Development of aquaporin based biomimetic membranes for desalination applications

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NANYANG
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APPLICATIONS**

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

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LIST OF PUBLICATIONS

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- [2] **Li, X.**; Wang, R.; Tang, C.; Vararattanavech, A.; Zhao, Y.; Torres, J. and Fane, A.G., Fusion behaviour of aquaporin Z incorporated proteoliposomes investigated by Quartz Crystal Microbalance with Dissipation (QCM-D). *Colloids and Surfaces B: Biointerfaces*. 2013, 111, (0), 446-452.
- [3] **Li, X.**; Wang, R.; Wicaksana, F.; Tang, C.; Torres, J. and Fane, A.G., Preparation of high performance nanofiltration (NF) membranes incorporated with aquaporin Z. *Journal of Membrane Science* 450(0): 181-188.
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- [6] Zhao, Y.; Vararattanavech, A.; **Li, X.**; Hélix-Nielsen, C.; Vissing, T.; Torres, J.; Wang, R.; Fane, A. G. and Tang, C. Y., Effects of proteoliposome composition and draw solution types on separation performance of aquaporin-based proteoliposomes: implications for seawater desalination using aquaporin-

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SUMMARY

Nature has contributed the best desalination material, aquaporin (AQP), for us. Attributed to its extremely high permeability for water molecules as well as high rejection to small solutes, in recent years there is a surge to mimic the natural cellular membranes by incorporating AQPs into various ultrathin films for water filtration applications. In this work, efforts are made to explore an effective way to design, prepare and evaluate highly permeable and selective AQP-based biomimetic membranes for water purification applications.

As a start, this thesis presents a comprehensive literature review on the AQPs and related AQP-based biomimetic membranes research. Basic information about AQPs, lipid and block copolymer systems is reviewed. Strategies for membrane protein reconstitution into lipid vesicles (liposomes) and polymer vesicles (polymersomes) are described. In addition, different types of AQP-based biomimetic membranes are reviewed and discussed in detail. Moreover, a series of characterization techniques commonly applied in protein/biomimetic membrane research are reviewed.

Equipped with fundamental knowledge and understanding of the state-of-the-art technology, a preliminary study was carried out to develop AQP-based biomimetic membranes. To make biomimetic membranes for water purification applications, preparing a defect-free platform for AQP incorporation on a suitable substrate is one of the critical steps. Two methods were used to prepare supported lipid membranes on a nanofiltration membrane surface under a benign pH condition of 7.8. One was direct vesicle fusion onto a hydrophilic membrane NF-270; the other was vesicle fusion facilitated by hydraulic pressure on a modified hydrophilic membrane NF-270, whose surface was spin-coated with positively charged lipids. Experiments revealed that the supported lipid membrane prepared by spin coating with vesicle fusion had a much lower defect density than that prepared by vesicle fusion only. It seems that the surface roughness and charge are main factors determining the quality of a supported lipid membrane. AQPs were successfully incorporated into 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes

and its water permeability was examined by stopped-flow experiments. However, after the proteoliposomes were fused onto the modified substrate, the AQPs function in the resultant membrane was not observed. Atomic force microscopy (AFM) images showed the presence of unfused proteoliposomes on the substrate. This may suggest that the presence of AQPs in the liposomes can significantly change the way that these vesicles interact with a surface.

In order to understand these problems that were encountered, different characterization techniques were employed to investigate the vesicle characteristics and the fusion behaviour of liposomes and proteoliposomes on solid surfaces. The results showed that after incorporation of aquaporin Z (AqpZ), the size and surface charge density of the proteoliposomes changed significantly compared with those of liposomes. Although the liposome could easily form a supported lipid bilayer on silica via vesicle rupture, it was much more difficult for proteoliposomes to fuse completely into bilayers on the same substrate. In addition, the fusion of proteoliposomes was further hindered after the density of incorporated AqpZ was increased, suggesting that the proteoliposomes with more proteins become more robust. However, both the liposome and proteoliposome had difficulty in fusing to supported lipid bilayers on the surface of a polyelectrolyte layer even though they carried an opposite charge, indicating that the polymer might play an important role in stabilizing the vesicles. It was also observed that a high concentration of AqpZ could be incorporated into the liposome even though its nominal permeability decreased.

Since it was difficult for proteoliposomes to fuse to bilayers completely because of the incorporated AQPs, we proposed a novel protocol to design an AQP-based membrane: the AqpZ incorporated proteoliposomes were immobilized in the inner surface of a hollow fibre membrane and subsequently encapsulated by a polyamide layer formed by interfacial polymerization, instead of being fused to bilayers. Different hollow fibre composite membranes with various lipid-to-AQP ratios were prepared and explored. The AQP-based hollow fibre composite membrane exhibited an increased water flux and salt rejection with an increase in the loading ratio of AQPs. The membrane with a high AQP-to-lipid ratio exhibited a $40 \text{ L} \cdot \text{m}^{-2}$

$^2 \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ flux at 5 bar, almost 200% more than the flux of a commercial reverse osmosis membrane. This membrane also exhibited 500 ppm NaCl rejection above 97.5%, which was also superior to the commercial membrane tested under the same conditions. The forward osmosis test of the AqpZ-based hollow fibre membrane also yielded a super high water flux. It showed that the most of introduced proteoliposomes were totally embedded in the polyamide layer, maintaining an intact spherical shape even after long-term pressure-driven filtration test. Series of experiments proved that it was the embedded AqpZ that enhanced the water flux and the integration between the embedded proteoliposomes and polyamide layer was entirely defect-free. It was evaluated that the AqpZ-incorporated hollow fibre composite membrane could cut the power consumption by up to 40% in a Newater plant for wastewater treatment, if these hollow fibres were assembled in a standard 8-inch commercial membrane module (assuming a 80% packing density, giving a 59 m^2 membrane area). The use of AQP within membranes has been taken beyond the concept stage and offers a novel means for tailoring the membrane materials and improving the membrane performance.

Based on the previous work, a modified method was proposed to prepare a nanofiltration (NF) membrane based on AQPs. The proteoliposome, incorporated with AqpZ, was embedded into the selective layer through crosslinking of a polyelectrolyte with the membrane substrate made by poly(amide-imide) (PAI). The proteoliposome with mutants of AqpZ, which had a low water permeability, was selected as a control group. The water flux of the AqpZ-based membrane was around 50% higher than the mutant one. At the optimal preparation conditions, the AqpZ-based membrane displayed a water flux of $36.6 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ with a MgCl_2 rejection of 95% at 1 bar. Results from our studies indicated that AqpZ could maintain a high water transport ability (about $250 \text{ } \mu\text{m/s}$ for proteoliposomes with LPR 400) even at high temperature ($70 \text{ } ^\circ\text{C}$ for 2 hours) and keep its activity in the presence of highly charged polyelectrolytes (polyethyleneimine). These results and findings may provide useful insights for developing the next generation of biomimetic membranes.

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A	Water permeation coefficient of membrane, $\text{L} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$
A_2	The second Virial coefficient
B	Solute permeation coefficient of the membrane, $\text{g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$
C	Concentration of all solutes, $\text{mol} \cdot \text{L}^{-1}$
C_f	Salt Concentration of feed, $\text{mol} \cdot \text{L}^{-1}$
C_p	Salt Concentration of permeate, $\text{mol} \cdot \text{L}^{-1}$
ΔC_f	The salt concentration change of feed, $\text{mol} \cdot \text{L}^{-1}$
D	Diffusion coefficient of particles in certain solution, $\text{m}^2 \cdot \text{s}^{-1}$
D_p	Dissipation
ΔD	Change in dissipation
ΔD_b	The dissipation change of bilayer obtained for the liposome.
ΔD_m	The actual measured dissipation change at saturation.
ΔD_s	The dissipation change at saturation when the surface is fully covered by intact vesicles.
E_{lost}	Energy lost during one oscillation cycle, J
E_{stored}	Total energy stored in the oscillator, J
Δf	Change in frequency, Hz
I_0	Modified Bessel function of zeroth
I_1	Modified Bessel function or first-order
J_v	Water flux, $\text{L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$
J_s	Salt flux, $\text{g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$
k	Initial rising rates of fitted stopped-flow light scattering, s^{-1}
K	Optical constant in light scattering
K_1	Constant in Sauerbrey equation
K_b	Boltzmann constant
M_w	Weight average molecular weight

Δm	Change in mass per unit area, $\text{g} \cdot \text{m}^{-2}$
Δ_{osm}	Difference in osmolarity, $\text{mol} \cdot \text{L}^{-1}$
P	Water permeability of membrane, $\text{m}^3 \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{Pa}^{-1}$
P_d	Diffusive permeability coefficient, $\text{m} \cdot \text{s}^{-1}$
P_f	Osmotic permeability coefficient, $\text{m} \cdot \text{s}^{-1}$
P_{net}	The net driving force in reverse osmosis process
$P(\theta)$	The angular dependence of the sample scattering intensity
ΔP	Hydrostatic pressure difference across the membrane, bar
ΔP_L	The pressure drop in a membrane module in reverse osmosis process
R_c	Resistance of fouling layer
R_g	Radius of gyration, m
R_h	Hydrodynamic radius of particles in certain solution, m
R_m	Resistance of membrane
R_θ	Rayleigh factor
r	Rejection of membrane (%)
S	Surface area of liposome, m^2
T	Temperature, K
V	Volume of liposome, m^3
V_f	Volume of feed, m^3
v	Volume of the hydrocarbon part of lipid molecule, m^3
V_w	Partial molar volume of water, 18 cm^3
Y	Recovery in reverse osmosis process

Greek

α	Bilayer coverage on solid surface
β	The constant ratio of ΔD_s to the vesicle diameter

κ	A constant related to the radius of vesicles in light scattering measurement
λ	Wavelength of light, nm
η	Viscosity, Pa·s ⁻¹
ω	Diameter of bleach area, m
π_0	Osmotic pressure of feed in reverse osmosis process
π_{ave}	Average osmotic pressure of feed in reverse osmosis process
$\Delta\pi$	Osmotic pressure gradient across the membrane, Pa
τ_D	Axelrod's time constant, s

GLOSSARY OF TERMS

Aquaporin	Aquaporins are a group of integral membrane proteins found in biological membrane and selectively transport water molecules while preventing the passage of other solutes
Cytoplasm	The gel-like substance that is enclosed within the cell membrane
Extrusion	Forcing a vesicle solution through a membrane to make its size distribution more uniform
Fusion	The wall of a vesicle breaks and make the vesicle becomes a bilayer
Hyperosmolar	The solution has a increased osmolar concentration
Liposome	The artificially-prepared vesicle composed of a lipid bilayer
Micelle	An aggregate of amphiphilic molecules in aqueous solution. Hydrophobic regions aggregate in centre and hydrophilic regions contacts the surrounding solvent
Periplasm	The space bordered by two permeable membranes.
Polymersome	The artificially-prepared vesicle composed of a plymer layer
Proteoliposome	The liposome with incorporated membrane proteins in bilayer
Proteopolymersome	The polymersome with incorporated membrane proteins in polymer layer
Vesicle	A small bubble enclosed by a lipid bilayer or polymer layer

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND

Water scarcity is now increasingly receiving attention worldwide and is becoming more challenging with the expanding population, climate change and socio-economic activities. The growing demand for safe drinking water is driving people to find more water supplies such as water reuse and seawater desalination. This will undoubtedly need more efficient water treatment technologies. Of these technologies, membrane-based technology has aroused much interest because of its cost-effectiveness over other water treatment technologies. In comparison to other membranes, reverse osmosis (RO) membranes can remove almost all impurities including ions and small organic molecules, and can directly supply high-quality water. The development of thin-film composite membranes has reduced the cost dramatically and made RO membrane technology a fast growing approach for seawater or brackish water desalination, including wastewater reclamation. Presently RO membrane technology is one of the most energy-efficient technologies for water purification and desalination.

However, one big obstacle for larger scale application of RO membrane technology is its high energy consumption as the energy cost normally takes half of the total desalination cost. Theoretically minimum energy of desalination for seawater that typically contains 35,000 ppm NaCl is 1.06 kWh/m^3 with a recovery of 50% and it is around 0.12 kWh/m^3 for the brackish water containing 1000 ppm NaCl with a recovery of 80%. However, the actual energy cost is much higher because of finite size of membrane plants and irreversible thermodynamic operation process (Elimelech and Phillip 2011). Extensive efforts have been made trying to reduce the cost, which include the optimization of membrane materials, process design, module design, feed pretreatment and energy recovery (Lee *et al.* 2011). In recent years, various hybrid membranes have been explored to improve the water purification efficiency such as vertically aligned nanotube membranes, thin-film nanocomposite membranes and aquaporin (AQP)-based membranes (Pendergast and Hoek 2011). These nanotechnology-featured or bio-inspired novel membranes

could potentially offer higher water productivity compared to conventional thin-film composite RO membranes. Of these novel membranes, the AQP-based membrane has attracted much attention because of the exclusive and efficient water transport ability of AQPs. It is believed that such an AQP-based membrane could allow a closer approach to the theoretical minimum energy dictated by thermodynamics (Elimelech and Phillip 2011). Since 2012, more than ten papers introducing how to design or fabricate a high performance AQP-based membrane have been published. Several companies have been established to explore the scale-up of this type of membrane (Buonomenna 2013), indicating that AQP-based biomimetic membranes are quite promising for desalination.

1.2 AQUAPORINS

AQPs are a group of very hydrophobic integral membrane proteins widely expressed in bacteria, animals and plants. The first AQP was expressed in *Xenopus laevis* oocytes by Peter Agre in the 1980s, who shared the Nobel Chemistry Prize in 2003 attributed to this great discovery. To date thirteen AQPs have been found in mammals and some other AQPs have been found in plants, yeast, bacteria and archaea (Kumar 2010b). Some AQPs only allow rapid passage of water molecules but reject all other solutes (Verkman 2005). Today, it is well-known that water transport across biological membranes is dominated by the AQPs. For example, the kidney has very high water permeability and the kidneys of one adult human are responsible for reabsorption of more than 150 litres water per day (Agre *et al.* 1998). It is reported that the osmotic permeability coefficient of AQPs could reach $5.43 \times 10^{-14} \text{ cm}^3 \cdot \text{s}^{-1} \cdot \text{channel}^{-1}$, which is equal to 1.8×10^9 water molecules $\cdot \text{s}^{-1} \cdot \text{channel}^{-1}$ (Heymann and Engel 1999). The high water permeability and selectivity of AQPs have been confirmed by many experimental results (Borgnia *et al.* 1999b, Kumar *et al.* 2007, Zhao *et al.* 2013). AQPs lower the activation energy for water permeation through the membrane from 10-20 kcal/mol to around 5 kcal/mol (Heymann and Engel 1999). This low activation energy can allow a high water flux when the AQP-based biomimetic membrane is exposed to a hydrostatic or osmotic pressure. The unique property of AQPs makes AQP-based membranes totally different from conventional thin-film composite membranes, in terms of the mechanism of water

transport through the membrane. The AQP-based membrane is exquisitely selective for the transport of water molecules because the structures of the AQPs provide a unique selective mechanism for water free permeation (Kozono *et al.* 2002). Therefore, it is very attractive to utilize the excellent permeability and selectivity of AQPs in water purification applications. It is estimated that an AQP-based membrane could offer a water permeability in a range of $10^{-11} \sim 10^{-9} \text{ m} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1}$, orders of magnitude higher than current commercial RO membranes (Kumar *et al.* 2007, Pendergast and Hoek 2011).

1.3 OBJECTIVES

Since the proof of concept for using AQPs to develop a high-performance biomimetic membrane (Kumar *et al.* 2007), many efforts have been made to fabricate a practical biomimetic membrane that can be applied in water filtration process. However, to date it is still quite challenging to obtain an AQP-based biomimetic membrane having a competitive performance with most commercial RO or nanofiltration membranes. Several main reasons are responsible for that. Firstly, the fundamental understanding of the biomimetic system is quite limited such as maintaining AQP's activity in a benign condition, the physicochemical properties of AQP-lipid systems, and interactions between vesicles and polymer materials. Secondly, a systematic study of the biomimetic system, especially when the thesis work was just started, was lacked on how to design, prepare and characterize an AQP-based biomimetic membrane. Thirdly, although many methods are proposed to design or prepare the AQP-based biomimetic membranes, most of them still have various difficulties to become promising or practical for water purification or desalination application.

In terms of these limitation and challenges, this study aims (1) to gain deep insight into the fundamental aspects of AQPs and AQP-based biomimetic membranes; and (2) to design, prepare, characterize and evaluate the highly permeable and selective biomimetic membranes based on AQPs. The specific objectives include:

- to reconstitute AQPs into liposomes and maintain their high activity in a benign condition.

- to investigate the properties of AQP-incorporated proteoliposomes and the integration of proteoliposomes with polymeric substrates to gain a deeper insight on the fundamental science of biomimetic membranes.
- to prepare a suitable polymeric substrate in the laboratory, and to design a high performance AQP-based biomimetic membrane for desalination application (high water flux and salt rejection) on the bench-scale.
- to explore the structure of AQP-based biomimetic membranes by employing different characterization techniques.
- to evaluate the desalination performance (permeate flux and salt rejection) of as-prepared AQP-based biomimetic membranes.

1.4 OUTLINE OF THESIS

This thesis contains seven chapters, which are highlighted as follows;

- Chapter 1: ***Introduction***

The background information, motivation and the objectives of this study are introduced.

- Chapter 2: ***Literature review***

This chapter provides a comprehensive review on the AQPs and related biomimetic membrane research. Basic information about AQPs, model lipid bilayer systems, model block copolymer membrane systems is introduced. Some recent studies about AQP-based membranes are briefly summarized in this chapter. In addition, a series of characterization techniques commonly applied to protein/biomimetic membrane research are reviewed.

- Chapter 3: ***Preparation of supported lipid membranes for aquaporin Z incorporation***

A preliminary study was carried out to develop AQP-based biomimetic membranes. Two methods of preparing a supported lipid layer for AQPs were proposed and implemented, and AQPs were also incorporated into the supported lipid layer. Details of the experiments and results are discussed in this chapter. This work

suggests that the presence of AQPs in the lipid vesicles can significantly change the way that these vesicles interact with a polymer surface.

➤ Chapter 4: *Fusion behaviour of aquaporin Z incorporated proteoliposomes investigated by the Quartz Crystal Microbalance with Dissipation (QCM-D)*

The work discussed in Chapter 3 indicated that the proteoliposomes with AQPs were difficult to fuse onto a polymeric substrate even under an applied pressure. A further study was conducted to elucidate this problem by employing the QCM-D technique. It was demonstrated that the proteoliposomes became quite different from the liposomes after being incorporated with AQPs. Some effects of the AQPs on proteoliposomes were also investigated.

➤ Chapter 5: *An ultra-high performance hollow fibre composite membrane based on aquaporin Z*

As it was confirmed that the proteoliposome was very difficult to fuse to bilayers on a polymeric substrate, one novel protocol was proposed to prepare the biomimetic membrane: instead of fusion to bilayers, the proteoliposome was encapsulated into a polyamide layer supported on a hollow fibre through interfacial polymerization. The resultant membrane displayed a super-high performance that combined water permeability and salt rejection. Preparation, characterization and performance evaluation of the membrane are described in this chapter.

➤ Chapter 6: *Preparation of high performance nanofiltration (NF) membranes incorporated with aquaporin Z*

A NF membrane was obtained by a modified method: the modified proteoliposome was encapsulated into a selective layer by crosslinking the substrate with a polyelectrolyte. The mutant was selected as the control group because it presented properties closer to those of AqpZ. The results showed that this membrane with AqpZ gave a superior separation performance to any other reported NF membrane based on AQPs.

➤ Chapter 7: *Conclusions, implications and recommended future research*

Some major conclusions of this work are given and some implications inspired from the research are discussed. Based on the thesis work and reports of others in this research filed, some recommendations are made for future study.

CHAPTER 2 LITERATURE REVIEW

2.1 AQUAPORINS

Aquaporins (AQPs) are a group of integral membrane proteins that dominate the transport of water molecules across a biological membrane. AQP exists in biological membrane in a tetramer form and each monomer acts as a water channel. Each monomer has six membrane spanning domains which contain six transmembrane α -helices and two half-membrane spanning helices (hemipores) arranged in a right-handed bundle with five connecting loops (Gonen and Walz 2006b). The hemipore loops do not span the whole bilayer, and each hemipore loop contains the signature motif Asn-Pro-Ala (NPA). The amino- and carboxy-termini are intracellular and the repeats are oriented at 180° to each other, as shown in Figure 2.1A. This structure is believed to be responsible for the selective transport of water molecules. The interaction between water molecules and the residues in the water channel are described in Figure 2.1B. It is proposed that three features may be mainly responsible for the high selectivity of AQP: (i) size restriction: the diameter of channel is around 8 Å above the midpoint of the channel; however, the pore becomes 2.8 Å which is just big enough for one water molecule; (ii) electrostatic repulsion: the conserved residue arginine-195 and histidine-180 provide a strong repulsion force to cations, including protonated water (H_3O^+); (iii) water dipole reorientation: the charged dipoles formed by the two partial helices in the water channel will reorient a water molecule when it passes through. The hydrogen bonds of the water molecules are disrupted in the water channel, which could also prevent proton conductance (Kozono *et al.* 2002).

Figure 2.1. Schematic architecture of the channel within an AQP1 subunit. (A) Membrane-spanning helices are denoted as H1-H6, loops are denoted as A-E; Two helices formed by loops B and E as HB and HE, respectively. (B) H, N, R represents Histidine, Asparagine and Arginine, respectively. The number beside the amino acid refer to their locations in the amino acid sequence. Adapted from (Kozono *et al.* 2002, Gonen and Walz 2006a).

The water transport of AQPs can be regulated by phosphorylation (Johansson *et al.* 1998), pH (Nemeth-Cahalan *et al.* 2004), osmolarity (Johansson *et al.* 1996) or some metal ions (Zelenina *et al.* 2004, Gunnarson *et al.* 2005). The regulation of AQPs was realized by the gating of water channels. Two main structural mechanisms had been proposed to understand the gating of AQPs. One is the “capping”: the rearrangement of cytoplasmic loop D caps the water channel to close the channel or open it by being displaced. This mechanism was found in some plant AQPs. Another one is “pinching”: some residues move and pinch in the (aromatic/arginine) constriction region, which restricts the transport of water molecules through channel. This mechanism was found in some mammalian AQPs and AqpZ (Hedfalk *et al.* 2006).

To date 13 types of AQPs have been discovered widely in different tissues of human body and some of them also transport some other solutes such as glycerol, urea besides water molecules. Some characteristics of AQPs were summarized in Table 2.1.

Table 2.1. Characteristics of some AQPs in human and *E.coli* (Borgnia *et al.* 1999a, Itoh *et al.* 2005, Gorelick *et al.* 2006, Laforenza *et al.* 2013)

	Molecules transported	Permeability	Major tissue distributions
AQP 0	Water	Low	Lens fibre cells
AQP 1	Water	High	Eyes, lung, kidney, red cells, vascular endothelium, the gastrointestinal tract, sweat glands.
AQP 2	Water	High	Kidney, some tissues in central nervous system and gastrointestinal system.
AQP 3	Water, glycerol, urea	High (water, glycerol) Low (urea)	Kidney, nasopharyngeal epithelium.
AQP 4	Water	High	Brain
AQP 5	Water	High	Alveolar pneumocytes, Salivary and lachrymal glands
AQP 6	Water	Low	Kidney
AQP 7	Water and glycerol	High	Spermatids and seminiferous tubules.
AQP 8	Water	High	Testis, pancreas and liver
AQP 9	Water, glycerol, urea	Low	Brain, liver, testis.
AQP 10	Water and glycerol	Low	Human subcutaneous adipose tissue
AQP 11	Unclear	N.A.	Kidney, liver, testes and brain
AQP 12	Unclear	N.A.	Pancreas
AQP Z	Water	High	<i>Escherichia coli</i>

The aquaporin Z (AqpZ), expressed in a wild-type *Escherichia coli* strain, was selected to conduct the entire research in this study because it is easier to express and purify in its native host. AqpZ is a type of markedly hydrophobic and tightly packed membrane protein, which has a relatively small molecular weight (~ 23k/monomer). Some studies indicated that the AqpZ tetramer, in contrast to the mammalian AQPs, was quite stable and not readily denatured even in a 1% sodium dodecyl sulfate solution (Calamita 2000). It was also reported that AqpZ could maintain its activity in lipid or polymer vesicles and present the highest water permeability among the AQPs examined (Borgnia *et al.* 1999b, Hashido *et al.* 2005, Kumar *et al.* 2007). The sequence and topology of AqpZ are shown in Figure 2.2. Similar to the structure of AQP1, the loops B and E including the typical NPA motif

are believed to play roles in the selective transport of the channel. The six transmembrane domains of AqpZ are located in the lipid bilayer which stabilizes those highly hydrophobic protein domains. The hydrophilic domains are located at the periplasm and cytoplasm, respectively. The lipid plays the role of a “cushion” among the membrane proteins. In the absence of this “cushion”, interactions between the membrane proteins may lead to their precipitation and inactivation (Kumar 2010a). In addition to the lipids, some lipid-like block copolymers are also used to construct an appropriate “cushion” for membrane proteins (Nardin *et al.* 2000a, Nardin *et al.* 2000b, Kumar *et al.* 2007).

Figure 2.2. Predicted membrane topology of *E.coli* AqpZ protein. Amino acid residues are represented as circles. Black circles represent the highly conserved NPA motifs. Adapted from (Calamita 2000)

2.2 MODEL LIPID BILAYER SYSTEMS

Lipids are generally used to construct a cell model with a bilayer structure in an aqueous environment because a biological membrane indeed is a lipid bilayer matrix. Cell models, such as liposomes, formed by lipid molecules in certain solvents, have the similar properties to a cell in terms of composition, selectivity and permeation. Besides liposomes, a series of other model lipid systems are designed, such as black lipid membranes, solid supported lipid membranes, polymer supported lipid membranes, etc. These systems possess different characteristics, providing more choices to design or study biomimetic systems.

Lipids comprise a large group of molecules which constitute a water-insoluble hydrophobic part and a water-soluble hydrophilic part, including waxes, fatty acids, fat-soluble vitamins, triglycerides, fatty-acid derived phospholipids, sphingolipids, glycolipids and terpenoids. As most lipids are amphiphilic molecules, they tend to self-assemble into various structures in polar solvents like water. Not all lipids can form a bilayer structure in water. Single chain lipids such as detergents can spontaneously assemble into micelles (Ulrich 2002). The most widely used lipids for preparing liposome and supported lipid membranes are glycerolphospholipids, some of which are shown in Figure 2.3.

Figure 2.3. Schematic representation of glycerolphospholipids and their most abundant representatives in biological cell membranes. Adapted from (Renner 2009).

Liposomes form spontaneously in an aqueous solution. The hydrophilic part of the lipids points out to the aqueous solution on either side of the bilayer while the hydrophobic part goes into the core of the bilayer (Bangham *et al.* 1965). The bilayer is an energetically stable structure. Liposomes can be divided into two main groups in terms of the number of walls: multilamellar vesicles (MLVs, 0.1-10 μm) and unilamellar ones. The unilamellar vesicles are widely studied because they provide a good model for cells and are relatively stable. The unilamellar vesicles can be divided into three major types in terms of the size: small unilamellar vesicles (SUVs, < 100 nm), large unilamellar vesicles (LUVs, 100-500 nm) and giant unilamellar vesicles (GUVs, $\geq 1 \mu\text{m}$).

In comparison to unilamellar vesicles, the application of MLVs is quite limited. They can be obtained by hydrating the dry lipid film under strong agitation. However, the resulting size distribution and lamellarity of the MLVs are very

heterogeneous. Some procedures such as sonication, extrusion, and freeze-thawing cycles, can be used to help produce liposomes with an uniform size (Lee *et al.* 2001). Sonication can break MLVs into SUVs or MLVs having a smaller size. Extrusion of an MLV suspension through a polycarbonate membrane with a certain mean pore size (usually 50-400 nm) produces an LUV with a mean diameter close to the nominal pore size. Freeze thawing can produce an equilibrium transmembrane ion concentration and increase the trapped volume and trapping efficiency. This is the most commonly used method to prepare unilamellar vesicles because of the absence of organic solvents or detergents, availability of high lipid concentrations and high encapsulation efficiencies of vesicles (Hope *et al.* 1985). Vesicles may maintain elongated elliptical shapes for some time after extrusion, which may affect the evaluation of the size or entrapped volume of vesicles (Jin *et al.* 1999). A rapid approach to prepare LUVs is dissolving the lipid in an organic solvent and then injecting into an aqueous buffer, producing a heterogeneous mixture of SUVs, LUVs or MLVs in a short time. The organic solvent can be removed by evaporating under a reduced pressure for certain period of time. However, the liposomes may have a poor stability and some organics may be hard to remove completely. These may be unfavourable in some applications.

Although vesicles are good models to study biological membranes, the closed environment, relatively poor stability and operability inhibit their research and some specific applications. A series of planar bilayer systems were created to provide excellent models for investigating the biomimetic membranes or some processes occurring at the biological membrane surface such as cell signalling, ligand-receptor interaction, enzymatic reactions (Castellana and Cremer 2006). In addition, more analytical techniques are available for these planar bilayer systems and these planar bilayer systems could also provide more choices for developing biosensors or biomimetic membranes (Tien *et al.* 1997, Trojanowicz and Miernik 2001, Bally *et al.* 2010, Kaufman *et al.* 2010).

2.3 MODEL BLOCK COPOLYMER MEMBRANE SYSTEMS

Amphiphilic block copolymer membranes have attained much attention during the past decades as they showed some superior properties to lipids. One of those is that they have more choices of monomers and allow functionalization of some functional groups (Belegrinou *et al.* 2011). Since the block copolymers and lipids share many characteristics, the research on block copolymer membranes basically follows the methodology as lipid membrane research.

Most block copolymers used in biomimetic membrane formation are linear amphiphilic block copolymers. The structure of amphiphilic block copolymers is similar to that of lipids. They consist of at least one hydrophilic and one hydrophobic polymer block, and most of them are diblock or triblock. They can be abbreviated by capital letters like AB, ABA, or ABC. A and C blocks are hydrophilic parts and B is a hydrophobic part. Similar to the lipids, block copolymers could also self-assemble into various structures in an aqueous solution, determined by some intrinsic characteristics or external conditions. Some fundamental parameters that affect the morphology of block copolymer vesicles include core stretching, interfacial energy between the core and solvent outside, and the corona-corona repulsion (Choucair and Eisenberg 2003). The block copolymers can also self-assemble to different morphologies or structures including micelles, tubes, vesicles or mixture of some aggregates (Blanazs *et al.* 2009) as shown at Figure 2.4.

Figure 2.4. Transmission electron microscopy (TEM) images (A, B) and optical micrograph (C) of different aggregation structures formed by PB-PEO block copolymers in different conditions including spherical micelles (A), cylindrical micelles (B) and vesicles (C). Adapted from (Antonietti and Forster 2003).

In order to be able to form vesicles in water, block copolymers should have a hydrophilic-hydrophobic volume ratio similar to lipids. The hydrophobic block chain length largely influences the membrane thickness. As the block copolymers have a much higher molecular weight (up to tens of kDa) than the lipids (less than 2 kDa), the thickness of block copolymer membranes is usually higher than that of lipid bilayers, up to 10 nm compared to ~5 nm of lipid bilayers. For a block copolymer, the most noteworthy characteristic should be its biocompatibility as a platform for membrane proteins. However, to date the most promising polymer for biomimetic application is the amphiphilic triblock copolymers poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA) which was synthesized in 2000 (Nardin *et al.* 2000a). OmpF and AqpZ were successfully incorporated into this polymersome (Nardin *et al.* 2000b, Kumar *et al.* 2007), which proved that this polymer had a good biocompatibility. Choi H J (Choi *et al.* 2005) also synthesized another triblock copolymer similar to the PMOXA-PDMS-PMOXA, poly(2-ethyl-2-oxazoline)-b-poly(dimethylsiloxane)-b-poly(2-ethyl-2-oxazoline) (PEtOz-PDMS-PEtOz), which also displayed a good biocompatibility with some membrane proteins (Choi *et al.* 2006). This group of ABA triblock copolymers, composed of a hydrophobic layer (B) set between two hydrophilic layers (A), can form a hydrophilic-hydrophobic-hydrophilic structure similar to a lipid bilayer. However, as for the triblock copolymers, the polymer vesicle has only one layer, which is different from a lipid bilayer. In comparison with the lipid membrane, the permeability and fluidity of membranes constructed by copolymers are lower because of the higher molecular weight, as shown in Figure 2.5. In the present study the membrane fluidity refers to the viscosity of a biological membrane or synthetic biomimetic membrane. These are favourable in some applications which need low permeability and relatively high mechanical strength such as water purification application by biomimetic membranes (Kumar *et al.* 2007) or ion-channel assisted reaction by nanoreactors (Langowska *et al.* 2013).

Figure 2.5. Schematic of membrane properties versus amphiphile molecular weight based on single vesicle measurements. Adapted from (Discher and Eisenberg 2002).

However, generally the thickness of a block copolymer membrane is much higher than that of a lipid membrane. The mismatch between the membrane thickness and membrane protein length may be unfavourable for incorporation of membrane proteins. Nevertheless, it was reported that such insertion was still possible through mean field calculations (Pata and Dan 2003), which is mainly attributed to the compressibility and polydispersity of polymer molecules. It has been widely reported that some membrane proteins can be incorporated into polymersomes successfully and maintain their activities or functions (Nardin *et al.* 2000b, Choi *et al.* 2006, Kumar *et al.* 2007, Langowska *et al.* 2013). These studies indicated that some types of polymersomes were good platforms for membrane proteins. The extensive application of liposomes in incorporating membrane proteins indicates that lipids have good biocompatibility, which is essential for biomimetic research and application. However, compared to liposomes, most polymersomes exhibit poor biocompatibility as only few types of block copolymers have been successfully used to incorporate membrane proteins (Nardin *et al.* 2000b, Kumar *et al.* 2007).

Some methods used for preparing the polymersomes are similar to those for preparing liposomes. The most commonly used solvent free method is the film rehydration method (Reeves and Dowben 1969) that is also commonly used to

prepare liposomes. The polymer is first dissolved in an organic solvent, which is then evaporated to yield a thin and uniform polymer film. Subsequently an aqueous buffer is added to hydrate the film. With strong agitation or long-time stirring, it can yield vesicles with a broad size distribution. In order to get small unilamellar vesicles with uniform size, techniques like sonication, freeze-thawing and extrusion could also be applied. Figure 2.6 shows the effects of different techniques on homogenizing the vesicle solution.

Figure 2.6. Polymersome radii distributions after sonication ($133 \pm 65 \text{ nm}$), sonication plus extrusion ($88 \pm 13 \text{ nm}$), or sonication followed by freeze-thawing plus extrusion ($93 \pm 8.6 \text{ nm}$), as measured by DLS. Adapted from (Lee *et al.* 2001).

Another commonly used method is the solvent displacement method. The block copolymer is first dissolved in an organic solvent (usually ethanol) and then added dropwise to an aqueous solution under vigorous stirring (Kremer *et al.* 1977, Nardin *et al.* 2000a). Polymersomes may have a broad size distribution prepared by this method, but it is fast and relatively easy to prepare. The organic solvent can be removed by low-pressure evaporation. However, the organic solvent is always difficult to be removed completely, which may denature the membrane proteins incorporated.

2.4 SUPPORTED BIOMIMETIC MEMBRANE SYSTEMS AND PREPARATION METHODS

In addition to the liposomes and polymersomes, supported biomimetic membrane systems are also widely constructed to model a biological system. Many approaches have been explored to prepare supported biomimetic membranes. These approaches include the methods for black lipid membranes (BLMs) (Mueller *et al.* 1962, Montal and Mueller 1972) or black polymer membranes (BPMs) preparation (Nardin *et al.* 2000c), for the preparation of supported lipid/polymer bilayers, such as the Langmuir-Blodgett/Langmuir-Schaefer method (Tamm and McConnell 1985, Ross *et al.* 2001), vesicle fusion (Cremer and Boxer 1999, Reviakine and Brisson 2000, Dorn *et al.* 2011) and the spin-coating method (Mennicke and Salditt 2002).

The BLM is the earliest model bilayer system (Mueller *et al.* 1962). A lipid bilayer is formed across a small aperture created in a hydrophobic material. This method can be used to prepare a larger BLM array incorporating membrane proteins. For instance, a perforated ethylene tetrafluoroethylene (ETFE) support was successfully used to prepare multiple BLMs (Hansen *et al.* 2009a, Hansen *et al.* 2009b, Vogel *et al.* 2009). However, these bilayers had poor stability and were easily broken in less than 20 hours, and none of these explorations were extended to the evaluation of the separation performance of the biomimetic membranes in terms of water flux and salt rejection. BPMs could be obtained by a similar method using block polymers (Nardin *et al.* 2000c). The electrochemical experimental results showed that the BPM gave a higher energy barrier for pore formation, indicating a higher mechanical stability of the BPM. The functional groups in the hydrophilic part of the polymer could be crosslinked, giving an even higher stability.

Lipid bilayers supported on planar supports can be prepared by the Langmuir-Blodgett/Langmuir-Schaefer method. In this process, a lipid monolayer is transferred to from the air-water interface a support surface by the Langmuir-Blodgett technique, and then another monolayer can be transferred by the Langmuir-Schaefer method through horizontally dipping the substrate (Tamm and McConnell 1985). However, it is difficult to incorporate membrane proteins into the

lipid bilayer by this method. The Langmuir-Blodgett and Langmuir-Schaefer methods were also employed to prepare supported polymer membranes on gold (Beleguinou *et al.* 2010).

Vesicle fusion onto a solid surface, such as mica, silica or glass, has been studied extensively over the past decades (Sackmann 1996, Tanaka and Sackmann 2005, Castellana and Cremer 2006). The basic characteristics of the substrate that were identified to affect the formation of the supported membrane include the materials, hydrophilicity and surface charge (Jass *et al.* 2000, Richter *et al.* 2006, Rakhmatullina and Meier 2008). However, it was also found that the incorporated proteins in the lipid bilayers may become immobilized after the vesicle fusion on a solid substrate due to the direct interaction between the proteins and the substrate (Salafsky *et al.* 1996). Some approaches had been proposed to try to maintain the activity of the proteins in the supported lipid layers, such as using a soft polymer as a cushion to avoid direct contact of the incorporated proteins from the substrate (Wong *et al.* 1999, Renner *et al.* 2010). For supported polymer membranes, the situation is quite different. As the thickness of a polymer membrane is larger than that of a lipid membrane and there is a mismatch between the polymer membrane and membrane protein, it may help prevent proteins from interacting with substrates. Therefore, it may be possible to directly support the biomimetic membrane incorporated with proteins by a solid support without using cushions. Polymersomes or protein incorporated polymersomes fusion on different substrates was investigated (Rakhmatullina and Meier 2008, Duong *et al.* 2012, Zhong *et al.* 2012). The results indicated that polymersomes showed similar behaviours to liposomes. The polymersomes tended to fuse on a hydrophilic solid surface and form a uniform polymer layer. The surface charge of the substrate is also favourable for the formation of a supported polymer membrane. In contrast to the lipid membrane, the polymer membrane can be anchored on the substrate through some specific chemical reactions (Rakhmatullina *et al.* 2009, Wang *et al.* 2012), attributed to their multiple functionality. However, the fluidity of the resulting membrane was reduced due to the immobilization on the substrate.

Compared to the vesicle fusion and Langmuir-Blodgett/Langmuir-Schaefer methods, the spin-coating method is an efficient way to prepare large areas of supported lipid layers (Mennicke and Salditt 2002, Mennicke *et al.* 2006). An organic lipid solution spreads onto a substrate surface and a lipid layer forms with the evaporation of the volatile organic solvent. It has been reported that there is only a single bilayer left on the support after hydration for a period of time (Simonsen and Bagatolli 2004). Thus, spin-coating combined with hydration may provide a new choice to prepare a supported lipid bilayer. However, it should be pointed out that transmembrane proteins cannot be incorporated into the lipid membrane since an organic solvent is used and moreover, the lipid layer prepared by this method may have many defects (Rappolt *et al.* 2004, Simonsen and Bagatolli 2004).

2.5 INCORPORATION OF MEMBRANE PROTEINS INTO BIOMIMETIC MEMBRANES

Incorporation of membrane proteins into a vesicle has been studied extensively as it can provide not only a useful protocol to study the properties of membrane proteins such as lipid-protein and protein-protein interactions, but also a basis to develop biosensors or other apparatus such as nanoreactors (Nikolelis *et al.* 1999, Graff *et al.* 2001, Langowska *et al.* 2013). Reconstitution of membrane proteins into biomimetic membranes is very important as the reconstitution protocol can directly influence the incorporation efficiency or the activity of the proteins. Here some commonly used strategies for protein reconstitution are introduced.

2.5.1 Strategies for proteins reconstitution into liposomes

➤ *Mechanical methods*

Addition of proteins into lipids during the formation of liposomes has been used for membrane protein reconstitution. It is rapid and no detergent is required. The disadvantages are irreproducibility, yielding small size proteoliposomes (20-40 nm) (Rigaud *et al.* 1995) and significant loss of material. The small size of proteoliposomes may be unfavourable in some applications. The freeze-thawing process was used to facilitate the reconstitution of proteoliposomes (Kasahara and

Hinkle 1976, Kasahara and Hinkle 1977). In this method, liposomes were mixed with proteins and then the mixture went through several freeze-thawing cycles. The freeze-thawing process could facilitate the self-assembly of a lipid bilayer, which helps reconstitute the proteins. However, the freeze-thawing process may also denature the proteins to some degree because of the thermal shock. As generally most membrane proteins are isolated and stabilized by detergents, it cannot absolutely avoid using detergent even by this method and it is necessary to remove the detergents in the subsequent procedures for some applications (Sun *et al.* 2013c).

➤ ***Detergent-mediated reconstitutions***

The most widely employed protocol for proteoliposome reconstitution is detergent-mediated reconstitution. The main reason is that most membrane proteins are isolated and purified from biological membranes using detergents. Moreover, detergents could protect membrane proteins in a benign environment. For this strategy, proteins in detergents are added into liposome solution to form a mixture of lipid-detergent-protein and lipid-detergent aggregation. The detergent in the mixture subsequently is removed gradually, which would result in the formation of proteoliposomes.

Detergents are amphiphilic molecules, which can form micelles in an aqueous solution. Most membrane proteins could be encapsulated in the micelles formed by detergents. In terms of their structures, detergents are divided into four major groups: ionic detergents, non-ionic detergents, bile acid salts and zwitterionic detergents. Their structures and some examples are shown in Figure 2.7.

Figure 2.7. Several types of detergents. CHAPS refers to 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and CHAPSO refers to 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate. Adapted from (Seddon *et al.* 2004).

As detergents have significant effects on the structure or function of membrane proteins, an optimal detergent should be selected to reconstitute the membrane proteins. The choice of detergent for a membrane protein is determined by the application and the membrane protein. Before selecting a detergent, the critical micelle concentration (*cmc*) of the detergent must be determined, which refers to the concentration of detergents above which micelles form and all additional detergents added into the system go to micelles. It is easier to remove the detergent having a high *cmc* while it becomes difficult to remove the detergent with a low *cmc* completely. Therefore, the *cmc* of a detergent determines the method selection for removing the detergent. Most importantly, the detergents used to reconstitute the proteoliposomes should have little effects on the activity of membrane proteins.

There are two common approaches of detergent-mediated reconstitution. The first one is a dilution approach. The lipid-detergent-protein mixture is gradually diluted by a liposome aqueous solution. When the concentration of detergent the *cmc*, the

detergent micelles start to dissociate and then the proteins encapsulated in the micelles transfer into the lipid layer. For the second approach, the detergent is dissolved into a liposome solution and it would make the liposome more permeable or disrupt the liposome into detergent-lipid micelles at a high detergent concentration level. Proteins can be incorporated into the liposome after removing the detergent. The detergent can be removed by several methods as the excess detergent may affect further experimental work.

Various removal methods can be applied to incorporate the membrane protein into a liposome based on the physicochemical properties of detergents. Detergents with a high *cmc* can be easily removed by the dialysis method (Allen *et al.* 1980). The mixture of lipid-detergent-protein was sealed in a tube or bag made of semi-permeable membrane and then dialyzed against a buffer. The pore size of membrane should be much larger than that of detergent but smaller than the membrane protein. The detergent would gradually diffuse into the external buffer through the semi-permeable membrane. Dialysis is a simple and low-cost way, which has been widely used to reconstitute various membrane proteins. However its application is also limited by some disadvantages such as poor reproducibility, time-consuming (~ days) and an uncontrolled rate of dialysis. In addition, the dialysis method is unsuitable for detergents with low *cmc*, which requires a relatively long time to remove most of the detergent. A long-time exposure is not favourable for membrane proteins.

Detergents with low *cmc* can be removed efficiently through polystyrene bead resins such as Bio-beads SM-2. Bio-beads SM-2 is a macroporous divinyl benzene cross-linked polystyrene bead with an average pore diameter of about 9 nm. For the removal of detergents, Bio-beads are added directly to the protein-lipid-detergent mixtures to absorb the detergent gradually. The rate of detergent removal can be easily controlled by controlling the relative amount of beads added to the lipid-protein-detergent mixtures or the time of mixing with beads and the mixture. Its addition into the mixture would not affect the solution attributed to its good chemical stability. The bead can absorb the detergent with a low non-specific adsorption (100-200 times lower adsorptive capacity for lipids and even less for

proteins). The surface area within the pore of a bio-bead is about 10^4 higher than the external surface of the beads and thus it provides a large specific surface area to absorb more small molecules (Rigaud *et al.* 1998). The average pore diameter of the beads is 90 Å while the sizes of micelles are close to or larger than the mean size of bead pore. Therefore, the diffusion of these micelles into the pores of the beads is inhibited because of their big sizes, resulting in a large difference in the adsorption rates of molecule and micelle. This procedure is now widely used and demonstrated to be a successful protocol for reconstitution of various membrane proteins into liposomes.

2.5.2 Strategies for proteins reconstitution into polymersomes

As the block copolymers share many similar properties with lipids, most of the strategies used to reconstitute the proteins into the lipid can also be used for reconstitution of proteopolymersomes. There are two commonly used strategies. One is the organic solvent (usually ethanol) mediated reconstitution: proteins are mixed with a polymer-ethanol solution and then the mixture is slowly added to a specific buffer solution to form a diluted aqueous solution (Nardin *et al.* 2000b). However, it was not reported whether the organic solvent would affect the activity of incorporated membrane proteins. Another method is the direct incorporation of protein during formation of polymersomes: the block copolymer vesicles are prepared by using the film rehydration method. A specific buffer solution solubilising proteins is added to rehydrate the dry polymer film (Kumar *et al.* 2007). Or the buffer solution containing proteins is added to solubilise the polymer powder directly (Choi *et al.* 2006). Proteins are incorporated into the polymersomes during the formation of vesicles. The direct incorporation of proteins into polymer membranes under an electrical potential was also studied (Ho *et al.* 2004, Wong *et al.* 2006). As stated previously, most of membrane proteins are isolated and purified by detergents. For some applications, it is necessary to remove detergents in the subsequent steps. To date there are many reported results of successful membrane protein incorporation into block copolymer membranes: OmpF (Nardin *et al.* 2000b, Langowska *et al.* 2013), maltoporin (Meier *et al.* 2000), LamB (Graff *et al.* 2002), bacteriorhodopsin, ATP synthase (Choi *et al.* 2006) and AqpZ (Kumar *et al.* 2007).

Generally, some integral membrane proteins have orientations with distinct intracellular and extracellular parts, which may be closely related to their distinct biological function. One shortcoming of ABA polymer membranes (and also lipid membranes) is that it is hard to guide membrane proteins with desired orientation into the symmetric artificial membranes. This problem may be solved by synthesizing the ABC block copolymer consisting of three different blocks. It has been demonstrated that this ABC type of block copolymer can guide the proteins to be incorporated with a preferable orientation (Stoenescu *et al.* 2004). However, it is not clear whether the activity of proteins was affected or not. By far the mechanisms of membrane protein incorporation into polymer membranes are still not fully understood.

2.6 CHARACTERIZATION TECHNIQUES

To gain a deep insight into this complicated biomimetic system, characterization techniques are essential to investigate and evaluate it. Some techniques commonly employed to characterize biomimetic membranes are reviewed as follows.

2.6.1 Light Scattering

Light scattering methods are mainly used to study the aggregation of the liposomes or polymersomes in a solution. Static light scattering (SLS) can use the scattered light to measure the weight average molecular weight M_w and the radius of gyration R_g of a particle or polymer molecule. Dynamic light scattering (DLS) can be employed to measure the size distribution of particles or aggregations in a stable suspension. Both methods are extensively employed to study the physical properties of vesicles in suspensions (Murphy 1997, Jin *et al.* 1999, Nardin *et al.* 2000a, Jansson *et al.* 2005). The mechanisms of light scattering are very complicated, and a brief introduction is provided below.

For static light scattering experiments, a laser light is used to detect the particles in a solution and many detectors are used to measure the light scattering intensity at one or many angles. The light scattering is described by the following equation

$$\frac{KC}{R_\theta} = P(\theta) \left[\frac{1}{M_w} + 2A_2C \right] \quad (2.1)$$

where M_w is the sample molecular weight, A_2 is the second Virial coefficient, C is the concentration of sample, $P(\theta)$ is the angular dependence of the sample scattering intensity and K is the optical constant. The Rayleigh factor R_θ can be determined by comparing with a standard reference such as toluene which has a known value of R_θ . However, if the mean size of the particles is much smaller than the wavelength of the laser light, multiple photon scattering will be avoided, known as Rayleigh scattering. Given these conditions, $P(\theta)$ will reduce to 1. M_w will be only related to the concentration C and R_θ . KC/R_θ is proportional to C at one angle. If the data are presented in one graph, this graph is called a Debye plot, which could help calculating the M_w and A_2 .

For DLS, particles in a solution are in constant random movement due to the Brownian motion. The particles move randomly in the solution, inducing intensity fluctuations of the scattered light. These time-dependent fluctuations of the scattered light are monitored. The movement of the particles is dependent on their sizes at a certain temperature and solvent. Small particles move faster than the bigger ones and therefore would reflect more light signals during the same time. A complex signal of scattered light is detected and analyzed by an autocorrelation function. By the autocorrelation function, the diffusion coefficient (D) can be attained, which has a certain relationship with the hydrodynamic radius (R_h) of a particle based on the Stokes-Einstein equation

$$D = \frac{K_b \cdot T}{6 \cdot \pi \cdot \mu \cdot R_h} \quad (2.2)$$

where K_b is the Boltzmann constant, T is temperature [K], μ is viscosity [Pa·s]. The difference between static and dynamic light scattering is mainly in the way the scattered photons are collected by the detector. If it is averaged over a certain period of time, then SLS is performed. If it is correlated with time, then DLS is performed. From SLS, several parameters of particles are attained, such as M_w , R_g , and A_2 . From the DLS, the hydrodynamic radius and translational diffusion coefficient of

the particles can be obtained. The light scattering technique is suitable to evaluate the size or size distribution of a monodispersed sample whereas it is quite difficult to analyze a polydispersed sample because of the mutual interference of multiple signals.

2.6.2 Zeta potential measurement technique

In a biomimetic membrane research, the Zeta potential is a key parameter to evaluate the stability of vesicle solution and analyze the surface properties of vesicles. It is well known that the Zeta potential is the potential difference between the disperse medium and the boundary of the ion diffuse layer around the particle. The particles in suspension carrying a large negative or positive Zeta potential repel each other strongly and the suspension will keep a stable state. However, the particles having a low Zeta potential value will be not very stable and may tend to aggregate. The Zeta potential value can be obtained by measuring the electrophoretic mobility of particles in suspension with an applied electric field. Particles would be driven towards the electrode having the opposite charge and meanwhile their velocity is monitored by laser Doppler velocimetry. The moving particles induced frequency or phase shift of the scattering light, which is related to the moving velocity of particles, is detected. Therefore, any factors affecting the electrophoretic mobility of the particles would affect the Zeta potential, such as pH, conductivity and temperature of the solution.

2.6.3 Transmission electron microscopy and cryogenic-transmission electron microscopy

Both transmission electron microscopy (TEM) and cryogenic-TEM (cryo-TEM) are the most frequently used methods for investigating liposomes or polymersomes in a solution (Almgren *et al.* 2000, Nardin *et al.* 2000a). TEM is applicable for directly observing the size and shape of vesicles attributed to its high resolution. The sample needs to be dried before conducting the TEM characterization. But the vesicle may shrink during the drying process which may change the morphology of the vesicles. Cryo-TEM can prevent this as samples are frozen at an extremely low temperature environment such as liquid nitrogen and they are examined in a hydrated state,

which is closer to their natural states. When a sample is frozen perfectly, the osmotic effects induced by chemicals or dehydration can be prevented. It is reported that these effects may induce particle aggregation or loss of biological materials during the preparation process (Kita-Tokarczyk *et al.* 2005).

2.6.4 Stopped-flow technique

The stopped-flow technique is generally used to study the chemical kinetics of a fast reaction in a solution, different reagents from two syringes are mixed together rapidly in a cell and this cell is irradiated with monochromatic light. The reaction may induce a fluorescence signal or the absorbance at a specific wavelength that could be detected. This technique is widely used in determining reaction rates or protein structures, investigating the reaction mechanisms or drug-binding processes. In biomimetic membrane research, it is employed to measure the permeability of vesicles and thus it can indirectly evaluate the activity of AQPs in vesicles (Borgnia *et al.* 1999b, Kumar *et al.* 2007). The water permeability of vesicles can be measured by rapid mixing hyperosmolar buffer, which contains the same buffer as that in vesicles and some large molecules not able to transport through vesicle walls such as sucrose. The difference between intravesicular and extravesicular would cause water to leave the vesicles. The rate of vesicle volume change can be monitored by light scattering. The relative vesicle radius change ($\Delta R/R_0$) is correlated to the relative light scattering change ($\Delta I/I_0$),

$$\Delta R/R_0 = -\kappa \Delta I/I_0 \quad (2.3)$$

κ is a parameter related to original radius of vesicles (Milon *et al.* 1986). The initial rate constant (k) were obtained by fitting the light scattering data. The osmotic water permeability (P_f) of AQPs was calculated using the following equation (Borgnia *et al.* 1999b, Mallo and Ashby 2006):

$$P_f = \frac{k}{S/V_0 \times V_w \times \Delta_{osm}} \quad (2.4)$$

where S/V_0 is the vesicle surface area to initial volume ratio, V_w is the partial molar volume of water (18 cm³), and Δ_{osm} is the osmolarity difference between the

intravesicular and extravesicular solutions. The permeability of a single AQP molecule can be estimated as the difference in permeability between proteoliposomes and liposomes, and then divided by the number of AQPs in a proteoliposome (Hovijitra *et al.* 2009). In addition to P_f , the diffusive permeability (P_d) is an also very important parameter to evaluate the transport properties of a water channel. Generally a smaller P_f and a higher P_f/P_d are favourable for a water channel because these two desirable parameters imply a single-file structure of narrower channel and thereby achieve a complete selectivity (Jensen and Mouritsen 2006). For example, for AqpZ, it was estimated that $7.7 \leq P_f/P_d \leq 13.0$ and $0.4 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1} \leq P_d \leq 1.3 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$. These two parameters could be estimated through molecular dynamic simulations because of the difficulty of measuring P_d (Xin *et al.* 2011a, Xin *et al.* 2012).

Usually the dead time of a stopped-flow apparatus is 1-2 ms or even less while the dead time refers to the time between the end of mixing the two solutions and the beginning of observing the signal. The shorter the dead time is, the faster the measurable reaction rate will be. The whole operation should be performed under a constant temperature as the temperature may affect the activity of the membrane proteins. The temperature can be adjusted by a temperature controller system.

2.6.5 Atomic force microscopy

Atomic force microscopy (AFM) is a very useful tool to characterize the surface of planar biomimetic membranes (Dufrene and Lee 2000, Berquand *et al.* 2003, Rakhmatullina and Meier 2008, Li *et al.* 2012) or evaluating the mechanical properties of a biomimetic membrane (Wang *et al.* 2011). The morphology of the membrane structure or vesicle deformation can be characterized by direct imaging using AFM. The morphology maps provide a relatively clear image of the surface of planar membranes. The coverage can be evaluated by AFM images qualitatively. The thickness of a planar lipid bilayer can be estimated by a high-resolution AFM (Kaufman *et al.* 2010). The unique capabilities of AFM include: (1) probing the surface morphology of a sample at a high resolution in a dry or hydrated environment and (2) measuring the mechanical properties of a soft layer. Because

the biomimetic membranes are soft samples, the contact mode may be unsuitable for analyzing surface morphology due to the high shear forces. To solve this problem, the tapping mode can be employed since it can minimize the damage to the sample. The hydrated samples can also be characterized by AFM in the liquid phase. Therefore, some biomimetic membranes like supported lipid bilayers or planar polymer membranes can be characterized without any pre-treatments like drying, which may impair the samples. For high-resolution characterization, the AFM needs a smooth surface as a rough surface will easily damage the probe or affect the resolution. Thus, most biomimetic membranes for AFM characterization are usually prepared on a relatively smooth support.

2.6.6 Quartz crystal microbalance with dissipation

A quartz crystal microbalance with dissipation (QCM-D) is an efficient tool to measure the mass deposited onto a surface. The traditional quartz crystal microbalance (QCM) is applied to analyze the mass change on a solid rigid surface. A voltage is applied to a quartz crystal and causes it to oscillate at a specific frequency. The QCM measures a mass deposited by measuring the change in frequency of the quartz crystal resonator. The resonance is affected by the mass deposited on the resonator. Addition or removal of a mass on the sensor surface can cause changes in frequency, which can be described by the Sauerbrey relationship (Kankare 2002)

$$\Delta m = -K_1 \cdot \Delta f \quad (2.5)$$

where Δm is the mass change per unit area; Δf is the change in frequency; K_1 is constant. The negative sign indicates that an increase in mass will produce a decrease in frequency. However, the Sauerbrey relation is only valid for a rigid, uniform and thin film. For a soft or highly viscoelastic film, the Sauerbrey relationship cannot measure the mass accurately and thus another modified relationship is necessary to analyze those deposited materials. Besides the frequency, QCM-D also can measure the dissipation of the quartz crystal. Dissipation D_p is defined as:

$$D_p = \frac{E_{lost}}{2\pi E_{stored}} \quad (2.6)$$

where E_{lost} is the energy lost during one oscillation cycle and E_{stored} is the energy stored in the oscillator. Dissipation measurements provide information on the structural properties of adsorbed film and the quantitative analysis of the mass, shear elastic modulus and viscosity of the deposited materials.

QCM-D has been developed to realize real-time, label-free measurements of molecular adsorption on a solid surface. This technique is a powerful analytical tool that has been widely used to investigate the fusion behaviour of liposomes (Keller and Kasemo 1998, Keller *et al.* 2000, Reimhult *et al.* 2002a, Reimhult *et al.* 2002b, Reimhult *et al.* 2003). QCM-D uses the f and D to determine the mass and viscoelasticity of the adsorbed vesicles or bilayer, respectively. The changes of these two parameters can indicate whether the vesicles have been fused into bilayers or remain intact. The bilayer can be treated as a rigid thin film; hence the mass and viscoelasticity will change slightly when vesicles fuse into bilayers. If the vesicle stays intact upon deposition, the trapped water inside the vesicle will cause substantial changes in the frequency and viscoelasticity. Richter *et al.* investigated the fusion behaviour of liposomes on a solid surface systematically and found several typical fusion behaviours of vesicles fusion on solid surfaces (Richter *et al.* 2003). Under some conditions, vesicles tend to directly fuse to bilayers or monolayers on the solid surface. However, under other conditions, vesicles would not fuse to bilayers until reaching a critical vesicular coverage. If the affinity force between the vesicles and substrate is not strong enough, vesicles would keep intact on a solid surface instead of fusing to bilayers. A series of QCM-D experimental results showed that many factors affect the fusion behaviour of a vesicle, such as electrostatic interaction, solid support, divalent ions and vesicle size (Reimhult *et al.* 2002b, Richter *et al.* 2006).

2.6.7 Fluorescence microscopy and fluorescence recovery after photobleaching

Fluorescence microscopy is widely used to image the vesicles or planar supported lipid membranes (Berquand *et al.* 2003, Kaufman *et al.* 2010, Li *et al.* 2012). It can

provide a qualitative two-dimensional image of a supported lipid membrane. Three-dimensional images can be attained by some fluorescence microscopy instruments. There are several types of fluorescence microscopy widely used, including wide-field fluorescence microscopy, total internal reflection fluorescence microscopy, and confocal fluorescence microscopy. For fluorescence microscopy, the excitation light irradiates a labelled lipid membrane and the fluorescent light is emitted which can be separated and detected. In most cases, some specific dyes are needed to label the biomimetic membranes because the lipids do not exhibit intrinsic fluorescence. Normally, the dye is functionalized lipids where headgroup or fatty acid is functionalized with a fluorescent function group. These dyes could be mixed with lipids and form the liposomes together. So far, there is no suitable synthetic fluorescent polymer to stain block copolymers.

The fluorescence recovery after photobleaching (FRAP) technique has been widely employed to analyzing the lateral mobility of labelled probes. When a FRAP is performed, a small area of sample is photobleached by an intense focused laser excitation in a very short time and the fluorescent of the bleached area would disappear. However, it would recover over time because of fluorescent lipids diffusion. The recovery of the photobleached region is recorded by the fluorescence intensity with a low laser power over a certain time. Generally a similar region without photobleaching but near the photobleached area is selected as a reference. Analysis of the FRAP curve could be performed with a standard diffusion model (Axelrod *et al.* 1976). The lateral mobility of a biomimetic membrane is a critical parameter as it could influence the function of membrane proteins to some degree.

2.6.8 Scanning electron microscopy

Scanning electron microscopy (SEM) is widely employed to investigate the topography and composition of samples. Different from many other microscopes, the SEM instrument use electrons to detect the images instead of using visible light. SEM can get very high-resolution images of a sample an average of nanometers. SEM uses an electron gun under a high voltage to produce an electron beam. The beam would hit the specimen and produce many types of signals. Of these signals, secondary electrons and backscattered electrons are commonly employed to

characterize the sample. Secondary electrons are produced when the high energy electron excites atoms of samples and causes ejection of some electrons outside of the nucleus. Some of the ejected electrons will move toward to sample surface. Those carrying sufficient energy will emerge from the surface of sample and be detected to provide the sample information. Because of their low energy, only those secondary electrons which are near the surface ($< 10\text{nm}$) can escape from the sample and be detected. The production of secondary electrons is correlated to the topography of the samples. Any subtle change in topography of the sample would affect the generation of secondary electrons. As for the back-scattered electrons, they are emitted from the primary beam and interact with the atoms of samples elastically. The number of back-scattered electrons detected depends on the atomic number of the elements. Therefore, the SEM is also widely used to analyze the element information of samples.

However, the SEM technique is not suitable for characterizing vesicles or membranes in a hydrated state. Nevertheless, the SEM is an efficient tool to explore the structure of an AQP-based membrane. It was found those bilayers or vesicles in the membranes could still maintain their structures to some degree in a fully dry state (Zhao *et al.* 2012, Zhong *et al.* 2012, Xie *et al.* 2013). These results indicated that the SEM was, to some extent, suitable to confirm the existence of the bilayer or vesicles after these vesicles were fused to bilayers (Zhong *et al.* 2012) or immobilized on membrane surface (Sun *et al.* 2013a, Sun *et al.* 2013c, Xie *et al.* 2013) or embedded in a polymer layer (Zhao *et al.* 2012). However, since these samples were all dried prior to characterization, the water encapsulated in the vesicles was totally evaporated, which could induce the change of vesicle morphology. The conventional SEM technique may not be able to obtain some original information of bilayers or vesicles at hydrated state such as thickness, size or morphology. However, currently a new type of SEM, environmental scanning electron microscope (ESEM), allows characterizing the sample in a hydrated state or liquid due to its special design. Nevertheless, the application of ESEM is limited because of its high cost and relative low resolution compared to conventional SEM (Chan and Chen 2004, Patterson *et al.* 2009).

2.6.9 Other characterization techniques

In addition to the characterization techniques stated above, there are some other techniques always used to characterize the biomimetic membrane. For example, ultraviolet light, Fourier transform infrared spectroscopy and nuclear magnetic resonance are widely used to analyze the concentration and structures of AQPs or to characterize the composition of polymer or lipid vesicles after synthesis or modification (Nardin *et al.* 2000a, Rakhmatullina *et al.* 2007, Rakhmatullina *et al.* 2009). Gel-size exclusion chromatography and the electrokinetic analyzer are powerful tools to examine the pore size and surface charge of the membrane substrate for biomimetic membranes.

2.7 STATE-OF-THE-ART AND CHALLENGES OF AQP-BASED BIOMIMETIC MEMBRANES

Ever since the proof-of-concept for use of AQPs for desalination applications (Kumar *et al.* 2007), a surge to fabricate a high performance membrane based on AQP has been emerging. At the early stage, people proposed one straightforward protocol to design an AQP-based membrane (Kaufman *et al.* 2010, Li *et al.* 2012): proteoliposomes incorporated with AQPs directly fused to a nanofiltration membrane to form a dense layer. It was proposed that this dense layer could function as a salt-rejection layer, meanwhile the incorporated AQPs could facilitate the water transport driven by an applied pressure. The nanofiltration membrane was selected to be the substrate because of its relative high permeability and low surface roughness which allows for a minimal distortion of the lipid bilayer (Pendergast and Hoek 2011). In addition, its small pores might ensure this thin planar lipid bilayer could be kept intact even under an external force. Wang *et al.* (Wang *et al.* 2012) reported a biomimetic membrane prepared by AQPs using the method of vesicle fusion on a track-etched polycarbonate membrane. A covalent-conjugation-driven reaction promoted the fusion of ABA block copolymer vesicles with AQPs. Due to the extremely thin polymer layer or the support with big pore sizes, the membrane performance was only evaluated in a forward osmosis (FO) mode to prevent the formation of defects induced by applied force. It was reported that this membrane

presented quite good FO performance, in comparison with the conventional FO membranes. Zhong *et al.* (Zhong *et al.* 2012) reported an AQP-based membrane prepared by fusing AQP-incorporated polymersomes on a silanization-modified ultrafiltration cellulose acetate membrane. Silanization modifications made the cellulose acetate membrane become more hydrophobic, which initiated vesicle rupture and formed a single layer on a substrate surface. Subsequently a multilayer (~100 nm) was formed on top by vesicle fusion. At a high protein-to-lipid ratio, this membrane gave a fairly good water flux and salt rejection, in a range of nanofiltration performance. Duong *et al.* (Duong *et al.* 2012) prepared another AQP-based membrane in a similar way. AQP-incorporated polymersomes were fused on a modified microfiltration membrane made of alumina to form a selective layer, which also gave good nanofiltration performance. Their results indicated that the selective layers of both membranes supported on an ultrafiltration or microfiltration substrate could still maintain their intact structures even under a 5-bar pressure, showing a good mechanical strength, which exceeded the previous expectation. However, although these membranes showed some interesting results and good performance, it was very difficult to scale up because of the ease of defect formation during vesicle fusion, especially in a sizeable membrane.

Sun *et al.* (Sun *et al.* 2013c) proposed another protocol to prepare AQP-based biomimetic membranes: proteoliposomes were immobilized on the membrane surface first and subsequently conjugated by a polydopamine layer. In contrast to the vesicle fusion protocol, the vesicles with AQPs were kept intact on membrane instead of being fused to a planar layer on the substrate surface. These immobilized vesicles were sealed by a polydopamine layer, which could function as a salt-rejection layer. This layer could be constructed by other chemicals that could display a good salt rejection, such as polyelectrolytes by layer-by-layer, as reported by Sun *et al.* (Sun *et al.* 2013b). Wang *et al.* (Wang *et al.* 2013) immobilized the crosslinked proteopolymersomes on a membrane substrate and used a layer-by-layer polydopamine-histidine coating process to seal these vesicles. Stabilizing these vesicles on the substrate surface is a critical step to prepare this membrane by this type of protocol. Conjugation reactions or electrostatic forces through modifying vesicles and substrates have been used to stabilize the vesicles. It was

also reported a surface imprinting method was employed to stabilize the proteopolymersomes on substrate (Xie *et al.* 2013). The amount of immobilized vesicles on the substrate determined the performance of prepared membrane as more AQPs could produce a higher water flux. Sun *et al.* (Sun *et al.* 2013a) managed to encapsulate magnetic particles into the proteoliposome and more proteoliposomes tended to move onto the substrate surface under a magnetic field. The water flux of resultant membrane with more AQPs increased up to 70% than that of control membrane. All membranes prepared by this protocol were nanofiltration-type membranes. Although some of these membranes showed relative good separation performance, it appeared it was still difficult to prevent the formation of defects during preparation. This is probably the main reason why all these membranes were prepared in an extremely small area ($< 1\text{cm}^2$).

Zhao *et al.* (Zhao *et al.* 2012) proposed another different protocol to prepare AQP-based biomimetic membranes: the proteoliposome was introduced into the polyamide layer of a thin-film composite membrane during interfacial polymerization on a flat-sheet membrane. The results showed that the introduced proteoliposomes were completely embedded into the polyamide layer. Compared to the control group, the flux of this membrane had increased by 20% and its salt rejection was improved as well. Additionally, it could be obtained a large membrane area by this protocol. This work demonstrated the feasibility of the utilization of AQPs into RO membranes in the future.

Over the past two years there have been more than ten papers published to introduce how to prepare a high performance AQP-based membrane, and many promising results have been obtained. Although there are still some concerns about the stability or commercial viability of the AQP-based membrane (Pendergast and Hoek 2011), it is believed that the research on bio-inspired membranes may lead to a revolution in membrane technology in the near future. However, to obtain a biomimetic membrane competitive with current conventional RO membranes is not an easy work. The concept of AQPs for water purification was proposed on 2007. There are still many unsolved or unexpected questions encountered during exploring this biomimetic system because of lacking adequate fundamental

understanding in this field. For example, it was found that there was an optimal ratio of AQPs in vesicles for the highest permeability (P_f) and the proteoliposome had quite different properties from the liposome. Such problems need further investigation. In addition, there are some well-recognized challenges for preparing a biomimetic membrane. Firstly, the AQPs are very sensitive to in-vitro environment and need a suitable biomimetic platform (a lipid or polymer layer) to maintain their activity. Secondly, this biomimetic membrane needs a good integration with a robust substrate which could supply a strong mechanical strength for water filtration application. In conclusion, there are three components (AQPs, lipids or polymers, substrate) and two processes (reconstitution of AQPs, integration of AQPs and substrate) critical for obtaining an acceptable biomimetic membrane. These important factors are schematically described in Figure 2.8.

Figure 2.8. Critical steps for preparing an AQP-based biomimetic membrane.

CHAPTER 3 PREPARATION OF SUPPORTED LIPID MEMBRANES FOR AQUAPORIN INCORPORATION

3.1 INTRODUCTION

Based on the literature review conducted in Chapter 2, it appears that, for the preparation of aquaporin (AQP) biomimetic membranes, AQPs have to be embedded in lipid bilayers or block copolymers that have a similar amphiphilic structure to biological membranes to form vesicles or films (Borgnia *et al.* 1999b, Kumar *et al.* 2007). Due to their thin thickness and poor stability, these AQP embedded vesicles or films need to be integrated with a robust water permeable support that provides mechanical strength in a practical configuration such as a flat sheet for applications. Inspired by the structure of reverse osmosis (RO) membranes, which normally have a thin dense selective layer supported by a porous substrate on a nonwoven fabric, AQPs could be incorporated into the top thin layer, or an AQP embedded film could be used as the selective layer on a porous substrate, and tested in a pressure driven mode. This system is similar to the supported biomimetic membrane system as reviewed in Chapter 2.4. There are many approaches to prepare the supported membrane. As for an AQP-based membrane supported on a porous structure, vesicle fusion is one commonly used protocol because of two main reasons: (i) it is very simple to operate and handle during the whole preparation process; (ii) it is relatively easy to incorporate AQPs into vesicles and meanwhile maintain the activity of AQPs in a biocompatible environment.

Kaufman *et al.* (Kaufman *et al.* 2010) prepared supported lipid bilayers (without AQPs) on commercial nanofiltration (NF) polymeric membranes by the vesicle fusion method in a feasibility study. It was found that a high lipid bilayer coverage (~97%) could be achieved at a very low pH value (pH 2) on a nanofiltration membrane, NTR-7450. While Kaufman and co-workers' work has provided important insights into vesicle fusion onto membrane substrates, they did not investigate the biocompatibility of the lipid with AQPs. For example, a low pH

environment may be harmful for AQPs. In addition, the NTR-7450 membrane may be not suitable for preparing supported lipid membranes as its hydrophobic surface is unfavourable to the hydrophilic liposome shells (Boussu *et al.* 2007).

In this chapter we reported on how to prepare a supported lipid layer on a water permeable substrate---NF-270 membrane under benign conditions suitable for AQPs. Two methods were compared: one was vesicle fusion on a virgin NF-270 membrane directly in a biocompatible environment, and the other was vesicle fusion on a modified NF-270 membrane that was spin-coated with positively charged lipids. The modification was expected to change the surface properties of the NF-270 membrane to facilitate vesicle fusion. Further trials were performed to incorporate AqpZ into the lipid bilayer to mimic the biological membranes.

3.2 EXPERIMENTAL

3.2.1 Chemicals and materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) dissolved in chloroform were purchased from Avanti Polar Lipids (Alabaster, USA). N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY® FL, SE) were purchased from Invitrogen (*Singapore*). The structures of these chemicals are shown in Figure 3.1. Sodium chloride, monopotassium phosphate, potassium chloride, ethanol, and n-hexane were purchased from Merck Chemical (*Singapore*). Disodium phosphate was purchased from Aik Moh Paints & Chemicals (*Singapore*). Detergent 1-n-octyl- β -D-glucopyranoside (OG) was purchased from Calbiochem® (*Singapore*). Sodium dodecyl sulphate (SDS) was purchased from Bio-Rad (*Singapore*). All other chemicals or materials were purchased from Sigma-Aldrich (*Singapore*) unless stated otherwise. All chemicals were used without further purification. Milli-Q water (*Millipore, integrated ultrapure water system*) with a resistivity of 18.2 M Ω •cm was used. A phosphate buffer with a pH value of 7.8 was prepared with 2.68 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄. The

ratios mentioned in the the present study are in molar fraction unless otherwise stated. Flat sheet samples of NF-270 membrane were provided by Dow Filmtec (*Minnesota, USA*). The top layer of the NF-270 membrane is made of semi-aromatic poly(piperazinamide) (Tang *et al.* 2009a). This layer was supported on a polysulfone microporous membrane reinforced with a non-woven fabric. The contact angle of the membrane is around 30° and its surface is negatively charged at neutral pH conditions (Boussu *et al.* 2006, Boussu *et al.* 2007). The average surface roughness of typical NF-270 membranes is around 3 nm (Boussu *et al.* 2007).

Figure 3.1. Chemical structures of DOPC, DOTAP, NBD-PE and BODIPY ® FL.

3.2.2 Preparation of liposome solutions

1.8 mmol of DOPC in chloroform was first dried in a N₂ stream and then vacuumed overnight. The dried lipid film was hydrated in phosphate buffer. After agitating for 10 min and three freeze-thawing cycles, the final solution was then extruded 21 times through a polycarbonate membrane with a mean pore size of 200 nm to get a liposome solution with a low polydispersity index (PDI). The final concentration of the DOPC vesicle solution was 1.5 mM. The liposome size and Zeta potential were measured by a Zetasizer Nano ZS (*Malvern, UK*) at room temperature of 22°C. The

DOPC liposome Z-average diameter was 151.8 nm (PDI =0.127) and the Zeta potential was around -1.22 mv in the phosphate buffer. The same procedure was used to prepare a 1.5 mM DOPC and DOTAP (in a mole ratio of 80% to 20%) mixed liposome solution in phosphate buffer, whose Z-average size was 141.2 nm (PDI=0.108) and the Zeta potential was around 40 mv. All solutions were used within 12 hours.

3.2.3 Incorporation of aquaporin Z into DOPC liposomes

A certain amount of the aquaporin Z (AqpZ) solution was added into a 1.5 mM DOPC liposomes phosphate buffer containing 1% OG in terms of a protein-to-lipid molar ratio (PLR) of 1/200. The solution was dialyzed in a 12000-14000 Da molecular weight cut-off (MWCO) dialysis bag (*Spectrum Labs*) against 3 L phosphate buffer for 3 days at room temperature. The buffer was refreshed every day. After dialysis, the proteoliposome solution was extruded through a polycarbonate membrane with a mean pore size of 200 nm for further use. The size of the proteoliposome was measured by a Zetasizer Nano ZS, which showed a Z-average size of 104.5 nm (PDI=0.160) and a Zeta potential of -12.5 mv for proteoliposomes with PLR 1/200; and a Z-average size of 111.3 nm (PDI=0.126) and a Zeta potential of -4.4 mv for proteoliposomes with PLR 1/800. An amount of positively charged lipid DOTAP was added to the lipids to investigate its charge effect on the proteoliposomes. The Zeta potentials were 26 mv and 34.8 mv for proteoliposomes (DOPC/DOTAP=80/20, mole/mole) with PLR 1/200 and 1/800, respectively.

3.2.4 Pretreatment and modification of NF-270 membrane substrates

NF-270 membrane has a relative small pore size but reasonably high water permeability (Boussu *et al.* 2007). Its high permeability, hydrophilic and smooth surface make it suitable for the substrate of a biomimetic membrane. NF-270 membranes were soaked in a 50% (v/v) ethanol/water solution for 1.5 h and then ultrasonicated for 2 min. After that, the membranes were rinsed with pure water and then soaked in pure water for at least one day before use.

The NF-270 membrane was modified by DOTAP. 1.5 mmol of DOTAP in chloroform was first dried in a N₂ stream and then vacuumed for several hours. After drying, a dried lipid film was spread on the surface of vial. This dried lipid film may be constructed by a sequence of normal bilayers resting on the support (Simonsen and Bagatolli 2004). The dried lipid film was dissolved in n-hexane to give a solution of 1.5 mM DOTAP hexane solution. 0.4 ml DOTAP hexane solution was then spin-coated onto a NF-270 membrane surface with a Spin 3000D spin coater (*E-flex, South Korea*). The membrane area was 28.26 cm². The spin-coating procedure involved the initial step of 60 rpm rotation for 15 s followed by 100 rpm rotation for 1 min. The low rotating speed was used to try to make the lipid fully cover the membrane surface. The coated membrane was then put in a chamber with 95~96% humidity for 3 h to inhibit the evaporation of water in the membrane. The modified membrane was spin-coated again with the same lipid solution by the same procedure in order to ensure a full coverage of the membrane surface. Then the membrane was put in a chamber with 95~96% humidity for 6 h to evaporate the n-hexane from the lipid multilayers. The modified membrane was then soaked in a phosphate buffered saline (PBS) solution for 15 h before further use.

3.2.5 Vesicle fusion on substrates

3 ml of 1.5 mM DOPC and DOTAP mixed liposomes in a phosphate buffer was spread onto an unmodified NF-270 membrane (12.57 cm²) and incubated at room temperature for 3 h. After fusion, a pipette was used to take the residual liposome solution away and pure water was carefully injected to rinse the membrane several times. The supported lipid layer prepared by this method was designated as lipid membrane #1.

For vesicle fusion on the modified NF-270 membrane surface, 4 ml 1.5 mM DOPC was spread on the hydrated NF-270 membrane modified with lipids (28.26 cm²) for 1 h under -500 mbar pressure. Some of the solution could transport through the membrane under this pressure, which would cause an increase in ionic strength on the membrane surface. In order to minimize this effect, the liposome solution was removed after the first hour of fusion, refilled to 4 ml by the phosphate buffer and spread on the membrane again for another 1 hour of fusion under -500 mbar. After

deposition, the membrane was rinsed following the same procedure as described previously. The resultant membrane was designed as the lipid membrane #2.

For the proteoliposome fusion on the modified NF-270 membrane, 4 ml proteoliposome solution was fused onto the modified NF-270 membrane (28.26 cm²) following the same procedure as described for lipid membrane #2. The resultant membrane was designated as lipid-AQPs membrane.

3.2.6 Water flux & rejection measurements and characterization

In the current study, the water flux and salt rejection of each membrane were measured before use. The effective area of the membrane was 12.57 cm². The measurements were performed in a batch stirred cell for about 1.5 h using a 1 mM NaCl solution at 1 bar and 22°C. The surface Zeta potential measurements of the membranes were performed with an electrokinetic analyzer (*SurPass, Anton Paar GmbH, Graz, Austria*) in a 10 mM NaCl solution.

Fluorescence images were obtained in a LSM 710 system (*Carl Zeiss, Germany*) with a 10× objective using the 488 nm excitation line. For fluorescence characterization, the lipids were mixed with 1% BODIPY dye (1:100, mole/mole) in chloroform before liposome preparation. Fluorescence recovery after photobleaching (FRAP) was performed in the same instrument with a 63× objective. Before FRAP characterizations, flux measurements were carried out for all samples. The 488 nm excitation line was used for FRAP bleaching at 100% intensity to obtain images at 0.4% intensity. A 11.84 μm² area was bleached and the same fluorescence area was chosen as a reference. For fluorescence and FRAP characterizations, the lipid was mixed with 1% NBD-PE (99:1, mole/mole) in all experiments. Atomic force microscopy (AFM) images were obtained at room temperature by an atomic force microscope in the non-contact mode (*XE-100, Park Systems*). Surface roughness values were attained from the AFM image processing and analysis program provided by Park systems. Before AFM characterization, samples were dried in desiccators for at least 24 h. The permeabilities of the liposomes and proteoliposomes were analyzed using a stopped-flow apparatus

(SX20, *Applied Photophysics*) at 22°C. Light scattering measurement was done with an emission line wavelength of 500 nm.

3.3 RESULTS AND DISCUSSION

3.3.1 Vesicle fusion on virgin NF-270 membrane

The electrostatic force is considered as the main driving force for lipid bilayer formation by vesicle fusion (Richter *et al.* 2006, Renner *et al.* 2008). As it was reported that the NF-270 membrane carried a relatively strong negative charge around a neutral pH value, the positively charged DOPC and DOTAP mixed liposome was used to fuse onto the membrane surface. Figures 3.2A-3.2C showed the AFM topography images of the virgin NF-270 membrane, lipid membrane #1 and lipid membrane #1 after flux measurement, respectively. Although the NF-270 membrane had a relatively smooth surface, it is not comparable with mica or glass (Brant *et al.* 2006, Seu *et al.* 2007), as shown in Figure 3.2A. However, after vesicle deposition on the membrane surface, it was observed that there were many protrusions on the membrane surface, shown in Figure 3.2B, indicating that most of those deposited vesicles did not completely fuse to bilayers on the membrane surface after a 3-hour fusion. However, after flux measurement at 1 bar for 1.5 hours, those observed protrusions disappeared and the membrane surface became smoother (Figure 3.2C).

Figure 3.2. AFM topography images of (A) virgin NF-270 membrane; (B) lipid membrane #1; (C) lipid membrane #1 after flux measurement. (area: 500 nm x 500 nm)

It was probable that the most of unfused vesicles fused to bilayers completely under the applied pressure. In order to confirm the existence of lipids on the membrane after flux measurement, a confocal image of lipid membrane #1 after flux measurement is shown in Figure 3.3. It was quite evident that the lipids had fully covered the membrane surface.

Figure 3.3. Confocal fluorescence images of NF-270 membrane without lipid layers (left image) and labeled lipid membrane #1 after flux measurement (right image). (Scale bar 200 μm)

For the supported lipid bilayers prepared by the vesicle fusion method, the transformation from vesicle to lipid bilayer on a hydrophilic, smooth and highly charged solid surface such as silica and mica could be completed in several minutes (Richter *et al.* 2003, Richter and Brisson 2005), while it would take a much longer time on a polymer surface, up to several hours in some cases (Tang *et al.* 2009b, Kaufman *et al.* 2010). According to the present experimental results, it was difficult to get a bilayer structure on the NF-270 membrane surface without an extra force. This was probably mainly due to the much lower surface charge density of the polymer than that of mica or silica (Boussu *et al.* 2007, Zimmermann *et al.* 2009). Nevertheless, it revealed that the applied hydraulic pressure during the permeation test could facilitate the fusion of vesicles, i.e., help to transform unfused vesicles to lipid bilayers.

Figure 3.4 showed the water flux and salt rejection of the lipid membrane #1 along with the virgin NF-270 membrane. During the test, the water flux of the lipid membrane #1 exhibited a relatively steady value even under stirring (~ 200 rpm),

suggesting a good stability of the lipid layer. However, the water flux only dropped ~46% compared to that of the virgin NF-270 membrane. Based on theoretical calculations (Kaufman *et al.* 2010), the water permeability of membrane (P) could be derived from the osmotic permeability (P_f) by using the following equation,

$$P = \frac{P_f V_w}{RT} \quad (3.1)$$

where V_w is the molar volume of water. As the osmotic permeabilities of most liposomes were less than 20×10^{-6} m/s (Mathai *et al.* 2008b), it could be derived that the water flux should be very low (< 1 LMH/bar) if the lipid layer, which is almost impermeable to water (Kaufman *et al.* 2010), has fully covered the substrate. The higher water flux suggested the existence of defects in the lipid layer. One reason for this may be the hydrophilicity of the NF-270 membrane. It is well known that hydrophilic membranes exhibit good anti-fouling tendencies to hydrophobic organic matter (Freger *et al.* 2002, Manttari *et al.* 2004). As an amphiphilic molecule, a lipid has a much longer hydrophobic “tail” than a hydrophilic “head”. This implies that the lipid may prefer to attach on a hydrophobic surface under the same conditions. A similar phenomenon was also found in a nanofiltration process of surfactants (Cornelis *et al.* 2005) and the polymer vesicle fusion process (Rakhmatullina and Meier 2008). During the vesicle fusion process, the electrostatic attraction force and hydrophobic repulsion may compete against each other, which might influence the degree of coverage of the lipid on the NF-270 membrane.

Figure 3.4. Fluxes and rejections of virgin NF-270 membrane and lipid membrane #1. (Dead-end filtration test, applied pressure: 1 bar; feed solution: 1 mM NaCl solution. Error bars were derived from three independent experiments.)

Another consideration is the relatively rough membrane surface that might affect the bilayer continuity and also the bilayer spreading during fusion (Nissen *et al.* 1999), as the lipids tend to self-assemble together by relatively weak hydration, hydrophobic and van der. waals forces. The scale of the ridge-and-valley structures of the NF-270 membrane (Figure 3.2A) is very close to the scale of a lipid bilayer thickness (4~8 nm) (Jass *et al.* 2000, Mathai *et al.* 2008a), suggesting the possibility that these ridge-and-valley structures might affect the unity of the lipid layer. Thus, surface modification of the NF-270 membrane was proposed and performed, aiming at enhancing the compatibility of the lipid with the support surface, meanwhile the surface modification should not impose a significant mass transfer resistance to the NF-270 membrane substrate.

3.3.2 Modified NF-270 membrane by spin-coating

Charged DOTAP lipids were introduced to modify the surface of the NF-270 membrane by a spin-coating process. Then the modified membrane was soaked in the PBS buffer solution for hydration. Figure 3.5 depicts the pure water flux of modified NF-270 membrane as a function of hydration time.

Figure 3.5. Pure water fluxes of virgin membrane and detergent washed membrane, and flux change of the modified NF-270 membrane with a soaking time in PBS buffer (Dead-end filtration test, applied pressure: 1 bar; feed solution: pure water; error bars were obtained from three independent measurements).

After an 18 hour hydration, the water flux tended to be constant, but the flux at the 24th hour was a bit lower than the flux of the virgin membrane (11.9 LMH vs. 16.9 LMH). It suggested that some lipids were still left on the membrane surface. Normally, one defect-free bilayer should impose a high resistance to the water permeation based on theoretical calculation and experiment (Kaufman *et al.* 2010). However, the experiment results showed a high pure water flux for the lipid modified membrane, suggesting the partial coverage of the lipid on the membrane. In order to make sure that the spin-coating process has little effect on the NF membrane substrate, the water flux and salt rejection measurements were carried out again after the modified membrane was soaked in a 4 g/L SDS solution for 2 hours to wash off the lipid from the membrane. It was found that the water flux of the detergent-washed membrane changed little as compared to the virgin membrane, as shown in Figure 3.5. Besides, the salt rejection was also changed little, ~40% for the detergent-washed membranes compared to the ~46% for the virgin membranes.

3.3.3 Vesicle fusion on modified NF-270 membranes by spin-coating

It was reported that charged vesicles could attach on the oppositely charged supported lipid layer, and finally the parts of the vesicles could fuse into the

supported lipid bilayer to form a mixed lipid bilayer through lipid transfer and diffusion (Wikstrom *et al.* 2008, Kunze *et al.* 2009). Since the surface of the modified NF-270 membrane was covered by positively charged lipids, the lipids used for vesicle fusion should be negatively charged or zwitterionic lipids. DOPC was chosen as it is a type of zwitterionic lipids which carries slightly negative charges in the phosphate buffer. Suction was introduced to facilitate the attachment of the vesicles on the supported lipid layer.

Figure 3.6A shows that fluorescence labelled DOPC attached on the modified NF-270 membrane. Similar to the one prepared by the vesicle fusion directly on the virgin NF-270 membrane (Figure 3.3), the lipid fully covered the membrane surface uniformly. Moreover, the AFM image (Figure 3.6B) shows that the NF membrane surface was covered by a flat lipid membrane which is quite similar to lipid membrane #1 (Figure 3.2C).

Figure 3.6. (A) Confocal fluorescence image (A) and AFM image (B) of lipid membrane #2 after flux measurement. (area: 500 nm x500 nm)

Figure 3.7 gives the flux and rejection measurement results of the lipid membrane #2. The flux of the lipid membrane #2 dropped by 78% compared to that of the virgin membrane. It is also much lower than its counterpart of the lipid membrane #1, suggesting a better coverage of the lipid layers on the membrane surface.

However, the salt rejection of the lipid membrane #2 also decreased, lower than the virgin membrane and the lipid membrane #1.

Figure 3.7. Fluxes and rejections of the virgin NF-270 membrane and lipid membrane #2. (Dead-end filtration test, applied pressure: 1 bar; feed solution: 1 mM NaCl solution. Error bars were derived from three independent experiments.)

The relationship governing the water flux and membrane resistance can be described as

$$J_v = \frac{\Delta P - \Delta \pi}{\mu(R_m + R_c)} \quad (3.2)$$

where J_v is the water flux, ΔP is hydrostatic pressure difference across the membrane, $\Delta \pi$ is the osmotic pressure, μ is the viscosity of water, R_m and R_c are the resistance of the membrane and the foulant layer, respectively. Here a low concentration of the salt solution (1 mM NaCl) and strong stirring were used to minimize the effect of osmotic pressure. Therefore, J_v is mainly determined by resistance of the NF-270 membrane and the lipid layer. The experiments showed that the flux of the lipid membrane #1 dropped about 46% relative to the virgin membrane flux. It indicated that the resistance of the lipid layer was about 85% of the resistance of the virgin membrane. In contrast, the resistance of the lipid layer of the lipid membrane #2 is up to 3.6 times that of the virgin membrane. Obviously, the lipid membrane #2 is much denser compared to the lipid membrane #1.

As it was widely reported that the rejection of NF membrane was closely related to the surface charge of membrane (Rautenbach and Gröschl 1990, Wang *et al.* 1997, Childress and Elimelech 2000), the primary reason for the decreased rejection may be due to the decreased surface charge of membrane after modification because of the neutralization by oppositely charged lipids, leading to a drop in the salt rejection. This is supported by characterization of the surface Zeta potential shown in Table 3.1. The results also indicated that lipid membrane #2 had a higher density of lipids than lipid membrane #1 as the charge density of lipid membrane #2 was much higher at the same pH value.

Table 3.1. Zeta potentials of membranes at different pH values

		Pretreated NF 270 virgin membrane	Lipid membrane #1	Lipid membrane #2
Zeta potential (mv)	pH 5	-27.6	-11.8	13.2
	pH 5.6	-42.3	-21.8	4.6
	pH 7	-53.4	-39.2	-14.7

For a NF membrane, the water flux J_v and salt flux J_s can be written as

$$J_v = A(\Delta P - \Delta \pi) = (\Delta P - \Delta \pi) / \frac{1}{A} \quad (3.3)$$

and

$$J_s = B \Delta C = \Delta C / \frac{1}{B} \quad (3.4)$$

where A and B are the water and salt permeabilities, respectively; and both A and B are closely related to the lipid bilayer coverage on NF membrane surface. ΔC is the salt concentration gradient across the membrane. The rejection r is determined by A , B , ΔP and $\Delta \pi$

$$r = \left\{ 1 + \frac{B}{A(\Delta P - \Delta \pi)} \right\}^{-1} \quad (3.5)$$

If ΔP is kept constant in each experiment and $\Delta\pi$ is negligible, r is determined only by B/A . It is reasonable to assume that the water and salt transfer resistances consist of the contributions from the lipid layer and supported membrane. According to the resistance-in-series model, it can be derived that

$$\frac{1}{A} = \frac{1}{A_1} + \frac{1}{A_2} \quad (3.6)$$

and

$$\frac{1}{B} = \frac{1}{B_1} + \frac{1}{B_2} \quad (3.7)$$

where subscripts 1 and 2 refer to the lipid layer and the support layers, respectively. Combining these two equations, one can write:

$$\frac{B}{A} = \frac{B_1 B_2}{A_1 A_2} \cdot \frac{A_1 + A_2}{B_1 + B_2} \quad (3.8)$$

The experiment results indicated that the water transfer resistance of the lipid layer ($1/A_1$) was much higher than that of support NF-270 membrane ($1/A_2$). When comparing the B/A values of the virgin membrane and the lipid membrane, the following equation could be obtained

$$\frac{B}{A} / \frac{B_2}{A_2} = \frac{B_1}{A_1} \cdot \frac{A_1 + A_2}{B_1 + B_2} = \frac{A_1 B_1 + B_1 A_2}{A_1 B_1 + A_1 B_2} \quad (3.9)$$

if the lipid coverage on membrane surface was relatively low, there would be many defects which have no resistance to salt. That is

$$\frac{1}{B_1} < \frac{1}{B_2} \quad (3.10)$$

And then it could be derived that

$$\frac{B}{A} > \frac{B_2}{A_2} \quad (3.11)$$

If the lipid coverage on membrane surface was high, the lipid bilayer could give an equivalent salt resistance with NF membrane. That is

$$\frac{1}{B_1} = \frac{1}{B_2} \quad (3.12)$$

It can be derived that

$$\frac{B}{A} > \frac{B_2}{A_2} \quad (3.13)$$

If the lipid coverage on membrane surface was very high, the lipid bilayer could contribute a higher salt resistance with NF membrane. The final rejection would be depends on the ratio of $B_1A_2/(B_2A_1)$. Assuming the coverage of lipid bilayer is 100%, that is to say, the lipid bilayer is defect-free and the rejection of this membrane would be close to that of RO membrane as the lipid bilayer is almost impermeable to salt (Paula *et al.* 1996). However, based on current experimental results, it can be concluded that many defects still existed in the lipid bilayer. That was probably also a main reason why a reduced rejection was obtained.

However, although two methods used in this work provided a large-area supported lipid layer on the NF-270 membrane suitable for flux and rejection measurements, it is still quite challenging to obtain a defect-free biomimetic membrane. Further optimization of the vesicle fusion condition may be needed to get a good supported lipid membrane.

3.3.4 Proteoliposomes permeability measurements

Before preparing a lipid membrane incorporated with AQPs, the activity of AQPs incorporated was examined. The permeabilities of DOPC liposomes and AQPs incorporated DOPC liposomes were examined by a stopped-flow apparatus. The liposome solutions were rapidly mixed with a hyperosmotic phosphate buffer with sucrose. The osmotic gradient is around 350 mosmol/l after solution mixing. The shrinkage rate of liposomes was obtained by measuring the change of scattered light at right angle with time. The wavelength of light used is 500 nm. The initial rise rate (k) was determined by fitting the average of five traces to a single exponential equation. The water permeability of the liposomes, P_f , was calculated from the equation 2.4.

Figure 3.8 gives the normalized stopped-flow light scattering curves of the liposomes and proteoliposomes. It can be seen that the vesicle shrinkage rate of the proteoliposomes was significantly faster than that of the liposomes. The estimated water permeabilities for the liposomes and proteoliposomes are 58.3 $\mu\text{m/s}$ and 356.8 $\mu\text{m/s}$, respectively. Such high water permeability for the proteoliposomes should be attributed to the existence of active AQPs. Assuming total incorporation of AqpZ molecules into the liposomes, around 415 monomers per proteoliposome can be estimated according to the area per DOPC lipid (Mathai *et al.* 2008a) and the area per AqpZ molecule (Scheuring *et al.* 1999). The osmotic water permeability (P_f) for the subunit of AqpZ was about $2.5 \times 10^{-14} \text{ cm}^3\text{s}^{-1}$, comparable to other reported values (Hovijitra *et al.* 2009).

Figure 3.8. Stopped-flow light scattering fitted curves for DOPC liposomes (red curve) and AQPs incorporated DOPC proteoliposomes (dark curve).

3.3.5 AQP-embedded lipid membrane

As the lipid membrane #2 can give a lower defect density, it was chosen as the platform for AQP incorporation. The proteoliposomes fused on the modified NF-270 membranes. Theoretically, the water flux of the lipid-AQPs membrane is expected to be higher than that of the lipid membrane #2, given the fact that the proteoliposome had a permeability 5 times higher than that of the liposome. Rejection of the lipid-AQPs membrane should also increase because of the higher

water permeability of the lipid-AQPs membrane. However, from Figure 3.9, it is observed that the flux and the rejection of the lipid-AQPs membrane were as low as those of the lipid membrane #2. It seemed that AQPs did not function in this platform.

Figure 3.9. Fluxes and rejections of virgin NF-270 membrane and lipid-AQPs membrane. (Dead-end filtration test, applied pressure: 1 bar; feed solution: 1 mM NaCl solution. Error bars were derived from three independent experiments.)

To explore possible reasons, the AFM image of the lipid-AQPs membrane was taken after flux measurement and shown in Figure 3.10. It displayed that there were a lot of big protrusions on the top of the membrane substrate, which was quite different from the situation of the lipid membrane #2 (Figure 3.6B) that shows almost complete fusion after a hydraulic pressure was applied. Some big protrusions could reach to 500 nm in diameter and 70 nm in height. The average roughness was 4.5 nm. Compared to these protrusions, one AQP molecule is only about 9 nm in side length and about 6 nm in height (Ringler *et al.* 1999, Scheuring *et al.* 1999, Fotiadis *et al.* 2001), while the proteoliposomes used were around 100 nm in diameter. It was probable that these big protrusions were unfused proteoliposomes. Regardless of the reasons, the results indicated that the AQPs could not work in this study, suggesting the function of AQPs might be inhibited in the present environment. In order to investigate the mechanism, proteoliposomes with a lower protein-to-lipid ratio (PLR) 1/800 were also fused onto the modified NF-270

membrane. Before fusion, the permeability of the proteoliposome was also tested by the stopped-flow spectrometer. The results show that the permeability was about $108.9 \mu\text{m/s}$, lower than that of proteoliposome with PLR 1/200 but higher than the value of liposome. AFM images (Figure 3.10B, 3.10C) show that the surface of lipid-AQPs membrane (PLR 1/800) was very smooth and large protrusions were not found. The average roughness was 0.4 nm , much smaller than that of the lipid-AQPs membrane with PLR 1/200. As the presence of active AQPs was proven by the stopped-flow, this meant that the large protrusions were probably unfused proteoliposomes and the proteoliposome with low PLR had a similar fusion behaviour to the liposome. However, the flux and rejection of the lipid-AQPs membrane dropped by 77% and 56% compared to the virgin NF-270 membrane respectively. This was as low as the lipid membrane # 2 and lipid-AQPs membrane with PLR 1/200. It appears that the AqpZ still did not have functionality on the membrane substrate.

Figure 3.10. AFM image of (A) lipid-AQPs membrane with PLR 1/200, (B) lipid-AQPs membrane with PLR 1/800 after flux measurement (area: $3 \mu\text{m} \times 3 \mu\text{m}$) and (C) lipid-AQPs membrane with PLR 1/800 after flux measurement with higher resolution (area: $1 \mu\text{m} \times 1 \mu\text{m}$).

3.3.6 Lipid Mobility on NF-270 Membrane

As the lateral mobility of the lipid layer is crucial to the dynamics and function of the membrane proteins (Lenaz 1987, Sackmann 1996), FRAP was employed to investigate the lateral mobility of as-prepared membranes. Analysis of FRAP data was completed with a standard diffusion model (Axelrod *et al.* 1976).

$$f(t) = \frac{F(t)-F(0)}{F(\infty)-F(0)} = \exp\left(-\frac{\tau_D}{t}\right) \left[I_0\left(\frac{2\tau_D}{t}\right) + I_1\left(\frac{2\tau_D}{t}\right) \right] \quad (3.14)$$

$F(t)$ is the emission intensity of the bleached area at time t . $F(0)$ is the intensity of bleached area immediately after photobleaching. $F(\infty)$ is the emission intensity of the bleached area after a long time corresponding to the fully recovered intensity. I_0 and I_1 are the modified zeroth and first-order Bessel functions, respectively. τ_D is the diffusion characteristic time. The lateral mobility of lipid can be determined by the diffusion coefficient of lipid, D , which can be obtained by the following equation

$$D = \omega^2 / 4\tau_D \quad (3.15)$$

where ω is the diameter of the bleached area at $t=0$. The FRAP results of the lipid diffusion coefficient for the lipid membrane #1 was $2.84 \pm 0.70 \times 10^{-10} \text{ cm}^2 \cdot \text{s}^{-1}$, which was slightly higher than the reported value of the lipid bilayer on the NTR-7450 membrane (Kaufman *et al.* 2010). The FRAP result of the lipid diffusion coefficient for the lipid membrane #2 on NF-270 membrane was $2.77 \pm 0.83 \times 10^{-10} \text{ cm}^2 \cdot \text{s}^{-1}$, which was very close to the value of the lipid membrane #1.

Compared to the NTR-7450 membrane, the NF-270 membrane is much more hydrophilic, according to the surface contact angle measurement (Boussu *et al.* 2006, Boussu *et al.* 2007). Therefore, NF-270 membrane may be able to hold more water on the membrane surface providing a lubricating layer for the supported lipid layer (Majewski *et al.* 1998, Tanaka and Sackmann 2005). Some experiments also showed that a decrease in hydrophilicity of polymer substrates correlated well with a decrease in lipid mobility (Renner *et al.* 2008). However, since the roughness of the NF-270 membrane is much higher than mica or glass, even higher than the NTR-7450 membrane (Boussu *et al.* 2007), it may slow down the lipid diffusion.

This may be the main reason why lipid membranes #1 and 2 exhibited a much lower diffusion rate for the lipid bilayer than on mica or glass, which could reach $\sim 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$ (Rubenstein *et al.* 1979, Scomparin *et al.* 2009). However, the lipid mobility on the NF-270 membrane is also much lower than the lipid mobility in the cell membrane, which was reported to be around $10^{-8} \sim 10^{-9} \text{ cm}^2 \cdot \text{s}^{-1}$ (Bloom and Webb 1983, Oghalai *et al.* 1999). Further investigations to delineate the exact mechanism may be needed.

3.4 CONCLUSIONS

To make biomimetic membranes, incorporating AQPs for water filtration applications, the preparation of a defect-free platform for AQP incorporation on a suitable substrate is a crucial step. Two methods were compared to prepare supported lipid membranes on NF-270 membrane surfaces under a benign pH condition of 7.8. One method used direct vesicle fusion on a hydrophilic NF-270 membrane; the other was vesicle fusion facilitated by hydraulic pressure on a modified hydrophilic NF-270 membrane whose surface was spin-coated with positively charged lipids. AqpZ proteoliposomes were also fused on the modified substrate following the second method to explore the possibility of making AQP biomimetic membranes.

Experiments revealed that lipid vesicles cannot fuse to bilayers on the NF-270 membrane without an extra applied force, and that hydraulic pressure could be employed to facilitate vesicle fusion on the membrane. Water flux measurements indicated that the supported lipid membrane prepared by spin coating plus vesicle fusion had a much lower apparent defect density than that prepared by vesicle fusion only. However, the rejection results showed that the supported lipid membranes prepared by both methods were not defect free. It is probable that the surface roughness and charge are the main factors determining the quality of the supported lipid membrane.

AqpZ molecules were successfully incorporated into DOPC liposomes and the resulting permeability was measured by stopped-flow experiments. The fusion of the proteoliposomes onto the modified substrate was attempted, but the AqpZ

function in the resultant membrane was not observed and AFM images showed large protrusions of aggregates on the substrate. Proteoliposomes with lower protein-to-lipid ratio were used to prepare lipid-AQPs membrane and the large protrusions were not found on its surface, which indicated that the protrusions seen previously were probably induced by unfused proteoliposomes. However, the membrane also gave a very low flux and poor rejection. Finally, FRAP characterization showed that the supported lipid membranes prepared by the two methods exhibited similar lipid diffusion rates, comparable to other supported lipid bilayers on NF membranes. However, they are still much lower than the lipid mobility in cell membranes. The inhibition of AqpZ functionality may be affected by the low lipid mobility on the NF-270 membrane surface.

As the proteoliposome fusion on a support is the most suitable protocol to prepare the supported lipid membrane incorporated with membrane proteins, it is essential to explore why proteoliposomes could not be fused to bilayers on a polymer support as was the liposome under an applied pressure. This may help further understand this biomimetic system.

CHAPTER 4 FUSION BEHAVIOUR OF AQUAPORIN Z INCORPORATED PROTEOLIPOSOMES INVESTIGATED BY QUARTZ CRYSTAL MICROBALANCE WITH DISSIPATION (QCM-D)

4.1 INTRODUCTION

In the previous study, proteoliposomes with aquaporins (AQPs) were used to fuse onto an NF membrane to prepare an AQP-based biomimetic membrane. However, it was found the AQP-incorporated proteoliposomes did not fuse to bilayers as the liposome did under the same conditions, indicating there are significant differences in their intrinsic properties. In order to identify these differences between the liposome and the proteoliposome, a quartz crystal microbalance with dissipation (QCM-D), in combination with the Zetasizer and stopped-flow spectrometer, have been employed to explore the characteristics of proteoliposomes and liposomes in this study. The information about the surface charge of the vesicles can be obtained by the Zetasizer. The activity of the AQPs in proteoliposomes can be examined by the stopped-flow spectrometer. Moreover, QCM-D is a powerful tool to investigate the fusion behaviour of vesicles on a solid surface as it is very sensitive and accurate for detecting the subtle mass change or viscoelasticity change and allows for a real-time monitor without the need for any specific label for the samples.

However, although extensive studies have been performed to investigate liposome fusion on various solids (Keller and Kasemo 1998, Reimhult *et al.* 2002b, Reimhult *et al.* 2003) and different theories have been proposed to elucidate the fusion mechanisms of vesicles, the study on proteoliposome fusion is quite limited to date (Granéli *et al.* 2003). In addition, it has been found that certain membrane proteins can significantly affect the fusion behaviour of proteoliposomes. Granéli *et al.* (Granéli *et al.* 2003) reported that the water-exposed hydrophilic domains of membrane proteins in proteoliposomes can hamper the bilayer formation on silica, while the membrane proteins without a hydrophilic domain had little effect on it.

Their work demonstrated that the fusion behaviour of certain proteoliposomes can be rather different from that of liposomes, depending on the structure of the incorporated membrane protein. As stated above, a similar phenomenon was also observed in our previous study on the preparation of aquaporin Z (AqpZ)-based biomimetic membranes by vesicle fusion (Li *et al.* 2012). Proteoliposomes with a relatively high content of AqpZ could not completely fuse into bilayers on the polymeric membrane surface even when it was subjected to a pressure that could efficiently assist the fusion of liposomes. However, in contrast to Granéli's findings, AqpZ does not have a large hydrophilic domain (Calamita 2000). Thus, the factors that affect the fusion behaviour of proteoliposomes have not been clearly identified due to limited fundamental research in this field.

In order to study the fusion behaviour of vesicles on a polymeric membrane surface, a hydrophilic polymer layer was selected to be deposited on the solid sensor surface. Commonly used polymers to modify the solid surface include polyethylene glycol (Kaufmann *et al.* 2009, Wang *et al.* 2011, Watkins *et al.* 2011), polysaccharide (Baumgart and Offenhausser 2003, Mulligan *et al.* 2011), and polyelectrolytes (Wong *et al.* 1999, Kugler and Knoll 2002, Fischlechner *et al.* 2008a, Tang *et al.* 2009b). Polyelectrolytes are the most widely used hydrophilic polymers for the support because their opposite charges can enhance vesicle deposition and facilitate their fusion on the polymer. Nevertheless, it was reported that the fusion behaviour of vesicles on certain polymers was significantly different from that on hydrophilic solids (Kohli *et al.* 2006, Tang *et al.* 2009b).

In order to elucidate the effect of the AqpZ content incorporated on the fusion behaviour, liposomes and proteoliposomes with different AqpZ-to-lipid ratios were prepared and characterized under the same conditions. Different substrates were selected to identify their effects on the fusion behaviour of vesicles. It is expected that this study can shed light on the fundamentals of proteoliposome fusion on different surfaces, ultimately facilitating the preparation of biomimetic membranes.

4.2 MATERIALS AND METHODS

4.2.1 Materials

1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was purchased from Avanti Polar Lipids (*Alabaster, AL*). DPhPC does not present a detectable gel-to-liquid crystalline phase transition over a large temperature range (-120°C to $+120^{\circ}\text{C}$) (Lindsey *et al.* 1979). Monopotassium phosphate (KH_2PO_4), potassium chloride (KCl), and disodium phosphate (Na_2HPO_4) were acquired from Merck Chemical (*Singapore*). Detergent OG and SDS were purchased from Calbiochem® (*Singapore*) and Bio-Rad (*Singapore*), respectively. All other chemicals or materials were purchased from Sigma-Aldrich (*Singapore*) unless otherwise stated. All chemicals were used without further purification. Milli-Q water (*Millipore, integrated ultrapure water system*) with a resistivity of $18.2\text{ M}\Omega\cdot\text{cm}$ was used. A phosphate buffer with a pH value of 7.8 was prepared with 2.68 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 in Milli-Q water.

4.2.2 Liposomes preparation

The preparation method for the liposomes is similar to the one described in Chapter 3. The DPhPC lipid in chloroform was dried by a N_2 stream and then vacuumed overnight. The dried lipid film was rehydrated in a phosphate buffer. After stirring for several minutes and three freeze-thawing cycles, the suspensions were extruded 21 times through a polycarbonate membrane with a mean pore size of 200 nm for further use. The lipid suspension was extruded at 22°C .

4.2.3 AqpZ reconstitution into DPhPC liposomes

A certain amount of AqpZ solution, according to the theoretical PLR, was added to the DPhPC liposome solution with 1 wt% OG. After incubation for 1 hour, 0.05 g of biobeads were added into 1 ml of the liposome/AQP solution and rotated for 1 hour. An additional 0.15 g of the biobeads were then added and rotated for another 1.5 h to remove the OG. In order to minimize the adsorption of lipids by the biobeads, all biobeads were incubated in the same liposome solution for 1 hour prior to addition for detergent removal. The proteoliposome solution was then

extruded through a polycarbonate membrane with a mean pore size of 200 nm for further use. Extrusion experiments were performed at 22°C.

4.2.4 Characterization of liposomes and proteoliposomes

The size and Zeta potential of the liposomes and proteoliposomes were characterized by a Zetasizer Nano ZS (*Malvern, UK*) at 22°C. The permeabilities of the liposomes and proteoliposomes were determined by a stopped-flow apparatus (*SX20, Applied Photophysics*) at 22°C. In this process the liposomes and proteoliposomes were rapidly mixed with hyperosmolar phosphate buffer containing 600 mM sucrose. The shrinkage rates of the liposomes and proteoliposomes were measured by light scattering. Each stopped-flow experiment gave five light scattering traces, which were subsequently averaged. The initial rise rate (k) was calculated by fitting the raw data of the averaged trace using Origin software. The water permeability of the liposomes, P_f ($\mu\text{m/s}$), was calculated using the equation 2.4.

4.2.5 Substrate preparation

Prior to each QCM-D measurement, the SiO_2 sensors (*Q-Sense*) were cleaned in a 2% SDS solution followed by a water rinse. The sensor was then dried with nitrogen gas and cleaned in a UV/Ozone ProCleaner (*Bioforce*) for 15 minutes.

4.2.6 Quartz Crystal Microbalance with Dissipation

The QCM-D measurements were performed with a QCM-D system from Q-sense (*Göteborg, Sweden*). The lipid concentration in all samples was 1.5 mM. All QCM-D measurements were performed at 22°C. Each measurement was performed at several independent overtones ($n=3, 5, 7, 9, 11$) and recognized as valid data only when all overtones presented the same trend. Furthermore, each measurement was performed at least three times to confirm its reproducibility. The frequency and dissipation data in the third overtone ($n=3$, i.e., 15 MHz) were provided in all QCM-D results.

4.3 RESULTS AND DISCUSSION

4.3.1 Characteristics of liposomes and proteoliposomes

Table 4.1 lists the size and Zeta potential data of AqpZ incorporated proteoliposomes. It shows that the surface charges of the proteoliposomes increased correspondingly with an increase of the AqpZ concentration in the proteoliposomes, while the Zeta potential of the liposome was only -1.64 mv. This phenomenon is attributed to the negatively charged AqpZ at the buffer pH. The size of proteoliposome also increased depending on the concentration of AqpZ incorporated. A similar phenomenon was also observed in another proteoliposome system (Granéli *et al.* 2003). Each group of proteoliposomes was also observed to be smaller than the liposome (~ 170 nm, obtained from pure liposome treated with the same procedure in the absence of AqpZ). While the exact mechanism is not clear, it has been suggested that the size of vesicles prepared by extrusion through track-etched membranes is determined by the pressure, membrane pore size and lipid properties (Friskén *et al.* 2000). Unlike liposome, proteoliposome is a more complicated system that comprises lipids and proteins. Apparently, the incorporated AqpZ had a major influence on the physicochemical properties of the vesicles.

Table 4.1. Sizes and Zeta potentials of proteoliposomes with different PLRs.

Vesicle	Mean size (diameter, nm)	Polydispersity Index (PDI)	Zeta potential (mv)
Proteoliposomes (PLR 1:400)	118.9	0.101	-4.5
Proteoliposomes (PLR 1:200)	111.4	0.111	-5.1
Proteoliposomes (PLR 1:100)	122.2	0.137	-9.7
Proteoliposomes (PLR 1:50)	156.7	0.149	-14.4
Proteoliposomes (PLR 1:25)	161.6	0.128	-23.5

4.3.2 Activity of AqpZ

The typical stopped-flow results of liposome and proteoliposome can be seen in Figure 4.1A. The shrinkage rate of proteoliposome was significantly greater than the one in liposome, which could be attributed to the higher water permeation of AqpZ. The permeability is comparable to our previous reported value (Li *et al.* 2012) indicating that the AqpZ maintained its intrinsic permeability in DPhPC lipids under the conditions tested in this study. However, the water permeability did not increase proportionally with the increase in PLR. Upon reaching a certain PLR value, a decrease in water permeability occurred with further increase in AqpZ concentrations (Figure 4.1B). Similar results have also been observed in other AqpZ incorporated proteoliposomes (Borgnia *et al.* 1999b, Wang *et al.* 2011). It has been suggested that the method of incorporation might be responsible for the lower reconstitution efficiency of AQPs and lower permeability at a high PLR (Kumar *et al.* 2007). However, no report in the literature has confirmed this yet.

Figure 4.1. (A) Normalized stopped-flow light scattering measurements of liposome and proteoliposome (PLR 1/200). (B) The permeability values (P_f) of proteoliposomes with different PLRs (error bars were obtained from three independent measurements).

4.3.3 Fusion behaviour of DPhPC liposomes on silica

Figure 4.2 shows the adsorption of DPhPC liposome on silica in the phosphate buffer. The initial buffer washing induced a minor change of f and D , which may be due to the difference of water and buffer in terms of viscosity and density. Upon

addition of the liposome solution, the Δf gradually decreased and reached saturation in approximately 3 minutes. The saturation value of the resonant frequency in the buffer solution was around -28.8 Hz. By taking into account the Δf caused by the buffer, the Δf induced by liposome adsorption was about -27.6 Hz, which is close to other reported values for the bilayer (Granéli *et al.* 2003, Reimhult *et al.* 2003). The minor discrepancy can be attributed to variations in the hydration mechanisms of the lipid bilayer in various solutions or incomplete formation of a bilayer (Granéli *et al.* 2003). Upon water rinsing the Δf increased and ΔD decreased, but their changes were almost the same as induced by buffer rinsing at the beginning. If there were unruptured liposomes on the surface, the osmotic shock caused by water rinsing would have helped rupture the liposomes, which would induce a big change in ΔD and Δf . However, this was not observed in the present study, suggesting most liposomes ruptured spontaneously upon interaction with silica and formed bilayers completely on the silica surface (Richter *et al.* 2006).

Figure 4.2. Changes in resonant frequency and dissipation versus time for exposure of silica to a DPhPC liposome solution.

However, the dissipation value for the bilayer ($\sim 1.6 \times 10^{-6}$) after the buffer rinse was relatively higher when compared to other reported values of lipid bilayers (Reimhult *et al.* 2003, Richter and Brisson 2005). It is speculated that the high viscoelasticity may be induced by the unique structural features of phytanoyl lipids

or incomplete fusion of few vesicles on the solid surface (Andersson *et al.* 2011).

4.3.4 Fusion behaviour of proteoliposomes with different PLRs on silica

As stated above, proteoliposome is relatively different from liposome because the incorporated membrane protein can significantly affect the properties of vesicles. In the present study, the vesicle fusion can be governed by the size, the surface charge and the composition of the vesicles. In order to further investigate the effects of AqpZ on vesicle fusion, proteoliposomes were divided into two groups based on their sizes and Zeta potential values. Since each group obtained similar vesicle sizes and Zeta potential values, as such, the proteoliposomes with lower PLRs (1/400, 1/200 and 1/100) are categorized as group A, while the rest of the proteoliposomes are classified as group B. Figure 4.3 presents the QCM-D results of both groups (A and B). Unlike the liposome, the resonant frequency of proteoliposomes in group A initially decreased. Upon reaching a minimum value, the frequency then increased to an equilibrium level. As for the dissipation values, an opposite trend was observed. The dissipation initially increased, and then decreased after reaching a maximum value before finally approaching an equilibrium level. This fusion behaviour was consistent with one pathway of vesicle fusion (Keller *et al.* 2000): the deposition of vesicles onto a surface induced a high mass and viscoelasticity change. When they reached a critical vesicular coverage upon adsorption, the vesicles ruptured and released the trapped water causing the mass and viscoelasticity to decrease. In contrast to liposome, which directly ruptured upon interaction with silica, proteoliposome might need to overcome an energy barrier to fuse. Although these proteoliposomes had a similar fusion pathway, some slight variations still existed between them. At critical vesicular coverage, proteoliposomes with higher PLR obtained a lower Δf value and a higher ΔD value. It is believed that the minor differences of size (Reimhult *et al.* 2002b) and surface charge (Richter *et al.* 2003) of proteoliposome in group A had little effect on the shifting of frequency and dissipation; therefore, it was more difficult for the proteoliposome with a higher PLR to rupture on the silica surface. Furthermore, proteoliposomes with higher PLRs exhibited lower and higher equilibrium values of Δf and ΔD , respectively. In addition, these two parameter values of all

proteoliposome samples were much higher than those of bilayers formed by liposome. Such phenomena are also observed in other proteoliposome systems (Granéli *et al.* 2003). For certain PLRs, it is estimated that AqpZ could supply around -9 Hz when proteoliposome with PLR 1/100 forms a supported bilayer.

Figure 4.3. (A-B) The changes in frequency and dissipation of QCM-D for liposome and the proteoliposomes with different PLRs adsorption on silica.

The dissipation shift should not be caused by the AqpZ as proteins have little contribution to produce substantial dissipation change on a solid surface (Höök *et al.* 1998, Lubarsky *et al.* 2007). Therefore, it should be attributed to the presence of unfused vesicles or semi-fused vesicles on the silica surfaces, suggesting that proteoliposomes carrying more proteins were considerably more difficult to fuse into bilayers. As the surface charge of proteoliposomes did not vary significantly, the only possible reason could be associated with the incorporated AqpZ molecules. It was proven that the hydrophilic parts of several specific proteins such as proton translocating nicotinamide nucleotide transhydrogenase (TH), could hamper the formation of bilayers for proteoliposomes (Granéli *et al.* 2003). However, unlike proteins with large hydrophilic domains, AQPs are a group of highly hydrophobic

membrane proteins with small hydrophilic parts and have a similar side length to the domain II of TH (Ringler *et al.* 1999, Bizouarn *et al.* 2000). Therefore, the most probable reason for the inhibition of proteoliposome fusion would be the presence of AqpZ with vesicles of increased mechanical strength. AFM force indentation tests have shown that the incorporated AqpZ could drastically improve the mechanical stability of lipid membranes (Wang *et al.* 2011). The increased mechanical strength of the lipid bilayer would increase the energy barrier for planar bilayer formation through vesicle rupture.

The resonant frequency of group B gradually decreased until it reached an equilibrium level, while the ΔD value increased until it approached an equilibrium level (Figure 4.3). These equilibrium values are lower (for Δf) and higher (for ΔD) than those of group A. As the proteoliposomes in group B carry more negative charge than those in group A, the surface charge should be taken into account as more negative charge could inhibit the fusion of vesicles on a negatively charged silica surface (Richter *et al.* 2003). However, when compared to other reported values, such a significant decrease of frequency and increase of dissipation for proteoliposome (PLR 1/25) could not be entirely attributed to the surface charge (Richter *et al.* 2003, Viitala *et al.* 2006). It is believed that the improved mechanical stability also contributed to the presence of unfused proteoliposomes. Although the proteoliposome with PLR 1/25 had a lower permeability than that with PLR 1/50, it seems the proteoliposome with PLR 1/25 carried more AQPs based on the QCM-D results. Thus, it should be determined whether the lower permeability of the proteoliposome with the higher PLR was caused by a lower incorporation efficiency of AqpZ. As mentioned previously, the proteoliposome with PLR 1/25 exhibited a higher mechanical stability than other proteoliposomes due to a higher loading density of incorporated proteins. Furthermore, a higher surface charge density corresponds to a higher density of AqpZ molecules (as displayed in Table 4.1). Therefore, the decrease in the intrinsic water permeability of AqpZ should not be due to the lower incorporation efficiency. Liposome could still incorporate a higher density of AqpZ beyond the optimal PLR ratio. One possible reason for the decrease in water permeability of the proteoliposome is that the strong interaction among AqpZ molecules at a high protein density has inhibited their function.

In order to compare the degree of fusion of individual proteoliposome groups, a bilayer coverage (α) is estimated from the equilibrium dissipation value based on the method described by Granéli (Granéli *et al.* 2003). The corrected equations are as follows (Granéli *et al.* 2003) :

$$\alpha = 1 - (\Delta D_m - \Delta D_b) / (\Delta D_s - \Delta D_b) \quad (4.1)$$

and

$$\Delta D_s = \beta \cdot d \quad (4.2)$$

where ΔD_s is the dissipation change at saturation when the surface is fully covered by intact vesicles, ΔD_m is the actual measured dissipation change at saturation, ΔD_b is the dissipation change of the bilayer obtained for the liposome, which is around 0.1×10^{-6} (Granéli *et al.* 2003, Richter *et al.* 2003), d is the diameter of the vesicle and β is the constant ratio of ΔD_s to the vesicle diameter, equal to 0.15 (Reimhult *et al.* 2002b, Granéli *et al.* 2003). The bilayer coverage versus PLR is shown in Figure 4.4. Interestingly, the bilayer coverage decreases with an increase in concentration of incorporated AqpZ, indicating that the incorporated AqpZ has inhibited the fusion of proteoliposomes.

Figure 4.4. Bilayer coverage formed by proteoliposomes on a silica surface as a function of PLR (error bars were obtained from three independent overtone measurements).

4.3.5 Fusion behaviour of liposomes and proteoliposomes on a polymer surface

As stated in Chapter 3, the lipid lateral mobility of supported lipid membrane is very important for the activity of incorporated membrane proteins and it was reported that the lipid bilayer deposited on polydiallyl dimethyl ammonium chloride (PDADMAC) had higher mobility than other polyelectrolytes (Fischlechner *et al.* 2008b), in this study PDADMAC was selected to investigate the fusion behaviour of vesicles. It was dissolved in the phosphate buffer and deposited on the silica surface. QCM-D results showed that the deposited PDADMAC layer only caused minor changes in frequency (~ -9.75 Hz) and dissipation ($\sim 1.38 \times 10^{-6}$), suggesting that this layer was very thin and rigid. Figure 4.5 depicts QCM-D results for liposome and proteoliposomes depositions on a PDADMAC layer. In contrast to the fusion behaviour of liposome on silica, a considerable decrease in frequency and increase in dissipation occurred throughout the very long deposition process. This indicates that the liposome deposited gradually and remained intact on the PDADMAC layer. Similar phenomena were obtained from the QCM-D measurements of zwitterionic liposome deposition on other polyelectrolyte layers (Fischlechner *et al.* 2008a, Tang *et al.* 2009b). A significant increase in frequency and considerable decrease in dissipation occurred during a buffer rinse before reaching a stable level, indicating that a large portion of the loosely bound vesicles had been washed away by the buffer. A low charge density of the liposome in the phosphate buffer may be responsible for the presence of unfused and loosely bound vesicles as the electrostatic force between the vesicles and surface is the main driving force to form bilayers (Richter *et al.* 2006). In contrast to a long equilibrium time (more than 1 hour) needed for liposome deposition, the proteoliposomes only required a few minutes to reach a stable state after depositing on the PDADMAC layer (Figure 4.5B and 4.5C), signalling a strong affinity between the proteoliposome and the polyelectrolyte, probably attributed to the greater negative charge density of the proteoliposome. However, a sudden decrease of the resonant frequency and increase of dissipation changes of proteoliposome with PLR 1/200 were observed upon deposition of the proteoliposomes. Once reaching a minimum/maximum level, a small increase in f and decrease in D occurred within a

short period of time before finally reaching an equilibrium level (Figure 4.5B). As described above, this phenomenon has been commonly found in vesicle fusion through a critical vesicular coverage. However, the equilibrium f and D values shifted greatly as compared to the values of bilayers, indicating that the majority of proteoliposomes remained intact after interacting with the polyelectrolyte layer though a small portion of vesicles had fused.

Figure 4.5. The changes in frequency and dissipation of QCM-D for (A) liposome, (B) proteoliposome with PLR 1/200 and (C) proteoliposome with PLR 1/25 deposition on PDADMAC layer.

However, vesicle fusion was not observed for the proteoliposomes with PLR 1/25, since the f and D gradually approached equilibrium levels. For liposome, the increase in surface charge could have facilitated its rupture and fusion on a polyelectrolyte layer (Tang *et al.* 2009b). Nonetheless, it was not the same case for proteoliposome in the present study. Although the proteoliposomes with PLR 1/25 carry a higher charge density, which should help vesicles fuse on charged polymer surface, fewer vesicles were fused on the polymer layer as compared to the proteoliposomes with PLR 1/200. As discussed above, an improved mechanical stability of vesicles by AqpZ might inhibit the vesicle fusion. Despite the

electrostatic force between the proteoliposome and the polyelectrolyte layer that could facilitate vesicle fusion, the effect of increased mechanical strength of the vesicles appears to be much more significant. In our previous study, it was observed that proteoliposomes with a higher protein density became more difficult to fuse on a polymer surface even when it was conducted under applied pressure, though the pressure could greatly facilitate the fusion of liposomes (Li *et al.* 2012). It is noticeable that the equilibrium frequency value for the proteoliposomes with PLR 1/200 or PLR1/25 on the PDADMAC layer was considerably higher than that of the same proteoliposomes on silica. Since the equilibrium frequency change was dominated by the mass trapped on the sensor surface, the water inside and between the vesicles, only slight deformation of proteoliposome on the polymer surface occurred, resulting in an increase of effective thickness (Reimhult *et al.* 2002b). This suggests that these deposited vesicles became less deformed at the equilibrium state. One probable explanation for this is that the polyelectrolyte played an important role in stabilizing the vesicles, which was also found in other vesicle-polyelectrolyte systems (Fischlechner *et al.* 2008a, Tang *et al.* 2009b). It appears that the stabilization effect by polyelectrolyte is not negligible.

4.4 CONCLUSIONS

Both the Zeta potential and QCM-D results revealed that a high concentration of AqpZ could be incorporated into the DPhPC liposomes even though its intrinsic permeability decreased. However, it was much more difficult for the AqpZ incorporated proteoliposomes to fuse into bilayers than liposomes on silica surfaces, especially with an increase in the density of AqpZ in the proteoliposome. The increased surface charge and mechanical strength of the proteoliposome may affect the fusion and hinder the bilayer formation upon interacting with the substrate. The QCM-D results also indicated that the proteoliposome was more difficult to fuse to bilayers on a polyelectrolyte surface, which apparently showed a stabilizing effect on the vesicles. Based on this part of work, it suggested that because of the incorporated membrane proteins, the proteoliposome had a different size, carried more surface charge and became more difficult to fuse to a bilayer, in compared with the liposome. If the proteoliposome fusion method is employed to prepare a

supported biomimetic membrane, especially on a polymeric substrate, further optimization may be required.

CHAPTER 5 AN ULTRA-HIGH PERFORMANCE POLYETHERSULFONE HOLLOW FIBRE COMPOSITE MEMBRANE BASED ON AQUAPORIN Z

5.1 INTRODUCTION

The Quartz crystal microbalance with dissipation (QCM-D) results demonstrated the proteoliposome became more robust because of the incorporated aquaporins (AQPs). In contrast to liposome, proteoliposomes became more difficult to fuse to bilayers on a solid surface. Additionally, polymeric membranes were commonly employed to be the substrates for biomimetic membranes. However, it was also demonstrated that the polymer had a stabilizing effect for the vesicle, indicating that it would be more difficult to prepare a defect-free AQP-based membrane through vesicle fusion on a polymer substrate. According to the work introduced in Chapter 3, it was also indicated that the activity of AQP molecules might be quite susceptible to the external environment. Based on these results, it was proposed that the intact vesicle might be employed to help design and prepare the AQP-based membrane, instead of fusing vesicles onto a porous support. In this study, proteoliposomes were managed to be embedded into the dense layer of a thin-film reverse osmosis (RO) membrane and thereby facilitated the water transport. This protocol is basically based on a bio-inspired technology and the conventional thin-film composite membrane technique.

The thin-film composite membrane technique was well acknowledged as a scientific breakthrough in membrane development. The thin layer coated onto a porous substrate could provide an enhanced water flux meanwhile maintaining a high salt rejection, while the support underneath could stand the applied high pressure and allowed the functionalization or optimization independently. This scientific breakthrough also makes the RO and nanofiltration (NF) membranes fabricated by this technique commercially available for water purification

applications (Lau *et al.* 2012). A common interfacial polymerization process is schematically illustrated in Figure 5.1.

Figure 5.1. Schematic drawing of the preparation of a composite membrane through interfacial polymerization.

One side of the support, usually an ultrafiltration membrane or microfiltration membrane, is uniformly covered by an aqueous solution containing an active monomer. Then this side of the support is covered by a water-immiscible solution containing another reactive monomer. These two monomers react with each other in an extremely short time in the interface between the two solvents and form a dense thin layer. One advantage of interfacial polymerization is that the polymerization reaction is self-inhibiting, which is favourable in forming a thin film (Mulder 1996). The resultant membrane consists of a porous support (~40-50 μm thick) and an ultra-thin polyamide layer (~ 200 nm thick) on top. Over the past decades, extensive work has been focused on performance improvement of thin-film composite membranes, with respect to the permeability, selectivity, chlorine tolerance, fouling resistance, etc. (Lau *et al.* 2012). The performance of a conventional thin-film composite membrane can be tailored by changing the formula of the interfacial polymerization. One typical chemical reaction in a formula for the preparation of thin-film composite membranes is displayed in Figure 5.2. The amine is dissolved in the aqueous phase while the chloride is in the organic phase. These two chemicals react immediately upon contacting with each other to form a dense polyamide layer. This polyamide layer generally has good chemical resistance and mechanical strength.

Figure 5.2. One typical chemical reaction of the preparation of a thin-film composite membrane.

In this study, proteoliposomes were introduced into a hollow fibre composite membrane during the interfacial polymerization process: the proteoliposome was first deposited on inner surface of a hollow fibre membrane and then encapsulated within a polyamide layer by a novel interfacial polymerization method. The whole procedure is schematically illustrated in Figure 5.3. During the whole preparation procedure, all proteoliposomes were restricted in a semi-closed system, the lumen of the hollow fibre, so that the loss of proteoliposomes was minimized. Moreover, aggressive chemicals were avoided in order to maintain the activity of AQPs. The results showed that the AQP-based biomimetic hollow fibre membrane achieved a super-high water flux with a reasonable salt rejection; different characterization techniques were conducted to investigate this biomimetic system.

Figure 5.3. Schematic of preparation of an AqpZ-based hollow fibre membrane. (not to scale)

5.2 MATERIALS AND METHODS

5.2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (*Alabaster, AL*). Polyethersulfone (PES, *Solvay*) was used as the membrane material for the hollow fiber substrate fabrication. N-Methyl-2-pyrrolidone (NMP, > 99.5%, *Merck Chemicals, Singapore*) was used as a solvent for the dope preparation. m-Phenylenediamine (MPD, $\geq 99\%$, *Sigma-Aldrich*) and trimesoyl chloride (TMC, > 99%, *Sigma-Aldrich*) were used as the monomers for the interfacial polymerization. NaCl (*Merck Chemicals, Singapore*) was used to prepare the draw solutions and the feed water for RO and forward osmosis (FO) tests. The BW 30 membrane was supplied by Dow Filmtec (*USA*). All other chemicals or materials were purchased from Sigma-Aldrich (*Singapore*) unless otherwise stated. All chemicals were used without further purification. Milli-Q water (*Millipore, integrated ultrapure water system*) with a resistivity of 18.2 M Ω •cm was used.

5.2.2 Liposome preparation

A DOPC liposome solution was prepared by a film rehydration method. 12 mmol DOPC lipid in chloroform was dried by a N₂ stream and then vacuumed for at least 12 hours. The dried lipid film was rehydrated in the water. After stirring for several minutes and three freeze-thawing cycles, the suspensions were extruded 21 times through a polycarbonate membrane with a mean pore size of 200 nm for further use. The lipid suspension was extruded at 23°C. The as-prepared liposome solution was used within 6 hours.

5.2.3 Aquaporin Z reconstitution into DOPC liposomes

The reconstitution of AQPs into liposome basically follows the strategy for membrane protein reconstitution developed by Rigaud J *et al.* (Rigaud and Levy 2003). After preparation of homogenous and unilamellar liposomes, n-octyl- β -D-glucoside (OG) was added to the liposome solution with a final concentration of 1%. OG can solubilise the liposome into micelles. A controlled amount of aquaporin Z

(AqpZ) solution, according to the required lipid-to-protein ratio (LPR), was added to the lipid-detergent mixture. 0.2 g biobeads were added into 1 ml liposome/AqpZ solution stepwise and kept stirred for 2.5 hours to remove the OG through adsorption. During the process of OG removal, the added AQPs can be incorporated into reassembled liposomes. The proteoliposome solution was then extruded through a polycarbonate membrane with a mean pore size of 200 nm for further use. Extrusion experiments were performed at 23°C.

5.2.4 Characterization of liposomes and proteoliposomes

The size of the liposomes and proteoliposomes were characterized by a Zetasizer Nano ZS (*Malvern, UK*) at 22°C. The permeability of the liposomes and proteoliposomes was investigated by a stopped-flow apparatus (*SX20, Applied Photophysics*) at 22°C. During this process the liposomes and proteoliposomes were rapidly mixed with water containing 600 mM sucrose. The shrinkage rates of the liposomes and proteoliposomes were measured by light scattering. Each stopped-flow experiment gave five light scattering traces, which were subsequently averaged. The initial rise rate (k) was calculated by fitting the raw data of the averaged trace. The water permeability of the liposomes, P_f ($\mu\text{m/s}$), was calculated using the equation 2.4.

5.2.5 AqpZ-based hollow fibre membranes

An ultrafiltration-type PES hollow fiber membrane fabricated by the dry-jet wet spinning method (Wang *et al.* 2010) was used as a substrate for the AQP-based composite membrane. The molecular weight cut-off of the lumen side in the hollow fibre is about 37 kDa while that of the shell side is about 500 kDa. As schematically displayed in Figure 5.3, the membrane substrate was first soaked and flushed with the MPD aqueous solution which was subsequently replaced by the aqueous solution containing liposomes or proteoliposomes. During this process, part of liposomes or proteoliposomes would stay on the substrate surface due to the intention of reducing surface Gibbs free energy. The excess aqueous solution was removed by gentle flushing. The TMC solution was then pumped into lumen of hollow fibres and would react with the MPD on the inner surface of the hollow fibre

immediately to form a polyamide layer. Those immobilized liposomes or proteoliposomes were eventually encapsulated in the polyamide layer. The resultant membranes were kept in water before characterization and evaluation. These AqpZ-modified hollow fibre membranes were sealed in a hollow fibre module for performance evaluation. The effective membrane area of one module was around 34.2 cm².

5.2.6 Characterization of AqpZ-based hollow fibre membranes

The scanning electron microscopy (SEM) characterization was conducted by a field-emission scanning electron microscope (*JSM-7600F*). Prior to SEM characterization, all samples were dried in a desiccator filled with desiccants for 12 hours.

5.2.7 Membrane performance evaluation

Pure water flux and low pressure RO tests were conducted using a bench-scale cross-flow setup. A high cross-flow rate was applied to minimize the effect of concentration polarization. The pure water flux test was carried out of one bar for at least one hour until reaching a stable water flux. The low pressure RO test was conducted under 5 bar with a 500ppm NaCl solution using the same cross-flow setup. The water flux, J_v (L·m⁻²·h⁻¹) was calculated according to equation 5.1:

$$J_v = \frac{\Delta w}{S \cdot \Delta t} \quad (5.1)$$

where Δw (kg) is the increase of permeate weight over a certain period of time, Δt (hour) is the time interval and S (m²) is the effective membrane area. The salt rejection, r (%) was calculated according to equation 5.2:

$$r = \frac{C_f - C_p}{C_f} \times 100\% \quad (5.2)$$

where C_f and C_p are the salt concentration in the feed and permeate, respectively. A lab-scale FO cross-flow setup the same as the unit in Ref. (Chou *et al.* 2010) was used to conduct the FO test. Two gear pumps (*Cole-Parmer, US*) were employed to supply the feed and draw solutions. One pump transported the feed through the shell

side of hollow fibre module while the other one was for the draw solution through lumen side. A flow meter and pressure transducer were installed between the pump and the membrane module to monitor the flow rate and pressure of the two circulations, respectively. The Reynolds numbers of the fluids flowing in the lumen and shell were controlled at around 2500 to minimize the external concentration polarization. The permeate flux was determined by measuring the weight change of the feed. The concentration of the draw solution was maintained constant by a doping system. A data logging system was built to record the conductivity, temperature, pressure of circulations and weight change of draw solution in real-time. The water flux of the FO test was calculated using equation 5.1 while Δw is the weight increase of the draw solution. The salt flux (J_s) was determined from the equation

$$J_s = \frac{\Delta C_f \cdot V_f}{S \cdot \Delta t} \quad (5.3)$$

Where ΔC_f ($\text{mol} \cdot \text{L}^{-1}$) is the salt concentration change of the feed water and V_f (L) is the volume of the feed water; Δt (hour) is the time interval; and S (m^2) is the effective membrane area of hollow fibre membrane. All tests were carried out at 23°C. The water permeability (A value) and salt permeability (B value) of the membranes were derived from equation 3.3 and equation 3.4.

5.3 RESULTS AND DISCUSSION

5.3.1 Permeability of proteoliposomes

The normalized light scattering curves related to the shrinkage rate of the vesicle size when the vesicles were imposed to an osmotic gradient are shown in Figure 5.4A. The AqpZ incorporated proteoliposomes exhibited a more rapid size change compared to the liposome. A faster size change was obtained when the LPR decreased to 100 (AQP-to-lipid ratio 0.01). It is estimated that the permeability of the proteoliposome with an LPR at 100 is almost 30 times as high as that of liposome alone. The enhanced permeability would be due to the presence of AqpZ molecules in the proteoliposomes. Although the buffer used in this study was

different, the permeability result was comparable to our previous report and other studies (Kumar *et al.* 2007, Li *et al.* 2012). It indicates that the incorporated AqpZ maintained its function in this synthetic environment and buffer, which directly led to the enhanced water permeability. However, it was also observed that further increasing the ratios of AqpZ in the proteoliposomes (decreasing LPR) could not provide a higher permeability, as displayed in Figure 5.4B. As discussed in Chapter 4, the proteoliposome with LPR 50 carried more AQPs than the one with LPR 100 although its permeability was lower, probably due to the inhibition of AQP's activity in a high PLR. Therefore, it appears there is an optimal LPR to reach the highest permeability of a proteoliposome; however, the reason for this have not been elucidated, although considerable research including our previous study reported similar trends (Borgnia *et al.* 1999b, Kumar *et al.* 2007).

Figure 5.4. (A) Stopped-flow curves of proteoliposomes with different LPRs (lipid-to-protein ratios) and (B) estimated water permeability of proteoliposomes with different AqpZ-to-lipid ratios (error bars were obtained from three independent measurements).

5.3.2 Water permeability and salt rejection of AqpZ-based hollow fibre membranes

To evaluate the separation performance of the AqpZ-based hollow fibre membranes, these membranes with different AqpZ ratios were tested at various conditions. The results (Figure 5.5A) showed that the pure water permeability of the membrane after adding proteoliposomes (LPR 200) was raised by 60%, in comparison to the

control group, the membrane with the vesicles without AqpZ. Furthermore, the membrane with a higher protein ratio (LPR 100) offered even higher pure water permeability, $\sim 8 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$, almost 3 times as high as the control group. These results are also consistent with the trend of proteoliposome permeabilities obtained from stopped-flow measurements, which suggested the incorporated AqpZ contributed to the enhanced pure water permeability of these membranes. Nevertheless, the salt rejection data are needed to confirm that the enhanced water permeability was not induced by defects after introducing proteoliposomes during the formation of a polyamide layer. The salt rejection of the membrane was tested by a 500ppm NaCl solution under 1 bar pressure. As shown in Figure 5.5B, all tested membranes exhibited high rejections to salt ($\sim 87\%$ - 90%) even under a low pressure. Additionally, the osmotic pressure effect was also observed where the water flux obtained from salt water was lower than the pure water permeability at the same pressure. Similar to the results for the pure water permeability, both the flux and the salt rejection increased in proportion to an increase in the AqpZ ratio in the proteoliposome. Compared with the other membranes, the one with an LPR of 100 presented the highest water flux and salt rejection, consistent with the results of stopped-flow measurements and pure water permeability of these hollow fibre membranes.

Figure 5.5. (A) Pure water permeability of AqpZ-based hollow fibre (AHF) membranes with different LPRs. (B) Salt water flux and rejection of AHF membranes with different LPRs (1bar, 500ppm NaCl). Error bars were obtained from three independent measurements.

As described in Chapter 3 by equation 3.5, the salt rejection of a membrane, r , is only determined by the B/A value for a given applied pressure and testing solution. For the AqpZ-based hollow fibre membrane, these introduced highly permeable proteoliposomes could increase the A value of the selective layer. The B value was mainly determined by the polyamide layer; theoretically introduced proteoliposomes did not increase the B value because both the lipid bilayer and the AqpZ molecules have a very high transport resistance to salt and other solutes (Kumar *et al.* 2007, Zhao *et al.* 2013). This could explain why an increasing salt rejection was observed by increasing the AqpZ ratio in the proteoliposomes that was introduced into the hollow fibre membranes. Based on these results, it is reasonable to conclude that the proteoliposome-modified polyamide layer was effectively defect-free.

5.3.3 Low pressure RO test and forward osmosis test

As this membrane showed a superior performance and it is proposed that the AqpZ-based hollow fibre membrane has a similar structure to conventional RO membranes and thin-film composite FO membranes, it should be quite promising in the application of low pressure RO or FO processes. The low pressure RO tests were conducted at 5 bar with a 500ppm NaCl solution for the hollow fibre membrane with LPR 100 and a commercial RO membrane (BW30). Figure 5.6 shows the AqpZ-based hollow fibre membrane achieved a water flux approximately $40 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$, which was about twice as the water flux of the BW 30 membrane or an AqpZ-based flat sheet membrane (Zhao *et al.* 2012) tested under the same or closely similar conditions. Probably attributed to the modified interfacial polymerization process employed and absence of some aggressive chemicals, the hollow fibre biomimetic membrane displayed a higher flux and rejection to flat sheet one.

Figure 5.6. Salt water flux and rejection of three different membranes. Test conditions: BW30 and hollow fibre membranes with AQPs were tested at 5 bar with a 500ppm NaCl solution; Flat sheet membrane with AQPs was tested at 5 bar with a 10mM NaCl solution (the circle indicated the estimated datum distribution from ref.(Zhao *et al.* 2012)).

Additionally, the salt rejection of our hollow fibre membrane was even higher than the other two membranes. It should be noted that all of these membranes were fabricated by the interfacial polymerization method. In contrast to the BW 30 membrane prepared by a conventional protocol, the AqpZ-based flat sheet membrane and hollow fibre membrane were based on a bio-inspired technology to achieve a better separation performance. However, in comparison to the flat sheet membrane, the hollow fibre membrane was able to achieve an ultra-high water flux with a higher salt rejection, which should be attributed to the particular structure or geometry of the hollow fibre and the novel interfacial polymerization method employed in the present study. These protocols, and the improved control offered by the lumen flow of reagents, also suggested that the AqpZ-based hollow fibre membranes could offer a more reproducible result, compared to the commercial RO membrane and flat sheet membrane as depicted in Figure 5.6. Along with the results displayed in Figure 5.5, it could be concluded that it was the introduced AqpZ that contributed to the enhanced water flux of the biomimetic hollow fibre membranes.

This conclusion was further confirmed by our FO test results showed in Table 5.1. To minimize the effect of internal concentration polarization, the AqpZ-based hollow fibre membranes were tested in the active layer facing the draw solution orientation, that is also referred to pressure-retarded-osmosis (PRO) mode. Compared to the hollow fibre membrane with liposome but without AqpZ, the water flux of the membrane with AqpZ was remarkably improved to $55.2 \text{ L}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ from $38.6 \text{ L}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$, meanwhile maintaining a high resistance to salt flux. This is superior to any other reported thin-film composite FO membrane tested in similar conditions (Chou *et al.* 2010).

Table 5.1. Water flux and salt flux in the forward osmosis process.
(Draw solution: 0.5M NaCl; feed solution: pure water; PRO mode; error bars were obtained from three independent measurements)

Membrane	Water flux J_w (LMH)	Salt flux J_s (gMH)
Membrane with AQPs (LPR 100)	55.2±4.5	4.5±0.2
Membrane without AQPs (control membrane)	38.6±4.2	4.9±0.4

5.3.4 Characterization of AqpZ-based hollow fibre membranes

To elucidate the exact structure of the polyamide layer modified by proteoliposomes, SEM characterization was performed to explore the location of the introduced proteoliposomes and the integration of the proteoliposomes and the polyamide layer. The SEM images are presented in Figure 5.7.

Figure 5.7. SEM images of (A,a) the membrane with proteoliposomes (LPR 100) and (B,b) the membrane with liposome (without AQPs). The scales in A and B are 100 μm and the scales in a and b are 100 nm.

The porous structure of the hollow fibre substrate (Figure 5.7A, 5.7B) provided a robust support for the selective layer, and meanwhile, minimizes the resistance to mass transport or the interfacial concentration polarization. The inner skin layer of this hollow fibre substrate has a very small mean pore size (Chou *et al.* 2010), which was smaller than the size of the proteoliposomes. This ensured that the bulk of the proteoliposomes were held at the inner surface of the substrate rather than going inside it. Both images revealed that most of the vesicles were located at the interface of the substrate and the polyamide layer in both membranes. The surface of the membranes also presented the “hill and valley” structure, commonly observed in a RO membrane prepared by interfacial polymerization. More critically, the vesicles were totally embedded into the polyamide layer as expected and the coverage of proteoliposomes in the polyamide layer was also extensive. The improved water flux and salt rejection of these membranes suggested that the integration of the proteoliposome and the polyamide layer was entirely defect-free.

This guarantees that a large area of this membrane prepared by this protocol could provide a reproducible high water flux and salt rejection, as presented in this study. Though all samples underwent the RO test prior to SEM characterization, the SEM images (Figure 5.7a, 5.7b) showed that these embedded vesicles retained their intact spherical shape even after experiencing long-term pressure tests. This polyamide layer and the porous substrate helped these embedded vesicles withstand the external applied force. In addition, the proteoliposome also had an intrinsic good mechanical strength to stand the applied pressure due to the incorporated AQPs, as discussed in Chapter 4.

5.3.5 Comparison of AqpZ-based hollow fibre membrane with other RO membranes

A comparison of the AqpZ-based hollow fibre membrane with several commercial membranes (BW30, CPA 2, ESPA 3, XLE and SW30HR) and one novel RO membrane (thin-film nanocomposite membrane) is summarized in the Figure 5.8. In comparison to other membranes, the AqpZ-based hollow fibre membrane displayed the best flux with a comparable salt permeability. As the salt rejection was determined by the A/B value for a given operation process, the AqpZ-based hollow fibre membrane still could offer a good quality permeate although this membrane had a higher B value than several other membranes. We believe the salt flux of this membrane would be further reduced by the optimization of the interfacial polymerization process. Additionally, extensive studies on thin-film composite membranes have suggested a trade-off phenomenon whereby it is quite challenging to further improve the water flux without sacrificing selectivity (Geise *et al.* 2011). The utilization of AQP in the membrane materials may promise to circumvent this trade-off limitation because of the fundamental difference in transport mechanisms between the AqpZ-based membrane and conventional thin-film composite polymeric membranes. The selectivity of conventional composite polymeric membrane relies on the difference of water and salt permeability through the dense membrane. Water and salt transport are related to the crosslinking degree of polyamide layer. Increased water permeability can be achieved by reducing the crosslinking degree of polyamide layer while the salt permeability would be

increased correspondingly. However, for AQP-based biomimetic membrane, the water channel transport water molecules and reject other solutes exclusively. The water permeability of membrane can be improved by water channels without sacrificing the salt rejection.

Figure 5.8. Water permeability (A) and salt (NaCl) resistance ($1/B$) of different membranes. The data of TFN was obtained from ref. (Lind *et al.* 2010). The data of ESPA 3 membrane and BW30 #2 membrane were obtained from ref. (Tang *et al.* 2007). The data of CPA 2, BW30 #3, SW30HR and XLE membranes was obtained from their product specifications provided by suppliers, respectively. The results of AHF and BW30 #1 were obtained in this study.

5.4 CONCLUSIONS

In this chapter, the preparation and characterization of an AQP-based hollow fibre membrane was introduced. A highly permeable and selective membrane based on AqpZ was obtained by embedding proteoliposome into a polyamide layer in the lumen of a hollow fibre substrate by a novel interfacial polymerization method. The water flux of the hollow fibre membrane with AqpZ at a high protein ratio could be

increased by 260%, compared with a control membrane embedded with liposomes but without AqpZ. A low-pressure RO test for this membrane exhibited a flux almost 200% of that for a typical commercial RO membrane, and an even higher salt rejection. Additionally, a FO test also gave a superior water flux and selectivity comparable to most of the reported thin-film FO composite membranes. SEM images clearly showed that most of the introduced proteoliposomes were entirely embedded in the polyamide layer and maintained their spherical shape even after a long-time filtration test. It is concluded that the improved water flux and salt rejection should be attributed to the presence of AQPs in the membrane. We believe that our proposed protocol offered a practical way to prepare a high-performance AQP-based composite membranes because of two main reasons: (i) the technique based on a hollow fibre membrane is quite simple and easy to scale up and operate; (ii) the completely embedded proteoliposomes and AQPs could be protected by the polyamide layer from external influences such as biofouling or cleaning during the water purification process, ensuring the stability of this membrane.

CHAPTER 6 PREPARATION OF HIGH PERFORMANCE NANOFILTRATION (NF) MEMBRANES INCORPORATED WITH AQUAPORIN Z

6.1 INTRODUCTION

The preparation and evaluation of a super high performance thin-film composite reverse osmosis (RO) membrane was described in Chapter 5. The introduced protocol was proven to be efficient to obtain a high performance biomimetic RO membrane. In addition to RO membranes, currently NF membranes are also widely used for water purification and seawater desalination. Compared to RO membranes, nanofiltration (NF) membranes have a more open network structure, which has a lower retention for monovalent salts such as Na^+ and Cl^- while still retaining a high retention for divalent ions such as Ca^{2+} and SO_4^{2-} as well as most organic pollutants. For some water purification processes, such as water softening, treatment of wastewater containing dyes, sugars or pesticides, an NF membrane is more suitable and efficient because it could contribute a higher water flux meanwhile maintaining a high retention. Therefore, NF membranes only require a relatively low operation pressure but still can provide a high water flux and a high rejection for multivalent salts and organic molecules. Besides, they may need lower investment and maintenance cost than RO membranes (Hilal *et al.* 2004).

In this study, a high performance NF membrane was prepared based on aquaporin Z (AqpZ) by incorporating the proteoliposomes into a selective layer formed by polymer crosslinking. The proteoliposomes were initially immobilized on a poly(amide-imide) (PAI) membrane substrate and subsequently encapsulated completely in a selective layer formed by polyelectrolyte crosslinking with the substrate. It was reported that the polyelectrolyte with primary amine groups, such as poly(ethyleneimine) (PEI), could crosslink with the PAI membrane at certain conditions, to form a rejection layer with a thickness of several hundred nanometers thickness (Setiawan *et al.* 2011, Fang *et al.* 2012). This layer could offer a fairly

good rejection to divalent ions, as well as a reasonable water permeability (Setiawan *et al.* 2012, Setiawan *et al.* 2013). If the introduced proteoliposomes were completely encapsulated by this layer, this polyelectrolyte layer with embedded proteoliposomes is expected to form a highly permeable selective layer. The procedure for the selective layer formation is schematically illustrated in Figure 6.1. This technique is relatively simple for preparation and scaling up as compared to other methods for aquaporin (AQP)-based NF membrane preparation, and thus it provides a potential way to design and fabricate an AQP-based biomimetic membrane for practical applications.

Figure 6.1. Schematic diagram of AQP-based biomimetic membrane preparation. (not to scale)

6.2 EXPERIMENTAL

6.2.1 Chemicals and materials

The chemicals used in this study were 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) in chloroform (*Avanti Polar Lipids, Alabaster, USA*), *n*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (*NBD-PE, Invitrogen, Singapore*), detergent 1-*n*-octyl- β -D-

glucopyranoside (OG) and biobeads (*Calbiochem*®, *Singapore*), poly(ethyleneimine) solution (average Mw ~750,000 by LS, 50 wt. % in H₂O) and dopamine hydrochloride (*Sigma-Aldrich*, *Singapore*). Other chemicals or materials were purchased from Merck chemicals (*Singapore*) unless otherwise stated. All chemicals were used without further purification. Milli-Q water (*Millipore*, *integral 10*) with a resistivity of 18.2 M Ω ·cm was used. Tris buffer solution was prepared by dissolving 10 mM Tris in Milli-Q water, the pH was then adjusted to 8 by HCl addition. Torlon® 4000T (copolymer of amide and imide) (PAI) was supplied by Solvay Advanced Polymers (*Alpharetta GA*). NF-270 and NTR-7450 flat sheet membranes were supplied by Dow Chemical Company (*USA*) and Nitto Denko Corporation (*Japan*), respectively.

6.2.2 Preparation of Liposome and Proteoliposome Solutions

DOPC chloroform solution was dried by slowly passing a pure N₂ stream and then vacuumed for at least 12 hours. The dried lipid film was then hydrated in Tris buffer. After agitating for 10 min and three freeze-thawing cycles, the solution was extruded 21 times through a polycarbonate membrane with a mean pore size of 200 nm by an extruder (*Avanti*, *US*). The final concentration of the DOPC vesicle solution was 8 mM. A certain amount of the AqpZ solution was added into 8 mM DOPC liposomes Tris buffer containing 1% OG at different lipid-to-protein ratios (LPRs). Stepwise addition of 0.2 g biobeads into 1 ml solution was performed. The mixture was kept stirring for 2.5 hours to remove the OG. The proteoliposome solution was then extruded through a polycarbonate membrane (200 nm mean pore size) 11 times for further use. The size and Zeta potential of the liposome were measured by a Zetasizer Nano ZS (*Malvern*, *UK*) at 22 °C.

6.2.3 Preparation of Substrates

Torlon® 4000T was dried in a 50 °C vacuum oven for at least one day to remove the moisture prior to the dope preparation. 14% (wt%) PAI and 3% (wt%) LiCl were dissolved in NMP using a mechanical stirrer at 70°C for 3 days. The PAI membrane was cast by a casting knife on a flat-sheet casting machine. The thickness was set at 200 μ m. The cast PAI membranes were then soaked in Milli-Q

water for at least one day.

6.2.4 PDA coating of proteoliposomes

2 ml of a 4 mM extruded proteoliposome solution was injected into 2 ml of a 0.1 mg/ml dopamine in Tris buffer. The mixed solution was continuously stirred for 12 hours and then extruded through a polycarbonate membrane with a mean pore size of 200 nm.

6.2.5 Preparation of nanofiltration membrane encapsulated with proteoliposomes

The extruded PDA coated proteoliposomes were deposited on the PAI membrane substrate for 1.5 hours. Upon removal of the proteoliposome solution, a 15 ml 0.5% (wt%) PEI in Tris buffer solution was deposited on the membrane surface for crosslinking in water bath at 70°C for 2 hours. At a temperature of 70°C, branched PEI molecules could react with PAI membrane surface to form a rejection layer that was several hundred nanometers thick (Setiawan *et al.* 2011).

6.2.6 Membrane characterization and water flux & rejection measurements

Fluorescence images were obtained with LSM 710 confocal microscopy system (*Carl Zeiss, Germany*) using a 40× objective lens (excitation light 458 nm). For fluorescence characterizations, the lipid was mixed with 1 mol% NBD-PE in all experiments. Samples for fluorescence microscopy characterization were prepared differently from other samples; these samples were heated at 70°C for only 20 minutes in order to control the thickness of PEI layer and to prevent the proteoliposomes from being fully covered by the PEI layer. Scanning electron microscopy (SEM) images were obtained at room temperature. Prior to the SEM characterization, samples were dried in desiccators for at least 24 hours. The permeability of the liposomes and proteoliposomes was analyzed by a stopped-flow apparatus (*SX20, Applied Photophysics*) at 22°C. In this process the liposomes and proteoliposomes were rapidly mixed with hyperosmolar phosphate buffer containing 600 mM sucrose. The shrinkage rates of the liposomes and proteoliposomes were monitored by light scattering. Light scattering was recorded

at an emission line wavelength of 500 nm. Each stopped-flow experiment gave five light scattering traces, which were subsequently averaged. The initial rise rate (k) was calculated by fitting the raw data of the averaged curve. The water permeability of the liposomes, P_f ($\mu\text{m/s}$), was calculated using the equation 2.4.

The water flux and salt rejection were measured by using a standard stirred dead-end cell (*Millipore*) and a 100 ppm MgCl_2 solution as feed under 1 bar pressure. The water flux was calculated based on the permeate weight over a certain period of time based on the equation 5.1. The salt rejection was attained by measuring the salt concentration in the feed and permeate based on the equation 5.2. The effective area of the membrane was 28.26 cm^2 . All flux and rejection measurements were carried out at 22°C .

6.3 RESULTS AND DISCUSSION

6.3.1 Properties of proteoliposomes with AqpZ or mutants

It has been proposed that water permeability of AqpZ channel was mediated by the 189th amino acid, arginine (Jiang *et al.* 2006, Xin *et al.* 2011b). The mutants used for the experimentation protocol were mutated in the arginine. The mutation of highly conserved arginine into a different amino acid like alanine resulted in inactivation of channel without change in its tetrameric state. The main advantage of using these mutants is maintaining the same structure as AqpZ but with a loss in functionality. Figure 6.2 shows typical normalized stopped-flow curves of liposome, proteoliposomes with mutants and proteoliposomes with AqpZ. In comparison with the liposomes and proteoliposomes with mutants, the proteoliposomes with AqpZ gave a notably higher shrinkage rate, which was similar to the observation in our previous work although the conditions were different. Their calculated permeability values are displayed in Table 6.1. Correspondingly, the AqpZ incorporated proteoliposomes provided the highest water permeability and their calculated permeability value per subunit was comparable to our previously reported value, which confirmed that AqpZ could maintain its activity in present conditions. In contrast, the mutant one gave a significantly lower water permeability due to the change in its channel structure (Zhao *et al.* 2012).

Nevertheless, its permeability was still higher than that of the liposome, indicating that the mutant still could facilitate water transport to some degree because the mutation did not make the water channel lose its function completely (Borgnia *et al.* 1999b).

Figure 6.2. Normalized stopped-flow curves of liposomes, proteoliposomes with AqpZ and mutant.

The incorporation of AqpZ/mutants into liposomes had altered the size of the proteoliposomes that became considerably smaller but more uniform (as shown in Table 6.1). Moreover, the proteoliposome carried more negative charges than the liposomes because the incorporated proteins were negatively charged under the buffer pH condition. It was also reported that the size and surface charge of the vesicle had substantial effects on the interaction between the vesicle and the solid surface (Reimhult *et al.* 2003, Tang *et al.* 2009b). In addition, the proteoliposome possessed a greater mechanical strength than the liposome and therefore the proteoliposome could nearly maintain its vesicle shape under pressure, while the liposomes would fuse into bilayers under the same conditions, which were reported by other researchers (Wang *et al.* 2011) and discussed in Chapter 4. In comparison to the liposome, the proteoliposome with mutants displayed properties more similar to the proteoliposome with AqpZ, except for the permeability. Therefore, in the

present study, the proteoliposome with mutants was selected as the control group instead of the liposome without AQPs.

Table 6.1. Properties of liposome, proteoliposomes with AqpZ and mutants in Tris buffer.

	Mean size (diameter/nm)	Polydispersity index (PDI)	Zeta potential (mv)	Permeability ($\mu\text{m/s}$)
Liposome	139.8	0.132	-15.8	34.9
Proteoliposome with AqpZ (LPR 400)	107.8	0.094	-20.3	266.9
Proteoliposome with mutants (LPR 400)	108.8	0.087	-21.5	84.6

6.3.2 PDA coating and thermal treatment effects on AqpZ

Before incorporating the proteoliposomes into this selective layer, the proteoliposomes were decorated with polydopamine (PDA) to enhance its affinity to the membrane substrate. PDA is a novel bio-compatible polymer that can adhere to many surfaces, including liposome (Westen *et al.* 2012) via covalent or non-covalent bonds (Lee *et al.* 2007). The effects of PDA and thermal treatment on the activity of AqpZ and the mutant were investigated by the stopped-flow apparatus. The results are shown in Figure 6.3. Upon coating with PDA, there was a little increase in the permeability of the proteoliposome with mutants, while no significant change in the permeability was observed even after being heated at 70°C for 2 hours. Slightly different from its mutant counterpart, the permeability of the proteoliposome with AqpZ did not significantly change after being coated with PDA. However, approximately a 10% decrease in the water permeability occurred after the thermal treatment. Although the thermal treatment caused different effects on the permeability of different proteoliposomes, the permeability values of the proteoliposomes with AqpZ were nearly twice the permeabilities of those with mutants after the same treatment. This result also demonstrated that the AqpZ

tetramers were fairly stable, even in a harsh in-vitro environment.

Figure 6.3. Water permeability of proteoliposomes with mutants (left group) and AqpZ (right group) after different treatments: non-coated, PDA coating, PDA coating and thermal treatment (70°C, 2h). Error bars were obtained from five independent measurements.

6.3.3 Performance and structure of the membranes with proteoliposomes

During the encapsulation of the proteoliposomes, PDA could serve as a bridge between the proteoliposomes and the membrane surface due to its excellent adhesion to a wide range of materials (Lee *et al.* 2007). Moreover, the internal gap between the proteoliposome and PEI layer could also be eliminated by PDA via amine-catechol reactions (Lee *et al.* 2007). In order to minimize experimental errors, PAI membranes with a water permeability of around $120 \pm 20 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1} \cdot \text{bar}^{-1}$ were selected as the substrates; this substrate had no rejection to salt. Figure 6.4 shows the results for the water flux and salt rejection of the membranes after PEI crosslinking with the PAI membrane. The flux of the PAI-PEI membrane without immobilized proteoliposomes in its selective layer was around $32.7 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ while the rejection was only 93.8%. The membrane encapsulated with proteoliposome with mutants had a lower flux of around $19.9 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ but had a higher salt rejection (~95.6%), which might be due to the high resistance of mutant to water

and any other solute. The AqpZ-incorporated membrane exhibited an equivalent salt rejection but with a much higher water flux ($30.5 \text{ L}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$), which was ~50% higher than that of the mutant. The AqpZ-based membrane exhibited a superior purification performance to the control group, which should be attributed to the high permeability of the active AqpZ molecules in the selective layer. As the crosslinked PEI layer carried positive charges at the measurement pH condition, the charge of membrane surface was one main contribution the high salt rejection (Setiawan *et al.* 2011).

Figure 6.4. Water flux and MgCl_2 rejection of different membranes. Membrane without vesicles represents the PAI-PEI membrane without vesicles. Membrane with mutants or AQPs represents the membrane crosslinked with vesicles with mutants or AQPs (LPR 400). Error bars were obtained from three independent measurements.

To understand the above results, it is necessary to analyze the structures of the selective layers of the different membranes. The introduction of the proteoliposomes into the PEI layer and the proteoliposomes integrated into the crosslinked PEI layer can be explained in two different ways: either proteoliposomes were partially embedded into the layer or entirely embedded. These would be determined by the size of proteoliposomes and the thickness of the crosslinked PEI layer. The SEM images in Figure 6.5A and 6.5B clearly show that

the proteoliposomes were completely embedded into the crosslinked PEI layer. Additionally, these embedded proteoliposomes maintained their spherical shape even after PEI crosslinking and flux measurements. The confocal fluorescence images (Figure 6.5a, 6.5b) also show a reasonable coverage of the proteoliposomes in the membrane.

Figure 6.5. SEM images (A and B, scale bar 100 nm) and confocal fluorescence microscopy images (a and b, scale bar 50 μ m) of different membranes with LPR 400.

In this study, a low concentration of salt solution and high stirring speed were used to minimize the effect of osmotic pressure. By assuming that all the introduced proteoliposomes were fully encapsulated into the selective layer, a simplified model is shown in Figure 6.6 to help understand our experimental results. After the proteoliposomes were encapsulated into the PEI layer, they would function as a part of the selective layer. J_1 , J_2 , J_3 represent the equivalent fluxes of the PEI layer, the proteoliposome with mutants and the proteoliposome with AqpZ in the selective layer, respectively. r_1 , r_2 and r_3 refer to their equivalent salt rejection correspondingly. According to our experimental results, one could obtain that

$J_1 > J_3 > J_2$ while $r_3 = r_2 > r_1$. As the mutants could not transport water molecules efficiently, a proteoliposome with mutants could be considered as an impermeable (or less permeable) vesicle (Figure 6.6B).

The relationship between the water flux and membrane resistance as well as the relationship between the rejection and trans-membrane pressure can be described as follows;

$$J_v = \frac{\Delta P - \Delta \pi}{\mu R_m} \times 100\% \quad (6.1)$$

where J_v represents the water flux, ΔP is hydrostatic pressure difference across the membrane, $\Delta \pi$ is the osmotic pressure, μ is the viscosity of water and R_m is the resistance of the membrane. The introduction of proteoliposomes with mutants increased the resistance of the selective layer, resulting in a decrease in the water permeance, according to the equation 6.1.

Figure 6.6. Schematic illustration of different membrane types. A represents the crosslinked PEI area with the same size as a vesicle; B represents the proteoliposome with mutants; C represents the proteoliposome with AqpZ. (not to scale)

In contrast, the AqpZ incorporated proteoliposome could be considered as a highly water-permeable vesicle which could facilitate the transport of water molecules (Figure 6.6C) and dramatically decrease the resistance of the selective layer, thus leading to a much higher flux than the mutant one. Since both the lipid bilayer and AqpZ had a high rejection to salt molecules (Zhao *et al.* 2013), a lower salt

permeability (B value) would be obtained by integrating the proteoliposomes with the PEI rejection layer, based on the equation 3.4. This was probably the reason why the rejection still increased although the flux decreased, based on the equation 3.3, equation 3.4 and equation 3.5. Although AqpZ could facilitate the transport of water molecules, the flux of the AqpZ-based membrane was still slightly lower than the PEI layer ($J_1 > J_3$). This indicated that the proteoliposome within this LPR (400) still had a higher water flux resistance than the crosslinked PEI layer.

6.3.4 Water flux and salt rejection of membranes with proteoliposomes with higher AqpZ density

As it has been demonstrated that the incorporated AqpZ could enhance the water permeability of the proteoliposome, the performance of an AqpZ-based biomimetic membrane may benefit from a further increase in the AqpZ density incorporated in the proteoliposome. Proteoliposomes with LPR 200 and LPR 100 were used to prepare the biomimetic membranes by the same procedures. The stopped-flow results had shown that these two groups of proteoliposomes owned much higher permeability even after PDA coating and thermal treatment, as shown in Figure 6.7A. The results displayed the similar values and trend to the ones investigated in Chapter 4 and Chapter 5, indicating the PDA and thermal treatment had little effect on the activity of the AqpZ. The membrane with LPR 200 produced the highest flux, $\sim 36.6 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ with similarly high salt rejection ($\sim 95\%$) to the one with LPR 400 (Figure 6.7B). This demonstrated that a greater amount of AqpZ molecules would provide more paths for the passage of water molecules. It was interesting to observe that when the AqpZ density increased to LPR 100, the water flux of the membranes did not increase; instead, on the contrary, the flux decreased to around $27.3 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ though the salt rejection still remained at high level.

Figure 6.7. (A) Permeability of proteoliposomes after PDA coating and thermal treatment (70°C , 2h) at different protein-to-lipid ratios and (B) water flux and salt rejection of membranes at different LPRs. Error bars were obtained from three independent measurements. (Testing conditions: 1 bar, 100 ppm MgCl_2)

However, the stopped-flow results showed that the proteoliposome with LPR 100 could still provide higher permeability even after PDA coating and thermal treatment (Figure 6.7A). Based on the previous analysis, the decrease of water flux was thought to be caused by the increase of resistance in the rejection layer. Thus it was suspected that the proteoliposomes with a higher AqpZ density (LPR 100) became less permeable than those with LPR 200 after being embedded into the crosslinked PEI layer. When the density of the AqpZ was further increased to LPR 50, the permeability of proteoliposome did not increase but decreased as shown in the stopped-flow test. A similar phenomenon was also reported by other researchers (Borgnia *et al.* 1999b, Wang *et al.* 2012) and the work introduced in Chapter 4 and Chapter 5. Nevertheless, this result showed that the permeability of the proteoliposome with LPR 50 was still higher than those of proteoliposomes with LPR 400 and LPR 200 for the same conditions. However, the membrane embedded with proteoliposome with LPR 50 gave the lowest water flux but a similarly high salt rejection. These results suggested that proteoliposome with the highest density of AqpZ became significantly less permeable but still kept a high rejection to salt, after being encapsulated into the PEI layer. In the present study, the LPR 200 might be close to the optimal ratio for the water flux and salt rejection. Although the same

proteoliposome was used in the stopped-flow test and for the preparation of the biomimetic membranes, two different optimal LPRs were found in two different systems. One probable reason was the crosslinking process between the PEI molecules and the PAI substrate may interfere with the interaction between the tetramers at a high density, leading to the proteoliposome with a high protein ratio losing its function. The high rejection maintained for the membranes with high protein ratios suggested that embedded proteoliposomes still kept an intact structure although the AQPs inside may lose their function. However, the exact mechanism in these two systems needs to be further investigated in the future study.

As the introduced proteoliposome was completely embedded in the crosslinked PEI layer, the composite layer could withstand a relatively higher pressure than the isolated or semi-embedded proteoliposome. In addition, this layer could also provide a shield to protect the proteoliposome from the impurities or toxic chemicals in the feed, which could help maintain the activity of the embedded AQP. Besides, the modification or post-treatment of membrane surface would probably have little effect on the completely embedded proteoliposomes.

Table 6.2 summarizes the permeation and separation performance of different AqpZ-based biomimetic NF membranes reported in some recent studies. It showed that many protocols were created and tried to produce an AQP-based membrane. In combination with the literature review in Chapter 2.6, it is suggested that vesicle fusion was the main method to design a biomimetic membrane in the early stage of research. But recently it appears that the research has started to focus on the utilization of intact vesicles instead of a planar lipid or copolymer membrane, indicating this protocol may be favourable in preparing the biomimetic membrane.

However, all other NF membranes based on AQPs reported were designed or prepared for an extremely small membrane area, which indicated that some difficulties might be encountered in scaling up. In contrast, the AqpZ-based biomimetic membrane obtained in this current work offered a superior performance to other biomimetic NF membranes, in terms of the effective membrane area, water flux and salt rejection under comparable testing conditions. The biomimetic

membrane designed in this current work still exhibited a promising performance as compared to selected commercial NF membranes under the same testing conditions. As discussed above, the performance of this membrane may be further enhanced by improving the loading amount of proteoliposomes in the crosslinked PEI layer or optimizing the crosslinked layer.

Table 6.2. Comparison of different AQP-based biomimetic NF membranes.

Approaches of preparing nanofiltration membranes	Permeability ($\text{L}\cdot\text{m}^{-2}\cdot\text{h}^{-1}\cdot\text{bar}^{-1}$)	Salt rejection (%) (test conditions)	Membrane area (cm^2)	Reference
Proteopolymersomes fused on silanized cellulose acetate membrane	34.19±6.90	32.86±9.12 (200 ppm NaCl, 5bar)	0.07065	(Zhong <i>et al.</i> 2012)
Proteopolymersomes fused on gold-coated porous alumina membrane	8.2±5.1	45.1±4.2 (200 ppm NaCl, 5bar)	0.20	(Duong <i>et al.</i> 2012)
Immobilized proteoliposomes were crosslinked on a pretreated PAN membrane	~2.8	~90 (200 ppm MgCl_2 , 5bar)	0.785	(Sun <i>et al.</i> 2013c)
Immobilized proteoliposomes were coated by a layer-by-layer assembled film on PAN	~6.1	~95.8 (200 ppm MgCl_2 , 4bar)	0.785	(Sun <i>et al.</i> 2013b)
Immobilized proteopolymersomes were coated by a layer formed by polymerization	22.9	~75 (200 ppm MgCl_2 , 5bar)	0.196	(Xie <i>et al.</i> 2013)
Proteoliposomes were totally embedded in a selective layer by crosslinking on a PAI	36.6±2.2	95.0±0.70 (100 ppm MgCl_2 , 1bar)	28.26	Present work
Commercial NF-270 flat sheet	15.9±1.1	98.5±0.5 (1mM Na_2SO_4 , 1bar)	–	Present work
Commercial NTR-7450 flat sheet	10.1±1.1	98.3±0.9 (1mM Na_2SO_4 , 1bar)	–	Present work

6.4 CONCLUSIONS

An NF-type biomimetic membrane based on AqpZ has been designed and developed in this present study. PDA coated proteoliposomes were first deposited onto the substrate surface and PEI was then crosslinked with the PAI substrate to embed these deposited proteoliposomes. The SEM images showed that the whole proteoliposome could be embedded into the PEI selective layer. The proteoliposome with mutants was selected to be the control group as it had properties more similar to the proteoliposome with AQPs, in comparison with liposome. Compared to the control group, the AqpZ-based membrane provided a much higher water flux. At LPR of 200, the water flux and the rejection to divalent salts could reach $36.6 \text{ L}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ and 95%, respectively. This performance was superior to any other reported AQP-based NF membranes to date in comparable conditions. However, further increase of the AqpZ density in the proteoliposomes could not improve the water flux any further, although the proteoliposome had a higher water permeability as shown in the stopped-flow measurements. Our experiment results revealed that the AqpZ molecules could maintain a relatively high activity even under harsh environmental conditions of thermal treatment at 70°C for 2 hours. More importantly, our findings provided useful protocols for the preparation of the next generation of biomimetic membranes in a simple and scalable approach.

CHAPTER 7 CONCLUSIONS, IMPLICATIONS AND RECOMMENDED FUTURE RESEARCH

In this chapter, some main conclusions and implications based on the entire thesis work are described and some recommendations for future research are provided.

7.1 CONCLUSIONS

This entire thesis has explored how to design and fabricate high performance biomimetic membranes based on aquaporins (AQPs) for desalination applications. Different protocols were employed in the study. The first protocol was to fuse proteoliposomes on a permeable polymer substrate. However, the results and further study conducted by quartz crystal microbalance with dissipation (QCM-D) showed that it was difficult to make proteoliposomes completely fused to bilayers on a polymer substrate. Thus, an alternative protocol was proposed to prepare the membrane. The intact proteoliposomes were completely encapsulated into the selective layer of a membrane. The resultant reverse osmosis (RO) membrane and nanofiltration (NF) membrane with AQPs presented high separation performances. The thesis work has led to some basic understanding regarding in AQP reconstitution into liposomes, fusion behaviour of vesicles, integration of proteoliposomes within a substrate and some critical principles for preparing a biomimetic membrane. From this work, some principal conclusions were obtained as follows:

- An externally applied pressure could assist the fusion of vesicles on a permeable substrate very efficiently.
- A lipid membrane with low defect density could be obtained by vesicle fusion in combination with a spin-coating method. However, it is very challenging to obtain a defect-free lipid biomimetic membrane with a large area by vesicle fusion because of the vulnerability of the thin lipid bilayer.
- Because of the incorporated membrane proteins, the proteoliposome had a different vesicle size, carried more surface charge and became more robust, in compared with the liposome.

- The mechanical strength of proteoliposomes increased with increasing content of the incorporated AQPs.
- More AQPs could be incorporated into the liposomes beyond the optimal lipid-to-protein ratio though the nominal permeability would decrease.
- Vesicles tended to keep intact instead of fusing to the bilayers upon interacting with a polymer surface. It appeared that the polymer had a stabilizing effect on the vesicles.
- Proteoliposomes could be encapsulated completely into the polyamide layer of a thin-film composite membrane and maintain their activity. The resultant membrane with AqpZ presented the highest water permeability and comparable salt rejection, in comparison with current commercial RO membranes.
- The results indicated that AqpZ could maintain its activity in the polyamide layer and it was the encapsulated AqpZ that enhanced the water permeability of the membrane.
- Embedding proteoliposomes into the selective layer of a membrane is a better alternative way to prepare AQP-based membranes because it could effectively prevent defect formation and protect the AQPs from external influences.
- The AQP-based hollow fibre composite membrane is a new type of membrane promising to help further reduce the energy cost for seawater or brackish water desalination significantly, attributing to its high water permeability and salt rejection.
- A high performance NF membrane could be obtained by encapsulating proteoliposomes into the selective layer formed by crosslinking poly(ethyleneimine) with a poly(amide-imide) substrate.
- The proteoliposomes with mutants had similar properties to the ones with AQPs except for the water permeability, which was more suitable for a control group.
- In contrast to the proteoliposome system, the biomimetic NF membrane required a different optimal lipid-to-protein ratio to obtain the highest performance.

- AqpZ molecules could maintain their activity even after a relatively long-time high-temperature thermal treatment, which proved that AqpZ had a fairly good stability.

7.2 IMPLICATIONS

7.2.1 What type of protocol is desirable to prepare a good biomimetic membrane?

The supported lipid membrane protocol was the first protocol used to prepare an AQP-based biomimetic membrane. However, in this work, it indicated that this protocol might not be a feasible way because of three main reasons: (i) a sizeable and defect-free membrane was difficult to obtain using this protocol as this supported lipid layer was easily distorted by the support and external influences. (ii) the incorporated AQPs were exposed to the external environment and thereby were vulnerable to foreign influences, such as fouling. (iii) The integration between the lipid layer and the support lacked a strong affinity to withstand the long-term filtration process. These challenges added too much uncertainty to make a commercially available membrane. Over the past two years, many approaches were proposed to design and prepare AQP-based biomimetic membranes and each one had its own merits and demerits. Here some considerations regarding to evaluating a good protocol for the design of an AQP-based biomimetic membrane are provided;

- The protocol can be readily scaled up for membrane fabrication.
- The incorporated AQPs must be maintained active (or to some extent) and have a good integration with their matrix.
- The long-term stability of the resultant biomimetic membranes should be ensured.
- The membrane should achieve (or potentially achieve) good performance in terms of water permeability, salt selectivity and fouling resistance.

7.2.2 Stability of aquaporin Z

In this work, aquaporin Z (AqpZ) was the only candidate for preparation of the biomimetic membranes because it is easy to express and purify using a recombinant *E.coli* strain. This work showed that the AqpZ presented not only a high water permeability but also good stability. In Chapter 5, the results indicated that AqpZ could still maintain its functionality after being embedded into a polymer matrix through interfacial polymerization. In Chapter 6, even after undergoing the crosslinking reaction in a high-temperature environment (70°C for 2 hours), apparently AqpZ still maintained its activity to some degree. In the different parts of this work, different buffers were used to prepare proteoliposomes including a phosphate buffer, Tris buffer and water. Stopped-flow results confirmed that AqpZ molecules maintained good activity in these buffers ranging from pH 5.0 to pH 8.0. In view of other studies of AqpZ, it can be found that the AqpZ could maintain its activity even in various types of lipids or polymers, or through chemical crosslinking, or undergoing layer-by-layer process by polyelectrolytes. Although a systematic study on AqpZ stability is still lacking, this finding may provide a guide to obtain a better AQP-based biomimetic membrane.

7.2.3 Application prospects for AQP-based hollow fibre membranes

It is believed that this highly permeable hollow fibre membrane based on AQPs could dramatically reduce the energy consumption of the reverse osmosis (RO) process. In terms of applications a high permeability thin-film composite hollow fibre based on AqpZ could potentially reduce the footprint and energy demand. Operation at an elevated flux may not be an option because it could lead to increased concentration polarization and fouling. However, hollow fibre modules can have a higher packing density than the spiral wound module. For example, based on the present study it is estimated that at least 19,000 AQP-based hollow fibres could be assembled in a standard 8-inch commercial membrane module (assuming a 80% packing density). The hollow fibre module can provide about 59 m² of membrane area, which is about 50% more than a commercial spiral wound module (~ 37 m²), allowing a smaller foot print. In terms of energy demand, for a targeted permeate flux or recovery, a lower net operating pressure would be

required by using this highly permeable membrane, and thereby the power consumption for the pump can be reduced. Due to the constraint of hollow fibre mechanical strength, the most likely applications are brackish water desalination and water reclamation. We estimated that, if this membrane is applied in a water reclamation plant, such as a NEWater plant in Singapore, to treat industrial and municipal effluent (Dow 2008), Assuming the salt concentration is 400 ppm and the applied pressure for original membrane module is 6 bar. The relation among the average osmotic pressure π_{ave} , the osmotic pressure of original feed π_0 , the net driving pressure (P_{net}), the pressure drop in one module (ΔP_L) and the recovery (Y) could be described by following equations (Zhu *et al.* 2008)

$$\Delta\pi = \pi_{ave} \cdot \frac{\ln\left(\frac{1}{1-Y}\right)}{Y} \quad (7.1)$$

$$P_{net} = \frac{\pi_{ave}}{1-Y} - \Delta\pi \quad (7.2)$$

$$\Delta P = P_{net} + \frac{\Delta P_L}{2} + \Delta\pi \quad (7.3)$$

If the recovery for this application is around 75%, the average osmotic pressure in the module is about 0.628 bar based on the equation 7.1. Under the same conditions, as the AQP-based hollow fibre membrane could offer a water flux 2 times as high as that commercial one, the net driving pressure required is about 2.26 bar. If the pressure drop is also about 1.7 bar, the applied pressure should be 3.74 bar. The osmotic pressure of feed at the end of module could reach 1.36 bar because of the high recovery, which would be still under the operation limitation. Therefore, the power consumption for the feed pump could be cut by up to 40%, in comparison to the commercial BW 30 membrane. Such a significant energy saving could make the AQP-based hollow fibre membrane become more attractive for low-pressure RO applications. For forward osmosis (FO) applications, the AqpZ-based hollow fibre membrane could assist in reducing the demand of the draw concentration to achieve an acceptable flux, thereby reducing the energy consumption for water extraction from the draw solution. For the thin-film composite hollow fibres described here, most of the proteoliposomes were totally embedded in the polyamide layer, and this

would help them withstand the high applied pressure and protect them from external environmental influences, such as membrane fouling or cleaning.

7.3 RECOMMENDED FUTURE RESEARCH

Based on the previous works, some recommendations on future research in this field are made as follows:

7.3.1 Optimization of the AqpZ-based hollow fibre membrane

The AqpZ-based hollow fibre membrane prepared in this work offered an excellent performance. It was believed that this membrane may benefit from further optimization of the preparation conditions. As discussed in Chapter 5, the loading amount of the proteoliposomes may determine the water permeability of this membrane. Some work would be required to increase the loading amount of proteoliposome in the polyamide layer. This could be achieved by enhancing the affinity between the proteoliposomes and the support through chemical modification. In comparison to the physical attractive forces such as van der Waals force and electrostatic forces, covalent forces or coordination bonding are stronger to stabilize the proteoliposome on a support. Additionally, the water permeability and salt selectivity of the membrane were also affected by the quality of the polyamide layer prepared through interfacial polymerization. As extensive work had been focused on how to get a high-quality polyamide layer over past decades, the experience in this field, in combination with AQP engineering, may provide more choices to attain a better membrane.

7.3.2 Other application prospects of the AQP-based hollow fibre membrane

With respect to the excellent performance in low-pressure RO test and FO test as shown in Chapter 5, it is believed this technique can also be applied to fabricate a nanofiltration (NF) membrane (Fang *et al.* 2013), or pressure retarded osmosis (PRO) membrane (Chou *et al.* 2012) which were also prepared by interfacial polymerization for the selective layer formation. To date most work on AQPs focused on their water-transport function. However, it was also reported that one member of the AQP family, AQP1, has a major pathway for CO₂ transport across

the biological membrane (Endeward *et al.* 2006). This important finding indicated that an efficient CO₂ separation membrane could be obtained through tailoring the membrane materials using AQP1. As a gas separation membrane is generally a membrane with a dense layer, the proteoliposome with AQP1 can be embedded into this dense layer by the protocol used in this work, to act as a selective channel for CO₂.

7.3.3 Selecting block copolymers as a platform for AQPs

In this work, lipids were the only candidate for the platforms of AQPs. Nevertheless, as reviewed in Chapter 2, the ABA type polymer has already been proven as a good platform for AQPs in many studies. Additionally, it is regarded as a better platform for AQPs because of its good mechanical and chemical stability, high selectivity, and ease of functionalization. These superior properties may provide more choices to tailor the membrane materials. For example, the high selectivity of block polymers could improve the salt rejection of the membrane. Polymer functionalization by other molecules could help immobilize or recognize the vesicles in the membrane. Therefore, the ABA type of block copolymer is probably a promising material for the AQP-based biomimetic membranes in future studies.

7.3.4 Fundamental understanding of the AQP biomimetic system

Although a surge is emerging in the engineering application of AQPs, the fundamental understanding of AQPs and biomimetic membranes is still quite limited. One example is that so far it is not clear why there is an optimal LPR in proteoliposomes or proteopolymersomes to attain the highest permeability; this phenomenon was observed in different experimental conditions. It was proposed that this phenomenon might be involved with the inefficient incorporation of AQPs, while our work introduced in Chapter 4 demonstrated that more AQPs could still be incorporated into a liposome even though its nominal permeability decreased. Further exploration on these problems may help understand more about this system and give us guidance in the design of biomimetic membranes.

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