Highly permeable artificial water channels that can self-assemble into two-dimensional arrays

Yue-xiao Shen, Wen Si, Mustafa Erbakan, Karl Decker, Rita De Zorzi, Patrick O. Saboe, You Jung Kang, Sheereen Majdf, Peter J. Butler, Thomas Walz, Aleksei Aksimentiev, Jun-li Houb, and Manish Kumara

*Department of Chemical Engineering, The Pennsylvania State University, University Park, PA 16802; **Department of Chemistry, Fudan University, Shanghai 200433, China; †Department of Physics, University of Illinois at Urbana–Champaign, Urbana, IL 61801; ‡Department of Cell Biology, Harvard Medical School, Boston, MA 02115; §Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115; and ¶Department of Biomedical Engineering, The Pennsylvania State University, University Park, PA 16802

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Bioinspired artificial water channels aim to combine the high permeability and selectivity of biological aquaporin (AQP) water channels with chemical stability. Here, we carefully characterized a class of artificial water channels, peptide-appended pillar[5]arenes (PAPs). The average single-channel osmotic water permeability for PAPs is 1.0±0.3×10−16 cm2/s or 3.5±1.0×108 water molecules per s, which is in the range of AQPs (3.4–40.3×108 water molecules per s) and their current synthetic analogs, carbon nanotubes (CNTs, 9.0×108 water molecules per s). This permeability is an order of magnitude higher than first-generation artificial water channels (20 to ~107 water molecules per s). Furthermore, within lipid bilayers, PAP channels can self-assemble into 2D arrays. Relevant to permeable membrane design, the pore density of PAP channel arrays (~2.6×105 pores per μm2) is two orders of magnitude higher than that of CNT membranes (0.1–2.5×105 pores per μm2). PAP channels thus combine the advantages of biological channels and CNTs and improve upon them through their relatively simple synthesis, chemical stability, and propensity to form arrays.

Significance

This study focuses on the design of highly permeable artificial water channels for the use in membrane-based separation materials. A platform was developed for the systematic characterization of the single-channel water conduction of artificial channels, which is based on permeability measurement by stopped-flow light-scattering experiments and single-molecule counting by fluorescence correlation spectroscopy. With this platform the water conduction of the redesigned peptide-appended pillar[5]arene channels was found to be similar to that of aquaporins, natural water channel proteins, and their synthetic analogs, carbon nanotubes, which is an order of magnitude higher than that of first-generation artificial water channels. The channel can also self-assemble into arrays in membranes, opening the possibility for materials that can be used in engineering applications such as liquid and gas separations.


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1To whom correspondence should be addressed. Email: manish.kumar@psu.edu.

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into materials suitable for use in engineering applications was not explored. To derive the most advantage from their fast and selective transport properties, artificial water channels are ideally vertically aligned and densely packed in a flat membrane. These features have been long desired but remain a challenge for CNT-based systems (4).

Here we introduce peptide-appended pillar[5]arene (PAP; Fig. 1) (30) as an excellent architecture for artificial water channels, and we present data for their single-channel permeability and self-assembly properties. Nonpeptide pillar[5]arene derivatives were among first-generation artificial water channels (1, 23). Pillar[5]arene derivatives, including the one used in this study, have a pore of ~5 Å in diameter and are excellent templates for functionalization into tubular structures (31–34). However, the permeability of hydrazide-appended pillar[5]arene channels was low (~6 orders of magnitude lower than that of AQPs; SI Appendix, Table S1). We addressed the challenges of accurately measuring single-channel water permeability and improving the water conduction rate over first-generation artificial water channels by using both experimental and simulation approaches. The presented PAP channel contains more hydrophobic regions (30) compared with its predecessor channel (23), which improves both its water permeability and its ability to insert into membranes. To determine single-channel permeability of PAPs, we combined stopped-flow light-scattering measurements of lipid vesicles containing PAPs with fluorescence correlation spectroscopy (FCS) (35, 36). Stopped-flow experiments allow the kinetics of vesicle swelling or shrinking to be followed with an excellent resolution and water permeability to be calculated, whereas FCS makes it possible to count the number of channels per vesicle (36, 37). The combination of the two techniques allows molecular characterization of channel properties with high resolution and demonstrates that PAP channels have a water permeability close to those of AQPs and CNTs. The experimental results were corroborated by molecular dynamics (MD) simulations, which also provided additional insights into orientation and aggregation of the channels in lipid membranes. Finally, as a first step toward engineering applications such as liquid and gas separations, we were able to assemble PAP channels into highly packed planar membranes, and we experimentally confirmed that the channels form 2D arrays in these membranes.

**Single-Channel Water Permeability**

The PAP channel was functional in phosphatidylcholine/phosphatidylserine (PC/PS) liposomes, but its water conduction rates were magnitude lower than that of AQPs; SI Appendix, Table S1). We found to be negligible (SI Appendix, Fig. S3).

To calculate the average single-channel permeability of PAP channels, an FCS technique was used to count the number of channels per vesicle (36, 37). The channels were tagged with a fluorophore, tetramethylrhodamine cadaverine, using dicyclohexylcarbodiimide as the crosslinking reagent (Fig. 2E). The labeling process was optimized to ensure that all channels were labeled (SI Appendix, Figs. S4–S6), and the labeled channels were incorporated into PC/PS liposomes. A two-species model was used to fit the autocorrelation curves of both liposomes and micelles to differentiate them from free fluorophores. Curve fitting yielded the number of fluorescent liposomes ($N_{Liposomes}$) (the inverse of the correlation function amplitude) in the confocal volume. The labels were then solubilized in 2.5% (wt/vol) octyl glucoside (OG) to release the labeled channels into micelles (Fig. 2E), and the number of the micelles ($N_{Micelles}$) was obtained by fitting.

As expected, the diffusion time ($τ_d$) for labeled channels in liposomes was longer than those for channels in detergent micelles (Fig. 2F). The size of detergent micelles with and without channels, as determined by both FCS and dynamic light scattering, was similar, which implies that most micelles contain only one channel molecule (Fig. 2F and SI Appendix, Table S2). The subsequently calculated insertion efficiency (SI Appendix, Fig. S7) shows that if micelles contained two or three channels, the insertion efficiency would be better than 100%, which is not possible. With these insertion efficiency considerations, together with size measurements and the amount of detergent used during

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**Fig. 1.** Structure of the peptide-appended pillar[5]arene (PAP) channel. (A) The PAP channel (C$_{325}$H$_{320}$N$_{30}$O$_{60}$) forms a pentameric tubular structure through intramolecular hydrogen bonding between adjacent alternating D-l-Phe (D-Phe-L-Phe-D-Phe-COOH). (B) Molecular modeling (Gaussian09, semiempirical, PM6) of the PAP channel shows that the benzyl rings of the phenylalanine side chains extend outward from the channel walls (C, purple; H, white; O, red, N, blue). (C and D) MD simulation of the PAP channel in a POPC bilayer revealed its interactions with the surrounding lipids. The five chain-like units of the channel are colored purple, blue, ochre, green, and violet, with hydrogen atoms omitted. In C, the POPC lipids are represented by thin tan lines; in D, water is shown as red (oxygen) and white (hydrogen) van der Waals spheres.
FCS experiments, it is most likely that most micelles contained only one channel. However, even if some micelles contained two channels per micelle, the experimentally determined single-channel permeability values would only drop by a factor of two, not affecting the order of magnitude of the determined permeability.

The lower correlation amplitude indicated that solubilization created a large number of fluorescent micelles (Fig. 2G). The number of labeled particles per vesicle (\(N_{\text{Channels}}\)) was calculated as the ratio \(N_{\text{Micelles}}/N_{\text{Liposomes}}\). Compared with the theoretical number of channels that could be inserted into a liposome of ~150 nm in diameter, the size used in our experiments, the calculated number corresponded to an insertion efficiency of ~45% (SI Appendix, Fig. S7). Because fluorophore labeling reduced the water conductance of PAP channels in the shrinking mode (SI Appendix, Fig. S8), single-channel permeability (Fig. 2H) was calculated using the number of channels determined by FCS and the permeability data obtained with unlabeled channels for both modes (Fig. 2D). In the shrinking mode, the PAP channel has a water permeability of 1.1(\(\pm\)0.3) \(\times\) \(10^{-16}\) cm/s, corresponding to 3.7(\(\pm\)1.2) \(\times\) \(10^6\) water molecules per s. In the swelling mode, the water conductance of the channel is ~2 orders of magnitude higher than that in the shrinking mode, with a measured permeability of 1.0(\(\pm\)0.3) \(\times\) \(10^{-14}\) cm/s, corresponding to 3.5(\(\pm\)1.0) \(\times\) \(10^8\) water molecules per s.

**MD Simulations**

MD calculations (39) were performed with a system of 25 regularly spaced PAP channels in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) bilayer that was surrounded by water molecules (Fig. 3A). The simulations revealed several interesting features. First, individual channels exhibited wetting–dewetting transitions, i.e., the average fraction of channels filled with water fluctuated over the simulation time (Fig. 3B and Movie S2). After 30 ns of unrestrained simulation, on average, 40% of the channels were filled with water, regardless of whether simulations were initiated with water-filled or empty PAP channels. Fig. 3C illustrates the occupancy of a typical PAP channel over a 150-ns segment of a MD trajectory, showing that it takes less than 5 ns for a channel to transition from completely wet to completely dry. The water occupancy probability of a single channel (Fig. 3C, Inset) averaged over all microscopic states observed in our MD simulations has two maxima, corresponding to the dry and wet states. The snapshots in Fig. 3D illustrate the microscopic conformation of the channel before and after the wetting–dewetting transitions. Such transitions have also been observed in other biological and artificial channel systems (40–42). Second, the PAP channels had a propensity to aggregate into clusters (Fig. 3E). For example, in the 240-ns simulation initiated with water-filled channels, the average number of channels being part of a cluster increased from 1 to 13 (Movie S3). Visual inspection identified hydrogen bonds between the carbonyl and amine groups at the peptide chains of the neighboring channels stabilized the aggregates. Third, the channels showed considerable dynamics in the lipid bilayer. Although the peptide side chains remained rigid in the

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Fig. 2. The water permeability of PAP channels was calculated using a combination of stopped-flow and fluorescence correlation spectroscopy (FCS) experiments. (A) Representative stopped-flow traces from experiments performed on liposomes formed with different molar channel-to-lipid ratios (mCLRs; 0, 0.001, 0.002, 0.003, and 0.005) after a rapid exposure to a hypertonic solution containing 400 mM sucrose. (B) The water permeability of PAP channel-containing liposomes was calculated with different mCLRs measured under hypertonic conditions. Data shown are the average of triplicates with error bars representing standard deviation (SD). (C) Representative stopped-flow traces from experiments performed on liposomes with mCLRs of 0 and 0.005 after rapid exposure to a buffer without the 100 mM PEG600 used to form the vesicles. (D) Net channel permeability measured for PAP channel-containing liposomes (mCLR = 0.005) under vesicle shrinking and swelling conditions. E) FCS was used to determine the number of PAP channels per vesicle. First, FCS was used to analyze vesicles containing fluorescently labeled channels (Left). The vesicles were then solubilized with detergent, releasing the channels into detergent micelles (Right), and the solution was again analyzed by FCS. (F) The normalized FCS autocorrelation curves of the free dye tetramethylrhodamine cadaverine, labeled channel PAP in detergent micelles, and vesicles incorporating labeled channel micelle. They have significantly different diffusion coefficients (\(D_x\), represents diffusion coefficient of \(x\) species, whereas \(D_x\) represents the estimated diameter, \(D_{\text{free dye}} = 4.6(\pm0.6) \times 10^{-6}\) cm/s, \(D_{\text{free dye}} = 1.0(\pm0.1)\) nm; \(D_{\text{Micelles}} = 5.3(\pm0.2) \times 10^{-7}\) cm/s, \(D_{\text{Micelles}} = 8.5(\pm0.4)\) nm; \(D_{\text{liposomes}} = 2.4(\pm0.2) \times 10^{-8}\) cm/s, \(D_{\text{liposomes}} = 191.3(\pm 18.2)\) nm). The FCS measurements give a diameter of the micelles containing PAP channel of 8.5 ± 0.4 nm, close to the value determined by dynamic light scattering (DLS) (8.4 ± 0.1 nm). The channel-containing micelles were slightly larger than pure OG micelles (7.8 ± 0.2 nm, determined by DLS), indicating that each micelle contains only a single channel. (G) The FCS autocorrelation curves for vesicles with labeled PAP channels (mCLR = 0.005) before and after solubilization with 2.5% (w/v) OG. The high correlation function amplitude \(G(0)\) obtained for the vesicles indicates a low number of fluorescent liposomes in the confocal volume \(N_{\text{liposomes}}\). After detergent solubilization, the number of free particles increased by more than 500 times (\(N_{\text{Micelles}}\)). The number of channels per vesicle was then calculated as \(N_{\text{Micelles}}/N_{\text{Liposomes}}\). (H) Single-channel water permeability of the PAP channel (obtained from the vesicles formed at an mCLR of 0.005; permeability data are from D). Data shown are the average of triplicates with error bars representing SD.
Gaussian model due to interchain hydrogen bonds (Fig. 1A and B), the side chains exhibited greater flexibility as they interacted with the hydrophobic core of the lipid bilayer (Movie S4). In addition, the PAP channels tended to be tilted with respect to the membrane plane, and although the angle varied considerably from channel to channel (SI Appendix, Fig. S9), the most probable angle was 25°. A few channels were observed to tilt so much that they no longer spanned the membrane.

The water permeability was calculated using a collective diffusion model for a system in equilibrium (43). To test its appropriateness, the method was first used to calculate the permeability of AQP1. The calculated value of $2.0(\pm0.1) \times 10^{-14} \text{ cm}^2/\text{s}$ was within the range of previously determined values ($1.4-2.9 \times 10^{-13} \text{ cm}^2/\text{s}$) (44). For simulations on PAP channels, the root mean square deviation (RMSD) of the coordinates of the channels with respect to their crystallographic coordinates (SI Appendix, Fig. S10) and the fraction of water-filled channels (Fig. 3B) showed that the system had converged to equilibrium after 30 ns. Hence, MD trajectory data from that time onward were used to calculate the water permeability. Simulations yielded a very similar average permeability irrespective of whether simulations were initiated with water-filled or empty channels, $1.9(\pm0.4) \times 10^{-14} \text{ cm}^2/\text{s}$ and $1.5(\pm0.5) \times 10^{-14} \text{ cm}^2/\text{s}$, respectively. Individual simulations exhibited considerable variability with permeability values ranging from 0.1 to $3.1 \times 10^{-14} \text{ cm}^2/\text{s}$ within the 240-ns simulation. The average tilt of a channel showed little correlation with its water permeability provided the channel remained accessible to water from both sides of the membrane. Of the 50 simulated channels, about 10 channels showed low water conductance ($<3 \times 10^{-15} \text{ cm}^2/\text{s}$) at any given moment of the MD simulations. At any time during the simulation, on average, approximately nine of these ten were devoid of water, and seven of these ten had the entrance blocked by a phenylalanine chain. For one low-conductance channel, one acyl chain of a lipid molecule entered the channel, blocking it completely for the remainder (>200 ns) of the simulation (Movie S5 and SI Appendix, Fig. S11). We speculate that an increase in the fraction of low-conductance channels in the shrinking mode by one of the mechanisms described above might reduce osmotic permeability compared with that under the swelling mode. However, simulations for the shrinking mode, in which the area occupied by channels and lipids was reduced, did not reveal an increase in the number of low-conductance channels. Longer and more detailed simulations will be required to investigate this effect.

**Self-Assembly Behavior**

Pillar[5]arene channels can be packed into lipid membranes with very high densities. A slow dialysis technique (45) was used to conduct self-assembly experiments with PAP channels and lipids over a wide range of mCLRs. Like for membrane proteins (46), the concentration of PAP channels in the membrane had a significant effect on the morphology of the resulting PAP channel–lipid aggregates as observed by negative-stain electron microscopy (EM) (Fig. 44). When the channel concentration was low (below an mCLR of 0.05), dialysis produced vesicles mostly with a diameter of 100–200 nm. When the mCLR was increased to ~0.5, the vesicles grew to approximately 1 µm in diameter. At mCLR of 0.714, sheet-like membranes began to form. At mCLRs higher than 1, channel aggregates were observed in addition to membrane-like morphologies (see SI Appendix, Fig. S12, for the different aggregate morphologies observed over the full mCLR range). Increasing the density of the channel in the membrane thus changed the curvature of the lipid bilayer and resulted in the formation of flat membranes. The mCLR at
which the PAP channel–lipid aggregates transition from curved to flat membranes was close to 0.5, compared with a range of 0.02–0.125 for most membrane protein–lipid aggregates (45). The transition at a lower mCLR for PAP channel–lipid aggregates can be explained by the cross-sectional area of the PAP channel, which is smaller than that of most membrane proteins.

To further analyze the packing of the channels in the membranes, PAP channel-containing vesicles produced at an mCLR of 0.909 were prepared by the carbon sandwich technique (47) and analyzed by cryo-EM (Fig. 4B, Left). Power spectra of the images showed blurry first-order diffraction spots, indicating the presence of very densely packed or even somewhat ordered regions with lattice constants of \(a = b = 21 \text{ Å}\) and \(\gamma = 120^\circ\) (Fig. 4B, Right). Considering that the entire cross-sectional area of the channel is \(\sim300 \text{ Å}^2\), a single unit cell within the 2D crystal can only accommodate one channel molecule. The PAP channels are most likely densely packed in a hexagonal arrangement. A molecular model of these densely packed PAP sheets is shown in Fig. 4C.

**Solute Rejection and Ion Selectivity**

Stopped-flow light-scattering measurements were also performed in the presence of osmolytes of different molecular weights (SI Appendix, Figs. S13–S15). The reflection coefficient for each osmolyte was calculated as the ratio of the measured water permeability in its presence to that in the presence of a completely retained osmolyte (Dextran500) (48). Based on a reflection coefficient of 0.9, the molecular weight cutoff of the PAP channel was determined to be \(\sim420 \text{ Da}\) (SI Appendix, Fig. S16), consistent with its pore diameter of \(\sim5 \text{ Å}\). The ion selectivity of the channel was determined by patch-clamp experiments. The voltage–current relationship showed PAP channels to be cation selective (SI Appendix, Fig. S17), which was expected as the channel has five carboxylic groups at each entrance. The order of ion selectivity was \(\text{NH}_3^+ > \text{Cs}^+ > \text{Rb}^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+ > \text{Cl}^-(\text{SI Appendix, Table S3)}\).

**Comparison of PAP Channels with AQPs and CNTs**

The water permeability of the PAP channel under swelling conditions is only one order of magnitude lower than that of AQP1, the AQP with the highest water permeability (11.7 \(\times 10^{-14} \text{ cm}^2/\text{s}\)) (49), and is close to that of CNTs (2.61 \(\times 10^{-14} \text{ cm}^2/\text{s}\)) (12). If water channels are to be used in separation materials, it is important to consider their effective cross-sectional area. AQPs occupy a large cross-sectional area in the membrane (\(-9 \text{ nm}^2\)), but the actual water pore represents only a very small portion of the occupied membrane area (SI Appendix, Fig. S18). This will offset the high water permeability of AQPs, even if a membrane is very densely packed with AQPs, such as in 2D crystals (1, 45). Taking into account the cross-sectional areas of the PAP channel (3.0 \(\text{ nm}^2\)), AQP1 (9.0 \(\text{ nm}^2\)) (50), and CNTs (2.1 \(\text{ nm}^2\)) (12, 51), the single-channel permeability of the PAP channel per cross-sectional area is similar to that of the most water-permeable AQPs and CNTs (SI Appendix, Fig. S18). Artificial water channels provide two additional advantages over channel proteins, namely, high physicochemical stability as well as solubility in and compatibility with organic solvents. The latter is particularly critical for membrane fabrication because organic solvents are used in polymer membrane processing. A method based on solvent casting to self-assembly artificial water channels in lamellar amphiphilic block copolymers may allow scalable manufacturing of high-permeability membranes.

PAP channels also present advantages over CNTs. First, the structure of the PAP channel could be further modified so that the pore size is compatible for use in water desalination. The majority of currently reported artificial water channels have a pore diameter of less than 0.6 nm (SI Appendix, Table S1), but CNTs with this pore size cannot yet be synthesized (4). Furthermore, artificial channels with specially designed pore sizes may not only be useful in water purification but also hold promise in other fields currently proposed for CNT-based systems, such as biomolecule sensing, gas separations, drug delivery, and protective fabrics (4–6, 15, 52). Second, most CNTs produced in bulk today suffer from disorganized architectures (4), which significantly limit their use in applications that depend on transport through their pores. The experimental and simulation studies presented here demonstrate that the PAP channels are aligned in lipid bilayers, and that PAP channels can form 2D arrays, mimicking crystalline protein arrays found in some biological membranes, such as the mammalian eye fiber cell membranes (53), retinal rod membranes (54), and photosynthetic membranes (55). The packing density of the PAP channels (2.6 \(\times 10^{12} \text{ molecules}/\text{cm}^2\)) is higher than those of AQP 2D crystals (8.6 \(\times 10^{10} \text{ molecules}/\text{cm}^2\) for AQP1 and 9.5 \(\times 10^{9} \text{ molecules}/\text{cm}^2\) for AQP0) (56, 57) and two orders of magnitude higher than those of current CNT-based membranes (0.1–2.5 \(\times 10^{12} \text{ molecules}/\text{cm}^2\)) (12, 58–61) (SI Appendix, Table S4). Taking into account the effective cross-sectional pore areas of the membranes, the packing of PAP channels at an mCLR close to 0.5 is also far more efficient than that of cyclic peptide nanotubes in block copolymer membranes (62). Third, pillar[n]arene-based artificial channels present the opportunity for simple modifications of their...
side chains to change their transport properties. Compared with hydrazide-appended pillar[n]arene (23), the presented peptide-appended pillar[5]arene channels have greatly improved water permeability and membrane insertion characteristics. A drawback of the current PAP channel is its substrate selectivity. With a molecular weight cutoff of 420 Da, the channel is not suitable to remove salt and other small solutes. However, the ability to attach different substituents to the pillar[5]arene scaffold may provide a way to enhance selectivity. Overall, this study shows the promise of pillar[5]arene-based artificial water channels to mimic the high permeability of biological water channels and carbon nanotubes.

Materials and Methods

SI Appendix provides detailed materials and methods, including preparation of lipid vesicles, water permeability measurements, water permeability of giant unilamellar vesicles (GUVs), labeling procedure and estimating labeling efficiency, FCS, MD simulation, assembly of PAP channellipid aggregates and EM imaging, and solute rejection.

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