En route to dynamic life processes by SNARE-mediated fusion of polymer and hybrid membranes

Lado Otrin1✉, Agata Witkowska2,6, Nika Marušić3, Ziliang Zhao4, Rafael B. Lira4,7, Fotis L. Kyrilis5, Farzad Hamdi5, Ivan Ivanov3, Reinhard Lipowsky4, Panagiotis L. Kastritis5, Rumiana Dimova4, Kai Sundmacher3, Reinhard Jahn2 & Tanja Vidaković-Koch1

A variety of artificial cells springs from the functionalization of liposomes with proteins. However, these models suffer from low durability without repair and replenishment mechanisms, which can be partly addressed by replacing the lipids with polymers. Yet natural membranes are also dynamically remodeled in multiple cellular processes. Here, we show that synthetic amphiphile membranes also undergo fusion, mediated by the protein machinery for synaptic secretion. We integrated fusogenic SNAREs in polymer and hybrid vesicles and observed efficient membrane and content mixing. We determined bending rigidity and pore edge tension as key parameters for fusion and described its plausible progression through cryo-EM snapshots. These findings demonstrate that dynamic membrane phenomena can be reconstituted in synthetic materials, thereby providing new tools for the assembly of synthetic protocells.

1 Electrochemical Energy Conversion, Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany. 2 Laboratory of Neurobiology, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany. 3 Process Systems Engineering, Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany. 4 Department of Theory and Bio-Systems, Max Planck Institute of Colloids and Interfaces, Potsdam, Germany. 5 Interdisciplinary Research Center HALOmem & Institute of Biochemistry and Biotechnology, Martin Luther University Halle-Wittenberg, Biozentrum, Halle/Saale, Germany. 6 Present address: Department of Molecular Pharmacology and Cell Biology, Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Berlin, Germany. 7 Present address: Moleculaire Biofysica, Zernike Instituut, Rijksuniversiteit Groningen, Groningen, Netherlands. ✉ email: otrin@mpi-magdeburg.mpg.de

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At the dawn of a new genesis, as envisioned and facilitated by humankind, artificial life in its current iterations appears to be vividly reminiscent of the zygotic stage of multicellular life—full of potential and of ever-increasing complexity. In analogy to natural cells, the current synthetic constructs (synthetic cells and organelles) that are being assembled from molecular building blocks in a bottom-up fashion, are predominantly envisioned as enclosed structures made of phospholipids, while cytosolic and membrane proteins (MPs) endow a plethora of natural functionalities to the otherwise passive containers. Even though these mimics rapidly develop complexity and elegance, they remain short-lived without the natural mechanisms for repair and replacement. Unsurprisingly, a good amount of effort is invested in increasing their stability and performance by replacing natural parts with man-made ones. Hereby, protein optimization via extremophilic sources and mutations is ultimately constrained by the intrinsic fragility of biomolecules, while chemical mimicking of complex MPs such as the rotary engine ATP synthase, nearly perfected through millions of years of evolution, is currently beyond our reach. On the other hand, significant progress can be achieved by augmenting the common delimiter and an essential component of artificial cells and organelles. In recent years, lipid membranes were successfully replaced with synthetic polymers, which even enabled successful insertion of ATP-synthesizing apparatus and retention of MP activity. This substitution led to extended functional lifetimes, by counteracting enzyme delipidation for instance, while providing enhanced chemical resistance and lower permeability. Altogether, the biocompatibility of the amphiphile to the MP is still explored on a case-to-case basis but a general roadmap is beginning to crystalize. For instance, the rigidity of the historical polymer poly(butadiene)-poly(ethylene oxide) (PBd-PEO) arrested the oxygen reduction by a proton pumping terminal oxidase in a purely polymeric environment, though blending with phospholipids resulted in prolonged activity. The latter strategy of membrane “hybridization” has been proposed to alleviate the drawbacks of natural and synthetic building blocks and such mixed membranes have been investigated in detail, e.g., with respect to phase separation. More fluid hydrophobic blocks like poly(dimethylsiloxane) (PDMS) combined with other hydrophilic chains such as poly(2-methoxyethanol) (PPO) and poly(2-ethylhexanol) (PEO) have alone facilitated the reconstitution of complex bioenergetic proteins, e.g., ATP synthase or complex I, next to plentiful channels like aquaporins. Thereby, membrane fluidity, which was found to scale with the length of the hydrophobic block, and the hydrophobic mismatch between the bilayer and the MP, i.e., the membrane thickness, were found to be crucial parameters for unrestricted enzyme activity. This said, polymer membranes are not by default less permeable than natural ones and light-driven proton pumps have also been reconstituted in “frozen” amphiphiles based on polystyrene. In fact, the mechanical properties of polymersomes vary greatly, whereby the expanded design freedom and tunability are the particular assets of synthetic intervention. Thus, the design of ABC-type triblocks has allowed for the directed insertion of MPs thanks to membrane asymmetry, while a variation of the phospholipid type in hybrid films enabled control of MP partitioning in raft-like domains due to varying fluidities.

The next developmental stage of synthetic constructs, beyond purely channel or enzyme properties, is associated with the reconstitution of dynamic processes. Natural membranes not only segregate biochemical reactions and serve as interfaces for MPs but are also constantly being remodeled by these MPs and the cytoskeleton in processes like proliferation, signaling, and motility. The fusion of vesicles, together with fission, constitute probably the most important membrane remodeling processes. This enables the trafficking of cargo in various secretory pathways as well as of MPs and lipids, the latter providing a mechanism for membrane expansion in eukaryotic growth scenarios. On a practical note, vesicle fusion also allows for the reconstitution of multiple MPs with controlled orientation—a critical point in the assembly of synthetic constructs that requires further attention. So far, the fusion of polymer membranes has been rarely addressed. Only physicochemical triggers have been applied to block copolymer vesicles with limited efficiency. Furthermore, the fascinating process of protein-mediated fusion remains to this day entirely unexplored in polymersomes.

To address this challenge, we designed a fusion platform based on graft copolymer PDMS-g-PEO membranes, next to hybrid polymer/lipid mixtures, functionalized with fusogenic SNARE proteins. The choice of the comb-type siloxane polymer was directed by its suitable properties with respect to MP reconstitution and not least of all, its commercial availability. We explored the SNARE-mediated fusion of polymer and hybrid membranes via membrane mixing, as well as through the functional coupling between two respiratory enzymes, bo1 oxidase and ATP synthase. To elucidate the reasons for successful and efficient fusion, we investigated the change of bending rigidity upon SNARE insertion and the pore opening dynamics. In addition, we observed key fusion intermediates via cryo-electron microscopy (cryo-EM), which led to further insights of the fusion process in this system.

Results and discussion

Correct orientation of SNAREs and low bending rigidity of the synthetic membranes as prerequisites for fusion. First, we reconstituted minimal SNARE fusion machinery—full-length synaptobrevin (syb) and the acceptor complex comprising syntoxin, SNAP-25, and a C-terminal syb fragment (referred to as “ΔN complex”)—into polymer and lipid/polymer hybrid vesicles, and in parallel into liposomes, which served as a natural benchmark. To achieve this, we employed the so-called co-micellization method (for details see Methods) by using mixed micelles of SNAREs and amphiphiles, with sodium cholate as the mediating detergent. SNARE-functionalized nano-sized proteovesicles (Figs. S1, S2) were spontaneously formed upon detergent removal via size exclusion chromatography. Bearing in mind that only SNAREs with a correct (outward) orientation at the membrane will contribute to membrane fusion, we next analyzed their alignment via proteolytic digestion in the absence or presence of detergent. Analysis of SNARE fragments by SDS-PAGE (Fig. S3) revealed syb insertion with at least 81% and ΔN complex with over 92% correct orientations in all types of examined membranes, with the best overall orientation achieved in polymer vesicles (Fig. 1a). A similar trend of a more uniform outward orientation of the ΔN complex was observed previously in lipid vesicles. Additionally, it is worth noting that only outwards-facing SNAREs could be observed via cryo-EM (Fig. S2). Furthermore, we assessed the stability and integration efficiency of inserted SNAREs with a flotation assay (Fig. S4). In this approach, proteovesicles are separated from the poorly incorporated/ unstable SNAREs on a density gradient via ultracentrifugation. We observed stable and efficient integration of SNAREs in all types of membranes with marginal protein loss upon ultracentrifugation across all systems (Fig. 1b).

Following the successful and stable incorporation of SNAREs with predominantly correct orientation in the synthetic membranes, we next placed the proteovesicles in an environment that kept the system minimal, well-defined, and versatile. Towards this end, we used (unless specifically stated otherwise) a moderately
buffered solution (20 mM) containing only HEPES, KCl, and dithiothreitol (DTT), at physiological pH, osmolarity, and ionic strength. We selected KCl since it is known for its relatively weak interactions with phospholipids as well as with PEO. This enabled a more accurate assessment of SNAREs as the predominant fusion mediator across all tested membranes by minimizing the contribution of ions/agitation towards membrane fusion. Since KCl was previously shown to induce membrane swelling, we probed for this effect by cryo-EM (Fig. 1c and Fig. S2) and measured the membrane thickness of polymersomes (N = 578) as well as of the polymer (Fig. 1c, single fuzzy contour; N = 976) and lipid (Fig. 1c, two parallel contours; N = 171) nanodomains of hybrid vesicles, with and without SNAREs. We determined a fairly constant thickness of PDMS-g-PEO in polymersomes (6.6 ± 0.6 nm) and in hybrids (6.1 ± 0.3 nm), while the lipid domains of the latter were on average 4.9 ± 0.3 nm thick. Interestingly, compared to vesicles formed in sucrose solution with low buffering capacity, the polymer swelled by nearly 20% (from 5.3 ± 0.2 nm), while the thickness of lipid bilayers remained unchanged. Furthermore, we observed no significant thickness change in any of the membrane compositions upon SNAREs insertion.

The presence of salts and the reconstitution of MPs can result in considerable changes of the membrane bending modulus — an important parameter governing the energy of a membrane and one of the several energetic barriers to fusion, with respect to the high curvature of the stalk. To assess the effect of KCl, lipid dyes and inserted SNAREs on the bending rigidity of polymer and hybrid vesicles, we scaled up the vesicle size to the micron range via fusion/electroformation. With this method, giant unilamellar vesicles (GUVs) were formed in 5 mM HEPES (pH 7.4) containing 5 mM KCl and their was determined via fluctuation (flickering) spectroscopy. Even though we had to considerably lower the salt concentration in order to facilitate the formation of GUVs, this setup enabled us to gain valuable information on the influence of the aforementioned factors. Indeed, we saw a nearly 50% decrease of the bending rigidity in protein-free polymersomes and hybrids formed in KCl compared to the ones grown in sucrose (from 11.7 ± 2.1 kBT determined previously to 6.1 ± 1.1, and from 11.6 ± 2.4 to 6.6 ± 0.7, respectively). This observation is consistent with the previously reported effect of salts on neutral and charged lipid membranes in conjunction with the proposed polymer loosening effect upon membrane insertion of various species.

The addition of the lipid dyes DOPE-N-(lissamine rhodamine B sulfonyl) (Rho) and DPPE-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD) to polymer and hybrid vesicles led to further substantial softening. This was likely due to increased salt screening via the additional membrane charge introduced by Rho and NBD. In contrast, the insertion of syb resulted in only a moderate increase of the bending modulus in both polymersomes and hybrids, while the ΔN complex had no statistically significant effect. This outcome does not correspond to our previous findings on membrane softening upon insertion of large transmembrane and multidomain proteins (ATP synthase and...
bo3 oxidase), likely reflecting considerable differences in protein size, architecture, charge, and concentration. In fact, a similar increase in the bending rigidity was previously reported for DNA, anchored in lipid membranes44, a system that is more reminiscent of our current setup.

Lastly, KCl enabled us to neutralize the charged membrane dyes that were introduced to the membranes (discussed separately in the following sections), which allowed us to observe the fusion of artificial membranes in their near-native state with respect to surface charge.

SNAREs prompt efficient membrane mixing of polymer and hybrid membranes. Next, we examined whether SNAREs are able to induce membrane mixing in synthetic membranes. Towards this end, we supplemented one population of each type of vesicles with the fluorescence resonance energy transfer (FRET) couple Rho/NBD and reconstituted the ΔN complex. Meanwhile, we inserted syb in vesicles containing no dyes. With this setup (Fig. S5), membrane mixing can be followed by the dequenching of NBD upon fusion, which results from the dilution of labeled proteovesicles with unlabeled ones. Thereby, KCl neutralized the additional surface charge from Rho/NBD and thus minimized the electrostatic repulsion between the vesicles (Figs. 2a and S6). In control experiments, we omitted SNAREs from the vesicles, while keeping everything else the same in order to assess the sole contribution of KCl under mild agitation.

Compared to proteoliposomes, a very similar initial fast stage of membrane mixing was observed in proteopolymersomes and proteohybrids (Fig. 2b–d). However, while the NBD fluorescence in proteoliposomes reached a plateau after about 2 h, we measured a progressive increase at a slower but steady rate in both polymer-containing systems. This led to higher membrane mixing on average, whereby no saturation was seen over the duration of the experiment (Fig. S7). Some degree of membrane mixing was also observed in SNARE-free vesicles, albeit absent of the rapid initial stage, characteristic for SNAREs, which substantiated their predominant role as the fusion mediator. Interestingly, the overall faster membrane mixing in proteohybrids was seemingly not a result from superimposition of unmediated fusion. On the other side, the proteins appear to expedite the anticipated saturation as evidenced by the comparison between SNARE-functionalized and SNARE-free liposomes (Fig. 2b). We also complemented the fluorescence-based measurements with dynamic light scattering (DLS) measurements before and after fusion. Similarly, DLS showed an increase in vesicle size in all tested systems after 2 h (Fig. 2e–g). Finally, variations in the total membrane mixing within identical systems were previously reported for proteoliposomes45. We probed for this effect in proteopolymersomes and measured the variability over several separate reconstitutions in different hands, with values ranging from 18 to 24% after the 2 h benchmark, defined by the proteoliposomes (Fig. 2d, inset).

Prolonged pore lifetime promotes content mixing. The observed membrane mixing in polymer and hybrid vesicles provides strong evidence for the early steps of fusion, namely docking and hemifusion. However, membrane mixing does not allow to draw definite conclusions about pore opening and expansion, which are crucial during the later stages of fusion and enable the lumen of the two vesicles to merge. To gain insights into these later steps, we again used micron-scaled vesicles and porated them in electric fields to observe the closure dynamics of micron-sized pores. This technique has been applied at the dawn of polymersome research, and for PBD-PEO, the pore dynamics were correlated to the membrane thickness by microscopic analysis46. Here, we expanded the analysis and quantified the pore edge tension47. Typical pore closure profiles of the three types of vesicles can be seen in Fig. 3a. The value of the edge tension for the lipid membranes used as a benchmark 16.4 ± 7.4 pN is in the lower range of values found for commonly used pure lipids like POPC (25.8 ± 6.4 pN), whereas the edge tension in polymersomes (7.9 ± 4.2 pN) and hybrids (8.9 ± 2.3 pN) was even lower (Fig. 3b).

During electroporation, we noticed that several minutes after the electric pulse was applied and microscopic pores closed, some polymer vesicles lost contrast originating from the loss of sugar asymmetry across their membranes. To examine the porated vesicles more closely for the presence of persistent submicron pores, we electroporated the vesicles in the presence of the watersoluble and membrane-impermeable dye sulforhodamine B (SRB) to observe a potential leak in. We found that about a third of the polymersomes remained porated up to several minutes after the application of the pulse. A representative course of the dye entry can be seen in Fig. 3c but the dynamics varied greatly (Fig. 3d), which suggested that the defects differ in number and size. Finally, we investigated whether the submicron pores were sealed at later times by adding a second dye of similar size (ATTO 647) 5–10 min after electroporation. In all instances, in which the polymersomes were permeable to SRB, ATTO 647 was not observed in the lumen (Figs. 3e and S8). This indicates that the remaining submicron pores in the PDMS-g-PEO membranes eventually closed.

Functional coupling as a crucial determinant for full fusion. Next, we examined the content mixing of fused polymer and hybrid vesicles, primed by the SNARE-induced pore opening and expansion. We approached this by co-reconstituting one vesicle population, containing the ΔN complex with ATP synthase and another population, containing syb with bo3 quinol oxidase (Fig. 4a). ATP synthase and bo3 oxidase are respiratory enzymes involved in bacterial oxidative phosphorylation. The reduction of bo3 oxidase via ubiquinol leads to translocation of protons across the membrane, establishing a proton gradient, which is then used by ATP synthase for the coupling of ADP and inorganic phosphate to ATP. In our setup, the reducing power is provided by DTT, while synthetic ubiquinone (UQ) 1 is used instead of bacterial UQ 8. The essence of this functional assay is that ATP synthesis can be achieved only when both enzymes are integrated into a shared compartment, thus enabling the bioenergetic coupling27. For a more reliable comparison, we carefully tailored the vesicle formation procedure so that all co-reconstituted vesicles described here would better match the ones employed in membrane mixing experiments (Figs. 4c–e and S9). Nevertheless, we had to make some changes in order to accommodate both respiratory enzymes, such as increasing the pH from 7.3 to 8.0 and lowering the temperature from 37 to 23 °C. We also supplied the necessary cofactors (phosphate, ADP) along with the reporter luciferin/luciferase system to enable functional testing. In this respect, we previously reported a decrease in the bending rigidity of liposomes, polymersomes, and hybrids upon insertion of bo3 oxidase11. All mentioned changes could affect the early stages of vesicle fusion, manifested by membrane mixing, therefore the main focus here was solely on content mixing.

We combined the two populations of vesicles and, following short incubation, initiated proton pumping by bo3 oxidase with the addition of DTT and UQ. As a result, we observed successful ATP production in both SNARE-functionalized and SNARE-free vesicles, although synthesis in the latter subsided rapidly (Fig. S10a–c), while the SNARE-mediated fusion resulted in a steady rate over a prolonged period of time (Fig. S10). The sole
presence of various salts and cofactors, in combination with the energy provided by mechanical agitation, appears to suffice for some degree of fusion (consistent with the membrane mixing experiments). However, in the absence of guided mechanism, binary to the enzyme integration, this fusion is less efficient and random with respect to the functional coupling, which altogether leads to transient activity that cannot be sustained. The several-fold higher ATP synthesis rates achieved via SNARE-mediated fusion (Fig. 4b) further support this claim. Finally, we observed comparable ATP synthesis rates between SNARE-integrated liposomes, polymersomes, and hybrids, with polymersomes exhibiting slightly higher activity than the other two platforms. However, this observation alone does not factor in the different protein integration efficiencies of both respiratory enzymes in these types of membranes. In fact, our previous findings\(^7\) suggested that the integration efficiency of ATP synthase was the lowest in polymersomes and the highest in liposomes. Accounting for these differences by normalizing the ATP synthesis to the amount of reconstituted enzyme will lead to even better performance of polymer membranes. In either case it can be concluded that suitable membrane properties like the lower edge tension led to easier pore opening and expansion,

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**Fig. 2 Membrane mixing upon SNARE-mediated fusion.**

a The nearly neutral zeta potential of lipid (Lip), polymer (Poly), and hybrid (Hyb) vesicles promotes membrane mixing. The surface charge, introduced by lipid dyes was neutralized with KCl. b-d Comparison of SNARE-induced and spontaneous membrane mixing in different amphiphiles. Mean values of at least two different reconstitutions with standard deviations are shown. Inset of (d): Variability of SNARE-mediated membrane mixing in polymersomes. Individual measurements, as well as their mean value with standard deviation, are shown. e-g Intensity-based size distribution with indicated peak values of different vesicles upon synaptobrevin (Syb) or ΔN complex (ΔN) reconstitution before and after fusion. Size increase corresponding to several rounds of fusion can be seen in the lowest panels.
facilitating efficient SNARE-mediated fusion in both polymer and hybrid vesicles. Similar membrane composition but different fusion progression. Lastly, with cryo-EM, we were able to capture fusion intermediates during the membrane and content mixing stages. Even though it is impossible to follow the process by destructive imaging, and the mixing of vesicle populations generates additional stochasticity, we reconstructed a plausible SNARE-mediated fusion progression in polymer and hybrid proteovesicles by comparison with natural systems45 and molecular simulations48,49. Another argument for the postulated sequence was the fact that later intermediates were prevalent upon longer incubation times (Fig. S11).

We observed a docking stage of proteopolymersomes (Figs. 5-I and S12), which likely had proceeded towards the exvagination of a small membrane portion of a single vesicle, leading to the formation of a unilateral stalk (Fig. 5-II). Thus, a local point contact, which was also observed in some cases of liposome fusion50, was established between the stalk and the proximal membrane (the act of “kissing”), which should, in turn, have initiated membrane mixing and led to the emergence of a narrow hemifusion diaphragm (Fig. 5-III). Considering the previously proposed organization of PDMS-g-PEO molecules (hairpin conformation) as a bilayer51,52, we note that we adopted the terminology from lipid bilayers to provide an analogy, although some dissimilarities between the two systems cannot be excluded. Presumably, the diaphragm expanded further, and thinning of the
**Fig. 4 SNARE-induced content mixing in different vesicles.**
a) Content mixing was assessed via functional coupling between two enzymes of the bacterial respiratory chain: the proton pump $b_o$ oxidase ($b_o$) and the proton gradient consumer ATP synthase (AS). Each enzyme was co-reconstituted separately with either synaptobrevin (syb) or the $\Delta N$ complex ($\Delta N$). Upon successful fusion, the gradient established upon activation of $b_o$ oxidase with DTT was used by ATP synthase for ATP production. ATP was converted to luminescence signal via the luciferase/luciferin reporter system (CLSII).

b) Comparison of ATP synthesis rates after SNARE-free or SNARE-mediated fusion of different vesicles indicated higher coupling efficiency in the presence of SNAREs. Each point corresponds to a single measurement. Also shown are mean values with standard deviations. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. The highest SNARE-mediated content mixing was achieved in polymersomes.

c–e Intensity-based size distribution of different vesicles co-reconstituted with synaptobrevin (syb) + $b_o$ oxidase ($b_o$) or with the $\Delta N$ complex and ATP synthase (AS).

**Fig. 5 Proposed fusion intermediates of SNARE-mediated polymersome fusion.** Following vesicle docking (I; for an enlarged image please see Fig. S12) local point contact is established between the vesicles (II), leading towards membrane mixing and the emergence of hemifusion diaphragm (III). While the latter is expanding (IV), membrane thinning (shown with white arrows, enlarged in Fig. S13) can be observed at the juncture, indicating the location of the eventual pore opening (V). Such position of fusion pore was previously observed in simulations. Slight lateral deformations in the newly fused vesicles along with less frequent inclusions in the lumen are likely remnants of the hemifusion diaphragm (VI). Scale bars represent 30 nm.
membrane at the juncture, likely at the point of initial contact, was observed (Figs. 5-IV and S13). Furthermore, the pore opening (Figs. 5-V and S14) at the diaphragm extremity (previously observed in simulations49) suggested potential colocalization of the points of initial contact, membrane thinning, and pore opening. Finally, the resulting fused polymersomes featured persistent indentations, as well as occasional inclusions (bits of polymer membrane), likely remnants of the fusion diaphragm (Fig. 5-VI).

In contrast to proteopolymersomes, we discovered different steps of the fusion process in proteohybrids (Fig. 6). The docking stage in the latter was superseded by a long contact between the fusing vesicles, at a distance of about 2.5 nm. Remarkably, such contact was most frequently established between the polymer-rich domain of one vesicle and the lipid-rich domain (in a form of a bilayer) of another one (Fig. 6-I). Seemingly, the membrane mixing between the outer lipid layer and polymer bilayer originated at the lateral edge of the protruding lipid domain, and was propagated towards the opposite side (Figs. 6-II and S15). Such “hybrid membrane zippering” likely resulted in a state of transient hemifusion, in which lipid-rich bilayer domains (Fig. 6-II, III, exhibiting higher contrast compared to the polymer) were stabilized within the polymer bilayer. This temporary state was potentially resolved by membrane reorganization and the formation of lipid domains outside of the contact, leading to the formation of the characteristic configuration of hemifusion diaphragm, as in polymersomes (Fig. 6-IV). In all hybrid cases, we observed pore opening in a more central region of the juncture (V), which continued with pore expansion, diaphragm dissolution, and, finally, membrane relaxation (Fig. 6-VI).

For mimicking natural membranes, we selected the graft copolymer PDMS-g-PEO, which previously enabled the insertion of complex MPs, while preserving their activity7,9,11. Furthermore, it is readily blended with lipids7 to form macrohomogenous hybrid vesicles, offering a versatile environment for the reconstitution of MPs. Importantly, this polymer considerably extended the functional lifetime of integrated enzymes9,11 and offered protection against oxidative damage11. We previously observed several PDMS-g-PEO characteristics of high potential to promote membrane fusion, in particular its low bending rigidity, sufficient fluidity, and comparatively low membrane thickness7,11. Here, we identified the low edge tension as another key material property that enabled easier pore opening and thus facilitated content mixing. Even though the structure and nanomechanics of the nascent fusion pore of potentially proteolipid character53 and the complex energy landscape of liposome fusion have not been fully resolved yet, there are indications that pore formation may be the dominating factor in the experimentally determined lumped activation energy of $\sim 30k_BT$ in phosphatidylcholines54. In any case, it is safe to conclude that the joint contribution of easier bending and pore stability leads to a lower apparent activation energy for fusion in the polymer membrane compared to lipids. Thus, the 5 $k_BT$55 of mechanical work, exercised by SNAREs at the conclusive stage of fusion will more easily overcome the energetic barrier and less cooperative effort would be required to achieve synaptic rates.

We believe that these advantages of PDMS-g-PEO are crucial merits in order to choose appropriate membranes for the construction of artificial cells and organelles, in particular with respect to membrane processes. The molecular basis for the beneficial properties of this particular polymer appears to be related to the flexible PDMS backbone and the grafted architecture, in contrast to the commonly used rigid diblocks based on PBD. In general, we do not expect that grafted arrangement will be the sole determinant of biomimicking since the mechanical properties can be tuned by variation of the hydrophilic/hydrophobic ratio and the molecular weight. On the other hand, high flexibility may present a trade-off and come at the expense of increased permeability, which would negatively affect bioenergetic, segregation, and signaling scenarios. However, this seems not to be an issue in the particular case since PDMS-g-PEO has similar water56 and proton11 permeability to lipids, while providing the emergent benefit of membrane resealing upon MP reconstitution in the case of mixed membranes. As stated above, the hospitality to MPs has to be ultimately proved in each specific case, while quantitative efforts for optimization of reconstitution protocols have already been undertaken57. Based on the current experimental evidences though, the commercially available PDMS-g-PEO accommodates optimal properties for the replacement of lipid membranes and further systematic studies may identify a general molecular roadmap for the design of nature-like or superior membranes.

The minimal fusion environment in the present setup was defined with as wide as possible biocompatibility in mind. Apart from HEPES, and DTT to reduce the SNAREs, the only other additive was KCl, which is commonly used to stabilize MP$s$ and was chosen due to its comparatively weaker interaction with membranes. Thereby, KCl neutralized the surface charges introduced by lipid dyes, which minimized the electrostatic

**Fig. 6** Proposed fusion intermediates of SNARE-mediated fusion in hybrids. Long contact is established between the lipid-rich domain (bilayer) of one fusing vesicle, and the polymer-rich domain of another (I). The initial contact between the fusing membranes occurs at the lateral edge of the protruding bilayer and it further develops into crosswise membrane mixing (the process of “hybrid zipperping”, II). Transient fusion diaphragm, exhibiting lipid-rich bilayer stabilized in polymer bilayer is observed (III) before stable lipid bilayers are formed in the newly mixed hybrid diaphragm (IV). The fusion pore was formed in the more central region of the juncture (V), as previously observed in simulations48. No inclusions or deformations can be observed in fused vesicles (VI). Scale bars represent 30 nm.
repulsion between vesicles, and additionally stimulated fusion by lowering the bending rigidity of polymersomes and hybrids. The latter effects led to measurable events of unmediated fusion, both in content and membrane mixing experiments, but the significantly slower initial kinetics of the latter do not lessen the orthogonality of the SNARE-based approach. Remarkably, we observed fusion of polymersomes and hybrids with identical or better efficiency than in lipid vesicles. This is particularly exciting with respect to the previously reported decrease in the functional integration of ATP synthase with increasing polymer proportion.7,11. In fact, we demonstrated that the current strategy can be employed as a useful practical tool for the integration of bioenergetic apparatus in all tested amphiphiles, paving the way to further applications for protein trafficking in natural and synthetic membranes. Furthermore, the biological congruity of SNAREs allows for immediate employment of natural NSF/SNAP recycling machinery to enable directed and sustained fusion, in contrast to one off physicochemical triggers such as electrostatic attraction, bulk control by osmotic pressure, or biomimetic strategies like DNA-mediated fusion.58. Sustained virtually inexhaustible progress will be particularly relevant for recursive phenomena like the growth of membrane in the context of proliferation. SNAREs instantly motivates exemplary scenarios for the polymer needs to be cellularly degradable and/or easily deliverable, SNARE mediation opens new ways for targeted strategies like DNA-mediated fusion58. Such a virtually inexhaustible source of dodecyl maltoside (Merck, 69227-93-6: D4641). The puriﬁed (GE Healthcare). All proteins with the exception of truncated syb-2 were solubilized with an equal concentration of cholesterol and trypsin (400:1 (mol:mol). Upon protein addition, reconstitution mixtures were mixed briefly with three short bursts (1000 RPM) and incubated at 23 °C for 5 min. Proteosomes were then formed spontaneously upon the detergent removal via size exclusion chromatography on the PD Minitrap® G-25 column (GE Healthcare) equilibrated with 20 mM HEPES (pH = 7.4/KOH), containing 150 mM KCl, 1 mM DTT. Eluted fractions containing lipid dyes were collected and pooled. Furthermore, volume-wise, all eluted fractions containing dye-free vesicles were collected and pooled.

For the content mixing experiments, dry thin films were resuspended in 20 mM HEPES (pH = 8.0/KOH), containing 150 mM KCl, 1 mM DTT, 5% Na-Cholate as described above. To one population of mixed micelles, first, ΔN complex was added at the lipid/polymer/hybrid mixture:ΔN complex ratio of 1000:1 (mol:mol) and to the dye-free micelles, syb was added at the lipid/polymer/hybrid mixtures:1:1 (mol:mol). Upon protein addition, reconstitution mixtures were mixed briefly with three short bursts (1000 RPM) and incubated at 23 °C for 5 min. Proteosomes were then formed spontaneously upon the detergent removal via size exclusion chromatography on the PD Minitrap® G-25 column equilibrated with 20 mM HEPES (pH = 8.0/KOH), containing 150 mM KCl, 1 mM DTT, and 40 mM K3HPO4 (Merck, 7778-77-0: P04555). All eluted fractions containing lipid dyes were collected and pooled.

For the cyto-EM imaging, vesicles were prepared in the exact same way as described for the membrane mixing experiments, except that the ΔN complex was reconstituted at the lipid/polymer/hybrid mixture:ΔN complex ratio of 1800:1 (mol:mol).

Preparation of SNARE-inserted nano-sized lipid, polymer, and hybrid vesicles with the co-micellization method. Proteosomes were formed from mixed reconstituted upon detergent removal and subsequently following the reconstitution strategy described previously68,69, with considerable modiﬁcations.

Soy phosphatidycholine (Avanti, 95%, 441601), polymer PDMS-g-PEO (Dow, DOWSIL® OFX-5329 Fluid), or hybrid mixture (polymer:lipid = 7:3, mol:mol—this composition produces homogeneous, well-mixed hybrid membranes7), dissolved in chloroform:methanol (2:1, V/V), were deposited into a round-bottom glass vial.

For the membrane mixing experiments, in addition to the dye-free vesicles, one population of vesicles was supplemented with 1.5 mol% of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (“Rhod” (Avanti, 801050)) and 1.5 mol% of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (ammonium salt) ("NBD") (Avanti, 801444) and the lipid content (liposomes, hybrids) or polymer content was reduced proportionally. Both dyes were dissolved in chloroform:methanol (2:1, V/V), at 1 mg ml⁻¹.

For the content mixing experiments, vesicles were supplemented with 0.5 mol% Rho (Avanti, 801150), dissolved as described above, and the lipid content (liposomes, hybrids) or polymer content was reduced proportionally.

Then, the solvent was evaporated under a gentle stream of nitrogen and the produced thin films were further dried under nitrogen for 1.5 h.

Next, for the membrane mixing experiments, dry thin films were resuspended at the final concentration of 5 mM in 20 mM HEPES (Merck, 7365-45-9: H6147) (pH = 7.4/KOH), containing 150 mM KCl (Merck, 7447-40-7: P9541), 1 mM DTT (Merck, 3483-12-3: D9779), and 5% (g [m]/100 ml) Na-Cholate, by rigorous vortexing (~1200 RPM) until all material was well-dispersed. Foamed lipid/Me-dye/detergent-mixed micelles. Next, to micelles containing Rho/NBD, ΔN complex was added at the lipid/polymer/hybrid mixture:ΔN complex ratio of 1000:1 (mol:mol) and to the dye-free micelles, syb was added at the lipid/polymer/hybrid mixtures:1:1 (mol:mol). Upon protein addition, reconstitution mixtures were mixed briefly with three short bursts (1000 RPM) and incubated at 23 °C for 5 min. Proteosomes were then formed spontaneously upon the detergent removal size exclusion chromatography on the PD Minitrap® G-25 column (GE Healthcare) equilibrated with 20 mM HEPES (pH = 7.4/KOH), containing 150 mM KCl, 1 mM DTT. Eluted fractions containing lipid dyes were collected and pooled.

Furthermore, volume-wise, all eluted fractions containing dye-free vesicles were collected and pooled.

For the content mixing experiments, dry thin films were resuspended in 20 mM HEPES (pH = 8.0/KOH), containing 150 mM KCl, 1 mM DTT, 5% Na-Cholate as described above. To one population of mixed micelles, first, ΔN complex was added at the lipid/polymer/hybrid mixture:ΔN complex ratio of 1000:1, following mixing with three short bursts. Next, after 5 min, ATP synthase was added at the lipid/polymer/hybrid mixture:ATP synthase ratio of 81000:1 (mol:mol) followed by brief mixing, as before. To another population of mixed micelles, syb was added at the lipid/polymer/hybrid mixtures:1:1 (mol:mol), and bo3 oxidase at the lipid/polymer/hybrid mixture:bo3 oxidase ratio of 27000:1 with intermediate mixing steps, as described above. Reconstitution mixtures were then incubated at 23 °C for 5 min. Proteosomes were then formed spontaneously upon the detergent removal size exclusion chromatography on the PD Minitrap® G-25 column equilibrated with 20 mM HEPES (pH = 8.0/KOH), containing 150 mM KCl, 1 mM DTT, and 40 mM K3HPO4 (Merck, 7778-77-0: P04555). All eluted fractions containing lipid dyes were collected and pooled.

For the cyto-EM imaging, vesicles were prepared in the exact same way as described for the membrane mixing experiments, except that the ΔN complex was reconstituted at the lipid/polymer/hybrid mixture:ΔN complex ratio of 1800:1 (mol:mol).

Determination of the orientation of reconstituted SNAREs. Orientation of SNARE proteins incorporated into liposomes, polymersomes, or hybrid vesicles was assessed with pro tease (trypsin) digestion of intact and detergent-solubilized vesicles as described from ref. 80. Briefly, 40 μl of vesicles were incubated either with 10 μl of buffer only (20 mM HEPES pH 7.4 and 150 mM KCl), buffer and trypsin (Novex, 00007-7, final concentration 0.1 mg ml⁻¹), or buffer and triton X-100 (Merck, 9002-93-1, final concentration 0.3%). After 2 h incubation at 37 °C samples were analysed by Tricine-SDS-PAGE57. With respect to this, the band intensity of samples, treated with buffer only (total amount of inserted SNAREs) was compared with the band intensities of trypsin-treated samples (amount of inserted intact SNAREs) and trypsin/Triton-treated ones (amount of indigestible intramembrane fragments of SNAREs). The fraction of outwards-facing SNAREs was then calculated by subtracting the inwards-facing SNAREs and indigestible fragments from the total amount of inserted SNAREs and dividing it by the latter.

Stability assessment of reconstituted SNAREs via the flotation assay. Integration stability and efficiency was evaluated with a flotation assay on a discontinuous Nyodexen (Progen, 1003242) gradient, as described 81. Briefly, Protein content was estimated using Coomassie solubilized vesicle pellets dissolved in 20 mM HEPES (pH 7/4/KOH), 150 mM KCl, and were overlaid first with 30% Nyodexen and then with said buffer only. Following ultracentrifugation,
reconstituted vesicles were partitioned in the uppermost (buffer) layer of the gradient, while the non-incorporated SNAREs as well as disassembled αN complex can be considered. The SNARE content of different layers was analyzed by Tricine-SDS-PAGE and the amount of SNAREs, inserted in a stable manner (upmost layer) was compared with the total amount of SNAREs in all layers.

Zeta potential determination. The Zeta potential of protein-free vesicles employed in the membrane and content mixing experiments was determined under experimental conditions with respect to solvent (buffer) composition, pH, temperature, and presence of various related cofactors (UQ, ATP, etc.). Towards this end, zeta-potential measurements were performed above 0.75% of both Rho/NBD for membrane mixing and with 0.5% Rho for the content mixing) were prepared as described above in either membrane mixing buffer (20 mM HEPES (pH 7.4/KOH), 150 mM KCl, 1 mM DTT; viscosity at 37 °C = 0.760 cP, the refractive index at 37 °C = 1.3364). Zeta-potential measurements consisted of 10–300 runs each. Reported are mean zeta potentials acquired in separate measurements.

SNARE-mediated membrane mixing and vesicles size changes following fusion. Vesicles intended for the membrane mixing experiments as described in section “Preparation of SNARE-inserted nano-sized lipid, polymer, and hybrid vesicles” were first incubated at 37 °C for 30 min following preparation to preheat them. Typically, total NBD dequenching was reached at 460 nm and a baseline fluorescence emission of NBD at 335 nm was recorded on a Varian Cary Eclipse (Agilent), with the exc./em. slits at the positions 10/10 and with the PMT voltage set at 480 V. Then, to initiate fusion, dye-free vesicles were added next. The final concentration of both vesicles was 0.4 mM each in a total reaction volume of 0.9 ml. Membrane mixing as an NBD dequenching was monitored until a plateau was reached. After that, 10% octyl glucoside resuspended in membrane mixing buffer was added stepwise (1–5 µl per addition) until maximal NBD dequenching was reached. Typically, total NBD dequenching was reached at 0.53% final concentration of OG in liposomes, 0.43% in hybrids, and 0.48% in polymersomes. Measured changes in NBD emission were normalized using the initial NBD fluorescence as 0% and the fluorescence of max. dequenched NBD upon OG addition as 100%. In control experiments, SNAREs were omitted from vesicles. Reported are average values of at least two separate vesicle preparation with standard errors. Each point in Fig. 2b: inset represents separate vesicle reconstitution.

Intensity-based size distribution of vesicles before and after fusion was recorded with the freshly prepared SLBs being relatively high, 17 mM. After size exclusion chromatography, said vesicles were diluted to about 5 mM, which we found to be optimal for the formation of GUVs. Microscopy of GUVs. Microscopy was performed according to the previously established fusion/electroformation approach, which we further modified and optimized for SNAREs. First, vesicles at about 5 mM following their size distribution was deposited onto ITO-coated glass slides (55 Ω) as seven droplets, 2 µl each, followed by partial dehydration of deposited samples over the course of 1 h. A snapshot was taken on an electroformation chamber (consisting of two sandwies, PCD Ag, Kelheim, Germany) with 200 µs exposure time and 20 fps frame rate (in phase-contrast mode and 40x (NA 0.6) objective on inverted microscope Zeiss Observer.D1). Vesicle fluctuations were analysed using custom-built software as previously reported. Vesicles containing inclusions, large buds or tubes, or ones that did not significantly fluctuate, were excluded from the analysis. A t-test (two-tailed, heteroscedastic) was conducted to determine the effects of dyes/SNAREs on the bending rigidity of polymer and hybrid vesicles.

Determination of the pore edge tension of vesicles and the analysis of dye entry dynamics. Protein-free GUVs were prepared using the previously described electroformation method. In short, lipid, polymer, or the hybrid mixture of the two (7:3, mol/mol) dissolved in chloroform at the concentration of 1 mg ml−1 was spread on a Teflon-coated Dahlquist-type (V212; Vision Scientific, USA) and the solvent was evaporated under a gentle stream of nitrogen over 1 h. Both slides were then connected via a 2-mm-thick Teflon spacer, which created a chamber with about 1.9 ml volume. Chamber was filled with 200 mM sucrose (Merck, 57-50-1: 57903) dissolved in milli-Q, and connected to a function generator. Vesicles were formed in the AC field of 1.5 V and 10 Hz, over 30 min. When lipid dyes were used, the electroformation was performed in the dark.

Membrane edge tension was measured according to the method reported in ref. 42, with the use of the pore closure as previously developed in ref. 43. In short, GUVs prepared as described above, but in 200 mM sucrose solution containing 0.1 mM NaCl (Merck, 7647-14-5: S7653) were dispersed in 180 mM glucose (Merck, 50-99-7: G7528) solution and observed under phase-contrast microscopy using an inverted microscope (Axiovert 135, Zeiss, Göttingen, Germany) equipped with a 40x (NA 0.6) Ph2 objective. Images were recorded using a high-speed camera (Vision Research, USA) and the images were recorded at 5 kfps. The obtained images were semiautomatically processed using ImageJ (NIH, USA) and analysed as described previously.27 The slow, linear third stage of the pore closure was used in the analysis. For the electroporation, an electroprobe chamber was formed by sticking two parallel copper strips (copper conductive tape, 1.6 mm width, 1101102, 3 M) onto a glass coverslip, separated by 0.5 cm. A closed electroprobe chamber was formed by placing parafilm perpendicular to the strips, forming a chamber approx. volume of 100 µl. Prepared GUVs were diluted 10–20 times in isosmolar glucose solution and placed into the chamber for electroporation. The end of each cap was connected to a multiproporator (Retech pulse generator, 50 kHz, Retech, Union, USA), and using the theory of pore closure as previously developed, the electrical pulse of 200 V and 4 ms duration was applied. If not mentioned otherwise, we applied a single DC pulse of 200 V and 4 ms duration. Since polymer GUVs often become permeable after the

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application of an electric pulse (see Results), we exchange the GUVs in the chamber every time a pulse was applied and so vesicles were subjected to only one pulse. The dye entry into electroporated GUVs was studied as in ref. 69 with minor modifications. Studied GUVs, labeled with 0.5 mol% of a green dye NBD were dispersed in a medium containing 2 µM of water-soluble dye sulforhodamine B (SRB)(3520-42-1; Sigma) and were electroporated as described above. To test the long-term membrane permeability 5–10 min after the original poration, a second dye, ATTO 647 (ATTO-TEC) was added to the external solution of GUVs at the final concentration of 2 µM. Images were acquired using a Leica TCS SP5 confocal microscope (Wetzlar, Germany) using a 63x water-immersion objective (1.2 NA) at 512 x 512 pixels, at 1 A.U., 400 Hz scanning speed, and three-line averages. To record fast electroporation dynamics in real time, videos were recorded with 128 x 128 pixels, at 1000 Hz scanning speed and one line average. NBD was excited at 488 nm using argon laser and its emission was detected at 495–550 nm. SRB and ATTO 647 were excited with a diode-pumped solid-state laser at 561 and 633 nm, respectively, and detected at 564–615 nm and 640–690 nm. As in the above, the GUV samples were discarded after single electroporation.

Cryo-electron microscopy. The SNARE-inserted polymer and hybrid vesicles were prepared as described in the section "Preparation of SNARE-inserted nano-sized lipid, polymer, and hybrid vesicles". After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. Preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. Preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C. After the formation, first, vesicles were preheated at 37 °C for 30 min. Then, both populations of vesicles were mixed 1:1 (V:V) to initiate fusion and were being continuously stirred at 600 RPM at 37 °C.
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Author contributions
L.O. and T.V.K.-T. conceptualized the study, L.O., A.W., N.M., Z.Z., R.B.L., F.L.K. and F.H. performed the experiments. L.O., A.W., N.M., Z.Z. and R.B.L. developed the methodology. L.O. and N.M. coordinated the research. R.L., P.L.K., K.S., R.D., R.J. and T.V.K.-T. provided funding and resources. All authors analyzed the data. L.O. visualized the data. L.I., R.L., P.L.K., R.D., K.S., and T.V.K.-T. supervised the research. L.O. and L.I. wrote the manuscript. All authors reviewed and edited the manuscript.

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Correspondence and requests for materials should be addressed to L.O.

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