

PEPTIDE-ATTACHED PILLAR[5]ARENE FOR HIGHLY PERMEABLE AND
SELECTIVE ARTIFICIAL WATER CHANNEL, AND ITS SYNTHESIS AND
CHARACTERIZATION

5 **Cross-Reference To Related Application**

[0001] This application claims the benefit of priority of Singapore Patent Application No. 10201802298R, filed 21 March 2018, the content of it being hereby incorporated by reference in its entirety for all purposes.

10 **Technical Field**

[0002] The present disclosure relates to a method of synthesizing hydrolysates of a peptide-attached pillar[5]arene. The present disclosure also relates to such hydrolysates and their uses.

15 **Background**

[0003] Aquaporins (AQPs) are water channel proteins composed of an hourglass-like structure with its narrowest constriction at 2.8 Å. This unique structure not only allows for a fast water transport (about 10^8 – 10^9 molecules/s/channel), but also prevents solute transport. Such transport properties have inspired an extensive study on reconstruction of AQPs into synthetic membranes for desalination and water-purification applications. Several types of AQPs-based biomimetic membranes have since been designed and fabricated with outstanding water flux and desalination performance. The high cost of AQPs production, AQPs' low stability and challenges in AQPs membrane fabrication, however, hinder large scale applications of AQPs.

25 [0004] As such, artificial water channels constructed to have a water permeable central pore and an outer hydrophobic shell that are compatible for incorporation into a lipid membrane, have been considered as a substitution of AQPs. Based on chemical synthesis and self-assembly, several types of artificial water channels have been designed and developed at improving their water conduction rate and selectivity.
30 However, none of the conventional water channels attains the water permeability and/or selectivity of AQPs.

[0005] Moreover, exploration of artificial water channels remains at a proof-of-concept stage, which is far away from practical applications.

[0006] There is thus a need to provide for a solution that ameliorates one or more of the limitations mentioned above. The solution should at least provide for a compound
5 configurable into an artificial water channel with high water permeability and improved selective transport properties.

Summary

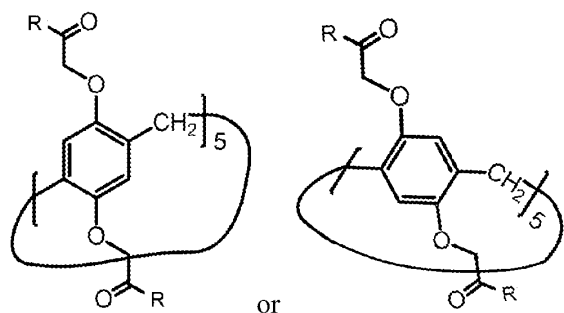
[0007] In one aspect, there is provided for a method of synthesizing a hydrolysate of a
10 peptide-attached pillar[5]arene, the method comprising:

forming a pillar[5]arene substituted with carboxyl groups from a dialkoxybenzene;

mixing the pillar[5]arene substituted with carboxyl groups and a tripeptide in an organic solvent to form the peptide-attached pillar[5]arene, wherein the tripeptide
15 has a terminal -NH₂ group and a terminal ester; and

subjecting the peptide-attached pillar[5]arene to hydrolysis in the presence of a base to obtain the hydrolysate of the peptide-attached pillar[5]arene.

[0008] In another aspect, there is provided for a hydrolysate of a peptide-attached pillar[5]arene configured as a water channel in a liposomal membrane, wherein the
20 hydrolysate of the peptide-attached pillar[5]arene is represented by the formula:

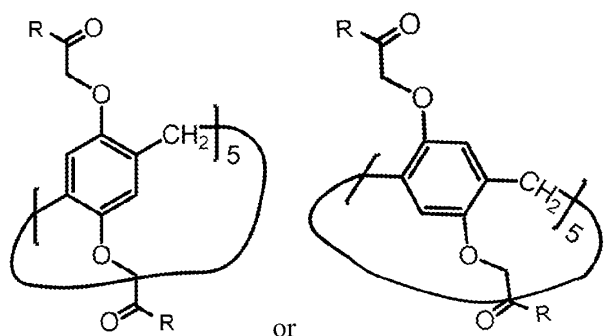


wherein R represents a tripeptide having a terminal -NH- group and a terminal -COOH group, wherein the terminal -NH- group forms part of an amide linkage, and wherein the terminal -COOH group extends away from the amide linkage.

[0009] In another aspect, there is provided for a method of synthesizing a liposomal membrane comprising a hydrolysate of a peptide-attached pillar[5]arene, the method comprising:

- synthesizing a hydrolysate of a peptide-attached pillar[5]arene according to the method described in various embodiments of the first aspect;
- mixing the hydrolysate and a plurality of lipids to form a mixture;
- forming a liposomal film from the mixture;
- contacting the liposomal film with a buffer to form a suspension comprising the hydrolysate and the plurality of lipids; and
- extruding the suspension through a membrane to form the liposomal membrane.

[0010] In another aspect, there is provided for a liposomal membrane comprising a plurality of lipids and at least one hydrolysate of a peptide-attached pillar[5]arene, wherein the at least one hydrolysate of the peptide-attached pillar[5]arene is represented by the formula:



wherein R represents a tripeptide having a terminal -NH- group and a terminal -COOH group, wherein the terminal -NH- group forms an amide linkage with the carbonyl carbon, and wherein the terminal -COOH group extends away from the amide linkage.

[0011] In another aspect, there is provided for a method of determining insertion efficiency of a hydrolysate of a peptide-attached pillar[5]arene obtained according to the method described in various embodiments of the first aspect, the method comprising:

- mixing a liposomal membrane obtained according to the method described herein with a buffer solution;

subjecting the buffer solution to UV-visible spectroscopy at an UV-visible absorbance wavelength of 293 nm to detect the UV-visible absorbance from π - π transition of a benzene; and

5 correlating the UV-visible absorbance to a UV-visible absorbance-concentration standard curve to determine the insertion efficiency.

[0012] In another aspect, there is provided for a method of determining insertion efficiency of a hydrolysate of a peptide-attached pillar[5]arene obtained according to the method described in various embodiments of the first aspect, the method comprising:

10 mixing a liposomal membrane obtained according to the method described herein with a buffer solution;

subjecting the buffer solution to circular dichroism at a circular dichroism absorbance wavelength of 306 nm to detect the circular dichroism absorbance from π - π transition of a benzene; and

15 correlating the circular dichroism absorbance to a circular dichroism absorbance-concentration standard curve to determine the insertion efficiency.

Brief Description of the Drawings

[0013] The drawings are not necessarily to scale, emphasis instead generally being
20 placed upon illustrating the principles of the invention. In the following description, various embodiments of the present disclosure are described with reference to the following drawings, in which:

[0014] FIG. 1 shows a synthesis procedure of peptide-attached (pS)- and (pR)-pillar[5]arenes (pS-PH and pR-PH).

25 [0015] FIG. 2A shows the (pS)-isomer and (pR)-isomer of planar chiral pillar[5]arene.

[0016] FIG. 2B shows the chemical structures of peptide-attached (pS)-pillar[5]arenes and peptide-attached (pR)-pillar[5]arenes (i.e. pS-PM and pR-PM, respectively), and their corresponding hydrolysates (pS-PH and pR-PH, respectively).

30 [0017] FIG. 2C shows partial ^1H NMR spectra of pS-PM, pR-PM, and a mixture of pS-PM and pR-PM, in DMSO- d_6 at 298 K.

[0018] FIG. 2D shows the circular dichroism (CD) spectra of pS-PM, pR-PM, pS-PH, and pR-PH, in tetrahydrofuran (THF) at 298 K.

[0019] FIG. 2E shows UV-vis spectra of pS-PM, pR-PM, pS-PH, and pR-PH, in THF at 298 K.

5 [0020] FIG. 3 shows a ^1H NMR spectrum of pS-PM in $\text{DMSO-}d_6$.

[0021] FIG. 4 shows a ^{13}C NMR spectrum of pS-PM in $\text{DMSO-}d_6$.

[0022] FIG. 5 shows a MALDI-TOF (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) mass spectrum of pS-PM.

[0023] FIG. 6 shows a ^1H NMR spectrum of pR-PM in $\text{DMSO-}d_6$.

10 [0024] FIG. 7 shows a ^{13}C NMR spectrum of pR-PM in $\text{DMSO-}d_6$.

[0025] FIG. 8 shows a MALDI-TOF mass spectrum of pR-PM.

[0026] FIG. 9 shows a ^1H NMR spectrum of pS-PH in $\text{DMSO-}d_6$.

[0027] FIG. 10 shows a ^{13}C NMR spectrum of pS-PH in $\text{DMSO-}d_6$.

[0028] FIG. 11 shows a MALDI-TOF mass spectrum of pS-PH.

15 [0029] FIG. 12 shows a ^1H NMR spectrum of pR-PH in $\text{DMSO-}d_6$.

[0030] FIG. 13 shows a ^{13}C NMR spectrum of pR-PH in $\text{DMSO-}d_6$.

[0031] FIG. 14 shows a MALDI-TOF mass spectrum of pR-PH.

[0032] FIG. 15A is a schematic illustration of stopped-flow light scattering test for shrinking experiment.

20 [0033] FIG. 15B shows stopped-flow light scattering curves of liposomes containing pS-PH channels with different channel-to-lipid molar ratios (CLRs) after exposure to a hypertonic solution of 400 mM sucrose at 10°C .

[0034] FIG. 15C shows stopped-flow light scattering curves of liposomes containing pR-PH channels with different channel-to-lipid molar ratios (CLRs) after exposure to
25 a hypertonic solution of 400 mM sucrose at 10°C .

[0035] FIG. 15D shows the net water permeability of pR-PH in liposomes with different channel-to-lipid molar ratios (CLRs) measured under hypertonic conditions at 10°C .

[0036] FIG. 15E shows Arrhenius plots for calculating activation energy.

30 [0037] FIG. 15F is a schematic illustration of the solubilization pR-PH channels in buffer solution containing octyl glucoside (OG).

[0038] FIG. 15G shows the single-channel water permeability of the pR-PH channels.

[0039] FIG. 15H shows reflection coefficients (or rejection ratios) of the pR-PH channels in liposomes.

[0040] FIG. 16A shows representative stopped-flow light scattering curves of liposomes containing pS-PH channels with different CLR_s after exposure to a
5 hypertonic solution of 400 mM sucrose at 25°C.

[0041] FIG. 16B shows representative stopped-flow light scattering curves of liposomes containing pR-PH channels with different CLR_s after exposure to a hypertonic solution of 400 mM sucrose at 25°C.

[0042] FIG. 16C shows the net water permeability of pR-PH in liposomes with
10 different CLR_s measured under hypertonic conditions at 25°C.

[0043] FIG. 17A is a schematic illustration of stopped-flow light scattering test for swelling experiment.

[0044] FIG. 17B shows representative stopped-flow light scattering curves of liposomes containing pS-PH channels (CLR for 0 and 0.0025) after exposure to a
15 hypotonic solution (the same buffer without 100 mM NaCl) at 25°C.

[0045] FIG. 17C shows net water permeability of pR-PH in liposomes (CLR for 0.0025 and 0.005) in swelling and shrinking mode at 25°C.

[0046] FIG. 18A shows UV-vis spectra of pR-PH channels solutions (from 0 μM to 15 μM).

[0047] FIG. 18B shows calibration curves of UV-vis absorbance against
20 concentration of pR-PH channels.

[0048] FIG. 18C shows CD spectra of pR-PH channel solutions (from 0 μM to 15 μM).

[0049] FIG. 18D shows calibration curves of CD absorbance against concentration of
25 pR-PH channels.

[0050] FIG. 18E shows size of a hypothetical liposome and surface area of phosphatidylcholine/phosphatidylserine (PC/PS) lipids and pR-PH channels.

[0051] FIG. 18F shows the insertion efficiency and number of pR-PH channels into liposomes at CLR = 0.005.

[0052] FIG. 19 shows representative stopped-flow light scattering curves of
30 liposomes containing PR-PH channels (CLR = 0.005) using different solutes as osmolytes. The liposomes were abruptly exposed to a hypertonic solution of 200 mM

NaCl, 400 mM glycine, 400 mM valine, 400 mM Glucose and 400 mM sucrose at 25°C

[0053] FIG. 20 shows a comparison of pR-PH channels to AQPs and other artificial water channels.

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Detailed Description

[0054] The following detailed description refers to the accompanying drawings that show, by way of illustration, specific details and embodiments in which the invention may be practised. These embodiments are described in sufficient detail to enable those skilled in the art to practice the invention. Other embodiments may be utilized and changes may be made without departing from the scope of the invention. The various embodiments are not necessarily mutually exclusive, as some embodiments can be combined with one or more other embodiments to form new embodiments.

[0055] Features that are described in the context of an embodiment may correspondingly be applicable to the same or similar features in the other embodiments. Features that are described in the context of an embodiment may correspondingly be applicable to the other embodiments, even if not explicitly described in these other embodiments. Furthermore, additions and/or combinations and/or alternatives as described for a feature in the context of an embodiment may correspondingly be applicable to the same or similar feature in the other embodiments.

[0056] The present disclosure relates to hydrolysates of peptide-attached pillar[5]arenes, which are derivatives of pillar[5]arenes, and a method of synthesizing such hydrolysates. The term “hydrolysate” used herein refers to a compound derived from hydrolysis of a peptide-attached pillar[5]arene. Advantageously, each of the hydrolysates derived from the present method provides for a water permeability of 1.3×10^9 water molecules/s, which is superior over conventional derivatives of pillar[5]arene, and is at least comparable to that of aquaporins in terms of having a same orders of magnitude (i.e. 10^9). The hydrolysates also provide substantial, or even complete, rejection of salts and solutes. The term “peptide-attached” and its grammatical variants thereof, as used herein, means that the pillar[5]arene is

covalently bonded to the peptide via an amide linkage. The term “amide” refers to a chemical group having the form of -NHC(=O)- .

[0057] The peptide-attached pillar[5]arenes can be used as templates to form tubular structures of water channels by functionalization, e.g. to form hydroxyl or carboxyl groups on the attached peptide. The term “hydroxyl” used herein refers to an -OH functional group. The term “carboxyl” used herein refers to a -COOH functional group. Such pillar[5]arene-based water channels, as disclosed herein, demonstrate water conduction ability with desirable salt rejection over conventional pillar[5]arene that has water conduction ability but no salt rejection. The phrase “water conduction ability” used herein refers to the ability to allow water molecules to pass through the channel defined by the tubular structure of the hydrolysates of the peptide-attached pillar[5]arene.

[0058] The hydrolysates of the peptide-attached pillar[5]arene disclosed herein possess planar chirality, which arises from rotation of the benzene units around methylene bridges of the pillar[5]arene. By incorporating bulky rigid peptides and substituents thereof onto pillar[5]arene, two of the most stable isomers (abbreviated as pS and pR) can be formed (FIG. 2A), and these two isomers are preferably formed, through the present method, due to their lowest energy. In fact, the planar chirality of the hydrolysates disclosed herein is a factor influencing the water conduction properties of the water channels. The present synthesis of hydrolysates of peptide-attached pillar[5]arenes disclosed herein isolates the (pS)- and (pR)-isomers of pillar[5]arene, leading to improved water conduction ability with desirable rejection of salts, as these isomers of pillar[5]arene are specifically synthesized.

[0059] In the present disclosure, the phrase “liposomal membrane” broadly refers to a membrane that comprises lipids and may be incorporated with the hydrolysate. The lipids may be in the form of a liposome. The liposomal membrane may be in the form of a membrane that is formed from lipids and is incorporated with the hydrolysate. The liposomal membrane may be in the form of a membrane comprising one or more liposomes incorporated with the hydrolysate. The liposomal membrane may alternatively be a liposome that is incorporated with the hydrolysate. Accordingly, the phrase “liposomal membrane” may refer to any form of the membranes described above, and may be used interchangeably with the phrase “lipid membrane” as the

membrane comprises lipids. The phrases “liposomal membrane” and “lipid membrane” may be termed “water membrane” as the liposomal membrane is usable for conducting water, e.g. improving water flux in filtration.

5 [0060] In the present disclosure, the word “substantially” does not exclude “completely” e.g. a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

10 [0061] In the context of various embodiments, the articles “a”, “an” and “the” as used with regard to a feature or element include a reference to one or more of the features or elements.

[0062] In the context of various embodiments, the term “about” or “approximately” as applied to a numeric value encompasses the exact value and a reasonable variance.

[0063] As used herein, the term “and/or” includes any and all combinations of one or more of the associated listed items.

15 [0064] Unless specified otherwise, the terms “comprising” and “comprise”, and grammatical variants thereof, are intended to represent “open” or “inclusive” language such that they include recited elements but also permit inclusion of additional, unrecited elements.

20 [0065] Details of the present method of synthesizing the hydrolysates, the hydrolysates and their uses, and methods of determining insertion efficiency of hydrolysates in a liposomal membrane, and the various embodiments are described as follows.

25 [0066] In the present disclosure, there is provided for a method of synthesizing a hydrolysate of a peptide-attached pillar[5]arene. The method comprises forming a pillar[5]arene substituted with carboxyl groups from a dialkoxybenzene, mixing the pillar[5]arene substituted with carboxyl groups and a tripeptide in an organic solvent to form the peptide-attached pillar[5]arene, wherein the tripeptide has a terminal -NH₂ group and a terminal ester, and subjecting the peptide-attached pillar[5]arene to hydrolysis in the presence of a base to obtain the hydrolysate of the peptide-attached
30 pillar[5]arene.

[0067] In the present method, forming the pillar[5]arene substituted with carboxyl groups may comprise mixing the dialkoxybenzene and a paraformaldehyde in the

presence of an organic acid catalyst to form a dialkoxypillar[5]arene. The term “dialkoxybenzene” used herein refers to a benzene having two alkoxy groups positioned opposite to each other, wherein each alkoxy group is of the form -O-alkyl and the “O” atom is attached directly to the benzene via covalent bonding. An example of such a dialkoxybenzene may be 1,4-dimethoxybenzene. Accordingly, the term “dialkoxypillar[5]arene” refers to a pillar[5]arene having two alkoxy groups positioned opposite to each other, wherein the alkoxy group has been defined above.

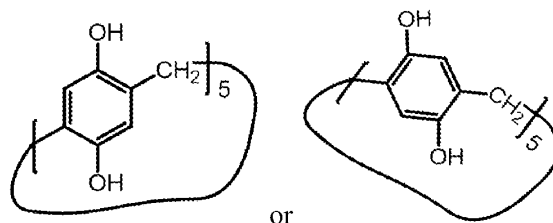
[0068] The term “alkyl” used herein as a group or part of a group refers to a straight or branched aliphatic hydrocarbon group, including but not limited to, a C₁-C₁₀ alkyl, a C₁-C₉ alkyl, C₁-C₈ alkyl, C₁-C₇ alkyl, C₁-C₆ alkyl, C₁-C₅ alkyl, a C₁-C₄ alkyl, a C₁-C₃ alkyl, and a C₁-C₂ alkyl. Examples of suitable straight and branched C₁-C₆ alkyl substituents include methyl, ethyl, n-propyl, 2-propyl, n-butyl, sec-butyl, t-butyl, hexyl, and the like.

[0069] By having the alkoxy groups positioned opposite to each other on the benzene ring, the hydrolysates of the peptide-attached pillar[5]arene are configured to define a channel without obstruction to water molecules passing through, as the position of the alkoxy groups forms the position at which the peptides can be attached. In various embodiments, the dialkoxybenzene may comprise 1,4-dimethoxybenzene. In embodiments where 1,4-dimethoxybenzene is used, the dialkoxypillar[5]arene may comprise 1,4-dimethoxypillar[5]arene.

[0070] In various embodiments, the organic acid catalyst may comprise boron trifluoride diethyl etherate, p-toluenesulfonic acid, or trifluoroacetic acid. The trifluoroacetic acid, as one example of the acid catalyst, can provide higher yield for the dialkoxypillar[5]arene, which in turn provide higher yield for the hydrolysates.

[0071] In the present method, forming the pillar[5]arene substituted with carboxyl groups may comprise mixing the dialkoxypillar[5]arene with boron tribromide to form the pillar[5]arene. Boron tribromide, as one example, may be used to completely remove the alkoxy groups to obtain higher yield for the pillar[5]arene substituted with carboxyl groups. Reaction with the boron tribromide converts the alkoxy groups on the dialkoxypillar[5]arene to hydroxyl groups. Thus, the pillar[5]arene of the present disclosure may have two hydroxyl groups positioned opposite to each other on the

benzene, and the advantage of such positioning has been described above. In various embodiments, the pillar[5]arene may be represented by the formula:



[0072] In various embodiments, forming the pillar[5]arene substituted with carboxyl groups may comprise mixing the pillar[5]arene and an alkyl acetate in the presence of a catalyst to form a pillar[5]arene substituted with carbonyl groups. The alkyl acetate is low in cost and easy for use in the present method. Forming pillar[5]arene substituted with carbonyl groups advantageously allows for conversion to carboxyl groups, which can then react with the “-NH₂” group of a peptide, for the peptide to be attached to the benzene of the pillar[5]arene.

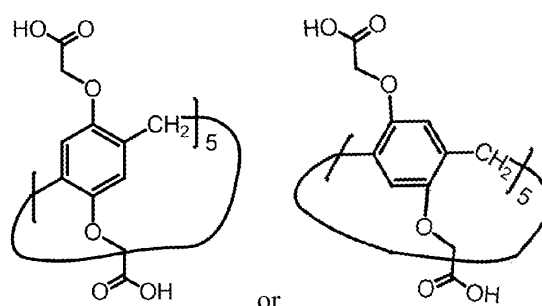
[0073] The term “alkyl acetate” refers to an acetate ion of the form alkyl-C(=O)O⁻. In various embodiments, the alkyl acetate may comprise butyl bromoacetate, ethyl bromoacetate, methyl bromoacetate, or propyl bromoacetate. The alkyl acetate reacts with the hydroxyl groups of the pillar[5]arene to form carbonyl groups.

[0074] Carbonyl groups in the context of the present disclosure refer to groups of the form “-R_mC(=O)R_n”, wherein R_m and R_n represent generic organic substituents, including hydrogen. In various embodiments, the pillar[5]arene substituted with carbonyl groups may comprise an ethoxycarbonylmethoxy-substituted pillar[5]arene.

[0075] In various embodiments, the catalyst may comprise potassium iodide or sodium iodide. Use of these iodides, or other suitable iodides, may help to improve yield of the pillar[5]arene substituted with carbonyl groups.

[0076] In the present method, forming the pillar[5]arene substituted with carboxyl groups may comprise mixing the pillar[5]arene substituted with carbonyl groups and a basic solution to form the pillar[5]arene substituted with carboxyl groups. As already mentioned above, when the pillar[5]arene becomes substituted with carboxyl groups, the carboxyl groups allow for the peptide to be attached thereon. The basic solution may comprise caesium hydroxide, lithium hydroxide, potassium hydroxide, or sodium hydroxide according to various embodiments.

[0077] In various embodiments, the pillar[5]arene substituted with carboxyl groups may be represented by the formula:



[0078] Once pillar[5]arene substituted with carboxyl groups are obtained, they can be mixed with peptides to form peptide-attached pillar[5]arene. The peptide may be a tripeptide. The term “tripeptide” refers to a peptide having three amino acids joined by amide linkages, has a terminal -NH₂ and a terminal ester. Such tripeptides render the resultant water channel compatible for incorporation into a liposomal membrane. The term “terminal” used herein means that the -NH₂ group and the ester are positioned at the end of a carbon chain of the peptide. This includes a positioning where the -NH₂ group and the ester are positioned at opposite to each other at the end of carbon chain of the tripeptide. The term “ester” used herein, whether as a group, or part of a group such as in an “ester linkage, refers to a compound having -C(=O)O-.

[0079] Mixing the pillar[5]arene substituted with carboxyl groups and the tripeptide may comprise mixing the pillar[5]arene substituted with carboxyl groups and the tripeptide at a temperature ranging from 45 to 70°C for 12 to 180 hours (hrs) under an inert atmosphere. The temperature may be 45 to 70°C, 50 to 70°C, 60 to 70°C, 45 to 60°C, 50 to 60°C, 45 to 50°C, etc. The duration may be 12 to 180 hrs, 50 to 180 hrs, 100 to 180 hrs, 150 to 180 hrs, 12 to 150 hrs, 12 to 100 hrs, 12 to 50 hrs, 50 to 150 hrs, 50 to 100 hrs, 100 to 150 hrs, etc.

[0080] In various embodiments, the tripeptide may comprise NH₂-L-Phe-L-Phe-L-Phe-O-methyl, NH₂-L-Phe-L-Phe-L-Phe-O-ethyl, NH₂-L-Phe-L-Phe-L-Phe-O-propyl, NH₂-D-Phe-D-Phe-D-Phe-O-methyl, NH₂-D-Phe-D-Phe-D-Phe-O-ethyl, or NH₂-D-Phe-D-Phe-D-Phe-O-propyl. In various embodiments, the tripeptide may comprise NH₂-L-Phe-L-Phe-L-Phe-O-methyl. This peptide, as an example, may render the water channel more stable, thereby forming a more stable open pore when incorporated into a liposome.

[0081] In various embodiments, the organic solvent may comprise anhydrous dimethylformamide, 4-dimethylaminopyridine and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride. These organic solvents are advantageous in that, for example, dimethylformamide can be used to dissolve the various compounds as disclosed herein, and 4-dimethylaminopyridine and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride can lead to higher yield for forming the peptide-attached pillar[5]arene.

[0082] The inert atmosphere may comprise or may be of nitrogen, argon, or any other non-reactive gases such as noble gases.

[0083] The present method may further comprise subjecting the peptide-attached pillar[5]arene to column chromatography to produce a fraction comprising a diastereomeric mixture of the peptide-attached pillar[5]arene, and filtering the diastereomeric mixture after mixing the diastereomeric mixture with acetone to obtain a residue and a filtrate, wherein the residue comprises a diastereomer pR and the filtrate comprises a diastereomer pS. The fraction as disclosed herein may be the first fraction that is eluted out of the chromatographic column. The acetone as used herein is advantageous as it is a good solvent for isolating the two diastereomers based on their different solubility. The diastereomer pS has good solubility in acetone while the diastereomer pR cannot be dissolved in acetone.

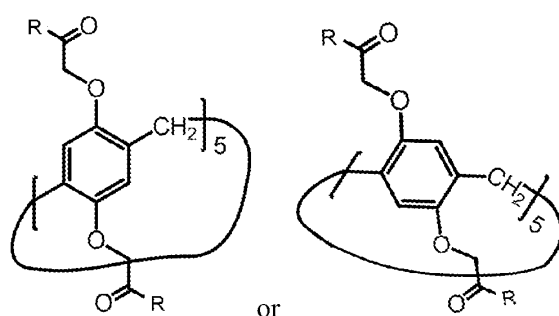
[0084] The term “diastereomer” used herein refers to a stereoisomer of the peptide-attached pillar[5]arene, including its hydrolysates, having different planar configurations due to one or more, but not all of the stereocenters, and are not mirror images of each other. The term “diastereomer pR” refers to a diastereomer of the peptide-attached pillar[5]arene having a pR configuration. Such a diastereomer is shown in FIG. 2A. The term “diastereomer pS” refers to a diastereomer of the peptide-attached pillar[5]arene having a pS configuration. Such a diastereomer is shown in FIG. 2A.

[0085] The isolated diastereomers of the peptide-attached pillar[5]arene may be subjected to hydrolysis to obtain the hydrolysates, and in various embodiments, subjecting the peptide-attached pillar[5]arene to hydrolysis may comprise mixing the peptide-attached pillar[5]arene and the base for 8 to 40 hours (hrs). The duration may

alternatively be, for example, 10 to 40 hrs, 20 to 40 hrs, 30 to 40 hrs, 8 to 30 hrs, 8 to 20 hrs, 8 to 10 hrs, 10 to 30 hrs, 10 to 20 hrs, 20 to 30 hrs, etc.

[0086] In various embodiments, the base may comprise lithium hydroxide, potassium hydroxide, or sodium hydroxide. These hydroxides selectively hydrolyze the ester and avoid unnecessary hydrolysis of the amide to form the resultant hydrolysates.

[0087] The present disclosure also provides for a hydrolysate of a peptide-attached pillar[5]arene configured as a water channel in a liposomal membrane, wherein the hydrolysate of the peptide-attached pillar[5]arene may be represented by the formula:



wherein R represents a tripeptide having a terminal -NH- group and a terminal -COOH group, wherein the terminal -NH- group forms part of an amide linkage, and wherein the terminal -COOH group extends away from the amide linkage. The terminal -NH- group of the tripeptide is attached to the -C(=O)- group extending away from the benzene, as shown in the above formula, to form the amide linkage. The hydrolysate is obtained or obtainable according to the method described in various embodiments of the first aspect.

[0088] Embodiments and advantages described in the context of the present method are analogously valid for the hydrolysate described herein, and vice versa. As the various embodiments and advantages have already been described above, they are not iterated for brevity.

[0089] In various embodiments, the tripeptide may comprise -NH-L-Phe-L-Phe-L-Phe-COOH or -NH-D-Phe-D-Phe-D-Phe-COOH. In such embodiments where the tripeptide comprises -NH-L-Phe-L-Phe-L-Phe-COOH or -NH-D-Phe-D-Phe-D-Phe-COOH, the hydrolysate may be of the pR configuration, pS configuration, or a mixture thereof.

[0090] In the present disclosure, a method of synthesizing a liposomal membrane comprising a hydrolysate of a peptide-attached pillar[5]arene is provided. Embodiments and advantages described in the context of the present method of synthesizing the hydrolysates are analogously valid for the method of synthesizing the liposomal membrane as described herein, and vice versa. As the various embodiments and advantages have already been described above, they are not iterated for brevity.

[0091] The method may comprise synthesizing a hydrolysate of a peptide-attached pillar[5]arene according to the method described in various embodiments of the first aspect, mixing the hydrolysate and a plurality of lipids to form a mixture, forming a liposomal film from the mixture, contacting the liposomal film with a buffer to form a suspension comprising the hydrolysate and the plurality of lipids, and extruding the suspension through a membrane to form the liposomal membrane.

[0092] In the present method, mixing the hydrolysate and the plurality of lipids may comprise mixing the hydrolysate and the plurality of lipids in a molar ratio of more than 0 and up to 0.02. Non-limiting examples of the molar ratio may range from 0.025 to 0.02, 0.005 to 0.02, 0.01 to 0.02, etc. Liposomal membrane, for example, in the form of liposomes made from these molar ratio advantageously provide for water permeability comparable to that of aquaporins without compromising the rejection of salts and solutes. The rejection of salts and solutes may be a substantial rejection, or even complete rejection. Substantial rejection includes at least rejecting 95%, 99%, or even 100%, of the salts and solutes. In certain embodiments, the molar ratio may be 0.02.

[0093] In the present method, mixing the hydrolysate and the plurality of lipids may comprise dissolving the hydrolysate in one or more organic solvents. The one or more organic solvents may comprise chloroform and/or methanol. Such organic solvents have low boiling point that allows ease of removal by evaporation. In various embodiments, the chloroform and/or methanol may be present in a volume ratio of 0:1 to 20:1. Where a mixture of chloroform and methanol is used in such ratios, all the various compounds used herein may be easily dissolved. In certain embodiments, the chloroform and methanol may be present in a volume ratio of 1:1.

[0094] For synthesizing the liposomal membrane, for example as liposomes, the plurality of lipids may comprise phosphatidylcholine and/or phosphatidylserine. In

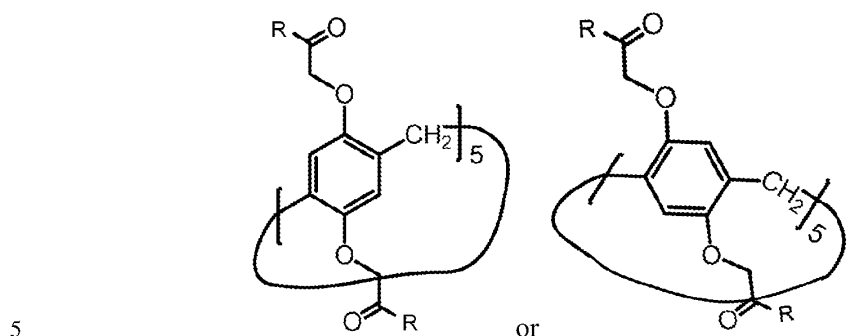
various embodiments, the plurality of lipids may comprise phosphatidylcholine and/or phosphatidylserine. Other lipids may be used. In embodiments where phosphatidylcholine and/or phosphatidylserine are used, the phosphatidylserine and/or phosphatidylcholine may be present in a molar ratio of 0:1 to 100:1. In certain
5 embodiments, the phosphatidylserine and phosphatidylcholine may be present in a molar ratio of 1:4. Advantageously, liposomes present in such molar ratios tend to be more stable.

[0095] After mixing the hydrolysate and plurality of lipids, a liposomal film may be formed. Forming the liposomal film may comprise drying the mixture to remove the
10 one or more organic solvents. The drying may be carried out by subjecting the liposomal film to vacuum. The vacuum pressure may be, for example, 0.08 MPa.

[0096] The liposomal film can then be mixed with a buffer to form a suspension. In the suspension, liposomes may be formed as a hydrophobic membrane surrounding an aqueous solution core, wherein the membrane is composed of a lipid bilayer. The
15 buffer may comprise (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sodium chloride, and sodium azide. The pH of this suspension may range from 4.5 to 9.0. In certain embodiments, the pH of the suspension may be 7. Any suitable reagents for preparing a buffer to form such a liposomal suspension may be used. In various embodiments, the present method may further comprise incubating the suspension at
20 0 to 50°C for 10 to 18 hours. In certain embodiments, the present method may further comprise incubating the suspension at 4°C for 10 to 18 hours. The duration can also be 12 to 18 hours, 14 to 18 hours, 16 to 18 hours, 10 to 12 hours, 10 to 14 hours, 10 to 16 hours, 12 to 16 hours, 14 to 16 hours, 12 to 14 hours, etc. These durations can lead to higher incorporation efficiency of the hydrolysates in the liposomal membrane, e.g.
25 a liposome.

[0097] In the present method, the liposomal membrane may then be obtained by extruding the suspension through a membrane. In various embodiments, extruding the suspension may comprise extruding the suspension for more than one time. Extruding the suspension through a membrane may help to obtain unilamellar liposomes, and
30 repeating the extrusion helps obtain monodispersed unilamellar liposomes that have uniform diameter.

[0098] In the present disclosure, there is also provided for a liposomal membrane comprising a plurality of lipids and at least one hydrolysate of a peptide-attached pillar[5]arene, wherein the at least one hydrolysate of the peptide-attached pillar[5]arene is represented by the formula:



wherein R represents a tripeptide having a terminal -NH- group and a terminal -COOH group, wherein the terminal -NH- group forms an amide linkage with the carbonyl carbon, and wherein the terminal -COOH group extends away from the amide linkage. The liposomal membrane is obtained or obtainable according to the method described above in various embodiments of the method of synthesizing the liposomal membrane disclosed herein.

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[0099] Embodiments and advantages described in the context of the present method of synthesizing the hydrolysates, the present method of synthesizing the liposomal membrane, and the present hydrolysate, are analogously valid for the liposomal membrane as described herein, and vice versa. As the various embodiments and advantages have already been described above, they are not iterated for brevity.

15

[00100] In the present liposomal membrane, the hydrolysate and the plurality of lipids may present in a molar ratio of more than 0 and up to 0.02. The molar ratio may also range from 0.025 to 0.02, 0.005 to 0.02, 0.01 to 0.02, etc.

[00101] In various embodiments, the tripeptide may comprise -NH-L-Phe-L-Phe-L-Phe-COOH or -NH-D-Phe-D-Phe-D-Phe-COOH.

20

[00102] In various embodiments, the plurality of lipids may comprise phosphatidylcholine and/or phosphatidylserine. As already described above, the phosphatidylserine and/or phosphatidylcholine may be present in a molar ratio of 0:1 to 100:1. As already described above, the phosphatidylserine and phosphatidylcholine may be present in a molar ratio of 1:4.

25

[00103] The liposomal membrane may comprise or may be a unilamellar liposome. The liposome may have a diameter of 80 nm to 250 nm. The liposome may have a diameter that is of this range.

[00104] The present disclosure further provides for a method of determining insertion efficiency of a hydrolysate of a peptide-attached pillar[5]arene obtained according to the method described in various embodiments of the first aspect. The method comprises mixing a liposomal membrane obtained according to various embodiments described in the method of synthesizing the liposomal membrane with a buffer solution, subjecting the buffer solution to UV-visible spectroscopy at an UV-visible absorbance wavelength of 293 nm to detect the UV-visible absorbance from π - π transition of a benzene, and correlating the UV-visible absorbance to a UV-visible absorbance-concentration standard curve to determine the insertion efficiency.

[00105] The buffer solution may comprise octyl glucoside. Octyl glucoside may be used as a surfactant to dissolve the hydrolysate easily. The buffer solution may also include a salt such a sodium chloride, and may be any suitable phosphate buffer solution.

[00106] In the present disclosure, there is further provided a method of determining insertion efficiency of a hydrolysate of a peptide-attached pillar[5]arene obtained according to the method according to the method described in various embodiments of the first aspect in a liposomal membrane. The method comprises mixing a liposomal membrane obtained according to the method according to various embodiments described in the method of synthesizing the liposomal membrane with a buffer solution, subjecting the buffer solution to circular dichroism at a circular dichroism absorbance wavelength of 306 nm to detect the circular dichroism absorbance from π - π transition of a benzene, and correlating the circular dichroism absorbance to a circular dichroism absorbance-concentration standard curve to determine the insertion efficiency.

[00107] The buffer solution may comprise octyl glucoside. The buffer solution may also include a salt such a sodium chloride, and may be any suitable phosphate buffer solution.

[00108] In both methods of determining the insertion efficiency of the hydrolysate in the liposomal membrane, the UV-visible absorbance-concentration standard curve and

circular dichroism absorbance-concentration standard curve may be prepared by subjecting solutions having different concentrations of lipids to UV-visible spectroscopy at a UV-visible absorbance wavelength of, for example, 280 nm. The concentrations may be, for example, 0 mM to 1.5 mM. The solution may be any
5 suitable phosphate buffer including octyl glucoside and a salt such as sodium chloride. The preparation of the calibration curve (i.e. standard curve) may involve the same steps as the methods of determining the insertion efficiency via UV-visible absorbance and circular dichroism absorbance. The UV-visible absorbance measurements, based on the wavelength of 280 nm, may be obtained for different
10 concentrations, and this may be used to set up a standard curve for the correlation.

[00109] In summary, the present disclosure includes a method of synthesizing and isolating (pS)- and (pR)-pillar[5]arenes containing peptides, wherein the abbreviations “pS” and “pR” refer to diastereomers having a planar chirality of the pS and pR configurations, respectively, which is shown in FIG. 2A. The method may include (a)
15 forming a solution of carboxylic acid groups-substituted pillar[5]arene and -NH₂ terminated tripeptide in DMF, (b) introducing 4-dimethylaminopyridine and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride into the solution, (c) reacting carboxylic acid groups-substituted pillar[5]arene with -NH₂ amine terminated tripeptide for a reaction time and a reaction temperature sufficient to produce the
20 peptide-attached pillar[5]arene, (d) purifying the pillar[5]arene containing peptide by column chromatography and isolating the (pS)- and (pR)-isomers based on their solubility difference in an organic solvent.

[00110] The -NH₂ terminated tripeptide may comprise a three L-phenylalanine. The reaction time may be within a range from 12 to 180 hrs. The reaction temperature may
25 be within a range from 45 to 70°C; The organic solvent used to isolate the (pS)- and (pR)-configuration diastereomers may be an acetone. The (pR)-configuration diastereomer may form an insoluble solid in acetone. The (pR)-configuration diastereomer may be the (pR)-pillar[5]arene attached with the peptide. The (pS)-configuration diastereomer may form a soluble solid in acetone. The (pS)-
30 configuration diastereomer may be the (pS)-pillar[5]arene attached with the peptide. The peptide-attached (pR)-pillar[5]arene are highly water permeable and usable selective water channel in a liposomal membrane.

[00111] The liposomal membrane may include peptide-attached (pR)-pillar[5]arene incorporated in the membrane along with liposomes.

[00112] The water permeability of the peptide-attached (pR)-pillar[5]arene may be measured by stopped-flow light scattering experiment. The present method may also include determining the single-channel water permeability by circular dichroism and UV-visible techniques. The present disclosure also provides for calculation of activation energies by measuring the water permeability at different temperatures, and evaluating the relative solute rejection by employing different solutes as osmolytes. The different temperatures may range from 10 to 25 °C. The solutes used as osmolytes may include sodium chloride, glycine, glycerol, glucose, and/or sucrose.

[00113] The liposomal membrane used, e.g. in the form of liposome, may include L- α -phosphatidylcholine and L- α -phosphatidylserine. The molar ratio of L- α -phosphatidylcholine to L- α -phosphatidylserine may be 4:1.

[00114] The obtained water permeability values can be within a range from 45 to 1200 $\mu\text{m/s}$. The single-channel water permeability can be $3.9 \times 10^{-14} \text{ cm}^3/\text{s}$, which corresponds to 1.3×10^9 water molecules/s. The activation energy for peptide-attached (pR)-pillar[5]arene channels can be $7.77 \pm 1.06 \text{ kcal/mol}$. The peptide-attached (pR)-pillar[5]arene can have a relative rejection for sodium chloride that is greater than 1.

[00115] While the methods described above are illustrated and described as a series of steps or events, it will be appreciated that any ordering of such steps or events are not to be interpreted in a limiting sense. For example, some steps may occur in different orders and/or concurrently with other steps or events apart from those illustrated and/or described herein. In addition, not all illustrated steps may be required to implement one or more aspects or embodiments described herein. Also, one or more of the steps depicted herein may be carried out in one or more separate acts and/or phases.

Examples

[00116] The present disclosure relates to artificial water channels comprising peptide-attached pillar[5]arene and a method of fabricating the same.

[00117] In particular, the artificial water channels can be made of peptide-attached pillar[5]arene in the pR configuration. These artificial water channels are found to have excellent water-conduction activity. These artificial water channels also showed substantial rejection of salt and small solutes. The water permeability for these artificial water channels ranges from 58 $\mu\text{m/s}$ to 783 $\mu\text{m/s}$, based on the lipid-to-channel molar ratio and the temperature at which the permeability measurements are derived.

[00118] The single water channel water permeability is found to be 1.3×10^9 water molecules/s/channel, which is comparable to that of aquaporin (4×10^9 water molecules/s/channel).

[00119] The artificial water channel includes a peptide-attached pillar[5]arene, wherein the peptide-attached pillar[5]arene is in the pR configuration, and the peptide having levorotatory (L) configuration. In certain embodiments, the peptide may be L-Phe-L-Phe-L-Phe-COOH.

[00120] As for the method of fabricating the artificial water channels, the method may include dissolving carboxylic acid group substituted pillar[5]arene in anhydrous dimethylformamide (DMF) to form a solution. A peptide, 4-dimethylaminopyridine (DMAP) and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) may be added into the solution to form a mixture, and the mixture may be stirred at 45 to 70°C for 12 to 180 hrs under an inert environment. The mixture may then be poured into hydrochloric acid (1 to 10 wt%) to obtain precipitates. The precipitates can be subjected to column chromatography to obtain a first fraction that comprises of pS-methylated pillar[5]arene and pR-methylated pillar[5]arene artificial water channels. The first fraction may be filtered and washed with acetone to obtain insoluble solids comprising the pR-methylated pillar[5]arene artificial water channels and a filtrate comprising pS-methylated pillar[5]arene. Lithium hydroxide monohydrate can be added into a solution of the pR-methylated pillar[5]arene artificial water channels, stirred at room temperature for 8 to 40 hrs, concentrated under reduced pressure and acidified with 1 to 5 wt% hydrochloric acid to form pR-nonmethylated pillar[5]arene artificial water channels.

[00121] The present disclosure also relates to a water membrane incorporated with the artificial water channels. The water membrane may include the artificial water

channels, wherein the artificial water channels comprise pR-nonmethylated pillar[5]arene. The water membrane may be a lipid membrane, wherein the lipid membrane is formed from lipids or may be in the form of a membrane comprising liposomes incorporated with the water channels.

5 [00122] The present disclosure further relates to a method of determining the insertion efficiency of peptide-attached pillar[5]arene in a liposomal membrane, e.g. a liposome. The method may include the steps of dissolving the peptide-attached pillar[5]arene and liposomal membrane in a phosphate buffer solution of octyl glucoside, and using a UV-vis (ultraviolet-visible) method to detect the presence of π - π transition of benzene at an absorbance signal at about 293 nm, or using circular
10 dichroism to detect the presence of π - π transition of benzene at an absorbance signal at about 306 nm.

[00123] Details of the artificial water channel, its synthesis, the liposomal membrane, and the method of determining the insertion efficiency, are further discussed, by way
15 of non-limiting examples set forth below.

[00124] Example 1: Materials

[00125] Dichloromethane (CH_2Cl_2) was distilled over CaH_2 under N_2 atmosphere. Trifluoroacetic acid (TFA), 1,4-dimethoxybenzene, paraformaldehyde, boron tribromide (BBr_3), ethyl bromoacetate, 4-dimethylaminopyridine (DMAP), anhydrous
20 DMF and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) were purchased from Sigma Aldrich.

[00126] L- α -phosphatidylcholine (chicken egg, PC) and L- α -phosphatidylserine (porcine brain, sodium salt, PS) were purchased from Avanti Polar Lipids.

[00127] O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)
25 were purchased from GL biochem (Shanghai) Ltd. Tripeptide (NH_2 -L-Phe-L-Phe-L-Phe-OMe) was also purchased from GL biochem (Shanghai) Ltd.

[00128] Water purified by a Milli-Q system (18 M Ω cm) was used.

[00129] Polyethersulfone (PES, E6020P) was purchased from BASF for support
30 membrane fabrication.

[00130] Materials were used as provided by supplier.

[00131] Example 2: Equipment

[00132] ^1H and ^{13}C NMR spectra were recorded on 500 MHz Bruker DRX NMR spectrometer, BrukerAvance III 400 (400MHz) (100 MHz) spectrometer or Bruker AV-300 (300 MHz). Low resolution mass spectra (LR-MS) were obtained on the ThermoFinnigan LCQ Fleet MS. The matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) spectra were obtained on a JMS-S3000 SpiralTOF (JEOL Ltd., Japan) at an accelerating potential of 20 kV in the positive spiral mode. LRMS and MALDI-TOFMS were reported in units of mass of charge ratio (m/z). UV-vis spectra were measured on a Cary Varian 5000 UV-vis spectrometer using 1 cm quartz cuvettes. Circular dichroism (CD) spectroscopy was conducted with a Jasco J-1500 CD spectrometer in a 1 cm path length cuvette at room temperature.

[00133] Example 3: Method of Synthesizing Peptide-Attached Pillar[5]arenes

[00134] The synthesis of peptide-attached (pS)-pillar[5]arenes and (pR)-pillar[5]arenes is described below, and with reference to the drawings. Specifically, the synthesis of (pS)-pillar[5]arenes (pS-PH) and (pR)-pillar[5]arenes (pR-PH) involves six steps, and an example of the synthesis is shown in FIG. 1.

[00135] The first step concerns synthesis of 1,4-dimethoxypillar[5]arene (DMP5).

[00136] DMP5 was prepared by using trifluoroacetic acid as a catalyst. Trifluoroacetic acid (15 mL) was added to a solution of 1,4-dimethoxybenzene (4.15 g, 30 mmol) and freshly ground paraformaldehyde (0.9 g, 30 mmol) in 1,2-dichloroethane (285 mL), and the mixture was refluxed at 90°C for 3 hrs. After cooling to room temperature, the mixture was poured into methanol (400 mL), and the resulting precipitate was collected by filtration and dissolved in CHCl_3 (70 mL). Acetone (70 mL) was added to obtain a precipitate from the CHCl_3 and the solid was washed with acetone to obtain the DMP5 as a white solid (3.25 g). Yield was 69%. ^1H NMR (500 MHz, CDCl_3): δ = 6.90 (s, 10H), 3.77 (s, 10H), 3.75 (s, 30H). LR-MS (EI): Calculated for $\text{C}_{45}\text{H}_{51}\text{O}_{10}$ $[\text{M}+\text{H}]^+$: 751.35. Found: 751.31.

[00137] The second step concerns the synthesis of pillar[5]arene (P5). In this step, boron tribromide (13.2 mL, 140 mmol) was added slowly to a solution of DMP5 (3.0 g, 4 mmol) in dry CH_2Cl_2 (100 mL). The resulting mixture was stirred at room temperature for 72 hrs. Water (100 mL) was then added at 0°C slowly and the mixture was stirred for a further 36 hrs at room temperature. The precipitate was filtered and

washed with water to result in an oyster white solid (2.4 g). Yield was 98%. ^1H NMR (300 MHz, CD_3COCD_3): δ = 7.98 (s, 10H), 6.68 (s, 10H), 3.60 (s, 10H). LR-MS (EI): Calculated for $\text{C}_{35}\text{H}_{34}\text{NO}_{10}$ $[\text{M}+\text{NH}_4]^+$: 628.22. Found: 628.26.

[00138] The third step concerns synthesis of ethoxycarbonylmethoxy-substituted pillar[5]arene (EP5). K_2CO_3 (7.0 g) was added to a solution of P5 (1.4 g, 2.3 mmol) in acetonitrile (60 mL). The mixture was stirred for 1 hr at room temperature and KI (80 mg) and ethyl bromoacetate (5 mL, 45 mmol) were then added. The mixture was heated at reflux under a nitrogen atmosphere for 24 hrs and then left to cooled at room temperature. The mixture was filtered and washed with CHCl_3 . The filtrate was concentrated under vacuum, and the residue was dissolved in CHCl_3 (15 mL). A white solid was formed by slow diffusion of methanol into the CHCl_3 solution. The white solid was collected by filtration, washed with methanol, and dried under vacuum to obtain the EP5 white product (2.74 g). Yield was 81%. ^1H NMR (300 MHz, CDCl_3): δ = 7.04 (s, 10H), 4.54 (q, $J=15$ Hz, 20H), 4.09 (m, $J=6$ Hz, 20H), 3.85 (s, 10H), 0.96 (m, $J=6$ Hz, 30H).

[00139] The fourth step is the synthesis of carboxylic acid group substituted pillar[5]arene (AP5). In this step, 20% aqueous sodium hydroxide (60 mL) was added to a solution of EP5 (2.7 g, 1.8 mmol) in THF (100 mL). The solution was heated to reflux for 15 hrs. After cooling, the solution was concentrated, diluted with water (100 mL), and acidified with HCl. The white precipitate was collected by filtration, washed with water, and dried under vacuum to obtain a white solid of AP5 (1.9 g). Yield was 87%. ^1H NMR (300 MHz, CD_3SOCD_3): δ = 7.03 (s, 10H), 4.64 (d, $J=18$ Hz, 10H), 4.41 (d, $J=15$ Hz, 10H), 3.72 (s, 10H). LR-MS (EI): Calculated for $\text{C}_{55}\text{H}_{49}\text{O}_{30}$ $[\text{M}-\text{H}]^-$: 1189.23. Found: 1189.33.

[00140] In the next step, the fifth step, pS-PM and pR-PM are synthesized. Firstly, AP5 (0.1 to 2 mmol, 1 equiv) was dissolved in anhydrous DMF. NH_2 -L-Phe-L-Phe-L-Phe-OMe (1 to 60 mmol, 10 to 30 equiv), DMAP (1 to 300 mmol, 10 to 150 equiv) and EDC (1 to 90 mmol, 10 to 45 equiv) were added to the solution of AP5 in anhydrous DMF. The term "equiv" in this step denotes the amount used with respect to the AP5. The mixture was stirred at 45 to 70°C for 12 to 180 hrs under a nitrogen atmosphere. After cooling, the solution was poured into aqueous HCl solution (1 to 10%). The precipitate was filtered off and washed with water to obtain the crude

product. The crude product was subjected to the column chromatography. The first fraction (the mixture of pS-PM and pR-PM) was collected and then dissolved in acetone. After filtration and washing thoroughly with acetone, the insoluble solid was collected to give pR-PM. The filtrate was evaporated to give pS-PM.

- 5 **[00141]** In certain examples for synthesis and isolation of pS-PM and pR-PM, NH₂-L-Phe-L-Phe-L-Phe-OMe (3.26 g, 6.8 mmol), DMAP (3.5 g, 28 mmol) and EDC (1.34 g, 7 mmol) were added to a solution of AP5 (0.44 g, 0.37 mmol) in anhydrous DMF (45 mL). The mixture was stirred at 55°C for 86 hrs under a nitrogen atmosphere. After cooling, the solution was poured into aqueous HCl solution (2%,
10 400 mL). The precipitate was filtered off and washed with water to obtain the crude product. The crude product was subjected to column chromatography (CH₂Cl₂:CH₃OH = 10:1). The first fraction (a mixture of pS-PM and pR-PM) was collected and concentrated to obtain a white solid (0.9 g). The solid was then dissolved in acetone (50 mL). After filtration and washing thoroughly with acetone,
15 the insoluble solid was collected to give pR-PM (0.3 g). The filtrate was evaporated to give pS-PM (0.58 g).

[00142] pS-PM: ¹H NMR (300 MHz, CD₃SOCD₃): δ = 8.47-8.44 (br, 20H), 7.56 (br, 10H), 7.15-6.96 (m, 150H), 6.73 (s, 10H), 4.74-4.66 (m, 20H), 4.54-4.44 (m, 10H), 4.35 (d, J=15 Hz, 10H), 4.20 (d, J=15 Hz, 10H), 3.50 (s, 40H), 2.98-2.77 (m, 60H).
20 ¹³C NMR (100 MHz, CD₃SOCD₃): δ=171.99, 171.39, 171.18, 167.89, 148.96, 137.68, 137.58, 137.30, 129.59, 129.41, 128.70, 128.38, 127.00, 126.69, 114.98, 54.15, 54.04, 53.62, 52.23, 38.55, 38.24, 37.21. MALD-TOF-MS: Calculated for C₃₃₅H₃₄₀N₃₀O₆₀Na [M+Na]⁺: 5768.4463. Found: 5768.5095.

[00143] pR-PM: ¹H NMR (300 MHz, CD₃SOCD₃): δ= 8.50-8.40 (br, 20H), 7.89 (br, 10H), 7.22-6.95 (m, 150H), 6.60 (s, 10H), 4.67 (br, 20H), 4.49 (br, 10H), 4.20-4.09 (br, 20H), 3.52 (s, 40H), 2.95-2.78 (m, 60H). ¹³C NMR (100 MHz, CD₃SOCD₃): δ=171.98, 171.39, 171.19, 167.93, 148.99, 137.65, 137.57, 137.28, 129.59, 129.41, 128.71, 128.39, 127.01, 126.70, 126.62, 115.02, 67.70, 54.15, 54.05, 53.64, 52.24, 38.50, 38.21, 37.25. MALD-TOF-MS: Calculated for C₃₃₅H₃₄₀N₃₀O₆₀Na [M+Na]⁺:
25 5768.4463. Found: 5768.5073.
30

[00144] For the sixth step, pS-PH and pR-PH were obtained by adding lithium hydroxide monohydrate (0.02-8 mmol, 20-80 equiv) and water to a solution of pS-PM

or pR-PM (0.001-0.1 mmol, 1 equiv) in THF. The term "equiv" in this step denotes the amount used with respect to the pS-PM or pR-PM. The mixture was stirred at room temperature for 8 to 40 hrs. The solution was then concentrated under reduced pressure and poured into water. After acidifying with aqueous HCl solution (1 to 5 wt%), the formed precipitate was filtered off, washed with water, and dried under a vacuum to give the white product.

[00145] An example for synthesis of pS-PH based on the above steps is described below.

[00146] Lithium hydroxide monohydrate (30 mg, 0.70 mmol) and water (2.5 mL) were added to a solution of pS-PM (80 mg, 0.014 mmol) in THF (7.5 mL). The mixture was stirred at room temperature for 20 hrs. Then, the solution was concentrated under reduced pressure and poured into water (30 mL). After acidifying with aqueous HCl solution (2%), the formed precipitate was filtered off, washed with water, and dried under a vacuum to give the white product pS-PH (72.6 mg, yield: 93%). ¹H NMR (300 MHz, CD₃SOCD₃): δ= 8.43 (br, 10H), 8.31 (br, 10H), 7.54 (br, 10H), 7.19-6.95 (m, 150H), 6.71 (s, 10H), 4.71 (br, 20H), 4.48 (br, 10H), 4.35 (br, 10H), 4.16 (br, 10H), 3.51 (s, 10H), 3.00-2.80 (m, 60H). ¹³C NMR (100 MHz, CD₃SOCD₃): δ=173.06, 171.33, 170.90, 168.15, 148.92, 137.62, 136.83, 129.59, 129.43, 128.58, 128.39, 126.80, 114.88, 67.62, 54.18, 53.07, 38.16, 38.08, 37.31. MALD-TOF-MS: Calculated for C₃₂₅H₃₂₀N₃₀O₆₀Na [M+Na]⁺: 5628.2898. Found: 5628.2896.

[00147] An example for synthesis of pR-PH based on the above steps is described below.

[00148] Lithium hydroxide monohydrate (30 mg, 0.70 mmol) and water (2.5 mL) were added to a solution of pR-PM (80 mg, 0.014 mmol) in THF (7.5 mL). The mixture was stirred at room temperature for 20 hrs. Then, the solution was concentrated under reduced pressure and poured into water (30 mL). After acidifying with aqueous HCl solution (2%), the formed precipitate was filtered off, washed with water, and dried under a vacuum to give the white product pR-PH (71.8 mg, yield: 92%). ¹H NMR (300 MHz, CD₃SOCD₃): δ= 8.35 (br, 20H), 7.88 (br, 10H), 7.19-6.94 (m, 150H), 6.59 (s, 10H), 4.64 (m, 20H), 4.45 (br, 10H), 4.20-4.10 (br, 20H), 3.01-2.76 (m, 60H). ¹³C NMR (100 MHz, CD₃SOCD₃): δ=173.13, 171.34, 171.19, 167.92,

148.95, 137.73, 137.56, 129.60, 129.52, 128.62, 128.38, 126.86, 126.67, 114.98, 67.61, 54.14, 53.60, 38.46, 38.17, 37.34. MALD-TOF-MS: Calculated for $C_{325}H_{320}N_{30}O_{60}Na$ $[M+Na]^+$: 5628.2898. Found: 5628.2880.

[00149] Example 4: Method of Preparing Liposomal Membrane

5 **[00150]** The liposomal membrane, e.g. in the form of liposomes, can be prepared by film rehydration described as follows.

[00151] The pR-PH or pS-PH channels in a chloroform/methanol mixture (v/v=1/1) were added to 4 μ mol PC/PS mixture with a molar ratio of 4/1. The solvent was slowly distilled on a rotary evaporator and subsequently dried under high vacuum to remove residual solvent. The dried film was rehydrated with 1 mL buffer containing
10 10 mM Hepes (pH=7), 100 mM NaCl and 0.01% NaN_3 . The suspension was further incubated with stirring at 4°C for 14 hrs and then extruded through 0.2 μ m track-etched membrane for 21 times (Whatman, UK). The sizes of liposomes were measured to be 160 ± 15 nm using a Nano Zetasizer (NanoZS, Malvern Instruments
15 Limited, UK).

[00152] Example 5: Stopped-Flow Light Scattering Test Procedures

[00153] Stopped-flow measurements were performed with a stopped flow apparatus (SX-20, Applied Photophysics) at a given temperature. The liposome solution was mixed rapidly with hypertonic osmolyte (400 mM sucrose) in the same buffer to
20 induce liposomes to shrink due to the osmotic gradient. The changes of light scattering were recorded at a wavelength of 500 nm. The stopped flow data was fitted to a single exponential function to obtain the rate constant (k). The water permeability of the liposomes (P_f) was calculated using the following equation (1):

$$P_f = \frac{k}{S_o/V_o \times V_w \times \Delta_{osm}} \quad (1)$$

25 **[00154]** where k is the rate constant, S_o/V_o is the ratio of the initial surface area to the volume of liposomes, V_w is the partial molar volume of water ($18 \text{ cm}^3 \text{ mol}^{-1}$), and Δ_{osm} is the osmolarity difference. All the osmolarities were measured with a freezing-point osmometer (Model 3250, Advanced Instruments, Inc.)

[00155] Example 6A: Insertion Efficiency of Channel

30 **[00156]** The liposomal membrane, e.g. in the form of liposomes, with and without pR-PH channels were first prepared in the phosphate buffer (10 mM sodium

phosphate, pH=6.4, 100 mM NaCl) using the film rehydration method described above, containing 2 mM PC/PS lipids with a molar ratio of 4/1. The formed liposomes were further extruded through 0.2 μm track-etched membrane for 21 times (Whatman, UK) to obtain monodispersed unilamellar liposomes. The water permeability of liposomes was studied by stopped-flow measurements. The liposomes were exposed to a hypertonic osmolyte (400 mM sucrose). The net water permeability for pR-PH in liposome (CLR = 0.005) was calculated to be $255.9 \pm 26.4 \mu\text{m/s}$.

[00157] For deriving the insertion efficiency of the channels: 500 μL pR-PH channel solutions (from 0 μM to 30 μM), in phosphate buffer containing 10 mM sodium phosphate (pH=6.4), 100 mM NaCl and 8% n-octyl- β -D-glucoside (OG), were mixed with 500 μL control liposomes. The obtained solutions (final concentration of channels: from 0 μM to 15 μM) were scanned on a UV-Vis spectrophotometer or CD spectrophotometer. It is noted that the channels' special UV-Vis absorbance at 293 nm and CD absorbance at 306 nm increased with the concentration linearly, to generate a standard curve (FIG. 18). For liposomes containing channels used for water transport studies, 500 μL liposomes were mixed with 500 μL the same phosphate buffer containing 8% OG. The insertion efficiency of channels into a liposome was evaluated based on a channel's special UV-vis absorbance at 292 nm or CD absorbance at 306 nm.

[00158] The insertion efficiency of lipids: PC/PS lipids solutions (from 0 mM to 1.5 mM) with a molar ratio of 4/1 were prepared in phosphate buffer containing 10 mM sodium phosphate (pH=6.4), 100 mM NaCl and 4% n-octyl- β -D-glucoside (OG). The solutions were scanned on a UV-vis spectrophotometer. The UV-vis absorbance of the lipid solutions at 280 nm increased with the concentrations and was proportional to the concentration, to generate a calibration curve. When the extruded liposomes (initially 1 mM) were measured at this wavelength, the actual concentration estimated according to the calibration curve was 0.96 mM. The insertion efficiency of lipids was 96%. These steps were used to generate the standard calibration curve.

[00159] Example 6B: Calculation of Single-Channel Water Permeability

[00160] The single-channel permeability was calculated based on the insertion efficiency of pR-PH channel and lipid. When channel-to-lipid molar ratio, i.e. CLR, is 0.005, the radius (r) of the liposome was 80 nm. Assuming that the bilayer thickness

was 5 nm, the sum of outer and inner surface areas was $4\pi \times r^2 + 4\pi \times (r-5)^2 = 151110 \text{ nm}^2$. The average cross-sectional area of a lipid in average was 0.7 nm^2 (the cross-sectional areas of PC and PS were about 0.7 nm^2), and that of pR-PH channel was estimated to be 4.5 nm^2 (FIG. 18E). The initial molar ratio of channels/lipids was 1/200. Provided 96% of lipids and 48% of the pR-PH channels remained in the purified vesicles, the real molar ratio of channels/lipids was 1/400. The insertion number of the channel was 523 per vesicle. If the overall net permeability by channels in liposomes was $255.9 \text{ } \mu\text{m/s}$, the single-channel permeability was $3.9 \times 10^{-14} \text{ cm}^3/\text{s}$ and 1.3×10^9 water molecules/s.

10 **[00161]** Example 7: Solute Rejection

[00162] If small solutes as osmolytes are imperfectly rejected by channel, the osmotic gradient and the measured water flux decrease. The solute rejection of a channel can be assessed from the reflection coefficient (σ). Sodium chloride, glycine, glycerol, glucose and sucrose were used as osmolytes (FIG. 19). Sucrose was used as the reference solute ($\sigma_{\text{sucrose}} = 1$) and all experiments were measured at 25°C . The reflection coefficient was calculated as the following equation:

$$\sigma_{\text{solute}} = \frac{J_{\text{solute}}}{J_{\text{sucrose}}} \quad (2)$$

[00163] where σ_{solute} is the reflection coefficient of the solute, J_{solute} and J_{sucrose} are the measured water fluxes when solute and sucrose are used as osmolytes, respectively.

20 **[00164]** Example 8A: Discussion on Method of Fabricating Water Channel

[00165] The synthesis and characterization of peptide-attached (pS)- and (pR)-pillar[5]arenes are discussed below with reference to FIG. 1 to 19.

[00166] The detailed synthesis of the peptide-attached pillar[5]arenes is shown in FIG. 1. The peptides were attached to pillar[5]arenes by an amide condensation reaction between carboxylic acid and amine. Through a silica gel column chromatography, the first fraction was collected and analysed by ^1H NMR spectroscopy (FIG. 2C). The ^1H NMR spectrum identified the presence of two diastereomers in the first fraction which was then further purified and separated to obtain (pS)- and (pR)-pillar[5]arenes containing peptide, designated as pS-PM and pR-PM, respectively (FIG. 2B). pS-PM was found as a major product in the present method, which is attributed to the stereoselectivity. The chemical structures of pS-PM

and pR-PM were confirmed by ^1H NMR, ^{13}C NMR and mass analysis, respectively (FIG. 3 to FIG. 8).

[00167] Example 8B: Discussion on Characterization Results

[00168] Circular dichroism (CD) spectroscopy and UV-vis spectroscopy were further used to characterize the structures of pS-PM and pR-PM. Both pR-PM and pS-PM showed a special UV-vis absorption band at 293 nm corresponding to $\pi-\pi^*$ transition of the aryl fragments of pillar[5]arenes (FIG. 2E). In the CD spectra (FIG. 2D), the positive Cotton effect for pR-PM and the negative Cotton effect for pS-PM were observed, which were in agreement well with that for previously reported pillar[5]arene derivatives. The Cotton effect refers to the characteristic change in optical rotatory dispersion and/or circular dichroism in the vicinity of an absorption band of a substance. The Cotton effect is referred to as positive if the optical rotation first increases as the wavelength decreases and negative if the optical rotation first decreases as wavelength decreases. These characterization results indicated a complete isolation and purification of the diastereomers pR-PM and pS-PM.

[00169] The methyl groups in pS-PM and pR-PM were then easily removed by lithium hydroxide hydrolysis to afford corresponding compounds pS-PH and pR-PH with free carboxylic acids which are expected to facilitate water transport by hydrogen-bonding interactions between carboxylic acids and water (FIG. 2B). The UV-vis and CD spectra of pS-PH and pR-PH confirmed that the conditions employed for hydrolysis did not result in any change of configurations, as shown in FIG. 2D. Furthermore, the ^1H NMR spectrum of pS-PH (FIG. 9) was consistent with that of peptide-appended pillar[5]arene.

[00170] Example 8C: Discussion on Water Permeability Results

[00171] To investigate the water-conduction behaviour of the two diastereomers, pS-PH and pR-PH were incorporated into phosphatidylcholine/phosphatidylserine (PC/PS) liposomes through lipid film rehydration in Hepes buffer and the water permeabilities of liposomes were investigated by using stopped-flow light-scattering experiments. In shrinking experiments, liposomes were rapidly exposed to a hypertonic solution to cause the shrinkage of liposomes, which led to the increase of light-scattering signal over time (FIG. 15A). The initial rise in the light scattering

curve was fitted to a single exponential equation, and the obtained exponential coefficient (k) was used to calculate water permeability.

[00172] To obtain the low lipid background permeability, the low temperature (10°C) was first used. Since liposomes containing pS-PH with different channel-to-lipid molar ratios (CLRs for 0.005 and 0.01) had nearly identical light scattering curve with pristine liposomes (curve was overlapped), as shown in FIG. 15B, it was difficult to differentiate the pS-PH channels' contribution to water transport, implying extremely low water-conduction activity of pS-PH channels. However, the incorporation of pR-PH channels increased dramatically the overall water permeability compared to the lipid background permeability (FIG. 15C). The calculated permeability of the liposome containing pR-PH channels at channel-to-lipid ratio (CLR) of 0.005 was $157.11 \pm 19.33 \mu\text{m/s}$, which was more than 3 times as high as that of the pristine liposomes ($46.83 \pm 4.16 \mu\text{m/s}$). When the CLR was increased from 0.0025 to 0.02, the water permeability reached up to $446.99 \pm 46.61 \mu\text{m/s}$, almost 9 times higher than that of the pristine liposomes. FIG. 15D shows that the net water permeability achieved by excluding lipid background permeability increased gradually.

[00173] To reconfirm the high water-conduction activity of pR-PH, the measured temperature was elevated to 25 °C (FIG. 16A to 16C), leading to a higher lipid background permeability. As expected, the higher water-conduction activity for pR-PH channel was observed in FIG. 16B. At CLR = 0.02, the net water permeability reached up to $782.57 \pm 124.78 \mu\text{m/s}$ (FIG. 16C), which is orders of magnitude higher than that of conventional artificial water channels. The dramatically enhanced water permeability of liposomes containing pR-PH channels implied an excellent water-conduction activity of pR-PH channels.

[00174] To explore the stabilization of water-conduction of pR-PH channel, the water permeability of pR-PH channel in swelling experiment was also investigated by exposing the liposomes into hypotonic solutions (FIG. 17A to 17C). As a result, the net water permeability values in the swelling mode and the shrinking mode were calculated to be in the same order of magnitude, as shown in FIG. 17C, implying the stabilization of water-conduction activity of pR-PH channel in the lipid bilayer membrane environment.

[00175] Example 8D: Discussion on Calculation for Activation Energies

[00176] The activation energies associated with water transport are assessed by measurement of the water permeability at different temperatures, and these energies can be used to determine whether the water transport across vesicle membranes is diffusion driven or channel-mediated. Shrinking experiments for pristine liposomes and liposomes containing pR-PH channels (CLR was 0.01) were carried out at different temperatures to produce an Arrhenius plot ($\ln k$ versus $1/T$) (FIG. 15E). The activation energy for pristine liposomes was calculated to be 11.64 ± 0.68 kcal/mol, which was close to the value of activation energy previously reported for lipid bilayer membranes (12.39 ± 2.21 kcal/mol). The high activation energy indicated that the water transport across the lipid bilayer was diffusion driven. After incorporation of pR-PH channels into the liposomes, the activation energy was lowered to 7.77 ± 1.06 kcal/mol, which was strong evidence for channel-mediated water through the pR-PH channel. This value is comparable to reported value for the activation energies from AQP0-mediated water conduction (7.60 ± 1.70 kcal/mol), wherein AQP0 is an example of the AQP family.

[00177] Example 8E: Discussion on Single-Channel Water Permeability Results

[00178] The pR-PH channels dissolved in phosphate buffer containing octyl glucoside (OG) showed a special UV-vis absorbance signal at 293 nm and a CD absorbance signal at 306 nm (FIG. 18A and 18C). Thus, the UV-vis and CD techniques were utilized to determine the insertion efficiency of pR-PH channels into a liposomal membrane, e.g. in the form of a liposome, which could be used to calculate the single-channel water permeability. As expected, the UV-vis absorbance at 293 nm and CD absorbance at 306 nm increased linearly with increasing the concentration of pR-PH channels (FIG. 18B and 18D). When CLR was 0.005, the insertion efficiencies of channels into liposomes determined by these two techniques presented similar values (48.13 ± 7.76 % for UV-vis and 49.20 ± 4.53 % for CD), as shown in FIG. 18F. Based on insertion efficiencies of channels and lipids, the single-channel water permeability was calculated to be about 1.3×10^9 water molecules/s/channel (FIG. 15G), which was comparable to that of AQP 1 (4×10^9 water molecules/s/channel) (also see FIG. 20).

[00179] Example 8F: Discussion on Rejection of Solutes Results

[00180] The reflection coefficient has been used to evaluate the relative rejection properties of channels. The determination of reflection coefficient was based on the stop-flow light-scattering experiments by employing different solutes as osmolytes (FIG. 19). Sucrose was selected as the reference solute due to its relatively large molecular size. For all solutes, the reflection coefficient was greater than 1 (FIG. 15F), indicating a nearly perfect rejection for these solutes. The rejective property was attributed to the narrow pore of pR-PH channel. The diameter in the narrowest region is about 3 Å, which is very close to that of AQP1 (2.8 Å) (also see FIG. 20).

[00181] Example 8G: Discussion on Comparison of pR-PH Channel to Other Water Channels

[00182] The outstanding properties of pR-PH channel allowed us to compare it with the biological water channel proteins and previously reported artificial water channels (FIG. 20). As a benchmark, aquaporin Z (AqpZ) was reconstituted into PC/PS liposomes in buffer, the properties of AqpZ are also listed in FIG. 20. The pR-PH channel was found to possess equivalently high water permeability to AQPs and at least one order of magnitude higher than other artificial water channels. It was noted that the water permeability of peptide-appended pillar[5]arene in the shrinking mode was two orders of magnitude lower than that in the swelling experiment due to the increase of low-conduction channels. Unlike peptide-appended pillar[5]arene, the pR-PH channel showed similar water permeability values in the swelling mode and shrinking mode, implying the stabilization of water-conduction activity of pR-PH channel in the lipid bilayer membrane environment. Furthermore, compared with other pillar[5]arene-based artificial water channels without salt rejection, another superiority of pR-PH channel is the excellent rejection for NaCl and small solutes, making it a feasible material for incorporation into membrane materials for water-purification applications.

[00183] Example 9: Summary

[00184] The present disclosure and the examples described herein relate to synthesis and characterization of peptide-attached (pR)-pillar[5]arene for use as a highly permeable and selective artificial water channel. (pS)- and (pR)-isomers of pillar[5]arene containing peptide have been synthesized and isolated.

[00185] One aspect of the present disclosure provides for a synthesis method of peptide-attached (pS)- and (pR)-pillar[5]arenes. Another aspect is to provide for a peptide-attached (pR)-pillar[5]arene for use as a highly permeable and selective water channel. The peptide-attached (pR)-pillar[5]arene may be incorporated into a liposomal membrane. Water permeability and selectivity may be measured by the stopped-flow light scattering experiments. Another aspect is to provide for a UV-vis method of determining the insertion efficiency of peptide-attached (pR)-pillar[5]arene in a liposomal membrane, e.g. in the form of a liposome. In a further aspect, there is provided for a circular dichroism (CD) method of determining the insertion efficiency of peptide-attached (pR)-pillar[5]arene in a liposomal membrane, e.g. in liposomes.

[00186] While the invention has been particularly shown and described with reference to specific embodiments, it should be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. The scope of the invention is thus indicated by the appended claims and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced.

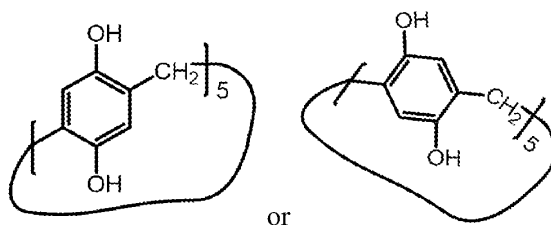
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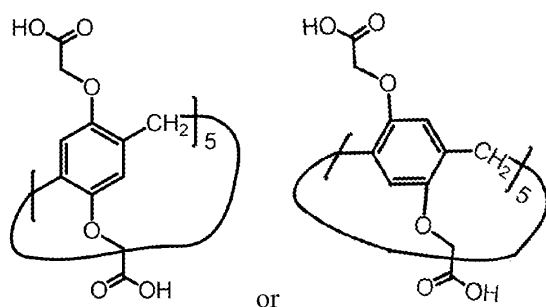
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CLAIMS

1. A method of synthesizing a hydrolysate of a peptide-attached pillar[5]arene, the method comprising:
- 5 forming a pillar[5]arene substituted with carboxyl groups from a dialkoxybenzene;
- mixing the pillar[5]arene substituted with carboxyl groups and a tripeptide in an organic solvent to form the peptide-attached pillar[5]arene, wherein the tripeptide has a terminal -NH₂ group and a terminal ester; and
- 10 subjecting the peptide-attached pillar[5]arene to hydrolysis in the presence of a base to obtain the hydrolysate of the peptide-attached pillar[5]arene.
2. The method of claim 1, wherein forming the pillar[5]arene substituted with carboxyl groups comprises mixing the dialkoxybenzene and a paraformaldehyde in
- 15 the presence of an organic acid catalyst to form a dialkoxypillar[5]arene.
3. The method of claim 1 or 2, wherein the dialkoxybenzene comprises 1,4-dimethoxybenzene.
- 20 4. The method of claim 2 or 3, wherein the organic acid catalyst comprises boron trifluoride diethyl etherate, p-toluenesulfonic acid, or trifluoroacetic acid.
5. The method of any one of claims 2 to 4, wherein the dialkoxypillar[5]arene comprises 1,4-dimethoxypillar[5]arene.
- 25 6. The method of claim 1 or 2, wherein the pillar[5]arene is represented by the formula:



7. The method of any one of claims 1 to 6, wherein forming the pillar[5]arene substituted with carboxyl groups comprises mixing the pillar[5]arene and an alkyl acetate in the presence of a catalyst to form a pillar[5]arene substituted with carbonyl groups.
8. The method of claim 7, wherein the catalyst comprises potassium iodide or sodium iodide.
9. The method of claim 7 or 8, wherein the alkyl acetate comprises butyl bromoacetate, ethyl bromoacetate, methyl bromoacetate, or propyl bromoacetate.
10. The method of any one claims 7 to 9, wherein the pillar[5]arene substituted with carbonyl groups comprises an ethoxycarbonylmethoxy-substituted pillar[5]arene.
11. The method of any one of claims 7 to 10, wherein forming the pillar[5]arene substituted with carboxyl groups comprises mixing the pillar[5]arene substituted with carbonyl groups and a basic solution to form the pillar[5]arene substituted with carboxyl groups.
12. The method of claim 11, wherein the basic solution comprises caesium hydroxide, lithium hydroxide, potassium hydroxide, or sodium hydroxide.
13. The method of claim 1 or 2, wherein the pillar[5]arene substituted with carboxyl groups is represented by the formula:



14. The method of any one of claims 1 to 13, wherein mixing the pillar[5]arene substituted with carboxyl groups and the tripeptide comprises mixing the pillar[5]arene substituted with carboxyl groups and the tripeptide at a temperature ranging from 45 to 70°C for 12 to 180 hours under an inert atmosphere.

15. The method of any one of claims 1 to 14, wherein the organic solvent comprises anhydrous dimethylformamide, 4-dimethylaminopyridine and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride.

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16. The method of any one of claims 1 to 15, wherein the tripeptide comprises NH₂-L-Phe-L-Phe-L-Phe-O-methyl, NH₂-L-Phe-L-Phe-L-Phe-O-ethyl, NH₂-L-Phe-L-Phe-L-Phe-O-propyl, NH₂-D-Phe-D-Phe-D-Phe-O-methyl, NH₂-D-Phe-D-Phe-D-Phe-O-ethyl, or NH₂-D-Phe-D-Phe-D-Phe-O-propyl.

15

17. The method of any one of claims 1 to 16, further comprising subjecting the peptide-attached pillar[5]arene to column chromatography to produce a fraction comprising a diastereomeric mixture of the peptide-attached pillar[5]arene, and filtering the diastereomeric mixture after mixing the diastereomeric mixture with acetone to obtain a residue and a filtrate, wherein the residue comprises a diastereomer pR and the filtrate comprises a diastereomer pS.

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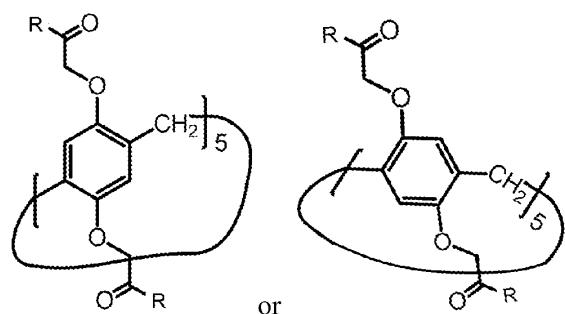
18. The method of any one of claims 1 to 17, wherein subjecting the peptide-attached pillar[5]arene to hydrolysis comprises mixing the peptide-attached pillar[5]arene and the base for 8 to 40 hours.

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19. The method of any one of claims 1 to 18, wherein the base comprises lithium hydroxide, potassium hydroxide, or sodium hydroxide.

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20. A hydrolysate of a peptide-attached pillar[5]arene configured as a water channel in a liposomal membrane, wherein the hydrolysate of the peptide-attached pillar[5]arene is represented by the formula:



5 wherein R represents a tripeptide having a terminal -NH- group and a terminal -COOH group, wherein the terminal -NH- group forms part of an amide linkage, and wherein the terminal -COOH group extends away from the amide linkage.

21. The hydrolysate of claim 20, wherein the tripeptide comprises -NH-L-Phe-L-Phe-L-Phe-COOH or -NH-D-Phe-D-Phe-D-Phe-COOH.

22. A method of synthesizing a liposomal membrane comprising a hydrolysate of a peptide-attached pillar[5]arene, the method comprising:

15 synthesizing a hydrolysate of a peptide-attached pillar[5]arene according to the method of any one of claims 1 to 19;

mixing the hydrolysate and a plurality of lipids to form a mixture;

forming a liposomal film from the mixture;

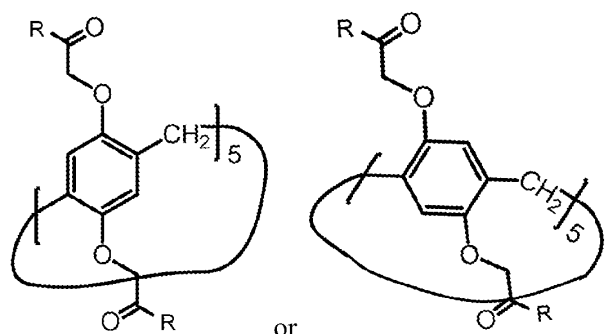
contacting the liposomal film with a buffer to form a suspension comprising the hydrolysate and the plurality of lipids; and

20 extruding the suspension through a membrane to form the liposomal membrane.

23. The method of claim 22, wherein mixing the hydrolysate and the plurality of lipids comprises dissolving the hydrolysate in one or more organic solvents.

24. The method of claim 23, wherein the one or more organic solvents comprise chloroform and/or methanol.
25. The method of claim 24, wherein the chloroform and methanol are present in a
5 volume ratio of 0:1 to 20:1.
26. The method of any one of claims 22 to 25, wherein the plurality of lipids comprise phosphatidylcholine and/or phosphatidylserine.
- 10 27. The method of claim 26, wherein the phosphatidylserine and phosphatidylcholine are present in a molar ratio of 0:1 to 100:1.
28. The method of any one of claims 22 to 27, wherein mixing the hydrolysate and the plurality of lipids comprises mixing the hydrolysate and the plurality of lipids in a
15 molar ratio of more than 0 and up to 0.02.
29. The method of any one of claims 22 to 28, wherein the buffer comprises (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sodium chloride, and sodium azide.
- 20 30. The method of any one of claims 22 to 29, further comprising incubating the suspension at a temperature ranging from 0 to 50°C for 10 to 18 hours.
31. The method of any one of claims 22 to 30, wherein extruding the suspension comprises extruding the suspension for more than one time.
- 25 32. A liposomal membrane comprising a plurality of lipids and at least one hydrolysate of a peptide-attached pillar[5]arene, wherein the at least one hydrolysate of the peptide-attached pillar[5]arene is represented by the formula:

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wherein R represents a tripeptide having a terminal -NH- group and a terminal -COOH group, wherein the terminal -NH- group forms an amide linkage with the carbonyl carbon, and wherein the terminal -COOH group extends away from the amide linkage.

33. The liposomal membrane of claim 32, wherein the tripeptide comprises -NH-L-Phe-L-Phe-L-Phe-COOH or -NH-D-Phe-D-Phe-D-Phe-COOH.

34. The liposomal membrane of claim 32 or 33, wherein the plurality of lipids comprise phosphatidylcholine and/or phosphatidylserine.

35. The liposomal membrane of claim 34, wherein the phosphatidylserine and phosphatidylcholine are present in a molar ratio of 0:1 to 100:1.

36. The liposomal membrane of any one of claims 32 to 35, wherein the hydrolysate and the plurality of lipids are present in a molar ratio of more than 0 and up to 0.02.

37. The liposomal membrane of any one of claims 32 to 36, wherein the liposomal membrane comprises a unilamellar liposome.

38. The liposomal membrane of claim 37, wherein the liposome has a diameter of 80 nm to 250 nm.