Extended Methods online

Single Vesicle Assaying of SNARE-Synaptotagmin Driven Fusion Reveals Fast and Slow Modes of Both Docking and Fusion and Intra-Sample Heterogeneity

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Vesicles

v- (PC/PS/Cholesterol//DiO-C18 73:15:10:2)) and t-vesicles (PC/PS/Cholesterol /PiP2/Dope-Biot./DiI-C18 58:25:10:5:0.1:2) were prepared by mixing the lipids in chloroform in a glass vial. Chloroform was evaporated under nitrogen flow followed by 15 min. incubation under vacuum. Vesicles were formed by addition of 0.5 ml hydration buffer (50 mM Tris pH 8, 150 mM NaCl, 2 mM DTT). The v-vesicles were extruded 21 times using the Avanti mini-extruder and a 50 nm filter. 20 µl of the vesicle suspension (lipid concentration of 10 mM) were added to 80 µl 50 µM vSNARE solution (1:50 protein to lipid ratio) and incubated for 15 min. at RT (vSNARE solution: 11.2 µl 358 µM syb were diluted to 50 µM in vSNARE buffer (25 mM HEPES pH 7.5, 100 mM KCl, 10 % Glycerol, 1 % OGP, 4 mM DTT)). The resulting mixture was diluted below the CMC of the detergent by adding 100 µl hydration buffer. t-vesicles were extruded 3 times using the Avanti mini-extruder and a 800 nm filter. 10 µl vesicles (20 mM lipids) were added to 90 µl 11.1 µM tSNARE solution (1:200 protein to lipid ratio) and incubated for 15 minutes at RT (tSNARE solution: a 1:1 syx:SNAP-25 complex was formed prior to incubation by mixing 5.25 µl 190 µM Syntaxin with 11.1 µl 90 µM SNAP25 and incubating for 1 hour at 4° C). The tSNARE concentration was adjusted to 11.1 µM in tSNARE buffer (50mM TRIS pH 8, 100 mM KCl, 10 % Glycerol, 0.8 % OGP, 5 mM DTT). After incubation the mixture was diluted below the CMC of the detergent by adding 100 µl hydration buffer. For both v- and t-SNARE vesicles the detergent was removed by dialysis over night against 2 l dialysis buffer (2 l 25 mM HEPES pH 7.5, 100 mM KCl, 5 % Glycerol, 2 mM DTT) and 10 g BioBeads at 4 °C. All fusion experiments were conducted in 25 mM HEPES pH 7.5, 100 mM KCl, 5 % Glycerol, 2 mM DTT. For the direct reconstitution the OGP concentrations were halved.

Microscopy

Microscopy was performed using a Leica TCS SP5 inverted confocal microscope

equipped with an oil immersion objective HCX PL APO, x100 magnification and NA 1.4. A 458 nm laser was used for excitation and fluorescence emission was acquired in two channels: 480-530 nm (donor) and 590-650 nm (acceptor). Scan speed was 1400 lines/s and the frame rate was either 1 Hz or 5 Hz, depending on the pixelation/zoom. The microscope was equilibrated to 20 $^{\circ}$C.

t-vesicles were immobilized and unbound vesicles were removed by wash. The microscope was then focused and an image of the t-vesicles was acquired (543 nm excitation) to extract vesicle coordinates used for ROI integration. 25 µl v-vesicle suspension (1 mM lipids) were mixed with 2.15 µl syt C2AB (355 µM) and injected into the microscope chamber that contained 75 µl buffer, resulting in a lipid concentration of 0.24 mM and 7.5 µM syt. Given an average v-vesicle radius of 37.5 nm and taking the headgroup area of a lipid to be 0.64 nm^2 this amounts to an approximate v-vesicle concentration of 4.3 nM in the chamber with the assumption that all lipids initially mixed end up in vesicles. After 3 minutes $CaCl_2$ was added at a concentration of 0.5 mM. After the fusion experiment an image of the t-vesicles was acquired to confirm that no t-vesicles had detached.

Cryogenic transmission electron microscopy

cryoTEM was performed at Biomikroskopienheten, Materialkemi, Kemi Centrum, University of Lund, Sweden using a Philips CM120 (BioTWIN Cryo) equipped with a GATAN CCD camera. Samples were prepared by applying 5 µl vesicles on a carbon grid and removing excess buffer by soaking onto a tissue. The grid was immediately frozen to -180 $^{\circ}$C according to the plunging method and imaged.

Ensemble lipid mixing

50 µl t- and v-vesicle suspension were mixed and, where indicated, 2.15 µl 355 µM syt was added. A concentration of 0.5 mM Ca^{2+} was achieved by injecting 2 µl 25 mM $CaCl_2$. The sample was immediately transferred to a temperature equilibrated cuvette at 20 °C. The fluorescence spectroscopy was conducted using a Horiba Jobin FluorLog Fluorometer. The specimen was excited at 458 nm and emission collected at 510 nm (donor) and 570 nm (acceptor). The percentage of lipid mixing was calculated by normalizing the crosstalk corrected acceptor signal of the fusion samples to the acceptor signal monitored on a control sample premixed with 1 mol % of donor and acceptor dyes.