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Tuning biomimetic membrane barrier properties by hydrocarbon, cholesterol and polymeric additives

Marta Espina Palanco‡, Nils Skovgaard§, Jesper Søndergaard Hansen†, Kirstine Berg-Sørensen ‡ and Claus Hélix-Nielsen *‡

‡Technical University of Denmark (DTU), Department of Physics. 2800 Kgs. Lyngby, Denmark, §Copenhagen University (KU), Department of Drug Design and Pharmacology.
9 2100 Copenhagen, Denmark, †Lund University, Department of Experimental Medical Science. 22100 Lund, Sweden, *‡Technical University of Denmark (DTU), Department of Environmental Engineering. 2800 Kgs. Lyngby Denmark & University of Maribor, Faculty of Chemistry and Chemical Engineering, 2000 Maribor, Slovenia. E-mail: clhe@env.dtu.dk

‡These authors contributed equally to this work.

ABSTRACT: The barrier properties of cellular membranes are increasingly attracting attention as a source of inspiration for designing biomimetic membranes. The broad range of potential technological applications makes the use of lipid and lately also polymeric materials a popular choice for constructing biomimetic membranes, where the barrier properties can be controlled by the composition of the membrane constituent elements. Here we investigate the membrane properties reported by the light-induced proton pumping activity of bacteriorhodopsin (bR) reconstituted in three vesicle systems of different membrane
composition. Specifically we quantify how the resulting proton influx and efflux rates are influenced by the membrane composition using a variety of membrane modulators. We demonstrate that by adding hydrocarbons to vesicles with reconstituted bR formed from asolectin lipids the resulting transmembrane proton fluxes changes proportional to the carbon chain length when compared against control. We observe a similar proportionality in single-component 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) model membranes when using cholesterol. Lastly we investigate the effects of adding the amphiphilic di-block copolymer polybutadiene-polyethyleneoxide (PB$_{12}$-PEO$_{10}$) to phospholipid membranes formed from DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), and 1,2-Dioleoyl-sn-glycero-3-phosphatidylserine (DOPS). The proton pumping activity of bR (measured as a change in extra-vesicular pH) in mixed lipid/PB$_{12}$-PEO$_{10}$ lipid systems is up to six-fold higher compared to that observed for bR containing vesicles made from PB$_{12}$-PEO$_{10}$ alone. Interestingly, bR inserts with apparent opposite orientation in pure PB$_{12}$-PEO$_{10}$ vesicles as compared to pure lipid vesicles. Addition of equimolar amounts of lipids to PB$_{12}$-PEO$_{10}$ results in bR orientation similar to that observed for pure lipids. In conclusion our results show how the barrier properties of the membranes can be controlled by the composition of the membrane. In particular the use of mixed lipid-polymer systems may pave the way for constructing biomimetic membranes tailored for optimal properties in various applications including drug delivery systems, biosensors and energy conservation technology.

1. INTRODUCTION

Fabrication of biosynthetic composite materials, incorporating biological constituents into synthetic matrices, is being actively investigated in basic research and for applications in biotechnology and medicine$^{1-3}$. The use of membrane proteins is of particular interest due to their unique biological properties$^4$. Biological cell membranes per se act as effective barriers
to the transmembrane flow of polar/charged solutes$^5$ and membrane proteins facilitate
selective transport of molecules across cell membranes$^6$. The molecular transport selectivity
of these proteins is so unique that functional replication using synthetic materials has proved
very challenging$^{7,8}$. Thus, harnessing this class of proteins in artificially made (biomimetic)
membranes could, by virtue of their unique transport properties, provide means to create
advanced materials with unprecedented properties and technological application potentials$^9$.

Despite considerable progress, accommodation of active membrane proteins in biomimetic
membranes remains a challenge, which thus far has limited the commercial use of membrane
proteins in large-scale technological applications$^{10}$.

The permeability barrier properties of biological membranes entail that membrane proteins
are inserted into the lipid bilayer without compromising these properties and/or
compromising protein function and stability. The function of any biological membrane
depends on its lipid composition and the controlled presence of proteins selected from the
thousands of possible membrane proteins, conferring stability and permeability to cellular
membranes$^{11,12}$. The most abundant membrane lipids are the phospholipids and sphingolipids.

Amongst these, a few lipid types predominate in the plasma membrane of many mammalian
cells and among those most frequent are phosphatidylcholine and sphingomyelin. Both are
neutrally charged lipids under physiological conditions and provide stable bilayers$^{13}$. The
lipid bilayers of biological cell membranes are not singularly composed of phospholipids, but
often also contain cholesterol and glycolipids. With its steroid structure, cholesterol differs
from both phospholipids and sphingolipids and functions as a precursor for hormone
production. Several studies show that the ring-based sterols, including cholesterol, greatly
enhance the permeability barrier properties of the lipid bilayer and therefore have an
inhibiting effect on passive diffusion of ions across membranes$^{11,13,14}$. Besides the amphiphilic
molecules of the bilayer, membrane barrier properties may also be modulated by
hydrocarbon-based solvents, which are naturally occurring in some biological membranes.

Dolichol in its hydrophobic ester form is found in high concentrations in the liver organelle membrane and similar isoprenoids are found in bacterial membranes, α-tocopherol is concentrated in mitochondrial and lysosomal membranes (1:64 phospholipid molecules) and ubiquinone is present in most eukaryotic cells, mainly in the mitochondria. The exact role of how these hydrocarbon-based solvents may influence the membrane barrier properties and membrane protein function is not well understood. However it is remarkable that mitochondrial membrane can retain transmembrane proton gradients as high as 1 pH unit across membranes delimiting mitochondrial compartments.

The microenvironment surrounding membrane proteins has before been reported to be modulated by lipid composition and hydrocarbon additives using aquaporins labeled with the polarity sensitive dye, Badan, as reporter system. In the work presented here we sought to investigate how different additives, i.e. hydrocarbon solvents, cholesterol and polymeric molecules, can modulate the membrane barrier properties as well as membrane protein activity. As a reporter, we used the light-driven proton pump Bacteriorhodopsin (bR). We established a pH-response assay, to monitor how modulation of the membrane matrix composition affects the transport of protons towards the interior of the vesicle (influx) and the exterior of the vesicle (efflux). One of the key properties of bR proton transporting dynamics is that bR only allows proton flux when activated and hence, it can be assumed that permeation of protons through the lipid membrane during the inactivation of bR is caused by either transport by other transmembrane proteins present or by passive diffusion through the lipid membrane.

To alter the composition and hence the barrier properties of lipid membrane containing bR a set of hydrocarbon solvents with varying chain length including squalene, decane, dodecane and hexadecane was used. These solvents were tested in diverse lipid environment primarily
consisting of soybean asolectin. In order to examine membrane barrier properties more precisely the permeability properties of 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) vesicles, a commonly used, simple model system, were investigated through addition of different concentrations of cholesterol, i.e. to see how the presence of sterols affects the proton flux. Lastly, lipid- mimicking polyethyleneoxide-polybutadiene (PEO10-PB12) di-block co-polymer (DBP) units was used as additives to investigate both their effect on the membrane barrier properties of vesicles containing bR due to the potential benefits in such mixed systems particularly in terms of their physical stability and biocompatibility.

Our results show that all systems showed significant effects on both membrane permeability and bR-activity in response to the additives as evidenced by extra-vesicular pH-measurements. Addition of hydrocarbons to asolectin vesicles showed that both influx and efflux kinetics changed significantly when solvents, such as decane and squalene, were added to the membrane. Thus addition of hydrocarbon solvents showed an inverse linear relationship between hydrocarbon chain length and the proton permeability for both active and passive transport. Similarly, addition of cholesterol resulted in a similar tendency with a decrease of proton permeability with increasing amounts of cholesterol showing how it is possible to increase and decrease membrane permeability simply by titrating with cholesterol.

Furthermore, we found that bR-activity and directionality are strongly altered by the addition of DBP to the lipid membrane. Interestingly, the presence of DBP also showed a strong effect on membrane permeability causing a tendency to retain the proton gradients and also to seemingly invert the bR-orientation at high polymer to lipid ratios. Our results show that bR reconstituted in mixed DBP/phospholipid systems results in a fast protein response with a large ΔpH upon illumination. This suggests that such mixed lipid-polymer systems may be advantageous in technological applications (e.g. light sensors) based on bR.
Generally, the results presented here addressing membrane barrier properties and the modulation hereof are relevant in biomimetic/biotechnological applications within drug delivery systems, separation technologies and chemical sensory techniques.

2. MATERIALS

2.1 Reagents. Lipids (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS)) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Octyl–β–D–glucopyranoside (OG) was acquired from Anatrace, Inc. (Maumee, OH, USA). Asolectin from soybean, cholesterol, squalene, n–decane, n-dodecane, and n-hexadecane were obtained from Sigma Aldrich Denmark (Brøndby, Denmark). Polybutadiene-polyethyleneoxide (PEO10–PB12) polymers were provided by Aquaporin A/S (Denmark). All other chemicals used were of analytical grade and purchased from commercial sources.

3. EXPERIMENTAL

3.1 Preparation of bR reconstituted large unilamellar liposomes and polymersomes. All vesicle systems were prepared accordingly to previously described work with small variations described further below. Lyophilized bR (~26kDa, extinction coefficient of 63000M⁻¹cm⁻¹ at A280nm) purple membranes were solubilized in a saline solution consisting of 0.15 M KCl with 1.3% OG. Large vesicles were created by dissolving soybean asolectin in 0.15 M KCl with 1.3% OG (8 mg/ml) and after extruded through a 400 nm polycarbonate filter. Lipid and protein was mixed in a lipid-to-protein ratio (LPR) of 300-600. The mixed protein–vesicles solution was dialyzed for 24 h in a dynamic microdialyzer dialysis device (Spectrum Laboratories Europe, Breda, The Netherlands) using a MWCO of 10,000 Daltons.
and a dialysate flow of 3 ml/min. Hydrocarbons were added in an approximately 33% vol/vol to the dissolved asolectin and incubated over night in the fridge while slowly rotating. Extrusion was performed as explained above.

Pure phospholipid vesicles were prepared by dissolving DOPC, DOPS or DOPE powder in chloroform. The lipid solution was dried under flowing nitrogen and liposomes were formed from film rehydration in a solution consisting of 0.15 M KCl with 1.3% OG for 2 days to a final lipid concentration of 10mg/ml. Extrusion was performed as explained above. In the cholesterol addition experiments cholesterol was added to the chloroform containing the dissolved lipids. The lipid-cholesterol films were then dried under flowing nitrogen and vesicles formed by rehydration and extrusion as described above.

DBP was mixed with DOPE, DOPS and DOPC and diluted in chloroform keeping a lipid-to-protein ratio (LPR) of 600. The polymer-lipid was dried under flowing nitrogen and the formed proteopolymersomes was then rehydrated in saline solution consisting of 0.15 M KCl with 1.3% OG for 2 days. Lyophilized bR purple membranes were added and the bR polymer vesicles were finally dialysed with BioBeads® for 24 hrs. Measurements of light induced proton flux were carried out in the same way as asolectin based bR vesicles. In addition, both liposomes and polymersomes with varying concentrations of phospholipids where prepared without protein as a control.

3.2 Bacteriorhodopsin photo induced pH-response assay. To assess the proton–pumping activity of bR 2.5 ml of the vesicle suspension was transferred to a 3 ml UV–cuvette with magnetic stirring and placed in a dark cabinet. A glass micro pH electrode (Microelectrodes Inc., Bedford USA) was placed into the vesicle suspension. The glass microelectrode was connected with an ORION 3 STAR pH-meter (Fisher Scientific) operated through Star Plus navigator software (Fisher Scientific), which enabled automated sampling of pH measurements. Illumination was provided with a cold–light generator equipped with an
incandescent halogen lamp of 200W with a light–guide (SCHOTT: model KL1500 LCD) with maximum emission spectrum at around 650 nm. The light produced was focused on the cuvette by means of a fiber optic cable placed 10 cm from the cuvette. The resulting light intensity on the sample were around 600 lumen. Before illumination, the sample was equilibrated in the dark for 30 min to permit pH stabilization and the pH baseline was subsequently recorded for 3 min. Illumination was carried out for 3 min followed by 7 min darkness while the pH was continuously sampled throughout the experiment (3 sec recording intervals).

For solvent partitioning experiments, proteoliposomes were mixed with hydrocarbon solvent as specified (1:3 oil phase:vesicles ratio) using gentle rotation mixing over night at room temperature. Solvent equilibrated proteoliposomes were transferred to a UV–cuvette and the bR light–induced proton activity assay repeated as described for the bR reconstituted proteoliposomes.

Polymersomes and proteopolymersomes were measured using dynamic light scattering (DLS) at the same concentrations as used for the light induced pH assay. DLS measurements were done using a Brookhaven BI200SM DLS system at an excitation wavelength of 636nm. Each measurement were set to 2 minutes and repeated 3 times. All data where averaged, exported in .txt format and plotted using Origin version 8 (OriginLab Corporation, Northampton, MA, US).

3.3 Data analysis. All pH-data obtained in the experiments was measured in discrete intervals of 3 seconds, during a 13 minutes time period, including influx and efflux periods. In order to quantify the proton flux rate across the membrane or through bR we obtained the influx and efflux time constants (τ) of each proteoliposome system by fitting normalized pH-values as a function of time to exponential decay functions (1) using Origin version 8:
\[ \Delta p\text{H} = A + B \exp \left( \frac{-t}{\tau} \right) \]  

where A is the final amplitude of the normalized curve, i.e. 1 for influx data and 0 for the efflux data, and B a negative constant value for influx data and a positive constant value for efflux. Thus, \( \tau \) describes the time for the \( \Delta p\text{H} \) value to reach 63% of the maximal value for influx and decrease to 37% of the maximal value for efflux.

4. RESULTS AND DISCUSSION

4.1 Influence of hydrocarbons as additive to bR-reconstituted liposomes.

Reconstitution of bR into asolectin lipid vesicles resulted in a preferential protein orientation where, upon illumination, protons were transported actively to the interior of the vesicles (\( H^+_{\text{out}} \rightarrow H^+_{\text{in}} \)) (Figure 1a). The net proton flux into the vesicles led to alkalization of the external bulk medium, which was measured by a pH micro-electrode. Inactivation of bR (by terminating illumination) inhibits the bR-proton pump effect resulting in passive proton diffusion across the membrane (\( H^+_{\text{in}} \rightarrow H^+_{\text{out}} \)) causing a pH decrease of almost 0.18 pH in the extra-vesicular bulk medium.

To assess how hydrocarbons of different carbon chain lengths affect protein activity and membrane barrier properties, bR-reconstituted asolectin liposomes were incubated over night with n-decane (C\(_{10}\)H\(_{22}\)), n-dodecane (C\(_{12}\)H\(_{26}\)), n-hexadecane (C\(_{16}\)H\(_{34}\)) and squalene (C\(_{30}\)H\(_{50}\)). pH-response assays of all the tested hydrocarbon proteoliposomes showed an active transport of protons through bR (Figure 1b) and passive proton diffusion across the membrane (Figure 1c) while no signal was observed for the control vesicles. Interestingly, in both cases the pH-response changed in a hydrocarbon chain length dependent manner. In order to examine their influx and efflux rates, the rate of alkalization of the medium (which starts after 180 seconds when bR is activated) and the rate of the acidification of the medium (which starts after 360
seconds, when bR is inactive) were extracted from Figure 1a. Our data indicates a linear relationship between hydrocarbon chain length and the influx time constants ($\tau$) where the decrease of the hydrocarbon chain length of the solvent leads to a concomitant increase in $\tau$ for proton influx (Figure 1d). Protein mediated proton influx notably slows down with a $>2$-fold increase in influx $\tau$ for liposomes containing n-decane compared to liposomes containing squalene, of which the influx rate resembled that of the natural asolectin membrane. Likewise, the proton efflux was diminished more than three-fold in n-decane containing liposomes indicating that addition of n-decane, the shortest chain length tested, results in a tighter membrane that decreases passive proton diffusion. For the hydrocarbon additives tested a somewhat similar inverse relation between tail length and permeability of the membrane appears to exist (with the exception of the lower $\tau$ observed for dodecane) (Figure 1d). Due to their smaller molecular size compared to lipids the alkanes (decane, dodecane and hexadecane) are likely to partition into the inner hydrophobic core of the bilayer resulting in a thicker inner hydrophobic membrane region\textsuperscript{20}, whereas the larger squalene may partition into the membrane to a lesser extent for steric reasons. Although we cannot ascertain the exact orientation, position, and degree of partitioning of the alkanes when embedded in the membrane, we note that they do indeed have significant influence on proton permeability barrier properties\textsuperscript{21}. 
Figure 1. Hydrocarbon additives affect bR-membrane properties in a chain-length dependent manner (a) Variations of pH across bR-asolectin model membranes influenced by light/darkness vesicle environment conditions with vesicle model membranes without bR working as a control. Normalized proton-influx upon illumination (b) and proton-efflux upon darkness (c) of bR-asolectin model membranes containing different mono-chain hydrocarbons. Black, blue, pink, red and green curves represent the proton transport through membranes made with pure asolectin, asolectin with n-decane, asolectin with n-dodecane, asolectin with n-hexadecane and asolectin with squalene respectively. (d) Comparison of time constants of the influx (black) and efflux (grey) rates based on an exponential decay function for each membrane model system. Standard deviations of τ from the curve fitting are in range of 1-4 seconds.
4.2 Influence of cholesterol on bR mediated proton transport in DOPC proteoliposomes. Similar to hydrocarbon solvents, the membrane barrier properties can also be modulated by cholesterol. In order to study cholesterol’s effect on the membrane barrier properties as well as on the protein activity, bR reconstituted in DOPC liposomes were incubated with cholesterol at different concentrations, i.e. 10%, 20% and 40% cholesterol molar concentrations. Our results showed that the addition of cholesterol to pure DOPC liposomes greatly influences the proton transport through both: the protein when bR is activated (Figure 2a) and the membrane due to the passive diffusion across the membrane (Figure 2b). Addition of cholesterol was found to have a dual role in active uptake of protons in DOPC vesicles. Modest amounts (10% and 20%) of cholesterol changed bR activity and inhibited the transport of protons, causing an increase in $\tau$ values, while high amounts (40%) resulted in a faster response of bR when compared to asolectin and DOPC model systems alone. Similarly, modest amounts of cholesterol decreased the rate of transport of protons through the plasma membrane drastically (Figure 3c), resulting in $\tau$ values that were up to two and a half times smaller than the control. Increasing the amount of cholesterol beyond 10% seemed to have a negative effect on the proton barrier properties of cholesterol, where addition of cholesterol to a final concentration of 20% or 40% reduced the otherwise improved barrier properties from low amounts of cholesterol. DOPC vesicles with 40% cholesterol resulted in the approximate same efflux rates as for the control system. Both influx and efflux showed a linear, inverse relationship between $\tau$ and the cholesterol content of DOPC membrane. With almost similar $\Delta$pH values as for the hydrocarbon solvent system, we conclude that cholesterol exhibit the highest decrease in permeability at a concentration around, or less than, 10%, resulting in a two- and three-fold slowing of proton flux when the proton pump is activated and deactivated respectively. It is also evident that the $\tau$ values for cholesterol containing liposomes are very close to that of hydrocarbon solvents in asolectin
based membranes despite the rather large difference in chemical structure between cholesterol and solvent hydrocarbon chains.

Figure 2. Cholesterol content efficiently alters bR-membrane properties. Normalized proton influx upon illumination (a) and efflux upon darkness (b) from phospholipids model

influx upon illumination (a) and efflux upon darkness (b) from phospholipids model
membranes with cholesterol. The black, red, blue, green, pink and light blue curves represent the proton transport through pure asolectin, pure DOPC, DOPC with 10% cholesterol, DOPC with 20% cholesterol and DOPC with 40% cholesterol and pure DOPC without any protein respectively. (c) Comparison of time constants of the influx (black) and efflux (grey) rates based on an exponential decay function for each membrane model system. Standard deviations of the inverse exponential curve fitting are in the range of 1-2 seconds.

4.3 Mixed amphiphilic di-block copolymer-lipid membranes. Recently, a promising synthetic biomimetic material has been introduced in the form of amphiphilic di-block copolymers (DBPs). Polymer based membranes have shown valuable properties in mimicking biological membranes as polymers generally have high stability when structured into vesicular forms\textsuperscript{22,23}. Compared to lipid bilayers, the permeability of DBP bilayers is decreased and overall polymeric membranes have been shown to be robust and highly stable membrane mimics\textsuperscript{22}. However, currently there are only a small number of examples where membrane proteins have been incorporated successfully into amphiphilic DBP based membranes\textsuperscript{24-27}. This is a reflection of the general challenge in protein reconstitution that the hydrophobic mismatch between the hydrophobic thickness of the membrane and the hydrophobic length of transmembrane proteins affect both insertion and function of proteins\textsuperscript{28-30}. In order to investigate how DBP affects the membrane barrier properties as well as the bR-activity, bR was reconstituted into either pure DBP or into mixed DBP/phospholipid membranes (Figure 3). In contrast to pure lipid bilayers, the proton pumping activity of bR in pure DBP systems resulted in a net efflux of protons inverse of what we observed for asolectin based proteoliposomes, causing acidification of the extra-vesicular medium when activated (Figure 3a). These results indicate that the preferential
orientation of bR is opposite to that of the proteoliposomes described in Figures 1 and 2. However, for the period of bR-activation, the amplitude of the pH varies in absolute values of at max only 0.10 compared to the values of almost 0.20 in asolectin and 0.38 in DOPC liposomes (see Figure S1), which may indicate that only a fraction of the bR is incorporated in a functional state. The persistence of the characteristic purple color of the bR-polymersomes indicated that some bR protein was intact, and the results indicate a diminished preferential bR-orientation. Interestingly, the pH value does not return to the resting (dark) level suggesting that the polymer membranes are able to maintain the proton gradient across the membrane. This is likely due to an increase of the stiffness and a concomitant decrease in the permeability of the polymer membrane.

The addition of DBP units into lipid membranes causes significant effects on the lipid membrane barrier properties and bR function. Figure 3b, 3c and 3d show the proton pumping activity of bR in mixed PEO<sub>10</sub>-PB<sub>12</sub>/DOPC, DOPS and DOPE membranes at different concentrations. For all three matrices, the addition of PEO<sub>10</sub>-PB<sub>12</sub> at high polymer to lipid molar concentrations (1 to 0.01) causes poor active transport of protons compared to non-DBP proteoliposomes. The activation of bR during illumination results in low pH-amplitude signals up to 0.07 pH values for DOPS membranes, 0.05 pH values for DOPE and less than 0.04 pH values for DOPC matrices (Figure 3b). As can be seen, the preferential orientation of bR is not only affected in pure polymer matrices but also with high polymer to lipid ratios.

Moreover, the presence of DBP in this case still seems to play an important role in terms of the membrane permeability maintaining the created pH gradient during darkness. When the polymer to lipid molar concentration is reduced 10-fold (1 to 0.1) (Figure 3c), the preferred natural bR-orientation is slowly restored in the DOPE membranes similar to that of the proteoliposomes described in Figure 1a, causing a positive amplitude signal of around 0.05 pH units. Still, the influence of PEO<sub>10</sub>-PB<sub>12</sub> in DOPS and DOPC liposomes, in terms of bR-
orientation and permeability, is strong enough to produce acidification of the medium and to maintain the ion gradient. Illumination of all three systems, even the lower polymer to lipid ratios (down to 1:1), results in a high proton translocation towards the vesicle interior (Figure 3d). In particular, DBP/DOPE membranes effectively re-establish a strong signal nearly double in size of the signal of asolectin proteoliposomes (Figure 1a). Among the three bR-reconstituted mixed membranes, DBP/DOPE polymersomes show the largest pH responses probably due to a better matching of the matrices with the membrane protein. The effect of higher bR-activity in DOPE systems indirectly supports the notion that non-lamellar lipids have an effect on the curvature of the membrane thereby regulating its activity. An interesting observation is that DBP/DOPS vesicles show an almost constant pH value when bR is activated suggesting an equal distribution between bR orientations towards both, the internal space and external phase. Remarkably, the permeability of the DOPS, DOPC, and DOPE systems, at low polymer to lipid ratios is considerably increased in the presence of PEO_{10}-PB_{12}, resulting in a fast kinetics of the pH-signal during influx and efflux.
Figure 3. bR orientation is greatly affected in pure polymeric and polymer/lipid composite membranes. Variations of pH across (a) bR pure PEO_{10}-PB_{12} based membranes compared to control, and (b-d) bR-mixed PEO_{10}-PB_{12} / phospholipids model membranes influenced by light/darkness vesicle environment conditions. PEO_{10}-PB_{12} / DOPE (black curve), PEO_{10}-PB_{12} / DOPS (red curve) and PEO_{10}-PB_{12} / DOPC (blue curve) model membranes are fabricated at (b) 100 polymer to phospholipid molar ratio, (c) 10 polymer to phospholipid molar ratio and (d) 1 polymer to phospholipid molar ratio.

4.4 DBP vesicle size distribution. The ability of the membrane protein to undergo conformational changes of the retinal complex upon irradiation, which allows for active transport, is influenced by membrane curvature and thus the size of the vesicles in which the proteins are embedded in^{34}. Therefore, in order to investigate how the long chains of DBP
affect the bR-activity into these proteoliposomes, the size distribution of the three mixed
DBP/lipid matrices were characterized by DLS. Figure 4 shows the relative size distribution
of DBP/DOPE, DBP/DOPC and DBP/DOPS based vesicles with and without protein at
different concentrations as well as the pure DBP vesicles. The size distribution of pure DBP
matrices results in a vesicle-population of around 350 nm (Figure 4a). However, there is a
clear reduction of the vesicle size, down to 50 nm as the polymer to lipid ratio decreases
underlining the effect that DBP exhibits on the lipid membrane (Figure 4b, 4c and 4d).
Therefore, a decrease of the polymer/lipid ratio not only improves the bR-activity, but is also
related with the vesicle-size distribution supporting the idea that lower radii of curvature
cause higher performance of the protein.
High polymer/lipid molar ratios, i.e. 100:1 and 10:1, notably affect the distribution of sizes
of the DOPE, DOPS and DOPC vesicles (Figure 4b - 4d). Consequently, the vesicle-size-
distribution shifts from 350 nm to lower diameter-values as a general tendency in the three
systems. This correlates with the results from Figure 3b and 3c where proteoliposomes at
these polymer/lipid ratios, showed a different behavior compared with pure polymer systems,
in terms of bR-activity and membrane barrier properties. Correlating DLS data and proton
influx and efflux rates we find that the decrease of the polymer/lipid ratios down to 1:1
ultimately causes the formation of small, monodisperse vesicles around 50 nm in diameter
with increased curvature which result in vesicle systems with higher bR-activity (Figure 3d).
To investigate any size effect caused by the presence of bR itself, DLS was performed on
polymersomes without bR and the vesicle size distributions were compared to the sizes of the
proteopolymersomes containing bR. For most of the systems, the size distributions were
found to be approximately the same where the most notable difference were observed for
DOPC and DOPE vesicle systems with high molar lipid content (Figure 4e and Figure 4k). It
is likely that the decrease of the rigidity in the high lipid content polymersomes caused by the
lipids can result in variations of the vesicle size depending on the constituents e.g. proteins.

The reorganizational role of the lipid in membrane organization correlates with our observations that bR in the lipid containing polymersomes also show much higher activity than for polymersomes with no or low amounts of lipids (Figure 4e-m).
Figure 4. Vesicle size distribution as determined by DLS of bR reconstituted in DOPE, DOPS and DOPC based membrane systems with and without additives. (a) Vesicle size distribution of pure bR-PEO$_{10}$-PBE$_{12}$ based membrane systems. (b-d) Vesicle size
distribution of bR in DOPE, DOPS and DOPC membrane systems respectively with PEO$_{10}$PBE$_{12}$ as additive at 100 (orange), 10 (green) and 1 (grey bars) polymer to phospholipid molar ratio. (e-m) proteopolymersomes compared to polymersomes without bR shows a similar size distribution. The largest change in sizes when bR is absent is found in the stoichiometric lipid/polymer mix, mainly for DOPC and DOPE vesicles and to smaller degree for DOPS which indicates that the curvature of polymeric membrane can change to improve protein reconstitution, but only when high amounts of lipids is present.

4.5 Future biomimetic membrane design strategies. Depending on the desired membrane functionality, one may tune the membrane to either induce larger influx and efflux across the membranes or to sustain the pH gradients by producing less permeable membranes. Figure 5 shows an overview of the different possibilities presented as a correlation between influx and efflux properties of liposomes with and without cholesterol and solvents, pure lipids, pure polymersomes as well as mixed polymer/phospholipid vesicles. There are four apparent correlation tendencies indicated by the blue, black, green, and red circles, which can be associated with specific membrane compositions.

The diffusion of protons through polymer/phospholipids mixed membranes (blue circle) improves protein activity and proton permeability of the membrane causing the lowest influx and efflux time constants reported here of 7-10 seconds and 8-10 seconds, respectively (Table 5b). A low value of $\tau$, for both influx and efflux, indicate a fast transport of protons through bR as well as a fast passive diffusion through the membrane, underlining how the polymer constituents diminishes the capability of the lipid membrane to act as proton barrier. The active diffusion of protons through bR in pure lipid membranes (black circle) seems to be smaller, compared to the mixed systems, resulting in influx time constants of around 20-25 seconds. In the same context, the pH-gradient created in these systems is dissipated slower through the lipid membrane compared to the mixed polymer/phospholipid vesicles where the
efflux time constants are around 26-31 seconds. As expected\textsuperscript{15}, the addition of hydrocarbon solvents into phospholipid vesicles seems to alter not only hydrophobic protein-lipid interactions but also reduce the ability of the vesicle membrane to let protons through (red circle) causing less permeable membrane systems. For this group, time constants increase to values of 20-65 seconds during influx and 40-100 seconds during efflux i.e., one order of magnitude higher than for the mixed lipid/polymer systems. As the hydrocarbon tail length increases (systems 2, 4, 5), there is a tendency towards lower time constants suggesting a faster diffusion than in system 3. This supports the idea that the hydrophobic interactions depend strongly on the solvent partitioning\textsuperscript{15}. For example, alkaline solvents such as n-decane more easily partition into the lipid membrane compared to squalene\textsuperscript{13} resulting in a thicker and thus less permeable membrane. The influx and efflux time constants of systems with hydrocarbons are generally higher than for both pure lipids and polymer/lipid mixed systems resulting in lower permeability towards protons. Also the increased cholesterol concentration in phospholipid membranes slows down the passive diffusion of protons through the membrane resulting in higher influx time constants of around 15-50 seconds and efflux time constants of around 33-80 seconds (green circle).
Figure 5. Comparison of membrane properties with investigated additives. (a) Comparison of influx and efflux rates of protons in bR-model membrane systems composed by pure lipids (number 1, 9, 10), asolectin membranes with mono-chain hydrocarbons (2, 3, 4, 5), DOPC membranes with cholesterol (11, 12, 13) pure polymers (14) as well as mixed polymer/phospholipid membranes (6, 7, 8). (b) Influx and efflux values for each model membrane system with their parametric standard deviations of the inverse exponential curve fitting.
5. CONCLUSIONS

Over the last decades especially structural studies on bR have been numerous resulting in bR becoming one of the most well described transmembrane proteins. The vast amount of information on bR has led to recent shift in focus where literature more and more describe the functionality of bR as a model membrane protein to study membrane effects on the protein.

In this context, many studies have reported the effect of membrane modulators including lipids, peptides and other proteins, but usually in an isolated context where the aim has been a detailed characterization of the effect of a specific membrane modulator on bR. In this work, we have analyzed the bR-activity in membranes of various compositions as well as the membranes capability to retain a proton gradient created by activation of bR to give a broader overview of the opportunities that lies within modulation of membrane permeability. We have shown how the barrier properties of pure phospholipid bilayers can be altered by additives including three structural different additives including hydrocarbons, cholesterol and polymers and correlated the resulting proton flux. Hydrocarbon solvents as well as cholesterol tend to decrease membrane permeability towards protons. The dose-dependency of cholesterol as well as the length-dependency of hydrocarbon solvents on membrane structure can be used to customize permeability properties in liposome models. DOPC vesicles with low amount of cholesterol changed both influx and efflux rates dramatically, however, when increasing the concentration of cholesterol further we observe a linear increase in proton uptake and in proton permeability. This suggests that membrane integrity as an ionic barrier can be improved with cholesterol, but only when added in modest amounts. We have found that our results on cholesterol in the lipid membrane express different degrees of consistency with previous studies among which it also has been discussed that it is difficult to generally conclude on the effect of cholesterol. The contrary beliefs suggest that the resulting membrane and protein properties is highly dependent on the
constituents of the membrane and that the lack of a unanimous understanding of how cholesterol influence the membrane properties needs to be studied further.

Using DBP as additive to phospholipid-based vesicles gave rise to faster efflux and influx by a factor of up to three compared to pure phospholipid systems (DOPC, DOPS or Asolectin proteoliposomes) diminishing the vesicles ability to retain protons in the intracellular compartment.

Polymeric materials as building blocks, in biotechnology, have recently gained a lot of interest. There are several applications based on synthetic biomimetic membranes where the influx/efflux kinetics play an important role, i.e. within medicine, energy production devices and fast responsive biosensors. The importance of delivering anticancer drugs, with a precise drug release, in treatments of cancer therapy is an example of how polymeric vesicles can be used. The ability to control efflux kinetics in respective treatments based on drug release is important in order to minimize adverse toxic effects. Also, polymer membranes that are able to retain ion gradients could potentially be a key constituent as a green alternative for the production of energy storage devices such as micro-batteries, or super capacitors.

Upon irradiation bR are able to promote an internal ionic condensation of polymeric vesicles that are able to retain the gradient. Thus, this system could potentially be used for energy storage by charging of the polymeric vesicle solution. A controlled release/discharge through destabilization or disruption of the polymeric membranes could provide as an energy source for small electronics or to provide a catalytic effect for biochemical reactions. Mixed polymer/lipid membranes, on the other hand, could be good building blocks in designing systems where fast bR mediated transport responses is desired such as biosensors. In summary, our result show how biomimetic membranes with a reconstituted proton pump can be tailored over a wide range of proton influx and efflux rates. This knowledge is useful in
design of biomimetic membrane systems aimed at a broad spectrum of applications based on
the use of reconstituted membrane proteins.

AUTHOR INFORMATION

Corresponding Author

*E-mail: clhe@env.dtu.dk

Author Contributions

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ABBREVIATIONS

bR, bacteriorhodopsin; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; PB12-PEO10,
polybutadiene-polyethyleneoxide; DOPE, 1,2-Dioleoyl-sn-glycero-3-
phosphatidylethanolamine; DOPS, 1,2-Dioleoyl-sn-glycero-3-phosphatidylserine; DBPs,
amphiphilic di-block copolymers; LPR, lipid-to-protein ratio.

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