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Combinatorial screening of water/proton permeation of self-assembled Pillar[5]arene artificial water channel libraries

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Abstract: Artificial water channels-AWCs that selectively transport water rejecting ions through bilayer membranes have potential to act as synthetic Aquaporins (AQPs). AWCs can have a similar osmotic permeability, better stability, with simpler manufacture on a larger-scale and have higher functional density and surface permeability when inserted into the membrane. Here, we report the screening of combinatorial libraries of symmetrical and unsymmetrical rim functionalized PAs **A-D**, that are able to transport $\sim 10^7$ - 10^8 water molecules/s/channel, which is within 1 order of magnitude of AQPs' and show total ion and proton rejection. Among four channels, **C** and **D** are 3-4 times more water permeable than **A**, **B** when inserted in bilayer membranes. The binary combinations of **A-D** with different molar ratios could be expressed as an independent (linear **ABA**), a recessive (inhibition **AB**, **AC**, **DB**, **ACA**) or a dominant (amplification, **DBD**) behavior of the water net permeation events.

Cells are the building blocks of any living organism, separating the intracellular space from the extracellular environment. From a functional point of view, the biological membranes have one of the most important functions of regulating the metabolites' transport for the cell membrane through natural protein channels.^[1] Water translocation across membranes is an important physiological process that can be selectively produced at accelerated rates via protein water-selective channels, such as Aquaporins-AQPs.^[2,3] Over time, the investigation of the water transport mechanism through natural AQP channels inspired the researchers into developing biomimetic Artificial Water channels-AWCs, involved in transmembrane water transport with total ion rejection.^[4-8]

A particular interest in the construction of self-assembled supramolecular channels for selective transport of water across the bilayer membranes are the easily-accessible Pillar[5]arenes (PAs) synthesized for the first time by one of us.^[9] PAs have a highly symmetrical pillar-shaped structure, tunable size of cavity, fine-tunable rims with easy functionalization. Hou et al. proposed them as suitable platforms to construct artificial water channels. The formation of columnar structures could be induced by the intramolecular hydrogen bonding between the side-chains on

pillar[5]arenes.^[5,10-13] Since their discovery, the water transport properties of peptide-PAs, as a bioinspired approach for artificial water channels, have been intensively studied for their high water permeability similar to that of AQPs.^[14-16] Another step into the design approaches for their construction are the self-assembly of rim-differentiated Pillar[5]arenes as expanded length-controlled tubular platforms for host-guest recognition materials or for the construction of directional channels within lipid bilayer or polymeric membranes.^[17-23] The natural AQPs are known to transport water at high rates ($\sim 10^8$ - 10^9 water water/s/channel) in a selective manner with a narrowest constriction active pore filter of 2.8 Å, that selectively accommodate and transport water wires.^[24-26] The tubular pillar[5]arenes have often an internal cavity ~ 5 Å, which acts as highly water permeating, but non-selective platform for single water-wire translocation over ions.^[14,27,28] Recently, we found that highly selective artificial water-channels may be obtained by self-assembling pillar[5]arene dimers, presenting fast transport of water and offering size restriction selectivity, unprecedentedly observed with the PA channels before.^[29] Within these PAs, classical relays of 5 Å diameter are superposed with a narrowest twisted hydrophobic pore of 2.8 Å diameter.^[30] This affords a size restricted control of water translocation along tubular channels at narrowest selectivity filter level, offering size restriction, similar to natural protein channels. Hou et al showed that positive charge groups at the entrances indicates that the positive charges enhance water transport.^[31] Pursuing these endeavors to conceive original and efficient water channels, herein we concentrate our efforts on investigation of water/ion transport abilities through bilayer lipid membranes of combinatorial libraries of dynamic self-assembled mixtures of rim-differentiated peralkyl-carboxylate-pillar[5]arene, **A** and peralkene-carboxylate-pillar[5]arene, **D** and symmetrical rim-functionalized peralkylamino-substituted pillar-[5]arene, **B** and percarboxylate-pillar-[5]arene, **C** (Figure 1a).

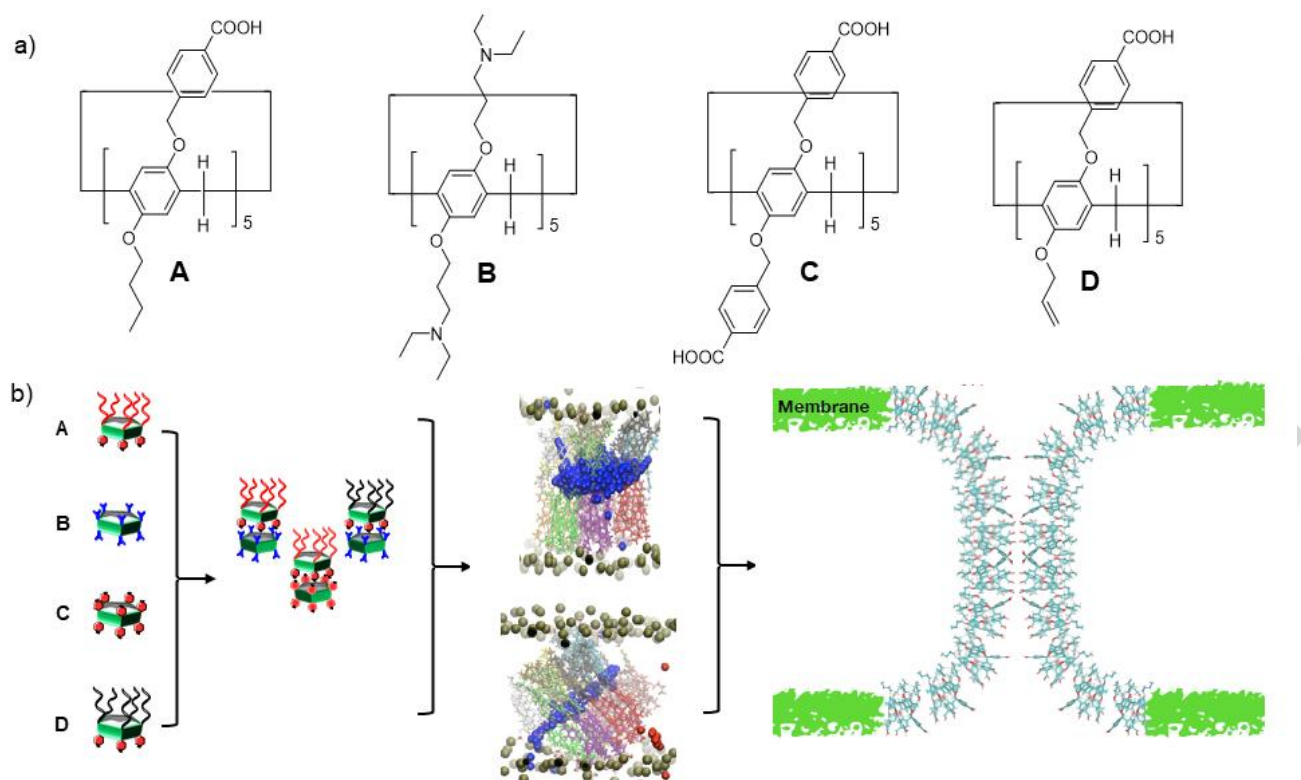


Figure 1. a) Structures of rim-differentiated and symmetrical pillar[5]arenes **A-D**. b) The self-assembly of PA channels via hydrogen bonding and charge interactions, resulting in the formation of combinatorial libraries of water channel architectures and water channels formation of aggregated PAs **A₂** dimers resulted for molecular simulation (reproduced from reference [30]) and resulting in the possible formation of toroidal pores of charged (COO^- and Et_2NH^+) inner hydrophilic surfaces when inserted in the bilayer membranes

The water transport properties are dependent on the nature of the functional groups grafted onto the PA platform as certain dimeric and trimeric combinations at different molar ratios (1:1 and 2:1) (Figure 1b), can be obtained from mixtures of mono- (**A**, **D**) and bis functional (**C**) negatively charged carboxylates or bis-functional positively charged ammonium (**B**) pillar[5]arenes that increase the overall water permeability relative to the single pillar[5]arenes components. The self-assembly of length-controlled discrete dimeric or trimeric tubular superstructures by homo-/hetero-/co-assembly via hydrogen bonds and salt bridges of rim-differentiated **A** and peralkylamino **B** pillar[5]arenes have been previously demonstrated by Ogoshi et al.^[29] by using NMR spectroscopy. Molecular simulations of the single crystal structure of dimers **A₂** embedded in bilayer membranes showed that the dimer is stable in the membrane, leading either to tilted or shifted side-by-side aggregated membrane-embedded structures, none of them featuring any water permeation.^[30] So we tested whether larger assemblies of honeycomb arrangement where a central dimer is surrounded by six other dimers, provided increased stabilization within the membrane environment.^[30] The honeycomb aggregate initiates water permeation events through the dimeric pore assembly with a continuous water accumulation around carboxylate groups located in the central membrane. When a maximal water permeation is observed, a tilted structure allows a toroidal pore formation that we observed transiently in the simulations (Figure 1b). An idealized toroidal pore is shown in Figure 1b, maximizing

favorable interactions with the environment for both water interacting with polar and membrane interacting with lipophilic moieties.^[30,32]

The water transport through unimolecular channels it is of utmost importance as proved by the works of Hou et al.^[18-20] However when the channels are unfavorably oriented in the membrane aggregated molecular self-assemblies may be considered. New mechanistic concepts can be used to describe the formation of multivalent pores. So we consider herein an innovative multivalent combinatorial strategy supposing that the exceptional channel PA platforms **A-D** combining variable pore dimensions and charged negative carboxylate or positive amino groups on their peripheries may allow: a) the simultaneous steric restriction, a key feature influencing channel selectivity and b) the charge driven enhanced water transport allowing water molecules to permeate the toroidal channels by mimicking the key functions of AQPs.

The lipid used for the water transport assays consists of egg yolk L- α -phosphatidylcholine (EYPC) used to prepare Large Unilamellar Vesicles (LUV) of 100 nm in diameter, maintaining the lipid background permeability of $\sim 50 \mu\text{m/s}$ at 20 °C. The functional pillar[5]arene **A-D** channels were combined and incorporated into this pre-formed vesicle suspension at different molar compound to lipid ratios (0.04%, 0.09%, 0.18%, 0.37%, 0.75%, 1.5% mCLR) in which channels were added by addition of dimethylsulfoxide (DMSO) solutions. The water transport

measurements through these channels were obtained by stopped-flow light-scattering experiments.

Under hypertonic conditions driven by outwardly 10 mM phosphate buffer solution and 100 mM KCl osmotic gradient, the shrinkage of the liposomes increased the light-scattering signal. The kinetics of the vesicle shrinkage indicated that PA channels' permeability increases when mCLRs increases. The net permeability (P_w) and single-channel permeability (P_s) for the single compounds present a maximum of $P_w=19.1 \mu\text{m/s}$ and of $P_s= 1.4 \cdot 10^8$ water molecules/s/channel (mCLR = 0.37 %) for compound **C** and of $P_w=11.6 \mu\text{m/s}$ and of $P_s= 3.54 \cdot 10^7$ water molecules/s/channel (mCLR = 0.75 %) for compound **D** which are the most permeable towards water and which are only one order of magnitude lower than that of AQP, whereas for **A**, and **B**, the maxima values are lower: $4.8 \mu\text{m/s}$ and of $P_s= 1.14 \cdot 10^7$ water molecules/s/channel and $5.4 \mu\text{m/s}$ and of $P_s= 7.4 \cdot 10^6$ water molecules/s/channel, respectively and they can be detected at higher mCLR = 1.5 % (Figure 2). Such a difference in performances explain that bis carboxylate (**C**) and rigid alkene (**D**) functionalized compounds are better fixed/oriented within membranes when compared with the most flexible compounds **A** and **B**. Several compounds or their combinations show a drop in overall activity at higher membrane loadings because of poor solubility or membrane partition, which might occur if higher order aggregates are formed at concentrations above 1 mM.

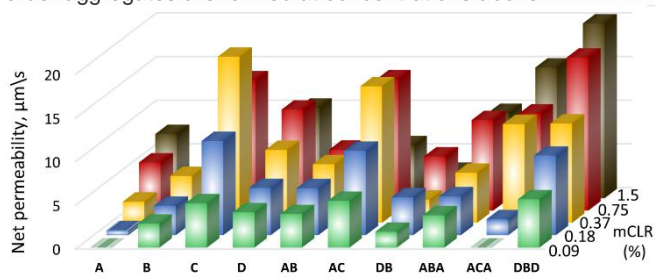


Figure 2. Water transport activity of PA channels and mixtures in liposomes. The water permeabilities of the liposomes containing different mCLRs of compounds **A**, **B**, **C** and **D**, and their combinations measured under hypertonic conditions from the post-mix assays. The liposomes (0.025 mg/mL of EYPC) were abruptly exposed to a hypertonic buffer solution of 100 mM KCl in PBS (10 mM, pH 6.4) at 20°C from the post-mix experiments.

AB and **ABA** aggregates present an additive behavior of their resulting permeability when compared with former components. Most probably electrostatic/H-bonding interactions between negatively (**A**) and positively (**B**) charged PAs are generating favorable heteromeric aggregation within hybrid toroidal channels maintaining high water permeability. Amazingly this assumption does not hold for **DB** dimers showing a strong recessive behavior when compared with former components. Increasing the content of **D** in the mixture, the **DBD** aggregates present the highest permeability of $20.3 \mu\text{m/s}$ (mCLR = 1.5 %) as proved by almost amplification behavior of the reference activity of the former components **D** and **B**. We can account that permeating waters favorably interacting with positive Et_2NH^+ and negative COO^- charges at the hydrophilic surface of the toroidal channel (Figure 1b) are of utmost importance.

The unsaturated alkene tail in **D** is most probably forming more favorable hydrophobic interactions with the lipids and

contributing to the fixation of the channels within membranes. A completely different expression of performances were observed with **AC** and **ACA** for which high permeability values of $15.0 \mu\text{m/s}$ (mCLR = 0.75-1.5 %) were measured, but these values correspond to dominant behavior of dicarboxylic compound **C** at the expense of mono carboxylic compound **A**. These results confirm that the strong activity of dicarboxylic compound **C** must be more related to its oriented H-bonding interactions with the lipids which are less effective in dimerization self-assembly with mono carboxylic compound **A**.

Ion (Na^+ and K^+) transport experiments performed on egg yolk L- α -phosphatidylcholine (EYPC) lipid vesicles (~100 nm in diameter) incorporating the monomers **A-D** and the mixtures **AB**, **AC**, **DB** and **ABA**, **ACA**, **DBD** of pillar[5]arenes at the same mCLRs as used for the water transport activity, were assessed using fluorescence spectroscopy. Inside the lipid vesicles we encapsulated 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), a pH-sensitive dye, added PA channels to the vesicle solution and monitored the fluorescence change over time upon exposure to high pH (Figure S12). The fluorescence intensity is monitored for 360 s and the fluorescence change rate followed a typical dose-response profile to the increase of the pH in the external medium. The PA channels were tested with NaCl and KCl on external buffer solution and it was registered a slight change in the fluorescence intensity after the compound addition at 20 s and the pH gradient at 40 s when the internal pH remains stable and no typical Na^+/H^+ and K^+/H^+ antiport conductance-states along the assembled channels were observed. In case of the bis-functional compounds **B** and **C** and the mixtures **AC** and **ACA** the addition of base in the extravascular media induces a rapid outward proton transport. Most probably the presence of carboxylate and ammonium relays at both entries of PA platforms help to transfer protons across within reconstituted channels across the bilayer membrane. A rapid deprotonation of the carboxyl groups of compound **C** or of the ammonium groups of compound **B**, induce HPTS deprotonation and generates the increasing of the fluorescence intensity. After the pH equilibrium was reached, dose-response profile displays constant absorbance behaviors, reminiscent of total rejection for Na^+ and K^+ and prevents the translocation of hydrated cations.

Potential proton transport acceleration is given in the case of coupling of valinomycin K^+ transporter with monomeric and mixtures of PAs. Electrogenic membrane polarization via Val- K^+ antiport, generate variable medium ($I= 0.5$, **C**, **AC** and **ACA**) to low ($I= 0.3$, **A**, **D**, **AB** and **DB**) proton antiport rates through these channels (Figure S13). Again, as for water transport experiments the use of dicarboxylic PA platforms **C** induces an increased transport of protons along most probably better oriented water filled channels.

Overall, the rim-differentiated and symmetrical carboxylic and ammonium pillar[5]arenes **A-D** and their mixtures, can be assembled into artificial channels suitable to transport water and protons. Their combinatorial self-assembly lead to synergic increase of water permeabilities, while preventing the translocation of hydrated alkali cations. Slight modifications to the number and disposition of rim carboxylic and ammonium functional groups surrounding the hydrophilic entries of the PA

channels can drastically change the water permeability. These channels are capable of transporting water at a rate that is one order of magnitude below the natural AQP. More importantly, salt rejection and their extremely low proton transport offer the closest functional mimic of the natural AQP through a synthetic scaffold. The present studies are of tremendous importance showing adaptive performances of mixtures combining simultaneous steric restriction and the charge driven enhanced water transport. Our findings show that the Artificial Water Channels story may reserve novel surprises, especially when channels are constructed from libraries with a multitude of members. The present study revealed a new paradigm: compounds may exhibit a dominant synergistic water transport activity in the presence of other co-active components. This contribution adds several new behaviors to the systematic rationalization of active artificial water channels mimicking the key functions of AQPs (Figure 2), towards the construction of industrial relevant membranes for desalination.^[33]

Acknowledgements

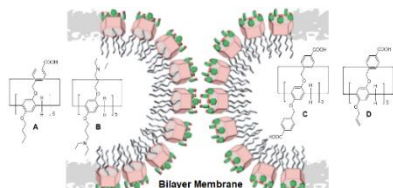
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Keywords: artificial water channels • pillar[5]arene • self-assembly • dynamic combinatorial libraries • bilayer membranes

Notes: The authors declare no competing financial interest.

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Entry for the Table of Contents

Binary combinations of pillararenes libraries have been used to construct selective artificial water channels presenting increased water transport activities across lipid bilayers than former components A-D.

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Combinatorial screening of water/proton permeation of self-assembled Pillar[5]arene artificial water channel libraries.
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