Constructing artificial respiratory chain in polymer compartments: Insights into the interplay between bo3 oxidase and the membrane

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Cytochrome bo3 ubiquinol oxidase is a transmembrane protein, which oxidizes ubiquinone and reduces oxygen, while pumping protons. Apart from its combination with F1F0-ATPase to assemble a minimal ATP regeneration module, the utility of the proton pump can be extended to other applications in the context of synthetic cells such as transport, signaling, and control of enzymatic reactions. In parallel, polymers have been speculated to be phospholipid mimics with respect to their ability to self-assemble in compartments with increased stability. However, their usability as interfaces for complex membrane proteins has remained questionable. In the present work, we optimized a fusion/electroformation approach to reconstitute bo3 oxidase in giant unilamellar vesicles made of PDMS-g-PEO and/or phosphatidylcholine (PC). This enabled optical access, while microfluidic trapping allowed for online analysis of individual vesicles. The tight polymer membranes and the inward oriented enzyme caused 1 pH unit difference in 30 min, with an initial rate of 0.35 pH·min⁻¹. To understand the interplay in these composite systems, we studied the relevant mechanical and rheological membrane properties. Remarkably, the proton permeability of polymer/lipid hybrids decreased after protein insertion, while the latter also led to a 20% increase of the polymer diffusion coefficient in copolymers. In addition, PDMS-g-PEO increased the activity lifetime and the resistance to free radicals. These advantageous properties may open diverse applications, ranging from cell-free biotechnology to biomedicine. Furthermore, the presented study serves as a comprehensive road map for studying the interactions between membrane proteins and synthetic membranes, which will be fundamental for the successful engineering of such hybrid systems.

Significance

Analogous to phospholipids, some polymers assemble into vesicles and can mimic cellular membranes. Apart from enabling compartmentalization in the context of artificial cells, amphiphiles may serve as interface for proteins. However, complex transmembrane proteins were reconstituted in polymers with limited success so far. We functionally integrated the proton pump bo3 oxidase (part of the bacterial respiratory chain) in synthetic membranes made of PDMS-g-PEO and demonstrated lumen acidification. We provided mechanistic insights into the interplay between the protein and the (semi) synthetic membrane by measuring bending rigidity, lateral diffusion and disorder, proton permeability, and protein partitioning. Polymer and hybrid membranes displayed favorable properties for the construction of artificial cells such as membrane rearrangement, enhanced stability and fluidity, while keeping the compartments proton-tight.

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bonds in phospholipids and degradation of repeating units would have a lower impact on the overall stability (3). In addition to chemical stability (8, 9), synthetic polymers offer increased resistance against aggregation (8, 10), broader chemical versatility (3), and increased tunability (11, 12). It is also possible to modify the size and morphology of the compartments by changing the hydrophilic/hydrophobic ratio (8, 13) or to functionalize the surface, e.g., for adhesion (14).

Amphiphilic assemblies serve another key role beyond compartmentalization; they act as interfaces for interactions with peripheral membrane proteins (MPs) and accommodate integral MPs. The latter take part in essential cellular processes such as selective transport and energy conversion, e.g., oxidative phosphorylation, in which electron transport chain (ETC) proteins pump protons across the membrane and the resulting proton-motive force (PMF) drives the synthesis of adenosine triphosphate (ATP). High PMF and reduced ETC have been linked to increased production of reactive oxygen species (ROS) in mitochondria (15, 16). ROS can induce peroxidation of lipids (unsaturated fatty acids) or cleave ester bonds and thus disrupt the bilayer arrangement, which may in turn inactivate MPs and increase the permeability (17, 18). With respect to this, partially or completely replacing the lipids with synthetic polymers, which are less prone to oxidation and hydrolysis, would increase the overall system stability.

For reconstitution of the energy machinery, it is crucial that the compartments have limited proton permeability in order to sustain a gradient. In addition, they have to offer a suitable environment for MPs, i.e., fluid and flexible membrane and thickness in the natural range (to avoid size mismatches, which may compromise protein folding). Until now, various MPs of the respiratory and photosynthetic ETC have been reconstituted in phospholipid microcompartments: bacteriorhodopsin (br) (19), photosynthetic reaction center (RC) (20), bo3 oxidase (21), and F1Fo-ATPase (22). On the other hand, MP reconstitution in polymer membranes is still rare and usually limited to nanosized compartments and less pretentious porins and channels (reviewed in ref. 23). In most cases, block copolymer membranes are too thick (2- to 10-fold thicker than the lipid bilayer), viscous, and rigid for the functional insertion of large and complex MPs (23–25). While in some cases the functional reconstitution was transparently demonstrated [proteorhodopsin in PMOXA-PDMS-PMOXA (26), for other cases, the protein functionality may require further confirmation (27, 28).

Recently, we coreconstituted functional ubiquinol bo3 oxidase and F1Fo-ATPase in PDMS-g-PEO polymer and hybrid large unilamellar vesicles (LUVs) (29). Thereby, we observed completely retained activity of the four-subunit heme copper oxidase from Escherichia coli [144 kDa (30)], which catalyzes the reduction of dioxygen to water, coupling the redox energy to proton translocation through the membrane (31). Provided that historically other polymers have not been as suitable for complex MPs, it appeared important to elucidate the mechanical characteristics of a successful platform, apart from the matching thickness. It is known that reconstituted MPs exert forces on membranes and change their mechanical properties. For instance, decrease in membrane stiffness (22, 32–34), membrane fluidity (35, 36), and increase in passive proton permeability (37) have been reported, whereby the latter is a decisive parameter for the cellular energetics. However, whether and to what extent the reconstituted MPs change the mechanical properties of polymer and hybrid membranes is still unknown. It is also largely unexplored how the graft copolymer affects the functional durability of such a system and whether it can improve the stability against chemicals.

To address these questions, we investigated the interactions between synthetic [polydimethylsiloxane-graft-poly(ethylene oxide) (PDMS-g-PEO) (for chemical structure, see SI Appendix, section S1. 1 and Scheme S1)] and hybrid (PDMS-g-PEO/soy PC) membranes with bo3 oxidase. First, we monitored the activity of the proton pump in these membranes over time and in the presence of ROS. To form compartments of tangible size and compatible with optical methods, i.e., in the micrometer range, we used electroformation from nanosized prereconstituted vesicles (19) and optimized the procedure for the particular MP and amphiphiles. In addition, we demonstrated the versatility of the protocol by applying it for F1Fo-ATPase, a MP with different size, hydrophobicity, and symmetry. Next, we analyzed the protein distribution and characterized its influence on the bending rigidity, membrane fluidity, and proton permeability, whereby in all cases the synthetic polymer membranes were benchmarked against the natural phospholipid analog. Microfluidic devices for hydrodynamic trapping (38) turned out particularly useful for permeability and pumping experiments, allowing control over the solution exchange and facilitating optical observations. Finally, the mechanistic understanding of the observed behavior was supported by cryo-TEM imaging, membrane order analysis, and protein partitioning analysis via fluorescence resonance energy transfer (FRET). Altogether, the scale-up of the functionalized compartments to the micrometer range enabled real-time visualization and detailed characterization of the interplay in the minimal respiratory chain module, while opening possibilities for the design of complex systems.

**Results and Discussion**

1. **PDMS-g-PEO Extends the Activity Lifetime of bo3 Oxidase and Prevents Enzyme Delipidation and Deactivation by ROS.** Reconstituted complex MPs are known to drastically lose activity with time, whereby the rate of the loss depends on the protein type and the environment. While block copolymers have been shown to increase the functional stability (24), graft copolymers are still largely unexplored in this context. In addition to the shelf life of individual MPs, collateral damage caused by other enzymes may present an issue in more complex systems, e.g., oxidative stress in ETC assemblies. To test whether PDMS-g-PEO can enhance the stability, we applied detergent-mediated reconstitution by slightly modifying our previous protocol (29) and inserted bo3 oxidase into ~100-nm nanocompartments made of soy PC, PDMS-g-PEO/soy PC (70:30 molar ratio) and PDMS-g-PEO at a constant lipid/polymer-to-protein molar ratio of 9,550:1. We determined activity and protein consumption was measured using a Clark-type electrode (SI Appendix, section S3.2). The protein was activated with the electron shuttle ubiquinone 1 (Q1) and DTT under nonlimiting concentration of the sacrificial electron donor (Fig. 1 A, Inset).

Interestingly, when keeping the same lipid/polymer-to-protein molar ratio, bo3 oxidase had the highest activity in hybrids, while activity in polymersomes and liposomes was virtually the same (day 1, SI Appendix, section S10 and Fig. S14). The superior properties of hybrids were reflected in stability tests as well. On day 2, they retained 90% of the initial activity, while liposomes and polymersomes decreased to 63% and 69%, respectively (Fig. 4A). Similar relation was present toward the end of the measurements. On day 11, the activity of liposomes decreased to 4%, while hybrids and polymersomes retained 25% and 15%, respectively (SI Appendix, section S10 and Fig. S14). Despite the scattering of the data, the qualitative trend (hybrids > polymersomes > liposomes) was maintained throughout the test. This was clearly evident also from the respective time constants of exponential decay (4.1 > 1.5 > 0.7), which reflected the days until the vesicles lost roughly one-third of their initial activities, even though the fit of liposome data might have led to some underestimation of the stability (SI Appendix, section S10

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and Fig. S1B). The loss of activity was ascribed to the protein due to the similar behavior in micelles, but the detailed mechanism of preservation should be clarified in a more detailed study. It is worth noting that the blending with graft copolymer resulted in a similar improvement (~20%) of the stability in comparison to pure lipids as in the case of PBd-b-PEO/POPC (75:25) hybrid vesicles after 1 wk (24). As previously emphasized (42), hybrids hold a great promise for the reconstitution of MPs, whereby the enhanced durability, stability, and shelf life will be essential for application. PDMS-g-PEO/PC hybrids in particular may not only have a stabilizing effect but also increase the activity (presumably via higher reconstitution efficiency, yet to be determined). On the other hand, PDMS-g-PEO seems to be the only polymer so far, which enables fully retained bo3 oxidase activity in addition to stabilization.

The incubation with ascorbate and ascorbyl radicals did not cause a change in the size distribution (SI Appendix, section S11.1 and Table S1), but it decreased enzyme activity. However, PDMS-g-PEO shielded the proteins against oxidative stress; after 30 min of exposure to radicals, polymersomes retained 73 ± 6% activity, while liposomes, only 10 ± 4% (Fig. 1B). In addition, it should be noted that the activity decrease in polymersomes was statistically nonsignificant. The chemical resistance of the graft copolymer substantiates its suitability to accommodate complex ETC and to counteract the detrimental influence of ROS (produced by Complex I in particular). The activity loss upon ROS exposure can be caused either by a direct attack to the reconstituted MP or by indirect deactivation via membrane disruption and enzyme delipidation. Since the vesicle size did not change, we sought for subtle changes associated with surface modifications and/or compromised tightness. Therefore, we measured the surface charge of the vesicles without reconstituted bo3 oxidase and tested the leakage of encapsulated carboxyfluorescein (CF) by its dequenching (43). The different conditions did not significantly alter the zeta potential results within the instrument accuracy (SI Appendix, section S11.2 and Fig. S2). On the other hand, while liposomes were permeabilized upon radical exposure (SI Appendix, section S11.3 and Figs. S3A, S4, and S5), no leakage was detected in polymersomes and hybrids (SI Appendix, section S11.3 and Fig. S3 B and C), indicating absence of membrane defects. In addition, we inspected protein-functionalized vesicles after oxidative treatment by cryogenic transmission electron microscopy (cryo-TEM). A portion of the liposomes exhibited bilayer defects manifested as either visible pores or irregular surface (SI Appendix, section S18.2 and Fig. S33). Such membrane disruptions by ROS likely lead to delipidation of bo3 oxidase and consequently to enzyme aggregation and deactivation (complete loss of activity in the absence of stabilizing amphiphiles is shown in SI Appendix, section S12.4 and Fig. S12). On the contrary, no difference between the cryo-TEM images of ROS-treated and untreated samples was observed in both hybrids and polymersomes.

Finally, we tested the effect of ROS on the enzyme alone (i.e., bo3 oxidase, which was not reconstituted in a membrane but stabilized in detergent micelles) and no loss of activity was observed. In fact, the oxygen consumption slightly increased, presumably due to direct reduction of hemes by ascorbate (25.5 ± 3.4 vs. 20.8 ± 3.5 μM·min⁻¹). These observations further emphasize the importance of a stable scaffold even when the integrated MPs are otherwise resistant to ROS. Furthermore, the structural integrity of the membrane is imperative for reaction compartmentalization or cargo delivery.

2. Scale-Up from Nano to Micro: Access to Single Vesicles in Controlled Environment.

2.1. Preparation of bo3-GUVs from pre-reconstituted lipid, hybrid, and polymer LUVs. The reconstitution of complex MPs into GUVs (44) is not a straightforward task due to the incompatibility of enzymes with organic solvents used in the traditional electroformation procedure (45) or in phase-transfer methods (46). Previously, detergent-stabilized bo3 oxidase has been inserted into preformed lipid GUVs by subsequent dilution of the detergent, which resulted in a protein density of ~30 proteins·μm⁻² (47). However, this approach turned out to be inefficient particularly for the polymer membranes because it substantially decreased the protein activity. In addition, remaining traces of detergent can change the membrane properties and increase the proton permeability (37, 48). We previously screened different mild detergents and optimized their concentrations and subsequent removal to obtain functional proteins in different types of LUVs (29). Seeking to reflect this performance on the GUV scale (e.g., retain a favorable orientation), we used the same reconstitution protocols and afterward converted the proteoLUVs into proteoGUVs via electroformation from a partially dried LUV film (19) (the main steps are presented in SI Appendix, section S4.2 and Scheme S4), whereby we note that the dehydration step required precise control.

For the subsequent tests, it was important that the GUVs were sufficiently large (minimum diameter of 10 to 20 μm or preferably larger). Therefore, we optimized the protocol to obtain vesicles in the desired size range (typical distributions in SI Appendix, section 12.3 and Fig. S8). To avoid phase separation in the hybrid GUV membranes (29) and potential budding (49), we used a ratio of 70 mol% PDMS-g-PEO to 30 mol% soy PC. This ensured a homogenous distribution on the micrometer scale (SI Appendix, section S12.1 and Fig. S6). To prove the successful protein incorporation, we labeled bo3 oxidase with ATTO 514 and analyzed its distribution in lipid, hybrid, and polymer GUVs via confocal microscopy (SI Appendix, section S12.2 and Fig. S8). No phase separation and aggregation were observed (Fig. 2A), suggesting uniform protein distribution.

We next probed whether the activity of bo3 oxidase is retained after the scale-up to GUVs because a complete dehydration may have a deleterious effect on the activity of MPs (19). For this, we
relied on measurement of the oxygen reduction. The oxygen consumption of different bo3-GUVs (see SI Appendix, section S12.3 for sample quality) and ~100-nm bo3-LUVs (SI Appendix, section S12.3 and Fig. S10) was determined from bulk samples. For results on enzymatic activity retention and homogeneity of the protein distribution, please see SI Appendix, section S12.4.

2.2. bo3 oxidase acidifies synthetic microcompartments. Following the populational activity characterization of the bo3-GUVs via oxygen consumption, we next probed the protein translocation on a single-vesicle level. Characterization of individual vesicles provides additional information, which could be overlooked and averaged in bulk tests due to vesicle rupture and size variance. Here, we were able to directly deduce the enzyme orientation by observing the net ΔpH upon chemical activation. Since it was previously shown that the orientation of a complex MP such as Ca2+-ATPase is retained upon the fusion/electroformation approach (19), we focused solely on the novel system, namely bo3 oxidase reconstituted in PDMS-PEO GUVs.

Similar observations were done only for bR (19, 50) and a RC (20) in conventional observation chambers. The downside of the latter setup is that the vesicles could move out of focus if not deliberately immobilized to the surface (which in turn can alter the membrane tension and local composition) and the external solution cannot be exchanged completely; instead, the samples are being diluted. While this effect is irrelevant for light-activated proton pumps, we preferred that oxygen and Q1/DTT were supplied to the membrane and diffusion to the enzyme [the latter is a rate-limiting step in the mitochondrial ETC (52)]. Interestingly, the rate did not decrease progressively but very sharply. Note that the traces are averaged and the scattering is due to the size variance. However, the high protein loading data were very reproducible; therefore, we could clearly identify a changing rate: 0.35 pH min⁻¹ and 0.64 × 10⁻² pH·min⁻¹. Decrease of the pumping rate has been observed also in the case of bR-liposomes [reaching a steady state after about 3 min (53) or 5 to 6 min (19) of illumination], which was ascribed to a retroinhibition by the established ΔpH (back-pressure effect) (53).

The magnitude of the pH gradient directly depends on the type and amount of reconstituted MPs. While light-driven proton pumps, such as bR, provide the virtue of orthogonality with respect to the energy source, chemically driven pumps such as bo3 oxidase have higher turnovers (500 to 341 s⁻¹ vs. 37 to 50 s⁻¹) (1) and introduce additional means for coupling with metabolic reactions. With respect to the reconstitution of the energy machinery, the enzyme orientation in the membrane plays a pivotal role as well. The latter can be controlled by the type of detergent and the experimental conditions, but it is largely determined by the membrane. Prevailing

Fig. 2. (A) Successful insertion and homogenous distribution of bo3 oxidase-ATTO 514 (green) in bo3-polymer-GUVs. Polymer labeled with rhodamine (PDMS-PEO-Rho, red) was used to visualize the membrane. (Scale bar, 10 μm.) Distribution of bo3 oxidase-ATTO 514 in hybrid and lipid GUVs is depicted in SI Appendix, section S12.2 and Fig. S8. (B) Scheme of activated bo3 oxidase with correct orientation (inward pumping). (C) bo3-polymer-GUVs (PDMS-PEO-bo3 oxidase, 2.980·mol/mol) with encapsulated pH-sensitive pyranine (green) trapped in microfluidic chip. Membrane was stained with 0.05 mol% PDMS-PEO-Rho (red). (Scale bar, 50 μm.) (D) Intravesicular pH change for negative control (protein-free vesicles; gray trace) and two types of GUVs with different polymer-to-protein molar ratios; 2,980:1 (purple trace) and 9,540:1 (green trace) upon activation with DTT/Q, reached bo3-polymer-GUVs trapped in the microfluidic chip. Each trace represents the mean of five to seven individual vesicles and their SD.
inward orientation of bo3 oxidase was previously deduced in DOPC SUVs (S4) and later on in PDMS-g-PEO LUVs (29) from the respiratory-driven ATP synthesis (possible only with favorable gradient direction). Here, we confirmed the utility of the reconstitution method and the synthetic membrane, alongside the scale-up procedure through direct visual observation of the pH change. In addition, it can be anticipated that even higher ΔpH could be established at the interface, where protons might accumulate before diffusing into the vesicle lumen. This has been postulated in phospholipid membranes at distances between bo3 oxidase and F1F0-ATPase shorter than 80 nm, in which the lateral proton transfer along the surface was faster than equilibration with the bulk water (47). With respect to the synthetic interface, bo3 oxidase has been previously reconstituted in PBD-b-PEO, but the protein was functional only in combination with POPC, i.e., in hybrid LUVs (42). The latter showed increased durability, but the random (symmetrical) orientation resulted in subtle pH changes (no data shown). The activity in the present study substantiates the choice of the graft copolymer for bo3 oxidase and provides the potential to extend this synthetic platform to other complex MPs, beyond ETC enzymes.

### 3. PDMS-g-PEO and Hybrid Membranes Are Softer than Lipid Ones, and the Bending Rigidity Decreases upon Protein Insertion.

The bending rigidity (κ) is an important membrane characteristic with implications on cellular mimics as it reflects the resistance to deformation (55), which vesicles may experience during division, fusion, or osmotic shock. Furthermore, there is a common notion that increased rigidity impedes the reconstitution and most importantly the activity of complex MPs. The bending rigidity of fluid lipid membranes is typically in the order of 10 to 20 kBT (56), while polymersomes can exhibit various stiffness [up to 400 kBT (3)], depending on the membrane thickness. Commonly used block polymersomes have usually higher rigidity, for example PEO-PBD [35 ± 6 kGT (11)], but there are also block copolymers that form much softer membranes such as Pluronic L121 [PEO2-PPO68-PEO8, ∼3 kGT (11)]. To unveil how susceptible to bending are PDMS-g-PEO and hybrid membranes, we tracked the membrane fluctuations in GUVs by means of flickering spectroscopy (57) as described in ref. S8. To allow for visible membrane fluctuations, we exposed GUVs to gentle hypertonic conditions (SI Appendix, section S6.1). A large fraction of the lipid and polymer GUVs exhibited tubes and buds [possibly resulting from buffer asymmetry (59)] and these vesicles were not analyzed, while the hybrid GUVs appeared less tubulated. PDMS-g-PEO GUVs exhibited twice lower rigidity than soy PC (Fig. S4). Rather than showing intermediate softening, hybrids exhibited lower bending rigidity than liposomes, comparable to that of pure polymersomes (i.e., 11.6 ± 2.4 kBT), which indicates that this property is largely determined by the prevailing membrane component.

Previous studies report lipid membrane softening upon MP insertion in the case of Ca2+-ATPase (33), while in other cases softening occurred only after protein activation: bR (32, 60, 61), Na+/K+-ATPase (34), and F1F0-ATPase (22) (summarized in SI Appendix, section S14 and Table S3). To check the influence of bo3 oxidase on the lipid and graft copolymer membranes, we performed fluctuation analysis upon reconstitution. Polymer GUVs and bo3-polymer-GUVs were on average tenser than protein-free and protein-functionalized hybrids and liposomes, which suppressed fluctuation. Therefore, further deflation was necessary (additional ∼5 min with open chamber to allow water evaporation). In addition, upon further deflation of the bo3-polymer-GUVs, the excess area almost exclusively formed tubes instead of enhancing fluctuation, enabling only a narrow window for analysis. We speculate that the reason for this phenomenon was the protein-induced asymmetry, as it is known that transiently bound or constitutively inserted MPs can alter the spontaneous curvature (33) and cause invaginations or tubulations (62). bo3 oxidase introduces slight asymmetry through its shape [predominantly hydrophobic truncated cone and small cytosolic fragment (63) (SI Appendix, section S19 and Fig. S41)].

Reconstitution of the proton pump caused membrane softening in all three types of membranes (20% for polymer, 26% for hybrid, and 30% for lipid membrane; Fig. S4 and SI Appendix, section S14 and Table S3). This indicates that the graft copolymer membrane responds to the inserted proton pump in the same manner as lipid membranes and no undesirable rigidification is taking place. Retention of the membrane softness after protein insertion is crucial for the reconstitution of additional MPs as well as for integration with other functional modules of artificial cells such as motility, division, and growth. Because it was previously demonstrated that nonactive integral MPs only slightly change the bending rigidity of lipid membranes, and to a considerable extent only when they were active (22, 33, 64), we expect a larger influence of pumping bo3 oxidase (in presence of activators). In addition, we speculate that the asymmetry will be enhanced by the unidirectional proton pumping if the proteins have adopted a preferred orientation. However, the assessment of activated membranes was not feasible due to very short window available for analysis during which DTT was already oxidized.

The difference in bending rigidity arises not only from the chemistry of the constituents but also from the arrangement they adopt in those membranes. Cryo-TEM analysis indicated that the protein-free membrane thickness slightly increased with increasing amount of polymer: 4.40 ± 0.16 nm for lipid, 4.86 ± 0.17 nm for hybrid, and 5.25 ± 0.17 nm for polymer membrane. Furthermore, we observed distinct structural differences between all three types of membranes. Lipid LUVs had a bilayer structure with sharp outer boundaries, while polymer membranes appeared as fuzzy monolayers (Fig. 3B). Meanwhile, two populations of hybrid vesicles were observed: one-third resembled polymersomes, while lipid nanodomains of different sizes (discussed in more detail in section 5) were observed in the rest (two-thirds) of the hybrid LUVs.

### 4. Protein Insertion Increases the Fluidity of the Polymer Membrane.

MPs can confer biological functionalities to synthetic membranes only when the former retain structural and functional integrity upon reconstitution. Key factors for this are the flexibility and fluidity of the membrane, which largely determine the lateral mobility of the protein (65, 66). In the case of bo3 oxidase, it is also important that the ubiquinone shuttle can readily diffuse to the active sites of the enzyme. Commonly used block copolymer membranes have significantly lower fluidity compared to lipid membranes [e.g., diffusion coefficients of 0.22 ± 0.06 μm2·s−1 for PBD70-b-PEO30 (67) vs. 10.0 ± 0.4 μm2·s−1 for DOPC (68)], which appears to be one of the reasons for the hindered functionality of MPs. Therefore, we checked whether PDMS-g-PEO provided similar fluidity to the natural environment. To this end, we determined the lateral diffusion of protein, polymer, and lipid by measuring the fluorescence recovery after photobleaching (FRAP). Lipid and polymer were labeled with rhodamine (Rho) and bo3 oxidase with ATTO 514.

The soy PC membrane, used as a benchmark in this study, has a similar fluidity to the commonly used POPC and DOPC membranes (SI Appendix, section S15 and Table S4). In hybrids and polymersomes, the diffusion coefficients of the polymer dye decreased to approximately one-half and one-third of the diffusion coefficient of the lipid dye measured in natural membrane, respectively. Fluidity of the PDMS-g-PEO membrane was in line with previous reports for the same polymer (69) and in fact, similar to that of SOPC (3.8 ± 0.2 μm2·s−1) (70). The high fluidity of the graft copolymer can be partially attributed to the relatively low molecular weight of PDMS-g-PEO (molecular weight, 3,000 g·mol−1) and the PDMS backbone (71).
Insertion of bo$_3$ oxidase led to a ~20% decrease of the diffusion coefficients of the labeled lipids in liposomes and the labeled polymers in hybrids (Fig. 4A and SI Appendix, section S15 and Table S4), while the diffusion coefficients of labeled lipids in hybrids and polymersomes decreased by 32%. Similar decrease in fluidity upon reconstitution of the E. coli outer MPs FhuA, LamB, NanC, OmpA, and OmpF was demonstrated in POPE/POPG bilayers (35). The latter effect was explained by the less smooth surface and the presence of concave regions in the MPs that trapped phospholipids, resulting in overall steric hindrance, which scenario should apply in the present case as well. Interestingly, this phenomenon was not observed in the case of the polymer dye in polymer membranes. On the contrary, the diffusion coefficient increased by 23%. We attributed this to the partitioning of the enzyme into the intertwined PDMS chains, which loosened their assembly and increased the diffusion of the tagged polymer (discussed below). In contrast, the repositioning of lipid molecules in the lipid and hybrid membranes could compensate for the structural disorder, caused by the insertion of bo$_3$ oxidase. The lower mobility of labeled lipids (0.3 mol%) in polymersomes (Fig. 4A) supported such a repositioning around the protein. The diffusion coefficients of proteins and lipids are known to decrease linearly with increasing protein concentration (35, 36). However, an excessive decrease of the membrane fluidity could potentially hinder the conformational changes of MPs. Therefore, the preserved fluidity of polymer membranes upon protein reconstitution may prove valuable for applications, which require higher protein density.

In the case of lipid and hybrid membranes, the lateral diffusion of bo$_3$ oxidase was faster than the diffusion of lipid and polymer dye, while in pure polymer we observed the opposite. Altogether, the protein diffusion slowed down from liposomes to polymersomes, which was analogous to the trend for the fluidity of protein-free membranes. The superimposition of properties of natural and synthetic materials resulted in the intermediate values observed for hybrids. The diffusion coefficient of labeled bo$_3$ oxidase in soy PC (9.9 ± 1.3 μm$^2$ s$^{-1}$) was identical to the previously determined by fluorescence correlation spectroscopy in DOPC and similar to the diffusion coefficients of smaller MPs in POPC (SI Appendix, section S15 and Table S4). On the other hand, the protein diffusion in PDMS-g-PEO (3.5 ± 0.9 μm$^2$ s$^{-1}$) was at least two times higher than the diffusion of porins and channels in a triblock copolymer membrane. For instance, the diffusion coefficient of KcsA decreased nearly sevenfold upon transition from lipid to polymer (PMOXA$_g$-b-PDMS$_{90}$b-PMOXA$_g$) environment (65), while for bo$_3$ oxidase in PDMS-g-PEO the decrease was less than threefold. We ascribed the favorable properties of the graft copolymer to the membrane thickness and the sufficient fluidity. In thicker membranes, the hydrophobic size mismatch between the membrane and the MP is more pronounced and the polymer molecules compress the MP stronger, which reduces its lateral mobility (65). In fact, the hydrophobic matching and slight compression of the PDMS-g-PEO membrane around the enzyme can be seen in cryo-TEM (see Fig. 8).

Since our overarching aim is the reconstitution of the entire ETC comprising several MPs, we checked whether the increase in polymer membrane fluidity upon protein insertion is bo$_3$ oxidase-specific or a general phenomenon. Toward this end, we labeled E. coli F$_1$F$_0$-ATPase with ATTO 620 and reconstituted it in hybrid and polymer GUVs by the same tailored fusion/electroformation protocol used for the proton pump, which demonstrated its utility for larger [F$_1$F$_0$-ATPase >500 kDa (72)] and highly asymmetric MPs (Fig. 4B and SI Appendix, section S15 and Figs. S19 and S20). Because of the lower reconstitution efficiency, which resulted in lower fluorescence signal, we were unable to obtain reliable data for the protein diffusion. Nevertheless, the analysis of the labeled lipids and polymers demonstrated that the loosening of PDMS-g-PEO after protein insertion is not protein specific: The diffusion of the polymer dye in polymersomes increased by 25% upon reconstitution of F$_1$F$_0$-ATPase (Fig. 4A).

Finally, we inspected the protein-induced loosening of the polymer membrane in more detail by analyzing the degree of disorder by a fluorescent probe in LUVs. Laurdan exhibits a red shift when set into more polar environment (presence of water in the membrane) (73), and the resulting generalized polarization (GP) values (for more details, see SI Appendix, section S6.3) range from +1 (most ordered membranes) to −1 (most disordered membranes) (74). Soy PC membranes exhibited similar order to DOPC membranes [GP of soy PC was −0.26 ± 0.01 vs. −0.24 ± 0.00 for DOPC (74)], while polymer membranes displayed significantly higher disorder or water content (about 50% lower GP values) (Fig. 5B). Meanwhile, intermediate GP values were determined for hybrid membranes. The reconstitution of bo$_3$ oxidase caused a slight decrease only in the case of PDMS-g-PEO, which was in line with the hypothesis of loosened polymer architecture.

5. Membrane Reorganization Reseals Hybrid Membranes after Protein Insertion. The passive proton permeability is arguably the most important membrane characteristic with respect to the reconstitution of proton pumps because it is fundamental for the establishment and sustainment of pH gradient. The insertion of bR has been shown to alter the tightness with respect to proton transport.
First, the GUVs, suspended in the electroformation buffer study, we used the same microfluidic setup as for proton permeability of lipid membranes with reconstituted bR was breached. Efforts in this direction have already been made: The attributes such as membrane curvature or compositional difference and the higher confidence of observations in the micrometer scale (79). In this regard, the convenience and the obtained values might not accurately depict the heterogeneity of the sample (79). In this regard, the convenience and the higher confidence of observations in the micrometer range could be potentially extended to account for individual attributes such as membrane curvature or compositional differences. Efforts in this direction have already been made: The permeability of lipid membranes with reconstituted bR was checked in GUVs but not quantified in detail (50). In the present study, we used the same microfluidic setup as for proton pumping in order to assess the permeability of individual GUVs. First, the GUVs, suspended in the electroformation buffer (1 mM Tris-HCl, pH 7.5, containing 100 mM sucrose and 10 μM pyranine), were trapped in the microfluidic chip. Next, non-encapsulated pyranine was flushed away with dye-free buffer, and finally, transmembrane pH gradient was induced by changing the external solution for isosmotic 1 mM MES, pH 6.0, containing ~100 mM sucrose (Fig. 6 A, Inset). Upon acidification in the microfluidic device, the luminal pH of the different types of GUVs decreased differently (SI Appendix, section S7.1-S7.3).

The reported values for proton permeability, which is more accurately denoted as net proton-hydroxyl permeability (80), vary over several orders of magnitude in liposomes (81). This discrepancy partially arises from different setups (pH dye, magnitude of pH gradient, temperature, etc.) and the calculation approaches; for details on the current data analysis, please see SI Appendix, section S7.4. The permeability coefficients at the nanometer scale slightly differed from the ones determined at micrometer scale (Fig. 6 C and D and SI Appendix, section S17.4 and Table S5), but the values did not vary by more than an order of magnitude (×5 at most), which is the range of usually reported precision. This variance may be attributed first to the fact that in the bulk assay the integrity of the LUVs is not always guaranteed, and second to the differences in curvature and packing density, which accompany the size variance. This second assumption was supported by the minor difference in passive proton permeability (~9%) in the case of protein-free polymersomes, in which the structure of the monolayer should be largely preserved, regardless of the size. In the case of bo3 oxidase-functionalized vesicles, the difference in proton permeability on nano and micro level may also arise from different membrane rearrangement and resealing after detergent removal (in the first case, detergent was removed by gel filtration, while in the second by Bio-Beads). Altogether, with respect to the comparison between different membranes, the trends at different scales were remarkably reproducible.

In the case of protein-free vesicles the proton permeability of polymersomes (~2 × 10−7 cm s−1) was slightly higher than that of liposomes in GUVs and 3.4x lower in LUVs (SI Appendix, section S17.4 and Table S5). In protein-free hybrids, we found different populations of vesicles; some exhibited slightly higher permeability than liposomes and polymersomes, while others exceeded it severalfold, which influenced the mean values correspondingly (Fig. 6C). This could obviously not be explained by the superimposition of properties of both membrane constituents, which would result in intermediate values. Instead, we hypothesize that the higher permeability to protons resulted from nanoscale phase separation. At 70 mol% polymer and 30 mol% lipid contents, the hybrid GUVs were exclusively homogeneous under the microscope. We found only a single GUV with observable microdomains in the entire population (500 to 600) of all sample preparations (SI Appendix, section 17.2 and Fig. S26) and no phase separation with time was observed (hybrids stayed microscopically homogenous for over a week). Therefore, we speculate that the permeability may be increased because of heterogeneity at the nanoscale, caused by slight size mismatches and different molecular architecture. This would make the membranes more prone to spontaneous pore formation, especially at the phase interfaces. Increased permeability of hybrid membranes in comparison to pure lipid or polymer vesicles was already reported for DOPC/PEO-h-PB blends (76), while nanodomain formation has been recently demonstrated for blends of PDMS-g-PEO and DPPC (82), and we further confirmed this in PDMS-g-PEO/soy PC hybrids with cryo-TEM. As mentioned in section 3, in larger portion of the hybrid vesicles, we observed nanodomains with lipid bilayer structure and surrounding fuzzy membrane resembling the structure of the polymer membrane (SI Appendix, section S18.3 and Fig. S36). Although the hybrids were prepared from 70 mol% polymer and
30 mol% lipid, the composition cannot be perfectly controlled as vesicles are out-of-equilibrium objects, which leads to formation of hybrids with disperse lipid content, and consequently lipid domains (83). Our observations of nanodomains (SI Appendix, section 18.3 and Fig. S36) and their size are in agreement with the previous report in PDMS-g-PEO/DPPC LUVs [3 to 7 nm, determined via small-angle neutron scattering and FRET (82)]. Importantly, lipid nanodomains in PDMS-g-PEO/PC were previously shown to be stable (no budding was observed in LUVs with nanodomains, while the budding occurred in phase-separated GUVs with microdomains) (83). Interestingly, despite the ~0.9- nm difference in the membrane thickness between the lipid and polymer membranes in the present case, the hybrid membrane had intermediate thickness and therefore no visible size mismatch was detected. A matching thickness between the lipid domains and the surrounding polymer membrane stabilizes the hybrid system by minimizing the line tension (83). Furthermore, the amphiphile mixing was previously shown to be more efficient for graft copolymer than for triblock with the same chemical composition and membrane thickness (PEO-b-PDMS-b-PEO) (82), giving additional reason for the utilization of PDMS-g-PEO for stable hybrid systems.

To further assess the influence of phase separation in hybrids, we deliberately formed microscopically heterogeneous GUVs because we expected that if nanodomains were the cause of increased permeability microdomains should, because of their lower stability, cause similar or even higher proton permeability. To obtain hybrids with microdomains we resorted to the classical electroformation procedure, in which the hybrid amphiphile mixture was deposited on ITO-slides in organic solvent and not in the form of preformed vesicles (SI Appendix, section S17.2 and Fig. S27). The permeability of phase-separated hybrid GUVs (with polymer-to-lipid molar ratio of 40:60) was about threefold higher than for homogeneous hybrid GUVs (7.1 ± 1.9 × 10⁻⁶ cm·s⁻¹) (SI Appendix, section S17.2 and Fig. S28), which confirmed that the proton permeability correlated with the dynamics of phase separation.

The reconstitution of bo3 oxidase increased the proton permeability of the polymer GUVs, which was supported at the LUV scale (Fig. 6D). The scattered data for bo3 oxidase-functionalized polymersomes and liposomes likely results from the different reconstitution efficiency (GUVs with lower permeability have likely lower protein density). We ascribe the decreased tightness of PDMS-g-PEO to the loosening of the polymer structure, as discussed in the case of the increased lateral diffusion (Fig. 4A) and membrane disorder (Fig. S5B). Permeability coefficients in the order of 10⁻⁹ cm·s⁻¹ at much lower lipid-to-protein weight ratios (40–160:1) were determined in egg PC/PA membranes with reconstituted br (37). One can anticipate an increase of permeability at higher protein loadings in the present system as well; however, we do not expect that such loadings will be required for efficient bioenergetics.

In contrast to single-component membranes, in hybrids bo3 oxidase caused an unexpected decrease of permeability for both GUVs and LUVs. To check whether this phenomenon was protein specific, we additionally tested the proton permeability at the LUV scale after insertion of E. coli F1F0-ATPase and observed the same behavior. The permeability of polymer membranes increased, while the permeability of hybrids decreased (Fig. 6D and SI Appendix, section S17.4 and Table S5), whereby we note that the type of detergent (sodium cholate [SC] for bo3 oxidase, octyl glucoside [OG] for F1F0-ATPase) used in the reconstitution apparently did not play a role either.

A plausible explanation for the decreased proton permeability of proteohybrid membranes relates to the reorganization of the membrane by protein insertion; lipid molecules rearrange to fill the protein insertion spots and thus counteract the loosening of the polymer chains. To explore the latter hypothesis in greater detail, we next analyzed the partitioning of either lipid (PE-Rh) or polymer dye (PDMS-g-PEO-Rh) with respect to bo3 oxidase-ATTO 514 in hybrid LUVs. In this scenario, the emission of the FRET donor ATTO 514 would be quenched by the FRET acceptor rhodamine when the lipid dye localizes in close proximity to the reconstituted enzyme. Next, the distance between the FRET couple was gradually increased by stepwise dissolution with OG and the ATTO 514 dequenching was monitored. The dequenching profiles differed significantly between the two systems. Most notably, the initial emission intensity of the protein dye was significantly lower with the lipid dye (i.e., quenched), hinting at preferred lipid localization in the vicinity of bo3 oxidase (Fig. 7A, mechanism presented in Fig. 7C). Moreover, the addition of OG up to the point of total vesicle solubilization (marked as OG Rsol) led to relatively small increase in the emission of ATTO 514 (Fig. 7A, steps 1 and 2). This was a further indication of persistent quenching by protein-associated lipids rather than by merely proximal ones (note that the distance between the latter and the enzyme should increase with increasing surfactant resulting in proportional dequenching, which was observed only partially and shortly). Furthermore, while rapid dequenching of the protein dye was observed upon total solubilization to mixed (lipid/polymer/detergent/bo3) micelles (Fig. 7A, “OG Rsol”), ATTO 514 was quickly requenched again (Fig. 7A, step 4), presumably due to lipid relocation to the protein. Finally, when OG was added at the critical micellar concentration (denoted as “OG CMC”), gradual ATTO 514 dequenching was observed, which was likely a result of PE-Rh leaching to detergent micelles as well as delipidation of bo3 oxidase. In contrast, the higher initial emission of ATTO 514 with PDMS-g-PEO-Rh (i.e., less quenching) indicated larger lateral distance between enzyme and polymer. The dilution of the hybrid membranes resulted in continuous linear dequenching of the polymer dye until the OG Rsol (Fig. 7B, step 2) and similar steady increase was observed in the micellar phase (Fig. 7B, step 3). Both the intensity and dynamics of the FRET experiment with labeled polymer are strong
indicating the presence of others. Since the scope of bottom-up synthetic biology extends beyond the mere segregation from the environment and aims to reconstitute essential life processes such as selective transport and energy transfer, we also seek for retaining those properties of the interfaces, which are necessary to interact with MPs. PDMS-g-PEO appears to accommodate these merits, while providing the virtually unlimited potential of further chemical functionalization. In addition, the polymer appears to increase the shelf life and resistance to ROS.

In this work, we studied the activity of the chemically driven proton pump bo3 oxidase in different types of compartments and determined its influence on relevant mechanical properties. To this end, we optimized the existing protocols and prepared GUVs (>10 μm) with homogenous protein distribution, whereby we demonstrated that the developed procedures can be used for the reconstitution of other complex MPs. The method resulted in active enzymes with predominantly inward orientation, which in combination with a well-sealed membrane, acidified the lumen of the synthetic compartments. The measurements of proton pumping and passive permeability were done in a microfluidic setup, which enabled better control of experimental conditions and individual tracking. While our main rationale is the establishment of PMF for ATP synthesis, the transmembrane proton gradient should not solely serve F1F0-ATPase, but could be also employed for transport, signaling, management of pH-dependent enzymatic reactions, or activation and inhibition of drugs.

The reason for the successful protein insertion and retained activity in PDMS-g-PEO is the suitable polymer chemistry, which results in a soft and fluid membrane and allows for unhindered conformational changes and lateral diffusion. The latter has direct implications on other potential scenarios in bottom-up synthetic biology such as the assembly of protein monomers and clusters, fission and fusion for trafficking, as well as equal distribution of membrane constituents upon division. Liposomes and hybrids have moderately low resistivity to bending deformation (χ = 11.7 ± 2.1 and 11.6 ± 2.4 kBT, respectively) and appear more elastic than lipids. This allows them to undergo deformations and to adjust to the environment for biomedical applications or withstand the mechanical forces exerted in

Conclusions
The replacement and augmentation of natural building blocks with synthetic alternatives may improve some characteristics (e.g., rigidity and tightness, crucial for drug delivery) at the expense of others. Since the scope of bottom-up synthetic biology extends beyond the mere segregation from the environment and aims to reconstitute essential life processes such as selective transport and energy transfer, we also seek for retaining those properties of the interfaces, which are necessary to interact with MPs. PDMS-g-PEO appears to accommodate these merits, while providing the virtually unlimited potential of further chemical functionalization. In addition, the polymer appears to increase the shelf life and resistance to ROS.

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biotechnology, for instance during agitation. We found that the studied membranes interacted differently with the reconstituted protein. While insertion of \( \text{bo}_{3} \) oxidase in soy PC decreased the fluidity, it exercised the opposite effect on the polymer by loosening its structure. The remarkable finding is that the characteristics of hybrid membranes are not always intermediate between lipid and polymer ones—blending the membrane led to increased permeability and broad distribution, but after proton pump insertion the compartments were surprisingly resealed. By combining biochemical and biophysical approaches, we showed that synthetic building blocks can successfully reproduce the demanding environment required by MPs, offering feasible platform for their integration toward the engineering of an artificial cell. This opens up the possibility of not only mimicking nature but also to the creation of functions that did not exist before.

**Methods**

**Fusion/Electroformation of GUVs.** GUVs were prepared from LUVs by modifying the previously published procedure (19). \( \text{bo}_{3} \) oxidase and \( \text{F}_{2} \text{F}_{2}-\text{ATPase} \) were reconstituted into LUVs via detergent-mediated reconstitution after optimization of our previous protocol (29). For details, see SI Appendix.

**Preparation of Microfluidic Chips.** The wafers were produced by conventional soft lithography methods (84). For chip fabrication, see SI Appendix. Each post in the trap has a dimension of 40 by 40 \( \mu \text{m} \), and a height of \( \sim 70 \mu \text{m} \); the gap distance between two posts is 5 \( \mu \text{m} \). Each post in the trap has a dimension of 40 by 40 \( \mu \text{m} \), and a height of \( \sim 70 \mu \text{m} \); the gap distance between two posts is 5 \( \mu \text{m} \). To prevent the adhesion of GUV to the surface and the resulting rupture, chips were coated with BSA solution.

**Oxygen Consumption Measurements.** Oxygen consumption measurements were performed with Oxytherm system (Hansatech Instruments). Steady-state activity of reconstituted \( \text{bo}_{3} \) oxidase was determined as described in ref. 29 with slight modifications. For details, see SI Appendix.

**Lateral Diffusion Analysis by FRAP.** FRAP experiments were performed using the FRAP booster mode in a Leica TCS SP8 (Wetzlar) microscope with a 63× (1.2 N.A.) water-immersion objective and at 1 Airy unit. Imaging and photobleaching were performed with a 561-nm diode-pumped solid-state laser. Images were acquired with 128 × 128 format at a speed of 1,400 Hz, with no line averaging. In this configuration, the time between frames was 54 ms. Ten prebleach images at low laser intensity were recorded and used as a reference, and then the laser intensity was increased to maximum for another four frames for photobleaching, after which the laser intensity was again decreased to recover photobleaching recovery. The nominal (user-defined) photobleaching radius (\( r_{\text{b}} \)) was set to 1.5 \( \mu \text{m} \).

**Membrane Order Analysis by Laurdan.** The emission spectra of Laurdan were monitored in fluorescence spectrophotometer Varian Cary Eclipse (Agilent) at excitation 530 nm and emission 400 to 600 nm. Final lipid/polymer concentration was 200 \( \mu \text{M} \), and final Laurdan concentration was 600 \( \mu \text{M} \).

**Passive Proton Permeability.**

**Passive permeability measurements of LUVs.** \( \text{bo}_{3} \) oxidase was reconstituted by 0.8% SC in lipid, hybrid, and polymer vesicles (40 mg mL\(^{-1}\)), at final lipid/polymer-to-protein ratios of 9,560:1, 9,550:1, and 9,540:1, respectively. \( \text{F}_{2} \text{F}_{2}-\text{ATPase} \) was reconstituted at the same ratios but via 2.4% SC in liposomes and 0.4% OG in hybrids and polymersomes. Passive proton permeability of LUVs was determined by adopting a reported protocol (85).

**Passive permeability measurements of GUVs.** The lipid/polymer-to-protein molar ratio in \( \text{bo}_{3} \)-GUVs was the same as in \( \text{bo}_{3} \)-LUVs. The experiments were performed in microfluidics, as described in main text.

**Determination of proton permeability coefficients from intravesicular pyranine fluorescence.** The data were fitted with a biexponential curve \( y = A_{1}e^{−k_{1}t} + A_{2}e^{−k_{2}t} + y_{0} \), where \( A \) is the amplitude of fluorescence signal, \( k \) is the first-order rate constant of proton influx, and \( y_{0} \) is the offset; \( k_{2} \) was used to calculate the permeability coefficient through the reported relation \( P_{\text{m}} = k(\text{R})^{-3} \), where \( R \) was the vesicle radius.

**Analysis of \( \text{bo}_{3} \) Oxidase Partitioning in Hybrid Membranes via FRET.** \( \text{bo}_{3} \) oxidase labeled with ATTO 514 was reconstituted in hybrid LUVs containing 2 mol% of either lipid dye (PE-Rho) or labeled polymer (PDMS-g-PEO-Rho). ATTO 514 fluorescence was monitored at excitation 511 nm and emission 533 nm at constant stirring and 5 °C. OG was added in 30 subsequent 2-μL 10% OG aliquots. After each addition, samples were vortexed for 5 s.

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Fig. 8. Cryo-TEM images of lipid, hybrid, and polymer bo3−LUVs. bo3 oxidase is marked with a red square. On the Left and Right image, inward orientation of the cytosolic part (pump in) can be observed; see SI Appendix, section S19 and Fig. S41 for bo3 oxidase dimensions and structure. The black arrows (Middle image) show increased membrane thickness around the protein (from 4.9 to −7.5 nm) in bo3−hybrid-LUVs. Lipid bilayer thickness did not change after protein insertion (SI Appendix, section S18.1 and Table S6). In bo3−polymer-LUVs (Right), the membrane compressed in the vicinity of the protein (−4.4 nm) and expanded at further distance (−6.3 nm), leading to increase in the average thickness (−5.8 nm). (Scale bar, 50 nm; defocus, approximately −2 μm.)

Data Availability. All relevant data and protocols discussed in the study are available in the main text and SI Appendix.

Cryo-TEM. The vitrification of the samples was carried out using Vitrobot Mark IV System (Thermo Fisher Scientific) and standard Vitrobot Filter Paper (i.e., OS2520 mm, Grade 955). For vitrification conditions, see SI Appendix. The grid was mounted onto a Thermo FEI Glacios 200 kV autoloader under cryo conditions. Images were acquired using Falcon 3EC direct electron detector in linear mode and a total dose of 50 e−/Å².

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1. L. Ottn et al., Artificial organelles for energy regeneration. Adv. Bioyst. 3, 1880023 (2019).
2. J. F. Le Meins, C. Schatz, S. Lecommandoux, O. Sandre, Hybrid polymer/lipid vesicles: State of the art and future perspectives. Mater. Today 16, 397–402 (2013).
3. E. Rideau, R. Dimova, P. Schwille, F. R. Wurm, K. Landfester, Liposomes and polymersomes: A comparative review towards cell mimicking. Chem. Soc. Rev. 47, 8572−8610 (2018).
4. J. S. Lee, J. Feijen, Polymersomes for drug delivery: Design, formation and characterization. J. Control. Release 161, 473−483 (2012).
5. D. E. Disher et al., Emerging applications of polymersomes in delivery: From molecular dynamics to shrinkage of tumors. Prog. Polym. Sci. 32, 838−857 (2007).
6. F. Meng, Z. Zhong, J. Feijen, Stimuli-responsive polymersomes for programmed drug delivery. Biomacromolecules 10, 197−209 (2009).
7. S. Egli et al., Biocompatible functionalization of polymersome surfaces: A new approach to surface immobilization and cell targeting using polymersomes. J. Am. Chem. Soc. 133, 4476−4483 (2011).
8. Y. Lee, J.-B. Chang, H. K. Kim, T. G. Park, Stability studies of biodegradable polymersomes prepared by emulsion solvent evaporation method. Macromol. Res. 14, 359−364 (2006).
9. A. Mata, A. I. Fleischman, S. Roy, Characterization of polydimethylsiloxane (PDMS) properties for biomedical micro/nanosystems. Biomach. Micro Devices 7, 281−293 (2005).
10. S. T. Poschenrieder, K. S. Schiebel, K. Castiglione, Stability of polymersomes with focus on their use as nano-reactors. Eng. Life Sci. 18, 101−113 (2018).
11. R. Rodríguez-García et al., Polymersomes: Smart vesicles of tunable rigidity and permeability. Soft Matter 7, 1532−1542 (2011).
12. J. F. Le Meins, O. Sandre, S. Lecommandoux, Recent trends in the tuning of polymersomes’ membrane properties. Eur. Phys. J. E Soft Mater 34, 14 (2011).
13. M. Xiao, J. Liu, J. Yang, R. Wang, D. Xie, Bioluminescent membrane control of block co-polymer vesicles with tunable wall thickness. Soft Matter 9, 2434−2442 (2013).
14. J. Pettit et al., A modular approach for multifunctional polymersomes with controlled adhesive properties. Soft Matter 14, 894−900 (2018).
15. B. Kadenbach, Intrinsically and extrinsically uncoupling of oxidative phosphorylation. Biochim. Biophys. Acta 1604, 77−94 (2003).
16. M. P. Murphy, How mitochondria produce reactive oxygen species. Biochem. J. 417, 1−13 (2009).
17. A. W. Grotti, Mechanisms of lipid peroxidation. J. Free Radic. Biol. Med. 1, 87−95 (1985).
18. E. Schnitzer, I. Pinchuk, D. Lichtenberg, Peroxidation of liposomal lipids. Eur. Biophys. J. 36, 499−515 (2007).
19. P. Girard et al., A new method for the reconstitution of membrane proteins into giant unilamellar vesicles. Biophys. J. 87, 419−429 (2004).
20. E. Altamura et al., Highly oriented photosynthetic reaction centers generate a proton gradient in synthetic protocols. Proc. Natl. Acad. Sci. U.S.A. 114, 3837−3842 (2017).
21. O. Biner, T. Schick, Y. Müller, C. van Ballmoos, Delivery of membrane proteins into small and giant unilamellar vesicles by charge-mediated fusion. FEBS Lett. 590, 2051−2062 (2016).
22. V. G. Almendro-Vedia et al., Nonequilibrium fluctuations of lipid membranes by the rotating motor protein F1F0-ATP synthase. Proc. Natl. Acad. Sci. U.S.A. 114, 11291−11296 (2017).
43. B. L. Mui, P. R. Cullis, E. A. Evans, T. D. Madden, Osmotic properties of large unilamellar vesicles prepared by extrusion. Biophys. J. 64, 442–453 (1993).

44. R. Dimova, C. Marques, The Giant Vesicle Book (CRC Press, ed. 1, 2019).

45. M. I. Angelova, S. Soleau, P. Mélard, F. Faucon, P. Bothorel, Preparation of Giant Vesicles by External AC Electric Fields. Kinetics and Applications (Steinkopff, 1992), pp. 127–131.

46. S. Pautot, B. J. Frisken, D. A. Weitz, Production of unilamellar vesicles using an inverted emulsion. Langmuir 19, 2870–2879 (2003).

47. J. Sjöholm et al., The lateral distance between a proton pump and ATP synthase determines the ATP-synthesis rate. Sci. Rep. 7, 2926 (2017).

48. B. Mattei, R. B. Lira, K. R. Perez, K. A. Riske, Membrane permeabilization induced by Triton X-100: The role of membrane phase state and edge tension. Chem. Phys. Lipids 202, 28–37 (2017).

49. M. Chemin, P.-M. Brun, S. Lecommandoux, O. Sandre, J.-F. Le Meins, Hybrid polymer/lipid vesicles: Fine control of the lipid and polymer distribution in the binary membrane. Soft Matter 8, 2867–2874 (2012).

50. N. R. Clement, J. M. Gould, Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate) as a probe of internal aqueous hydrogen ion concentration in phospholipid vesicles. Biochemistry 20, 1534–1538 (1981).

51. S. Gupte et al., Relationship between lateral diffusion, collision frequency, and electron transfer of mitochondrial inner membrane oxidation-reduction components. Proc. Natl. Acad. Sci. U.S.A. 81, 2606–2610 (1984).

52. M. Seigneuret, J. L. Rigaud, Analysis of passive and light-driven ion movements in large bacteriorhodopsin liposomes reconstituted by reverse-phase evaporation. 2. Influence of passive permeability and back-pressure effects upon light-induced proton uptake. Biochemistry 25, 6723–6730 (1986).

53. C. von Ballmoos, O. Biner, T. Nilsson, P. Brzezinski, Mimicking respiratory phosphorylation using purified enzymes. Biochim. Biophys. Acta 1857, 321–331 (2016).

54. P. Mélard et al., Mechanical properties of model membranes studied from shape transformations of giant vesicles. Biochimie 80, 401–413 (1998).

55. R. Dimova, Recent developments in the field of bending rigidity measurements on membranes. Adv. Colloid Interface Sci. 208, 225–234 (2014).

56. P. Mélard, T. Pott, H. Bourrais, J. H. L. Ipsen, Advantages of statistical analysis of giant vesicle flickering for bending elasticity measurements. Eur. Phys. J. E Soft Matter 34, 116 (2011).

57. R. S. Gracia, N. Bezylepkina, R. L. Knorr, R. Lipowsky, R. Dimova, Effect of cholesterol on the rigidity of saturated and unsaturated membranes: Fluctuation and electro-deformation analysis of giant vesicles. Soft Matter 6, 1472–1482 (2010).

58. M. Karimi et al., Asymmetric ionic conditions generate large membrane curvatures. Nano Lett. 18, 7816–7821 (2018).

59. D. W. Deamer, J. W. Nichols, Proton-hydroxide permeability of liposomes composed of archaeal tetraether lipids. Biochim. Biophys. Acta 106, 321–331 (1986).

60. J. B. Manneville, P. Bassereau, S. Ramaswamy, J. Prost, Active membrane fluctuations studied by micropipet aspiration. Phys. Rev. E Stat. Nonlin. Soft Matter Phys. 64, 021908 (2001).

61. J. B. Manneville, P. Bassereau, D. Lévy, J. Prost, Activity of transmembrane proteins induces magnification of shape fluctuations of lipid membranes. Phys. Rev. Lett. 82, 4356–4359 (1999).

62. P. Bassereau, B. Sorre, A. Lévy, Bending lipid membranes: Experiments after W. Helrich’s model. Adv. Colloid Interface Sci. 208, 47–57 (2014).

63. K. M. Berman et al., The Protein Data Bank. Nucleic Acids Res. 23, 235–242 (2000).

64. J. B. Manneville, P. Bassereau, S. Ramaswamy, J. Prost, Active membrane fluctuations studied by micropipet aspiration. Phys. Rev. E Stat. Nonlin. Soft Matter Phys. 64, 021908 (2001).

65. F. Itel, A. Najer, C. G. Pailivan, W. Meier, Dynamics of membrane proteins within synthetic polymer membranes with large hydrophobic mismatch. Nano Lett. 15, 3871–3878 (2015).

66. A. G. Lee, How lipids affect the activities of integral membrane proteins. Biochim. Biophys. Acta 1666, 62–87 (2004).

67. J. Nam, P. A. Beales, T. K. Vanderlick, Giant phospholipid/block copolymer hybrid vesicles: Mixing behavior and domain formation. Langmuir 27, 1–6 (2011).

68. F. Heinemann, V. Betanell, F. A. Thomas, P. Schwille, Quantifying lipid diffusion by fluorescence correlation spectroscopy: A critical treatise. Langmuir 28, 13395–13404 (2012).

69. T. P. T. Dao et al., The combination of block copolymers and phospholipids to form giant hybrid unilamellar vesicles (GUVs) does not systematically lead to “intermediate” membrane properties. Soft Matter 14, 6476–6484 (2018).

70. J. C.-M. Lee, M. Santore, F. S. Bates, D. E. Discher, From membranes to melts, route to re- plication: Diffusion in polymersome versus lipid bilayers. Macromolecules 35, 323–326 (2002).

71. F. Itel et al., Molecular organization and dynamics in polymersome membranes: A lateral diffusion study. Macromolecules 47, 7588–7596 (2014).

72. D. Okuno, R. Iino, H. Noji, Rotation and structure of FoF1-ATP synthase. J. Biochem. 149, 655–664 (2011).

73. L. A. Bagatolli, To see or not to see: Lateral organization of biological membranes and fluorescence microscopy. Biochim. Biophys. Acta 1758, 1541–1556 (2006).

74. H.-J. Kaiser et al., Order of lipid phases in model and plasma membranes. Proc. Natl. Acad. Sci. U.S.A. 106, 16645–16650 (2009).

75. A. Wiedemann, P. Dimroth, C. von Ballmoos, Deltapl and DeltapH are equivalent driving forces for proton transport through isolated F0 complexes of ATP synthases. Biochim. Biophys. Acta 1777, 1301–1310 (2008).

76. W. F. Paxton, P. T. McAninch, K. E. Achuthan, S. H. R. Shin, H. L. Monteith, Monitoring and modulating ion traffic in hybrid lipid/polymer vesicles. Colloids Surf. B Biointerfaces 159, 268–276 (2017).

77. M. G. Elferink, J. G. de Wit, A. J. Driessen, W. N. Konings, Stability and proton-permeability of liposomes composed of archaeal tetraraphic lipids. Biochim. Biophys. Acta 1193, 247–254 (1994).

78. R. H. Gensure, M. L. Zeidel, W. G. Hill, Lipid raft components cholesterol and spin-gomyltin increase H4OH permeability of phosphatidylycholine membranes. Biochim. J. 398, 485–495 (2006).

79. C. L. Kuypers, J. S. Kuo, S. A. Mutch, D. T. Chiu, Proton permeation into single vesicles occurs via a sequential two-step mechanism and is heterogeneous. J. Am. Chem. Soc. 128, 3233–3240 (2006).

80. J. W. D. W. Nichols, D. W. Deamer, Net proton-hydroxide permeability of large unilamellar liposomes measured by an acid-base titration technique. Proc. Natl. Acad. Sci. U.S.A. 77, 2038–2042 (1980).

81. D. W. Deamer, J. W. Nichols, Proton-hydroxide permeability of liposomes. Proc. Natl. Acad. Sci. U.S.A. 80, 165–168 (1983).

82. T. P. T. Dao et al., Mixing block copolymers with phospholipids at the nanoscale: From hybrid polymer/lipid wormlike micelles to vesicles presenting lipid nanodomains. Langmuir 33, 1705–1715 (2017).

83. T. P. T. Dao et al., Phase separation and nanodomain formation in hybrid polymer/lipid vesicles. ACS Macro Lett. 4, 182–186 (2015).

84. S. Deshpande, Y. Caspi, A. E. C. Meijering, C. Dekker, Octanol-assisted lipid assembly on chip. Nat. Commun. 7, 10447 (2016).

85. S. V. Albers, W. N. Konings, A. J. M. Driessen, Membranes of thermophiles and other extremophiles. J. Microbiol. Methods 35, 161–171 (2006).
Supporting Information

Constructing artificial respiratory chain in polymer compartments: insights into the interplay between bo3 oxidase and the membrane

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Materials and Methods

SECTION S1: Materials

Section S1.1: Chemicals

Soy PC (95 %) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (PE-Rho) were purchased from Avanti Polar Lipids. Pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt, HPTS) and 5(6)-carboxyfluorescein were purchased from Sigma-Aldrich. NHS-rhodamine, NHS-fluorescein and Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) were purchased from Thermo Fisher Scientific. NHS-ATTO 514 was purchased from Merck. PDMS-g-PEO was a kind gift from Dow Corning. The viscosity-average molecular weight of 3000 g mol⁻¹, the 47% weight fraction of ethylene oxide (2 arms of PEO per PDMS chain, on average) and the average degree of polymerization of 12 are reported in the data provided by the supplier. PDMS-g-PEO labeled with fluorescein (PDMS-g-PEO-Fluo) or rhodamine (PDMS-g-PEO-Rho) was synthesized following a previously described procedure (1) and was used as a fluorescent polymer marker for the visualization of hybrid and polymer membrane in confocal microscopy. The E. coli bo3 oxidase was expressed from plasmid pETcyo in E. coli strain C43 (DE3)(ΔcyoABCDE) and purified as described (2). The E. coli F₁F₀ ATP synthase was expressed from plasmid pBWU13-βHis in E. coli strain DK8 (ΔuncBEFHAGDC) and purified as previously described (3).

Scheme S1. Chemical structure of PDMS-g-PEO (m=2).

Section S1.2: Abbreviations

PC, L-α-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; PA, phosphatidic acid; PE-Rho, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl); PDMS-g-PEO, polydimethyl-
siloxane-g-poly(ethylene oxide); PEO-b-PBD, poly(ethylene oxide)-b-poly(butadiene); PEO–PPO–PEO, poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide); PMOXA-b-PDMS-b-PMOXA, poly(2-methyl-2-oxazoline)-b-polydimethylsiloxane-b-poly(2-methyl-2-oxazoline); PEtOz-PDMS-PEtOz, poly (2-ethyl-2-oxazoline)-b-poly(dimethylsiloxane)-b-poly(2-ethyl-2-oxazoline); PDMS-g-PEO-Fluo, PDMS-g-PEO-Fluorescein; PDMS-g-PEO-Rho, PDMS-g-PEO-Rhodamine.

**Section S1.2: Protein labeling**

**Labeling of bo3 oxidase with ATTO 514**

bo3 oxidase was labeled with the amine-reactive ATTO 514 N-hydroxysuccinimidyI(NHS)-ester. The enzyme was dissolved to 2.2 mg ml\(^{-1}\) in 20 mM HEPES, containing 0.05% DDM at pH = 8.2, which ensured sufficient concentration of unprotonated (and thus reactive) amino groups. Eightfold molar access of ATTO 514 dye, dissolved to 2 mg ml\(^{-1}\) in anhydrous DMSO, was added to the enzyme solution and the reaction mixture was incubated for 1.5 h at room temperature, under agitation (250 rpm). Unbound dye was removed from the conjugate solution via size exclusion chromatography on a column (internal diameter of 1.5 cm and length of 20 cm) filled with Sephadex G-25 resin, equilibrated with 20 mM HEPES (pH = 7.5), containing 0.05% DDM. The average number of dye molecules coupled to the enzyme (degree of labeling, DOL), was estimated via absorption spectroscopy, and can be described with the equation, provided by the manufacturer:

\[
DOL = \frac{A_{\text{max}} \times \varepsilon_{\text{prot}}}{(A_{280} - A_{\text{max}} \times \varepsilon_{280}) \times \varepsilon_{\text{max}}}
\]

where \(A_{\text{max}}\) is the absorbance of conjugate at the absorption maximum \(\lambda_{\text{abs}}\) (for ATTO 514 \(\lambda_{\text{abs}} = 511\) nm), \(\varepsilon_{\text{prot}}\) is the molar extinction coefficient of enzyme (\(\varepsilon_{\text{bo3}} = 184720\) M\(^{-1}\) cm\(^{-1}\), denatured, ExPASy/ProtParam), \(A_{280}\) is the absorbance of conjugate at the absorption maximum of proteins (280 nm), \(\varepsilon_{280}\) is the correction factor for the dye (\(\varepsilon_{\text{ATTO 515}} = 0.07\)) and \(\varepsilon_{\text{max}}\) is the extinction coefficient of the dye at the absorption maximum \(\lambda_{\text{abs}}\) (\(\varepsilon_{\text{max}} = 115000\) M\(^{-1}\) cm\(^{-1}\)). The DOL was determined to be 1.7 molecules of dye per enzyme.

**Labeling of F₁F₀-ATPase with ATTO 620**

Purified F₁F₀-ATPase was labeled with the fluorescent dye ATTO 620 NHS via conjugation to the primary amino groups of the enzyme. Towards this end, the ATP synthase was first diluted to 2 mg ml\(^{-1}\) in 50 mM HEPES (pH = 8.3) containing 100 mM KCl, 250 mM sucrose, 5 mM MgCl\(_2\), 0.1 mM EDTA, 0.2 mM DTT, 2.5 % glycerol, 0.5 % sodium cholate, 0.8 % phosphatidylcholine, 1.5 % octyl glucoside and
0.5% sodium deoxycholate. Next, ATTO 620 NHS dissolved at 1 mg ml\(^{-1}\) in DMSO was added to the enzyme at 8-fold molar excess and the conjugation mixture was thoroughly but gently mixed. The enzyme and the dye were incubated for 1.5 h at room temperature under agitation (shaking at 600 RPM). Following incubation, nonconjugated dye was removed via size exclusion chromatography following the manufacturer’s recommendations. The eluted fraction containing labeled enzyme was collected and concentrated using Amicon® Ultra centrifugal filter devices (Merck Millipore).

**Section S1.3: Preparation of LUVs**

Liposomes were prepared from soy PC, hybrid (lipid/polymer) vesicles were prepared from 70 mol% PDMS-g-PEO : 30 mol% soy PC, and polymersomes were prepared from PDMS-g-PEO. For confocal microscopy, FRAP, passive proton permeability and proton pumping experiments liposomes were supplemented with 0.016–0.05 mol% Rho-PE, while hybrids and polymersomes were supplemented with 0.05–0.5 mol% PDMS-g-PEO-Rho. 5 mg of lipid/polymer mixture (for hybrids), 5 mg of polymer (for polymersomes) or 10 mg of lipid (liposomes) in chloroform : MeOH (2:1, v/v) were deposited in a glass vial and the solvent was removed by evaporation under a gentle stream of nitrogen for ~30 min. The thin lipid, lipid/polymer or polymer film was rehydrated with 100 mM sucrose, 1 mM Tris-HCl (pH 7.5) and resuspended to a final lipid concentration of 5 mg ml\(^{-1}\) (hybrids and polymersomes) or 10 mg ml\(^{-1}\) (liposomes) by gentle vortexing. If polymersomes were used for reconstitution, the rehydration buffer was supplemented with 0.1% sodium cholate. The suspension of multilamellar vesicles (MLVs) was subjected to 5 freeze-thaw cycles (1 min LN\(_2\), then water bath at 35 °C until thawed completely, followed by 30 s vortexing). The freeze-thaw step was skipped for polymersomes. Finally, the size and lamellarity of vesicles was unified by extrusion (21 times) through a 100 nm pore (polycarbonate membrane, Mini Extruder).

**Section S1.4: Preparation of microfluidic chips**

**Wafer design and fabrication.** The pattern on the wafer was designed with AutoCAD 2017. The wafers were produced by conventional soft lithography methods (4). Typically, a desired thickness of SU 8-3050 was spun-coated onto a 4-inch silicon wafer and pre-baked at different temperatures, then a mask aligner (EVTG-620) was employed for writing the desired pattern onto the coated wafer, and the wafer was immediately post-baked. SU-8 developer was used for dissolving the remaining portion of photoresist in order to obtain the final pattern on the wafer.

**Chip fabrication.** Microfluidic chips were produced by pouring degassed PDMS precursor and curing agent (Sylgard 184, Dow Corning GmbH) onto the wafer at a weight ratio of 10:1 and baked at 80 °C for
2 h. The PDMS block was peeled off from the wafer, cut into pieces with a razor, inlet and outlet holes were punched by a biopsy punch. Glass coverslips were cleaned by detergent, water and ethanol, then blow-dried with nitrogen gas. The PDMS device and the coverslip were then exposed to oxygen plasma for 1 min and the two parts were immediately bonded after plasma treatment, followed by further baking at 80 °C to accelerate the process. The microfluidic chips were stored in a closed box until use. Each post in the trap has a dimension of 40 μm by 40 μm, and a height of ~70 μm, the gap distance between two posts is 5 μm.

**Chip coating.** The microchannels of the device were filled with 2% (w/v) bovine serum albumin (BSA) solution by centrifugation and incubated for 30 min at room temperature. This prevents the adhesion of GUV to the surface and the resulting rupture. After incubation, the BSA solution was replaced with 100 μl of buffer (100 mM sucrose, 1 mM Tris-HCl (pH 7.5)) or buffer with added 1 mM KCl using a syringe pump in withdraw mode at 10 μl min⁻¹.

**Scheme S2.** Network design of the microfluidic chip (5) (left) used for trapping GUVs in proton pumping and proton permeability experiments. The channels split into 8 separate lines, each with 17 traps totaling 136 per device. Bright filed image of one trap with posts of dimensions 40 × 40 μm (right).

**SECTION S2: LUV characterization**

**Section S2.1: Size and dispersity**

Size and dispersity of LUVs and proteoLUVs was determined by dynamic light scattering (DLS). DLS experiments were performed using a Zetasizer Nano ZS (Malvern, Worcestershire, UK) with a 633 nm helium-neon laser with back-scattering detection. Undiluted LUVs/proteoLUVs in 100 mM sucrose, 1 mM Tris-HCl (pH 7.5) were measured at a fixed 173° scattering angle in a 50 μl quartz cuvette.
Section S2.2: Zeta potential

Zeta potential measurements were done with Zetasizer Nano ZS (Malvern, Worcestershire, UK). One ml of vesicle suspension, liposomes (10 mg ml\(^{-1}\)), hybrids (20 mg ml\(^{-1}\)) or polymersomes (40 mg ml\(^{-1}\)), was transferred with 1-ml glass syringe into disposable folded capillary cell and zeta potential was measured with the following settings: model Smoluchowski, 23 °C, equilibration time 120 s, DTS 1060/DTS 1061 cell, data processing auto mode. Each sample was measured three times (each time in 60–160 runs).

SECTION S3: Durability and chemical stability

Section S3.1: Reconstitution of bo\(_3\) oxidase into LUVs

For stability experiments, bo\(_3\) oxidase was reconstituted at lipid/polymer to protein molar ratio of 9550:1. To achieve this ratio and at the same time nearly similar molar concentration of bo\(_3\) oxidase in the reconstitution mixture, we reconstituted the protein in 5 mg ml\(^{-1}\) liposomes, 10 mg ml\(^{-1}\) hybrids and 20 mg ml\(^{-1}\) polymersomes. The final protein concentration was 0.675 µM for liposomes, 0.449 µM for hybrids and 0.698 µM for polymersomes. For reconstitution in liposomes 0.1 % sodium cholate was added to the vesicle suspension previous to addition of protein, and to hybrids 0.2 % sodium cholate. In the case of polymersomes 0.4 % sodium cholate was added already during the vesicle preparation. Detergent was removed by addition of Bio-Beads in three steps (90 mg for 200 µl of reconstitution mixture). In order to maintain the reconstitution conditions and homogeneity of the sample, we performed four separate reconstitutions for each sample and afterwards combined the samples.

Section S3.2: Activity lifetime measurements

The activity lifetime of lipid, hybrid and polymer bo\(_3\)-LUVs was determined via the oxygen consumption of bo\(_3\) oxidase in bulk samples. Oxygen consumption measurements were performed with Oxytherm system (Hansatech Instruments). Oxytherm system consists of S1/Mini oxygen electrode disc, Oxytherm control unit, Oxytherm electrode chamber and OxyTrace software. The principle of oxygen detection is described in Scheme S3.
**Scheme S3.** Principle of oxygen concentration measurement. S1/MINI Clark-type oxygen electrode disc consists of a platinum cathode and silver anode set into an epoxy resin disc and it traps a layer of 50 % saturated KCl solution beneath an oxygen-permeable PTFE membrane. When a small voltage is applied across these electrodes, the current which flows is at first negligible and the platinum becomes polarized (i.e. it adopts the externally applied potential). As this potential is increased to 700 mV, oxygen is reduced at the platinum surface, initially to hydrogen peroxide $\text{H}_2\text{O}_2$ so that the polarity tends to discharge as electrons are donated to oxygen. The current which then flows is stoichiometrically related to the oxygen consumed at the cathode.

Steady state activity of reconstituted $\text{bo}_3$ oxidase was determined as described by (6, 7), with slight modifications. Total measurement volume was 1000 µl. The volume of $\text{bo}_3$-LUVs was adjusted to achieve theoretically the same molar concentration of $\text{bo}_3$ oxidase, i.e. ~13.5 nM. $\text{bo}_3$ oxidase was activated by addition of DTT (final concentration 8 mM) and $\text{Q}_1$ (final concentration 40 µM). The samples were measured for 11–13 days every 24 h and kept at 4 °C at all time. The oxygen consumption rates were reported as the average of 2–3 measurements, with standard deviation. All measurements were done at 22 °C while stirring.

**Section S3.3: Chemical stability of $\text{bo}_3$-LUVs**

To study the potential $\text{bo}_3$-LUVs damage under the influence of oxidative stress we induced a peroxidation process by the addition of iron(II)sulfate ($\text{FeSO}_4$) together with ascorbate, as it was done previously for mitochondrial membranes (8) and liposomes (9). $\text{FeSO}_4$ stock solution (1.25 mM) was prepared by dissolving $\text{FeSO}_4\cdot7\text{H}_2\text{O}$ in the buffer, used for preparation of $\text{bo}_3$-LUVs (100 mM sucrose, 1 mM Tris-HCl, pH 7.5). Sodium L-ascorbate stock solution (25 mM) was prepared in the same buffer, but the solution was prepared freshly (on the day of measurement). The influence of the ascorbyl free radical (A•) on $\text{bo}_3$-LUVs was tested by incubating 24 µl $\text{bo}_3$-LUVs with 0.5 µl $\text{FeSO}_4$ (final concentration 25 µM) and 0.5 µl sodium L-ascorbate (final concentration 500 µM). The solution was well mixed by vortexing and incubated for 30 min and the $\text{bo}_3$ oxidase activity was determined via oxygen consumption.
(the same volumes used as for shelf-life measurements). We measured oxygen consumption in absence of both chemicals as a reference (100 % protein activity). The experiments were carried out in duplicates or triplicates and the $t$-test ($p < 0.05$) was conducted for statistical analysis. The influence of ascorbyl radicals on vesicle stability was tested also by determining size distribution (DLS) before and after addition of both chemicals.

Section S3.4: bo$_3$ oxidase in micelles

Activity of bo$_3$ oxidase stabilized in n-dodecyl β-D-maltoside (DDM) was monitored via oxygen consumption in Oxytherm. Protein was diluted in 20 mM HEPES (pH 8.0), 0.05 % DDM. Activity was determined for untreated protein and treated protein (30 min incubation with ascorbyl radicals). Both samples were measured three times and the average value was given with standard deviation.

Section S3.5: Vesicles leakage of carboxyfluorescein

Vesicle leakage after exposure to ascorbate and ascorbyl radicals was monitored via the dequenching of encapsulated carboxyfluorescein (CF). LUVs were prepared in buffer (100 mM sucrose, 1 mM Tris-HCl, pH 7.5) supplemented with 20 mM CF. To remove the non-encapsulated CF, the vesicle suspension was first run through a Sephadex G-25 column and additionally purified with Nycodenz gradient separation. The vesicle suspension at the top of the Nycodenz gradient was carefully collected. Fluorescence of CF was monitored at excitation 492 nm and emission 517 nm (2.5/2.5 nm, 0.1 s) in a fluorescence spectrophotometer Varian Cary Eclipse (Agilent) at constant stirring. Fluorescence was blanked with buffer (100 mM sucrose, 1 mM Tris-HCl, pH 7.5) and baseline was monitored with vesicle suspension diluted 1:2 (v/v). After around 10 min, ascorbate solution was added and after around 40 min FeSO$_4$ solution was added, which initiated formation of ascorbyl free radicals. To obtain 100% fluorescence (complete dequenching), vesicles were solubilized with Triton X-100 (at concentration that we previously determined as solubilization point).

SECTION S4: Preparation and quality analysis of bo$_3$-GUVs

Section S4.1: Reconstitution of bo$_3$ oxidase into LUVs

The reconstitution protocol for bo$_3$ oxidase in liposomes, hybrids and polymersomes was a slight modification of our previous protocol (7). Briefly, sodium cholate was added to liposomes (10 mg ml$^{-1}$ LUVs, final conc. of sodium cholate 0.2 %) or hybrids (5 mg ml$^{-1}$ LUVs, final conc. of sodium cholate 0.1 %). Polymersomes already contained sodium cholate, therefore detergent was not added in this step.
Next, bo$_3$ oxidase was gently added to liposomes, hybrids and polymersomes at final conc. 1.35/0.45/0.35 µM, respectively. The reconstitution mixture was incubated at 4 °C for 30 min with mild agitation, followed by detergent removal via Bio-Beads SN-2 (Bio-Rad). For preparation of proteoliposomes, for 200 µl of reconstitution mixture 90 mg was added at once and incubated for 1.5 h at room temperature on a rocking platform. Meanwhile, for preparation of 200 µl of proteohybrids and protopolymersomes suspension, the beads were added in 3 subsequent additions, 30 mg of beads each, followed by 30 min incubation period, at room temperature on a rocking platform. After that, beads were pelleted and the supernatant (proteovesicles) was collected and stored on 4 °C. If the proteoLUVs were not used for preparation of proteoGUVs the same day, the vesicle suspension was frozen in LN$_2$ and aliquots of 20 µl were stored at −80 °C.

Section S4.2: Preparation of GUVs and bo$_3$-GUVs

Preparation of bo$_3$-polymer-GUVs and bo$_3$-hybrid-GUVs. Droplets (2 µl) of 100 nm proteohybrids or protopolymersomes (5 mg ml$^{-1}$) mixed with 100 nm hybrids or polymersomes (5 mg ml$^{-1}$; usually containing 0.05 mol% PDMS-g-PEO-Rho) in volume ratio 1:1 or 4:1 were deposited on ITO-coated glass slides (55 Ω). The LUV film was partially dehydrated for ~40 min at room temperature. Afterwards, an electroformation chamber (consisting of two sandwiched ITO-coated glass slides separated by a 1.81-mm-thick silicone spacer) was assembled and filled with 100 mM sucrose, 1 mM Tris-HCl (pH 7.5). For pH experiments buffer was supplemented with 10 µM pyranine. Electroformation in buffer without pyranine was performed by applying the following sinusoidal electric field: 50 Hz, 50, 100, 200, 300, 500, 700, and 900 mV for 6 min each; 50 Hz, 1.1 V for 2 h; and 4 Hz, 2 V for 30 min; when buffer was supplemented with 10 µM pyranine, frequency was increased to 500 Hz for the first two steps. These bo$_3$ oxidase-GUVs could be kept on ice for 48 h without losing activity; all the experiments, in which protein activity was essential, were done in this time frame.

Preparation of bo$_3$-lipid-GUVs. A sample with 400 µl volume of 100 nm proteoliposomes (10 mg ml$^{-1}$) was pelleted at 200000 ×g for 2 h at 4 °C and resuspended in 40 µl of buffer (100 mM sucrose, 1 mM Tris-HCl (pH 7.5)). Droplets (0.2 µl) of highly concentrated proteoliposomes were deposited on ITO slides. The LUV film was partially dehydrated overnight in desiccator in saturated NaCl environment at 4 °C. Afterwards, electroformation chamber was assembled and filled with 100 mM sucrose, 1 mM Tris-HCl (pH 7.5) and the same electroformation protocol as for proteopolymersomes and proteohybrids was applied.
**Preparation of GUVs.** All protein-free GUVs studied for proton permeability and FRAP were prepared by fusion/electroformation from partially dehydrated film of protein-free SUVs. The dehydration and electroformation protocols were the same as for bo$_3$-GUVs.

**Scheme S4.** Scheme illustrating preparation steps of bo$_3$-GUVs: 1) bo$_3$ oxidase was reconstituted into preformed LUVs (liposomes, hybrids or polymersomes) by detergent-mediated reconstitution. 2) Droplets of bo$_3$-LUVs were deposited onto ITO-coated glass slides and were partially dehydrated, which caused the bo$_3$-LUVs to fuse together and form a protein-polymer/lipid film (stacked membranes). 3) bo$_3$-GUVs were formed from bo$_3$-LUVs film by electroformation.

**Section S4.3: Monitoring protein incorporation and size distribution of GUVs**

The incorporation of ATTO 514-labeled bo$_3$ oxidase in GUVs was analyzed by a Leica SP8 confocal laser scanning microscope equipped with a water immersion 63× objective. bo$_3$ oxidase-ATTO 514 was excited at 511 nm and emission was monitored at 530 nm. Commercial confocal software (Leica) was used for image analysis. For statistical evaluation of the size distribution of GUVs, 30–60 images were taken per sample and the size of 100–250 GUVs was evaluated in ImageJ.

**SECTION S5: Protein activity in GUVs**

**Section S5.1: Retention of enzymatic activity in GUVs and sample homogeneity**

For bo$_3$-hybrid-LUVs and bo$_3$-polymer-LUVs (starting with 0.65 μM bo$_3$ oxidase in the reconstitution mixture), the baseline (210–230 nmol ml$^{-1}$ O$_2$) was recorded with 492.5 μl of 100 mM sucrose, 1 mM Tris-HCl (pH 7.5) buffer solution containing 1.5 μl of bo$_3$-SUVs and 4 μl of 1 M DTT (8 mM final concentration). To keep approx. the same amount of bo$_3$ oxidase in the final measurement volumes, the baseline for bo$_3$-GUVs was recorded with 478 μl of 100 mM sucrose, 1 mM Tris-HCl (pH 7.5) buffer
solution containing 16 µl of bo3-GUVs and 4 µl of 1 M DTT. For bo3 oxidase in liposomes (starting with 2.7 µM bo3 oxidase in reconstitution mixture), the baseline was measured with 0.4 µl of bo3-lipid-SUVs in 493.6 µl of buffer, and 21 µl of bo3-lipid-GUVs in 473 µl of buffer. In all cases, the reaction was initiated by the addition of 2 µl of 10 mM ubiquinone Q1 (40 µM final concentration) and the enzyme turnover was recorded for 10–20 min. In the control experiments, the activity of bo3 oxidase in micelles after the purification was determined as well as the activity of aggregated bo3 oxidase (negative control). The oxygen consumption rates were reported as the average of 3–4 measurements, with standard deviation. All measurements were done at 22 ºC while stirring.

Section S5.2: Monitoring pH changes in bo3-polymer-GUVs

The proton pumping by bo3 oxidase reconstituted into polymer GUVs was monitored through the fluorescence change of the encapsulated pH-sensitive dye pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt, HPTS). bo3-polymer-GUVs in buffer (100 mM sucrose, 1 mM Tris-HCl (pH 7.5)) with 10 µM pyranine were loaded into the chip reservoir and flown through the channels to occupy the traps. After trapping 30–60 µm bo3-polymer-GUVs, the outer pyranine was flushed away by exchanging the outer solution with pyranine-free buffer. Proton pumping was then activated by introducing a solution of 8 mM DTT and 40 µM Q1. Pyranine was excited at 405 nm and 458 nm, and the emission intensity in a region inside the GUV was measured in the range 499–551 nm. The ratio of intensities emitted when exciting at the two wavelengths correlates with pyranine deprotonation and thus pH, which was quantified with a calibration curve for intravesicular pyranine (equation shown below, calibration curve shown in Fig S13). Control experiment was performed on protein-free GUVs, exposed to the same conditions as bo3-GUVs (buffer solution containing DTT/Q1). For GUVs with reconstituted protein as well as for control experiments 5–6 GUVs were evaluated.

Calibration of the intravesicular fluorescence of pyranine to pH. The pH vs. fluorescence calibration curve (6.5–8.5) was obtained with confocal image analysis (excitation = 458 nm, 405 nm and emission = 499–551 nm) of five samples of polymer GUVs containing 10 µM pyranine and 100 mM sucrose in different buffering solutions (1 mM MES (pH 6.5), 1 mM MOPS (pH 7.0) and 1 mM Tris (pH 7.5, 8.0, 8.5)). For each pH 8 samples were analyzed. The calibration curve (Figure S13) was generated by taking a ratio of fluorescence intensity at 499–551 nm with 458 and 405 nm excitation (I458/I405). Comparing this ratio with measured pH the data was fitted to the following equation, using OriginPro:

\[
\frac{I_{458}}{I_{405}} = a \, pH + b
\]
SECTION S6: Mechanical characterization

Section S6.1: Fluctuation (flickering) spectroscopy

Polymer \( bo_3 \)-GUVs for fluctuation analysis were prepared from fused \( bo_3 \)-LUVs in lipid/polymer-to-protein molar ratio 9540:1. Protein-free GUVs were prepared by typical electroformation (not from fused LUVs) and proteoGUVs by prolonged overnight electroformation protocol. Here, 60 \( \mu \)l of outer solution (70 mM sucrose, 30 mM glucose, 1 mM Tris-HCl pH 7.5; 119 mOsmol kg\(^{-1}\)) was deposited on glass slide of the observation chamber and 5 \( \mu \)l of \( bo_3 \)-GUVs (in 100 mM sucrose, 1 mM Tris-HCl pH 7.5; 121 mOsmol kg\(^{-1}\)) was pipetted directly into the droplet of outer solution and gently mixed with the pipette. To deflate the vesicles, chamber was left open for 7–10 min (7 min for liposomes, 10 min for hybrids and polymersomes).

Fluctuation analysis was performed following the protocol described earlier (10). The data was acquired at room temperature (~23 °C). The acquisition of 1800 snapshots was done by high-resolution camera (pco.edge, PCO AG, Kelheim, Germany) with 200 \( \mu \)s exposure time and 15 fps frame rate (in phase contrast mode and 40× objective on inverted microscope Zeiss Observer.D1). Vesicle fluctuations were analyzed using a custom-built software as previously reported (10). Vesicles that had inclusions, large buds or tubes or did not significantly fluctuate were excluded from the analysis.

Section S6.2: Fluorescence recovery after photobleaching (FRAP)

FRAP was performed on protein-free GUVs and \( bo_3 \)-GUVs, and diffusion coefficients of lipid dye (Rho-PE), polymer dye (PDMS-g-PEO-Rho) and labeled protein (\( bo_3 \) oxidase-ATTO 514) were determined. Lipid GUVs contained 0.05 mol% Rho-PE, and \( bo_3 \)-lipid-GUVs contained 0.016 mol% Rho-PE. Hybrid and polymer GUVs contained 0.5 mol% PDMS-g-PEO-Rho, and \( bo_3 \)-hybrid-GUVs and \( bo_3 \)-polymer-GUVs contained 0.45 mol% PDMS-g-PEO-Rho. The maximum amount of dye was decided based on the quality of obtained GUVs (homogenous distribution of the dye, unilamellar, no internal structures); GUVs containing more than 0.5 mol% polymer dye contained smaller vesicles inside and the dye was not uniformly distributed. FRAP experiments were performed using the FRAP booster mode in a Leica TCS SP8 (Wetzlar, Germany) microscope with a 63× (1.2 NA) water immersion objective and at 1 Airy unit, as described in details in (11) and (12). Imaging and photobleaching were performed with a 561 nm diode-pumped solid-state laser. Images were acquired with 128×128 format at a speed of 1400 Hz, with no line averaging. In this configuration, the time between frames was 54 ms. Ten pre-bleach images at low laser intensity were recorded and used as a reference, and then the laser intensity was increased to maximum for
another 4 frames for photobleaching, after which the laser intensity was again decreased to record photobleaching recovery. The nominal (user-defined) photobleaching radius \( r_n \) was set as 1.5 \( \mu \text{m} \). The obtained curves were pre-analyzed using the Leica LAS X (Wetzlar, Germany) software and the FRAP curves were exported to Origin Pro 2015. FRAP was analyzed taking into account the diffusion that occurs during photobleaching (13). In short, due to the diffusion that occurs during the photobleaching, the measured effective bleaching radius \( r_e \) is larger than \( r_n \). To obtain \( r_e \), the line profile intensity on the first image after photobleaching was fitted using the equation

\[
f(x) = 1 - Ke^{\frac{-2x^2}{r_e^2}}
\]

where \( x \) is length of the profile and \( K \) is the photobleaching depth. The diffusion coefficient was hence obtained from

\[
D = \frac{r_n^2 + r_e^2}{8t_{1/2}}
\]

where \( t_{1/2} \) is the half-time of fluorescence recovery \( (i.e., \) the time to reach \( F_{1/2} = (F_o + F_\infty)/2 \), where \( F_o \) and \( F_\infty \) are the fluorescence intensities in the first post-bleach image and after full recovery, respectively. The first frame after photobleaching was normalized to \( t = 0 \).

To facilitate FRAP, the GUVs were immobilized in an agarose 0.2 wt% solution (final concentration) prepared in buffer (1 mM Tris-HCl (pH 7.5), 100 mM sucrose) using a method recently described by us (11). In short, the vesicles were mixed with previously heated agarose solution in the liquid phase and cooled down at room temperature for ~10 min, until the polymer solution jellified, after which the vesicles were ready for experiments.

**Section S6.3: Membrane order analysis by Laurdan**

To study the disorder of the membranes we used a polarity-sensitive fluorescent probe Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene), which shows a phase-dependent emission spectral shift (14). The relationship between the emission intensities obtained on the blue and red side of the Laurdan emission spectra is called generalized polarization (GP) (14):

\[
GP = \frac{I_{\text{blue}} - I_{\text{red}}}{I_{\text{blue}} + I_{\text{red}}} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}
\]

The respective emission spectra were monitored in fluorescence spectrophotometer Varian Cary Eclipse (Agilent) at excitation 530 nm and emission 400–600 nm (10/10 nm). First, fluorescence was blanked with
lipid (soy PC, 95%), hybrid (PDMS-g-PEO:soy PC = 70:30, mol%) or polymer (PDMS-g-PEO) (boi-
)LUVs suspension in 1 mM Tris-HCl (pH 7.5), 100 mM sucrose (final lipid/polymer concentration was
200 µM). Next, small amount of Laurdan (working stock solution 100 µM in DMSO) was added to LUVs
suspension (final concentration of Laurdan was 600 nM), well-mixed by vortex and incubated for 1 h at
room temperature. After 1 h sample was mixed again and the spectrum of Laurdan was recorded in
triplicates. Measurements were done in 700-µl quartz cuvettes. The GPs values were calculated from the
emissions at 440 and 490 nm according to the equation above and the average values are presented with
standard deviation.

Section S6.4: Statistical analysis

Statistical hypothesis test (Student’s t-test) was applied, in which the difference between two samples is
not significant (ns) for p-value >0.05, significant for p-value ≤0.05 (*), very significant for p-value ≤0.01
(**) and extremely significant for p-value ≤0.001 (***(**). For p-value ≤0.05, null hypothesis was rejected.
P-values were determined in Excel with t-test, considering two-tailed distribution and two-sample
assuming unequal variances.

SECTION S7: Passive proton permeability

Section S7.1: Passive proton permeability of LUVs

Passive proton permeability of LUVs was determined by adopting protocol previously applied for
liposomes consisting of lipids isolated from Thermophiles (15). Lipids/polymer were dried under N2 for
~30 min and hydrated in 50 mM 3-morpholinopropanesulfonic acid (MOPS), pH 7.0, 75 mM KCl, and 25
mM choline (inner buffer) to a final concentration of 40 mg ml⁻¹. Liposomes and hybrids were obtained
by 5 consecutive freezing in N2 and thawing steps, followed by extrusion through 200 nm polycarbonate
membrane filters. Polymersomes were extruded through 200 nm polycarbonate membrane filter as well,
but the freeze-thaw cycles were skipped. These vesicles are unilamellar with an average size that is close
to the pore size of the filter used. To exchange the external buffer for one with a lower buffering capacity,
0.5 mM MOPS, pH 7.0, 75 mM KCl, and 75 mM sucrose (outer buffer), the vesicles were passed through
a Sephadex G-25 column equilibrated with outer buffer, and collected. Vesicles were diluted to 1.5 mg
ml⁻¹ in 1.5 ml outer buffer and added to a 1.5 ml quartz cuvette with magnetic stirring. For exact
calculation of material concentration, we considered lipid/polymer loss during extrusion and dilution
during gel filtration. Next, the potassium ionophore valinomycin (1 nmol mg⁻¹ lipid/polymer) was added,
which facilitated potassium counterflux and prevented the establishment of inhibitory transmembrane
electrical potential ($\Delta \psi$), caused by the electrogenic influx of protons. Pyranine (10 μM) was added to monitor the changes in the external pH. Wavelength of 450 nm and 508 nm for excitation and emission was used, respectively. After equilibration, 100 nmol H$^+$ (from a 50 mM H$_2$SO$_4$ stock solution) was added to lower the external pH. The influx of H$^+$ into the liposomes can be monitored in time as an increase of the pyranine fluorescence. Finally, nigericin (1 nmol mg$^{-1}$ lipid), a protonophore, was added to equilibrate the H$^+$ gradient across the membrane. The signal was calibrated after the addition of nigericin by adding small aliquots of base or acid.

Section S7.2: Passive proton permeability of bo$_3$-LUVs

Polymersomes for reconstitution were prepared by hydrating polymer film with inner buffer with 0.8% sodium cholate (to avoid buffer dilution, dry detergent was suspended in buffer). All other vesicle preparation steps were as described above. The bo$_3$ oxidase was reconstituted by 0.8% sodium cholate in lipid, hybrid and polymer 200 nm vesicles (40 mg ml$^{-1}$) in inner buffer, at final lipid/polymer-to-protein ratios of 9560:1, 9550:1 and 9540:1, respectively. The concentration of bo$_3$ oxidase in the reconstitution mixture were 5.4 μM for liposomes, 1.8 μM for hybrids and 1.4 μM for polymersomes. Since polymersomes already contained detergent in the membrane, before adding the protein, detergent was added only to preformed liposomes and hybrids. After 30 min of incubation at 4 °C (with mild agitation), detergent was removed and outer solution was exchanged by passing the bo$_3$-LUVs through a Sephadex G-25 column equilibrated with outer buffer. In addition to loss of material during extrusion and dilution during gel filtration, dilution during reconstitution was considered as well. We previously tested reconstitution of bo$_3$ oxidase by detergent removal with gel filtration instead of Bio-Beads. The activity of bo$_3$ oxidase was completely retained; therefore, we expect similar reconstitution efficiency by both methods. All following steps for proton permeability experiment were performed the same way as for protein-free LUVs. Size of (bo$_3$)-LUVs was determined by DLS.

Section S7.3: Passive proton permeability of GUVs and bo$_3$-GUVs

The passive proton permeability of protein-free GUVs and bo$_3$-GUVs was monitored for 60 min via the changes in the luminal pyranine fluorescence. The lipid/polymer-to-protein molar ratio in bo$_3$-GUVs was 9560:1, 9550:1 and 9540:1 for liposomes, hybrids and polymersomes, respectively. To achieve those molar ratios, the following concentrations of bo$_3$ oxidase were used in the reconstitution mixture: 0.35 μM for polymersomes, 0.45 μM for hybrids and 1.35 μM for liposomes. bo$_3$-polymer-GUVs and bo$_3$-hybrid-GUVs were prepared from dehydrated film of mixture of bo$_3$-LUVs and LUVs (1:1, v/v). GUVs and bo$_3$-GUVs in buffer (100 mM sucrose, 1 mM Tris-HCl (pH 7.5), 10 μM pyranine) were flushed into the
microfluidic chip with a flow speed of 1 µl min⁻¹ and GUVs >10 µm were trapped. Next, non-encapsulated pyranine was washed away with 60 µl of the same buffer, lacking pyranine with a flow speed of 2 µl min⁻¹. In the last step we exchanged the outer solution with 60 µl of isotonic solution ~100 mM sucrose, 1 mM MES (pH 6.0) with a flow speed of 1 µl min⁻¹ and started the monitoring of proton transport. For data analysis images were taken every 15 s.

**Section S7.4: Determination of proton permeability coefficients from intravesicular pyranine fluorescence**

In the absence of diffusion potentials, the permeability coefficient can be calculated through the flux (16, 17), while accounting for the surface-to-volume ratio and the buffering capacity of all species, including the phospholipids (18). In the present case however, we neglected the buffering capacity of the buffer and of the phospholipids because of its low ionic strength (1 mM) and the lower surface-to-volume ratio of the GUVs, respectively. In addition, two mechanisms have been proposed for treatment of the experimental data: transient pore and solubility-diffusion mechanism (17). The initial fast change of pH has been attributed to the stochastic nature of pore formation showing a Poisson distribution of the decay rates, while the following slower phase corresponded to a solubility-diffusion pathway (19). Since in the present case the pH changes were more accurately described by such a two-stage process, we fitted the data with a biexponential curve \( y = A_1e^{-tk_1} + A_2e^{-tk_2} + y_0 \), where \( A \) is the amplitude of fluorescence signal, \( k \) is the first order rate constant of proton influx and \( y_0 \) the offset; we used the second (smaller) rate constant \( k_2 \) to calculate the permeability coefficient through the reported relation \( P_{H^+} = k_2 \frac{R}{3} \) (19), where \( R \) was the vesicle radius. It should be noted that in some cases of \((b0_{12})\)GUVs the data could be fitted with a single exponential function, which resulted in similar \( k_1 \) and \( k_2 \). The biphasic behavior was more pronounced in LUVs than in GUVs, therefore, we applied the same biexponential analysis.

The solubility-diffusion rate, characterized by \( k_2 \), has been shown to reflect the limiting counter flux of charge-compensating co-ions such as \( K^+ \) (19, 20). We did not introduce an ionophore to the GUVs to counteract the potential build-up of \( \Delta \psi \) because we could not ensure its homogeneous partitioning and calculate its loading in the membrane (valinomycin can become a protonophore at high concentration (21)). The lower surface-to-volume ratio should prolong the establishment of electrochemical potential. On the contrary, we added the required amount of valinomycin to the LUVs, based on the mass of lipids and polymer and corrected by the material losses during extrusion (Section S17.5).
SECTION S8: Analysis of \(b\alpha_3\) oxidase partitioning in hybrid membranes via FRET between \(b\alpha_3\)-ATTO 514 and PE-Rho/PDMS-g-PEO-Rho

Hybrid LUVs, prepared as described in Section S1.3, containing 2 mol% of either lipid dye PE-Rho or polymer dye PDMS-g-PEO-Rho (amount of non-labeled lipid/polymer was reduced proportionally) were reconstituted with \(b\alpha_3\) oxidase labeled with ATTO 514 in a same way as described in Section S4.1. For measurements, 20 µl of proteohybrids containing either of two dyes were mixed with 0.8 ml of 1 mM Tris-HCl (pH = 7.5), 100 mM sucrose (both precooled and kept at 5 °C at all times) and the fluorescence of ATTO 514 was monitored (excitation = 511 nm, emission = 533 nm, slits position = 10/10, PMT = 800 V) in a fluorescence spectrophotometer Varian Cary Eclipse (Agilent) at constant stirring and thermostated at 5 °C. Next, octyl glucoside (OG) was added in 30 subsequent steps, 2 µl of 10% OG each, and the change in ATTO 514 emission based on its interaction with rhodamine quencher was recorded. After each addition of OG, the samples were vortexed for 5 s.

SECTION S9: Cryo-TEM

Cryo-TEM was performed on lipid (soy PC), hybrid (PDMS-g-PEO/soy PC) and polymer (PDMS-g-PEO) LUVs and \(b\alpha_3\)-LUVs. 3.5 µl droplet of the LUVs colloidal suspension was applied on a glow-discharged R2/1 type 200 Mesh Quantifoil holey carbon grid. The vitrification of the samples was carried out using Vitrobot Mark IV System (Thermo Fisher Scientific) and standard Vitrobot Filter Paper (i.e. Ø55/20mm, Grade 595). The chamber of Vitrobot was held at 4 °C and 95 % relative humidity. For vitrification, a blot force of 2 and blotting time of 6 sec were applied. The grid was mounted onto a Thermo FEI Glacios 200 kV autoloader under cryo conditions. Images were acquired using Falcon 3EC direct electron detector in linear mode and a total dose of 50 e⁻/Å².
Additional Experimental Data

SECTION S10: Functional stability of bo3-LUVs

The shelf-life measurements of bo3 oxidase, reconstituted in lipid, hybrid and polymer LUVs, alongside the controls in micelles, were terminated when the oxygen consumption of the liposomes activity dropped close to zero (after day 11 there was no significant difference with baseline, i.e. ± 0.5 µM min⁻¹). It should be noted that the reconstitution of bo3 oxidase was performed slightly differently for the shelf-life experiments in comparison to the protocols for preparation of bo3-GUVs (different starting vesicle concentrations, see Section S4.1), however the lipid/polymer-to-protein molar ratio was kept constant. At these specific conditions, bo3 oxidase exhibited the highest activity in hybrids (day 1; Figure S1A). The time courses of the oxygen consumption rates were fitted by exponential decay (Figure S1B) in OriginPro. The respective time constants were: 0.72 for bo3-lipid-LUVs, 4.06 for bo3-hybrid-LUVs, 1.54 for bo3-polymer-LUVs and 1.6 for bo3 oxidase in micelles.

Figure S1. A) Activity of bo3 oxidase reconstituted in lipid, hybrid and polymer LUVs on days 1 and 11 of the shelf-life measurements. bo3 oxidase was reconstituted in all three types of vesicles at lipid/polymer-to-protein molar ratio 9550:1 and at ~13.5 nM protein in the measurement chamber. B) Exponential fitting of the experimental data on activity retention of bo3 oxidase in LUVs and n-dodecyl-β-D-maltoside micelles.
SECTION S11: Chemical stability of LUVs

Section S11.1: Size distribution

Table S1. Average size of lipid, hybrid and polymer vesicles (extruded through 100 nm-pore size membrane) before and after exposure to sodium-L-ascorbate and FeSO₄, in 100 mM sucrose, 1 mM Tris-HCl, pH 7.5.

|                  | Before exposure | After 30 min incubation with Na-L-ascorbate | After 30 min incubation with ascorbyl free radical |
|------------------|-----------------|---------------------------------------------|---------------------------------------------------|
|                  | Average size (nm) | PDI                         | Average size (nm) | PDI                         | Average size (nm) | PDI                         |
| Liposomes        | 116              | 0.051                        | 115              | 0.067                        | 116              | 0.094                        |
| Hybrids          | 83               | 0.145                        | 84               | 0.138                        | 84               | 0.125                        |
| Polymersomes     | 110              | 0.121                        | 111              | 0.107                        | 117              | 0.161                        |

Section S11.2: Surface charge

Figure S2. Change in zeta potential before and after exposure to Na-L-ascorbate and ascorbyl radical (30 min incubation with Na-L-ascorbate and additional 30 min incubation with FeSO₄).
Section S11.3: LUVs permeability for carboxyfluorescein

Table S2. Size of vesicles prepared in buffer (100 mM sucrose, 1 mM Tris-HCl, pH 7.5) with 20 mM carboxyfluorescein in subsequent preparation and purification steps, and after vesicle solubilization with Triton X-100.

|                  | Extrusion (100 nm) | Gel filtration (Sephadex G-25) | Gradient separation (Nycodenz) | Solubilization |
|------------------|-------------------|--------------------------------|--------------------------------|----------------|
|                  | Average size (nm) | PDI                            | Average size (nm)              | PDI                         |
| Liposomes        | 116               | 0.080                          | 126                            | 0.228                       |
| Hybrids          | 85                | 0.222                          | 86                             | 0.232                       |
| Polymersomes     | 107               | 0.105                          | 104                            | 0.113                       |

Figure S3. A) Changes in fluorescence intensity of carboxyfluorescein (CF) encapsulated in liposomes. At the end of the measurement liposomes were solubilized with Triton X-100 (TTX) to achieve 100% dequenching of CF. Dye leakage with two different permeation rates was observed: initial slower rate after the addition of Na-L-ascorbate and
faster second rate after the addition of FeSO₄. B) Changes in fluorescence intensity of carboxyfluorescein (CF) encapsulated in hybrids after the last purification step (gradient separation). In the end the vesicles are solubilized with Triton X-100 (TTX) (1ˢᵗ volume corresponding to the solubilization point, 2ⁿᵈ double amount). C) Changes in fluorescence intensity of CF encapsulated in polymersomes. No dye leakage was observed after addition of Na-L-ascorbate or FeSO₄. Complete solubilization of polymersomes was achieved with TTX (Section S11.3/Table S2).

**Figure S4.** Dequenching of carboxyfluorescein (CF) encapsulated in lipid LUVs (diluted 1:2). Leakage is calculated in % (100 % dequenching was obtained by completely solubilizing the vesicles).

**Figure S5.** Dequenching of carboxyfluorescein (CF) encapsulated in lipid LUVs at different dilutions (1:40, 1:10 and 1:2). The higher amount of vesicles results in higher CF concentration and dequenching is delayed.
SECTION S12: GUV characterization

Section S12.1: Distribution of lipids and polymer in the hybrid GUV membrane

Hybrid GUVs formed from 100 nm hybrids had homogenous distribution after electroformation. For visualization of lipid/polymer distribution we tagged the hybrid membrane with lipid (Rho-PE) and polymer (PDMS-g-PEO-Fluo) dye (Figure S2). Homogenous distribution was observed throughout the whole sample, in contrast to hybrids (>60 mol% of PDMS-g-PEO) formed with typical electroformation (not from LUVs), where a few phase-separated GUVs could be found. Hybrids with two membrane dyes were on average smaller than hybrids with one of those two dyes, therefore for all the experiments, in which the hybrid membrane had to be tagged, we used only polymer dye, i.e. PDMS-g-PEO-Rho. Polymer labeled with rhodamine was used instead of polymer labeled with fluorescein, to avoid interference with pyranine and bo3 oxidase-ATTO 514 fluorescence. Interestingly, homogenous and phase-separated hybrids with lipid and polymer dye formed from rehydration of hybrid film, from hybrid solution in chloroform:MeOH (2:1, v/v), under AC were formed readily (similar diameter as with one membrane dye) (Figure S6). We assume, that the smaller size is attributed to the lower fusion efficiency of LUVs – the same charge of membranes due to the charged dyes might repel the LUVs and prevent their fusion. Furthermore, vesicle swelling was restrained in buffer solution in comparison to sucrose solution.

Figure S6. Hybrid GUVs (70 mol% PDMS-g-PEO : 30 mol% soy PC) with lipid dye (0.05 mol% PE-Rho; red) and polymer dye (0.1 mol% PDMS-g-PEO-Fluo; green) prepared by fusion/electroformation from 100 nm LUVs in 100 mM sucrose (above) and buffer solution (1 mM Tris-HCl, pH 7.5, 100 mM sucrose, below). Typical size of GUVs with two membrane dyes in sucrose was ~10 µm, and in buffer ~5 µm; hybrids with one membrane dye had a diameter in the range 10–30 µm.
Figure S7. Phase-separated hybrids (domain formation) (above) and homogenous hybrids (below) formed by rehydration of hybrid film, from hybrid solution in chloroform:MeOH (2:1, v/v), under AC in 100 mM sucrose.

Section S12.2: Protein incorporation in GUVs

Figure S8. Typical soy PC (top), PDMS-g-PEO : soy PC (middle) and PDMS-g-PEO (bottom) bo3-GUVs. bo3 oxidase is labeled with ATTO 514 (green), the membrane is labeled with fluorescent lipid analogue (Rho-PE) for liposomes or polymer dye (PDMS-g-PEO-Rho) for hybrids and polymersomes (red). From left to right: ATTO 514 channel (green), rhodamine channel (red), overlay of both channels.
Section S12.3: Size distribution of \( bo_{2} \)-GUVs and \( bo_{2} \)-LUVs

*Quality (size, unilamellarity, intravesicular structures) of \( bo_{2} \)-GUVs.* The drawback of working with sensitive MPs is that during reconstitution, the protein can be deactivated (by aggregation, denaturation, or loss of cofactors). To retain the activity of \( bo_{2} \) oxidase, the dehydration step had to be strictly controlled. Longer dehydration steps caused complete fusion of 100 nm vesicles but at the expense of protein activity. Therefore, with goal to largely retain protein activity, we rather shortened the dehydration, and only partially dried \( bo_{2} \)-LUVs, which caused their incomplete fusion and the appearance of ~700–1000 nm vesicles in the final sample). Furthermore, all hybrid and polymer GUVs were unilamellar. In contrast, \( bo_{2} \)-lipid-GUVs, as well as protein-free lipid GUVs sample always contained a portion of multilamellar vesicles; those vesicles were not included in mechanical characterization. Also, a small portion of the GUVs contained internalized vesicles (e.g. upper panel in Figure S6); again, this was hardly observed for synthetic vesicles. In addition, a portion of the liposomes was aggregated, which occasionally caused blockage of the microfluidic channel, while no aggregation was observed for hybrids and polymersomes.

**Figure S9.** Typical size distribution of \( bo_{2} \)-GUVs prepared by the fusion/electroformation technique. Vesicles with diameter lower than 4 \( \mu \text{m} \) were not counted, along with small fraction of 40–60 \( \mu \text{m} \) GUVs that was occasionally observed. From left to right: \( bo_{2} \)-lipid-GUVs, \( bo_{2} \)-hybrid-GUVs, and \( bo_{2} \)-polymer-GUVs.
Size distribution of bo$_3$-LUVs.

**Figure S10.** Size distribution by DLS intensity of vesicles extruded through 100 nm pore-sized membrane and vesicles with reconstituted bo$_3$ oxidase. Top: polymersomes and proteopolymersomes; bottom: hybrids and proteohybrids.

**Section S12.4: Oxygen consumption by bo$_3$ oxidase**

**bo$_3$ oxidase activity retention after dehydration and electroformation.** The oxygen consumption of different bo$_3$-GUVs and bo$_3$-LUVs was determined from bulk samples using a Clark-type electrode (Section S6.1), whereby we always aimed for the same amount of protein (~1.95 nM) in order to avoid the accumulation of errors from rescaling. For that purpose, we adapted the sample volumes to the final volume, under the assumption that no enzyme was lost during the formation of bo$_3$-GUVs from bo$_3$-LUVs. In the case of small vesicles, the initial oxygen consumption rates were 3.2 ± 0.2 µM min$^{-1}$ for liposomes, 5.3 ± 1.1 µM min$^{-1}$ for hybrids and 4.0 ± 0.6 µM min$^{-1}$ for polymersomes. The reconstitution protocol for hybrid and polymer LUVs differed from the protocol for lipid LUVs (see Section S4.1): liposomes required higher concentrations of phospholipid and protein, which may have led to less efficient reconstitution. This could explain the lower activity of the latter, while in the case of polymer-containing vesicles rates were comparable (non-significant difference, $p$-value = 0.0949), as it was previously reported (7). When referred to the respective bo$_3$-LUVs, the conversion to bo$_3$-GUVs resulted in decrease of the initial rates. We found that 82 ± 8 % of the activity was retained in liposomes, 74 ± 5 % in hybrids, and 57 ± 6 % in polymersomes after the scale-up. During the dehydration step, the hybrid and polymer bo$_3$-LUV films adhered more strongly to the ITO slides than the lipid ones and during the electroformation only lipid GUVs detached completely (polymer/hybrid residue could be seen with the naked eye). Thus, we believe that the apparent decrease of protein activity is largely due to overestimation of the protein amount rather than by enzyme deactivation. Potentially, the loss during the electroformation could be
reduced by decreasing the drying duration. However, insufficient drying negatively affected the GUV formation, therefore, finding a compromise was unavoidable.

**Homogeneity of bo3-GUVs samples.** By correlating the increase in oxygen consumption rates with incremental volumes of the vesicle suspensions, *i.e.* by proportional increase of the protein amount in a constant reaction volume, we assessed the homogeneity of protein distribution. The average oxygen consumption rates of lipid and polymer bo3-GUVs increased by a factor of roughly 0.6 (see below), while the bo3-hybrid-GUVs exhibited lower proportionality. The deviations may be attributed to the rather wide size range of formed bo3-GUVs and the less homogenous distribution of bo3 oxidase in hybrid GUVs. The latter membranes showed larger scatter also with respect to other properties.

![Figure S11](image)

**Figure S11.** Homogeneity of the bo3-GUVs suspension. A) Increase of oxygen consumption with increased amount of protein (concentration of bo3-GUVs), starting with ~ 2 nM bo3 oxidase in the measurement chamber. B) Oxygen consumption (normalized to the first aliquot) of bo3-GUVs with increasing concentration of bo3-GUVs in the total measurement volume. Slopes: 0.57 (*R*^2^ > 0.93), 0.17 (*R*^2^ > 0.96), 0.54 (*R*^2^ > 0.94) for bo3-lipid-GUVs, bo3-hybrid-GUVs and bo3-polymer-GUVs, respectively.

**Aggregated bo3 oxidase.** The proton pump bo3 oxidase is active in micelles. Therefore, to confirm that detergent is completely removed, and no micelles are left after reconstitution and detergent removal, bo3 oxidase was exposed to the same condition as during reconstitution (amount of added Bio-beads and time of incubation), but in the absence of vesicles (the same volume of buffer was added instead).
Figure S12. Oxygen consumption by $b_{03}$ oxidase reconstituted in GUVs (measured in population) and aggregated $b_{03}$ oxidase (negative control, absence of stabilizing amphiphiles (detergent/lipid/polymer)). At ~220 s mark, the reaction was initiated by DTT and Q1.

Labeled $b_{03}$ oxidase. We ruled out the possible negative effect of the fluorescent tag (ATTO 514) on the enzyme based on the identical activity of labeled and native protein in polymer LUVs.

SECTION S13: Proton pumping

Section S13.1: Calibration of intravesicular fluorescence of pyranine with pH

Figure S13. Standard curve for intravesicular pyranine (pyranine encapsulated in protein-free polymersomes).
Section S13.2: Proton pumping by $b_{03}$-polymer-GUVs at different polymer-to-protein molar ratios

**Figure S14.** Polymersomes evaluated for proton pumping. Left: Protein-free polymersomes (negative control). Right: Polymersomes with reconstituted $b_{03}$ oxidase (PDMS-g-PEO:$b_{03}$ oxidase = 9540:1).

**Figure S15.** $b_{03}$-polymer-GUVs (2980:1, polymer-to-protein molar ratio) with encapsulated pyranine trapped in microfluidic chip. Pyranine fluorescence (green) was monitored at two excitation wavelengths (405 nm and 458 nm). For better visualization, membrane of proteoGUVs was tagged with polymer dye (PDMS-g-PEO-Rho, red).

**Figure S16.** Intravesicular pH change for $b_{03}$-polymer-GUVs (2980:1, polymer-to-protein molar ratio) in first 7 min.
Figure S17. pH change in bo3-polymer-GUVs after 30 min. Scale bar: 10 μm. Initial pH in bo3-polymer-GUVs was 7.5 ± 0.2 (n = 8), 7 min after activation with DTT/Q, pH decreased to 6.8 ± 0.2 (n = 9) and after 30 min further decreased to 6.5 ± 0.1 (n = 8).

SECTION S14: Bending rigidity – comparison of different membranes

Table S3. Bending rigidity of protein-free and protein-functionalized membranes of different compositions. Passive protein denotes the absence of the respective triggers.

| Type of membrane | Type of reconstituted protein | Lipid/polymer-to-protein molar ratio | Bending rigidity κ (k_BT) | Source |
|------------------|--------------------------------|-------------------------------------|--------------------------|--------|
|                  |                                |                                     | Protein-free membrane    | Protein-free membrane in presence of activators | Passive membrane | Active membrane |        |
|                  |                                |                                     | (Klipid/polymer)         | (Klipid/polymer)                      | (Kpass)         | (Kactive)      |        |
| Egg PC           | -                              | -                                   | 10 ± 1                   | -                                       | -                | -             |        |
| bR               | 1000–60                        | -                                   | 10.2 ± 1.5               | 10.4 ± 1.1                             | 9.4 ± 1.0       | 4.1 ± 0.8     | Niggemenn 1995 (22) |
| Ca2+-ATPase      | 1300                           | -                                   | 11.6 ± 0.7               | 11.2 ± 1.0                             | 10.2 ± 1.1      | 4.4 ± 1.0     | Manneville 2007 (23) |
| SOPC             | 1300                           | -                                   | 22.93 ± 0.63             | 23.80 ± 0.63                           | 26.40 ± 0.75    | 19.45 ± 1.02  | Girard 2005 (24)   |
| DOPC/Chol        | Na+/K+-ATPase                  | 2698                                | 22.30 ± 0.6              | 23.80 ± 0.63                           | 26.40 ± 0.75    | 19.45 ± 1.02  | Bouvrais 2012 (25) |
| DOPC/DOPS/Chol   | F1F0-ATPase                    | 2128                                | 28.88 ± 0.97             | 20.40 ± 0.60                           | 28.88 ± 0.97    | 20.40 ± 0.60  | Almendrov-Vedia 2017 (26) |
| E. coli TLE      | F1F0-ATPase, n_o = 10^{14}      | 13 ± 2                              | -                        | 14 ± 3                                 | 2 ± 1           |                |        |
| (total lipid extract) |                                      |                                     |                          |                                        |                  |                |        |
| Lipid/Polymer | Protein | # | Typical FRAP Curve | Normalized Intensity | Time (s) | This study |
|---------------|---------|---|-------------------|---------------------|---------|------------|
| Soy PC        | bo$_3$ oxidase | 9560 | 23.3 ± 4.1 (n = 5) | - | 16.4 ± 4.4 (n = 11) | - |
| PDMS$_{26g}$- (PEO$_{12}$)$_2$ /soy PC | 9550 | 11.6 ± 2.4 (n = 9) | - | 8.6 ± 1.3 (n = 11) | - |
| PDMS$_{26g}$- (PEO$_{12}$)$_2$ | 9540 | 11.7 ± 2.1 (n = 5) | - | 9.4 ± 2.7 (n = 10) | - |

**SECTION S15: Lateral diffusion of lipid/polymer dye and protein**

*Figure S18.* Typical FRAP curve for polymer dye and labeled protein in membrane of bo$_3$-polymer-GUV.
**Figure S19.** Successful insertion of F₁F₀-ATPase-ATTO 620 (magenta) in F₁F₀-hybrid-GUVs. Polymer dye PDMS-g-PEO-Rho (red) was used to visualize the membrane.

**Figure S20.** Successful insertion of F₁F₀-ATPase-ATTO 620 (magenta) in F₁F₀-polymer-GUVs. Polymer dye PDMS-g-PEO-Rho (red) was used to visualize the membrane.
Table S4. Comparison of the diffusion coefficients of protein-free and protein-functionalized membranes of different compositions, and diffusion coefficients of the reconstituted proteins.

| Type of membrane | Molecular weight (g mol⁻¹) | Membrane thickness (nm) | Membrane diffusion coefficient (μm² s⁻¹) | Reconstituted protein | Source |
|------------------|-----------------------------|-------------------------|------------------------------------------|----------------------|--------|
|                  |                             |                         | Protein-free | Protein-functionalized | Type of protein | Diff. coeff. | |
| DMPC             | 677.933                     | 5.0                     | 8.8 ± 1.3 (36 °C) | -                     | Rhodopsin (37 kDa) | 3.3 ± 0.3 (36 °C) | Vaz 1982 (27) |
| TLE (total lipid extract) | | | 14 ± 0.1 (37 °C) | - | SR-ATPase (100 kDa) | 1.8 ± 0.3 (36 °C) |
| Soybean lipids   |                             |                         | 11 ± 0.1 (37 °C) | - | AchR (250 kDa) | 2.4 ± 0.8 (36 °C) |
| DMPC             | 677.933                     | 6.9 (32 °C)             | 4.3 (32 °C) | bR (26 kDa) | 2.3 (32 °C) | Peters 1982 (28) |
| DOPC/DOPG (75:25, mol/mol) | 788.841                  | -                       | 11.3 ± 0.6 | 11.4 ± 0.7 | LacY (monomer, 45 kDa) | 4.3 ± 0.4 | Ramadurai 2009 (29) |
| DOPC/DOPS (75:25, w/w) | 792.091                    | -                       | 7.7 ± 0.8 | - | LasS (dimer, ~140 kDa) | 3.0 ± 0.3 | Doeven 2005 (30) |
|                  |                             |                         | - | MscL (pentamer, ~70 kDa) | 3.9 ± 0.3 |
| POPC             | 760.076                     | 5.0 ± 0.4               | 9.8 ± 1.7 (31) | 12.5 ± 0.5 (32) | KcsA-OG488 (tetramer) | 8.5 ± 0.8 (33) | Itel 2015 (33) |
|                  |                             |                         | - | | AqpZ-OG488 (tetramer) | 7.9 ± 0.5 (33) |
| DOPC             | 786.113                     | 3.87 ± 0.05 (34)       | 10.0 ± 0.4 (35) | - | bov oxidase-ATTO647N | ~9.5 (36) | Sjöholm 2017 (36) |
|                  |                             |                         | - | bov oxidase-ATTO594 | ~10.5 (36) |
| Soy PC           | 775.037                     | 3.49 ± 0.03 (37)       | 11.3 ± 1.5 | 8.8 ± 1.7 | bov oxidase-ATTO514 | 9.9 ± 1.3 | This study |
| PDMS250g-(PEO12)2/soy PC | 2333                  | -                       | 6.2 ± 1.4 | 5.0 ± 0.6 | bov oxidase-ATTO514 | 6.9 ± 1.7 |
|                  |                             |                         | - | 6.6 ± 1.3 | FiFø-ATPase-ATTO620 | - |
| PDMS250g-(PEO12)2 | 3000                     | 5 (38) – 5.4 (39)       | 3.6 ± 0.7 | 4.4 ± 0.4 | bov oxidase-ATTO514 | 3.5 ± 0.9 |
|                  |                      | 4.4 ± 1.1 | F:F\textsubscript{0}-ATPase-ATTO620 | -               |                  |
|------------------|----------------------|-----------|------------------------------------|-----------------|------------------|
| PMOXA\textsubscript{6,12}-b- | 3800 – 6900          | 9.2 ± 0.5 – | 2.4 ± 0.2 –                       | -               | AqpZ-OG488       |
| PDMSS\textsubscript{4,63}-b- | 13.4 ± 0.9 (32)      | 1.0 ± 0.1 (32) | -                                 | 1.7 ± 0.1 – (33) | Itel 2015 (33)   |
| PMOXA\textsubscript{6,12} |                      |           |                                    |                 |                  |
| PMOXA\textsubscript{7}-b- | 5100                 | 12.1 ± 1.0 (32) | 1.6 ± 0.2 (32)                       | -               | KcsA-OG488       |
| PDMSS\textsubscript{5,63}-b- |                      |           |                                    | 1.3 ± 0.1 (33) |                  |
| PMOXA\textsubscript{7} |                      |           |                                    |                 |                  |

**SECTION S16: Membrane order (Laurdan)**

**Figure S21.** Fluorescence emission spectra of Laurdan in protein-free and \(b\textsubscript{O_3}\) oxidase-functionalized hybrid membrane. Emission spectrum in lipid LUVs is presented with dark purple trace and emission spectrum in \(b\textsubscript{O_3}\)-hybrid-LUVs with light purple trace.

**Figure S22.** Fluorescence emission spectra of Laurdan in protein-free and \(b\textsubscript{O_3}\) oxidase-functionalized polymer membrane. Emission spectrum in polymer LUVs is presented with dark purple trace and emission spectrum in \(b\textsubscript{O_3}\)-polymer-LUVs with light purple trace. Protein insertion caused higher degree of membrane disorder (3.8 % increased disorder; GP decreased from \(-0.52 ± 0.01\) to \(-0.54 ± 0.00\)).
SECTION S17: Passive proton permeability

Section S17.1: Passive proton permeability of GUVs

Figure S23. Proton permeability in lipid, hybrid and polymer GUVs (A) and bo3-GUVs (B) before and 1 h after buffer exchange. Presented GUVs are examples of typical GUVs evaluated for pH change; “n” refers to the number of vesicles used for evaluation from single experiment (1 trap). Scale bar: 10 µm.
Figure S24. Fluorescence intensity ratio inside GUVs and bo$_2$-GUVs after external buffer exchange (from initial pH 7.5 to pH 6.0). Pyranine was excited at 405 nm and 458 nm, and the emission intensity measured in the range 499-551 nm. The ratio of intensities inside GUV correlates with pyranine deprotonation and thus pH. The curves present mean and standard deviation for 2–7 GUVs from single experiment (1 trap).
Figure S25. Examples of trapped $b\alpha_3$-GUVs evaluated for proton permeability (GUVs of 6 traps were evaluated for each system, one trap for each type of GUVs is shown as an example). All GUVs (including protein-free ones) were prepared by fusion/electroformation from LUVs. Scale bar: 50 µm.
Section S17.2: Passive proton permeability of phase separated hybrid GUVs

Figure S26. Protein-free hybrids: hybrid with domains (left) and hybrid with homogenous distribution of lipid and polymer (right). In all experiments, where polymer/lipid (70:30, molar ratio) hybrid GUVs were prepared by fusion/electroformation, we observed only one single phase separated hybrid GUV. All hybrids analyzed for proton permeability were homogenous (no microdomains were observed). Preparation of hybrid GUVs from fused LUVs increased mixing of lipid and polymer (in hybrids prepared from lipid/polymer film, i.e. typical electroformation, occasional phase separated hybrid was observed).
Figure S27. Phase-separated hybrid GUV (polymer/lipid = 40:60, molar ratio) prepared by classical electroformation procedure (in which hybrid mixture is deposited on ITO-slides in organic solvent and not in the form of pre-formed vesicles) evaluated for proton permeability of heterogeneous hybrids. Membrane is labeled with PDMS-g-PEO-Rho, which partitions primarily into polymer domains. White arrows show lipid domains (darker part, lower presence of polymer dye).
**Figure S28.** Permeability coefficients \( (P_{H^+}) \) of protein-free homogenous (polymer/lipid = 70:30, molar ratio) and heterogeneous (polymer/lipid = 40:60, molar ratio) hybrid GUV prepared by classical electroformation from dry film.

**Section S17.3: Passive proton permeability of LUVs**

**Figure S29.** Typical examples of pyranine fluorescence intensity curves for protein-free and protein-functionalized LUVs after addition of acid in the extravesicular solution. Black trace represents double-exponential decay fitting.
### Section S17.4: Summary of proton permeability for different membranes

#### Table S5. Passive proton permeability of protein-free and protein-functionalized membranes. For reconstituted bR is stated lipid-to-protein weight ratio and for bo3 oxidase and F1F0-ATPase lipid/polymer-to-protein molar ratio.

| Type of membrane | Type of protein | Lipid/polymer-to-protein ratio | Proton permeability – GUVs (cm s⁻¹) × 10⁻⁷ | Proton permeability – LUVs (cm s⁻¹) × 10⁻⁷ | Source |
|-----------------|----------------|-------------------------------|------------------------------------------|------------------------------------------|--------|
| Egg PC/PA (9:1, mol/mol) | bR | | | | Seigneuret 1986 (21) |
| Soy PC | bo3 oxidase | 5 | - | - | 60 | ~120 |
| PDMS26-g-(PEO12)2 / F1F0-ATPase | 9550 | 24.2 ± 2.0 (n=21) | 2.6 ± 0.2 (bo3) (n=38) | 11.1 ± 1.4 | 7.9 ± 2.5 (bo3) | 5.7 ± 1.0 (F1F0) |
| PDMS26-g-(PEO12)2 | 9540 | 2.3 ± 0.1 (n=46) | 29.3 ± 1.6 (bo3) (n=45) | 2.1 ± 1.1 | 11.9 ± 3.0 (bo3) | 6.4 ± 1.5 (F1F0) |

### Section S17.5: Loss of material during extrusion

The LUVs in proton permeability experiments were extruded through 200 nm-pore size membrane and GUVs were prepared from LUVs extruded through 100 nm pore-size membrane. Material loss for both cases was considered. In addition, we tested if the material loss changes with the different concentration of lipid/polymer, since for GUVs preparation we started with 5 mg ml⁻¹ and for LUVs with 40 mg ml⁻¹. The difference in material loss was less than 1%; therefore, we decided to neglect this effect. Since the vesicles were prepared in different buffers, we tested the effect of buffers as well because certain salts might cause increased lipid/polymer binding on the membrane. We compared the loss of material in buffer and in MiliQ water, and the loss was only around 2% higher in buffer. Therefore, we decided to determine all losses in MiliQ water. Material loss was determined as follows: first, the mass of the dry lipid/polymer (in glass vial) was weighed after evaporating the solvent (choforom:MeOH = 2:1, v/v). After rehydration of the lipid/polymer film, freeze-thaw and extrusion, a suspension of LUVs in 1.5 ml Eppendorf tube was freeze-dried and weighed and the material loss was calculated from the following equation:

\[
\% \text{ material loss} = 100\% - \frac{100\% \ m_{\text{dry material after extrusion}}}{m_{\text{dry material before extrusion}}}
\]
The loss of material during extrusion through 100 nm-pore size membrane was 21% for liposomes (determined by phospholipid assay), 25% for hybrids (determined by weighing freeze-dried material) and 31% for polymersomes (determined by weighing freeze-dried material). The loss of material during extrusion through 200 nm-pore size membrane was lower: 17% for liposomes, 17% for hybrids and 15% for polymersomes.

SECTION S18: Cryo-TEM

Section S18.1: LUVs size and membrane thickness

Table 6. Size distribution and membrane thickness of LUVs and bo3-LUVs extruded through 100 nm-pore size membrane. For cryo-TEM, vesicle diameter and membrane thickness was analyzed in ImageJ (Fiji).

| Type of membrane | DLS | Cryo-TEM |
|------------------|-----|----------|
|                  | Diameter (nm) | PDI | Diameter (nm) | Membrane thickness (nm) |
|                  | w/o bo3 | w/ bo3 | w/o bo3 | w/ bo3 | w/o bo3 | w/ bo3 | w/o bo3 | w/ bo3 |
| Soy PC           | 106   | 96 | 0.066 | 0.053 | 80.7 ± 29.4 (n=138) | 70.2 ± 23.1 (n=138) | 4.4 ± 0.16 (n=30) | 4.5 ± 0.14 (n=30) |
| PDMS26-g-(PEO12)2/soy PC | 101 | 71 | 0.077 | 0.160 | 86.0 ± 39.6 (n=91) | 61.0 ± 23.9 (n=158) | 4.9 ± 0.17 (n=30) | 5.3 ± 0.54 (n=66) |
| PDMS26-g-(PEO12)2 | 103 | 85 | 0.108 | 0.107 | 97.4 ± 34.7 (n=36) | 81.9 ± 39.0 (n=61) | 5.3 ± 0.17 (n=30) | 5.8 ± 0.42 (n=47) |
Section S18.2: Cryo-TEM of lipid LUVs

Figure S30. Cryo-TEM of soy PC LUVs. White square shows zoom in the bilayer structure. Defocus: ~−2 μm.

Figure S31. Cryo-TEM of aggregated soy PC LUVs. A few lipid LUVs were aggregated during the observation. White square shows zoom in the junction of two aggregated vesicles (double bilayer). Defocus: ~−2 μm.
**Figure S32.** Cryo-TEM of large unilamellar and multilamellar soy PC vesicles (left: protein-free, right: functionalized with bo3 oxidase). White arrows indicate multilamellar vesicles (majority of vesicles was unilamellar). Defocus: ~2 µm.

**Figure S33.** Cryo-TEM of untreated soy PC LUVs with reconstituted bo3 oxidase (left) and ROS-treated sample (after 30-min exposure to ascorbyl free radical) (right). In some of the treated bo3-lipid-LUVs parts of the bilayer are disrupted (potential leakage areas, see carboxyfluorescein experiments). White arrows indicate the damaged spots through bilayer and membrane pores. Defocus: ~2 µm.
Section S18.3: Cryo-TEM of hybrid LUVs

**Figure S34.** Representative Cryo-TEM images of PDMS-g-PEO/soy PC LUVs (prepared from polymer/lipid mixture 70:30, mol%). ⅓ of hybrids had homogenous distribution of lipid and polymer, where lipid content was lower; membrane had fuzzy structure of intertwined polymer chains (no obvious lipid bilayer structure observed). Defocus: ~2 μm.

**Figure S35.** Cryo-TEM images of PDMS-g-PEO/soy PC LUVs (prepared from polymer/lipid mixture 70:30, mol%). The white arrows show lipid nanodomains. Defocus: ~2 μm.
Figure S36. Cryo-TEM images of PDMS-g-PEO/soy PC LUVs (prepared from polymer/lipid mixture 70:30, mol%). The white arrows show lipid nanodomains. Defocus: $\sim -2 \mu m$.

Figure S37. Cryo-TEM images of PDMS-g-PEO/soy PC LUVs (prepared from polymer/lipid mixture 70:30, mol%). The white square in the left image shows lipid nanodomain in upper vesicle. The black square in the right image shows the junction of two aggregated vesicles. Defocus: $\sim -2 \mu m$. 
Section S18.4: Cryo-TEM of polymer LUVs

Figure S38. Cryo-TEM of typical PDMS-g-PEO LUVs. Defocus: $\sim -2$ $\mu$m.

Figure S39. Cryo-TEM of nested polymer (PDMS-g-PEO) LUV – occasionally observed in the sample. Defocus: $\sim -2$ $\mu$m. PDMS-g-PEO vesicles shape transformation from spherical into nested ones was previously observed by Salva et al. (2013) after exposure to hypertonic shock (39).
Figure S40. Cryo-TEM of PDMS-g-PEO LUVs with reconstituted bo3 oxidase. In the middle and right micrograph are unilamellar nested vesicles. The red square shows bo3 oxidase inserted into polymer membrane. On the left and right image inward orientation of the cytosolic part (pump in) can be observed; Figure S41 for bo3 oxidase dimensions and structure. Defocus: ~ −2 μm.

SECTION S19: Structure of ubiquinol bo3 oxidase

Figure S41. The structure of ubiquinol bo3 oxidase from E. coli, front (left) and back (right) (PDB (40)). The shape asymmetry originates from membrane part (size L₁ ≈ 8 nm, L₂ ≈ 7.2 nm) and cytosolic part (ranging ~3.8 nm out of the membrane).
REFERENCES

1. Chemin M, Brun P-M, Lecommandoux S, Sandre O, & Le Meins J-F (2012) Hybrid polymer/lipid vesicles: fine control of the lipid and polymer distribution in the binary membrane. *Soft Matter* **8**(10):2867.

2. Frericks HL, Zhou DH, Yap LL, Gennis RB, & Rienstra CM (2006) Magic-angle spinning solid-state NMR of a 144 kDa membrane protein complex: E. coli cytochrome bo3 oxidase. *J Biomol NMR* **36**(1):55-71.

3. Ishmukhametov RR, Galkin MA, & Vik SB (2005) Ultrafast purification and reconstitution of His-tagged cysteine-less Escherichia coli F1Fo ATP synthase. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1706**(1):110-116.

4. Deshpande S, Caspi Y, Meijering AEC, & Dekker C (2016) Octanol-assisted liposome assembly on chip. *Nature Communications* **7**:10447.

5. Yandrapalli N & Robinson T (2019) Ultra-high capacity microfluidic trapping of giant vesicles for high-throughput membrane studies. *Lab on a chip* **19**:626-633.

6. Nilsson T, *et al.* (2016) Lipid-mediated Protein-protein Interactions Modulate Respiration-driven ATP Synthesis. *Sci Rep* **6**:24113.

7. Otrin L, *et al.* (2017) Toward Artificial Mitochondrion: Mimicking Oxidative Phosphorylation in Polymer and Hybrid Membranes. *Nano Lett* **17**(11):6816-6821.

8. Khailova LS, Dedukhova VI, & Mokhova EN (2008) Cations SkQ1 and MitoQ accumulated in mitochondria delay opening of ascorbate/FeSO4-induced nonspecific pore in the inner mitochondrial membrane. *Biochemistry (Moscow)* **73**(10):1121-1124.

9. Schaffazick SR, Pohllmann AR, de Cordova CAS, Creczynski-Pasa TB, & Guterres SS (2005) Protective properties of melatonin-loaded nanoparticles against lipid peroxidation. *International Journal of Pharmaceutics* **289**(1):209-213.

10. Gracià RS, Bezlyepkina N, Knorr RL, Lipowsky R, & Dimova R (2010) Effect of cholesterol on the rigidity of saturated and unsaturated membranes: fluctuation and electrodeformation analysis of giant vesicles. *Soft Matter* **6**(7):1472-1482.

11. Lira RB, Steinkuhler J, Knorr RL, Dimova R, & Riske KA (2016) Posing for a picture: vesicle immobilization in agarose gel. *Sci Rep* **6**:25254.

12. Poojari C, *et al.* (2019) Behavior of the DPH fluorescence probe in membranes perturbed by drugs. *Chemistry and Physics of Lipids* **223**:104784.

13. Kang M, Day CA, Kenworthy AK, & DiBenedetto E (2012) Simplified equation to extract diffusion coefficients from confocal FRAP data. *Traffic (Copenhagen, Denmark)* **13**(12):1589-1600.

14. Bagatolli LA (2006) To see or not to see: Lateral organization of biological membranes and fluorescence microscopy. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1758**(10):1541-1556.

15. Albers SV, Konings WN, & Diessen AJM (2006) 7 Membranes of Thermophiles and Other Extremophiles. *Methods in Microbiology*, (Academic Press), Vol 35, pp 161-171.

16. Nichols J.W. DDW (1980) Net proton-hydroxyl permeability of large unilamellar liposomes measured by an acid-base titration technique. *Proc. Natl. Acad. Sci. USA* **77**(4):2038-2042.

17. Dao TPT, *et al.* (2017) Mixing Block Copolymers with Phospholipids at the Nanoscale: From Hybrid Polymer/Lipid Wormlike Micelles to Vesicles Presenting Lipid Nanodomains. *Langmuir* **33**(7):1705-1715.

18. Grzesiek S & Dencher NA (1986) Dependency of delta pH-relaxation across vesicular membranes on the buffering power of bulk solutions and lipids. *Biophysical Journal* **50**(2):265-276.
19. Christopher L. Kuyper JSK, Sarah A. Mutch, and Daniel T. Chiu (2006) Proton Permeation into Single Vesicles Occurs via a Sequential Two-Step Mechanism and Is Heterogeneous. *J. Am. Chem. Soc.* **128**(10):3233-3240.

20. Paxton WF, McAninch PT, Achyuthan KE, Shin SHR, & Monteith HL (2017) Monitoring and modulating ion traffic in hybrid lipid/polymer vesicles. *Colloids Surf B BioInterfaces* **159**:268-276.

21. Seigneuret M & Rigaud JL (1986) Analysis of passive and light-driven ion movements in large bacteriorhodopsin liposomes reconstituted by reverse-phase evaporation. 1. Factors governing the passive proton permeability of the membrane. *Biochemistry* **25**(21):6716-6722.

22. G. Niggemann MKaWH (1995) The Bending Rigidity of Phosphatidylcholine Bilayers: Dependences on Experimental Method, Sample Cell Sealing and Temperature. *J. Phys. II France* **5**(3):413-425.

23. Jean-Baptiste Manneville PB, Daniel Lévy, Jacques Prost (1999) Magnification of Shape Fluctuations of Active Giant Unilamellar Vesicles. *Perspectives in Supramolecular Chemistry*, eds Luisi PL & Walde P, *Vol. 6*.

24. Girard P, Prost J, & Bassereau P (2005) Passive or active fluctuations in membranes containing proteins. *Phys Rev Lett* **94**(8):088102.

25. Bouvrais H, Cornelius F, Ipsen JH, & Mouritsen OG (2012) Intrinsic reaction-cycle time scale of Na+,K+-ATPase manifests itself in the lipid-protein interactions of nonequilibrium membranes. *Proc Natl Acad Sci U S A* **109**(45):18442-18446.

26. Almendro-Vedia VG, *et al.* (2017) Nonequilibrium fluctuations of lipid membranes by the rotating motor protein F1F0-ATP synthase. *Proc Natl Acad Sci U S A* **114**(43):11291-11296.

27. Vaz WLC, Criado M, Madeira VMC, Schoellmann G, & Jovin TM (1982) Size dependence of the translational diffusion of large integral membrane proteins in liquid-crystalline phase lipid bilayers. A study using fluorescence recovery after photobleaching. *Biochemistry* **21**(22):5608-5612.

28. Peters R & Cherry RJ (1982) Lateral and rotational diffusion of bacteriorhodopsin in lipid bilayers: experimental test of the Saffman-Delbrück equations. *Proc Natl Acad Sci U S A* **79**(14):4317-4321.

29. Sivaramakrishnan Ramadurai AH, Victor Krasnikov, Geert van den Bogaart, J. Antoinette Killian, and Bert Poolman (2009) Lateral Diffusion of Membrane Proteins. *J. Am. Chem. Soc.* **131**(35):12650-12656.

30. Doeven MK, *et al.* (2005) Distribution, lateral mobility and function of membrane proteins incorporated into giant unilamellar vesicles. *Biophys J* **88**(2):1134-1142.

31. Nam J, Beales PA, & Vanderlick TK (2011) Giant Phospholipid/Block Copolymer Hybrid Vesicles: Mixing Behavior and Domain Formation. *Langmuir* **27**(1):1-6.

32. Itel F, *et al.* (2014) Molecular Organization and Dynamics in Polymersome Membranes: A Lateral Diffusion Study. *Macromolecules* **47**(21):7588-7596.

33. Itel F, Najer A, Palivan CG, & Meier W (2015) Dynamics of Membrane Proteins within Synthetic Polymer Membranes with Large Hydrophobic Mismatch. *Nano Lett* **15**(6):3871-3878.

34. Pokorna S, Jurkiewicz P, Cwiklik L, Vazdar M, & Hof M (2013) Interactions of monovalent salts with cationic lipid bilayers. *Faraday discussions* **160**(43):341-358; discussion 389-403.

35. Heinemann F, Betaneli V, Thomas FA, & Schwille P (2012) Quantifying lipid diffusion by fluorescence correlation spectroscopy: a critical treatise. *Langmuir* **28**(37):13395-13404.

36. Sjoholm J, *et al.* (2017) The lateral distance between a proton pump and ATP synthase determines the ATP-synthesis rate. *Sci Rep* **7**(1):2926.

37. Rawicz W, Olbrich KC, McIntosh T, Needham D, & Evans E (2000) Effect of chain length and unsaturation on elasticity of lipid bilayers. *Biophysical journal* **79**(1):328-339.
38. Lin Z, Hill RM, Davis HT, Scriven LE, & Talmon Y (1994) Cryo transmission electron microscopy study of vesicles and micelles in siloxane surfactant aqueous solutions. *Langmuir* **10**(4):1008-1011.
39. Salva R, et al. (2013) Polymersome Shape Transformation at the Nanoscale. *ACS Nano* **7**(10):9298-9311.
40. Gilliland G, et al. (2000) The Protein Data Bank. *Nucleic Acids Research* **28**(1):235-242.

**Supporting video files**

**Video S1.** A polymer GUV during fluctuation analysis. The data was acquired at room temperature (~23 °C). The acquisition of 1800 snapshots was done by high-resolution camera (pcol.edge, PCO AG, Kelheim, Germany) with 200 µs exposure time and 15 fps frame rate (in phase contrast mode and 40× objective on inverted microscope Zeiss Observer.D1). Scale bar 10 µm.

**Video S2.** Proton pumping experiment. The proton pumping by bO3 oxidase reconstituted into polymer GUVs was monitored through the fluorescence change of the encapsulated pH-sensitive dye pyranine. bO3-polymer-GUVs in buffer (100 mM sucrose, 1 mM Tris-HCl (pH 7.5)) with 10 µM pyranine were loaded into the chip reservoir and flown through the channels to occupy the traps. After trapping 30–60 µm bO3-polymer-GUVs, the outer pyranine was flushed away by exchanging the outer solution with pyranine-free buffer. Proton pumping was then activated by introducing a solution of 8 mM DTT and 40 µM Q1. Pyranine was excited at 405 nm and 458 nm, and the emission intensity in a region inside the GUV was measured in the range 499–551 nm. The ratio of intensities emitted when exciting at the two wavelengths correlates with pyranine deprotonation and thus pH, which was quantified with a calibration curve for intravesicular pyranine. Scale bar 50 µm.