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One SNARE complex is sufficient for membrane fusion

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Abstract

In eukaryotes, most intracellular membrane fusion reactions are mediated by the interaction of SNARE proteins that are present in both fusing membranes. However, the minimal number of SNARE complexes needed for membrane fusion is not known. Here, we show unambiguously that one SNARE complex is sufficient for membrane fusion. We performed controlled *in vitro* Förster resonance energy transfer (FRET) experiments and found that liposomes bearing only a single SNARE molecule are still capable of fusion with other liposomes, or with purified synaptic vesicles. Furthermore, we demonstrate that multiple SNARE complexes do not act cooperatively, showing that synergy between several SNARE complexes is not needed for membrane fusion. Our findings shed new light on the mechanism of SNARE-mediated membrane fusion and ask for a revision of current views of fusion events such as the fast release of neurotransmitters.

SNAREs are an evolutionary conserved superfamily of small transmembrane or membrane-anchored proteins that possess a conserved domain of about 60 to 70 amino acids, termed the SNARE motif. SNARE proteins play a vital role in eukaryotic life as they mediate all intracellular fusion reactions (except mitochondrial fusion). Thus they are essential for a wide range of cellular processes, including cell growth, cytokinesis and synaptic transmission^{1,2}. Isolated SNARE motifs are unfolded and have no secondary structure. When membranes with complementary sets of SNARE proteins are mixed, the SNAREs assemble in *trans*. This assembly proceeds from the N-terminal end to the C-terminal membrane anchors and results in a tight coil-coiled complex (core-complex). This process pulls the membranes together and initializes fusion, resulting in the SNARE proteins being present in a single membrane (*cis*-complex)^{1,2}.

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Author Contributions

G.v.d.B. purified the proteins, designed, performed and analyzed the FRET and sequential photobleaching experiments, and programmed the software for the data analysis. M.G.H. purified the synaptic vesicles and performed the partial proteolysis and flotation experiments. G.B. and F.W. assisted with the microscopy. D.R. performed the electron microscopy. G.v.d.B. and R.J. designed the study and wrote the paper. All authors discussed the results and commented on the manuscript.

Membrane fusion can be reproduced *in vitro*, using purified SNARE proteins reconstituted in liposomes. For instance, liposomes that bear the neuronal SNARE proteins syntaxin 1 and SNAP-25 from the plasma membrane fuse with liposomes containing synaptobrevin 2 from synaptic vesicles³. While this indicates that SNARE proteins are sufficient to mediate membrane fusion, mechanistic details of the fusion reaction are lacking^{1–3}. Most importantly, it is still unknown how many SNARE proteins are needed for membrane fusion. Although single molecule studies showed that a single SNARE complex is sufficient for membrane docking^{4,5}, the only estimates available for membrane fusion range from 3 to 15 SNARE complexes^{6–9}. These numbers are largely based on the *in vivo* titration of inhibitors while measuring fusion kinetics. An estimate of at least 3 SNARE complexes was obtained when exocytosis of PC12 cells was inhibited with a soluble fragment of synaptobrevin⁶. In contrast, a much higher number (10–15 SNARE complexes) was inferred when neuronal exocytosis was inhibited by titrating botulinum neurotoxins, which inactivate SNAREs by specific proteolysis⁷. Lastly, an electrophysiology study in PC12 cells involving syntaxin mutants indicated that between 5 and 8 SNARE complexes are needed for membrane fusion, based on a model of steric hindrance⁸. The wide range of estimates is explained by the fact that all determinations have so far been based on indirect approaches. No direct measurements of the minimal number of SNARE complexes needed for membrane fusion have been carried out.

Here, we have measured SNARE-mediated fusion directly, using liposomes in which the number of SNAREs was progressively reduced to an average of below one molecule per liposome. In these experiments, we have taken advantage of the observation that fusion is greatly accelerated when a 1:1 acceptor complex of syntaxin 1 (residues 183–288) and SNAP-25 is stabilized with a synaptobrevin 2 fragment (residues 49–96)¹⁰. Employing fluorescence correlation spectroscopy in combination with FRET measurements, we recently demonstrated that docking of the liposomes proceeds even faster (>10-fold) than membrane fusion with the stabilized acceptor complex¹¹. With this complex the formation of a so-called 2:1 complex is avoided, where the binding site of synaptobrevin is erroneously occupied by a second syntaxin, resulting in a kinetically trapped dead-end^{12,13}. In addition, the truncated version of syntaxin lacks the N-terminal Habc domain that downregulates its capability to enter SNARE complexes¹⁴. Thus, the stabilized acceptor complex ensures that all SNAP-25 and syntaxin molecules can participate in core-complex formation, therefore allowing the measurement of SNARE-mediated fusion at very low protein-to-lipid (p/l-) ratios, and hence the determination of the minimum number of SNAREs needed for fusion.

Results

Characterization of the proteoliposomes

In order to prepare liposomes with on average less than one SNARE molecule per liposome, variants of synaptobrevin 2 and the stabilized acceptor complex (from rat) containing single cysteines were purified and labeled with Texas-red. For our experiments, a high labeling efficiency was essential to rule out the possibility that the number of SNAREs was underestimated due to unlabeled protein. Indeed, for Texas-red, the labeling was stoichiometric, with efficiencies of 90–110% as assessed by UV-vis spectroscopy (Fig. 1a–

c). A second important point was that the proteins were reconstituted in a monodisperse liposome population and this was confirmed by electron microscopy, where the average radius of the liposomes was 17.9 ± 5.8 nm (s.d.; Fig. 1d). Density gradient flotation experiments indicated that all proteins were fully incorporated in the membrane. Furthermore, partial proteolysis experiments showed that 50–80% of the molecules were correctly oriented, *i.e.* with their cytoplasmic domains on the outside (Fig. 1e–f). We have shown in the past that all correctly oriented synaptobrevin in the proteoliposomes is capable of complex formation¹⁵. Subsequently, the distribution of fluorescently labeled SNARE proteins over the liposomes was determined.

We measured the number of labeled SNARE proteins in individual liposomes using sequential photobleaching. This single molecule technique has been employed to determine the number of fluorescently labeled proteins encapsulated in lipid vesicles¹⁶ and allows to sequentially resolve up to approximately 8 fluorophores¹⁷ through discrete photobleaching steps. For the sequential photobleaching, we first embedded the liposomes containing the Texas-red labeled proteins in an agarose matrix and recorded a time series of two-photon excitation microscopy images (Fig. 2a). Control experiments with unlabeled protein confirmed that the fluorescence signal was specific for the Texas-red label. Furthermore, experiments with the fluid phase marker calcein encapsulated in the lumen of the liposomes showed that the liposomes remained intact during the embedding procedure. Subsequently, the liposomes were selected for by employing an offset above the level of the background signal, as described¹⁸ (Fig 2b–c). The number of fluorescently labeled proteins was then determined for each liposome by counting the discrete photobleaching steps in the time trace (Fig. 2d).

Importantly, the distribution of the SNARE proteins was well described by a Poisson function, suggesting that no protein homo-oligomerization occurred before reconstitution. For a molar p/l-ratio of 1:4,000, an average of 1.4 synaptobrevin and 1.2 syntaxin molecules per liposome was measured (Fig. 2e–f), which is within the range calculated for 100% protein incorporation when assuming an average surface area per lipid headgroup of 0.7 nm^2 (2.8 copies per liposome)¹⁹. In addition, the number of proteins scaled to the p/l-ratio and an average of 0.4 synaptobrevin and 0.35 syntaxin molecules were present per liposome when the liposomes were prepared at a p/l-ratio of 1:16,000, again demonstrating that the SNAREs were completely, yet randomly, distributed over the liposomes. At this ratio, only a small fraction (~5%) of the liposomes contained more than one SNARE, of which only a proportion was correctly oriented (Fig. 1e–f). Hence, the number of SNAREs that can participate in fusion is effectively reduced to a single SNARE per liposome. These liposomes were used to determine the effect of the SNARE density on the fusion kinetics.

A single SNARE complex is sufficient for membrane fusion

First, we employed classical cuvette-based lipid mixing (FRET) experiments to measure membrane fusion. In these experiments, each population of liposomes is labeled with Oregon-green-phosphatidylethanolamine (PE; donor fluorophore) or Texas-red-PE (acceptor fluorophore). Upon membrane fusion, these spectrally separated fluorescent lipid analogs mix and this results in a FRET signal (Fig. 3a). The effect of lowering the concentration of

the stabilized acceptor complex and of synaptobrevin was measured by the quenching of the Oregon-green donor fluorophore (Fig. 3b–d). The fluorescent crosstalk of Texas-red in the Oregon-green channel was negligible (< 1%) and therefore the quenching of the Oregon-green donor fluorescence could be immediately used as a measure of lipid mixing. We varied the p/l-ratio from 1:1,000 to 1:16,000 and lipid mixing was observed in all cases, even at the lowest SNARE density. SNAP-25 enhanced lipid mixing, although with syntaxin only (in the absence of SNAP-25) approximately 10% lipid mixing was observed compared to the acceptor SNARE complex (Fig. 3e). Membrane fusion without SNAP-25 has been previously reported^{4,20}. Importantly, in all cases, lipid mixing was SNARE specific and could be blocked by competitive inhibition using the soluble SNARE domains: both the addition of the cytoplasmic domain of synaptobrevin 2 (residues 1–96) or of a combination of SNAP-25 and the soluble SNARE domain of syntaxin 1 (residues 183–263) resulted in a complete blocking of fusion. In addition, no lipid mixing was observed with empty liposomes in the absence of either the acceptor complex or synaptobrevin.

The observed lipid mixing was not caused by the small fraction of liposomes that contained multiple SNAREs, because the increase in FRET decreased linearly to the fraction of empty liposomes that did not contain any SNARE molecules, whereas it did not scale linearly to the fractions containing multiple SNAREs (Fig. 4a). This is further confirmed by electron microscopy analysis of the fusion product, where a slight increase in size was observed upon fusion of 1:1,000 synaptobrevin to 1:16,000 acceptor complex liposomes (Fig. 4b). The increase in size is relatively small, because only 30% of the acceptor complex liposomes contained SNARE proteins and were capable of fusion and because the radius increases only 1.4-fold upon fusion. Moreover, we can exclude that lipid mixing is a consequence of liposome aggregation, because no aggregates were observable using either electron microscopy or fluorescence correlation spectroscopy¹¹. In addition, at a p/l-ratio of 1:16,000 sequential photobleaching experiments indicated that the distribution of fluorophores over the liposomes did not significantly change upon fusion. Together, these results show that a single SNARE complex is sufficient to induce lipid mixing.

We next asked whether single SNARE complexes are also sufficient to mediate fusion with a native biological membrane. In contrast to proteoliposomes, such membranes are crowded with a large variety of proteins and probably contain a complex, asymmetric lipid composition. We have shown previously that synaptic vesicles isolated from rat brain are capable of fusion with liposomes containing syntaxin and SNAP-25 in a SNARE-dependent manner²¹. Here we measured fusion of purified synaptic vesicles with liposomes containing increasing dilutions of the acceptor SNARE complex. The liposomes contained membrane lipids labeled with Oregon-green and Texas-red, and fusion was recorded by donor fluorophore dequenching. At a p/l-ratio of 1:16,000 for the acceptor SNARE complex, specific lipid mixing with synaptic vesicles was still observed (Fig. 5a–b). These results demonstrate that a single SNARE complex is sufficient to propagate lipid mixing not only of liposomes but also of complex native biological membranes.

Lipid mixing does not distinguish between fusion and hemifusion, and it is thus conceivable that with a single SNARE complex, the reaction does not progress beyond the hemifusion state. To distinguish between fusion and hemifusion, we employed a content mixing assay,

where liposomes with encapsulated calcein were fused with empty (calcein free) liposomes, resulting in calcein dequenching (Fig. 5c)^{22,23}. At both a p/l-ratio of 1:1,000 and 1:16,000 of the acceptor SNARE complex, SNARE-specific content mixing was observed (Fig. 5d). The kinetics of the calcein dequenching overlapped reasonably well with that of the lipid mixing (Fig. 3b), indicating that the progression from hemifusion to full fusion occurred faster (<10 s) than could be resolved with our time resolution. To exclude that the signal was caused by leakage of calcein from liposomes, external calcein was quenched by cobalt ions (5 μ M). Only a small amount of leakage was detectable when liposomes at a p/l-ratio of 1:1,000 were used ($4.1 \pm 1.4\%$ (s.d.)), whereas no leakage was observed during fusion of liposomes with a p/l-ratio of 1:16,000. Because full fusion was observed at p/l-ratios where the average number of correctly oriented SNARE molecules per liposome is well below one (Fig. 2), we conclude that a single SNARE complex is sufficient to mediate full membrane fusion.

No cooperativity in SNARE complex formation

If one SNARE complex is sufficient for membrane fusion, the question arises why fusion is faster at higher SNARE densities in the membrane (Fig. 3b). Since docking precedes fusion, it is conceivable that the docking rate (Fig. 5e, step A) increases with the SNARE density, causing faster fusion (step B). If this were the case, one would expect an effect of the liposome concentration on the fusion kinetics. However, when using a p/l-ratio of 1:1,000 for both the acceptor complex and synaptobrevin, no or only minor effects on the lipid mixing rate were observed when the liposome concentrations were varied almost 20-fold (Fig. 5f–g). This indicates that under these conditions, membrane fusion is slow relative to the docking of the liposomes. Consequently, this shows that the sigmoidal fusion kinetics (e.g. in Fig. 3, 5–6) are not caused by slow membrane docking, but rather by multiple steps progressing after the docking, likely the slow dissociation of the synaptobrevin 49–96 fragment^{10,11}.

Subsequently, we checked whether the correlation between the fusion rate and the SNARE density was caused by cooperativity in SNARE complex formation e.g. whether increased SNARE densities would promote the formation of *cis*-complexes. Syntaxin and synaptobrevin were labeled with Alexa-fluor 488 and Texas-red at their respective C-terminal ends. Upon complex formation, the respective C-terminal ends come in close proximity and this results in a FRET signal. FRET was measured by the emission of the Texas-red acceptor fluorophore (sensitized emission; Fig. 6a–c). The sensitized emission was used, because the initial levels of fluorescence were proportional to the concentration of acceptor complex and the increase in signal corresponds to *cis*-complex formation (Fig. 6d). No effect on the rate of complex formation was observed upon changing the p/l-ratios, indicating that there is no cooperativity in the formation of the core-complex. Moreover, this shows that docking of the liposomes proceeds faster than the fusion also at the low p/l-ratios. Together, these results indicate that higher SNARE densities lead to faster fusion via a simple stochastic scheme, where the chance of a fusion event increases due to an increased population of docked liposomes and multiple SNAREs participating in the docking events.

Discussion

Our results show that liposomes containing a single SNARE molecule are capable of propagating membrane fusion, both with their cognate partner liposomes and with purified synaptic vesicles. This is an unexpected and surprising finding, considering previous estimates have been much higher (3–15)^{6–9}. Importantly, our findings ask for a reconsideration of popular models that invoke ring-shaped arrangements of multiple SNARE complexes surrounding a central patch of membranes⁹, or models proposing fusion pores that are lined by multiple SNARE transmembrane domains⁸. If multiple SNARE complexes interact at the site of fusion, our data suggest that interaction is stochastic, *i.e.* the resulting complexes may be randomly distributed across the interaction plane, with single SNARE complexes being sufficient to cause the opening of a fusion pore. Our findings extend previous observations showing that a single complex suffices for membrane attachment^{4,5}. They are also compatible with energetic considerations suggesting that the free energy estimated to be liberated during core complex formation ($33\text{--}43 k_B T$)^{4,24–27} may suffice for membrane fusion^{24,25}. It needs to be pointed out that the activation energy needed to fuse two membranes is not known, with estimates ranging from 40 to 200 $k_B T$ ^{28,29}. A further consideration is that the average radius of the liposomes used in this study was 17 nm, which is slightly smaller than synaptic vesicles (20 nm). Larger membranes may have a higher energy barrier for fusion and may consequently require multiple SNARE complexes for fusion or alternatively use chaperones such as synaptotagmin to locally change the curvature of the membrane and facilitate fusion³⁰.

Our findings have several implications for biological fusion events. First, they provide an explanation for the fact that in many intracellular membranes undergoing frequent and rapid fusion, the density of SNAREs is surprisingly low. For instance, it has recently been shown that in recycling endosomes the density of the relevant SNAREs is more than 100-fold lower than that of the synaptic SNAREs in nerve terminals, and even if the concentration of these SNAREs is further downregulated by 90%, the residual concentrations suffice to maintain fusion at near normal rates³¹. Considering that SNARE pairing is preceded by the cooperation of tethering and docking factors that recruit the SNAREs to the prospective fusion site³², fusion mediated by single complexes can thus remain highly efficient.

On the other hand, specialized membranes such as synaptic vesicles and synaptic plasma membranes contain extraordinarily high densities of SNAREs. Synaptic vesicles contain on average 70 active synaptobrevin molecules³³, while the 25 nm fusion sites on the plasma membrane of PC12 cells contain on average 75 clustered syntaxin molecules³⁴. What are the advantages of such high SNARE densities when considering that a single complex is sufficient to execute fusion? First, SNAREs are dynamic and can diffuse through the membrane³⁵, thereby moving away from the fusion site. Therefore, a high density of SNAREs will increase the likelihood for a SNARE being present when the assembly of the fusion machinery is triggered by external signals. Second, SNAREs are known to form promiscuous *cis*-complexes in the plane of the membrane²¹ that need to be reactivated by the disassembly ATPase NSF, thus lowering the effective concentration of active SNAREs. Consequently, a large redundancy might act as a safety mechanism to ensure that there are always active SNAREs available at the fusion site irrespective of NSF activity.

In addition, it needs to be considered that neuronal docking and Ca^{2+} -dependent secretion are tightly regulated, both spatially and temporally, by a multitude of proteins. Thus the complexity is vastly increased compared to our *in vitro* system. Many of the regulatory proteins, such as Munc18, complexin and synaptotagmin, interact with the SNAREs^{1,2}. It is conceivable that binding of these proteins siphons away some of the energy of SNARE assembly, and that under these conditions one SNARE complex may not suffice anymore to bring about fusion. However, there are some indications that the number of SNARE complexes required for neuronal exocytosis is low. For instance, experiments with a temperature sensitive NSF mutant (comatose) in *Drosophila* showed that synaptic transmission in the absence of active NSF was only blocked over a slow time course, while core complexes accumulated on the synaptic vesicles³⁶. This indicates that only a small number of synaptobrevins available on the synaptic vesicle participates in the fusion event. More recently, the number of synaptobrevin molecules required for exocytosis has been directly measured using cultured hippocampal neurons from synaptobrevin knockout mice. Here, the reintroduction of only two synaptobrevin molecules per vesicle was sufficient for full recovery of stimulation-dependent synaptic vesicle fusion (Sinha, R. and Klingauf, J., submitted). In summary, we cannot exclude that under *in vivo* conditions more than one SNARE complex is needed to effect fusion and that there may be differences in the number of required SNAREs between cell types and trafficking reactions. However, in a ‘bare-bones’ minimal system, a single SNARE complex is unequivocally sufficient for membrane fusion. This observation is of fundamental importance for our understanding and calls for a re-evaluation of our current models of SNARE-dependent membrane fusion.

Methods

Protein purification and labeling

Synaptobrevin 2 C117, synaptobrevin 2 (residues 1–96), Cys-less SNAP-25, syntaxin 1 (residues 183–288), syntaxin 1 (residues 183–263) and the stabilized complex consisting of syntaxin 1A (residues 183–288) with an additional cysteine at the C-terminus (289C), Cys-less SNAP-25A, and synaptobrevin 2 (residues 49–96) (all from rat) were expressed and purified as described¹⁰, except that buffers contained 2% (w/v) CHAPS as detergent and 0.2 mM TCEP instead of DTT. For fluorescent labeling, a 2-fold molar excess of Alexa-fluor 488 C₅-maleimide (485/515 nm) or Texas-red C₂-maleimide (596/604 nm, Invitrogen) was incubated for 2 hr followed by gel-filtration. The endogenous cysteines of synaptobrevin (C103) and syntaxin (C91, C92), all located in the transmembrane helices, are inaccessible for maleimide labeling. Labeling efficiencies were determined with UV-vis spectroscopy.

Protein reconstitution in liposomes

Proteoliposomes were prepared as described³⁷ in 20 mM Hepes pH 7.4 with 150 mM KCl. Lipids were used in a 5:2:1:1 ratio of brain L- α -phosphatidylcholine, L- α -phosphatidylethanolamine (PE), L- α -phosphatidylserine, and cholesterol (Avanti). For the lipid mixing experiments, PE was substituted with 1.5% of Oregon-green 1,2-dihexadecanoyl-sn-glycero-3-PE (496/524 nm) or Texas-red 1,2-dihexadecanoyl-sn-glycero-3-PE (Invitrogen). Calcein (495/515 nm) was encapsulated as described²³. Fusion was measured on a Fluorolog (Horiba) at 20.0°C with 1 nm bin-width at a final volume of

1.2 ml. For microscopy, liposome suspensions were mixed with 3 volumes 5% low melting temperature agarose (Sigma). Synaptic vesicle protocols have been described³³.

Sequential photobleaching

Microscopy was carried out on a FV1000 laser scanning microscope with an UPlanSApo 60×1.35NA objective (Olympus) and a Chameleon-Ultra II tuneable IR laser (Coherent) running at 890 nm, 80 MHz, and 15 mW. Fluorescence was recorded with a MPD-PDM avalanche photodiode coupled to a PicoHarp-300 (Picoquant). For the sequential photobleaching, a time series of images (516×516 pixels) was recorded, with a pixel step of 200 nm and a dwell time of 2 μs. For the Chung-Kennedy filtering³⁸, we used 60 equiprobable forward moving unbiased predictors, an analysis window of 20 data points, and 20 as a weighting factor.

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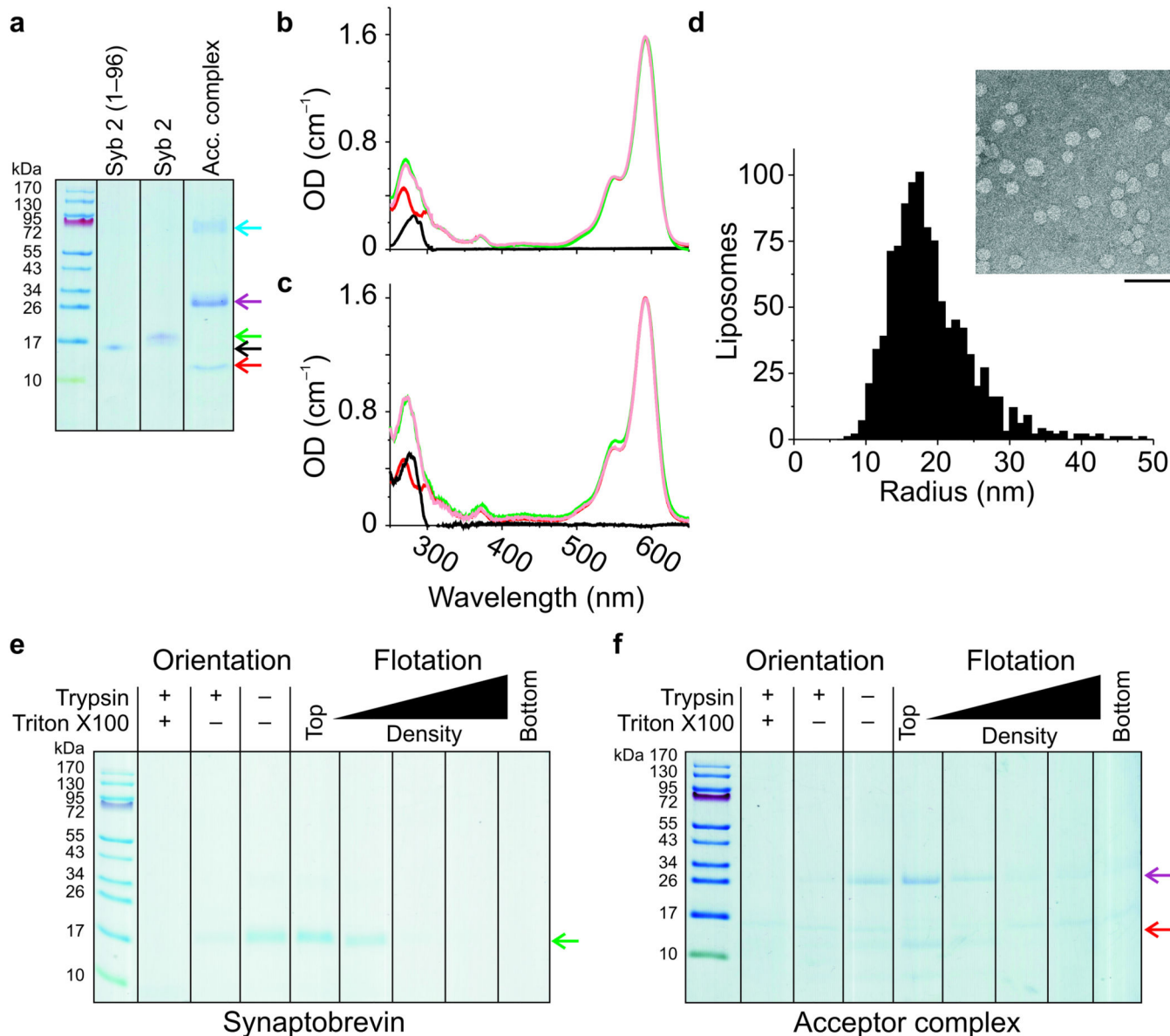


Figure 1. Characterization of the SNARE-containing liposomes. **(a)** Synaptobrevin 2 (1-96; black arrow), synaptobrevin 2 (green) and the synaptobrevin 2 (49-96) stabilized acceptor complex (blue) consisting of SNAP-25 (purple) and syntaxin 1A (183-288, 289C; red) were free of contaminants when analyzed by SDS-PAGE (Coomassie Blue staining). **(b-c)** The absorption spectrum of 18 μ M synaptobrevin (b) and the acceptor complex (c; green curves), both labeled with Texas Red. The absorption of 18 μ M unlabeled protein (black) and Texas-red (red) and the sum of both (pink) are also shown. The overlap between the pink and the green curves indicates stoichiometric labeling. **(d)** Negative staining electron microscopy (inset; scale bar 100 nm) showed that the proteins were reconstituted in monodisperse liposomes with an average radius of 17.9 ± 5.8 nm (s.d.). **(e-f)** All proteins were fully incorporated in the membrane as verified by density gradient flotation on a 30-80%

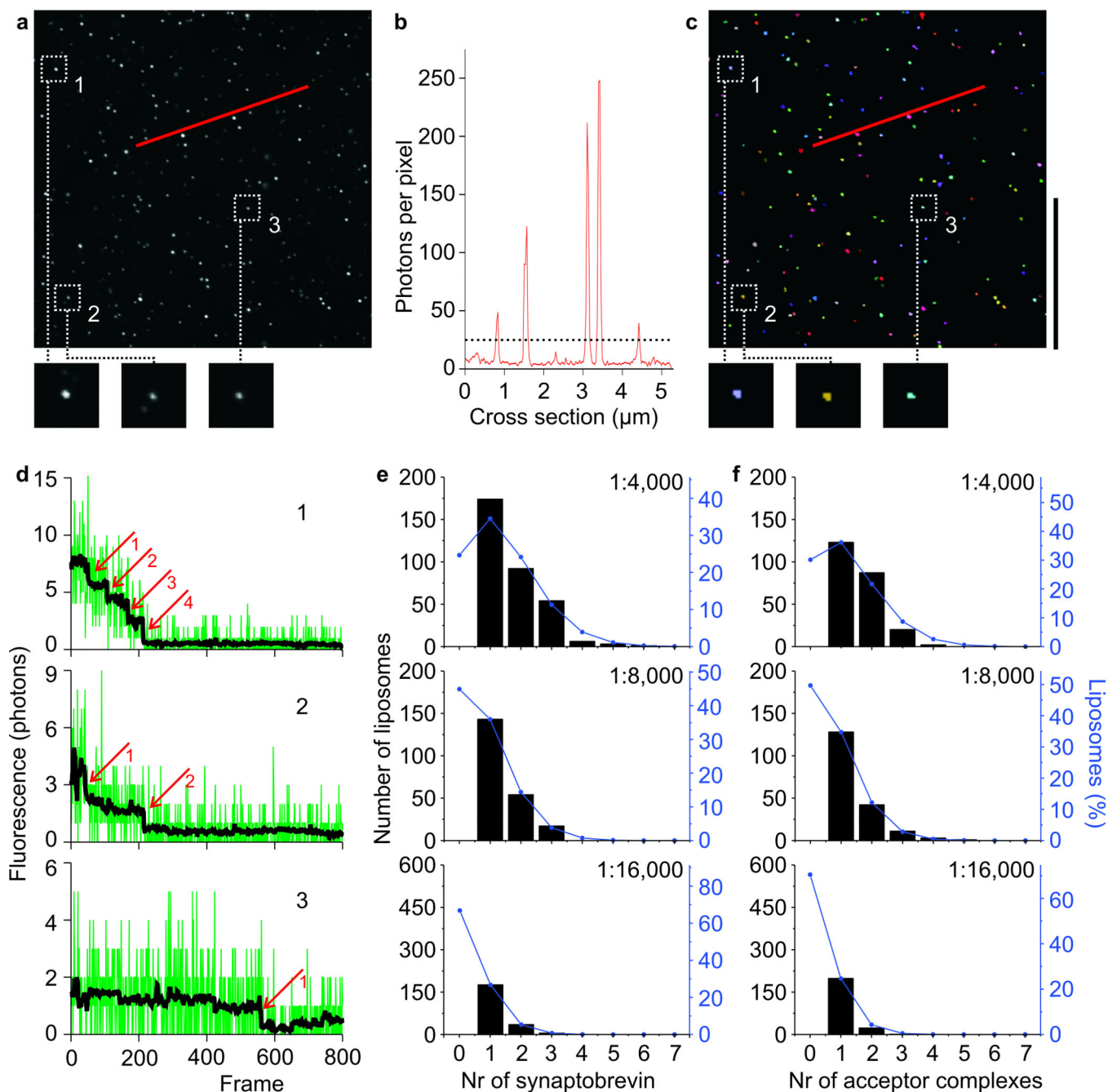
nycodenz gradient. Partial proteolysis was used to determine the orientation of the proteins in the liposomes. In the absence of detergent, only correctly oriented SNAREs (*i.e.* with their cytoplasmic domains outside) are cleaved by trypsin, whereas all proteins are proteolysed in the presence of Triton X-100. About 50% of the synaptobrevin (e) and 80% of the acceptor complex (f) were correctly oriented in the liposomes. Electron microscopy, flotation gradients, and trypsin digestion were described previously³⁷.

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**Figure 2.**

SNARE distribution over the liposomes. **(a)** Two-photon excitation microscopy image of liposomes containing Texas-red labeled synaptobrevin. **(b)** Cross-section of the image (red line in a). The liposomes were selected using an offset (dotted line) as described¹⁸. **(c)** Liposome recognition; each liposome was assigned a random colour. Scale bar, 5 µm. **(d)** Sequential photobleaching of the liposomes indicated in c (green). A Chung-Kennedy non-linear filtering technique³⁸ corrected for fluorescence intermittency (black). These liposomes contained 4, 2, and 1 synaptobrevin molecules (red arrows). **(e–f)** Distribution of synaptobrevin (e) and syntaxin (f) for the p/l-ratios indicated in the figure (bars; left axis).

Fitting the data with Poisson distributions (blue curves; right axis) indicated that the SNARE molecules were randomly distributed over the liposomes, with averages of 1.4, 0.8 and 0.4 synaptobrevin and 1.2, 0.7 and 0.35 syntaxin molecules per liposome, respectively.

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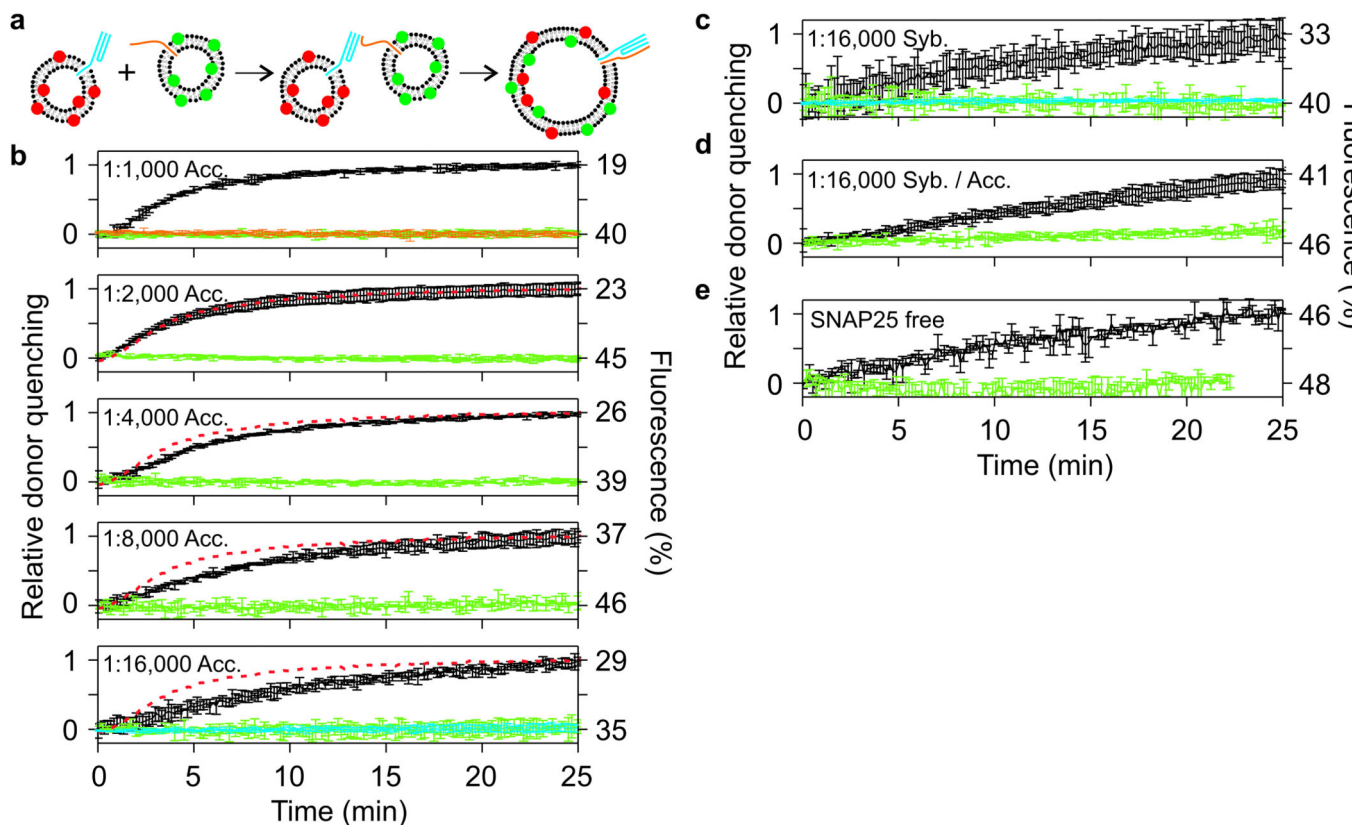


Figure 3.

Bulk lipid mixing as a function of SNARE density. **(a)** Scheme of the lipid mixing experiment where liposomes containing Texas-red-PE (red) and the acceptor SNARE complex (blue) fuse with liposomes containing Oregon-green-PE (green) and synaptobrevin (orange), resulting in quenching of the Oregon-green donor fluorophore. **(b)** Lipid mixing of 1:1,000 synaptobrevin liposomes with acceptor complex liposomes at the p/l-ratios indicated. The total lipid concentration in the cell was 50 μ M, corresponding to approximately 4 nM liposomes as determined by fluorescence correlation spectroscopy. Recordings were made at 20.0°C. **(c–d)** Fusion of 1:16,000 synaptobrevin to **(c)** 1:1,000 and **(d)** 1:16,000 acceptor complex liposomes; in all cases lipid mixing was observed. **(e)** Lipid mixing experiment of 1:1,000 synaptobrevin to 1:1,000 syntaxin 1 (residues 183–288) liposomes. In the absence of SNAP-25, 10% fusion compared to the acceptor complex (panel b) was observed. Curves are normalized (left axis); real Oregon-green fluorescence is indicated as a fraction of Triton-X100 controls (right axis). For reference, the shape of the highest concentration curve is shown in all subsequent panels (red). Lipid mixing was SNARE specific and could be inhibited with 10 μ M synaptobrevin 2 (residues 1–96; green curves) or with a combination of 10 μ M SNAP-25 and the soluble SNARE domain of syntaxin 1 (residues 183–263; orange). Moreover, liposomes containing no acceptor SNARE complex or synaptobrevin did not show membrane lipid mixing (blue curves; b and c, respectively). Error bars indicate triplicates (s.d.).

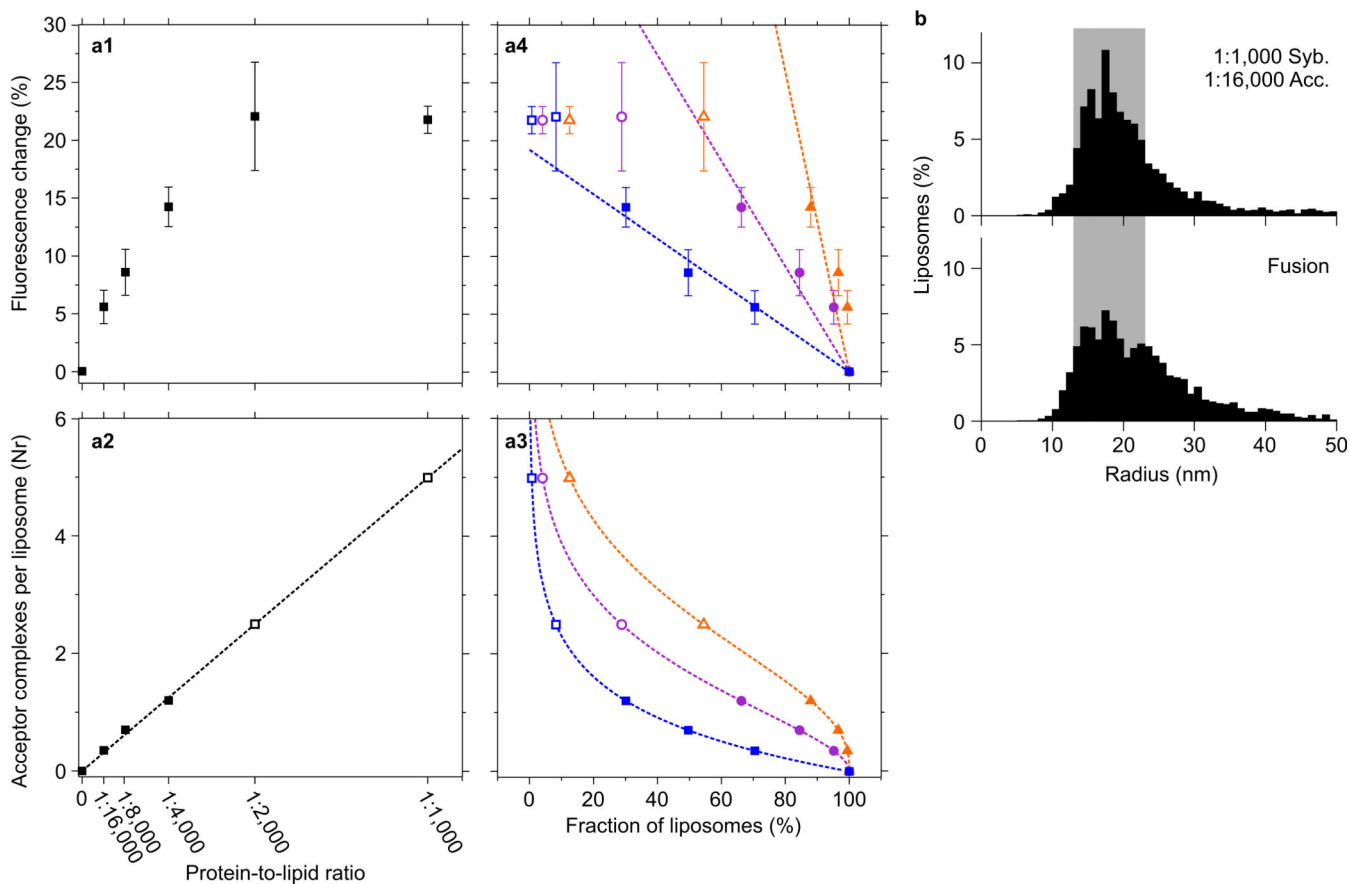


Figure 4.

Liposomes containing a single SNARE participate in membrane fusion. **(a1)** The fluorescence change after 25 min for the lipid mixing reactions of figure 3b and **(a2)** the number of acceptor complexes from figure 2f as a function of the protein-to-lipid ratio. Note that the figures are arranged counter-clockwise. **(a3)** The number of acceptor complexes relative to the fraction of liposomes containing less than 1 (blue), 2 (purple), or 3 (orange) SNAREs (data from Fig. 2f). The number of SNAREs for the 1:1,000 and 1:2,000 acceptor complex liposomes could not be determined with sequential photobleaching, because at these high protein-to-lipid ratios the error of the sequential photobleaching increases progressively. Therefore, these were estimated using Poisson distributions (a3, dotted lines) [correct? There are 3 dotted lines in a3: YES 3 dotted lines, one for each distribution (e.g. less than 1, 2, or 3)] with the averages linearly extrapolated from the 1:4,000 liposomes (a2, dotted line) [correct? There is 1 dotted line in a2: yes only 1 dotted line here]. Closed symbols indicate measured and open symbols indicate extrapolated data points. **(a4)** The fluorescence change as a function of the fraction of liposomes containing no (blue), 1 or less (purple), or 2 or less (orange) SNAREs. The data for multiple SNAREs (purple and orange) did not scale linearly (dotted lines; fits of measured data), whereas the data for empty liposomes (blue) scaled relatively well. Thus, the fluorescence change decreased linearly (dotted line) to the fraction of empty liposomes, indicating that all liposomes that contained one or more SNARE molecules participated in fusion. **(b)** Size distribution of a 1:1 ratio of 1:1,000 synaptobrevin and 1:16,000 acceptor complex liposomes determined with negative

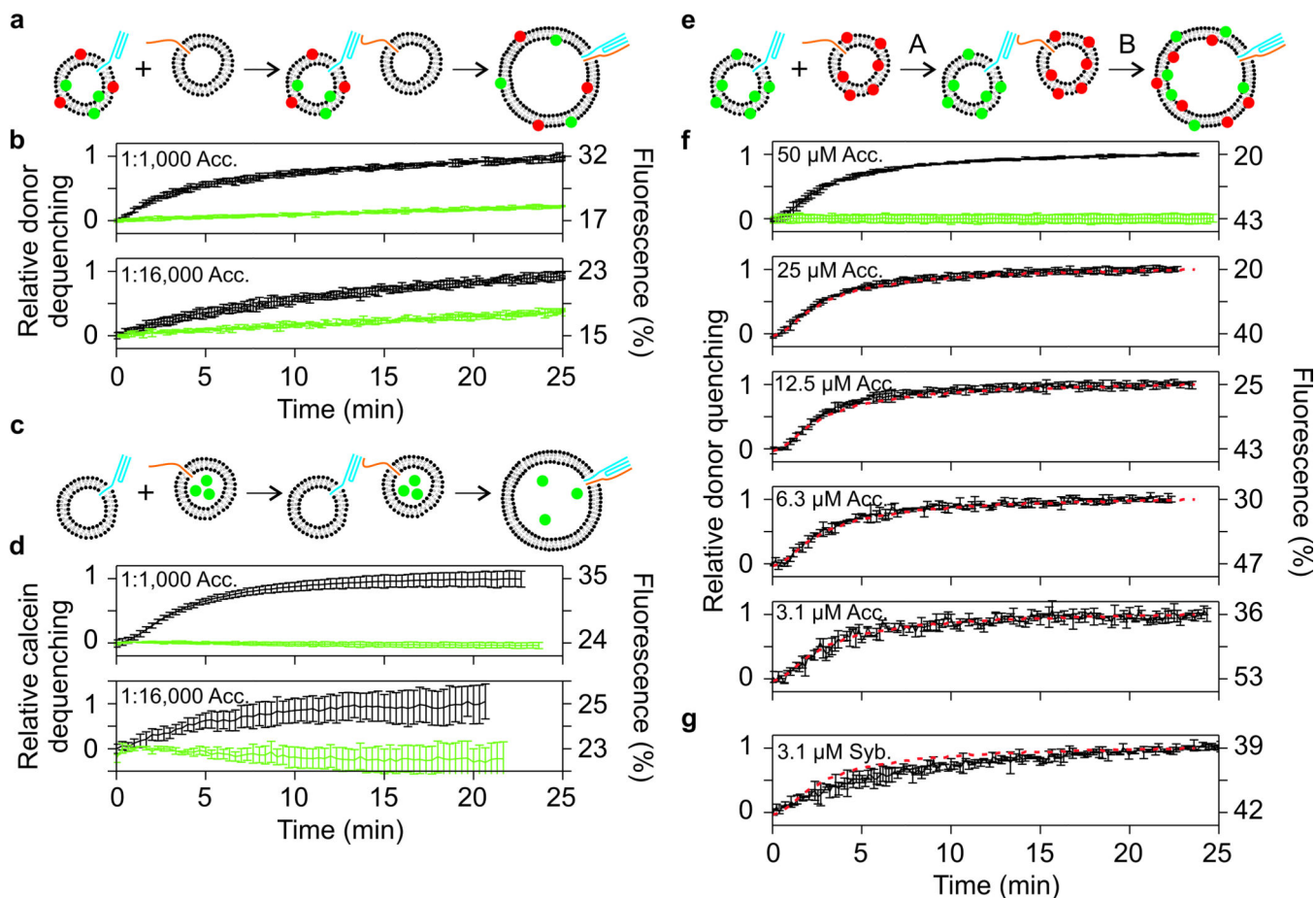
staining electron microscopy ($n = 2,887$). A slight increase in size was observed upon fusion ($n = 1,774$). The increase was only small, because the radius increases only 1.4-fold (2) upon fusion and because only 30% of the acceptor complex liposomes contained SNARE proteins (Fig. 2f).

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**Figure 5.**

Fusion with purified synaptic vesicles, content mixing, and lipid mixing as a function of liposome concentration. **(a–b)** Lipid mixing experiment, where 1:1,000 and 1:16,000 acceptor SNARE complex liposomes (50 μM total lipid) containing both Texas-red-PE and Oregon-green-PE were fused to purified synaptic vesicles (8.5 μg total protein). Fusion results in lipid mixing and dequenching of the Oregon-green donor fluorophore. The experiment indicates that a single SNARE complex is sufficient to drive fusion of native biological membranes. **(c)** Scheme of the content mixing assay based on calcein (green) fluorescence dequenching upon membrane fusion. **(d)** At p/l-ratios of 1:1,000 and 1:16,000 of the acceptor SNARE complex and 1:1,000 of synaptobrevin, content mixing was observed. The experiment shows that a single SNARE complex is sufficient not only for lipid mixing but also for content mixing. **(e–f)** Lipid mixing experiment where 50 μM (total lipid) of Texas-red-PE synaptobrevin liposomes were fused with Oregon-green-PE acceptor SNARE complex liposomes at the lipid concentrations indicated in the figure. **(g)** Fusion of 50 μM Oregon-green-PE acceptor complex to 3.2 μM Texas-red-PE synaptobrevin liposomes. These experiments indicate that the rate of membrane fusion is only weakly dependent on the liposome concentration. For reference, the shape of the highest concentration curves is shown in all subsequent panels (red). Curves are normalized (left axis); real fluorescence is indicated as a fraction of Triton-X100 controls (right axis). Fusion

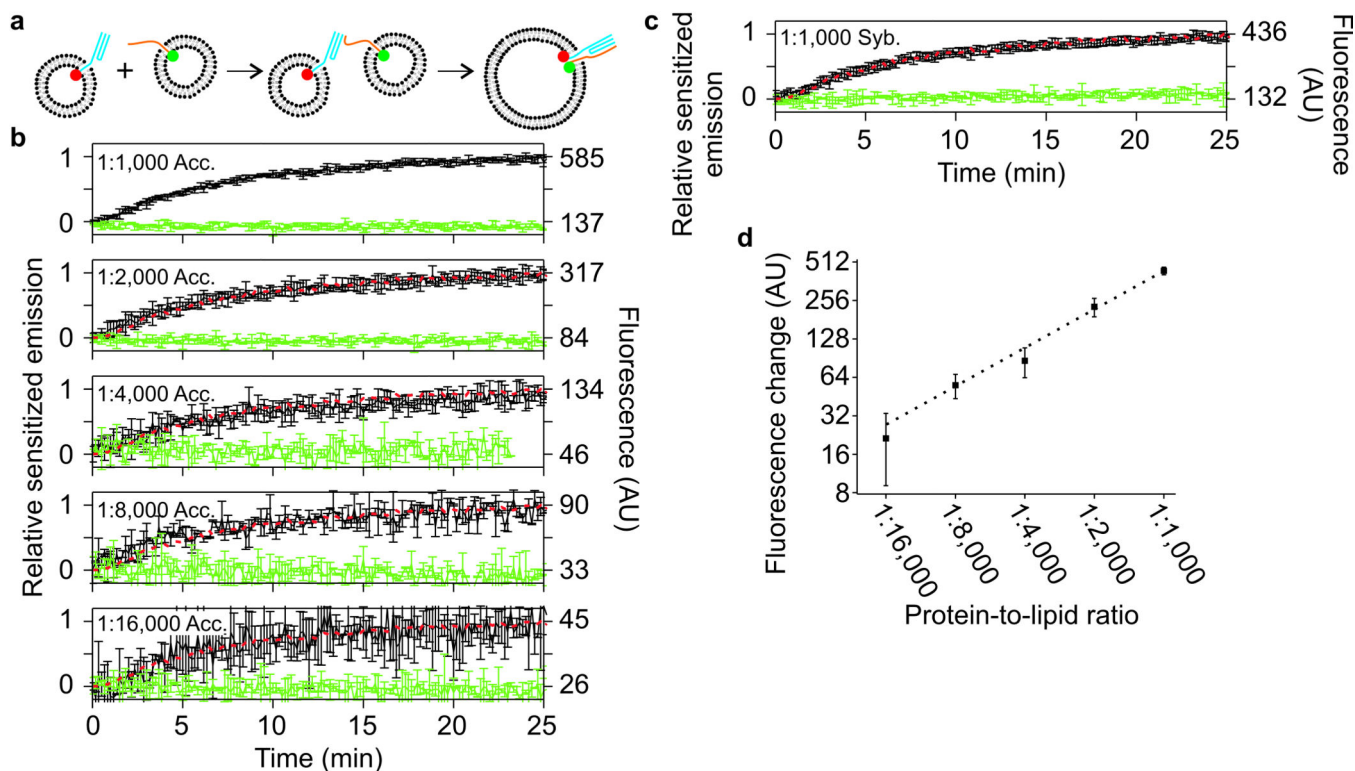
was inhibited with 10 μ M synaptobrevin 2 (residues 1–96; green curves). Error bars indicate triplicates (s.d.).

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**Figure 6.**

Formation of SNARE complexes monitored by C-terminal FRET. **(a)** Scheme of the FRET experiment to measure complex formation of Texas-red labeled syntaxin with Alexa-fluor 488 labeled synaptobrevin. **(b)** Complex formation of 1:1,000 synaptobrevin liposomes with the stabilized acceptor complex at the p/l-ratios indicated in the figure (50 μ M total lipid). Data is presented as emission of the Texas-red acceptor fluorophore (sensitized emission), so initial fluorescence is proportional to the acceptor complex levels. **(c)** Reversing the label did not influence the curves. The experiment shows that the rate of complex formation is not dependent on the SNARE density and indicates that there is no cooperativity in SNARE complex formation. Curves are normalized (left axis); real fluorescent signals of the acceptor fluorophore are indicated (right axis). Fusion was inhibited with 10 μ M 1-96 synaptobrevin (green curves). For reference, the shape of the highest concentration curves is shown in all subsequent panels (red). **(d)** The increase in the Texas-red emission after 25 min for the reactions of panel b as a function of the p/l-ratio. The linear correlation (dotted line) indicates that the total amount of core-complex formed was directly dependent on the concentration of SNAREs in the cell. Error bars indicate triplicates (s.d.).