7.5 on a shake table for at least one hour before testing.

#### **4) Molecular cut-off experiments**

**[0081]** The 25mm samples of step (2) tested for their ability to retain high molecular weight materials, by measuring their molecular weight cut-off, i.e. the point at which at least 90% of molecules of a given molecular weight are retained by the membrane.

**[0082]** Phosphate buffer (0.03M Na<sub>2</sub>HPO<sub>4</sub> + 0.03M KH<sub>2</sub>PO<sub>4</sub>) was pre-filtered using a 0.2  $\mu$ m membrane and the pH was adjusted to 7.2 prior to use for preparation of solutions. Dextran (DXT) standards were dissolved in phosphate buffer (DXT 165 kDa, 325 kDa, 548 kDa, 1300 kDa, and 5000 kDa, DXT 0.505 kDa, 4 kDa, 6 kDa, 11 kDa, 20 kDa, and 28 kDa). All of the dextran solutions were diluted to 0.5 mg/ml with phosphate buffer and pre-filtrated using a 0.2  $\mu$ m PES membrane prior to use. All filtration experiments were conducted in a 10 ml Amicon stirred ultrafiltration cell (Model 8010, Millipore Corp.)

**[0083]** All samples were evaluated according to the protocol described below:

- Filtered 10 ml volume of deionised water at 20 psi to wet the pore structure and the whole system.
- Connected the feed line with dextran solution feed to a digital peristaltic pump (Thermal Fisher Science Inc.), re-pressurized the cell to 20 psi, set the filtrate flux to 5  $\mu$ m/s.
- Obtained 800  $\mu$ L samples of the filtrate solution after filtration of 2,000  $\mu$ L of water for equilibration and washing out the dead volume downstream of the membrane.
- Obtained 1 ml permeate samples directly from the cell after filtration.
- Cleaned and rinsed the whole system with deionised water.
- The stirring speed was kept at 600 rpm and all experiments were performed at room temperature (22 $\pm$ 3  $^{\circ}$ C)

**[0084]** Permeate was further evaluated using high-pressure liquid chromatography (HPLC columns PL1149-6840, MW 10,000 to 200,000, PL1120-6830, MW 100 to 30,000, PL1149-

6860, MW 200,000 to > 10,000,000). Comparison of the feed to the permeate chromatograms allowed for calculation of retention coefficients and membrane molecular cut-off.

**[0085]** The results are shown in Figure 2, which shows that all of the membranes according to the invention retained all of the higher molecular weight molecules, while the control membrane demonstrated significantly poorer performance, with a molecular weight cut-off in excess of 3,000kDa.

### **5). Flow Testing**

**[0086]** The 25mm membranes of Step (2) were tested for their ability to transmit pure water using a stirred test cell (Amicon 10 ml, (Model 8010, Millipore Corp.) in which the feed was pure water. The system was closed and set to stir for at least 5min before testing. Subsequently the pressure was gradually increased from 1 to 5bar and data points representing the volume of pure water passing through the surface of the membrane in 1 minute were collected at 1 bar intervals (with permeate collected separately at each pressure). The experiment also included the best commercially available water filtration membrane currently on the market, Biomax 30 kDa from Millipore, for comparison.

**[0087]** The results are shown in Figure 3, in which LMH/bar is litre/m<sup>2</sup>/hour/bar of pure water, i.e. is a pressure-corrected flow rate, and PoPr represents polymer:protein ratio (note that the higher the PoPr, the lower the content of aquaporin protein).

**[0088]** The control membrane prepared in step 2 with a coating of vesicles but no aquaporin protein, had the lowest flow rate of all the membranes tested. All the membranes according to the invention performed significantly better, with a higher content of aquaporin leading to higher fluxes, and the membrane with the highest content of aquaporin significantly outperforming the commercially available membrane.

**[0089]** Figures 4 and 5 show SEMs of the membranes according to the invention. In Fig. 4 (magnification 1000) the lower layer having a sponge-like appearance is the polysulfone support, having a macrovoid due to the casting process. The upper layer is the continuous coating comprising a coherent mass of aquaporin-containing vesicles. In Fig. 5 (magnification 20,000), the lower portion of the SEM having a textured appearance is the polysulfone support, while the thin uppermost layer is the continuous coating comprising a coherent mass of aquaporin-containing vesicles. The bright line at the boundary between these two layers is a boundary layer where the vesicle layer is covalently bound to the polysulfone.

### **Examples 2 and 3**

**[0090]** Model experiments were carried out to confirm the suitability of various polymer end-

groups for the preparation of vesicles and the covalent linking of vesicles to each other. The alternative polymers were prepared as follows.

**(a) Carboxylic-terminated poly-2-methyloxazoline-poly-di-methyl-siloxane-poly-2-methyloxazoline (PMOXA-PDMS-PMOXA)**

**[0091]** Hydroxyl-terminated polymer Mn=4262 g/mol (PDMS) synthesized as in step (a) of Example 1 was used in the synthesis of poly PMOXA-PDMS-PMOXA amphiphilic block copolymer. In a three-neck round bottom flask 50 grams (0.01173 mols) of PDMS was kept under high vacuum for 24 h. In the next step reaction the flask was filled with dry argon and polymer was dissolved in dry hexane (200 ml) added to the three-neck flask via septum. Cooled (0-5 deg C) PDMS was then activated by drop-wise addition of 6.62 g (0.02346 mols) of trifluoromethanesulfonic anhydride in presence of 2.45 g (0.024 mols) of triethylamine and allowed to post-react for 3 hours. The activated PDMS was then filtered under argon and hexane was removed under reduced pressure. 250 ml of dry ethyl acetate was added to redissolve the activated polymer and ring-opening polymerization of 2-methyloxazoline was started upon addition of 23.5 g (0.27 mols) dried 2-methyl oxazoline at 40 deg C. After 12 h reaction under argon, deprotonated malonic acid was added in 1.3x excess as terminating agent 3.12 g (0.030 mols) in the presence of triethylamine 3.05g (0.030 mols). Product was recovered under high vacuum and evaluated for molecular weight by proton NMR and molecular weight distribution by GPC in chloroform.

**(b) Hydroxy terminated poly-2-methyloxazoline-poly-di-methyl-siloxane-poly-2-methyloxazoline (PMOXA-PDMS-PMOXA) (Outside of the claims)**

**[0092]** Hydroxyl-terminated silicon Mn=4262 g/mol (PDMS) synthesized as described in step (a) of Example 1 above was used in the synthesis of poly PMOXA-PDMS-PMOXA amphiphilic block copolymer.

**[0093]** In a three-neck round bottom flask 50 grams (0.01173 mols) of PDMS was kept under high vacuum for 24 h. In the next step reaction flask was filled with dry argon and polymer was dissolved in dry hexane (200 ml) added to the three-neck flask via septum. Cooled (0-5 deg C) PDMS was then activated by drop-wise addition of 6.62 g (0.02346 mols) of trifluoromethanesulfonic anhydride in the presence of 2.45 g (0.024 mols) of triethylamine and allowed to post-react for 3 hours. The activated PDMS was then filtered under argon and hexane was removed under reduced pressure. 250 ml of dry ethyl acetate was added to redissolve activated polymer and ring-opening polymerization of 2-methyloxazoline was started upon addition of 23.5 g (0.27 mols) dried 2-methyl oxazoline at 40 deg C. After 12 h reaction under argon, potassium hydroxide was added in 1.3x excess as terminating agent (1.68 g (0.030 mols) in 50 ml of methanol). Product was recovered under high vacuum and evaluated for molecular weight by proton NMR and molecular weight distribution by GPC in chloroform.

## Example 2

[0094] 250 $\mu$ L of vesicles made from amine-terminated polymer as prepared in Example 1 were placed in a 64mL clear glass vial, and protected from light by wrapping the vials in aluminum foil. The varying amounts (0, 1, 5, 10, 25 and 50  $\mu$ l) of the difunctional linker sulfo-SANPAH, (10mM Sulfo-SANPAH in 100mM Na.MOPS pH 7.5) was added and mixed by gentle shaking. Reaction was allowed to take place for 15 minutes, following which 100 $\mu$ L of solution was placed into a cuvette for dynamic light scattering (DLS) measurement, DLS being a technique for the measurement of the size of particles in solution. The sample was placed about 5cm below the UV lamp, the lid and foil were removed, the lamp was switched on, and the whole was covered with a foil tent. In all cases the attenuator was fixed at 6. After 15 minutes under UV,

[0095] Prior to reaction with sulfo-SANPAH, DLS showed the diameter of the vesicles to be 200nm. After UV irradiation to cause reaction with sulfo-SANPAH, large aggregates were formed which could be seen with the naked eye. The DLS results are shown in Figure 6. These aggregates were stable under sonication, indicating the presence of covalent bonding.

[0096] As a comparison, a similar experiment was carried out using hydroxyl-terminated polymer, which is not expected to be reactive with sulfo-SANPAH. As expected, no crosslinking occurred, and therefore no increase in diameter measured by DLS occurred.

## Example 3

[0097] Experiments were carried out using vesicles made from polymers having activated carboxylic acid groups as end groups.

### Materials

#### [0098]

- EDC, Pierce (Product No. 22980)
- NHS, Pierce (Product No. 24500)
- Malvern ZetasizerNANO DLS
- Sonication Bath
- pH Meter with micro probe
- Carboxyl terminated polymer vesicles prepared as described above
- Amine terminated polymer vesicles prepared as described above

## Experimental

[0099] Vesicles were prepared according to above described thin-film hydration protocol using deionised water. The average diameter of the resulting polymer vesicles was shown to be around 200nm using DLS.

[0100] Carboxylic vesicles activated with EDC and NHS were prepared by addition of 950 µg of EDC and 570 µg of NHS to 1 ml of carboxylic vesicles. The solution was then adjusted to pH 5 using HCl and allowed to react for 30 minutes at room temperature resulting in EDC-NHS activated vesicles.

[0101] Solutions of (control) carboxylic vesicles (1ml) and EDC-NHS activated vesicles (1ml) were allowed to react with equal amount of amine-functional vesicles (1ml). Subsequently the pH of all solutions was adjusted about 7.5 with a dilute solution of NaOH in deionised water and allowed to react for at least 90minutes. 100µL of the resulting samples were tested by DLS using a static attenuator setting of 5. After testing, the cuvettes were sonicated for 1 minute and then retested.

[0102] It was found that reaction of equal amounts of amine and carboxylic vesicles resulted in the formation of large aggregates (around 2000 nm by DLS). However, when sonicated, these aggregates dispersed, showing that the bonding was ionic rather than covalent. In contrast, reaction of equal amounts of amine and EDC-NHS activated carboxylic vesicles resulted in formation of large aggregates (about 3600 by DLS) which were not dispersed when sonicated, indicating that the forces holding aggregates together were covalent.

### **Example 4**

[0103] A series of experiments using the diblock copolymer polybutadiene-PMOXA was carried out.

#### **Step (a): PB Synthesis**

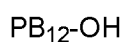
[0104] Polybutadiene was synthesized following the protocol of Hillmyer, M. A.; Bates, F. S. 1996, 9297, 6994-7002 with some modifications. The anionic polymerization of butadiene was carried out in THF at -60 to -50 °C using sec-butyl-butyllithium as the initiator. A dry 2 neck flask was dried in the oven overnight and a line was attached to one port with a septum to another. The flask was flame dried and a stir bar was added. 30 ml of Dry Solv THF was added to the 2 neck flask using a cannula. 11 ml butadiene (0.13 mol) was condensed in a condensing flask. Liquid nitrogen was first used to condense polybutadiene and then melted using a dry ice-acetone bath. This was transferred to the 2 neck flask using a cannula. 7 ml

(0.0098 moles) of 1.4 M sec-butyl lithium initiator was swiftly added. The polymerization was allowed to proceed for 3 h. End capping was accomplished by adding 2ml (0.051 moles) of ethylene oxide at  $-60^{\circ}\text{C}$  upon complete conversion of the butadiene. Acidic methanol (5 ml HCl: 50 ml methanol) was then used to liberate the polybutadiene alcohol which was isolated by evaporation of the solvent. Inorganic salts were removed by extraction of a cyclohexane solution of the polymer with distilled water. Polymer was left on high vacuum to remove water. Further drying was achieved by refluxing the polymer in dry hexane using molecular sieves in soxhlet extractor.

### Step (b): PB-PMOXA synthesis

**[0105]** 20 g (0.0260M) of polybutadiene ( $M_n$  769 g/mol) were functionalized with 7.33g (0.0260M) triflic acid anhydride (SigmaAldrich 176176-5G) in the presence of 2.63g (0.0260M) of triethylamine (SigmaAldrich T0886) at  $-10$  deg C under argon. Organic salts were further filtered out. Triflate-functionalized PB served as a macro-initiator of cationic ring opening polymerization of 2-methyl-2-oxazoline (SigmaAldrich 137448). Polymerisation was allowed to proceed in anhydrous ethyl acetate (SigmaAldrich 270989) at  $40$  deg C for 12 h. Reaction was terminated with ethylene diamine 0.4g (SigmaAldrich 03550). This provided primary- and secondary-amine terminated PB-PMOXA polymer.

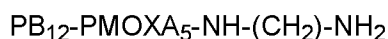
**[0106]** Polymer characterization:



NMR

5.45 ppm  $-\underline{\text{CH}}=\text{CH}_2$  (repeating unit), 4.94 ppm  $-\text{CH}=\underline{\text{CH}}_2$  (repeating unit), 2.12 ppm CH (repeating unit - backbone), 1.27 ppm  $\text{CH}_2$  (repeating unit - backbone),  $\text{CH}_2$  and  $\text{CH}_3$  3.65 ppm 0.82 ppm - end groups.

Polymer	Solvent	$M_n$	$M_w$	PDI
$\text{PB}_{12}$	$\text{CHCl}_3$	526	602	1.14
$\text{PB}_{12}\text{PMOXA}_5$	$\text{CHCl}_3$	632	738	1.19



NMR

PB: 5.45 ppm  $-\underline{\text{CH}}=\text{CH}_2$  (repeating unit), 4.94 ppm  $-\text{CH}=\underline{\text{CH}}_2$  (repeating unit), 2.12 ppm CH (repeating unit - backbone), 1.27 ppm  $\text{CH}_2$  (repeating unit - backbone),  $\text{CH}_2$  and  $\text{CH}_3$  3.65 ppm 0.82 ppm - end groups. PMOXA: 3.45 ppm  $(-\underline{\text{CH}}_2\text{-CH}_2\text{-N-})$ , 2.11 ppm  $(-\text{N-CO-}\underline{\text{CH}}_3)$

### Step (c) Vesicle preparation

**[0107]** PB<sub>12</sub>-PMOXA<sub>5</sub>-NH-(CH<sub>2</sub>)<sub>2</sub>-NH<sub>2</sub> polymer (50 mg) was dissolved in 1 ml chloroform in a round bottom flask (Pyrex 200ml). Solvent was evaporated on a rotary evaporator under reduced pressure producing a thin film of polymer. Subsequent 3 h high vacuum treatment removed the traces of chloroform. 5 ml of water was further added and stirred at 600 rpm. This way a 10mg/ml suspension of vesicles was prepared. Upon sampling for characterization (LSM, Stopped-Flow, DLS), the suspension was extruded successively through polycarbonate Track ached filters (Millipore) of 1 µm, 800nm, 400nm, 200nm. At each of the extrusions, the suspension was sampled for characterization.

**[0108]** The vesicles were characterised as follows. Cryogenic transmission electron microscopy (cryo-TEM) was used for particle imaging, and surface functionalization was studied using LSM imaging.

**[0109]** For the cryo-TEM, the microscope was FEI TecnaiG2, TF20. Samples were vitrified using a vitrification robot, Vitrobot™FEI. Magnification used was 25000x (calibrated 31625x) = scale bar 200 µm.

**[0110]** For the LSM imaging, the amine end groups present on the surface of the vesicles prepared as above were allowed to react with tetramethylrhodamine isothiocyanate fluorescent dye (1:1000 molar ratio) and dialyzed against deionized water. Dialysis was performed until dialysate showed no signs of fluorescence, followed by additional change of DI water to eliminate unspecific binding . The vesicles were visualized using a Zeiss LSM 710 Inverted Confocal Microscope with Apochromat 63x/1.4 Oil DIC M27 objective and 561nm Laser line. Pinhole was varied from 50µm to 70µm. This allowed for the confocal plane to "see through" the vesicles, which thus appear as rims of light (center of vesicle in the center of confocal point) or discs of light (top of the vesicle in confocal point) in suspension where a vesicle floated in and out of focus dynamically. Figs. 7A and 7B show two sample micrographs clearly showing vesicles.

#### **Step (d): Insertion of protein into vesicles**

**[0111]** Water permeability of polymer vesicles was enhanced by reconstitution of water channel membrane protein - aquaporin Z. Film hydration procedure was modified to accommodate addition of protein at PoPr 400. Shortly: to the hydrating vesicles protein solution is added at PoPr 400. Next steps follow the protocol of standard vesicles formation.

**[0112]** PB<sub>12</sub>-PMOXA<sub>5</sub>-NH-(CH<sub>2</sub>)<sub>2</sub>-NH<sub>2</sub> polymer (50 mg) was dissolved in 1 ml chloroform in a round bottom flask (Pyrex 200ml). Solvent was evaporated on a rotary evaporator under reduced pressure producing a thin film of polymer. Subsequent 3 h high vacuum treatment removed the traces of chloroform. 5 ml of 100 mM Na-MOPS buffer containing 0.1245 mg of

aquaporin Z (Applied Biomimetic) and 0.5% octyl glucoside (0311 - n-Octyl- $\beta$ -D-Glucopyranoside, Anagrade, Anatrace) and was further added and stirred at 600 rpm. 10mg/ml suspension of proteo-vesicles was extruded through 200 nm polycarbonate Track-etched filter (Millipore). Permeability measurements were performed using stopped-flow spectrometer.

**[0113]** Stopped flow spectroscopy was used to evaluate protein insertion. This is measured as increase in water permeability of vesicles reconstituted with aquaporin water channel. With the amount of protein added as little as PoPR (polymer to protein ratio) of 400 the increase in water permeability over control vesicles was measured to be 46 times. The results are shown in Fig. 8.

#### **Step (e): Membrane preparation**

**[0114]** In this Example, the concentration of deposited vesicles was kept constant and monitored by matching the count rate (250kcps) in Dynamic Light Scattering (Malvern Zetasizer Nano) with static attenuator.

**[0115]** Sulfo-SANPAH (SS) solution (10mM in 100mM NaMOPS pH 7.5) was allowed to react with previously prepared PB-PMOXA-NH-(CH<sub>2</sub>)<sub>2</sub>-NH<sub>2</sub> vesicles in the absence of light (250 $\mu$ L of vesicle solution combined with 50 $\mu$ L SS for 15-minutes). A series of 47mm polysulfone membranes (hand casted) were cut by punch press and placed into Teflon® membrane holders and rinsed with deionized water. Excess water was removed by compressed air and 300 $\mu$ L (each) of SS-activated vesicle suspensions were placed onto the polysulfone support membranes. The membrane holders were then placed under UV light approximately 5cm from the source and covered with foil for protection for 30 minutes. Excess reactants were then removed from the membrane surface using a 1 ml pipette without touching the membrane surface. The above steps were repeated three times, following which the membranes were removed from the holders and 25mm diameter membrane samples were cut from the coated area using a punch press. These were then rinsed in excess 100 mM NaMOPS pH7.5 on a shake table for at least one hour before testing.

**[0116]** Fig. 9 is a micrograph of the resulting membrane, showing a coherent mass comprising a plurality of vesicles cross-linked on the surface of the support membrane.

**[0117]** Membranes prepared in the step described above were subject to treatment with either 10 or 150  $\mu$ L of free radical initiating solution composing of:

25mM Iron(II) Sulfate Heptahydrate,

25mM Sodium Metabisulfite,

25mM Potassium Persulfate

**[0118]** The treatment resulted in crosslinking of the PB hydrophobic core.

**[0119]** The resulting membrane samples were tested for pore size distribution using a standard molecular weight cut-off analysis technique. The 25mm samples prepared in the previous step were tested for their ability to retain high molecular weight materials, by measuring their molecular weight cut-off, i.e. the point at which at least 90% of molecules of a given molecular weight are retained by the membrane. Phosphate buffer (0.03M Na<sub>2</sub>HPO<sub>4</sub> + 0.03M KH<sub>2</sub>PO<sub>4</sub>) was pre-filtered using a 0.2 um membrane and the pH was adjusted to 7.2 prior to use for preparation of solutions. Dextran (DXT) standards were dissolved in phosphate buffer (DXT 165 kDa, 325 kDa, 548 kDa, 1300 kDa, and 5000 kDa, DXT 0.505 kDa, 4 kDa, 6 kDa, 11 kDa, 20 kDa, and 28 kDa). All of the dextran solutions were diluted to 0.5 mg/ml with phosphate buffer and pre-filtrated using a 0.2 um polyethersulfone membrane prior to use. All filtration experiments were conducted in a 10 ml Amicon stirred ultrafiltration cell (Model 8010, Millipore Corp.) All samples were evaluated according to the protocol described below:

- Filtered 10 ml volume of deionised water at 20 psi to wet the pore structure and the whole system.
- Connected the feed line with dextran solution feed to a digital peristaltic pump (Thermal Fisher Science Inc.), re-pressurized the cell to 20 psi, set the filtrate flux to 5 µm/s.
- Obtained 800 µL samples of the filtrate solution after filtration of 2,000 µL of water for equilibration and washing out the dead volume downstream of the membrane.
- Obtained 1 ml permeate samples directly from the cell after filtration.
- Cleaned and rinsed the whole system with deionised water.
- The stirring speed was kept at 600 rpm and all experiments were performed at room temperature (22±3 °C)

**[0120]** Permeate was further evaluated using high-pressure liquid chromatography (HPLC columns PL1149-6840, MW 10,000 to 200,000, PL1120-6830, MW 100 to 30,000, PL1149-6860, MW 200,000 to > 10,000,000). Comparison of the feed to the permeate chromatograms allowed for calculation of retention coefficients and membrane molecular cut-off. The results are shown in Fig. 10, which shows that molecular cut-off of the control membrane was reduced to half when coated with vesicles. Molecular weight cut-off of the vesicle-coated membrane decreased to 4000 Ka upon core-crosslinking of the polybutadiene using initiator. Reduction in molecular cut-off is shown to be dependent on the amount of the cross-linker used.

## REFERENCES CITED IN THE DESCRIPTION

## Cited references

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## Patent documents cited in the description

- [WO0132146A \[0003\] \[0071\]](#)
- [WO2004011600A \[0003\]](#)
- [WO2009076174A \[0004\]](#)
- [WO2010146365A \[0004\]](#)
- [WO2013043118A \[0004\] \[0071\]](#)
- [WO2013180659A \[0005\]](#)

## Non-patent literature cited in the description

- **DONG et al.**J. Mem. Sci., 2012, 409-41034-43 [\[0004\]](#)
- **ZHAO et al.**J. Membrane Sci., 2012, 422-428 [\[0004\]](#)
- **XIE et al.**J. Mater. Chem A, 2013, vol. 1, 7592- [\[0005\]](#) [\[0071\]](#)
- **DUONG et al.**J. Memb. Sc., 2012, vol. 409-410, 34-43 [\[0005\]](#)
- **WANG et al.**J. Memb. Sc., 2013, vol. 434, 130-136 [\[0005\]](#)
- **XIEZHAO et al.**J. Membrane Sci., 2012, 422-428 [\[0071\]](#)

## Patentkrav

1. Filtreringsmembran, som omfatter en porøs bærer og, kovalent bundet til en overflade deraf, et kontinuerligt lag omfattende en flerhed af vesikler med
- 5 transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af en amfifil blokcopolymer, og transmembranproteinerne er udvalgt blandt aquaporiner og glyceroporiner; beta-barrel-transmembranproteiner; og alfa-helix-transmembranproteiner; **kendetegnet ved at** vesiklerne i laget er kovalent forbundet med hinanden til dannelse af en sammenhængende masse, hvori laget af
- 10 vesikler har en tykkelse, der er større end den gennemsnitlige diameter af vesiklerne, hvor den amfifile blokcopolymer er en diblok-copolymer AB med en hydrofil og en hydrofob blok eller en triblok-copolymer ABA med hydrofile endeblokke og en hydrofob indre blok, hvor filtreringsmembranen kan fremstilles ved en fremgangsmåde, der omfatter tilvejebringelse af en vandig suspension
- 15 af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af en amfifil blokcopolymer med reaktive endegrupper; afsætning af suspensionen af vesikler på en overflade af en porøs bærer; og tilvejebringelse af reaktionsbetingelser, således at der dannes kovalente bindinger mellem forskellige vesikler og mellem vesikler og overfladen, hvor fremgangsmåden omfatter:
- 20
- (i)
- (a) tilvejebringelse af en første vandig suspension af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af amfifile blokcopolymerer med reaktive endegrupper X;
- 25
- (b) tilvejebringelse af en anden vandig suspension af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af amfifile blokcopolymerer med reaktive endegrupper Y, som er reaktive med polymerendegrupper X;
- (c) afsætning af suspensionerne af vesikler på en bærer med en overflade,
- 30 som er reaktiv med enten polymerendegrupper X eller Y; og
- (d) fremkaldelse af reaktion af endegrupper X med endegrupper Y og enten endegrupper X eller endegrupper Y med overfladen af bæreren;
- eller hvor fremgangsmåden omfatter:
- (ii)

- (a) tilvejebringelse af en vandig suspension af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af amfifile blokcopolymerer med reaktive endegrupper X;
- 5 (b) tilvejebringelse af et multifunktionelt bindingsmiddel med mindst to reaktive grupper Y, som er reaktive med polymerendegrupper X;
- (c) afsætning af suspensionen af vesikler og den multifunktionelle linker på en bærer med en overflade, som er reaktiv med enten polymerendegrupper X eller reaktive grupper Y; og
- 10 (d) fremkaldelse af reaktion af endegrupper X med grupper Y og enten endegrupper X eller grupper Y med overfladen af bæreren, og hvor, i enten (i) eller (ii), grupperne X er amingrupper, og grupperne Y er carboxylsyre-, aktiveret carboxylsyre- og/eller azidgrupper; eller grupperne X er carboxylsyre-, aktiveret carboxylsyre- og/eller azidgrupper, og grupperne Y er amingrupper; eller grupperne X og Y er click-kemi-grupper.
- 15
- 2.** Membran ifølge krav 1, hvori laget af vesikler har en tykkelse, der større end 10 gange den gennemsnitlige diameter af vesiklerne.
- 20 **3.** Membran ifølge krav 1, hvori laget af vesikler har en tykkelse på mindst 0,04 mikron.
- 4.** Membran ifølge krav 3, hvori laget af vesikler har en tykkelse på mindst 0,2 mikron.
- 25 **5.** Membran ifølge et hvilket som helst af kravene 1 til 4, hvori vesiklerne har en gennemsnitlig diameter i området fra 50 til 100 nm.
- 30 **6.** Membran ifølge et hvilket som helst af kravene 1 til 5, hvori den hydrofile blok A er udvalgt fra gruppen af polymerer bestående af polyoxazolin, polyethylenglycol, polyethylenoxid, polyvinylalkohol, polyvinylpyrrolidon, polyacrylamid, poly(meth)acrylsyre, polyethylenoxid-co-polypropylenoxid-blokcopolymerer, poly(vinylether), poly(N,N-dimethylacrylamid), polyacrylsyre, polyacylalkylenimin, polyhydroxyalkylacrylater såsom hydroxyethyl-

- methacrylat, hydroxyethylacrylat, hydroxypropylacrylat og polyoler og copolymerer deraf, og den hydrofobe blok B er udvalgt fra gruppen af polymerer bestående af polysiloxaner, f.eks. polydimethylsiloxan eller polydiphenylsiloxan, perfluorpolyether, polystyren, polyoxypropylen, polyvinylacetat, polyoxybutylen, polyisopren, polybutadien, polyvinylchlorid, polyalkylacrylater, polyalkylmethacrylater, polyacrylonitril, polypropylen, polytetrahydrofuran, polymethacrylater, polyacrylater, polysulfoner, polyvinylethere og poly(propylenoxid) og copolymerer deraf.
- 5
- 10 **7.** Membran ifølge et hvilket som helst af kravene 1 til 6, hvori den amfifile blokcopolymer omfatter mindst en hydrofil blok omfattende (poly)2-C<sub>1-3</sub>alkyl-2-oxazolin og mindst en hydrofob blok omfattende (poly)dimethylsiloxan.
- 15 **8.** Membran ifølge krav 7, hvori den amfifile blokcopolymer er ((poly)2-C<sub>1-3</sub>alkyl-2-oxazolin)<sub>a</sub>-((poly)dimethylsiloxan)<sub>b</sub>-((poly)2-C<sub>1-3</sub>alkyl-2-oxazolin)<sub>a</sub>, hvori hvert a uafhængigt er et tal mellem 5 og 100, og b er et tal mellem 5 og 150.
- 20 **9.** Membran ifølge et hvilket som helst af de foregående krav, hvori bæreren omfatter en polyolefin, polyethersulfon, polysulfon eller polyacrylonitril.
- 25 **10.** Membran ifølge et hvilket som helst af de foregående krav, hvori transmembranproteinet er en aquaporin.
- 30 **11.** Membran ifølge et hvilket som helst af de foregående krav, hvor, i enten (i) eller (ii), grupperne X og Y er click-kemi-grupper, og grupperne X er azidgrupper, og grupperne Y er alkyngrupper, eller grupperne X er alkyngrupper, og grupperne Y er azidgrupper.
- 12.** Fremgangsmåde til fremstilling af en filtreringsmembran, hvilken filtreringsmembran omfatter en porøs bærer og, kovalent bundet til en overflade deraf, et kontinuerligt lag omfattende en flerhed af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af en amfifil blokcopolymer, og transmembranproteinerne er udvalgt blandt aquaporiner og glyceroporiner; beta-barrel-transmembranproteiner; og alfa-helix-transmembranproteiner;

- kendetegnet ved at** vesiklerne i laget er kovalent forbundet med hinanden til dannelse af en sammenhængende masse, hvori laget af vesikler har en tykkelse, der er større end den gennemsnitlige diameter af vesiklerne, hvor den amfifile blokcopolymer er en diblok-copolymer AB med en hydrofil og en hydrofob blok eller en triblok-copolymer ABA med hydrofile endeblokke og en hydrofob indre blok, hvilken fremgangsmåde omfatter tilvejebringelse af en vandig suspension af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af en amfifil blokcopolymer med reaktive endegrupper; afsætning af suspensionen af vesikler på en overflade af en porøs bærer; og tilvejebringelse af reaktionsbetingelser, således at der dannes kovalente bindinger mellem forskellige vesikler og mellem vesikler og overfladen, hvor fremgangsmåden omfatter enten:
- (i)
    - (a) tilvejebringelse af en første vandig suspension af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af amfifile blokcopolymerer med reaktive endegrupper X;
    - (b) tilvejebringelse af en anden vandig suspension af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af amfifile blokcopolymerer med reaktive endegrupper Y, som er reaktive med polymerendegrupper X;
    - (c) afsætning af suspensionerne af vesikler på en bærer med en overflade, som er reaktiv med enten polymerendegrupper X eller Y; og
    - (d) fremkaldelse af reaktion af endegrupper X med endegrupper Y og enten endegrupper X eller endegrupper Y med overfladen af bæreren; eller
  - (ii)
    - (a) tilvejebringelse af en vandig suspension af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af amfifile blokcopolymerer med reaktive endegrupper X;
    - (b) tilvejebringelse af et multifunktionelt bindingsmiddel med mindst to reaktive grupper Y, som er reaktive med polymerendegrupper X;
    - (c) afsætning af suspensionen af vesikler og den multifunktionelle linker på en bærer med en overflade, som er reaktiv med enten polymerendegrupper X eller reaktive grupper Y; og

5 (d) fremkaldelse af reaktion af endegrupper X med grupper Y og enten endegrupper X eller grupper Y med overfladen af bæreren, hvor, i enten (i) eller (ii), grupperne X er amingrupper, og grupperne Y er carboxylsyre-, aktiveret carboxylsyre- og/eller azidgrupper; eller grupperne X er carboxylsyre-, aktiveret carboxylsyre- og/eller azidgrupper, og grupperne Y er amingrupper; eller grupperne X og Y er click-kemi-grupper.

10 **13.** Fremgangsmåde ifølge krav 12, hvor, i enten (i) eller (ii), grupperne X og Y er click-kemi-grupper, og grupperne X er azidgrupper, og grupperne Y er alkyngrupper, eller grupperne X er alkyngrupper, og grupperne Y er azidgrupper.

**14.** Fremgangsmåde ifølge krav 12, hvori grupperne X er amingrupper.

15 **15.** Fremgangsmåde ifølge krav 14, som er en fremgangsmåde (ii), hvori der anvendes et multifunktionelt bindingsmiddel, hvor det multifunktionelle bindingsmiddel omfatter en gruppe Y, som er en aktiveret carboxylsyregruppe, og en anden gruppe Y, som er en azidgruppe.

20 **16.** Fremgangsmåde ifølge krav 15, hvori det multifunktionelle bindingsmiddel er N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoat.

**17.** Fremgangsmåde ifølge et hvilket som helst af kravene 12 til 16, som er en fremgangsmåde (ii), og som omfatter:

25 (a) tilvejebringelse af en vandig opløsning af vesikler med transmembranproteiner inkorporeret deri, hvor vesiklerne er dannet af amfifile blokcopolymerer med reaktive endegrupper X;

30 (b) tilvejebringelse af et multifunktionelt, fortrinsvis difunktionelt, bindingsmiddel med mindst to reaktive grupper Y, som er reaktive med polymerendegrupper X, herunder en første reaktiv gruppe Y(1), som er i stand til reaktion med polymerendegrupper X under et første sæt af reaktionsbetingelser, og en anden reaktiv gruppe Y(2), som ikke er reaktiv med polymerendegrupper X under det første sæt af reaktionsbetingelser, men som er reaktiv med polymerendegrupper X under et andet sæt af reaktionsbetingelser;

(b') blanding af den vandige opløsning af vesikler med det multifunktionelle bindingsmiddel under det første sæt af reaktionsbetingelser, således at den reaktive gruppe Y(1) reagerer med polymerendegrupper X;

5 (c) afsætning af den resulterende opløsning på en bærer, som er reaktiv med den anden reaktive gruppe Y(2), i en mængde, der er tilstrækkelig til at frembringe det ønskede lag af vesikler; og

(d) fremkaldelse af en reaktion af endegrupper X med den anden reaktive gruppe Y(2), og anden reaktive endegrupper Y(2) med overfladen af bæreren, ved anvendelse af det andet sæt af reaktionsbetingelser.

10

**18.** Fremgangsmåde ifølge krav 17, hvori det multifunktionelle bindingsmiddel har en første reaktiv gruppe Y(1), som er i stand til reaktion med polymerendegrupper X ved kontakt, og en anden reaktiv gruppe Y(2), som er i stand til reaktion med polymerendegrupper X ved fotobestråling; og trin (d) udføres ved anvendelse af fotobestråling.

15

**19.** Fremgangsmåde ifølge krav 18, hvori blokcopolymeren har terminale aminogrupper, det multifunktionelle bindingsmiddel er N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoat, og trin (d) udføres ved anvendelse af UV-lys.

20

## DRAWINGS

Figure 1

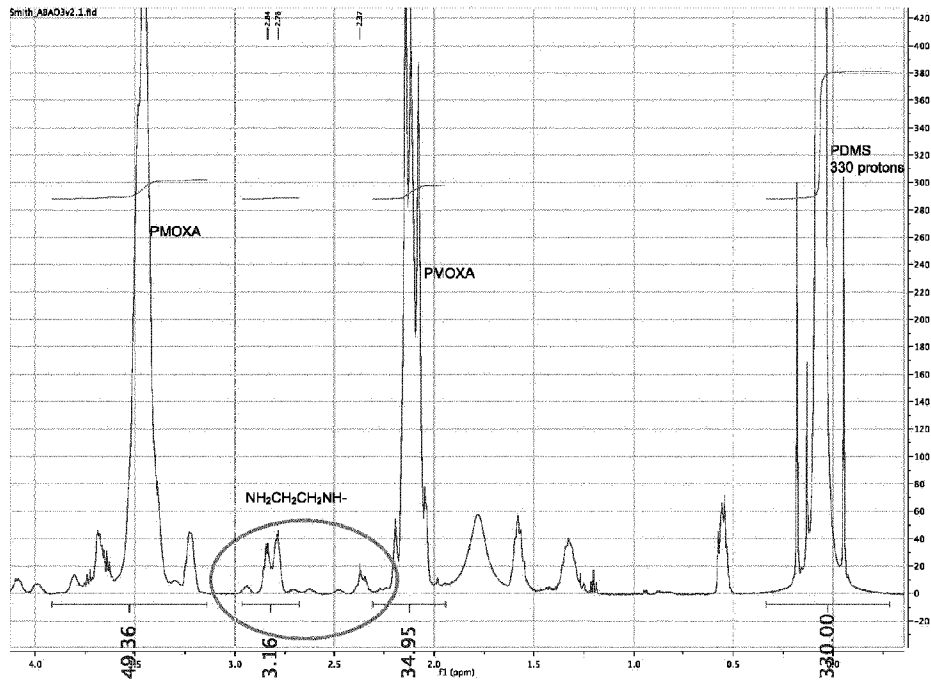
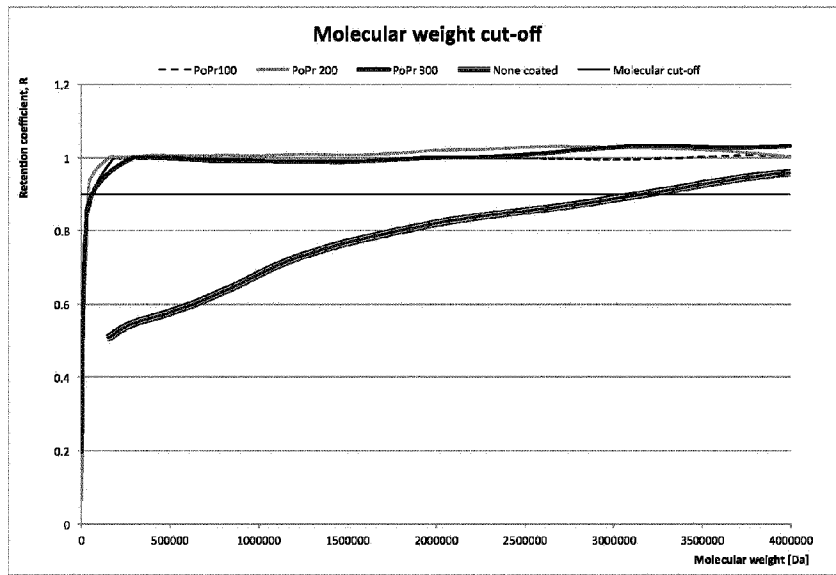


Figure 2



1

Figure 3

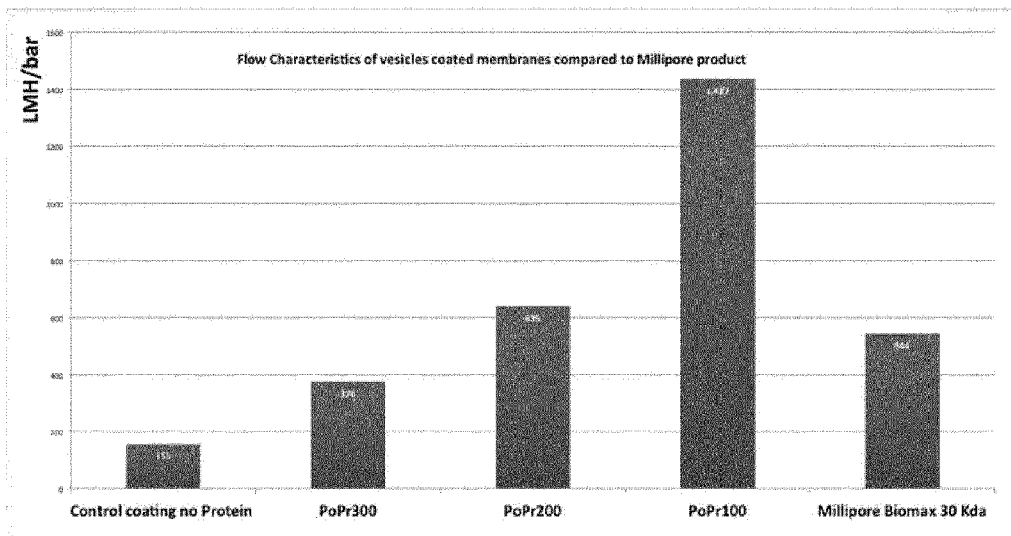


Figure 4

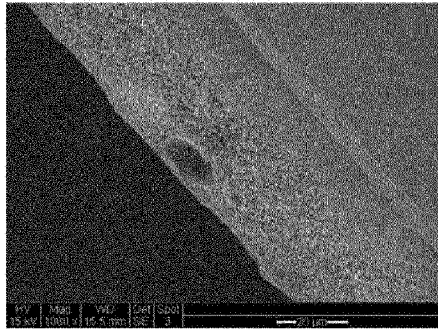


Figure 5

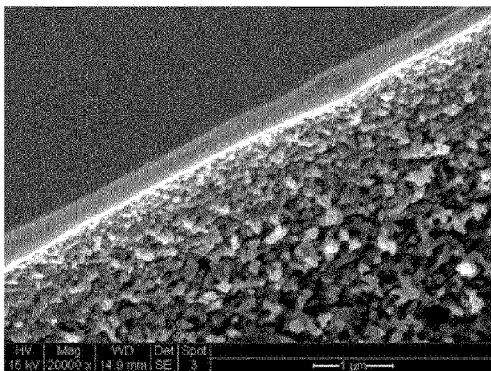


Figure 6

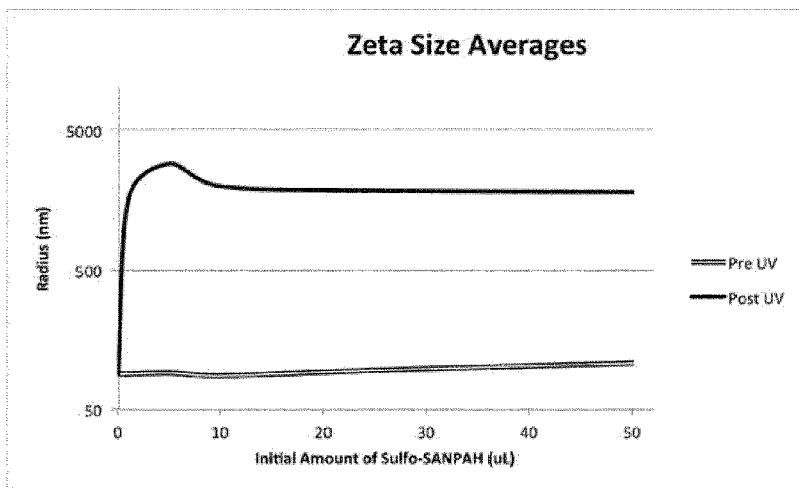


Figure 7A

Figure 7B

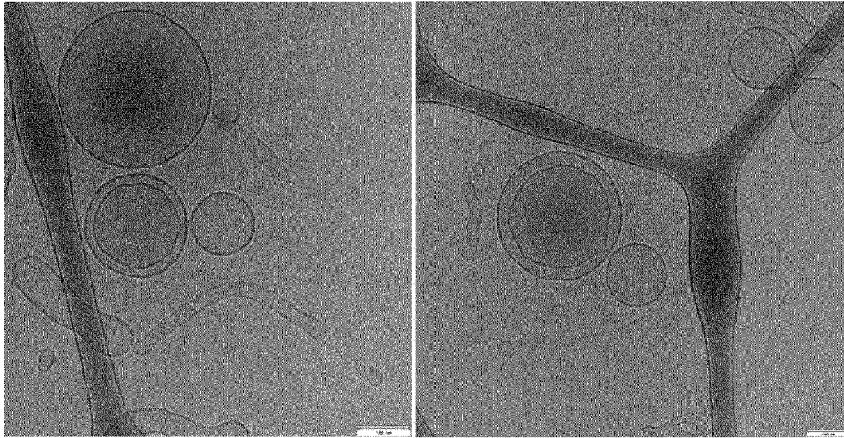


Figure 8

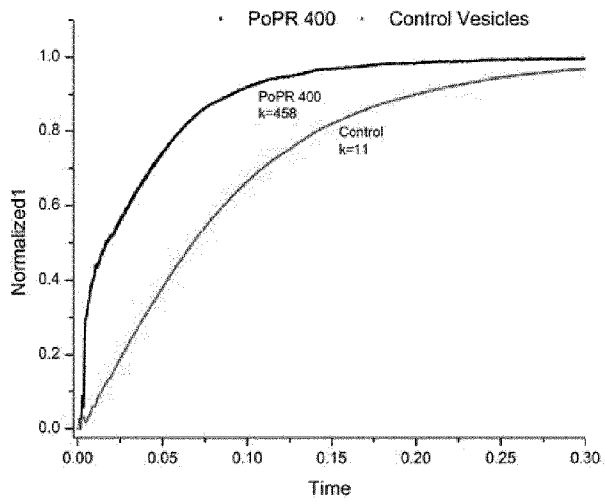


Figure 9

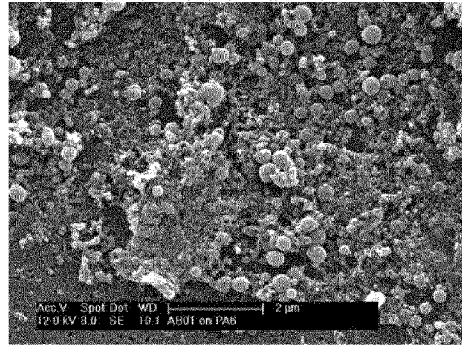


Figure 10

