

Supporting Information

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SI Materials and Methods

Protein Constructs and Purification. Synaptobrevin 2 (syb) and the 1:1 syntaxin 1-A:SNAP-25A acceptor complex (all from rat) stabilized by syb 49–96 (referred throughout the text as the ΔN complex) were purified and assembled as described (1, 2). An important distinction is that during the final ion-exchange purification step soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) used for the reconstitution in small liposomes were purified in CHAPS detergent (1%), whereas those destined for large liposomes were purified in the presence of *n*-octyl- β -D-glucopyranoside (OG) (34 mM; Glycon), as already noted (3).

Preparation and Reconstitution of SNAREs into Liposomes for Ensemble Lipid Mixing Measurements. Small and large SNARE liposomes used for ensemble lipid mixing experiments consisted of the same lipid composition (brain PC:PE:PS and cholesterol in 50:20:20:10 ratio; Avanti Polar Lipids) and were prepared as described in detail for small (1) and large (3) liposomes. Briefly, small SNARE liposomes were prepared according to the comicellization method using excess CHAPS as the solubilizing detergent. After removal of the detergent by gel filtration, bilayer formation and reconstitution of the SNAREs takes place in a single concerted step. For reasons that are not entirely understood but that seem to depend on both the detergent type and the lipid/detergent ratio (4), this procedure gives rise to very uniformly distributed SNARE liposomes of a mean diameter of ~ 30 – 45 nm (2, 5). For incorporation of SNAREs into large liposomes, reconstitutions occurs via the “two-step” method where preformed liposomes of ~ 100 -nm diameter prepared by extrusion are exposed to a critical amount of OG and a micellar solution of SNAREs (6). At an optimized lipid/OG ratio (see supplementary information in ref. 3), progressive removal of OG by dialysis results in efficient incorporation of SNAREs into large liposomes as confirmed by light scattering analysis.

For both small and large labeled liposomes, either the PE or PS fluorophore conjugate was used at a mole percentage of 1.5% (n/n), in which case the fraction of unlabeled PE or PS was correspondingly reduced. When preparing a set of syb liposomes with different densities to be used for leveling docking experiments, the following remarks require further note. For small liposomes, because lower concentrations of syb were added to the mixture to prepare SNARE liposomes of lower density, an equal amount of buffer containing CHAPS (1%) was added to keep the volume constant. Likewise, for large liposomes additional buffer containing an equivalent amount of OG was added as syb concentration was reduced, thus keeping the total amount of detergent constant for all preparations. The size distributions of liposomes were regularly checked by field flow fractionation coupled to multiangle laser light scattering (FFF-MALLS) (3).

Ensemble Liposome Lipid Mixing Dequenching Assay. All lipid mixing kinetics were monitored by fluorescence dequenching with the use of lipid-conjugates consisting of the Förster resonance energy transfer (FRET) pair of rhodamine-PE (RHO-PE) and nitrobenzoxadiazole-PE/PS as previously described (3). Reactions were performed at 30 °C in cuvette volumes of 1.2 mL. SNARE-liposome volumes were established based on the reference reaction (the reaction with the highest syb density) and varied between 8–12 μ L (for both labeled and unlabeled liposomes). The decisive factor was the fluorescence intensity of the labeled ΔN complex liposomes of the reference reaction, because intensity variations

(by as much as 50%) were observed depending on the preparation, but a minimum cutoff of 80×10^3 counts per second was used. Volumes for complete reaction sets were then up-scaled according to the relationship described in the text to level docking rates. For example, if 10 μ L (of each liposome) was used for syb liposomes of lipid/protein (l/p) = 400:1, 80 μ L was used for both liposomes for the reaction containing syb at l/p = 3,200:1. To keep reaction volumes approximately constant among the different reactions in a set, excess buffer from the cuvette was removed before mixing for SNARE liposomes containing lower densities. All dequenching signals were normalized to the initial fluorescence F_0 . Controls for all sets of experiments were performed with soluble syb (1–96, 2–4 μ M) confirming the SNARE specificity of the reactions (not shown).

Quantification of Syb and Lipid Phosphate in Liposomes. Syb concentration on small and large liposomes was quantified by Western blot analysis [mouse monoclonal 69.1, ascites (7)] and immunofluorescence using an FLA-5000 imaging system (FujiFilm). Recombinant syb of known concentration was used to generate a calibration curve, making sure the signal from liposome samples was within the linear range of the curve. Phosphate lipid content was quantified according to ref. 8 with error bars representing SDs from triplicates. Graphs in Figs. 2B and 3B are representative of three independent preparations.

SNARE Reconstitution into Liposomes for Single Vesicle Docking and Fusion Assay. Large SNARE liposomes were prepared as described above with changes to the lipid composition as described for small liposomes below. Small SNARE liposomes containing ΔN complex or syb were reconstituted by rapid dilution of micellar protein/lipid/detergent mixtures followed by dialysis as described (9, 10). Briefly, lipids from brain PC:PE:PS and cholesterol (Avanti Polar Lipids) in a ratio of 55:20:5:20 were mixed, respectively, and organic solvents evaporated under a stream of N_2 gas and placed in a vacuum desiccator for at least 1 h. In the case of syb liposomes, 1% (n/n) of RHO-PE was added instead of brain PE. The lipid films were dissolved in buffer containing 25 mM sodium cholate, 20 mM Hepes, and 150 mM KCl, pH 7.4. An appropriate amount of ΔN complex was added to obtain a final l/p ratio of 1,000:1 for ΔN complex liposomes or the desired ratios for each syb experiment (this solution was ~ 180 μ L in volume). After 1 h of equilibration at room temperature, the concentration of sodium cholate detergent was diluted in 20 mM Hepes, 150 mM KCl, and pH 7.4 buffer to a final volume of 550 μ L. The sample was then dialyzed at 4 °C overnight against 500 mL of the same buffer with one exchange after 4 h.

Preparation of ΔN Complex in Planar Supported Lipid Bilayers. The planar supported bilayer was prepared using the Langmuir–Blodgett/liposome fusion technique as previously described (9–11). Quartz slides were cleaned in a vigorous Contrad detergent boil for 10 min followed by bath sonication in the same heated detergent solution for 30 min. Slides were then extensively rinsed with milliQ water. Before use slides were then placed in an argon plasma sterilizer (Harrick Scientific). The first monolayer was prepared by a Langmuir–Blodgett transfer. This was done by spreading a lipid monolayer from a chloroform solution onto a pure water surface in a Nima 611 Langmuir–Blodgett trough (Nima). The solvent was allowed to evaporate for 10 min and the monolayer compressed at a rate of 10 cm^2/min until a surface pressure of 32 mN/m was reached. After a 5-min equilibration

period, a clean quartz slide was dipped at a rate of 100 mm/min into the trough and then removed at a rate of 5 mm/min while at a constant surface pressure of 32 mN/m. Monolayers were stored in a desiccator for no more than ~24 h before use. The outer leaflet of the planar supported bilayer was then formed by incubating ΔN complex liposomes (77 μM total lipid) with monolayers in a holding cell for 2 h at room temperature. Excess unfused liposomes were removed by gentle flushing with 20 mM Hepes, 150 mM KCl, and pH 7.4 buffer. This method forms a fluid lipid bilayer with correctly oriented and mobile ΔN complex as previously characterized (9, 10).

Membrane Docking and Fusion by Total Internal Reflection Fluorescence Microscopy. Total internal reflection fluorescence (TIRF) microscopy experiments were carried out on two fluorescence microscopes as described (12) and fusion monitored as previously reported (9, 12–14). Supported bilayers containing ΔN complex were perfused with 3 mL of syb liposomes (total lipid ~0.8 μM) labeled with RHO-PE. Images were acquired every 50.3 ms with exposure times of 50 ms for 4.2 min (5,000 images) and spooled directly to the hard drive. Two series were collected for each supported membrane preparation. Fusion data were analyzed as described in ref. 12 and were defined as the complete decrease in fluorescence, indicating diffusion of the RHO-PE dye. For measuring liposome docking, supported bilayers containing ΔN complex were perfused with 3 mL of syb liposomes (total lipid ~5 μM) of a defined SNARE density. The depicted time is from start of image acquisition which has a 30-s delay from mixing owing to injection of syb liposomes into the TIRF chamber. Docking was monitored by an increase in the average fluorescence per pixel (referred to as a count) within the field of view (127×127 pixels²). The initial rates of docking were determined by fits of first-order kinetics to the docking curves or, if docking rate was slow, by linear fits to the first 500 s of the docking curves.

Formal Derivation of the Principle of Leveled Docking Rates. The idea behind this principle is to level docking rates across a series of SNARE densities by increasing the liposome concentrations as the densities are reduced. To find the proportionality relation between liposome concentration and SNARE density, we inspect the rate equation (based on the law of mass action) describing the generation of docking:

$$\frac{d[D]}{dt} = k_d(\text{syb}, \Delta N) [L_{\text{syb}}] [L_{\Delta N}]. \quad [\text{S1}]$$

We define a new rate constant $k'_d(\text{syb}, \Delta N)$, which is altered by a change in SNARE density on one of the SNARE-liposomes by n , where n is any real number greater than zero. We relate this new rate constant to the reference rate constant $k_d(\text{syb}, \Delta N)$ by

$$k_d(\text{syb}, \Delta N) = n \cdot k'_d(\text{syb}, \Delta N). \quad [\text{S2}]$$

If liposome concentrations remain constant, the new docking rate will change by a factor of n according to the rate equation

$$\frac{d[D]}{dt} = n \cdot k'_d(\text{syb}, \Delta N) [L_{\text{syb}}] [L_{\Delta N}]. \quad [\text{S3}]$$

The goal is to find a way to prevent the change in the docking rate as a result of variations in the docking efficiency [i.e., changes in $k_d(\text{syb}, \Delta N)$]. According to Eqs. S1 and S3, this can be done by altering the concentration of one or both sets of liposomes L_{syb} and $L_{\Delta N}$ in a way that counteracts the change in $k_d(\text{syb}, \Delta N)$. To test this formally, we introduce the following variable substitutions so that both liposome concentrations (and correspondingly the

concentration of docked liposomes) are changed simultaneously by a factor of $1/n$:

$$[L_{\text{syb}}] = [L'_{\text{syb}}]/n \quad [\text{S4}]$$

$$[L_{\Delta N}] = [L'_{\Delta N}]/n \quad [\text{S5}]$$

$$[D] = [D']/n. \quad [\text{S6}]$$

Combining and rearranging Eqs. S3–S6 the following rate equation is obtained:

$$\frac{d[D']}{dt} = k'_d(\text{syb}, \Delta N) [L'_{\text{syb}}] [L'_{\Delta N}] = \frac{d[D]}{dt} / n. \quad [\text{S7}]$$

Although the original and new docking rates differ by a factor $1/n$, Eq. S7 has the exact form of that of the reference rate Eq. S1. In relative terms (i.e., normalized to $1/n$), it can be seen that a loss (or gain) in docking efficiency can be counter balanced by proportionally increasing (or decreasing) the concentration of both liposomes. Therefore, all lipid mixing traces were normalized to their initial fluorescence value F_0 to allow quantitative comparison of the different density reactions.

Calculation of Average Number of Sybs on a Liposome. We took average diameters from representative small and large liposome samples previously determined by field-flow-fractionation coupled to multiangle laser light scattering (FFF-MALLS) (3). These were 40 and 90 nm for small and large liposomes, respectively, and were used to estimate the number of sybs per liposome in Fig. 4B. To calculate the number of lipids per liposome, we used the formula

$$\frac{V_{\text{outer}} - V_{\text{inner}}}{L_V} = \frac{\frac{4}{3}\pi(r^3 - (r-b)^3)}{L_V}, \quad [\text{S8}]$$

where V_{outer} is the spherical volume defined by the outer radius of the liposome, V_{inner} is the spherical volume defined by the inner radius, r is the liposome radii, b is the bilayer thickness, and L_V is the molecular volume of a lipid. Unless stated otherwise, we assumed a membrane bilayer thickness of 4 nm and used a molecular volume of a lipid of 1.25 nm³, which is close to the volume of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine in a membrane containing 10% cholesterol at 30 °C (15). With the number of total lipids and the corrected number of sybs per lipid from Fig. 4A, we can determine the average number of sybs per liposome via the following calculation:

$$\text{syb/liposome} = \text{syb/lipid} \times \frac{\frac{4}{3}\pi(r^3 - (r-b)^3)}{L_V}. \quad [\text{S9}]$$

Geometric Model for Calculating Number of SNARE Complexes Simultaneously Forming at the Docking Interface. For the development of a geometric model to estimate the number of SNARE complexes at the docking interface, we considered the situation when the first SNARE complex nucleates at the N terminus, allowing docking between two membranes. We asked how many additional SNARE complexes would be readily available for SNARE complex formation in the vicinity of the first SNARE complex formed. Because in our experiments ΔN complex is in excess, we make the assumption that any syb within this vicinity will readily form a complex. The determining factor of whether neighboring sybs can engage in complex assembly is h , the minimum distance between opposing membranes needed for the N termini of syb and ΔN complex to be able to physically interact. Using surface force measurements, this has been determined to be ~8 nm (16).

To define the liposome area that contains all additional sybs able to form a SNARE complex, we first consider the situation of a sphere of radius r as it approaches a wall, so that its center is $r + d$ units of length from the wall (Fig. S5A). We want to know the surface area, S_{h_s} , of the sphere that is less than h units away from the wall. We consider the surface area S of the entire sphere in polar coordinates:

$$S = 4\pi r^2 = \int_0^{2\pi} \int_0^{\pi} r^2 \sin\phi d\phi d\theta, \quad [\text{S10}]$$

where θ belongs to the range $[0, 2\pi]$ and ϕ belongs to the range $[0, \pi]$. The surface area $S_{h_{\text{wall}}}$ is then given by

$$S_{h_{\text{wall}}} = 2\pi r^2 \int_0^{\phi_h} \sin\phi d\phi. \quad [\text{S11}]$$

From the geometry shown in Fig. S5A, the angle ϕ_h delimited by h is given by

$$\phi_h = \cos^{-1} \frac{r - h + d}{r}. \quad [\text{S12}]$$

Substituting and rearranging Eqs. S11 and S12 gives

$$S_{h_{\text{wall}}} = 2\pi r^2 \left[\cos \left(\cos^{-1} \frac{r - h + d}{r} \right) - 1 \right] = 2\pi r^2 \frac{(h - d)}{r} = 2\pi r(h - d). \quad [\text{S13}]$$

To model the fusion between two liposomes, we used the geometry portrayed in Fig. S5B to derive the expression for ϕ_h . In this case where the radii of two spheres of radius r are separated by $2r + d$ units, ϕ_h is given by

$$\phi_h = \cos^{-1} \frac{r - h/2 + d/2}{r}. \quad [\text{S14}]$$

Substituting this expression into Eq. S11 then gives the surface area $S_{h_{\text{sphere}}}$:

$$S_{h_{\text{sphere}}} = 2\pi r^2 \frac{(h - d)}{2r} = \pi r(h - d). \quad [\text{S15}]$$

The number of additional sybs confined within the area delimited by h (syb_h) that are readily within reach for SNARE complex formation is then given by

$$\text{syb}_h = (\text{syb}_{\text{total}} - 1) \times S_{h_{\text{sphere}}} / S = (\text{syb}_{\text{total}} - 1) \times \frac{(h - d)}{4r}, \quad [\text{S16}]$$

where $\text{syb}_{\text{total}}$ is the total number of sybs on a liposome as estimated according to Eq. S9. Because in our model one syb is already engaged in SNARE complex assembly, which mediates the docking, the number of sybs that would be required for efficient lipid mixing assuming a cooperative mechanism is $\text{syb}_h + 1$.

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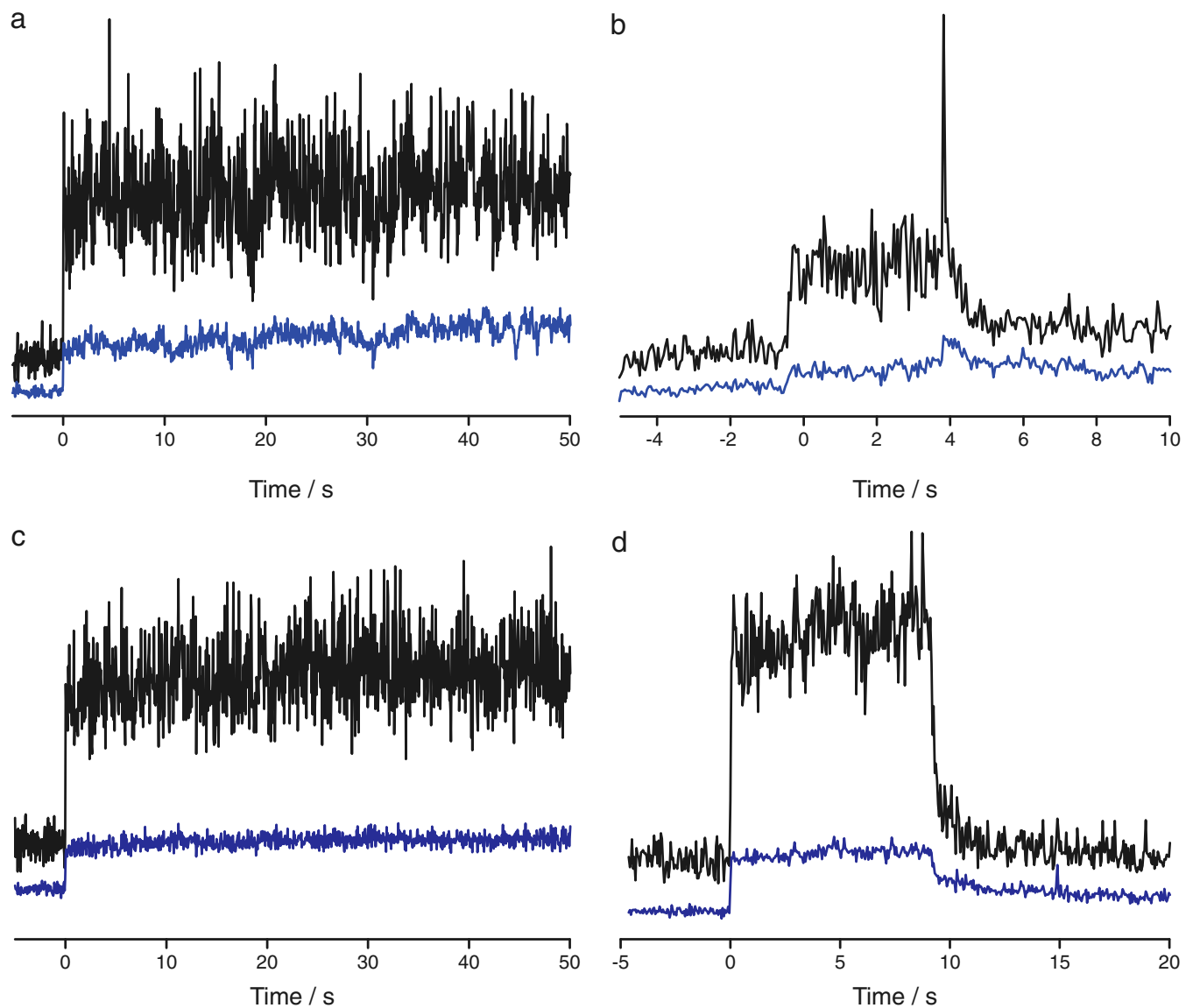


Fig. S1. Examples of single docking and fusion events of small and large syb liposomes ($l/p = 300:1$) to a planar supported bilayer containing ΔN complex ($l/p = 1,000:1$) using TIRF microscopy. Peak fluorescence (black) and mean fluorescence of a small region of interest (blue) of single docking/fusion events of small liposomes fusing with supported membranes. Docking occurs at time point zero and is characterized by a sharp increase of the observed fluorescence. (A) Docking event without subsequent fusion of a small liposome. (B) Single fusion event of a small liposome. The onset of fusion ~ 4 s after docking is characterized by a sudden increase of the intensity owing to the orientation change of the fluorophores and the s-polarization of the laser used in this experiment (14). (C) Docking event without subsequent fusion of a large liposome. (D) Single fusion event of a large liposome. The onset of fusion ~ 10 s after docking is characterized by a sudden decrease of the intensity owing to the orientation change of the fluorophores and the p-polarization of the laser used in this experiment (14).

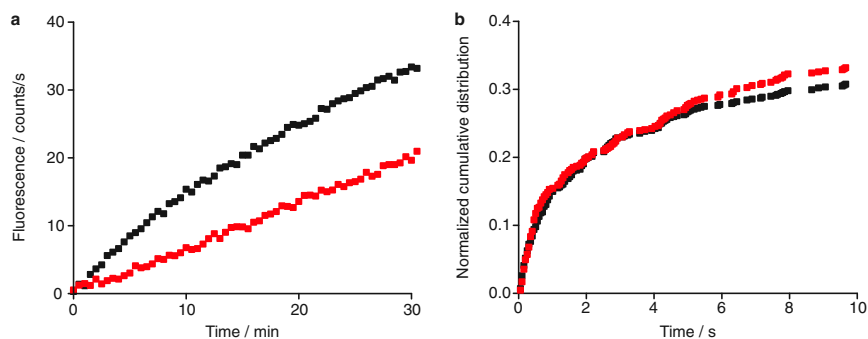


Fig. 52. Single liposome fusion events measured by TIRF microscopy confirm no effect on fusion when the syb density is halved. (A) Docking kinetics of a small syb liposome with $l/p = 300:1$ (black) and $l/p = 600:1$ (red) to a planar supported bilayer reconstituted with ΔN complex ($l/p = 1,000:1$) determined by total fluorescence using TIRF microscopy. As expected, the docking rate is reduced at a lower l/p ratio. (B) Comparative cumulative distributions of single liposome fusion events to a planar supported bilayer using TIRF of the same small liposomes in A. The kinetics of fusion are essentially the same.

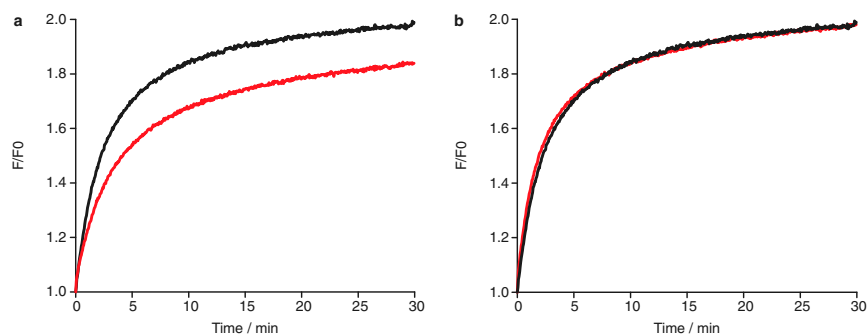


Fig. 53. Halving the density of ΔN complex has no effect on fusion. (A) Small FRET-labeled liposomes containing syb ($l/p = 200:1$) were mixed to ΔN complex-containing unlabeled liposomes with $l/p = 500:1$ (black) and $1,000:1$ (red) in the conventional way. (B) When liposome concentration was doubled for ΔN complex at $l/p = 1,000:1$, lipid mixing recovered to the reference trace. Thus, leveled docking reveals there is no effect on fusion when ΔN complex density is reduced to $l/p = 1,000:1$.

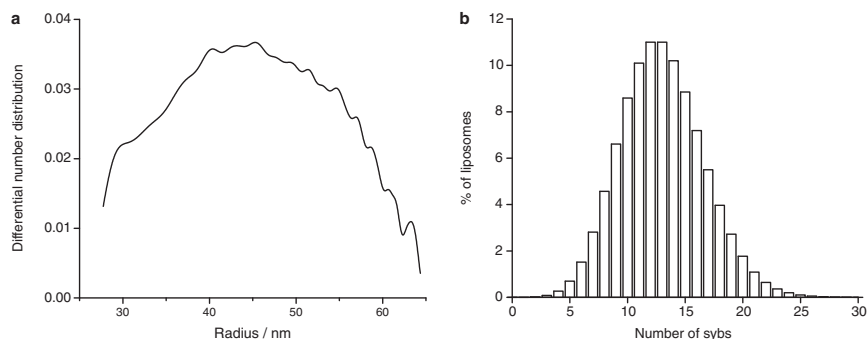


Fig. 54. The population of large liposomes without any sybs is negligible at the lipid mixing efficiency threshold and cannot explain the drop in lipid mixing on large liposomes. (A) Differential number distribution of a representative large syb liposome at $l/p = 400:1$ obtained by FFF-MALLS. (B) To evaluate whether a population of large liposomes containing no sybs could explain the lipid mixing decline on large liposomes, we assumed sybs are distributed randomly and calculated a hypothetical Poisson distribution of the number of sybs per liposome using the R-statistical software package (www.r-project.org). To be conservative, we used the corrected syb/lipid ratio at the lower bound threshold (3.1×10^{-4} , blue dashed line in Fig. 4A) and assumed sybs were reconstituted in 60-nm-diameter liposomes, which is at the lower end of the size distribution in A. Assuming a bilayer thickness of 4 nm, the average number of sybs per liposome is 13 (Eq. S9). Even in this conservative scenario there are virtually no liposomes without sybs.

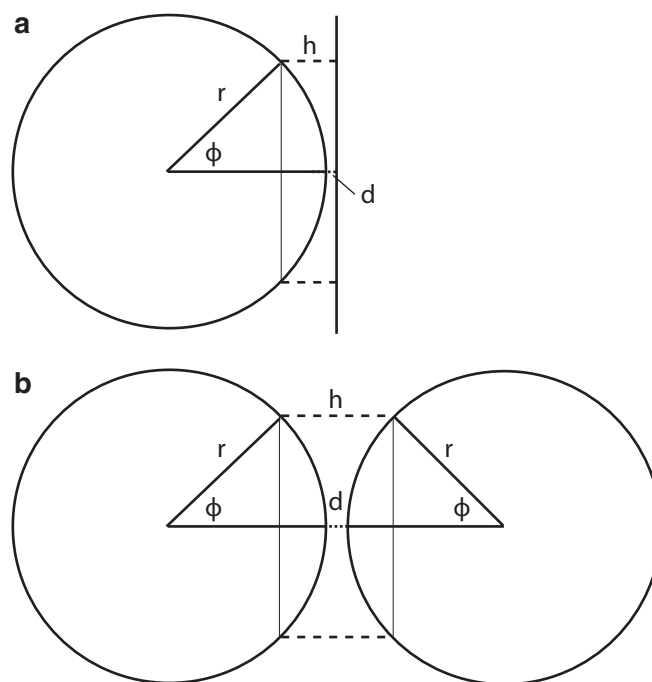


Fig. S5. Geometric model for calculating the surface area of the syb liposomes that is delimited by the maximum distance of the membranes by which SNARE partners can interact. (A) Geometry between a sphere and a wall and (B) between two spheres of equal radii r whose surfaces are separated by a distance d . We designed the liposome fusion assay so that the ΔN complex is present in excess and covers a large portion of the surface. The estimated number of SNARE complexes that can potentially form in this model is based on the assumption that, as the distance between the membranes d approaches zero, all sybs in the area confined by the parameters h , r , and ϕ will initiate SNARE complex assembly. However, the model is limited by dynamic and steric effects of the SNAREs themselves (which would decrease this estimate) or lateral diffusion (which would increase the estimated number), and as such should be treated as only a rough approximation (see also *SI Materials and Methods*).

Table S1. Estimation of number of SNARE complexes that would readily form at the docking interface delimited by the maximum distance over which SNAREs can interact

Scenario	Sybs per lipid at efficiency threshold (from Fig. 4A)	Average radius, nm	Bilayer thickness, nm	No. of sybs per liposome	Maximum distance SNARE interaction h , nm	No. of sybs at fusion site ($\text{syb}_h + 1$)
Lower boundary	3.1×10^{-4}	40	4	18.2	5	1.5
Average	3.6×10^{-4}	45	4	26.8	8	2.2
Upper boundary	4.2×10^{-4}	50	5	47.7	12	3.8

We used Eqs. S9 and S16 to evaluate how many sybs would form *trans* SNARE complexes in a local and concerted fashion under three distinct scenarios. For lower- and upper-bound scenarios, we used the syb/lipid lipid mixing thresholds from the lower and upper limits of the fitting (dashed lines in Fig. 4A) and the average fitting for the average scenario (blue solid line in Fig. 4A). In addition, we tested different model parameters appropriate for each scenario that include the radius r , the bilayer thickness b , and the maximum distance of SNARE interaction h to evaluate the variability of the output of our model. Based on this analysis, we estimate that between two and four sybs would form SNARE complexes assuming a localized and concerted cooperative mechanism. Therefore, between two and four SNARE complexes would be required for efficient lipid mixing.