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Supporting Material

Asymmetric GUVs Prepared by MβCD-Mediated Lipid Exchange: An FCS Study

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Chemicals

Dioleoylphosphatidylcholine (DOPC), sphingomyelin from porcine brain extract (bSM), phosphatidylethanolamine from porcine brain extract (bPE), phosphatidylcholine from porcine brain extract (bPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-DOPE) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Rho-DPPE), N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-DPPE), BODIPY® FL C_5-ganglioside G_{M1} (Bod-GM1) were from Invitrogen (Eugene, OR). Trehalose dehydrate, methyl-ß-cyclodextrin (mβCD) and sodium hydrosulfite were from Sigma-Aldrich (St. Louis, MO). The conjugation of the Nile Red fluorophore with an anchor group to obtain NR12S was performed as described in ref. 10.

Formation of the asymmetric GUVs

Asymmetric GUVs were formed with a protocol adapted from that described in (8).

First, we prepared symmetric GUVs using ITO-coated round coverslips (thickness #1.5, ZC&R coatings for optics, Torrance, CA) assembled in a home-built perfusion chamber, with ca. 250 µl total volume. Since the GUVs were never transferred, the same chamber was used to perform optical microscopy. The overall lipid composition at this step was the desired final composition for the inner leaflet of the asymmetric GUVs (e.g. DOPC, with or without fluorescent probes). The lipid solution, usually in chloroform at 10 mg/ml, was spread on the glasses for a total amount of ca. 20 nmol lipids and dried. Vesicles were formed using the electroformation method (see Angelova and Dimitrov, 1986, Faraday Discuss. Chem. Soc. 81, 303) using 1.2 V, 10 Hz for a minimum of 90 minutes at room T (22°C) in a solution of trehalose in water (concentration ranging from ca. 50 to 90 mM, see below). Trehalose here was only needed to counterbalance the osmotic pressure at later stages of the process and might be substituted by any other sugar or buffer, in the proper amounts and conditions. The vast majority of the vesicles obtained with this method are unilamellar. The few samples showing a significant amount of multilamellar vesicles were discarded without further analysis.

Second, we prepared a *donor* solution containing the lipid to be delivered to the outer leaflet of the GUVs (in this case bSM, with or without fluorescent probes). We formed bSM multilamellar vesicles (MLVs) by drying the lipid in a film and re-hydrating it at 14 mM lipid concentration (unless otherwise noted). After initial vortexing at 65°C, mβCD was introduced in the suspension at ~70 mM concentration. The sample was then kept at 65°C and vortexed for 2 hours. At this point, the donor solution should contain residual bSM MLVs and bSM-mβCD complexes. In order to remove any lipid aggregates and residual MLVs, we centrifugated the donor solution at 54000g, 4°C for at least 15 minutes (or until the supernatant was clear). It is worth noting that this step will remove most of the bSM, therefore reducing the maximum amount of lipid exchanged later in the outer leaflet. Nevertheless, we preferred to remove the MLVs to obtain cleaner asymmetric GUV samples, as bright slowly diffusing fluorescent lipid aggregates might disturb FCS measurements. The osmolarity of the donor solution was, at this step, between 100 and 120 mOsm/Kg (measured with a Micro-Osmometer 210, Advanced Instruments, Norwood, MA). For sake of simplicity, we assumed the mβCD to still have a nominal concentration of 70 mM. We normally stored the donor solution at -20°C until needed.

Third, we diluted the donor solution to 310 µl at the desired nominal mβCD concentration (usually between 40 and 65 mM) and flushed the GUV chamber with it. Note that the GUVs were initially formed in a sugar solution with osmolarity matching that of the diluted donor solution. Occasionally, we observed undulating/flaccid vesicles before the lipid exchange. In order to allow FCS measurements (i.e. to assure a tight focus on the membrane), those samples were washed with a hypo-osmotic medium (90% of usual osmolarity) before the introduction of mβCD. After 30 minutes at room temperature, the sample was delicately washed with 2 mL of the same trehalose solution used in the first step. Prolonging the exposure of the GUVs to the donor solution up to 1 hour did not appear to dramatically influence the final results. The yield of GUVs at the end of the exchange procedure ranged from ~50% (~40 mM mβCD) of the initial amount of vesicles to ~10% (~70 mM mβCD).

Leaflet-specific labeling

Labeling of the outer leaflet was performed by either introducing the fluorophore with the bSM when preparing the donor solution (e.g. Rho-DOPE 0.004 mol % compared to bSM) or washing the asymmetric GUVs with the fluorescent lipid dissolved at very low concentration (e.g. ~20 nM NR12S from a 70 μM DMSO stock solution) in the trehalose solution.

Labeling of the inner leaflet was performed by adding either NR12S or NBD-PE at 0.01% mol concentration to the lipid mixture in the first step of the asymmetric GUV preparation. After the final wash, the residual dye in the outer leaflet (some was extracted by mβCD together with DOPC) was quenched by adding 1 mg/mL sodium hydrosulfite (sodium dithionite) for 90 seconds and then washing carefully the sample. The use of NR12S is preferred in the case of FCS measurements in ordered bilayers with a low lipid diffusion coefficient, since NBD is not very bright and has a low photochemical stability. It is worth noting that, apart from the above mentioned issue, our results were qualitatively not influenced by the choice of the specific fluorescent probe (i.e. NBD-DOPE, NBD-DPPE or NR12S for the inner leaflet and Rho-DPPE, Rho-DOPE, NR12S, NBD-DPPE, NBD-DOPE or Bod-GM1 for the outer leaflet). Finally, although labeling of both leaflets in the same GUV preparation is possible and sometime advantageous (see e.g. figs. S1 and S2), we preferred in general to prepare each time two separate asymmetric GUV samples, one labeled with NR12S or NBD-PE in the inner leaflet and the other with the same label in the outer leaflet (e.g. for the results presented in Fig. 3). The reason for this is that the FCS experimental setup is simpler and the results are in general more reliable when only one type of fluorescent probe is used in a single sample.

Delivering Cholesterol to Asymmetric GUVs with mβCD

Cholesterol- mβCD complexes were prepared similarly to the procedure described in Bacia et al. (Bacia et al., 2004, Biophys. J. 87, 1035). Briefly, 2.2 mL of a ~60 mM mβCD solution in water were slowly mixed with 80 μL of a ~15 mM solution of cholesterol in isopropanol. The turbid suspension was sonicated for 15 seconds and vortexed at 60°C for 1 hour. After centrifugation (40000g at 4°C for 15 minutes), the supernatant was filtered with a 0.2 μm filter (Sarsted, Nümbrecht, Germany) and stored at 4°C until used. The cholesterol-mβCD complexes were eventually diluted in ~600 μL of a trehalose solution (to 12-6 mM mβCD, with a final osmolarity matching that of the solution in which the GUVs where originally prepared) which was then introduced in the GUV chamber. Delivery of cholesterol to the vesicles could be observed already after 5-10 minutes. In the case of symmetric DOPC GUVs, we measured D* before the introduction of the cholesterol-mβCD complexes and 40 minutes after it. In the case of asymmetric GUVs (DOPC inner leaflet, DOPC:bSM outer leaflet previously prepared using a 43 mM mβCD-bSM donor solution), we measured D* in the inner leaflet before the introduction of the cholesterol-mβCD complexes and 10 to 45 minutes after it.

Imaging and FCS measurements

Confocal imaging and single-spot FCS measurements were performed on an laser scanning microscope (LSM) Meta 510 system with a ConfoCor 2 module (Carl Zeiss, Jena, Germany) using a 40X NA 1.2 UV-VIS C Apochromat water-immersion objective. For both imaging and FCS, the sample was excited either by a 488 nm (green channel, e.g. NBD) or a 543 nm (red channel, e.g. rhodamine) laser. The fluorescence was then collected through either a 505-530 band pass filter and a 70 μm pinhole or a 560 long pass filter and a 80 μm pinhole. Unless differently noted, samples were usually produced in duplicates (i.e. two independent preparations). In each preparation, we typically analyzed 10 GUVs in different locations of the observation chamber. For each GUV, we acquired 6 to 10 independent fluorescence intensity tracks (corresponding to an equal amount of FCS curves), each 10 to 6 seconds long, on top of the vesicle. The FCS correlation curves were calculated using the Zeiss ConfoCor2 software and fitted using a nonlinear least squares algorithm to single-component 2D diffusion model. Triplet-state blinking contribution was not taken into account, due to the long diffusion times measured in the lipid bilayer (≥1ms). Typical normalized average correlation curves and fitting curves are shown in Fig.S5 for both a symmetric and an asymmetric GUV. We calculated a diffusion time for the fluorescent lipids in each GUV and repeated the procedure for several GUVs in the preparation, thus allowing us to average ~10 different diffusion times for each preparation. In order to transform the diffusion times into diffusion coefficients, the system was calibrated daily using DOPC GUVs with a known diffusion coefficient of ca. 10 $\mu m^2/s$ at room temperature (22°C), as we measured by scanning FCS (see below). Although this approach cannot provide absolute or precise values for D, we were satisfied with measuring the diffusion coefficient D*, i.e. the relative diffusion compared to a DOPC GUV with nominal D=10 $\mu m^2/s$. In other words, a value of e.g. D*=5 $\mu m^2/s$ for a specific GUV preparation simply indicates a diffusion time 2-fold longer than what we measured in a DOPC GUV at room temperature. It is worth noting that the experimental error bars (i.e. standard deviations of measurements in several GUVs) are usually larger for asymmetric samples than symmetric ones, possibly reflecting a sub-optimal mixing of the different components during lipid exchange. Alternatively, the presence of an inner-leaflet-lipid reservoir might also affect the composition of single vesicles, thus slightly enhancing the compositional heterogeneity of the GUV preparation

Finally, we confirmed the trend observed e.g. in Fig. 1 by using scanning FCS (as described by Ries et al., 2009, Biophys. J. 96, 1999 using a Zeiss ConfoCor3 LSM) on selected samples, the results being comparable to what measured with single-spot FCS.

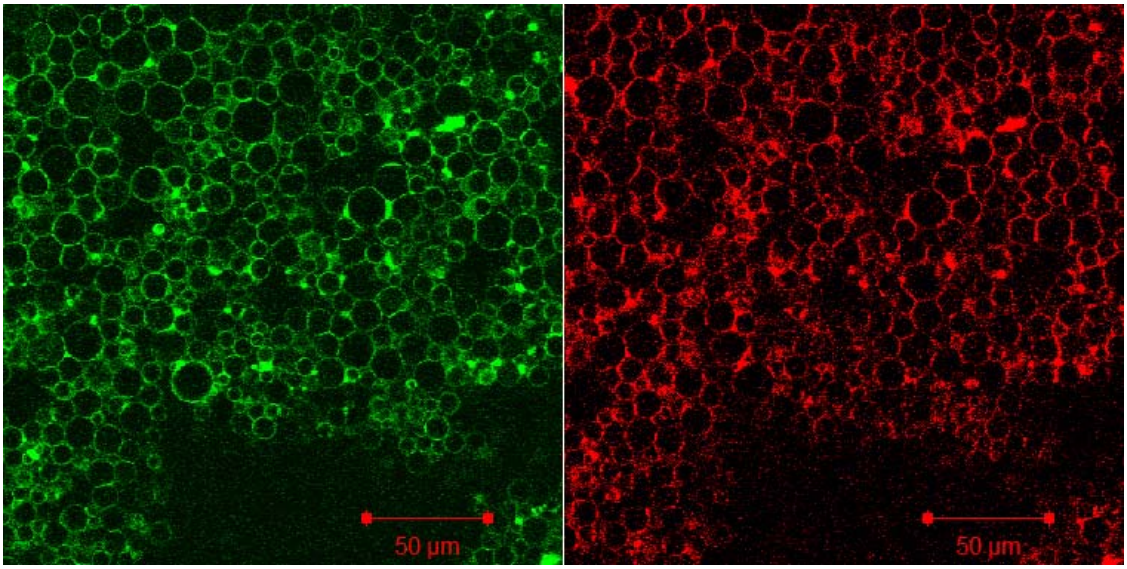


Figure S1 Asymmetric GUVs with inner leaflet composition DOPC co-labeled with NBD-DOPE (green channel, upper left panel) and outer leaflet composition DOPC:bSM ~2:1 labeled with Rho-DOPE (red channel, upper right panel). The lower panel shows the overlay of the two channels. These GUVs were formed starting from a preparation of symmetric DOPC vesicles stained with 0.01% mol NBD-DOPE, exchanged with a donor solution of 43 mM mβCD and 14 mM bSM+0.004 mol % Rho-DOPE. Although many GUVs were destroyed during the exchange and washing, the high initial yield of the electroformation method provided a more than sufficient number of vesicles to be visualized and further analyzed. The NBD in the outer leaflet was then quenched with a 90 second long exposure to 1 mg/mL sodium hydrosulfite. A rough estimation of the amount of dye in the bilayer, also derived from FCS measurements (data not shown), would yield values between 10 and 1 ppm for NBD-DOPE in the inner leaflet and Rho-DOPE in the outer leaflet. These low fluorophore concentrations are preferred for FCS measurements but make fluorescence imaging difficult, requiring higher excitation power and contrast enhancing. When better image quality is required, the dye concentration can be easily increased at least one order of magnitude.

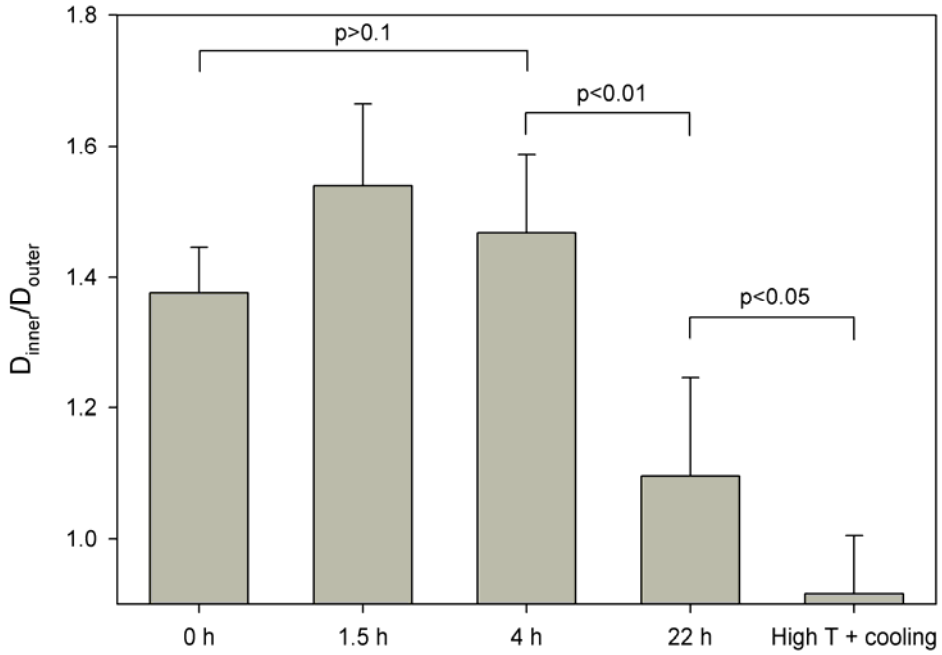


Figure S2 Stability of asymmetry as a function of time. In order to monitor the stability of the asymmetric GUVs, we labeled the inner leaflet with NBD-DOPE and the outer leaflet with Rho-DOPE. We monitored different samples, keeping the inner leaflet composition constant (i.e. DOPC+ ~0.005% NBD-DOPE) while varying the amount of bSM exchanged in the outer leaflet. All samples showed similar time dependence and in this figure we show samples prepared under comparable conditions. We measured the ratio between the diffusion times (i.e. time needed by the fluorescent molecule to diffuse through the focal volume) of the dyes in the two leaflets. We then used a calibration sample (i.e. symmetric DOPC GUVs labeled with both dyes) to account for the different sizes of the focal volumes in the green channel (NBD, inner leaflet) and in the red channel (rhodamine, outer leaflet) and thus obtained the ratio between the diffusion coefficients in the outer and in the inner leaflet. Our results indicate no significant change in the asymmetry of the vesicles in the first few hours after the lipid exchange. After one day (18-22 hours), most of the asymmetry was lost. Incubation of the sample at 65°C for 1 hour and slow cooling down to room temperature caused a drastic decrease in the D_{inner}/D_{outer} ratio, which was then close to unity. We assume this reflects a loss of asymmetry. It is worth noting that the flip-flop rates of the unlabeled lipids and the labeled ones (accounting for a very small part of the total mass of the bilayer) can be very different. If the unlabeled lipids flipped faster than their labeled counterparts, the drop in D_{inner}/D_{outer} that we measure would be a consequence of the effective scrambling of the membrane, both leaflets being in a similar physical state. On the other hand, if the labeled lipids flipped faster than the unlabeled ones, the decrease in D_{inner}/D_{outer} would be caused by a symmetric distribution of fluorescent probes in an asymmetric bilayer. In this last case, our measurement would underestimate the stability of membrane asymmetry. T-test probability results are shown for selected dataset pairs.

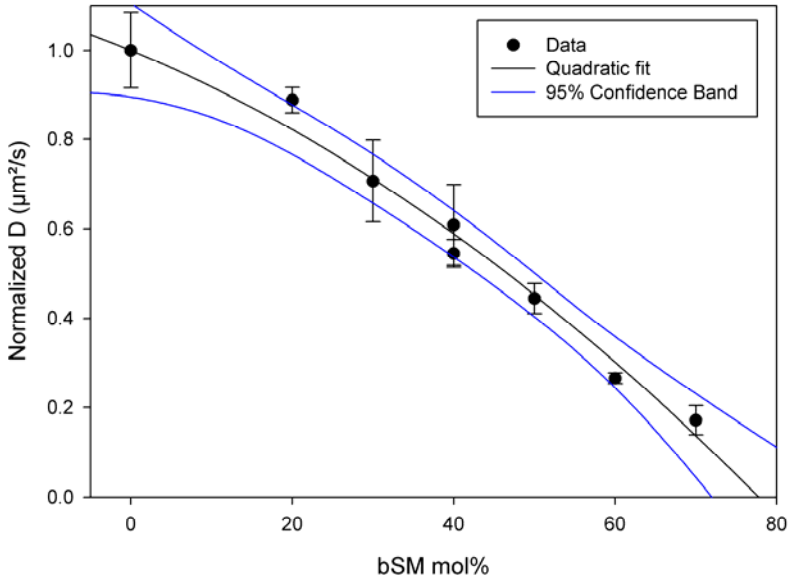
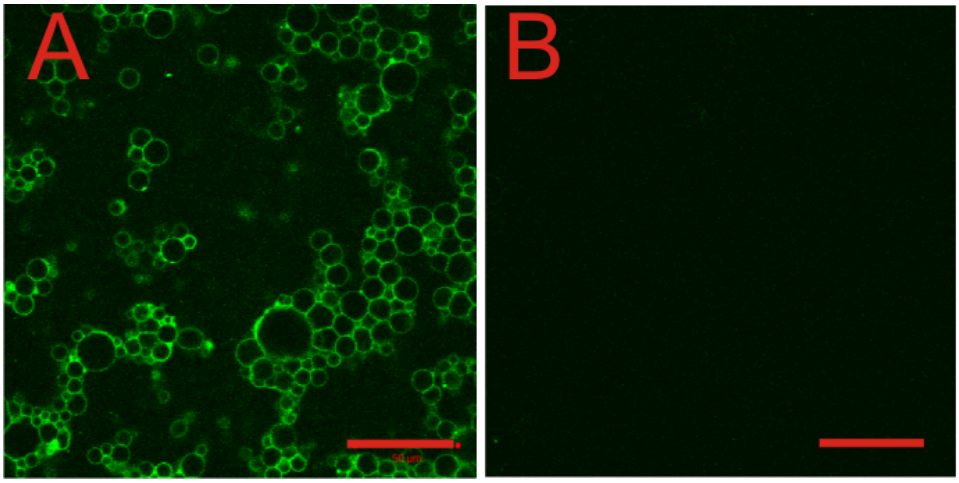


Figure S3 Effect of bSM on the lipid diffusion measured in symmetric DOPC GUVs at room temperature. NR12S was added at ca. 20nM concentration (in the aqueous medium) to symmetric DOPC-bSM GUVs and its diffusion coefficient was measured as a function of the amount of bSM in the bilayer. All the PC-SM mixtures we analyzed, either symmetric or asymmetric, appeared to be in a single homogenous bilayer phase. The diffusion coefficient in DOPC vesicles without bSM (ca. 10 $\mu m^2/s$) was normalized to 1. Each point represents the average of measurements collected from ~10 different vesicles in a GUV preparation. Error bars are the corresponding standard deviations. The data are then fit to an empirical curve (rather than to a precise physical model) which was then used to roughly calculate the bSM concentration in the DOPC:bSM outer leaflet with known D*. An underlying assumption is that the behavior of D* in the bSM:DOPC outer leaflet is similar to that in symmetric DOPC-bSM vesicles with the same amount of bSM. This is supported by the observation that the melting temperature of bSM in the outer leaflet of asymmetric vesicles was not affected by the nature of the lipids in the inner leaflet (8).



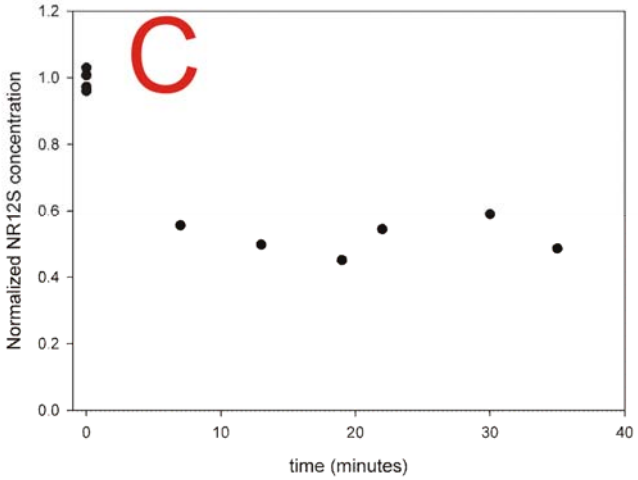


Figure S4 Assessing the asymmetry of bPC GUVs by measuring the asymmetric distribution of a fluorescent probe. Panel A shows asymmetric GUVs with bPC in the inner leaflet and bPC:bSM in the outer leaflet (exchange made using 43 mM mβCD). The outer leaflet contains also NBD-DOPE which was delivered together with the bSM (0.03 mol%) in the mβCD solution. The asymmetric localization of the NBD-DOPE in the outer leaflet was checked by sodium hydrosulfite reduction (0.5 mg/mL, ca. 5 minutes), 30 minutes after the end of the lipid exchange procedure. As shown in panel B, very low fluorescence from the NBD could be detected after fluorophore reduction, implying that the localization of the lipid probe delivered by mβCD was effectively restricted to the outer leaflet of the GUV. The presence of intact non-fluorescent vesicles after reduction was checked by differential interference contrast (DIC) imaging (data not shown). The complete NBD reduction might alternatively be explained assuming that the GUVs were symmetric and leaky to sodium hydrosulfite. To rule out this possibility, we prepared symmetric GUVs made of bPC:bSM 1:1 with 0.02 mol% NR12S in both leaflets. If these vesicles were leaky, we would observe a complete loss of fluorescence upon sodium hydrosulfite treatment. Panel C shows a time course experiment in which such vesicles were exposed to 0.5 mg/mL sodium hydrosulfite. The concentration of the fluorescent probe was measured in randomly chosen vesicles using FCS. After more than 30 minutes, there was no sign of sodium hydrosulfite leaking into the lumen of the vesicles, as the concentration of the fluorescent probe was still around half of the initial value (i.e. the NR12S in the inner leaflet was protected from the reduction). Similar results were observed using asymmetric GUVs with DOPC in the inner leaflet (data not shown). Scale bars are 50 µm.

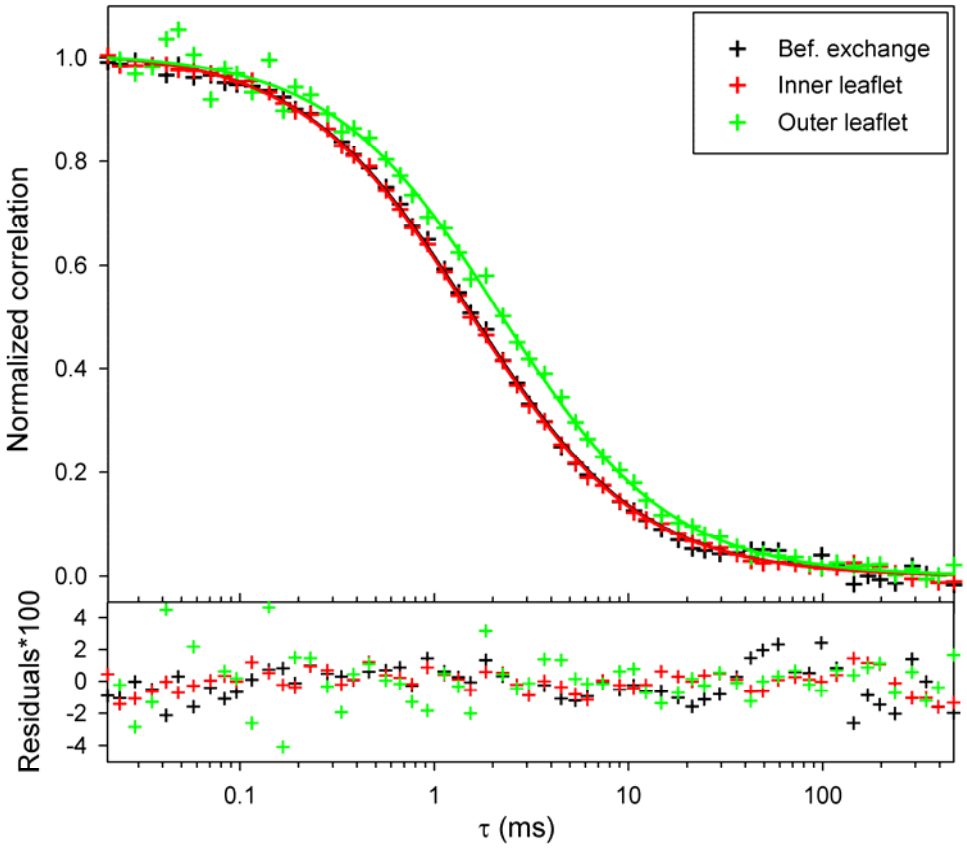


Figure S5 Typical normalized correlation curves in symmetric (before exchange) and asymmetric GUVs (inner and outer leaflet). Each curve is the average of ~10 correlation curves obtained in a single GUV. The time correlation for the symmetric GUV (black) was obtained monitoring the fluorescence of NR12S (0.01 mol%) in both leaflets of a DOPC GUV at 22°C. The asymmetric GUV was obtained through lipid exchange of DOPC GUVs with a donor solution containing 43 mM mβCD and 14 mM bSM. The correlation curves in the asymmetric GUV were acquired for the same fluorophore either in the outer (green) or in the inner leaflet (red). The solid lines are the best fit of the data to a single-component 2D diffusion model. The lower panel shows the fit residuals.