Supporting Information

Encapsulation efficiency measured on single small unilamellar vesicles.

Brian Lohse, Pierre-Yves Bolinger and Dimitrios Stamou*.

Bio–Nanotechnology Laboratory, Department of Neuroscience and Pharmacology & Nano–Science Center, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen, Denmark.

1. Materials and Methods

1.1 Chemicals:

CoroNa™ Green Sodium Indicator from Molecular Probes™; 2 % Hellmanex from Hellma, Müllheim, Germany; DSPE-$PEG_{(2000)}$Biotin 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanol amine-N-[Biotinyl(Polyethylene Glycol)$_{2000}$] (Ammonium Salt); 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC); 1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (Sodium Salt) (DOPG) from Avanti Polar Lipids Inc. (Alabaster, AL) as chloroform solutions; 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indodicarbocyanine perchlorate (DiD oil) from Molecular Probes™. Streptavidin was purchased from Invitrogen Inc. (Taastrup, DK). Bovine Serum Albumin (BSA) and biotinylated bovine serum albumin (BSA-biotin) were purchased from Sigma Aldrich (Brondby, DK). PBS (pH 7.4) buffer composed of 100 mM NaCl, 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, Ultrapure water (MilliQ). A Tris-HCl buffer 1 M (pH 7.5 m/HCl) from Bie & Berntsen A/S. A solution of 200 mM Sorbitol in ultrapure water (MilliQ).

1.2 Vesicle Preparation and immobilization:

A solution of DOPC (93.2 mol %), DOPG (6 mol %), DSPE-PEG_{2000}-biotin (0.3 mol %), C_{18}-DiD oil (0.5 mol %) in chloroform was dried under nitrogen flow for 15 minutes, and the lipid film (1 mg) was stored in vacuum over night. 100 µl of 100 mM PBS buffer (pH 7.4) was mixed with 1 µl of CoroNa Green (0.85 M) and this mixture was gently added to the lipid film, and incubated at 37^{o}C overnight.

After incubation the vesicle solution was transferred to an eppendorf tube, and 15 freeze/thaw cycles were applied by immersion in liquid nitrogen for 30 seconds, and thereafter immersion in water (45^{o}C) until the solution was completely thawed. The vesicle solution was extruded using the Avanti® mini extruder and following the procedure from the manufacturer, using a pore size filter of 800 nm.

The glass cover slips (thickness 170 +/- 10 µm), Assistant, Sondheim, Germany were sonicated in Hellmanex 2 vol % solution for 10 minutes, then flushed x 5 with milliQ water, this step was repeated 3 times. The glass cover slips were then sonicated in milliQ water for 10 minutes, and flushed x 5 with milliQ water, this step was repeated 3 times. Finally, the glass cover slips were sonicated and then stored in methanol. They were dried under nitrogen flow before use.

The vesicles were attached to glass cover slips the following way: first 200 µl 1.0 g/l BSA-biotin/BSA solution (1:10) was added to the glass cover slip, and incubated at r.t. for 10 minutes. The chamber was flushed 5 times with the 100 mM PBS buffer, and then 200 µl 0.025 g/l streptavidin solution in MilliQ was added to the chamber, and incubated at r.t. for 10 minutes. The chamber was gently flushed 5-10 times with the 100 mM PBS buffer and incubated for 10 minutes, and once again the chamber was gently flushed 5-10 times with the 100 mM PBS buffer. 1 µl of the vesicle preparation was then added to the chamber containing a volume of 200 µl of 100 mM PBS buffer.

1.3 The Experimental setup:

The experiments were carried out on a commercial microscope (Leica DMI 6000B), using a 12 V/ 100 W Halogen lamp as a light source. For the detection of the excited DiD dye inserted into vesicles lipid bilayer a ET Cy5 filter cube ET620/60x; ET700/75m; T660lpxr was used and for the CoroNa Green dye in the vesicle lumen, a ET GFP filter cube ET470/40x; ET525/50m; T495lpxr, was used. By using two dyes (DID and CoroNa Green) with λ_{max} far apart, with absorption/ emission at 644 nm/ 665 nm and 492 nm/ 516 nm respectively, we completely rule out crosstalk. The

microscope objective used was: HCX PL APO 100 x 1.46, oil immersion. The camera was a Photometrics Cascade 512B, CCD digital camera with the following settings (in all experiments); 16 bits, EM Gain lumen channel 3600 and lipid channel 3600. All measurements were performed at r.t.

1.4 Digital image treatment:

In order to properly quantify the micrographs, the quality of the images is optimized during acquisition by enhancing the signal to noise ratio. Thus, the region of interest is acquired two times sequentially giving two images, one for each channel. The digital images recorded are treated and quantified, using an in-house developed software routine for Igor developed by Andreas Kunding. With this software routine it is possible to extract the characteristics of individual vesicles.

The vesicle particles on each image are identified by applying two criteria. (i) The intensity of the vesicle particles should be higher than a calculated threshold value. This enables you to discriminate each vesicle from the background. Thus, the threshold value is chosen slightly higher than the background intensity. (ii) The area of the vesicle particles should be larger than a minimum particle area which is determined according to the area of the smallest visible vesicle. This enables us to exclude the noise higher than the threshold value. In addition vesicles too close to each other are also omitted by applying an elliptic data filter; the ratio of the minor axes divided by the major axes should be close to the value one as it is the case for a normal circular vesicle particle. For example, two vesicles with overlapping intensity distributions are removed from the data, since they appear as one elliptic particle instead of two circular particles. Finally, the coordinates of the vesicles are calculated with a centroid algorithm which allows corresponding lumen and membrane intensities to be associated in the two respective images.

1.5 Vesicle diameter:

The integrated intensity from the fluorophores in each vesicle provides useful information about the size of the vesicles and the concentration of their content. Effectively, since the fluorescent intensity is proportional to the number of fluorescent molecules, the intensity from the dye incorporated in the lipid membrane is proportional to the vesicle surface area and thus to the square of the vesicle diameter. We briefly describe the size calibration process below though full details can be found at Kunding et al. (ref. 6b) By analyzing the same vesicle reference sample, extruded with pore diameters of 50 or 100 nm, both with dynamic light scattering (DLS) and with the fluorescent microscope, a correction factor (F) is determined to calculate the absolute diameter of an individual vesicle from its membrane fluorescent intensity (equation 1).

$$F = \frac{r_{mean}}{\sqrt{I_{mean}}} \Rightarrow d_{ves} = \sqrt{I_{memb}} \cdot 2F \qquad (1)$$

Where: F is the correction factor, r_{mean} is the mean radius from DLS and I_{mean} is the mean intensity from the membrane of all vesicles of the reference sample, and I_{memb} is the intensity from an individual vesicle with a corresponding diameter $d_{ves.}$ Both I_{mean} and I_{memb} are recorded with the microscope using the same acquisition settings.

Note: Fig. 2B shows the ratio of two dyes tethered in the bilayer through anchors (DHPE and C_{18}) of different physicochemical character. Any dye segregation or phase separation (size dependant or not) would most certainly be completely different for the two dyes. However we consistently recorded a constant ratio that assured us that no such phenomena are involved and that indeed the intensity of the lipid marker is a reliable indicator of size.

1.6 Lumen dye concentration:

Once we know the volume V_v of a single vesicle we only need a calibration/conversion factor which relates the intensity from the encapsulated fluorophore to the number of molecules *N. This allows us to accurately determine the concentration of the fluorophores* $c_v = N / V_v$ *within each vesicle*,. We describe here this calibration that is also the main subject of a manuscript in preparation. Briefly, we record an image with the same microscope settings in a solution with a known concentration (c_{calib}) of the same dye. Knowledge of the illumination volume (V_{ill}) of the microscope allows us to relate the total recorded intensity to the total number of fluorophores N_{tot} ($N_{tot} = c_{calib} \times V_{ill}$) and thus extract the average intensity per molecule.

The trend line in figure 2a (red line), was fitted by introducing an additional parameter, allowing us to obtain the lowest average between the fit and every single data point. As explained in the main text we expect a 1/*d* relation, where y = c/x, where c is a constant. In order to move the fitted curve in the program Igor, we introduced the following

equations: $Y = c/(x-x_0)$, in order to move the fit right or left and $Y = y_0 + c/(x-x_0)$ to move the fit up and down, hereby obtaining the optimal fit as shown in figure 2a.

1.7 Characterizing the lamellarity of the sample:

In order to characterize the degree of lamellarity we first did CryoTEM and then an online experiment using 488 labeled streptavidin, which in addition would reveal miscalibration of the vesicle size due to dye segregation.

Cryogenic transmission electron microscopy:
Transmission electron microscopy was performed at Biomikroskopienheten, Materialkemi, Kemi Centrum, University of Lund, Sweden using a Philips CM120 (BioTWIN Cryo) equipped with a GATAN CCD camera (1024 x1024 pixels). Samples were prepared by applying 5 µl vesicles on a carbon grid where the excess buffer was removed by tissue. The grid was immediately frozen to -180^{o}C according to the plunging method and kept at this temperature during imaging. Several regions of at least two separate grids were inspected to ensure representative data collection.
Two samples were prepared with the following lipid composition: DOPC/DOPG/DSPE-PEG_{2000}-biotin/ C_{18}-DiD (93.2:6:0.3:0.5 mol %). These lipid mixtures were freeze-thawed up to 5 times and extruded through a 100 nm and a 200 nm pore filter respectively (see figure S1).
The degree of multilamellarity was determined by thorough review of 10 images per sample, counting the number of MLV's and dividing that number with the total number of intact spherical vesicles, giving less than 6% of MLV. The morphology of the vesicles investigated with CryoTEM was found to be spherical and no fractured vesicles were found. The same samples were found to contain ~ 20% empty vesicles based on the fluorescence investigation.

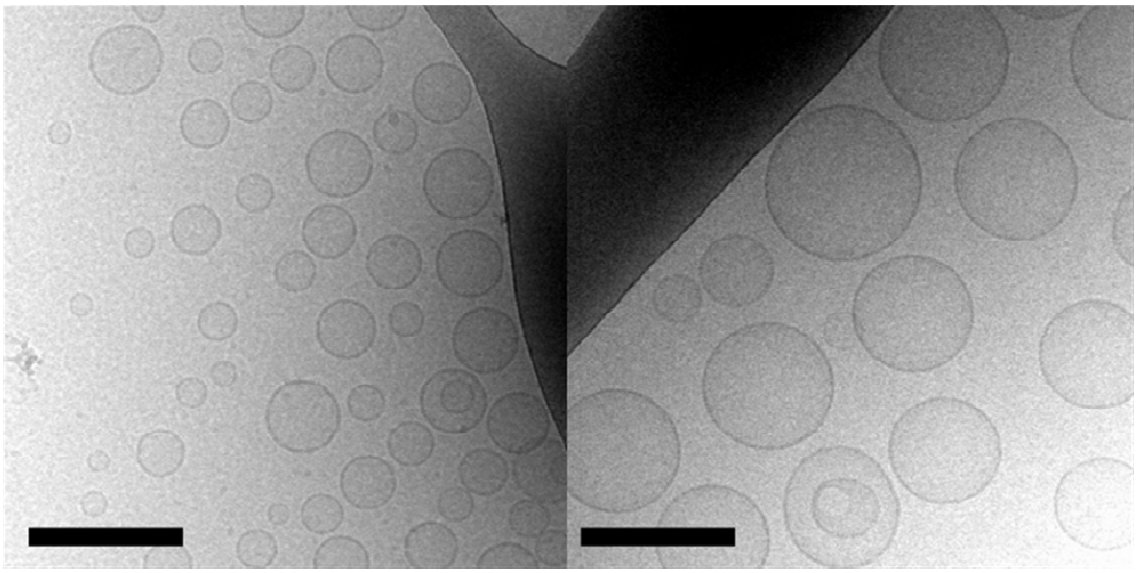

Figure S1: Cryogenic Transmission Electron Micrographs of Extruded Vesicles: (left) Representative micrographs of vesicles extruded through a polycarbonate membrane with filter pore diameters of 100 nm and (right) 200 nm. Both scale bars are 200 nm.

On-line characterization of MLV.
We used the materials and methods described earlier in this SI in order to obtain a sample containing surface attached small unilamellar vesicles. To this sample we added a solution (40 nM) of fluorescently labeled streptavidin (SAv488) in the PBS buffer. The sample was allowed to equilibrate for 20 min., thereby allowing the unbound biotin to tether with the labeled streptavidin. The sample containing the tethered vesicles was washed 10 times with the PBS buffer, to remove unbound SAv488. By dividing the measured intensity of the SAv488 (I_{SAv}) with the measured intensity of the membrane dye (I_{mem}) we obtain the density of SAv488 as a function of size (see figure S2). Using this relation $D_{(a.u.)} = I_{SAv} / I_{mem}$ it is possible to see the degree of homogeneity of the sample. As is evident from figure S2 a high degree of homogeneity within a sample is observed, which would not be the case if a significant number of MLV's were present.

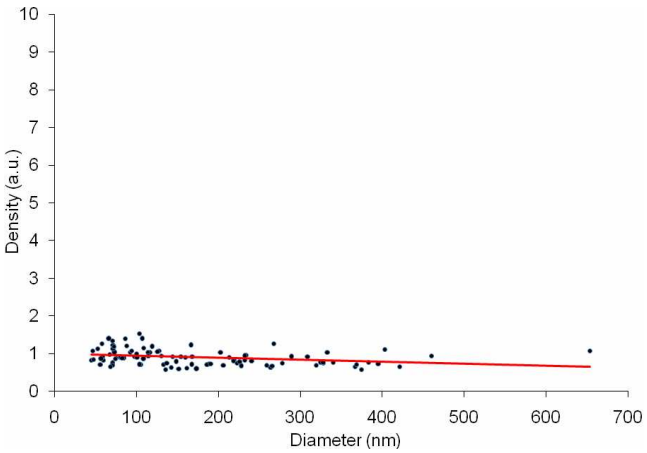

Figure S2: Intensity of a 488 labeled streptavidin divided with the membrane dye (C_{18}-DiD) intensity, yielding the density in arbitrary units, as a function of size.

1.8 The effect on EE upon freeze-thaw/ extrusion as a function of size:

On a sample prepared as described earlier in this SI, we did a study of the effect on EE upon freeze-thaw/ extrusion as a function of size. CoroNa green dye mixed with PBS buffer was added to the dry film rehydrating the sample and allowing for the CoroNa green dye to be encapsulated. We then analyzed the sample and determined the EE, as a function of size, before freeze-thaw and extrusion (see figure S3, black line connecting diamonds). The data is binned from the middle of two diamonds e.g. 150 nm ± 25 nm and the spread in EE is shown as standard deviations, for each binned size shown. The data from after 15 freeze-thaw and 15 extrusions was treated exactly the same and are plotted in figure S3 (blue line connecting squares). For each binned data point there are a minimum of 20 vesicles, ensuring a solid foundation for evaluation.

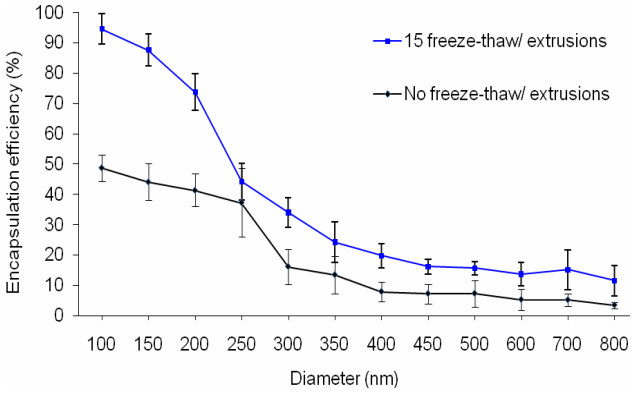

Figure S3: The effect on EE upon no (black line connecting diamonds) and 15 (blue line connecting squares) freeze-thaw/ extrusion as a function of size.

As can be concluded from figure S3, an overall increase in the EE is observed, however taking the single vesicles with the highest and lowest EE within the 100 nm and 800 nm binned vesicles groups, we see a doubled increase at 100 nm (highest EE 99.68 %, lowest EE 44.42 %) but a 7 fold increase at 800 nm (highest EE 16.55 %, lowest EE 2.47 %), before and after freeze-thaw and extrusion.