Short communication

Stabilization and immobilization of aquaporin reconstituted lipid vesicles for water purification

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Abstract

Aquaporins are water channel proteins in biological membranes that have extraordinary water permeability and selectivity. In this work, we have demonstrated that one of their family members, Aquaporin Z (AqpZ), can be possibly applied in a pressure-driven water purification process. A nanofiltration membrane was designed and fabricated by immobilization of AqpZ-reconstituted liposomes on a polydopamine (PDA) coated microporous membrane. Amine-functionalized proteoliposomes were first deposited via gentle vacuum suction and subsequently conjugated on the PDA layer via an amine-catechol adduct formation. Due to the existence of a polymer network within the lipid bilayers, the membrane could sustain hydraulic pressure of 5 bar as well as the strong surface agitation in nanofiltration tests, indicating a relatively stable membrane structure. In comparison with membrane without AqpZ incorporation, the membrane with AqpZ-to-lipid weight ratio of 1:100 increased the water flux by 65% with enhanced NaCl and MgCl 2 rejections of 66.2% and 88.1%, respectively. With AqpZ incorporation, the vesicle immobilized membrane exhibits a promising strategy for high productivity water purification.

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1. Introduction

Membrane technology plays a significant role in water purification. In recent years, water scarcity has become an increasingly pressing issue in many countries. Developing high-performance membranes for desalination and water reuse have received worldwide attention. Amongst the different membrane filtrations, membranes for reverse osmosis and nanofiltration are the most value-added products because of their effectiveness in removing low molecular weight impurities such as ions and small organic compounds for drinking water production [1]. However, reverse osmosis and nanofiltration are still energy-intensive processes, and innovations to improve their productivity are urgently needed.

Inspired from biological membranes where aquaporins provide extraordinary water permeability and selectivity [2–4], the use of aquaporin proteins has recently attracted worldwide attention [5–8]. Aquaporins are a group of water channel proteins that can transport water molecules across the cell membranes through an osmotic gradient and selectively reject ions and other solutes. The reported permeability of each aquaporin channel is higher than 10 × 10⁻¹⁵ cm² s⁻¹, which is equal to 3 billion water molecules per second [3,4]. If this protein can be successfully incorporated into synthetic membranes, there can be an increase in membrane efficiency of several folds. Although many efforts have been made to realize this target, most of these efforts have focused on aquaporin incorporation in planar lipid membranes, either supported lipid bilayers [9–11] or black lipid membranes [12,13]. The stability of pore-suspending planar lipid membranes formed is questionable under high hydraulic pressure and turbulence because the lipid bilayer thickness is only few nanometers [14]. Therefore, the challenge in this current work is to design a stable aquaporin embedded water selective layer that can maintain its structure and perform under high pressure and surface agitation. Herein, we aim to develop a novel biomimetic composite membrane for nanofiltration, by immobilizing AqpZ incorporated liposomes on a porous polymeric support.

Liposomes are typically unstable and tend to rupture on hydrophilic surfaces such as mica and silica, which result in the development of different approaches for their stabilization. Cross-linkable lipids like diynyl lipids can be polymerized easily in the bilayer but the lateral mobility of the lipids that are required for the protein functioning are largely diminished after cross-linking [15]. Liposomes stabilized by layer-by-layer (LBL) polyelectrolytes have been designed as hollow nanocapsules [16]. However, adsorption of polyelectrolyte chains on the surface of liposomes may create defects on the lipid membrane surface. Hence, we choose to use methacrylate monomers to cross-link the liposomes, which has also been suggested by earlier studies [17–19]. With the aid of
UV-induced free radical polymerization, a hydrophobic polymer mesh can be formed to stabilize the bilayer without much detrimental effects on the lipid mobility [17].

Polydopamine (PDA) is known to spontaneously react with amine- and thiol-containing compounds which makes it viable for grafting of an additional layers [20,21]. Polyethylene glycol (PEG) conjugated lipids or PEGylated lipids were terminated with amine groups and blended with normal lipids to form PEGylated liposomes in this work. The PEGylated liposomes have been shown to be more stable than normal liposomes [22]. The PEG can also behave like a cushion between the bilayer and the substrate such that direct contact of embedded proteins to the substrate can be avoided [23]. Fig. 1 shows the schematic diagram of membrane fabrication procedure. The amine-functionalyzed vesicles are first stabilized by forming a 2D polymer network inside the lipid bilayer. Then these vesicles are immobilized on a PDA coated porous membrane using a pressure-assisted adsorption method. In the final step, the membrane is further cross-linked with glutaraldehyde (GA) to enhance the vesicle stability on the membrane surface. Membranes of different aquaporin incorporation ratios are investigated for nanofiltration performance. For the first time, membranes covered with aquaporin-reconstituted proteoliposomes are designed and fabricated for pressure-driven water purification purposes. From the preliminary nanofiltration results, these polymerized vesicles prove to be stable under pressure and stirring shear.

2. Experimental

2.1. Materials

The lipids, 1,2-dioleyl-sn-glycero-3-phosphocholine (DOPC), and the ammonium salt of 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol])-2000] (DSPE-PEG-NH₂) were purchased from Avanti Polar Lipids. Ammonium salt of 1,2-dimyristoyl-sn-glycero-phosphoethanolamine-N-[lissamine-Rhodamine B sulfonyl], Rh-PE, was obtained from Invitrogen. Ethylene glycol dimethacrylate (EGDMA), 1-Hydroxycyclohexyl phenyl ketone (Irgacure184), 6-Carboxyfluorescein (CF), dopamine hydrochloride, isopropyl alcohol (IPA), glutaaldehyde (GA) solution (50 wt% in H2O), chloroform, sodium chloride (NaCl) and magnesium chloride (MgCl₂) were from Sigma-Aldrich. N-methylpyrrolidone (NMP) was purchased from Merck. The detergent, n-dodecyl beta-D-maltoside (DDM) was a product of Acros Organics. 10× phosphate buffered saline (PBS, pH = 7.4) and sucrose in ultra high purity were purchased from 1st Base, Singapore. Ultrapure water was produced by the Millipore Reference A+ system. Bio-Beads SM-2 Absorbents and tris(hydroxymethyl) aminomethane (Tris) were purchased from BIO-RAD. Polyacrylonitrile (PAN) for substrate preparation was from Tong-Hua synthesis fiber Co. Ltd. (Taiwan). A 10-histidine residual tagged (His-tagged) Aquaporin Z (AqpZ) used in this work was synthesized according to the methods of Borgia et al. [3]. The AqpZ stock solutions contain ~1 mg/mL protein and 18 mM DDM in PBS buffer.

2.2. Preparation of vesicles

DOPC, DSPE-PEG-NH₂, EGDMA and UV initiator Irgacure 184 were mixed at a molar ratio of 20:1:40:20. A multimellar vesicle suspension in PBS buffer was prepared using the film rehydration method. Small unilamellar vesicles (SUV) with a uniform pore size were produced by extruding the suspension through a polycarbonate Nuclepore track-etch membrane (Whatman) of 100 or 200 nm pore size. For AqpZ reconstitution experiments, AqpZ stock solution was added during the film rehydration step and the mixture was agitated for at least 8 h. Then, Bio-Beads were added into the mixture to remove the DDM completely. The suspension was protected with purified argon and kept away from light source throughout the experiment. Prior to the photo-polymerization of vesicles, the suspension was bubbled with purified argon to remove oxygen. Radical polymerization was then initiated by incubating the suspension in a UV cross-linker (Vilber Lourmat, BLX-E234, 245 nm, 40 W) for 30 min. The vesicle solution was maintained at a distance of 4 cm from the UV bulbs.

2.3. Characterization of polymerized vesicles

To verify that the vesicles underwent polymerization, the DOPC/DSPE-PEG-NH₂/EGDMA/Irgacure184 mixture was doped with 0.1 wt% Rh-PE (excitation/emission = 560 nm/583 nm) before dry film formation. In the film rehydration buffer, 0.01 mg/mL CF (excitation/emission = 494 nm/521 nm) was into vesicles. The resultant suspension was separated into two batches, i.e. with and without UV cross-linking. Both batches of vesicles were dialyzed 48 h against pure PBS buffer so as to remove the non-encapsulated CF. The Rh-PE doped vesicles with CF encapsulation were imaged using a confocal laser scanning microscope (Nikon A1 Confocal). After depositing the suspension on the glass coverslip, confocal imaging was performed at two wavelengths simultaneously, 488 nm and 514.5 nm (both from an argon ion laser), to excite CF and Rh-PE, respectively. It should take note that all the vesicles for confocal imaging were non-extruded.

![Fig. 1. Schematic presentation of immobilization of the cross-linked proteoliposome on a PDA coated membrane (not to scale).](image-url)
2.4. Vesicle permeability measurements

The permeability of vesicles was determined using a stopped-flow apparatus (Chirascan, Applied Photophysics). By rapidly mixing the vesicle solution with a hypertonic buffer (1.2 mol/L sucrose), water would permeate outwards of the vesicles, causing them to experience a sudden shrinkage. The rapid reduction of vesicle volume was recorded as an increase in the light scattering intensity at an emission wavelength of 577 nm. To improve the signal to noise ratio, all the experiments were performed at a temperature of 8 °C. Data can be fitted with an exponential decay equation shown in Eq. (1), where $Y$ is the intensity of light scattering signal, $A$ is a negative constant, $t$ is the time of recording, and $k$ is the initial rate constant (s$^{-1}$). The final osmotic permeability ($P_l$) of vesicles was calculated with Eq. (2), where $V_0$ is the vesicle initial volume, $S$ is the vesicle surface area, $V_w$ is the partial molar volume of water (18 cm$^3$/mole), and $\Delta_{osm}$ is the osmolarity difference that drives the size change of vesicles. The vesicle diameter was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments).

$$Y = A \exp(-kt)$$  \hspace{1cm} (1)

$$P_l = \frac{k}{(S/V_0)V_w\Delta_{osm}}$$  \hspace{1cm} (2)

2.5. Preparation of vesicle immobilized membranes

Flat sheet PAN substrates were prepared by casting a 12 wt% PAN solution in NMP directly on glass plates with a 100 μm casting knife. The membranes were then stored in ultrapure water until use. PDA deposition on the top surface of PAN substrates was performed by mounting the membranes on a dead-end modification cell. The dopamine solution (0.02 mg dopamine dissolved in 100 mL 10 mM Tris buffer with pH = 8.5) was added into the dead-end modification cell and stirred at 350 rpm for 3 h PDA deposition. After deposition, the membranes were immersed in IPA for 30 min to remove any unbound PDA and again rinse thoroughly with water.

The surface area of PDA-PAN membrane available for vesicle immobilization was 0.785 cm$^2$. 150 μL of the vesicle solution (6 mg/mL) was filtered onto the membrane at a transmembrane pressure of 40 mbar for 10 min. The membranes were then incubated with the vesicle solution at 4 °C for 3 h. Afterwards, the vesicle solution was removed from membrane surface and 0.5 wt% aqueous GA was filtrated to cross-link the immobilized vesicles at a pressure of 100 mbar for 10 min.

2.6. Field-emission scanning electron microscopy (FESEM)

The morphology of freeze dried membranes was observed by FESEM (JSM-6700F, JEOL). To image the membrane cross-section, the freeze dried samples were immersed in liquid nitrogen and fractured. Before imaging, a layer of platinum was coated on the membrane using a JEOL JFC-1300 Platinum coater.

2.7. Nanofiltration studies

The nanofiltration tests were performed using dead-end permeation cells. Pure water permeability, $PWP$ (Lm$^{-2}$ bar$^{-1}$ h$^{-1}$) measurements were conducted at 23 °C, 5 bar, which was calculated using Eq. (3).

$$PWP = \frac{Q}{\Delta P \cdot A}$$  \hspace{1cm} (3)

$Q$ is the volumetric flow rate of water (L/h), $\Delta P$ is the transmembrane pressure difference (bar) and $A$ is the membrane surface area (m$^2$). Rejections of NaCl and MgCl$_2$ solutions (200 ppm in ultrapure water) were tested with a surface mixing speed of 700 rpm at 23 °C, 5 bar. The membranes were flushed thoroughly with ultrapure water between tests of different solutes. The salt rejection was calculated using the equation shown below

$$R (\%) = \frac{C_t - C_p}{C_t} \times 100$$  \hspace{1cm} (4)

where $C_t$ and $C_p$ are the concentrations of the feed solution and the permeate, respectively. Data were obtained from at least three different preparations.

3. Results and discussion

3.1. Characterization of polymerized vesicles

To study the polymerization of cross-linker EGDMA within the lipid bilayer, CF was encapsulated in the designed vesicles labeled with 0.1% Rh-PE. Due to the high fluidity of DOPC bilayers, the CF could diffuse out of the bilayer if there was a concentration gradient. Therefore, by reducing the CF concentration in the bulk solution via dialysis, the dye can be slowly removed from the hydrophilic core of the liposomes. Using confocal laser scanning microscopy, CF and Rh-PE were excited at the same time to obtain an overlapped red and green fluorescence image, where the red refers to Rh-PE while the green refers to CF. Fig. 2(a) corresponds to the images of liposomes without UV polymerization, which did not display green fluorescence from both inside and outside the liposomes. As expected, most CF experienced diffusion out of the liposome through the DOPC bilayers. When a cross-linked polymer network was formed in the lipid bilayer, the green dye was trapped inside the red vesicles as shown in Fig. 2(b). The formation of the hydrophobic network in the bilayer caused the vesicles to be much less permeable (or may be impermeable) to hydrophilic molecules like CF, thus these hydrophilic molecules ended up being encapsulated within liposomes. Similar findings were also observed in the study carried by a field emission transmission electron microscopy (see Figure S1).

3.2. Water permeability of polymerized vesicles

The water permeability of vesicles at different AqpZ-to-lipid ratios was investigated using a stopped-flow spectrometer. The time-dependent traces of stopped-flow signal were plotted in Fig. 3(a) for vesicles with different AqpZ incorporation ratios. Fig. 3(b) shows the calculated permeability of the vesicles in the presence of EGDMA monomers and after UV cross-linking. Since the permeability of DOPC liposomes increases strikingly with temperature [24], all the permeability tests on stopped-flow were performed at 8 °C to reduce the permeability noise derived from the lipid bilayer. Without AqpZ incorporation (control), the permeability of cross-linked liposomes has been observed about to be approximately 40% lower than that of the non-cross-linked liposomes. It indicates that the formation of the polymethacrylate network in the hydrophobic phase of liposomes could resist water permeation across the lipid membrane. Sizes of control liposomes with and without cross-linking were measured by DLS on the 1st and 2nd day of extrusion (see Table S1). The cross-linked liposomes show an almost unchanged polydispersity index (PDI) on the 2nd day, while the non-polymerized liposomes displayed an obvious increased PDI. Since plain liposomes have the tendency to aggregate and fuse together, the cross-linked polymer mesh has stabilized the vesicle structure and prevented fusion of vesicles.

With AqpZ incorporation, the water permeability of non-cross-linked vesicles shows a significant improvement, from 29.4 μm/s of the control to 241.6 μm/s of the sample containing a
protein-to-lipid weight ratio of 1:100. This result is lower than the findings from Borgnia et al. in 1999 [3] in which they reconstituted AqpZ into liposomes of E. coli total lipid extract and observed a water permeability of about 400 µm/s. Apart from intrinsic differences in AqpZ, the lower permeability of our proteoliposomes could be owing to a lower AqpZ incorporation efficiency. This may be caused by PEG brushes on the vesicle surface and/or hydrophobic-hydrophobic interactions between EGDMA and AqpZ. The permeability of proteoliposomes was increased as the protein-to-lipid ratio was increased, but not proportionally. This may be attributed to the fact that, at a ratio of 1:50 and 1:10, more detergent was added into the vesicle solution together with the AqpZ stock solution. A large amount of detergent may cause solubilization of lipid membranes and result in a low AqpZ incorporation efficiency [2].

At the lower AqpZ incorporation ratio, saying 1:100, the vesicle permeability was reduced by 25% after UV cross-linking. It has been shown that the polymer network formation could induce an internal stress in the lipid bilayer [17]. Thus, AqpZ structure may be indirectly affected and function may be compromised. However, at higher AqpZ incorporation ratios, the permeability did not change significantly after UV cross-linking. This could be explained by an incomplete polymer network formation at a higher AqpZ content. Since a large amount of detergent was added into the DOPC/DSPE-PEG-NH₂/EGDMA/Irgacure184 mixture together with the AqpZ stock solution, hydrophobic monomers EGDMA can get solubilized by the excess detergent. During the detergent removal process, the detergent-EGDMA micelles may be also adsorbed and removed by BioBeads. Thus, at a higher AqpZ content, it is more likely to lose EGDMA in the detergent removal process, and the polymerization effect on AqpZ function is less prominent due to the incomplete polymerization within the bilayer.

3.3. Vesicle immobilized nanofiltration membranes

In comparison with the blank PAN membrane (Fig. 4(a)), both the surface pore size (Fig. 4(b)) as well as the membrane permeability (Table 1) were reduced by the 100 nm PDA coating on the

<table>
<thead>
<tr>
<th>Membrane</th>
<th>PWP (LMH/bar)</th>
<th>tested under 5 bar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank PAN (12%)</td>
<td>970</td>
<td></td>
</tr>
<tr>
<td>3 h-PDA-PAN</td>
<td>455</td>
<td></td>
</tr>
<tr>
<td>PDA-vesicle-glutaraldehyde</td>
<td>No AqpZ</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>AqpZ:lipid = 1:100</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>AqpZ:lipid = 1:50</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>AqpZ:lipid = 1:10</td>
<td>9.1</td>
</tr>
</tbody>
</table>

* The AqpZ-to-lipid weight ratio is used here.
PAN surface (see Fig. 4(c)). The cross-linked DOPC/DSPE-PEG-NH₂ vesicles were then immobilized on the PAN surface. The immobilization has been demonstrated successfully on a PDA coated silicon surface by directly incubating the vesicles on the substrate and imaging with AFM (see Supporting Information and Figure S2). For nanofiltration membrane fabrication, a slight vacuum was applied under the PDA-PAN substrate to drive the vesicles to block the top surface pores, and at the same time these vesicles were more likely to bond with the PDA layer. With this method, the vesicle solution on the membrane surface can be concentrated to a much higher concentration. Finally, the immobilized layer was further cross-linked with GA. Fig. 4(d) and (e) shows the PDA surface covered with polymerized vesicles after performance test at 5 bar. Interestingly, the structure of many vesicles was still maintained; showing that these vesicles were stable under the pressure. Some fractured vesicles on the membrane surfaces displayed as hollow spheres with diameters of 100–300 nm, and the wall thickness of the cross-linked vesicles were estimated at less than 20 nm.

For immobilization of vesicles without UV polymerization, very few vesicles were found on the membrane surface after the filtration tests (Fig. 4(f)). Under a testing pressure of 5 bar, the permeability of such membrane deviates from 10 to 20 LMH/bar and rejection was below 10% for both NaCl and MgCl₂. AqpZ did not play its role in the filtration process because of the heavy leakage in the selective layer. It was suspected that without polymerization, most of the vesicles were ruptured into a planar lipid film and this thin film was not strong enough under the hydraulic pressure.

PWP data of the membrane before and after immobilization of polymerized vesicles are shown in Table 1. Both blank and PDA coated membranes demonstrate zero rejections to NaCl and MgCl₂. After vesicle immobilization, the membrane flux drops dramatically indicating a high coverage of the surface pores by vesicles. The salt rejection and permeate flux results for both NaCl and MgCl₂ after vesicle immobilization are shown in Fig. 5. Compared to the membrane without AqpZ incorporation, the membrane comprising the AqpZ-to-lipid weight ratio of 1:100 increases the PWP of 65% with enhanced NaCl and MgCl₂ rejections of 66.2% and 88.1%, respectively. Clearly, proteoliposomes incorporated with AqpZ possess high selectivity to ions. The vesicles without AqpZ behave as a dense selective layer providing a great resistance to permeation flow, while AqpZ opens up the flow channels as well as controls the rejection. However, a trade-off between membrane
flux and salt rejection was observed when AqpZ incorporation ratio increases. As mentioned previously, at higher AqpZ incorporation ratios, loss of EGDMA during detergent removal process could result in incomplete formation of polymer networks. In other words, the polymer network in the bilayer would be “defective”. During the NF testing, the un-cross-linked region may become the weak points and generate defects under pressure or perturbation. Therefore, at higher AqpZ-to-lipid ratios, especially 1:10, these defects would lead to membrane flux increase and salt rejection decrease.

Moreover, although GA reacted with the membrane for only 10 min, there exists the possibility that AqpZ may get denatured. Since GA is a relatively active cross-linker, when cross-linking the amine-ended PEG, it may also cross-link the protein inside the vesicles, leading to the malfunction of AqpZ. In future works, better membrane stabilization methods will be studied. For example, the immobilized liposomes can be covered with a layer of hydrogel or a layer of PDA, and hopefully this layer is able to seal the defects among the vesicles and at the same time extend membrane lifetime in the nanofiltration tests.

4. Conclusion

In this study, we have explored the possibility of fabricating a stable aquaporin reconstituted biomimetic membrane for nanofiltration. We have shown that, with UV polymerization of EGDMA in the bilayer membrane, AqpZ can be still functionally reconstituted into the vesicles. The membrane formed by immobilizing the designed vesicles on a porous membrane via amine-catechol adduct formation can withstand hydraulic pressure up to about 5 bar as well as stirring shear. The preliminary nanofiltration data demonstrate that, compared to the control, the membrane with AqpZ incorporation exhibits a higher pure water permeability as well as enhanced NaCl and MgCl2 rejections. Obviously, AqpZ opens up the flow channels as well as controls the rejection. Although there could be a few ways to improve the membrane performance, this work demonstrates that AqpZ incorporated proteoliposomes may potentially modify ultrafiltration membranes for nanofiltration.

Acknowledgements

This work was financially supported by the Environment and Water Industry Programme Office (EWI) (NUS grant number: R-279-000-293-272) under the Singapore National Research Foundation (NRF). The authors would like to give thanks to Dr. Yi Li, Dr. Wenyuan Xie, Dr. Qingsong Lin, Ms. Honglei Wang, Mr. Zhou Hu, Ms. Peishan Zhong, Ms. Phuoc Hoang Hanh Duong, and Ms. Bingfang Wang.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2012.08.009.

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