Supporting Methods

Preparation of Biotinylated Phosphatidylethanolamine

Biotinylated phosphatidylethanolamine was synthesized from brain-phosphatidylethanolamine (PE, Avanti Polar Lipids, Alabaster, AL) and 6-((6-((biotinoyl) amino)hexanoyl)amino) hexanoic acid succinimidyl ester (biotin-X-X-SE, Molecular Probes). Biotin-X-X-SE (10 μmol) was mixed with 5 μmol of phycoerythrin in 1 ml of tetrahydrofurane (THF) /methanol (2:1 vol/vol) and 300 μl of 2,5-diaminopyridine (100 mM in THF) were added. After 15 min, the solvent was removed and the reaction mixture was redissolved in 1 ml of acetic acid methyl ester/methanol (3:1 vol/vol). The product was purified by column chromatography on a 12 × 0.5 cm column filled with silica gel 60 equilibrated in this solvent mixture. Fractions were taken and controlled by thin layer chromatography. Fractions containing the reaction product were pooled, and solvent was removed. The product was redissolved in HB100 containing 5% sodium cholate to yield a 1 mM solution.

Determination of the Diameter Distribution of Liposomes

For diameter analysis, ≈1,000 individual liposomes were analyzed for each sample by using the images obtained from cryo-electron microscopy. Because the shape of the liposome in all samples deviated from the classical circular sphere probably due to the presence of reconstituted proteins in the bilayer, both the short and the long diameter of the liposomes was determined and statistical distribution of the mean liposome diameter for all four samples was calculated.

Determination of the Diffusion Constants and Concentration of Vesicles by FCS

The diffusion constants D(Syx) and D(Syb) are quantified for vesicles containing syxH3 and synaptobrevin labeled with Alexa488. The evaluation is based on fluorescence

correlation spectroscopy (FCS), using a Multiparameter Fluorescence Detection (MFD) setup (1).

The fluorescently labeled sample is excited in a confocal microscope (Olympus, objective UPlanApo, ×60, 1.2 numerical aperture, pinhole diameter 100 μm) by an actively mode-locked argon-ion laser operated at 496.5 nm (pulse width 190 ps, repetition rate 73.6 MHz, mean excitation intensity within the detection volume 6.8 kW/cm^2) (Sabre, Coherent, Palo Alto, CA; APE, Berlin). A dual-band dichroic mirror separates the excitation wavelength from the fluorescence signal (488/636PC, AHF Analysentechnik, Tübingen, Germany). The fluorescence signal is divided into its parallel and perpendicular component with respect to the linearly polarized excitation beam by a polarizing beam splitter cube (VISHT11 Gsänger, Planegg, Germany). Subsequently, band pass filters (HQ535/50) discriminate the collected fluorescence from scattered laser light and from Raman scattered light from the solvent sample. Two avalanche photodiodes (AQR 14, EG&G Vandreuil, Quebec) are used for the detection of the fluorescence signal. The output pulses are processed online by a PC-based correlator board with a time resolution of 12.5 ns (ALV5000 with fast option, ALV GmbH, Langen, Germany).

For the evaluation of the collected data, the two detected fluorescence signals are cross-correlated to avoid artefacts due to detector dead times and after pulsing (2). The experimental data curves $G(t_c)$ can be fitted by using the following model function, which takes into account the three dimensional Gaussian diffusion of the molecules and the population of a triplett fraction:

$$G(t_c) = 1 + \frac{1}{N} \cdot \frac{1}{1 + \frac{t_c}{t_D}} \cdot \frac{1}{\sqrt{\left(1 + \frac{t_c}{(z_0/\omega_0)^2 \cdot t_D}\right)}} \cdot \left(1 - T_{eq} + T_{eq} \cdot \exp(-t_c / t_T)\right)$$

t_c denotes the correlation time of the correlation function $G(t_c)$. The amplitude of the diffusion part, $1/N$, displays the inverse average number of fluorescent particles in the

detection volume which is given by $V_D = \pi^{3/2}\ \omega_0^2\ z_0$. From this parameter, the concentration of fluorescent particles can be calculated. ω_0 and z_0 signify the $1/e^2$ distances in the radial and axial dimensions of the detection volume, respectively. t_D is the characteristic time for translational diffusion, t_T the characteristic time for the triplett population. T_{eq} denotes the mean fraction of fluorophors populating the triplett state.

The calibration of the experimental setup is done with the known diffusion constant of Rhodamine 6G, $D(Rh6G) = 2.8 \times 10^{-6}$ cm^2/s (3), resulting in a translational diffusion time $t_D = 0.26$ ms for our setup. This yields calculated values of the axial and radial focus of $z_0 = 312.5 \times 10^{-6}$ cm and $\omega_0 = 53.9 \times 10^{-6}$ cm, respectively, and corresponds to a detection volume of ~2 fl. FCS measurements provide the translational diffusion times $t_D(syx/syb)$ of our samples, the diffusion constant is then calculated by $D(Syx/Syb) = \frac{\omega_0^2}{4\, t_D(x/y)}$.

Calculation of the Collision Frequency Between Liposomes Using a Simple Diffusion Model.

Spherical donor and acceptor particles diffuse in a solution. Upon collision of a donor with an acceptor particle, the particles either combine to a new particle type (fusion), or they do not react with each other (elastic collision). Fused particles do not react anymore, i.e., multiple rounds of fusion are excluded.

Given are

D = diffusion coefficient (equal for donor and acceptor particles)

R = particle radius

$K_D(0)$ = concentration (number of particles N in a given volume V) of donor particles at the begin of the reaction ($t = 0$)

$K_A(0)$ = concentration of acceptor particles at the begin of the reaction ($t = 0$)

The concentration of fused particles at time t ($\boldsymbol{K_{fus}(t)}$) is being calculated.

For a fixed sphere with radius r and infinitely small particles that are randomly distributed at a concentration k at time $t = 0$ and that exhibit diffusional coefficient D, the number or particles colliding with the sphere during the time interval $[t,t+\Delta t]$ is given by (4):

[1] $$4\pi \cdot D \cdot r \cdot k \cdot (1 + r / \sqrt{\pi D t}) \cdot \Delta t$$

For large t, the term $r / \sqrt{\pi D t}$ can be neglected.

When this formula is applied to a diffusing donor particle instead to a fixed sphere, the diffusional coefficient must be multiplied by 2. The critical radius at which an interaction takes place is $2R$. The number of acceptor particles interacting with the given donor particle during the time interval $(t,t+\Delta t)$ is given by:

[2] $$16\pi \cdot D \cdot R \cdot K_A(t) \cdot \Delta t$$

Applying Smoluchoswki's formula (4) does not take into account that due to the movement of the donor particle the relative movements of the acceptor particles become correlated, and thus the assumption of independent movements is not entirely accurate, resulting in a slight overestimation of the number of interactions. Effects caused by the fact that the acceptor particles have finite dimensions can be neglected because of the low fractional volume occupied by the particles.

With $c = 16\pi \cdot D \cdot R$ as constant, the number of collisions per volume unit in a time interval $(t,t+\Delta t)$ is given by:

[3] $$c \cdot K_A(t) \cdot K_D(t) \cdot \Delta t \,.$$

If p denotes the probability by which a collision results in fusion, the concentration of active particles is reduced:

[4] $$K_A(t+\Delta t) - K_A(t) = K_D(t+\Delta t) - K_D(t) = -c \cdot p \cdot K_D(t) \cdot K_A(t) \cdot \Delta t$$

This results in the following differential equations:

[5] $$\mathrm{K}_A'(t) = -c \cdot p \cdot K_A(t) \cdot K_D(t)$$

$$\mathrm{K}_D'(t) = -c \cdot p \cdot K_A(t) \cdot K_D(t)$$

If the concentration of donor and acceptor particles is identical at the beginning of the reaction ($K_A(0) = K_D(0)$), then $K_A(t) = K_D(t) = K(t)$, the differential equations simplify to:

[6] $$\mathrm{K}'(t) = -c \cdot p \cdot K(t)^2$$

This equation is solved by:

[7] $$K(t) = \frac{K(0)}{K(0) \cdot c \cdot p \cdot t \; + \; 1}$$

and the concentration of fused particles is

[8] $$K_{fus}(t) = K(0) - K(t) = \frac{K(0)^2 \cdot c \cdot p \cdot t}{K(0) \cdot c \cdot p \cdot t \; + \; 1}$$

thus, the fusion probability p, for $t \neq 0$, can be calculated as

[9] $$p = \frac{1}{K(0)\cdot c\cdot t}\left[\frac{K_{fus}(t)/K(0)}{1-K_{fus}(t)/K(0)}\right]$$

If the starting concentration are not identical

$$K_{diff} = (K_A(0) - K_D(0)) \neq 0$$

Eq. **5** can be written as:

[10] $$K_A'(t) = -c\cdot p\cdot K_A(t)^2 + K_{diff}\cdot c\cdot p\cdot K_A(t)$$

which is solved by the function

[11] $$K_A(t) = ((K_A(0)^{-1} - K_{diff}^{-1})e^{-cpK_{diff}t} + K_{diff}^{-1})^{-1}$$

and for $t \neq 0$ p can be determined by:

[12] $$p = -\frac{1}{c\cdot K_{diff}\cdot t}\cdot \ln((K_A(t)^{-1} - K_{diff}^{-1})/(K_A(0)^{-1} - K_{diff}^{-1}))$$

We can express p also in terms of $K_{fus}(t)$, by replacing $K_A(t)$ with $K_A(0)$ - $K_{fus}(t)$.

Experimentally, the following parameters were determined:

$D = 3.3\ 10^{-8}$ cm^2/s

$R = 3.5\ 10^{-5}$ cm

which results in a constant $c = 5.8\ 10^{-11}$ cm^3/s

Applying Eq. **9** to two independent experiments in which the proportion of fused vesicles was determined by particle counting at different time points, the fusion probability/collision is calculated to $p \approx 10^{-7}$

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2. Widengren, J. & Rigler, R. (1998) *Cell. Mol. Biol.* **44,** 857-879.

3. Widengren, J. & Rigler, R. (1996) *Bioimaging* **4,** 149-157.

4. Smoluchoswki, M. (1917) *Z. Phys. Chem.* **92,** 129-168.