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A framework for accurate evaluation of the promise of aquaporin based biomimetic membranes

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ABSTRACT

Aquaporin based membranes (ABMs) are considered a promising biomimetic desalination technology and have been intensively studied over the last few years. The most common strategy to synthesize ABMs is to deposit the aquaporin incorporated lipid or block copolymer (BCP) vesicles onto porous substrates or more recently to integrate them within the active layer of polyamide membranes. However, ABMs with orders of magnitude improvement in permeability and perfect salt rejections proposed in initial work have not been realized. Early results were based on materials and methods that were rudimentary, especially considering the progress that has been made in this field. In particular, low signal to noise ratios (SNRs <50) of stopped flow measurements for vesicle-based assays have led to large inaccuracies in permeability estimation. We show that such low SNRs can result from using vesicle samples with a high concentration of micelles and provide a connection between morphology and data quality. We have conducted a comprehensive evaluation of the true promise of these membranes using improved methods for polymer synthesis, self-assembly, experimental evaluation as well as calculations that more directly compare the outcome of biophysical evaluations to those used in the desalination membrane industry. We propose these as standard methods for use in ABM research. The role of concentration polarization in introducing error into vesicle based permeability measurements is identified. We further describe a simple technique to calculate the expected flux from a membrane synthesized using vesicle immobilization on a permeable substrate that can be used to estimate realistic membrane fluxes from stopped flow data. These calculations show that it is possible to achieve permeabilities one to two orders of magnitude higher than current membranes using ABMs but several innovations will be needed to reach this potential.

1. Introduction

Biological cells facilitate efficient solute and water transport across their cell membranes via various membrane proteins. Due to their unique structures, membrane proteins are capable of transporting ions, solutes and water with high permeability while maintaining selectivity. These membrane proteins are promising for biomimetic applications [1,2] such as sensors, drug delivery and separations. Biological water channel proteins, Aquaporins (AQPs), are excellent model membrane proteins due to their high stability and the wide availability of structural and functional data. Because classical AQPs only transport water transport while rejecting all other solutes [3], they have been intensively studied for synthesizing biomimetic desalination membranes [1,4].

The widespread application of Aquaporin based membranes (ABMs) face several challenges. First, the production of AQPs and other membrane proteins is central to the success of these membranes but the purity and quality of these hydrophobic proteins cannot be easily controlled [5] due to their narrow range of stability in defined aqueous environments where the presence of specialized detergents is required. The second critical challenge is their reconstitution into artificial systems where these proteins need to be kept intact and functional. Lipid vesicles or liposomes are ideal analogues to biological cells and were the first model systems for the reconstitution of AQPs [6,7]. However, natural lipids are chemically unstable and are proposed to be replaced by amphiphilic block copolymers (BCPs) [8] in many ABM studies. Several studies have shown the functionality of AQPs in BCPs systems [9]. In this paper, we report on several challenges facing BCP based ABM membranes and propose solutions. The most common procedure utilized in current ABMs is depositing AQPs incorporated vesicles onto porous substrates [1]. In this paper, we focus on accurate functional characterization of AQPs in BCP

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vesicles because this is being widely used to characterize the first step in formation of ABMs. From the experimental perspective, research still seems to be in the process of development and optimization of techniques to accurately measure AQP permeability in polymersomes, and reported data is still of poor quality. Data with low signal to noise ratios (SNRs) in the range of 10–20 have been reported in nearly all publications utilizing stopped flow light scattering techniques on BCP vesicles (see Supplementary information for procedure proposed to calculate SNRs and data on published studies, Table S1). Low SNRs (<20) in stopped flow studies reported can make permeability data calculated from fits meaningless or have high error ranges (estimated using 95% confidence intervals) of 40–600% (see Table S1). These data also indicate that it has been more difficult to measure AQP permeability consistently and accurately in BCP systems (SNRs <20) compared to in liposomes (SNRs $\sim$200 to $>$1000, Supplementary information Table S1).

The lack of high quality data points to a need to evaluate the procedures involved in synthesizing and charactering ABMs. In this paper, we present a systematic method of characterizing AQPs in BCP systems. A comprehensive evaluation was conducted via strictly controlling the processes of BCP synthesis, polymersome self-assembly, protein incorporation and water transport measurements. We propose the use of a SNR metric to evaluate stopped flow data. We also propose the use of a method that directly compares the osmotic permeability of AQPs measured in BCP vesicles using stopped flow techniques to the intrinsic water permeabilities commonly used in desalination membrane literature. The intrinsic permeability in pressure driven membranes is given by the value of the "*A*" parameter in the following equation.

$$J_w = A(\Delta P - \Delta\pi) \quad (1)$$

where, J_w is the water flux through the membrane (volume per unit area per unit time). ΔP is the difference in pressure across the membrane and $\Delta\pi$ is the difference in osmotic pressure across the membrane.

Based on the calculations conducted in this work, we predict the upper limit of the permeability of current vesicle based ABMs based on our own measurements and the possible upper limit that can be reached using such a technology using a highly packed two-dimensional crystal form of the membrane. This paper provides a standard platform for evaluating experimental work in this emerging and promising desalination technology and its ultimate promise for improving performance.

2. Materials and methods

2.1. Protein expression and purification

E. coli JM109 containing an N-terminal histidine-tagged *E.coli K12* Aquaporin Z (AqpZ) plasmid was propagated following a previously published procedure [10]. Details are provided in Supplementary Information.

2.2. Polymer synthesis

The polymers were synthesized according to a previously published protocol [11]. In the first step, α,ω-Bis(4-hydroxybutyl)polydimethylsiloxane (PDMS) was synthesized, by polycondensation of dimethoxydimethyl silane, water and 3-bis(4-hydroxybutyl)tetramethyl disiloxane [12]. PDMS was further activated with trifluoromethanesulfonic anhydride and resulting triflic acid was quenched by triethylamine in cold anhydrous hexane [11]. Triflate-activated PDMS was than filtered, hexane was removed under high vacuum and dry ethyl acetate was added. Activated PDMS served as macro-initiator of ring opening polymerization of 2-methyloxazoline. The termination of the 'living' polyoxazoline chain in KOH, MeOH or other nucleophiles results in the termination with appropriate end-groups including carboxylate, methacrylate and amine [13]. The resulting triblock polymers ($PMOXA_8$–$PDMS_{55}$–$PMOXA_8$ and $PMOXA_8$–$PDMS_{60}$–$PMOXA_8$) are referred to as ABA polymers in the rest of the paper.

2.3. Liposome, polymer and protein polymer vesicle formation

Polymersomes were prepared using the film rehydration method. 50 mg of polymer was dissolved in $CHCl_3$ and was dried on a rotary vacuum evaporator to form a uniform thin film. After rehydration with either 5 ml of buffer (100 mM MOPS, pH=7) or protein solution in buffer (100 mM MOPS, pH=7), the suspension was incubated with stirring at 4 °C for 12 h. The detergent in proteopolymersome samples was removed by adding adequate amount of Bio-Beads (Bio-Rad) [14] and then subjected to extrusion through track-etched membrane filters (20 times through 0.2 μm membranes, Isopore, Millipore) to obtain monodisperse unilamellar vesicles, the size of which was characterized by dynamic light scattering (DLS, Zetasizer Nano, Malvern Instruments Ltd., UK). Proteopolymersome suspensions were further subjected to size exclusion chromatography using Sepharose 4B media.

Lipid stocks (used in preparation of liposomes) were prepared by sonication of 40.5 mg of *E.coli* Polar Lipid Extracts (Avanti Polar Lipids, Alabaster, AL) in 900 μl of 2 mM BME in DI water. 250 μl aliquots were added drop-wise to either 875 μl of 1.25% OG solution in 100 mM Na-MOPS (pH 7.5) or 875 μl OG solution containing appropriate amount of protein (lipid to protein ratios by weight, LPRs 100–1000) to form liposomes. After one hour incubation the detergent was removed by dialysis against 2 l of 100 mM Na-MOPS (pH 7.5). Liposomes were subsequently extruded through 0.2 μm membranes (Isopore, Millipore) to obtain monodisperse unilamellar vesicles

2.4. Permeability determination using stopped flow light scattering

Permeability tests were conducted on a stopped-flow instrument (SF-300X, KinTek Corp., Snowshoe, PA). Exposure to hypertonic solutions results in shrinkage of vesicles due to the outwardly directed osmotic gradient created and drives water transport out of the vesicles. The abrupt changes in vesicle size lead to the increase in the light scattering at 90° according to the Rayleigh–Gans theory applied to this system [15]. The changes of light scattering caused by vesicle shrinking were recorded at a wavelength of 600 nm. The data were fitted to a double exponential equation to account for the fast water transport through AQPs and slower but still rapid transport though the liposome or polymersome membranes. We then used the calculated faster exponential coefficient (k) for the initial change of the light scattering curve to calculate and the osmotic water permeability (P_f) using the following expression [10]:

$$P_f = \frac{k}{\left(\frac{S}{V_0}\right) \times V_W \times \Delta_{osm}} \quad (2)$$

where S is the initial surface area of the vesicles, V_0 is the initial volume of the vesicles, V_w is the molar volume of water, and Δ_{osm} is the osmolarity difference in driving the shrinking of the vesicles. This osmolarity coefficient can be calculated using the Van't Hoff equation [31] but a more accurate approach is to use an osmometer to measure osmolarity values for the osmotic agent and the background buffer solutions that are used for vesicle synthesis.