Calcium-Mediated Liposome Fusion to Engineer Giant Lipid Vesicles with Cytosolic Proteins and Reconstituted Mammalian Proteins

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Giant unilamellar lipid vesicles (GUVs) are widely used as model membrane systems and provide an excellent basis to construct artificial cells. To construct more sophisticated artificial cells, proteins—in particular membrane proteins—need to be incorporated in GUVs. However, current methods for protein reconstitution have limited throughput or are not generally applicable for all proteins because they depend on detergent solubilization. This limitation is addressed here by introducing calcium-mediated membrane fusion to transfer proteins between negatively charged GUVs and cell-derived plasma membrane vesicles (CDVs), derived from HEK293T cells overexpressing a membrane receptor protein. Fusion conditions are optimized using large unilamellar vesicles and GUVs containing phosphatidylserines and fusogenic lipids. The approach is then applied to induce lipid mixing and subsequent transfer of the overexpressed membrane receptor from CDVs into GUVs. The membrane receptor is detected by immunofluorescence on GUVs that underwent lipid mixing with CDVs. Those GUVs also exhibit esterase activity because cytosolic esterases entrapped in the CDVs are transferred during membrane fusion. Thus, content mixing is demonstrated. Using CDVs circumvents the need to purify or solubilize proteins. Moreover, calcium-mediated fusion allows transfer of lipids, water-soluble and membrane bound proteins in one step, resulting in a semi-synthetic cell.

1. Introduction

Giant unilamellar lipid vesicles (GUVs) are widely used as cell membrane models or to reconstitute cellular reactions in a synthetic and simplified system. Thus, they can be considered as a basic building block in bottom-up synthetic biology to build an artificial cell. For a living cell, it is estimated that proteins make up 50% of the dry weight of an average cell membrane, the other 50% being lipids. Membrane proteins are fundamental for cellular communication, transport of molecules across the cell membrane, cell adhesion and are also important targets for pharmaceutical drugs. However, integrating membrane proteins into the artificial membrane of GUVs remains challenging and tedious. Most methods require purified proteins and are inefficient and not suited to integrate several proteins at once. In order to advance giant vesicles as artificial cell model, simple reconstitution methods for membrane proteins are necessary. GUVs are cell-sized liposomes measuring several micrometers in diameter and are therefore used as basis to engineer artificial cells. Current methods to reconstitute proteins in GUVs to form proteo-GUVs are labor intensive and depend on submicron proteo-liposomes (small or large unilamellar vesicles). To generate these protein-carrying small or large unilamellar vesicles (SUbs or LUVs), membrane proteins are extracted and purified from host cells overexpressing the protein of interest. The purified proteins are solubilized in amphiphilic agents, for example, in detergent micelles. Mixing the solubilized proteins with lipids, followed by a detergent removal step, yields submicron proteo-liposomes. To prepare proteo-GUVs, the submicron proteo-liposomes are either (i) dried to a lipid film from which GUVs are swollen by hydration or (ii) fused to GUVs. The fusion approach can be done without possible disturbances such as detergents or proteins. However, in both cases (i and ii) the proteins of interest need to be solubilized with specific detergents, which limits these methods to proteins that can withstand the process. Also, the processes are usually optimized for a specific kind of protein, therefore the reconstitution of several proteins at once is labor intensive.

Other methods to directly reconstitute membrane proteins in GUVs involve spontaneous or targeted insertion into the
membrane proteins. Toxins and other proteins, for example the pore-forming toxin alpha-hemolysin, can spontaneously insert into lipid membranes with the appropriate lipid composition to enable diffusion of hydrophilic molecules into GUVs. Further, membrane-bound proteins can be engineered to facilitate spontaneous insertion in a favored direction into a GUV. This was achieved by adding fusion peptides or hydrophilic groups to one end of bacteriorhodopsin. In a similar vein, fusion proteins of the SNARE family and derivatives thereof were successfully incorporated in GUVs to enable controlled fusion with smaller liposomes carrying the matching SARE-receptors. Using this method the reconstitution of GFP–SNARE fusion proteins, carried in SUVs, in GUV membranes was demonstrated.

The above methods are limited to purified proteins. Reconstituting whole protein complexes, for example, membrane receptors and associated messenger proteins, requires integration of natural membranes or parts thereof into GUVs. For example, membrane protein function was partially preserved in GUVs prepared from red blood cell membrane extract. Another approach is to directly harvest giant plasma membrane vesicles (GPMVs) from cells. GPMVs are of similar size as GUVs but are entirely composed of plasma membrane and contain all membrane-associated proteins of the original cell. While these approaches are useful to study membrane proteins in a simplified but near-native environment, they are usually limited to cellular compounds and not suited to study interactions of synthetic membranes and allow not for addition of compounds into the lumen. Also, GPMV preparation requires the use of cross-linking agents such as paraformaldehyde that might impair protein and membrane integrity.

Alternatively, cell-derived vesicles (CDVs) are comprised of native cell membrane with all its associated proteins and enclose part of the cytosol. Thus, CDVs are ideal candidates to shuttle proteins into the synthetic membrane of GUVs by membrane fusion. Cell-derived (native) vesicles from E. coli were already fused to GUVs as a way to reconstitute functional membrane proteins. However, the method required detergents to induce membrane fusion, which can lead to denaturation of membranes or proteins.

In this work we present a detergent-free method to induce membrane fusion between GUVs and CDVs to engineer semisynthetic proteo-GUVs ("hybrid cells"). Our approach is based on calcium-induced membrane fusion. Calcium interacts with the negatively charged phospholipids, notably with phosphatidylserine (PS), which leads to aggregation and subsequent fusion of adjacent membranes. Calcium-mediated membrane fusion has been previously studied and used to great extent. Hence, ideal buffer and lipid composition, especially calcium concentration (at least 5 mM) and the ratio of PS to neutral phospholipids (at least equimolar or higher in favor of PS) were determined by literature research. Addition of fusogenic lipids (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, DOPE) in the GUVs greatly enhanced the fusion process and subsequent reconstitution of a single transmembrane-domain protein. Calcium induced membrane fusion improves on detergent-dependent methods and provides a tractable method to reconstitute proteins in GUVs. Potentially, many proteins entrapped in the CDVs are transferred at once.

Calcium-mediated fusion is therefore highly attractive for eukaryotic protein transfer from CDVs to GUVs.

2. Results

2.1. Assay Development for Calcium-Induced Membrane Fusion between Giant and Cell-Derived Vesicles

The aim of this study is the fusion of CDVs with GUVs to transfer cell membrane molecules into a synthetic membrane with a detergent-free method. Cell surface protein transfer into GUVs was tested by generating CDVs from a HEK293T cell line that overproduced a receptor protein with a single transmembrane domain and an extracellular V5 tag (V5-receptor, Supporting Information). The extracellular V5 tag enabled detection of receptor protein by immunofluorescence on both cell and GUV membranes (Figure 1) as antibodies with high affinity against V5 are readily available. CDVs carrying the V5-receptor were prepared with the cytochalasin B method (Figure 1a). The CDVs were added to GUVs containing the negatively charged phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) in buffer with 5 mM calcium. CDVs accumulated on the artificial membranes through electrostatic interaction induced by Ca\(^{2+}\) which eventually lead to lipid mixing and membrane fusion. As a result, the V5-receptor diffused onto the GUV membrane as shown by immunostaining (see Figure 1b). A PDMS microfluidic device as shown in Figure 1c facilitated imaging, buffer exchange and labeling. The GUVs were physically trapped in the center area of the microfluidic device. Exchange of fluids was feasible without removing the GUVs. Additionally, two inlets and a meandering mixing channel (mixer) allowed the addition of calcium-containing buffer and liposomes from separate inlets. Thus, clogging by liposome aggregation in the inlets was prevented.

2.2. Optimization of Lipid Composition

To evaluate the capability of calcium ions to induce liposome aggregation or fusion, we incubated POPS GUVs with LUVs in the presence of 5 mM Ca\(^{2+}\) (see Experimental Section). The LUVs were prepared to contain a biotinylated lipid, which could be labeled with a streptavidin-Alexa Fluor 488 conjugate. Thus, LUVs that adhered to the GUV membranes after the last washing step or underwent lipid mixing, that is, the transfer of biotinylated lipids onto GUV membranes, were detectable.

Following the procedure described in the Experimental Section, we tested GUVs with three different lipid compositions with respect to DOPE. We measured Alexa Fluor 488 fluorescence on the GUV membranes using confocal microscopy (Figure 2a). All three GUV configurations showed increased fluorescence compared to controls without Ca\(^{2+}\). To test whether Ca\(^{2+}\) was sufficient to provoke unspecific binding of the streptavidin-Alexa Fluor 488 conjugate, we measured a sample without LUVs and recorded only background signal. Thus, the biotin-streptavidin binding system was not impaired by calcium. We concluded that Ca\(^{2+}\) was required to aggregate by-flowing LUVs onto the trapped GUVs.
To differentiate between aggregation and lipid mixing, we investigated the confocal images qualitatively (Figures 2b–e). In case of GUVs without DOPE, the streptavidin-Alexa Fluor 488 conjugate staining on the GUVs was heterogeneous and resembled an accumulation of fluorescent granules on the GUV membrane. In contrast, DOPE containing GUVs contained large membrane regions where streptavidin-Alexa Fluor 488 was homogeneously distributed, and clusters of strong fluorescence usually aligned in direction of the flow (toward the outlet of the microfluidic device). We concluded that the homogeneously stained regions indicate lipid mixing, where the biotinylated lipids diffused unhindered. The heterogeneous clusters of fluorescence indicated the presence LUVs or imperfections of the membrane, where diffusion of lipids was hindered. Due to higher content of homogeneously stained regions on DOPE-containing GUVs, we concluded that DOPE enhanced lipid mixing in our system.
2.3. Preparation and Characterization of Cell-Derived Vesicles Containing an Overexpressed Membrane Protein

From stably transfected HEK293T cells we selected a clone with high cell surface receptor expression (Figure S1, Supporting Information). In Figure 3a, cells from the high producer clone are shown. To validate the presence of V5-receptor on the cell membrane, we stained fixed cells with fluorescently-labeled antibodies. We used an antibody against the extracellular V5-tag (anti-V5-PE, see Experimental Section and Figure 3a iii) and an intracellular domain (anti-IL6-FITC, see Experimental Section and Figure 3a iv). The latter staining only worked for permeabilized cells, confirming the correct folding of the modified receptor protein. HEK293T cells that were not transfected with the gene for the modified V5-receptor could not be immuno-stained (Supporting Information).

Next, we tested if the V5-receptor was displayed by CDVs originating from the receptor-expressing HEK293T. Additionally, we investigated the integrity of the CDVs, that is, leakiness, by exploiting therein entrapped intracellular enzymes. As in cells, cytosolic esterases in CDVs catalyze the conversion of calcein–AM, a non-fluorescent calcein derivate, to fluorescent calcein by acetoxymethyl ester hydrolysis.[31] For this, CDVs were immobilized on a glass slide in a microchannel by micro-contact printing using a biotinylated cholesterol-tether (Supporting Information and Figure S2, Supporting Information).[39] Subsequent staining with anti-V5-PE and calcein–AM revealed the presence of CDVs carrying the V5-tagged receptor as well as cytosolic esterases (Figures 3b,c). Compared to the micro contact-printed patches without CDVs, the fluorescent signals of those with CDVs were 5 times greater, confirming that the receptor on CDVs was present in detectable amounts using immunostaining.

To investigate the stability of CDVs for later use in fusion with GUVs, CDVs were encapsulated in GUVs. We prepared GUVs with a POPC:cholesterol 2:1 lipid composition with additionally 0.1 mol% Lissamine rhodamine B labeled DOPE (Rho-DOPE). CDVs were added to the inner solution of the GUVs during the preparation. The GUVs were flushed into the microfluidic device presented in Figure 1c. We added calcein–AM, which can permeate the synthetic GUV membrane as well as the CDV membrane. CDVs turned fluorescent as shown in Figures 3d, e, indicating that the CDVs were intact and contained functional cytosolic esterases. The calcein was contained by the CDVs and we observed no major leakage into the GUV lumen. We found that calcein–AM was only converted to fluorescent calcein if CDVs were encapsulated inside the GUVs (Figure 3f). GUVs prepared without CDVs showed only background fluorescence. In summary, these results confirm that CDVs and therein entrapped cytosolic esterases remained intact and, moreover, any calcein leakage was insignificant. Therefore, the calcein–AM assay could be used to investigate the transfer of esterases, that is, content mixing, during membrane fusion.
To test how CDVs interact with Ca\(^{2+}\), we measured the zeta potential of CDVs in presence and absence of Ca\(^{2+}\). Zeta potential is an arbiter of the surface charge and indicator of electrostatic repulsion. We expected Ca\(^{2+}\) to weaken the negative surface potential of CDVs by occupying the negatively charged membrane. In absence of calcium (pure buffer 1), a mean zeta potential of \(-14 \pm 1\) mV was determined. The zeta potential of CDVs suspended in buffer with 5 mM Ca\(^{2+}\) (a 1:1 mixture of buffer 1/buffer 2) was increased to 

\(-10.7 \pm 0.2\) mV. The shift indicates that Ca\(^{2+}\) ions interacted with the CDVs and lowered the electrostatic repulsion. This finding was consistent with our experience that CDVs aggregated and could clog microfluidic channels in Ca\(^{2+}\)-containing buffer. The reduced surface charge of CDVs, in presence of Ca\(^{2+}\), facilitated close contact with GUV membranes, a prerequisite for membrane fusion.

### 2.4. Transfer of Membrane Receptor from CDVs to GUVs in the Presence of Calcium

To induce lipid mixing between POPS-GUVs and CDVs, we simultaneously introduced CDVs and 5 mM CaCl\(_2\) to the microfluidic device. Trapped GUVs (encapsulating calcein) with varying membrane compositions were exposed to calcium and CDVs and immunostained with anti-V5-PE (see Experimental Section). Finally, the buffer was exchanged with buffer 1 and unbound antibody was flushed out. We expected to observe a relatively homogenous immunostaining on GUVs as an indication for lipid mixing with receptor-carrying CDVs.

DOPE containing GUVs were immunostained after exposure to CDVs and Ca\(^{2+}\) (Figures 4a,b), while GUVs without DOPE (0% DOPE) had very low signal. To evaluate the ideal lipid composition of GUVs for lipid mixing with CDVs, we compared the immunostaining signals from confocal images of GUV membranes. The signals of GUVs with 10 mol% and 20 mol% DOPE were elevated compared to GUVs without DOPE (Figure 4c). Mean and median fluorescence intensities of GUVs containing 20 mol% DOPE were higher than those of GUVs with 10 mol% DOPE. We conducted further controls to check for unspecific binding of the antibody or protein transfer independent of calcium. GUVs that were either not exposed to CDVs or Ca\(^{2+}\) were tested and we found that the fluorescence was decreased compared to the positive samples, confirming that the antibody assay was specific and calcium was required to induce protein reconstitution. The fluorescence of samples without Ca\(^{2+}\) was slightly increased, compared to samples without CDVs. We assume that sodium was sufficient to induce weak interactions between CDVs and GUVs in absence of divalent cations. We compared the means of the positive results (10 and 20 mol% DOPE) with those of the other results with a randomization test (see Experimental Section) and found that they were different under an \(\alpha = 0.05\) significance level.

### 2.5. Content Mixing between CDVs and GUVs after Membrane Fusion

Immunostaining of the GUV membranes confirmed the occurrence of lipid mixing and subsequent transfer of the V5 tagged receptor protein. To find out if this observation was a result of membrane fusion, we tested for content mixing between CDVs and GUVs. The most common content mixing assays rely on de-/quenching assays and are not suited for our system due to the vast volume difference between GUVs and CDVs. However, the CDVs obtained by cytochalasin B treatment of HEK293T cells encapsulate cytosolic esterases (Figure 3). We therefore reasoned that after membrane fusion with CDVs, a GUV would exhibit esterase activity and convert membrane-permeable esterases.
calcein–AM to calcein. This enzymatic assay would circumvent commonly used content mixing assays, which rely on similarly sized liposomes and were not applicable to our study.

To demonstrate the transfer of membrane-bound V5-receptor and luminal esterases, GUVs were stained with calcein–AM and V5 antibody PE. We prepared GUVs with 20 mol% DOPE in the outer leaflet (Experimental Section) but without encapsulating any fluorophore to avoid interference with the phycoerythrin antibody conjugate and calcein–AM assay. The GUVs were trapped in the microfluidic device and CDVs and Ca\(^{2+}\)-containing buffer were added. Next, calcein–AM and anti-V5-PE were added and then exchanged with buffer 1 to stop the supply of calcein–AM (Experimental Section). GUVs with immunostained membranes were imaged with confocal microscopy to determine calcein fluorescence (Figures 5a,b). Only GUVs that were exposed to both, CDVs carrying the V5-receptor and 5 mM Ca\(^{2+}\) were immunostained with anti-V5-PE (Figure 5c). Samples that lacked either CDVs or calcium exhibited no immunofluorescence and the negative control with V5-deficient CDVs also barely showed immunofluorescence confirming the selectivity of the immunofluorescence assay (Figure S3, Supporting Information). The median of the anti-V5-PE signal measured in fused GUVs was 8.8 and comparable to the previous measurements of V5-receptor in GUVs (Figure 4). In this experimental series, two very strongly stained GUVs were observed, indicating a large heterogeneity of initial CDV attachment to the GUVs.

As for the calcein–AM content mixing assay, samples without either CDVs or calcium showed only background fluorescence (Figure S3, Supporting Information). Only GUVs exposed to calcium and CDVs were green fluorescent (Figure 5d), indicating calcein accumulation in their lumen over the course of the experiment (30 min). The calcein signal from GUVs exposed to the V5-deficient CDVs showed a broader distribution than the immunostained GUVs (exposed with the V5-receptor carrying CDVs) albeit a similar upper range (Figure S3, Supporting Information). An explanation for the broadened calcein signal distribution was the lack of immunofluorescence of those GUVs making the detection of “fused” GUVs more difficult. In summary, these results confirmed the co-occurrence of lipid and content mixing during fusion of POPS/DOPE GUVs with CDVs, indicating successful calcium-mediated membrane fusion. Further, the presented content mixing assay worked also with V5-deficient CDVs from another HEK293T cell line. We immobilized 136 GUVs, but selected only those that had clearly detectable amounts of reconstituted V5-receptors and esterases, that is, underwent unambiguously fusion with CDVs (N = 7, 5.15%).

3. Conclusion

We demonstrated the reconstitution of a membrane protein in giant vesicles by inducing detergent-free membrane fusion with LUVs and CDVs. Compared to other methods that rely on the use of detergents, our method is very mild and potentially applicable to a wide range of proteins. The only prerequisite is a cell line overexpressing the proteins of interest to prepare CDVs. Additionally, we showed that the reconstitution of a membrane protein is concurrent with the transfer of a functional cytosolic protein; calcium-mediated fusion bears the potential to reconstitute receptor proteins along with messenger proteins. Such a method was previously not available and therefore advances the possibility to engineer artificial cells with functional protein networks. Compared to methods that only use cell membrane, like GPMVs, the approach here is semi-synthetic as we use artificial GUVs as a compartment scaffold. Moreover, we demonstrated that lipids and membrane proteins as well as cytosolic proteins are transferred to GUVs by the same method. This approach potentially enables the reconstitution of whole signaling pathways, from membrane receptor to messenger proteins with one method.

By isolating the GUVs in microfluidic traps, we were able to avoid uncontrolled aggregation caused by millimolar concentrations of calcium. To adopt the presented method in bulk...
experiments, the GUVs could be anchored on a solid surface or on porous membranes, which would also facilitate washing and labeling steps and increase throughput.

We believe that this system can be used to engineer fully functional hybrid liposomes, as we know from previous studies with CDVs that the functionality of the membrane proteins is preserved in the CDVs.[31,40] While we here showed the functionality of the cytosolic protein only, future studies will focus on functionality tests of the reconstituted membrane proteins. In case of the modified rtk, the functionality could be tested by exploiting the receptor kinase activity. We believe that other membrane proteins such a G-protein coupled receptors (GPCRs), which are important drug targets, could be reconstituted on CDVs, simply by changing the cell line from which the CDVs are derived.

4. Experimental Section

Materials: DMEM high glucose (#41966-029) containing pyruvate, and RPMI 1640 medium without glutamine nor phenolred (#32404-014), puromycin dihydrochloride (#A11138-03), fetal bovine serum (FBS, #10207-106), trypsin-EDTA 0.05% (#25300-054), and V5 Tag Monoclonal Antibody PE conjugate (#12-6796-42) were bought from ThermoFisher Scientific. Cytochalasin B from Drechslera dematiodae (#C2743), calcium- and magnesium-free phosphate buffered saline (DPBS, #D8662), mineral oil (#M3516), sodium chloride, calcium chloride, OptiPrep density gradient medium (#D1556), and bovine serum albumin (#BSA, #69896) were purchased from Sigma Aldrich. Lissamine Rhodamine B Sulfonyl Chloride (SRB, #L20), Phosphoethanolamine-N-lysine rhodamine B sulfonyl (Rho-DOPE, #810150), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt (POPS, #840034), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl (Rho-DOPE, #810150), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt (POPS, #840034), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl sodium salt (biotinyl DOPE, #708282), and cholesterol (#70000) were purchased from Avanti Polar Lipids. All phosphoethanolamines (#2500153) were purchased from PAA. 1-Palmityl-2-oleylphosphatidylcholine (POPC, Biosolve Chemicals. Non-essential amino acids (100x, #M11-003) were purchased from Invitrogen. Calcein (#21030) was purchased from Fluka. Tris(hydroxymethyl)aminomethane (#200923) was purchased from Sigma Aldrich. Serum albumin (BSA, #A7906) were purchased from Sigma Aldrich. Phoethanolamine, OptiPrep density gradient medium (#D1556), and bovine serum albumin (BSA, #69896) were purchased from Sigma Aldrich. Lissamine Rhodamine B Sulfonyl Chloride (SRB, #L20), Phosphoethanolamine-N-lysine rhodamine B sulfonyl (Rho-DOPE, #810150), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt (POPS, #840034), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl sodium salt (biotinyl DOPE, #708282), and cholesterol (#70000) were purchased from Avanti Polar Lipids. All phosphoethanolamines (#2500153) were purchased from PAA. 1-Palmityl-2-oleylphosphatidylcholine (POPC, Biosolve Chemicals. Non-essential amino acids (100x, #M11-003) were purchased from Invitrogen. Calcein (#21030) was purchased from Fluka.

Solutions: Experiments were performed in Tris-buffered saline (buffer 1) and a calcium-containing counterpart (buffer 2). Buffer 1 contained 125 mM NaCl, 10 mM Tris and the pH was adjusted to 7.5. Buffer 2 contained 100 mM NaCl, 10 mM CaCl₂, 10 mM Tris and the pH was adjusted to 7.8. The final molar ratio of the outer membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet was a mixture of POPC:cholesterol:POPS in a 5:3:2 molar ratio. For the composition of the outer membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2. For the composition of the inner membrane leaflet, the amount of DOPE was varied from 0 to 20 mol%. The final molar ratios of the outer leaflet were POPC:cholesterol:POPS:DOPE (i) 1:1:1:0, (ii) 3:3:3:1, and (iii) 2:3:3:2.
Experimental Procedure: 30 µL of GUV-containing solution (15 µL per inlet) was flushed through the microfluidic device at 1 µL mL⁻¹ for 30 min. Eventually, GUVs were trapped between PDMS posts in the trapping area (see Figure 1c). After a washing step with buffer 1, 20 µL of both, LUVs or CDVs and buffer 2 (5 mM CaCl₂) were each added to a separate inlet to prevent aggregation prior to the experiment and withdrawn through the microfluidic device at 1 µL mL⁻¹ for 40 min. The solutions were mixed shortly before reaching the trapped GUVs in a mixer (a serpentine channel). The LUV/CDV solution with buffer 1 in one of the inlet was exchanged and withdrew with 0.1 µL mL⁻¹ for another 30 min. It was used as an incubation step and the low residual flow rate prevented the GUVs from escaping the traps. Increasing the flow rate back to 1 µL mL⁻¹ for 10 min removed unbound LUVs/CDVs. To detect lipid mixing of GUVs and LUVs, 30 µL of 20 µg mL⁻¹ Streptavidin Alexa Fluor 488 conjugate (in buffer 1) was added to the GUVs with 1 µL mL⁻¹ for 20 min. For membrane fusion with CDVs, depending on the experiment, either both staining reagents, calcein–AM (10 µM in buffer 1) and anti-VS-PE (20 ng mL⁻¹ in buffer 1) was added, or only anti-VS-PE to both inlets and withdrew with 1 µL mL⁻¹ for 30 min. To remove residual fluorophores, the GUVs were washed by flushing buffer 1 for 1 min with 1 µL mL⁻¹. Experiments were conducted at 25 °C.

Microscopy: A spinning disk confocal microscope (Visitron, Germany) was used for fluorescence (confocal) imaging. Images were taken with a 60 × 1.2 NA water immersion objective (Nikon). The spinning disk confocal microscope was equipped with 488 nm and 561 nm diode lasers to excite green and red fluorophores. 460/50 nm and 525/50 nm emission filters were used.

Image Processing: Confocal images were processed with FIJI image analysis software. Background subtracted grey values of the raw images were measured in regions of interest, e.g. GUV membranes.

Statistical Analysis: Data from the image analysis was plotted and analyzed (e.g. randomization test) using MATLAB (MathWorks). Boxplots used here indicate the median by a red line, and the edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers indicate the most extreme data points and outliers are shown as red pluses. Bar plots represent arithmetic means and the whiskers ± standard deviation. For the statistical testing of population means, a randomization test was performed, resampling all samples 1000 times for a significance level α = 5%.[61]

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
Y.R.F.S. performed the experiments and evaluated the data. L.S. prepared the cells used in the study. S.B. helped to optimize the microfluidic device and assay. Y.R.F.S., and P.S.D. developed the concept and wrote the manuscript.

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