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Gas-tight triblock-copolymer membranes are converted to CO_2 permeable by insertion of plant aquaporins

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We demonstrate that membranes consisting of certain triblock-copolymers were tight for CO_2. Using a novel approach, we provide evidence for aquaporin facilitated CO_2 diffusion. Plant aquaporins obtained from heterologous expression were inserted into triblock copolymer membranes. These were employed to separate a chamber with a solution maintaining high CO_2 concentrations from one with depleted CO_2 concentrations. CO_2 diffusion was detected by measuring the pH change resulting from membrane CO_2 diffusion from one chamber to the other. An up to 21 fold increase in diffusion rate was determined. Besides the supply of this proof of principle, we could provide additional arguments in favour of protein facilitated CO_2 diffusion to the vivid on-going debate about the principles of membrane gas diffusion in living cells.

Aquaporins are membrane-spanning pore-forming proteins, facilitating the transport of water and certain uncharged solutes across biological membranes in almost all living organisms. Aquaporin proteins exhibit a characteristic conserved structure. Hydropathy plot analyses of the primary sequence predicted a topology of six transmembrane helices (I–VI) connected by five loops (loops A–E). The highly conserved loops B and E dipping into the membrane include a conserved signature motif, asparagine-proline-alanine (NPA motif), which is directly involved in the water transport mechanism[1]. Inserted into a membrane, aquaporins arrange in tetramers, in which four monomers giving four individual water conducting pores form a putative fifth pore in the centre of the protein complex. In plants, aquaporins were subdivided into distinct groups related to their localisation in the cell. The largest group is called PIP for plasma membrane intrinsic protein. The PIP group was further divided into PIP2, which are highly water selective, and PIP1, which are almost impermeable to water.

Aquaporin function was determined mainly in living cells, and it is generally accepted that aquaporins facilitate membrane water diffusion. A possible function concerning facilitating CO_2 membrane diffusion has caused an on-going scientific debate. This debate accounts for publications concerning significant differences of CO_2 diffusion rates in animals and plants with or without specific aquaporins. Conversely, theoretical considerations, and experimental data obtained from lipid bilayer CO_2 diffusion as well as CO_2 diffusion rate comparisons in animals and plants in the presence or absence of aquaporins in some cases showed no, or just slight, differences. Most profoundly, a protein facilitated CO_2 diffusion seems to be a violation of the so-called Meyer-Overton rule, which implies that lipid bilayer membranes such as biomembranes do not impose resistance to diffusion of small hydrophobic molecules like CO_2. A consequence of this solubility-diffusion model is that any protein, even if it is highly permeable for CO_2, would reduce the rate of CO_2 diffusion. In fact, Gutknecht and co-workers, for example, could show that an artificial bilayer consisting of egg lecithin and cholesterol does not constitute a substantial barrier to diffusion of CO_2 (permeability coefficient $P_{CO2} \approx 0{,}35$ cm/s)[2]. Combining CO_2 diffusion studies and an analytical model Missner *et al.* predicted a P_{CO2} in lipid bilayers of 3,2 cm/s[3]. However, studies with biomembranes report 10 to 1000 times reduced CO_2 diffusion rates[4–7]. Under these conditions, the Meyer-Overton rule is not applicable and protein facilitated CO_2 diffusion would make sense if high CO_2 transport rates are required.

One of the major pitfalls of the debate from both sides is that these could only rely on data from CO_2 permeable membranes. In this case the membranes, such as artificial bilayers, were highly permeable to CO_2, the figures were close to the theoretical considerations confirming Meyer-Overton. In the case of distinct cell membranes, the figures were orders of magnitude below the theoretical value possibly due to intrusion of biological components or coverage with proteins and other substances. These may cause a decrease in overall CO_2 diffusion rates and aquaporin facilitated CO_2 diffusion becomes necessary. To obtain data about aquaporin CO_2 conductivity,

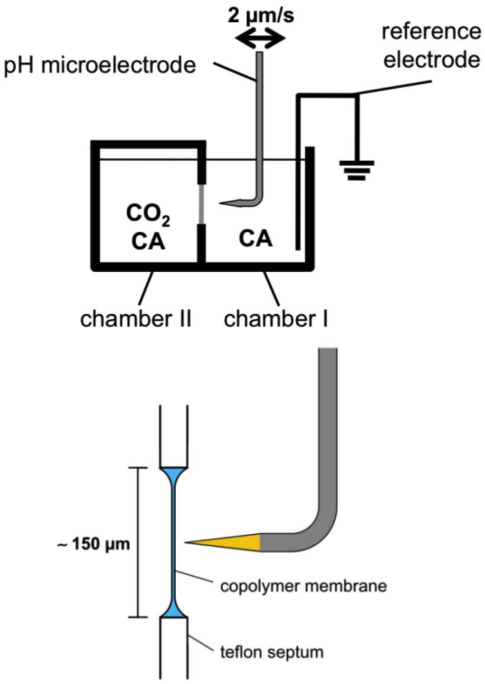

Figure 1 | **Modification of the two chamber system.** A PTFE septum with small hole (~150 µm diameter) has been clamped between two half chambers. A copolymer membrane has been spread across the hole. Chamber II contains a CO_2 and carbonic anhydrase containing buffer solution. Both compartments contained carbonic anhydrase. To minimize loss of CO_2 from the reservoir chamber II has been closed with a glass slide. Diffusion of CO_2 from chamber II across the membrane into chamber I is measured as a function of pH.

besides circumventing the background and disadvantage of a CO_2 permeable membrane, we decided to analyse a possible aquaporin dependent CO_2 flux in a CO_2 tight membrane. These conditions were provided by an artificial membrane consisting of block-copolymers. Thus, we are using a highly sensitive approach to analyse if specific biological components, the aquaporins, have the capacity to increase CO_2 diffusion rates.

Results

Measurement of CO_2 transport. In the range of maximal theoretical values for CO_2 diffusion, the so-called unstirred water layers (USL) on both sides of the membrane will restrict CO_2 diffusion rates. Therefore, it is required to analyse membrane transport of CO_2 with a tool capable of considering USL effects. The scanning pH electrode as described by Missner *et al.*[3,8,9] is an appropriate device to analyse such high diffusion rates. The approach was adapted to determine CO_2 diffusion over artificial planar membranes. Block-copolymer membranes were introduced into a modified two-chamber system (Fig. 1). In this two-chamber system, a gradient driven flux of CO_2 across a membrane from one compartment with a high CO_2 concentration to another with a lower concentration was measured. A defined CO_2 concentration was maintained by the addition of carbonic anhydrase. The enzyme was added to both chambers in a concentration ensuring that the conversion reaction to/from bicarbonate was not rate limiting. Increase or decrease of bicarbonate as a result of CO_2 concentration changes caused changes in pH and this was recorded by the scanning pH electrode. Both compartments have a volume of approximately 2 ml. Under the conditions applied, the CO_2 gradient across the membrane remained stable for more than an hour, admitting measurements comparatively independent of time.

Table 1 | Configuration of ABA block copolymers used for the present study

	A (PMOXA)	B (PDMS)	A (PMOXA)
ABA1	20	41	20
ABA2	12	55	12
ABA3	15	110	15

Triblock-copolymer membranes as a biomimetic model system to study membrane transport. Triblock-copolymer membranes exhibiting defined permeability properties became an attractive biomimetic system with properties relevant for nanotechnological applications. Immobilisation of functional biological molecules such as membrane proteins is conceivable[10]. Depending on the nature of the employed copolymer, these artificial membranes can exhibit characteristics comparable to that of lipid bilayer membranes. According to the copolymer's attributes the resulting membrane has distinct intrinsic permeability properties[11]. As mentioned above, lipid bilayers or cell membranes, exhibit a substantially high background CO_2 permeability representing an error source. For discovery of a suitable copolymer as source for the construction of a membrane with a low CO_2 conductivity, a set of different triblock-copolymers based on poly-2-methyloxazoline (PMOXA) and polydimethylsiloxane (PDMS) with different A/B ratios were tested. Triblock-copolymers used in preliminary experiments for this study are listed in Table 1. The substances were dissolved in n-decane and were spread across the aperture of the two chamber PTFE septum. The scanning pH microelectrode was advanced towards the resulting copolymer membrane patch and the pH decrease was documented. pH decrease was related to the rate of CO_2 flux ($J_{M,CO2}$) from the chamber with a higher apparent CO_2 concentration to that with the lower one. The CO_2 concentration in the vicinity of the membrane increased and consequently, a gradual decrease in pH was detected for ABA3 copolymer membranes. $J_{M,CO2}$ was determined as described by Missner *et al.* (2008). For ABA1 and ABA2 membranes no change of pH has been observed (Fig. 2a) indicating CO_2 impermeability (Fig. 2b). ABA3 membranes showed a substantial CO_2 permeability. Accordingly, ABA1 was chosen for further experiments.

Reconstitution of aquaporin proteins into planar polymeric membranes. Although block-copolymer membranes are two- to three-fold thicker than conventional lipid bilayers, they can be used as a matrix for membrane-spanning proteins. The proteins often remain functional, despite the thickness of the membranes in comparison to lipid bilayers and the polymerisation reaction of the reactive block-copolymers[12]. As an example, functional protein integration was successfully performed with the *E. coli* aquaporin AQPZ. In this case, water transport rates were found to be increased after integration of the aquaporin[11]. For the analysis of protein facilitated CO_2 transport, the tobacco PIP1 aquaporin NtAQP1 was employed. From data obtained in the heterologous expression/analysis system yeast and from physiological observations in plants concerning photosynthesis, NtAQP1 was considered to increase CO_2 transport rates. For comparison, NtPIP2;1, a PIP2 aquaporin from tobacco, which was found to be permeable for water and to a much lesser degree for CO_2 was also subjected to the analysis. Nontheless, all these conclusions came from data relying on membranes with a high internal CO_2 permeability. Prior to integration, the protein has to be synthesized to a considerable concentration. For this purpose, it was expressed as a 6xhis tagged fusion protein in *S. cerevisiae*. Via His-tag affinity to Ni^{2+}, NtAQP1 and NtPIP2;1 were purified according to Otto *et al.*[5]. The purified protein was inserted into copolymer membranes (80 µg

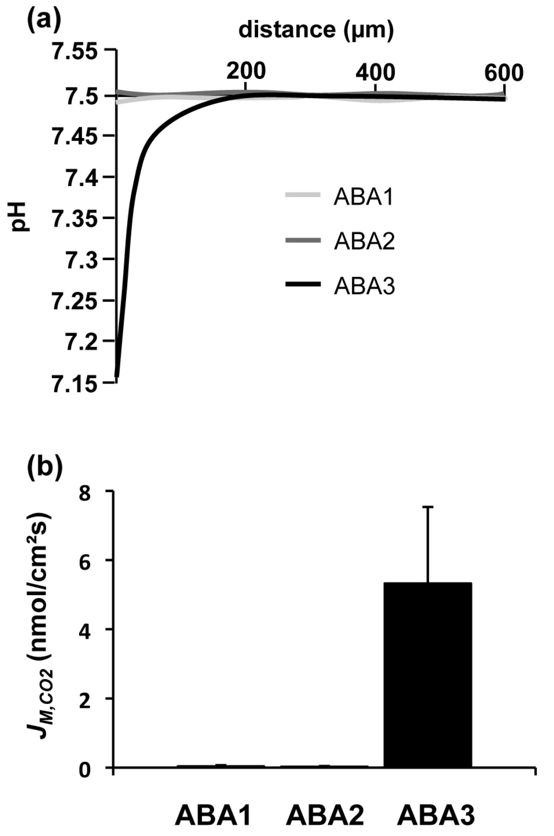

Figure 2 | CO_2 flux across block-copolymer membranes. (a) Experimental pH profiles in response to CO_2 diffusion across three types of poly-methyloxazoline-poly-dimethylsiloxane-poly-methyloxazoline based triblock copolymer membranes. (b) Average membrane flux of CO_2 (± S.E.; n=10 each) calculated from the slope of the pH traces within 50 µm from the membrane.

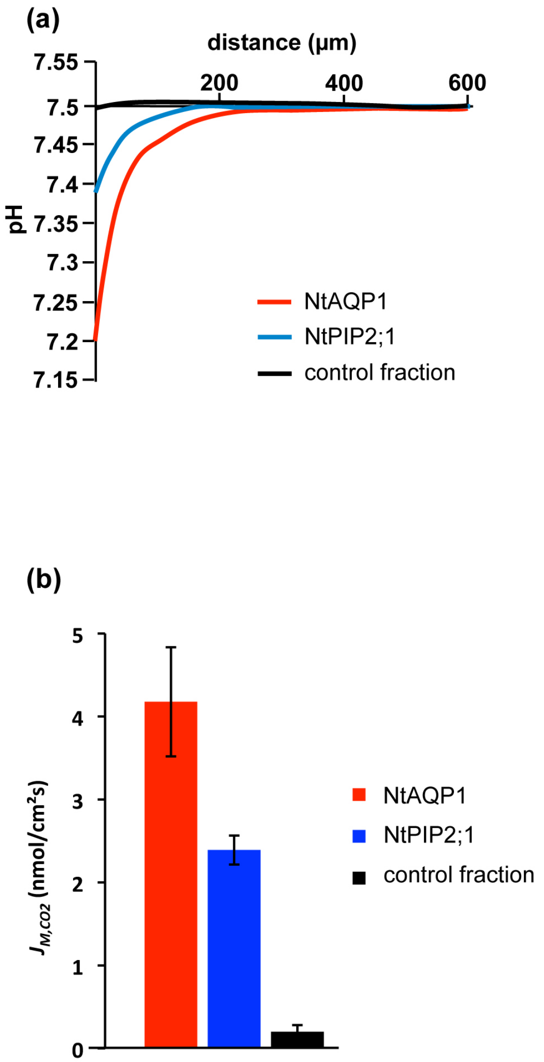

Figure 3 | Aquaporin facilitated CO_2 diffusion across ABA1 block copolymer membranes. (a) Experimental pH profiles in response to CO_2 diffusion across ABA1 membranes containing NtAQP1 or NtPIP2;1 as well as control membranes. Insertion of NtAQP1 protein reduces the membranes resistance to CO_2 diffusion dramatically, NtPIP2;1 to a minor extent. (b) Average membrane flux of CO_2 (± S.E.) calculated from the slope of the pH traces within 50 µm from the membrane (n=6 for NtAQP1; n=14 for NtPIP2;1; n=10 for control fraction).

protein per 6 mg triblock-copolymer) as described in the methods section. Both pH and reference electrodes were inserted into chamber I in order to detect a decrease of pH due to membrane CO_2 flux. The pH electrode was moved perpendicularly towards the copolymer membrane and the pH signal was continuously recorded. The CO_2 concentration in the vicinity of the membrane increased and consequently, a gradual decrease in pH was detected for copolymer membranes containing NtAQP1 and NtPIP2;1, but not for membranes treated with control protein fractions containing neither of the proteins (Fig. 3a). When the PIP1 aquaporin NtAQP1 was inserted into the copolymer membrane, average membrane flux of CO_2 was increased 21-fold compared to control membranes (4.18 ± 0.66 nmol CO_2/m^2s compared to 0.2 ± 0.08 nmol CO_2/m^2s for control measurements). Insertion of the NtPIP2;1 increased the CO_2 permeability of the membrane 12-fold (2.39 ± 0.17 nmol CO_2/m^2s; Fig. 3b).

Discussion

The present work describes an apparent discrepancy of membrane diffusion studies in an artificial system with measurements in biological systems. The latter revealed in general no, or just a small, increase in CO_2 diffusion by PIP2 aquaporins, compared to NtAQP1 or human aquaporin 1[4,5,13–16]. It might, however, indicate the different sensitivity of the deployed systems. NtAQP1 facilitated CO_2 transport has been studied in yeast cells or *Xenopus* oocytes as well as plant cells and the overall CO_2 transport was much lower in these studies[4,5,13] than presented here. These are biological membranes and, as mentioned above, have a certain level of background CO_2 permeability. In contrast, the planar block-copolymer system used for the present study has no detectable background CO_2 permeability and therefore is able to unravel the full CO_2 transport capacity of NtAQP1. The effect of NtPIP2;1 on membrane CO_2 permeability as measured in block-copolymer membranes could be covered by the intrinsic CO_2 membrane permeability of biological membranes. Additionally, the present CO_2 transport studies were done at room temperature whereas studies employing yeast cells are generally performed at 10°C. This may additionally mask the lower CO_2 transport activity of NtPIP2;1 in yeast cells due to thermal effects on the protein flexibility.

The results from this investigation provide clear experimental evidence that aquaporins have the capability to increase CO_2 transport, although to different extents. Unstirred water layers close to the membrane may affect the overall CO_2 transport to some extent, but were not limiting in our experiments as it was the case in the study by Missner et al.[3]. Thus, not only the theoretical considerations about the gas permeability of aquaporins could be confirmed, but also a missing link in the chain of evidence in favour of the CO$_2$ facilitating function is provided. Now, the observations supporting protein facilitated CO$_2$ diffusion start from theory, extend to molecular evidence, and reach into physiological changes. Considering that biological membranes in some cases show very low permeability for CO$_2$[17] and that relatively high CO$_2$ transport rates must be assured for a cell to survive, our studies indicate for the requirement of these proteins for the exchange of CO$_2$. Concerning biophysical characteristics, other gasses are similar to CO$_2$ and it is possible that also for these, a protein facilitated membrane transport has to be considered.

Uncovering the molecular basics of membrane gas transport and understanding how gasses move into and within cells, tissues and whole living organisms bears potential scientific and technical implications for environmental engineering and sensor technology. The novel findings and biomimetic membrane systems described above, in future may be used to improve separation processes important for technical and medical applications like sensing and purification of technical gasses. The presented system allows studies on gas conductivity properties of candidate membrane proteins in a system with minimal background permeability.

Methods

Microelectrode measurements. The pH measurements were performed as described by Missner *et al.*[3]. A scanning pH-sensitive microelectrode was moved by a motorized hydraulic micromanipulator (MHW-103, Narishige, Tokyo, Japan) within the stagnant water layer with a velocity of 2 µm s^{-1} towards or away from the cells[8]. The travel speed of the micromanipulator was regularly checked using a digital sliding caliper. The electrodes had a sensitivity of 56±0.3 mV/pH, which was determined by making a three-point calibration in buffer solutions with a defined pH before and after each experiment. The addition of carbonic anhydrase (CA) did not affect the sensitivity of the electrode. Voltage recordings were performed each second using an electrometer (Duo 773, World Precision Instruments, Berlin, Germany). The filtered signal (LHBF-48x, npi Electronic GmbH, Tamm, Germany) was recorded on a personal computer via an A/D converter box (USB-6008, National Instruments, Austin, Texas, USA). The electrodes were manufactured from borosilicate glass (GB 150F-10, Science Products GmbH, Hofheim, Germany) pulled to a tip size of 2–4 µm, silanized (Dichloro-dimethylsilane, VWR International GmbH, Darmstadt, Germany), and then filled with a proton-sensitive mixture (Hydrogen Ionophore II - Cocktail A, Selectophore, Fluka). A leaf without a lower epidermis was connected to the chambers (Fig. 1). The chambers were filled with a CO$_2$ and carbonic anhydrase (Sigma; 1 mg ml-1)-containing buffer solution (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 1.3 mM CaCl$_2$, 1.0 mM MgSO$_4$, 30 mM NaHCO$_3$, 30 mM HEPES pH 7.5). The liquid in both chambers was continuously agitated by magnetic stirring bars. $J_{M,CO2}$ was calculated as described by Missner *et al.*[3].

Production of NtAQP1 and NtPIP2;1 protein. Recombinant NtAQP1 and NtPIP2;1 protein was heterologously expressed in yeast a 6xhis tagged fusion protein and was purified essentially as described by Otto *et al.*[5]. In brief, isolation of plasma membrane fractions from yeast cells was performed according to Panaretou and Piper[18]. Total membranes from glass bead lysed yeast cells were collected by centrifugation at 22,000 x g for 30 minutes and subsequently fractionated on a sucrose-step gradient (1.1, 1.65, and 2.25 M sucrose in 2 mM EDTA, 25 mM imidazole/HCl, pH 7; 9 ml each) by centrifugation for 15 h at 80,000 × g. Plasma membranes were obtained from the 2.25 M/1.65 M interface. The combined plasma membrane fractions were washed with 20 mm Tris/HCl, pH 7.5, 150 mm NaCl, 10% glycerol. Aquaporins were solubilized with 2% dodecylmaltoside (DDM). Via His-tag affinity to Ni^{2+} NtAQP1 and NtPIP2;1 were purified using an Äkta prime chromatography system (GE Healthcare). Identity of the aquaporin containing fractions and purity of the proteins was confirmed by Western blot analysis with an NtAQP1 or NtPIP2;1 specific antibody raised in chicken or rabbit, respectively. Chromatography fractions not containing aquaporin protein were used as control fractions for CO$_2$ transport studies (see below).

Synthesis of ABA Polymer. Symmetric poly-(2-methyloxazoline)-block-poly-(dimethylsiloxane)-block-poly-(2-methyloxazoline) (PMOXA$_n$-PDMS$_m$-PMOXA$_n$) polymers of different block lengths were synthesized by the approach described by Nardin et al.[19], except for ABA1, for which the PDMS (Mw = 3000 g/mol, PDI = 1.12) was obtained from ABCR GmbH, Germany. Polymers were verified by ^{1}H NMR. Molecular weights and polydispersity indices (PDI) were determined by gel permeation chromatography (GPC) and were 6550 g/mol for ABA1 (PDI = 1.61), 6325 g/mol for ABA2 (PDI = 1.64) and 10700 g/mol for ABA3 (PDI = 1.62).

Production of aquaporin protein containing triblock-copolymer membranes. 6 mg ABA1 copolymer were dissolved in 100 µl n-decane and mixed with aquaporin fraction (80 µg aquaporin protein) at 4°C for 2 h. After phase separation by centrifugation the decane phase was collected and membranes were spread across the Teflon septum using the painting technique. Presence of a membrane was verified by microscopic inspection and by resistance measurements. For control experiments ABA1/decane was treated with fractions from the His-tag affinity chromatography not containing aquaporin protein.

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Author contributions

N.U. and R.K. designed the study. B.O., A.E. and N.U. performed the experiments. W.M. and F.I. provided materials and know-how. R.K. and N.U. drafted and edited the final manuscript.

Additional information

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