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Giant unilamellar vesicles : an efficient membrane biophysical tool and its application in drug delivery studies

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Chapter VII

Coiled coil driven membrane fusion on GUV–LUV: biophysical model evaluated at physiological ionic strength conditions

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Introduction

Membrane fusion is a key process naturally occurring in cells. Firstly, vesicles dock at target membranes captured by proteins and tethering factors.^{1, 2} Next, those two initially separated membranes come into molecular contact and they merge forming a continuous single membrane facilitating the transport of cargo between and within cells.³ Highly specific biological processes such as neuronal exocytosis,^{4, 5} fertilization,⁶⁻⁸ and viral infection,^{9, 10} are membrane fusion driven, thus understanding and mimicking the fusion of membranes is scientifically relevant. One of the most extensively studied biological systems is the synaptic vesicle fusion system, which is controlled by a coiled-coil interaction between three complementary proteins forming the SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) complex.^{11, 12} This family of neuronal SNARE proteins involves the formation of a highly stable four-helix coiled-coil bundle which brings two opposing membranes into close proximity, followed by local disruption of the lipids and merging of the membranes.^{3, 4} Moreover, it has been proposed that SNAREs, in combination with additional proteins, trigger fusion between synaptic vesicles and the plasma membrane.^{13, 14} Because *in vivo* membrane fusion is a highly specific and controlled process, mimicking naturally occurring membrane fusion has resulted in the development of several artificial model systems that use a variety of molecules as the fusogens, such as double stranded DNA,^{15, 16} covalent motifs,¹⁷ hydrogen bonding motifs,¹⁸ and coiled-coil peptides.^{19, 20} In practice, these models have to fulfill fundamental requirements such as: specific molecular recognition to trigger the merging of membranes; leakage free content mixing; size increase in liposomal studies; and should be independent of curvature stress.^{21, 22} However, all these requirements are often not achieved by all models systems.

We use two complementary peptide amphiphiles located in different membranes to mimic some of the aspects of SNARE driven membrane fusion.^{21, 23, 24} The synthetic model is designed to fulfill all the functional aspects of native membrane fusion. Our model system is constructed from two complementary coiled-coil peptides K₄ [(KIAALKE)₄] and E₄ [(EIAALEK)₄] as recognition motifs coupled to a cholesterol anchor through a flexible polyethylene glycol linker (**Figure 1**). The formation of hetero dimeric coiled-coils by these peptides brings the two opposing membranes into close proximity, thereby inducing efficient leakage-free membrane fusion. The dimeric coiled coil acts as a molecular zipper by binding of two α -helical peptide strands, while the cholesterol anchor ensures the tight insertion of the peptides into synthetic

or cellular membranes. The efficiency of this system has been proven by performing lipid mixing and content mixing assays with liposomes, where efficient leakage-free fusion has been obtained.²¹ Extension of these vesicle fusion assays has allowed for the study of the effect of altering the number of heptad repeats in the recognition motif,²⁵ changing the lipid anchor,²⁶ and the examination of the response to temperature and pH in the membrane fusion process with our synthetic coiled-coil peptide system. In those previous studies Large Unilamellar Vesicles (LUVs), with sizes ranging between 100–200 nm, were used as a robust biophysical tool for understanding the fusion of membranes. Yet, LUV–LUV interaction exhibits an inherent high degree of membrane curvature and tension, which may have an effect in the energetics of the membrane fusion process. Moreover, the relative small size of LUVs does not allow the visualization of the budding and fusion steps in the membrane fusion process, restricting those studies to bulk measurements. In this study, we used time lapse fluorescence microscopy to monitor and provide additional insights of membrane fusion triggered by the synthetic coiled-coil peptide system at physiological ionic strength conditions. We used peptide-labeled Giant Unilamellar Vesicles (GUVs, sizes 10–20 μm), as a biophysical model of the plasma membrane of cells, and follow fusion upon the addition of LUVs bearing the complementary lipopeptide. We tested both the specific molecular recognition of the coiled-coil system by the mixing of the membranes and the mixing of the inner aqueous contents by content mixing assays in two-color fluorescence microscopy experiments (**Figure 1**).

Results and Discussion

GUVs with the lipid composition 50 mol% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 25 mol% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 25 mol% Cholesterol were prepared by hydration of hybrid lipid/DexPEG hydrogel films substrates.²⁷ The lipid mixture was supplemented with ATTO 488 DOPE ($\lambda_{\text{abs}}=501$ nm, $\lambda_{\text{fl}}=523$ nm) for fluorescence imaging in lipid mixing experiments. The use of DexPEG substrates allows the growth of GUVs in good yields, reaching physiological ionic strength conditions (see **Chapter I** and **Chapter II**), which are required for an efficient binding and formation of a dimeric coiled coil. The lipid film deposited on DexPEG substrates was hydrated with phosphate buffer solution (PBS), supplemented with CaCl_2 (1 mM), MgCl_2 (0.5 mM) and sucrose (200 mM) at room temperature. After GUV formation, the vesicles were transferred to a solution containing PBS supplemented with CaCl_2 (1 mM), MgCl_2 (0.5 mM), glucose (200 mM) and the

lipopeptide CP₄E₄ or CP₄K₄ (1 mol% with respect to the lipids). The lipopeptides are spontaneously inserted into the GUV membrane, via the cholesterol anchor (**Figure 1**), simulating the function of the transmembrane domain of SNARE proteins. Finally, peptide-labeled GUVs were transferred to a microscopy chamber where they were immobilized via streptavidin-biotin binding to the bottom of the chamber (see details in *Experimental Section*), and the integrity of GUVs was verified by fluorescence and optical microscopy (~20 μm diameter). In parallel, peptide-labeled LUVs (1 mM, ~120 nm diameter) were prepared by sonication with the same lipid composition used for GUVs, but doped with a different dye (ATTO 633 DOPE, $\lambda_{\text{abs}}=629$ nm, $\lambda_{\text{fl}}=657$ nm) in order to avoid signal overlapping of the fluorescence emission between GUVs and LUVs in the lipid mixing assay.

Next the lipid mixing assay was initiated by treating peptide labeled GUVs with 30 μL peptide labeled LUVs as represented in **Figure 1**. LUVs were directly added to a microscope chamber containing 300 μL of immobilized GUVs in PBS solution supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and glucose (200 mM). The lipid mixing was detected in time by dual color fluorescence imaging of peptide-labeled GUVs doped with ATTO 488 DOPE and peptide-labeled LUVs doped with ATTO 633. The fluorescence signal was simultaneously monitored from GUVs at 500-550 nm and from LUVs at 650-700 nm in a Leica TCS SPE confocal microscope every minute for one hour. The arrival of LUVs to GUVs was observed 15–20 minutes after LUV addition to the GUVs solution. LUV docking on GUVs was detected after 25-30 minutes of liposome arrival to the GUVs and resulted in appearance of spotty places and non-homogeneously distributed fluorescence signal on edges of the GUVs. After 30 minutes, most of the GUVs showed homogeneously distributed fluorescence signal over the entire GUV. The fluorescence became even more intense and the bilayer more homogeneous after 60 min (**Figure 2**). The presence of intact GUVs was verified by observing fluorescence in the green channel.

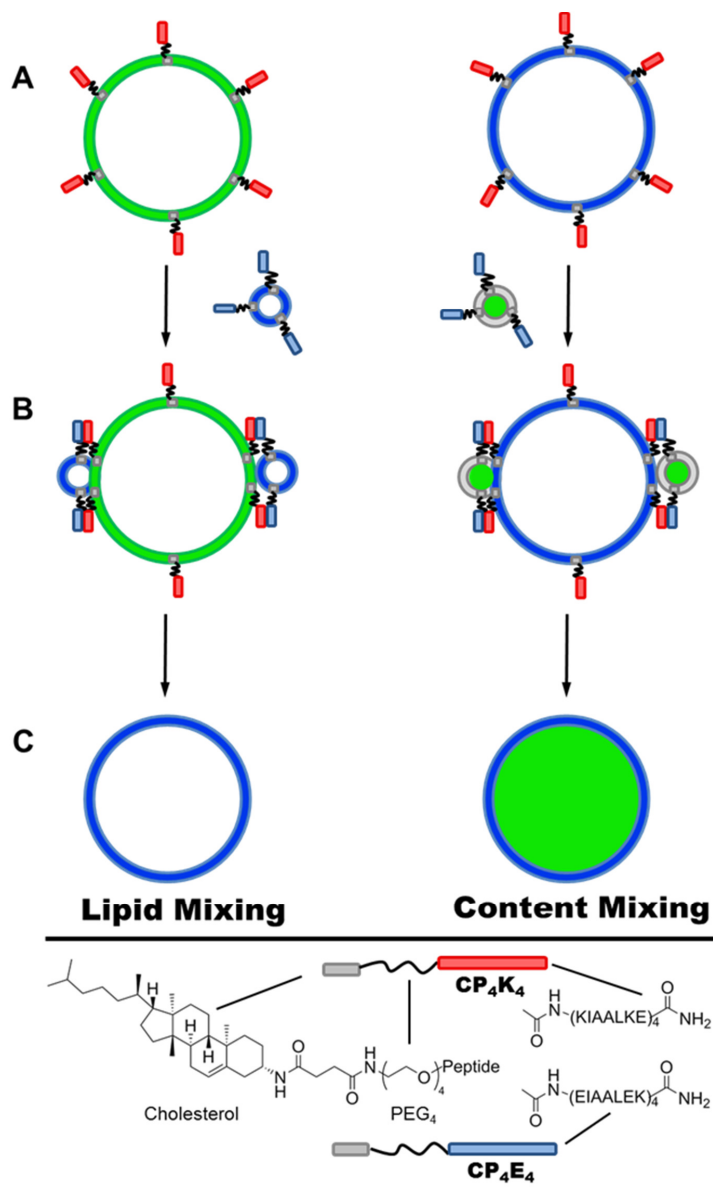


Figure 1. Schematic representation of coiled-coil peptide mediated membrane fusion between GUVs and LUVs. GUVs are labeled with lipopeptide CP₄K₄ (red) and LUVs with the lipopeptide CP₄E₄ (blue). The lipid mixing is shown in the left and in the right content mixing assay. **A**) Spontaneous incorporation of the lipidated peptide in the lipid membrane via cholesterol anchor results in the labeling of GUVs with either CP₄K₄ or CP₄E₄. **B**) Addition of CP₄E₄ labeled LUVs to CP₄K₄ labeled GUVs. The formation of a coiled-coil complex triggers the fusion of membranes. **C**) Transfer of the fluorescent lipid and mixing of inner aqueous contents in the GUV after membrane fusion with LUVs.

The mixing of non-exchangeable fluorescent lipids (ATTO 633 DOPE) in the target membrane of GUVs demonstrates the lipid mixing of membranes between LUVs and GUVs, independently of the selection of the peptide motif used in the target membrane of GUVs or LUVs, respectively. **Figure 2A** shows time lapse micrographs before the arrival (time=0 minutes) and after the arrival of CP₄E₄ peptide labeled LUVs to GUVs containing lipopeptide CP₄K₄ 10, 30 and 60 minutes after the start of the experiment. Interchanging the lipopeptides, i.e. labeling GUVs with CP₄E₄ and LUVs with CP₄K₄, is presented in the **Figure 2B**. Whereas the GUVs showed docking 10 minutes after CP₄K₄ lipopeptide insertion into the target membrane, switching the lipopeptide to CP₄E₄ showed earlier docking and full lipid mixing already after 10 minutes. This result suggests that the interaction of CP₄K₄ labeled LUVs is stronger than CP₄E₄ labeled LUVs. Similar experimental conditions were used for mixing non-labeled GUVs and non-labeled LUVs (**Figure 2C**). Some docking of LUVs was observed in the target membrane after one hour, promoted by the negative curvature of the membrane due to the presence of DOPE in the lipid composition of both GUVs and LUVs.

As an additional control experiment, the lipopeptide was omitted from the target membrane of GUVs and the experiment was performed by mixing CP₄E₄ peptide labeled LUVs (**Figure A1**, in *Annex Chapter VII*). Fluorescence imaging showed that CP₄E₄ labeled LUVs have minimal interaction with the non-labeled target membrane, similar to the lipid mixing assay in the absence of both lipopeptides. Moreover, we performed the lipid mixing assay keeping the lipopeptide CP₄K₄ in the membrane of GUVs and mixing with non-labeled LUVs (**Figure A1**, in *Annex Chapter VII*). The fluorescence imaging showed that plain LUVs strongly interact with the CP₄K₄ labeled target membrane of GUVs, transferring the fluorescent lipid (ATTO 633 DOPE) to the membrane of GUVs in approximately 30 minutes. The same peptide-membrane interaction is expected by switching peptide CP₄K₄ to LUVs and targeting to non-labeled GUVs. Those control experiments reveal that peptide K₄ has interaction with the membrane of both GUVs and LUVs. This interaction has been recently reported in lipid monolayer studies combined with surface sensitive infrared reflection absorption spectroscopy (IRRAS).²⁸ In those studies, a snorkel mechanism is proposed to enhance the hydrophobic interactions between the amphiphatic helix and the lipid monolayer.

In summary, the use of the complementary coiled-coil membrane fusion system produces efficient lipid mixing between GUVs and LUVs, similar to lipid mixing detected in GUV-LUV studies where efficient lipid mixing was observed only in the presence of the SM protein Munc18-1 when t-SNARE complexes were reconstituted in the membrane of LUVs and

GUVs.² Moreover, the imaging of lipid in GUVs is in line with the lipid mixing results reported in LUV-LUV studies, where lipid mixing was assayed by fluorescence spectroscopy.^{23, 25}

In addition to lipid mixing, the coiled-coil system was evaluated for content mixing between LUVs and GUVs (**Figure 1**, right). GUVs were prepared as described above for lipid mixing experiments, but now the lipid membrane of GUVs was doped with ATTO 633 DOPE ($\lambda_{\text{abs}}=629$ nm, $\lambda_{\text{fl}}=657$ nm). A concentrated solution of carboxyfluorescein (50 mM, pH=7) was encapsulated within LUVs by freeze-thawing, followed by extrusion, and the non-encapsulated carboxyfluorescein was removed by size exclusion chromatography.²⁹ The resulting LUVs were labeled with lipopeptides via incubation. Because the LUV solution was diluted by a factor of two after size exclusion, 60 μL of liposomes were added to the microscopy chamber to keep similar experimental conditions with respect to the lipid mixing assay. GUVs were imaged for 60 minutes, taking an image every 60 seconds, by dual fluorescence imaging. The fluorescence signal was simultaneously monitored for GUVs (633 nm laser line, filter detection 650-700 nm) and LUVs (488 nm laser line, filter detection 500-550 nm). All the experiments were performed in duplicate, imaging as many GUVs as possible for each experiment. Detailed analysis for each experiment is presented in traces of normalized fluorescence versus time (**Figures A2-A15** in *Annex Chapter VII*). The size of individual GUVs was measured directly from the micrographs before and after the membrane fusion assay and no change in the diameter of GUVs was observed during the time course of the experiments.

The content mixing experiment between CP₄K₄ labeled GUVs and CP₄E₄ labeled LUVs is presented in **Figure 3A**. A considerable background increase was detected immediately after the addition of carboxyfluorescein loaded LUVs. This background increase might be attributed to free carboxyfluorescein from the permeable membrane of highly loaded LUVs (50 mM).³⁰ Docking of LUVs to the target membrane of GUVs was observed after 10 minutes, even when the lipid membrane of LUVs was not supplemented with a fluorescent lipid for this assay. This result suggests that after the merging of LUVs with the target membrane, a fraction of inner carboxyfluorescein from LUVs is distributed along the lipid bilayer, depicting the boundaries of the GUVs (**Figure 3A**). This docking is in good agreement with the lipid mixing results obtained for this experiment. Inner content mixing was observed after 30 minutes of the addition of LUVs in some of the GUVs with small sizes (5-10 μm) and after 60 minutes in bigger GUVs (>15 μm); however the inner content mixing was not observed in all the GUVs.

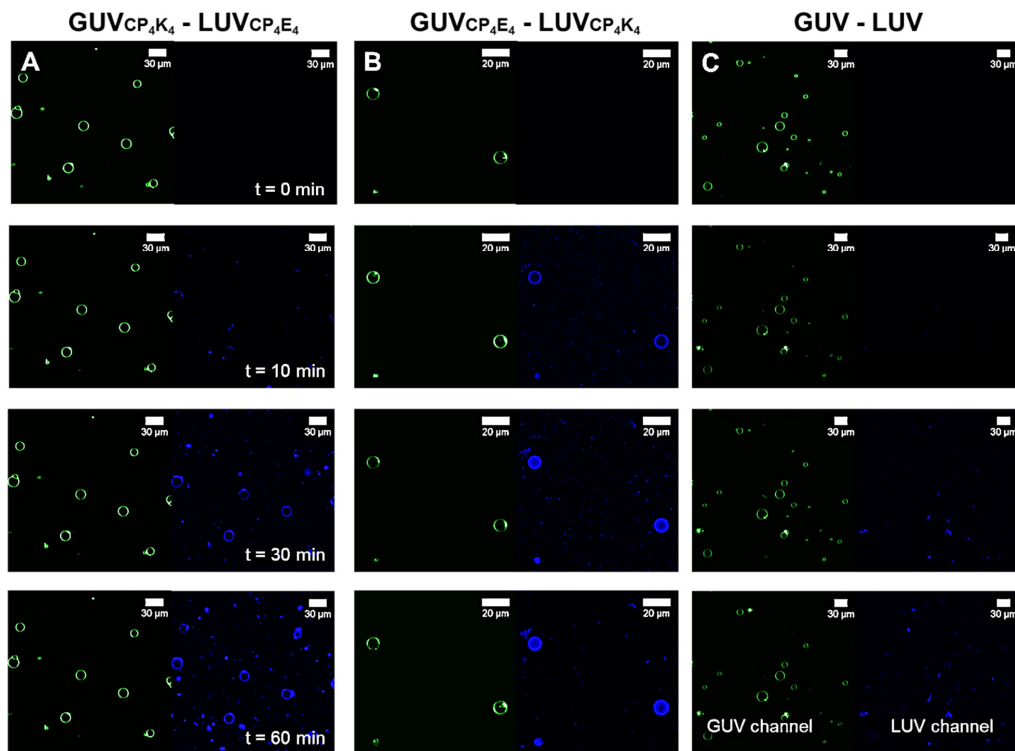


Figure 2. Time lapse micrographs of the lipid mixing assay between peptide labeled GUVs and peptide labeled LUVs before (time=0) and after (time=10, 30 and 60 minutes) appearance of LUVs in the confocal volume. The GUVs are excited at 488 nm and the emission of fluorescence is detected between 500-550 nm (green color), while LUVs are excited at 633 nm and the emission is detected between 650-700 nm (blue color). **A)** Lipid mixing assay between CP_4K_4 labeled GUVs and CP_4E_4 labeled LUVs, **B)** Lipid mixing assay between CP_4E_4 labeled GUVs and CP_4K_4 labeled LUVs and **C)** Lipid mixing assay between non-labeled GUVs and non-labeled LUVs. Imaging was performed every minute during one hour using a Leica TCS SPE microscope.

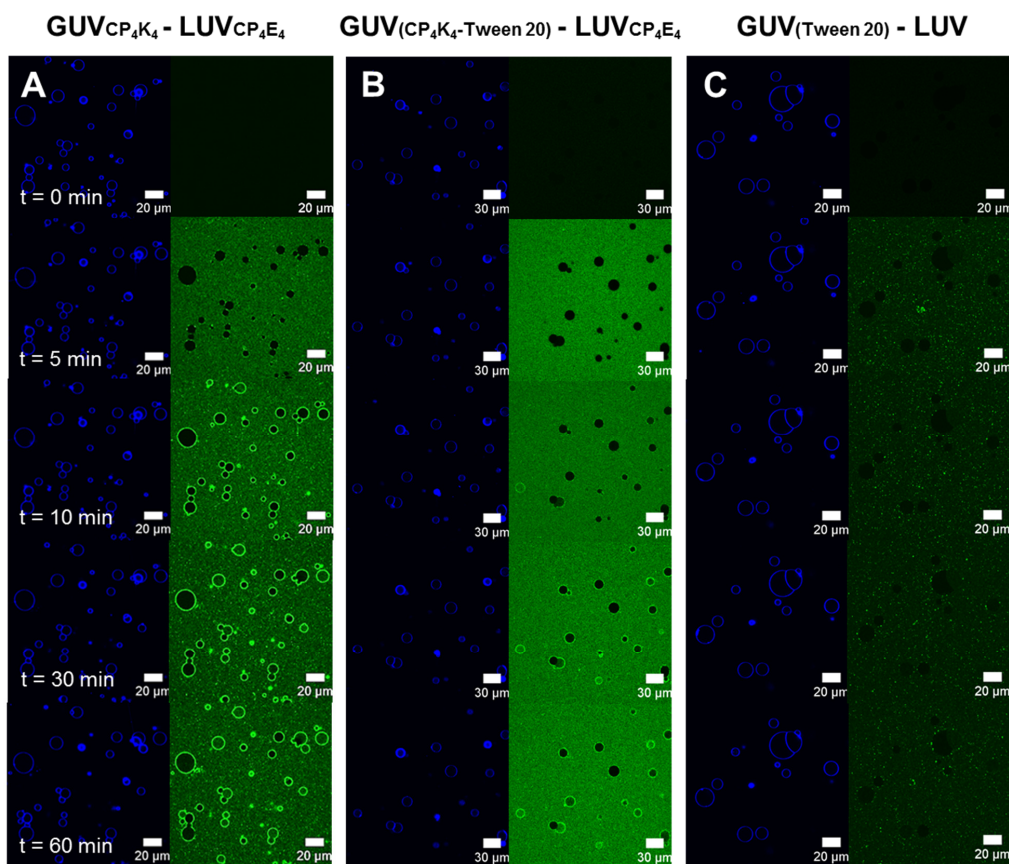


Figure 3. Time lapse micrographs of the content mixing assay between peptide-labeled GUVs and peptide-labeled LUVs before (time=0) and after (time=5, 10, 30 and 60 minutes) appearance of LUVs in the confocal volume. The GUVs are excited at 633 nm and the emission of fluorescence is detected between 650-700 nm (blue color), while LUVs are excited at 488 nm and the emission is detected between 500-550 nm (green color). **A)** Content mixing assay between CP₄K₄ labeled GUVs and CP₄E₄ labeled LUVs, **B)** Content mixing assay between, CP₄K₄-Tween 20 labeled GUVs and CP₄E₄ labeled LUVs and **C)** Lipid mixing assay between Tween 20-GUVs and non-labeled LUVs. Imaging was monitored every minute during one hour using a Leica TCS SPE microscope.

Analysis of individual GUVs after the membrane fusion showed that GUVs without content mixing exhibit large clustering of LUVs on the target membrane (*vide infra*). Our hypothesis is that CP₄K₄ creates clusters in the target membrane upon the insertion of the lipopeptide, decreasing the fusion efficiency of the peptide coiled-coil system. Polyoxyethylene (20) sorbitan monolaurate (Tween 20) is a non-ionic surfactant which has been proposed as a potential stabilizing agent for drug delivery systems because of its low toxicity,³¹ and used in

this work to decrease the clustering of CP₄K₄ improving its incorporation into the lipid membrane of GUVs. Mixtures of CP₄K₄-Tween 20 at two different concentrations of the non-ionic surfactant (0.4 and 1 mol% respect to CP₄K₄) were tested for its ability to reduce CP₄K₄ clustering in the lipid membrane of GUVs. Fluorescence microscopy images are presented in **Figure 3B** for the experiment with the mixture containing 1 mol% of Tween 20, which presented better performance in comparison to 0.4 mol% in the mixture with lipopeptide (**Figures A4-5** vs **Figures A6-7** in *Annex Chapter VII*). Clustering of LUVs detected with the mixture CP₄K₄-Tween 20 was less after 10 minutes in comparison to the experiment in the absence of Tween 20. After 60 minutes of imaging most of the small GUVs presented the mixing of inner contents while bigger vesicles showed less or no fluorescence, in good agreement with the experiment without Tween 20; however we observed higher quantity of GUVs with content mixing. We evaluated the effect of Tween 20 in the content mixing assay by incubating GUVs only with Tween 20 and performing the assay with carboxyfluorescein loaded LUVs in the absence of the coiled-coil peptide (**Figure 3C**). After 60 minutes there was no transfer of inner content detected in the interior of GUVs, showing that the addition of Tween 20 does not promote content mixing between GUV and LUVs.

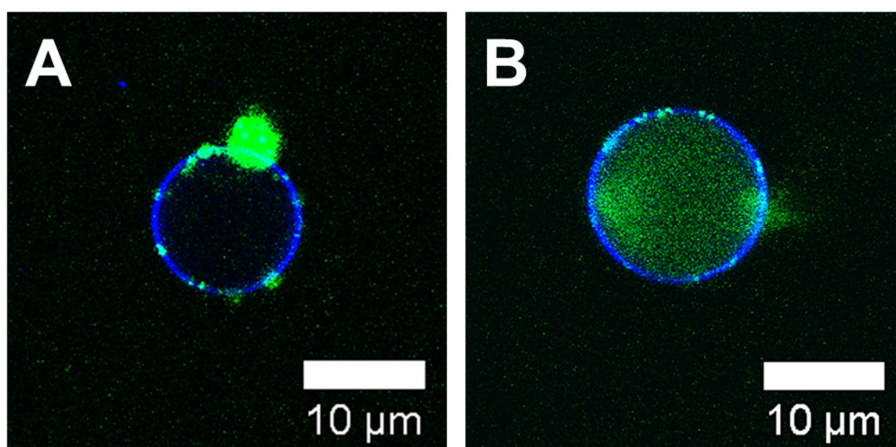


Figure 4. Overlay of fluorescence confocal microscopy images for 488 and 633 nm channels. **A)** Content mixing assay after one hour of incubation of CP₄K₄ peptide-labeled GUVs and CP₄E₄ peptide-labeled LUVs. The micrograph shows a single GUV (blue) without content mixing and large clustering of liposomes (green). **B)** Content mixing assay after one hour of incubation of CP₄K₄-Tween 20 peptide labeled GUVs and CP₄E₄ peptide labeled LUVs. The micrograph shows a single GUV (blue) with content mixing.

Furthermore, content mixing experiments without time lapse imaging between CP₄K₄ labeled GUVs and CP₄E₄ labeled LUVs in the presence and absence of the non-ionic surfactant Tween 20 were performed in microcentrifuge tubes to improve the mixing of GUVs and LUVs. After 60 minutes incubation, the GUVs were transferred from the tube to a microscope chamber containing fresh PBS solution supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and glucose (200 mM) for dual fluorescence imaging. This experiment allowed the removal of the background signal associated to carboxyfluorescein leakage from LUVs during the time lapse imaging and the imaging of single GUVs at higher magnification. The micrograph for a single CP₄K₄ labeled GUV after membrane fusion is presented in **Figure 4A** (no content mixing observed) and the micrograph of a single CP₄K₄-Tween 20 labeled GUV is presented in **Figure 4B** (content mixing observed). This result confirms that mixing CP₄K₄ and Tween 20 makes the insertion of the lipopeptide into the target membrane presumable more homogeneous, producing a positive effect in the GUVs undergoing membrane fusion.

The clustering of CPK in GUVs was further studied by dual color fluorescence correlation spectroscopy (FCS). CP₁₂K₄, which is an analogue of CP₄K₄, but has a longer linker between the peptide strand and cholesterol anchor, was fluorescently labeled with ATTO 488 dye via maleimide-thiol chemistry. The diffusion coefficient of CP₁₂K₄ in a free standing lipid bilayer was determined as a function of the amount of a lipopeptide added to the GUVs (**Figure 5**, red bars), and compared with the mobility of the lipid tracer 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine Perchlorate (DiD) (**Figure 5**, pattern red bars) in the same GUV. The diffusion coefficient of CP₁₂K₄ drops from 9 μm²/s to 6 μm²/s when the amount of CP₁₂K₄ added to the GUVs reaches 1 mol%. This concentration corresponds to the concentration used in all lipid and content mixing experiments. The drop in the CP₁₂K₄ diffusion coefficient is greater than that observed for DiD. Such behavior supports the hypothesis that CP₁₂K₄ aggregates in/on the lipid bilayer. In contrast, the diffusion coefficient of both CP₁₂E₄ (**Figure 5**, blue bars) and DiD (**Figure 5**, blue patterned bars) keeps constant while CP₁₂E₄ is added to the GUVs, indicating no clustering and homogeneous incorporation of the lipopeptide. These results are in line with the lipid and content mixing assays, which showed interaction of CPK with the bilayer but no interaction of CPE.²⁸

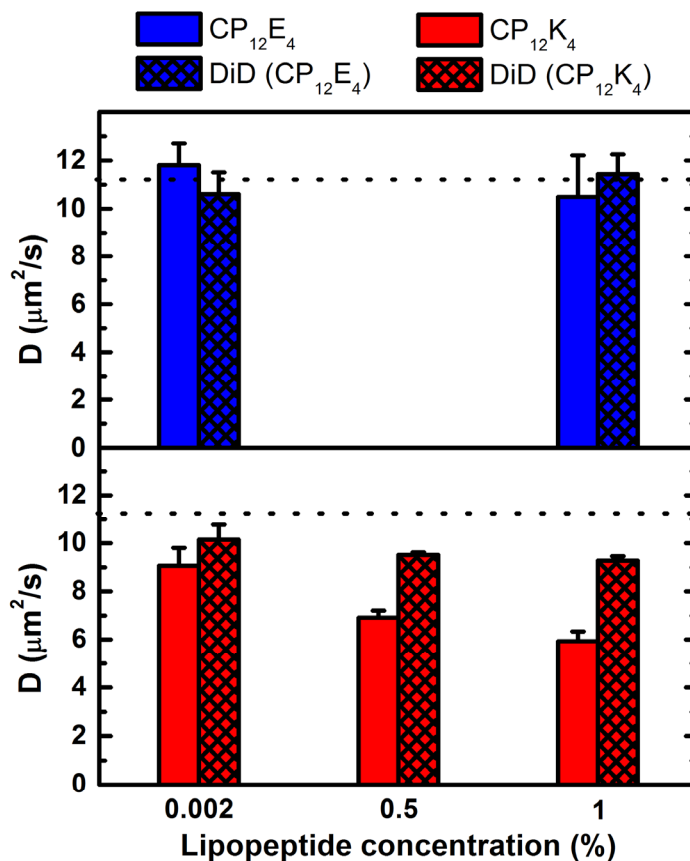


Figure 5. Diffusion coefficient of CP₁₂E₄ (blue, no pattern) and CP₁₂K₄ (red, no pattern) and membrane marker DiD (pattern) in the presence of lipopeptides for GUVs composed of DOPC/DOPE/Cholesterol (50/25/25). The black dotted line shows the reference value of the diffusion coefficient of DiD in GUVs with above mentioned lipid composition, but without any lipopeptide. Fluorescent lipopeptides labeled with Atto 488 were mixed with unlabeled lipopeptides to lipid ratio 1/50 000.

The effect of Tween 20 on the incorporation of CP₁₂K₄ into the lipid membrane is presented in **Figure 6**, as a function of the concentration of CP₁₂K₄. It has already been mentioned in the previous paragraph that diffusion of CP₁₂K₄ is slowed down significantly at 1 mol% lipopeptide concentration in the GUVs membrane. Using a mixture of CP₁₂K₄ and Tween 20 for the labeling of GUVs leads to slightly slower diffusion of both CP₁₂K₄ and DiD in comparison to the experiment in the absence of Tween 20. This finding might support the hypothesis that

Tween 20 improves the incorporation of CP₄K₄ into the GUVs. This effect may be more evident for the lipopeptide CP₄K₄, which is more hydrophobic due to a shorter PEG linker.

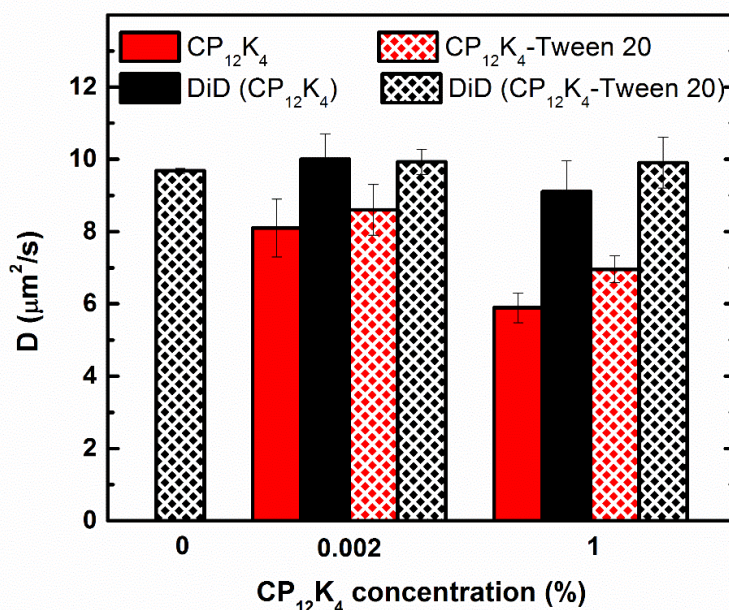


Figure 6. Diffusion coefficient of CP₁₂K₄ (red, no pattern), CP₁₂K₄-Tween 20 (red, pattern) and membrane marker DiD (black) in GUVs composed of DOPC/DOPE/Cholesterol (50/25/25). Tween 20 concentration is 0.01% of total lipid (Tween 20 1 mol% of 1 mol% CP₁₂K₄) in all CP₁₂K₄-Tween 20 samples.

In addition, several control experiments were performed to validate coiled-coil driven membrane fusion in the presence of Tween 20 (**Figures A8-13** in *Annex Chapter VII*). i) Removing CP₄K₄ from LUVs produced similar results to the experiment between non-labeled GUVs and non-labeled LUVs with minimal docking and no transfer of the aqueous content from LUVs to GUVs. On the other hand, docking of non-labeled LUVs on the target membrane of CP₄K₄-Tween 20 labeled GUVs was visible, which is in good agreement with the lipid mixing experiment. However, visual content mixing was not obvious due to a high background fluorescence originating from the liposomes. Development of fluorescence in time showed a

similar profile inside and outside GUVs (**Figures A10-11** in *Annex Chapter VII*). ii) GUVs previously incubated only with Tween 20, were transferred to a microscope chamber, followed by addition of carboxyfluorescein. The final concentration of carboxyfluorescein in the chamber was adjusted to 1 μM , which gives similar background fluorescence to the background detected in the membrane fusion experiments. Quantification of fluorescence inside individual GUVs showed that there was no leakage of carboxyfluorescein to the interior of the GUVs 60 minutes after the experiment was started (**Figures A14-15** in *Annex Chapter VII*). This result confirms that Tween 20 does not alter the GUV membrane properties. On the other hand, the strong interaction of CP₄K₄ with lipid bilayer may cause destabilization of the target lipid membrane. Those finding suggest that CP₄E₄ and CP₄K₄ are necessary for efficient content mixing and the mixture CP₄K₄-Tween 20 leads to an increase of GUVs with observable content mixing events. Therefore surfactant-lipopeptides interactions should be considered in further studies for understanding these interactions.

Conclusion

In conclusion we successfully visualized coiled-coil driven membrane fusion under physiological conditions. The use of GUVs as a target membrane allowed the visualization of the first steps of liposome docking and membrane fusion by dual color fluorescence microscopy. Time lapse imaging resulted in additional clues to the mechanism of membrane fusion, enabling optimization of the fusion efficiency under conditions that can be applied in the future to obtain fusion between liposomes and cells. Aggregation of CPK lipopeptides was observed in the membrane of GUVs, thereby decreasing the efficiency of membrane fusion. The use of Tween 20 as a co-surfactant solubilizing CPK improved the incorporation of the lipopeptide in the GUV membrane without affecting the properties of the membrane and improving the efficiency of the coiled-coil mediated membrane fusion between LUVs and GUVs. These results raise the hope that the coiled-coil membrane fusion system can be applied as a fast and efficient drug delivery system to cells *in vitro* and *in vivo*.

Experimental Section

Chemicals.

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl) (sodium salt) (DOPE-Biotin), were purchased from Avanti Polar Lipids. Cholesterol (CH), Bovine Serum Albumin (BSA), biotin-labeled bovine albumin (Biotin-BSA), Streptavidin from Streptomyces avidinii, Poly(ethylene glycol) sorbitan monolaurate (Tween 20), Calcium chloride anhydrous (CaCl₂), Magnesium chloride hexahydrate (MgCl₂·6 H₂O), 5(6)-carboxyfluorescein, Sodium hydroxide (NaOH), sucrose and glucose were purchased from Sigma-Aldrich. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-ATTO 488 (ATTO 488 DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-ATTO 633 (ATTO 633 DOPE), ATTO 488 maleimide and ATTO 655 maleimide were purchased from ATTO-TEC GmbH. Fmoc-protected amino acids were purchased from Novabiochem and Biosolve. Sieber amide resin was purchased from Agilent Technology. Phosphate Buffered Saline (PBS, pH 7.4) was supplemented with CaCl₂ (1 mM) and MgCl₂ (0.5 mM) for all GUV studies. Lipid stock solutions of DOPC:DOPE:CH (50:25:25 molar ratio, 1 and 14 mM) were prepared in chloroform.

General methods for liposomes and GUV studies

Synthesis of lipopeptides CP₄K₄ and CP₄E₄.

The spacer N₃-PEG₄-COOH, cholesteryl-4-amino-4-oxobutanoic acid, and the lipopeptides CP₄K₄ and CP₄E₄ were synthesized and utilized following procedures previously reported.^{21, 26, 32} The peptide segments E₄: NH₂-(EIAALEK)₄-CONH₂ and K₄: NH₂-(KIAALKE)₄-CONH₂ were synthesized using standard Fmoc chemistry on a peptide synthesizer (CEM-Liberty 1) and the spacer N₃-PEG₄-COOH was coupled to the N-terminus of the peptide segments. Next, the azide terminal group (N₃) on the spacer (PEG₄) was reduced to obtain an N-terminal free amine using 8 eq. of P(CH₃)₃ (1 M in toluene) in dioxane/H₂O (4:1 mixture). In the final step cholesteryl-4-amino-4-oxobutanoic acid (cholesterol anchor) was coupled to the terminal free amine in the PEG₄ spacer using 5 eq. of DIPEA and 4 eq. of PyBOP in DMF:DCM (2:1 mixture) over 72 h. Finally, the lipopeptides were purified by RP-HPLC with a Gemini C4 column to yield the pure product (Yield: 20-25%). CP₄K₄: LC-MS m/z Calcd. [1867.8

$M+2H]^{2+}$, found 1868.2. Calcd. $[1245.6 M+3H]^{3+}$, found 1246.2. Calcd. $[934.4 M+4H]^{4+}$, found 934.5). CP₄E₄: LC-MS m/z Calcd. $[1869.5 M+2H]^{2+}$, found 1869.9. Calcd. $[1246.7 M+3H]^{3+}$, found 1247.1. Calcd. $[935.2 M+4H]^{4+}$, found 935.5.

Synthesis of lipopeptides CP₁₂K₄ and CP₁₂E₄.

The peptide segments E₄: NH₂-(EIAALEK)₄-GC-CONH₂ and K₄: NH₂-(KIAALKE)₄-GC-CONH₂ were synthesized using standard Fmoc chemistry on a peptide synthesizer (CEM-Liberty 1). After synthesis, the resin was washed with DMF and Fmoc-PEG₁₂-COOH (1.1 equivalents) was coupled to the N-terminus of the peptides using 3 equivalents of HCTU and 4 equivalents of DIPEA. The reaction was left to proceed overnight. The resin was then washed with DMF, before the N-terminus was Fmoc deprotected using a 20% piperidine in DMF solution. Deprotection was achieved by incubating the resin with the piperidine/DMF solution for 10 minutes. This process was repeated 3 times, after which the resin was washed with DMF. Cholesteryl hemisuccinate was coupled using the same methodology as for the PEG coupling, (3 equivalents of HCTU and 4 equivalents of DIPEA, overnight reaction). The resin was washed with DMF, followed by DCM, before the product was cleaved from the resin using a mixture of TFA:TIPS:EDT (95:2.5 :2.5). The cleavage solution was left for one hour before the peptide was precipitated in ice-cold diethyl ether. The peptide was collected by centrifugation before being dissolved in water and freeze dried. Finally, the lipopeptides were purified by RP-HPLC with a Gemini C4 column to yield a pure product (Yield: 20-25%). CP₁₂K₄: MALDI-TOF m/z Calcd. $[4260 M+H]^+$, found 4260. CP₁₂E₄: MALDI-TOF m/z Calcd. $[4264 M+H]^+$, found 4270.

Fluorescent lipopeptides CP₁₂K₄-Atto488 and CP₁₂E₄-Atto488.

Lipopeptides were fluorescently labeled with ATTO 488 dye via maleimide-thiol reaction. Cysteine N-terminated lipopeptides (1 mg) were dissolved in 1 mL DCM and 1.3 fold molar excess of ATTO maleimide dye solution (1 mg/mL) was added to the reaction vessel. The reaction mixture was stirred for 1 hour protected from the light. Then the solvent was evaporated in vacuum and the lipopeptides were purified by RP-HPLC with a Gemini C4 column. CP₁₂K₄-Atto488: MALDI-TOF m/z Calcd. $[4970 M+H]^+$, found 4970. CP₁₂E₄-Atto488: MALDI-TOF m/z Calcd. $[4975 M+H]^+$, found 4986.

Formation of GUVs.

Giant Unilamellar Vesicles (GUVs) were grown on Dex-PEG hydrogel (1:1 molar ratio) coated microscope glass slide substrates as described previously.²⁷ Lipid solution (10 μ L) with the lipid composition DOPC:DOPE:CH (2:1:1 molar ratio, 14 mM), DOPE-Biotin (0.2 mol% relative to [lipids]) and ATTO 488 DOPE (0.1 mol% relative to [lipid]) for lipid mixing experiments or ATTO 633 DOPE (0.1 mol% relative to [lipid]) for content mixing experiments was deposited on a hydrogel coated glass slide. Next the chloroform was evaporated under a gentle stream of N₂ and placed in a vacuum oven overnight at 40 °C. A liquid chamber was made by placing a glass O-Ring (diameter = 15 mm and height = 3 mm, Agar Scientific) on top of the hydrogel and sealed with high vacuum silicon grease. The lipid film was hydrated by adding 400 μ L of PBS supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and sucrose (200 mM) into each chamber and the GUVs were formed overnight at room temperature.

Labeling of GUVs with lipopeptides CP₄K₄ and CP₄E₄.

GUVs were labeled with 1 mol% CP₄K₄ or CP₄E₄ (relative to [lipid]). A stock solution of CP₄K₄ or CP₄E₄ (15 μ L, 50 μ M in CH₃OH:CHCl₃ 1:1) was dried by evaporating the solvent under a gentle stream of N₂ and placed in a vacuum oven overnight at 40 °C. The lipopeptide film was hydrated by adding 700 μ L of PBS supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and glucose (200 mM), vortexed and transferred to a micro centrifuge tube. Subsequently 300 μ L of the solution with free floating GUVs was transferred into the micro centrifuge tube containing the CP₄K₄ aqueous solution. The GUVs were incubated during 60 minutes in the lipopeptide solution and finally 300 μ L of GUVs were transferred to a microscopy chamber for experiments.

Labeling of GUVs with lipopeptide CP₄K₄ and Tween 20.

GUVs were labeled with a mixture of CP₄K₄ (1 mol% relative to [lipid]) and depending on the experiment, 0.4 or 1 mol% of Tween-20 with respect to CP₄K₄. A stock solution of CP₄K₄ (15 μ L, 50 μ M in CH₃OH:CHCl₃ 1:1) and Tween 20 (5.6 μ L or 14 μ L, 0.001 mM in CH₃OH) were mixed and dried by evaporating the solvent under a gentle stream of N₂. Next, the lipid film was placed in a vacuum oven overnight. The CP₄K₄-Tween 20 film was hydrated by adding 700 μ L of PBS supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and glucose (200 mM), vortexed and transferred to a micro centrifuge tube. Next 300 μ L of a free floating GUVs solution was transferred into the micro centrifuge tube containing the mixture CP₄K₄-Tween

20 mixture. The GUVs were incubated during 60 minutes in this solution and finally 300 μL of GUVs were transferred to a microscopy chamber for experiments.

Formation of liposomes with lipopeptides CP₄K₄ or CP₄E₄ for lipid mixing experiments.

Peptide labeled Large Unilamellar Vesicles (LUVs) were formed with 1 mol% CP₄K₄ or CP₄E₄ (relative to total [lipid]). For this a lipid solution (1 mL) with the lipid composition DOPC:DOPE:CH (2:1:1 molar ratio, 1 mM), ATTO 633 DOPE (0.5 mol% relative to [lipid]) and lipopeptides CP₄K₄ or CP₄E₄ stock solution (50 μM in CH₃OH:CHCl₃ 1:1). The lipid solution was dried by evaporation of the organic solvents under a gentle stream of N₂ and placed in a vacuum oven overnight at 40 °C. The lipid film was hydrated by addition of 1 mL of PBS supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and sucrose (200 mM) for lipid mixing experiments with GUVs. Finally, the LUVs were formed by sonication at room temperature for 2-4 minutes with an average diameter of 120 nm determined by dynamic light scattering (Zetasizer Nano-S, Malvern).

Formation of carboxyfluorescein loaded liposomes with lipopeptides CP₄K₄ or CP₄E₄ for content mixing experiments.

Large Unilamellar Vesicles (LUVs) were labeled with 1 mol% CP₄K₄ or CP₄E₄ (relative to total [lipid]). A lipid solution (1mL) with the lipid composition DOPC:DOPE:CH (2:1:1 molar ratio, 1 mM) and ATTO 633 DOPE (0.5 mol% relative to [lipid]). The lipid solution was dried by evaporation of the organic solvents under a gentle stream of N₂ and placed in a vacuum oven overnight at 40 °C. The lipid film was hydrated by the addition of 1 mL of carboxyfluorescein (50 μM) in PBS solution supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and sucrose (200 mM). The LUVs were formed by extrusion (0.4 μm polycarbonate membrane) with 17 passes in a mini extruder with 250 μL syringes. Free carboxyfluorescein was separated from the liposome-encapsulated carboxyfluorescein by size exclusion using a Sephadex column (2.5 mL) eluting with PBS solution supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and glucose (200 mM). Liposome formation was verified by dynamic light scattering. Next the LUVs were labeled with 1 mol% CP₄K₄ or CP₄E₄ (relative to [lipid]). A stock solution of CP₄K₄ or CP₄E₄ (200 μL , 50 μM in CH₃OH:CHCl₃ 1:1) was dried by the evaporation of the organic solvent under a gentle stream of N₂ and placed in a vacuum oven overnight at 40 °C. The lipopeptide film was hydrated by the addition of 200 μL of PBS supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and glucose (200 mM). Subsequently, the

solution of LUVs (~ 2.5 mL) was transferred into the micro centrifuge tube containing the CP₄K₄ or CP₄E₄ aqueous solution and vortexed. The LUVs were incubated for 60 minutes in the lipopeptide solution and used immediately in content mixing experiments with GUVs.

Lipid and content mixing assays LUVs - GUVs.

The visualization of GUVs after lipopeptide labeling was achieved with a microscopy chamber (μ -Slide 8 well, Ibidi) which was pre-treated firstly with an aqueous mixture of BSA (0.9 mg/mL) and biotin-BSA (0.1 mg/mL) for 30 minutes, with streptavidin (0.1 mg/mL) for another 30 minutes and finally rinsed with water. Next 100 μ L of PBS solution supplemented with CaCl₂ (1 mM), MgCl₂ (0.5 mM) and glucose (200 mM) and 200 μ L of the solution with peptide labeled GUVs were transferred into the microscopy visualization chamber. The GUVs were left to sediment for at least 30 minutes before imaging. During imaging of the GUVs in a time lapse experiment, either 30 μ L of peptide labeled LUVs for lipid mixing experiment or 60 μ L of peptide labeled LUVs loaded with carboxyfluorescein for content mixing experiments were added to the microscopy well and the imaging was performed during 120 minutes after the arrival of LUVs to the bottom of the microscopy chamber.

Imaging of the GUVs and data analysis.

Imaging of GUVs was performed on a Leica TCS SPE confocal microscope system. Illumination was provided by a solid state laser using 488 nm laser line (15% laser power) for irradiation of carboxyfluorescein and ATTO 488 DOPE, detection 500-550 nm and 635 nm laser line (15% laser power) for irradiation of ATTO 633 DOPE, detection 650-700 nm. Fluorescence confocal microscopy was carried out using either 40 \times air or 63 \times water objectives. The analysis of the images was performed in ImageJ software,³³ by measuring the average intensity of an area corresponding to one GUV for the series of time lapse microscopy image frames.

Z-Scan Fluorescent Correlation Spectroscopy (z-scan FCS).

FCS measurements were performed on an inverted home-built confocal microscope (IX71 Olympus, Hamburg, Germany). The excitation was achieved by two pulsed diode lasers at 470 nm (LDH-P-C-470) and 635 nm (LDH-D-C-635) produced by PicoQuant, Germany. The laser light (approx. 10 μ W intensity in front of the objective) was pulsed alternatively in order to avoid artefacts caused by signal bleed-through. The emitted light was detected by two single

photon avalanche diodes using 515/50 and 697/58 band pass filters (Chroma Rockingham, VT). Z-scan measurements were performed on the top of a selected vesicle. The membrane was vertically scanned in 15 steps spaced 200nm apart from each other. A measurement at each point took 60s.

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Annex

Chapter VII

Full Data sets of lipid and content mixing assays.

Time lapse lipid mixing control experiments between GUVs and liposomes.

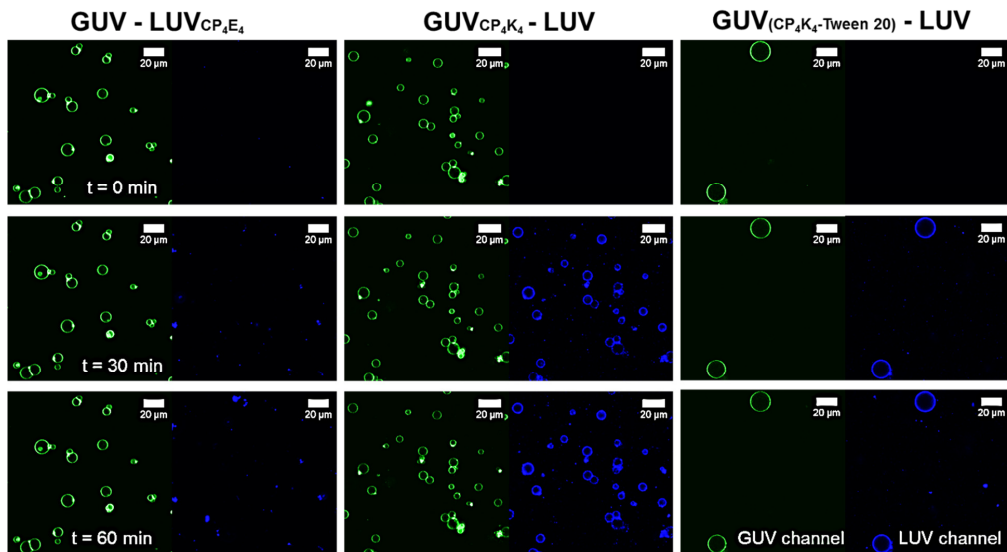


Figure A1. Time lapse micrographs of the lipid mixing control assay between GUVs and LUVs before (time=0) and after (time=30 and 60 minutes) appearance of LUVs in the confocal volume. The GUVs are excited at 488 nm and the emission of fluorescence is detected between 500-550 nm (green color), while LUVs are excited at 633 nm and the emission is detected between 650-700 nm (blue color). A) Lipid mixing assay between GUVs and CP_4E_4 labeled LUVs, B) Lipid mixing assay between CP_4K_4 labeled GUVs and non-labeled LUVs and C) Lipid mixing assay between CP_4K_4 -Tween 20 labeled GUVs and CP_4E_4 labeled LUVs. Imaging was performed every minute during one hour using a Leica TCS SPE microscope.

Datasets for the time lapse content mixing experiments between GUVs and liposomes.

Content mixing between CP_4K_4 labeled GUVs and CP_4E_4 labeled LUVs loaded with carboxyfluorescein.

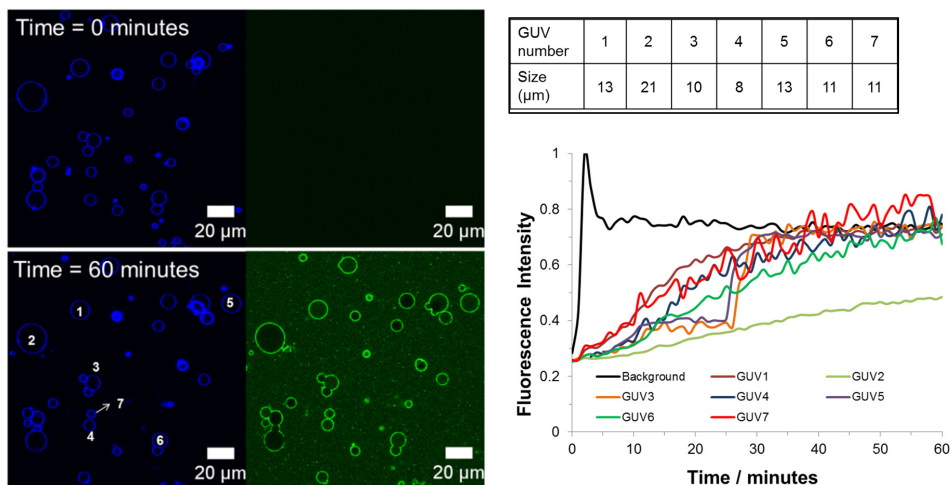


Figure A2. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP_4E_4 liposomes to CP_4K_4 GUVs . GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs .

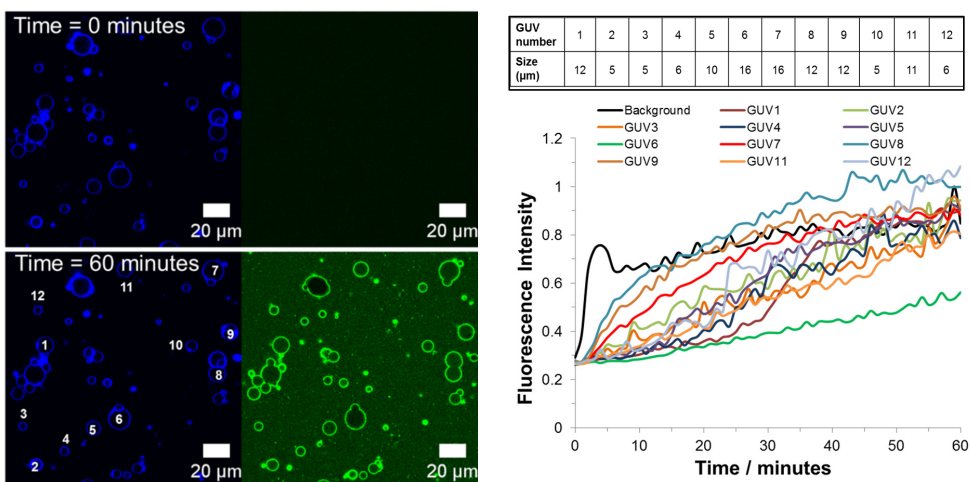


Figure A3. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP_4E_4 liposomes to CP_4K_4 GUVs . GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs .

Content mixing between 0.4 mol% Tween 20-CP₄K₄ labeled GUVs and CP₄E₄ labeled LUVs loaded with carboxyfluorescein.

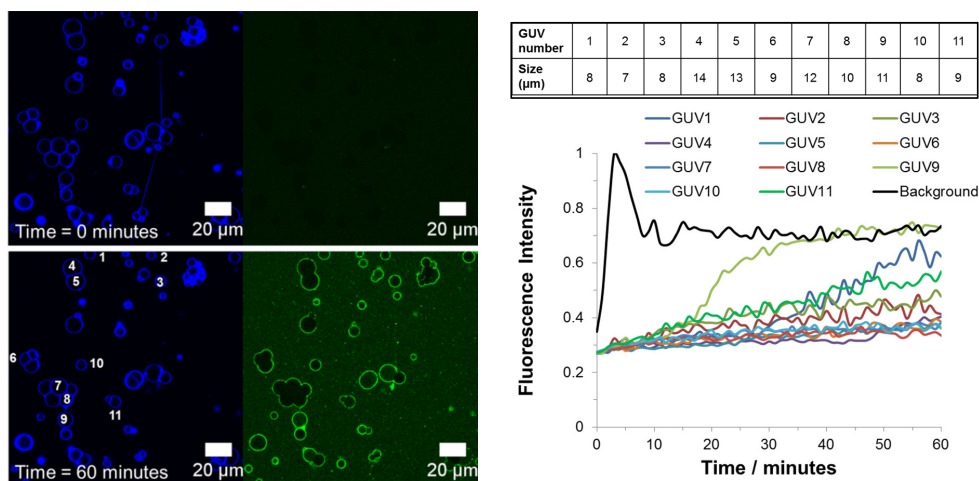


Figure A4. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

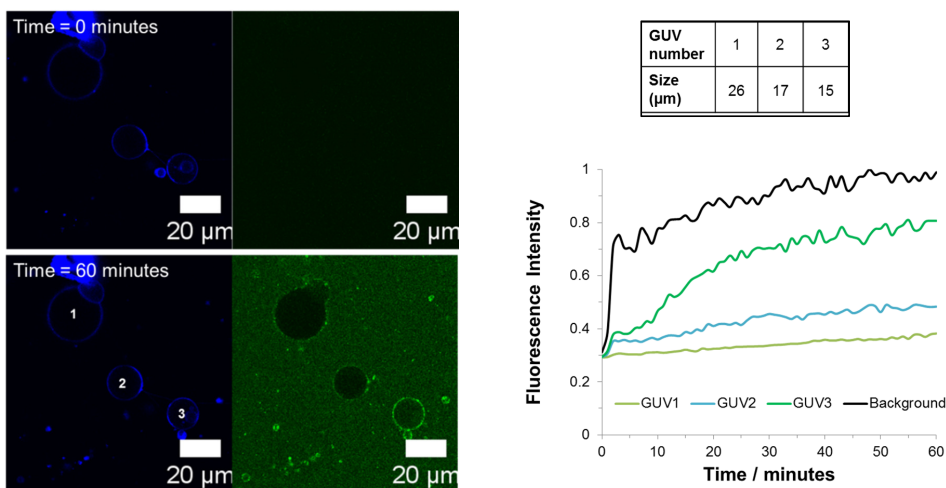


Figure A5. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

Content mixing between 1 mol% Tween 20-CP₄K₄ labeled GUVs and CP₄E₄ labeled LUVs loaded with carboxyfluorescein.

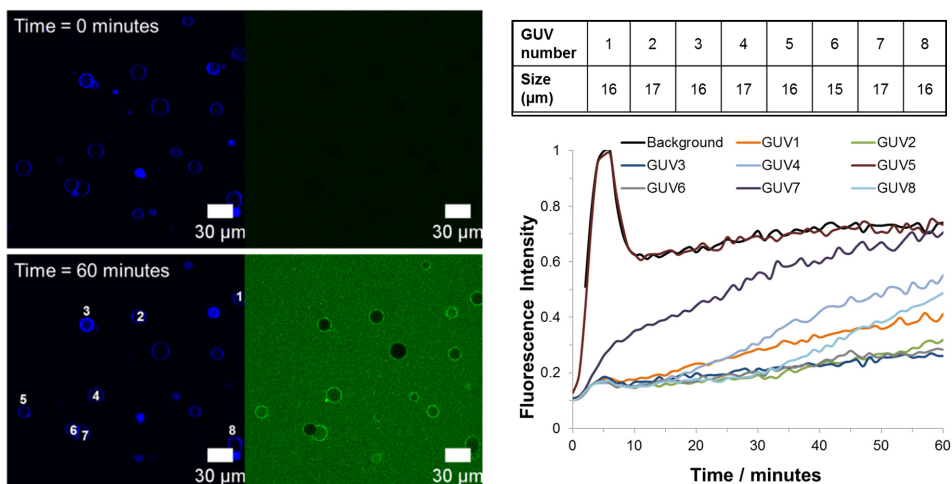


Figure A6. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

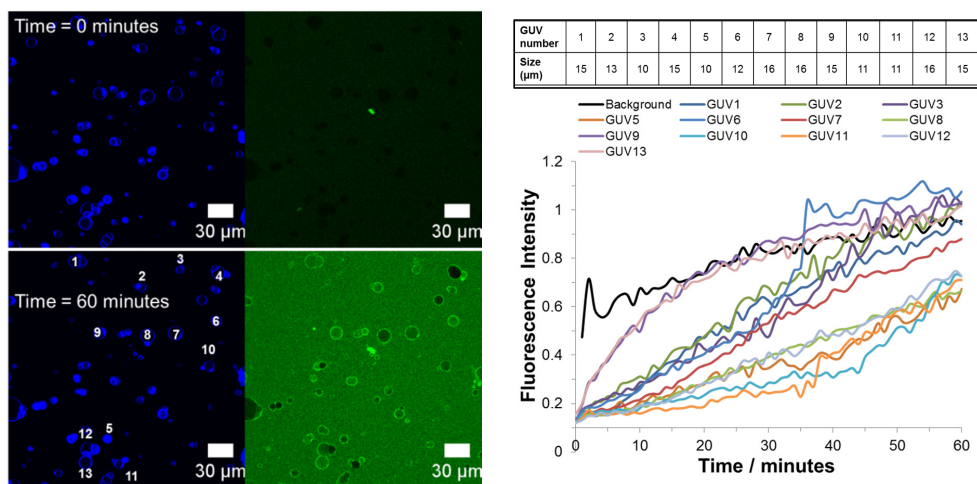


Figure A7. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

Datasets for the time lapse control experiments between 1 mol% Tween GUVs and LUVs.

Control experiment between 1 mol% Tween GUVs and LUVs loaded with carboxyfluorescein.

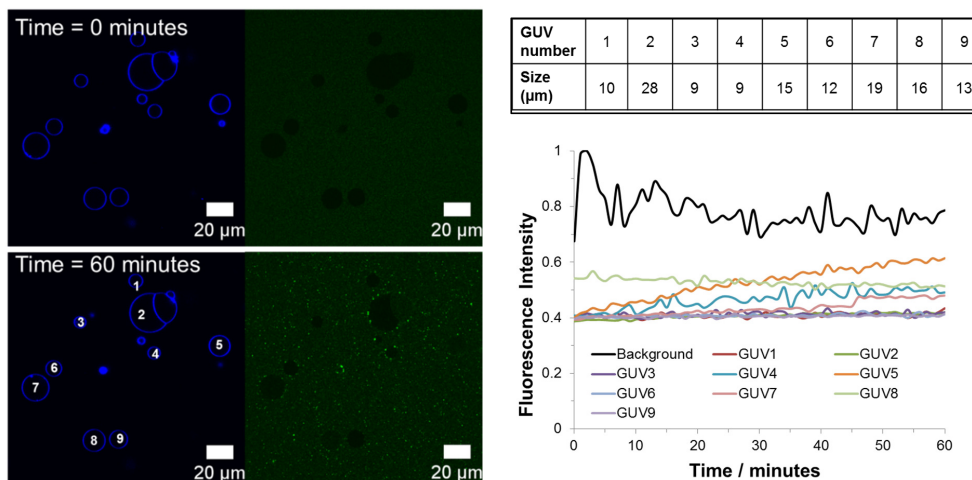


Figure A8. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

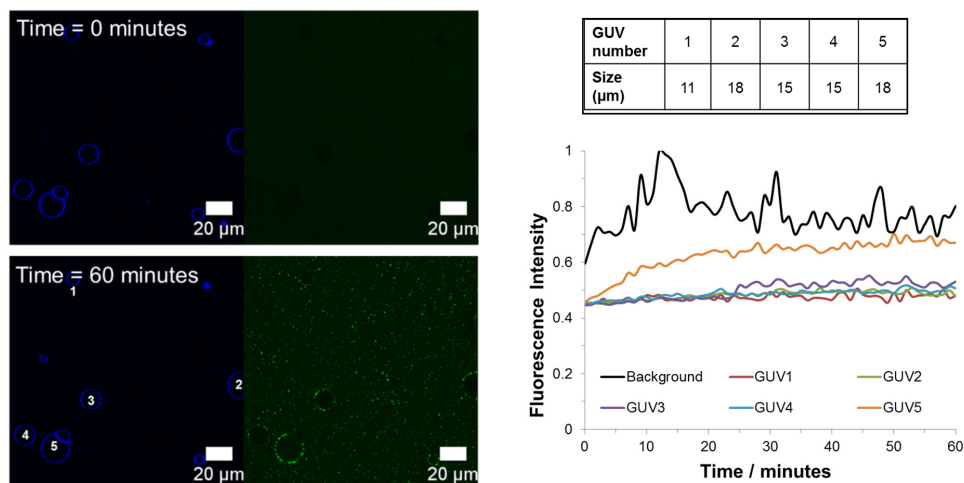


Figure A9. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

Control experiment between 1 mol% Tween-CP₄K₄ labeled GUVs and LUVs loaded with carboxyfluorescein.

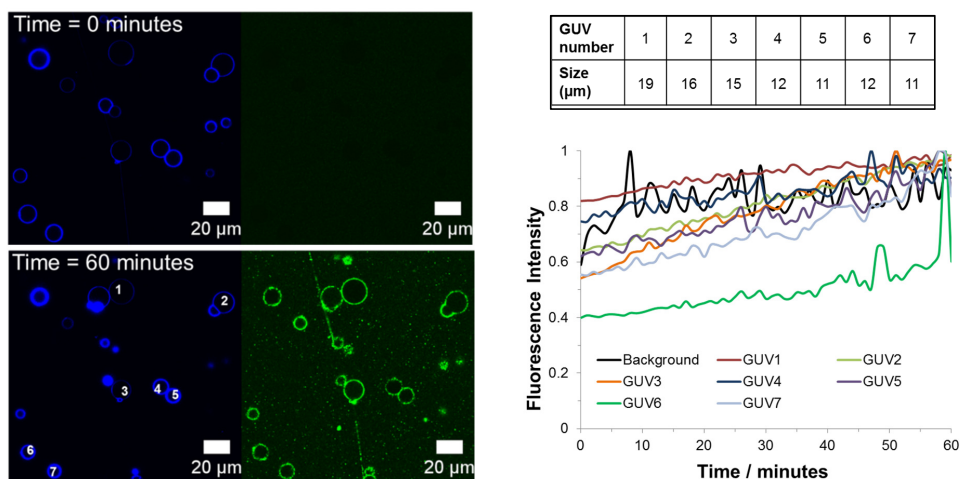


Figure A10. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

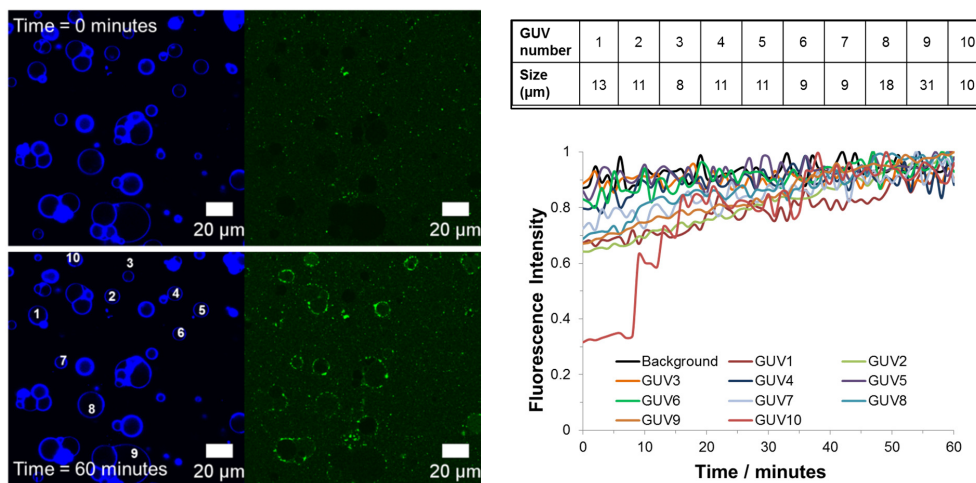


Figure A11. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

Control experiment between 1 mol% Tween GUVs and CP₄E₄ LUVs loaded with carboxyfluorescein.

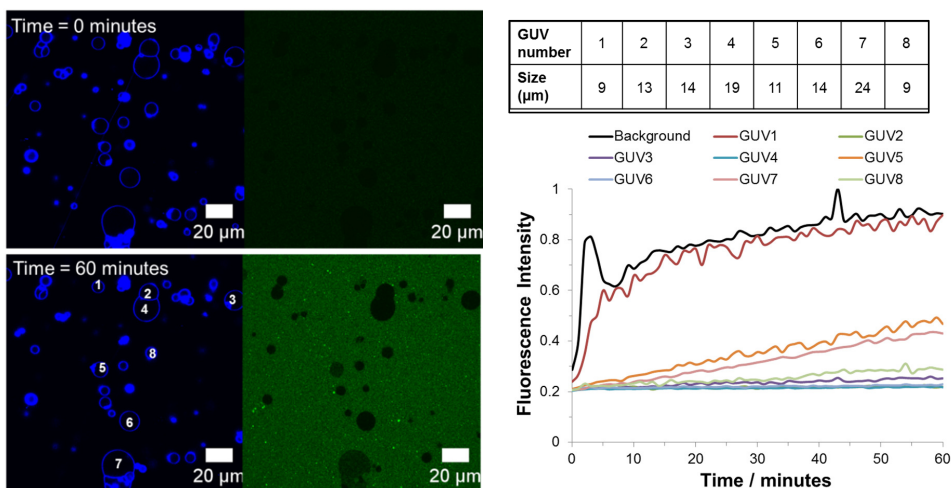


Figure A12. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

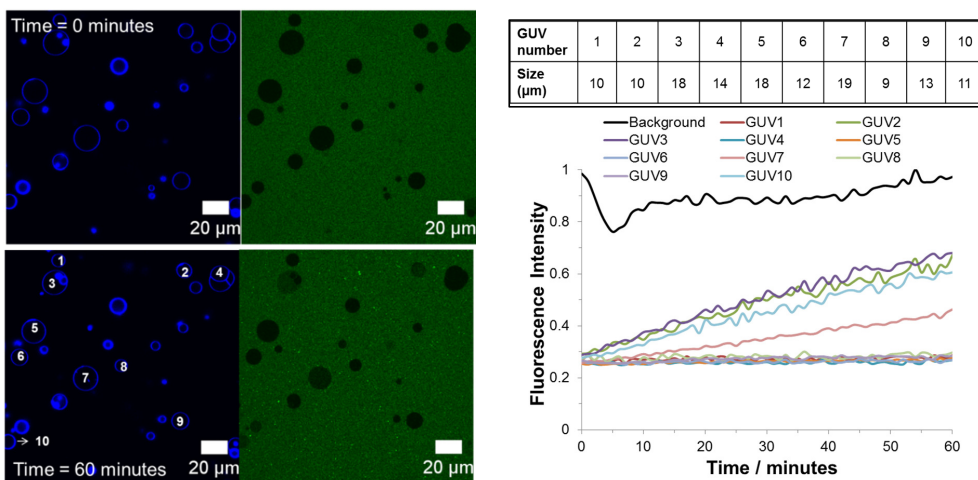


Figure A13. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP₄E₄ liposomes to CP₄K₄ GUVs. GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs.

Datasets for the time lapse leakage of carboxyfluorescein into 1 mol% CP_4K_4 -Tween 20 labeled GUVs and LUVs .

Leakage of 1 μM carboxyfluorescein into 1 mol% Tween- CP_4K_4 labeled GUVs .

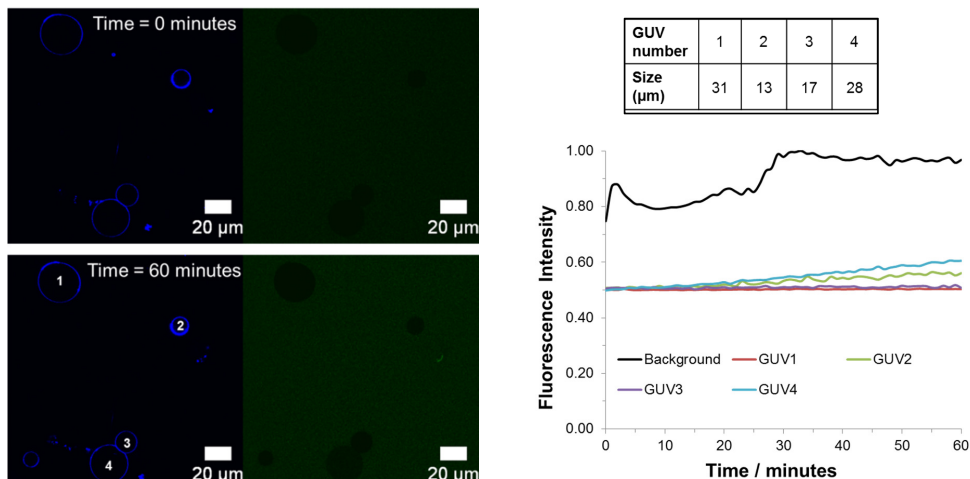


Figure A14. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP_4E_4 liposomes to CP_4K_4 GUVs . GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs .

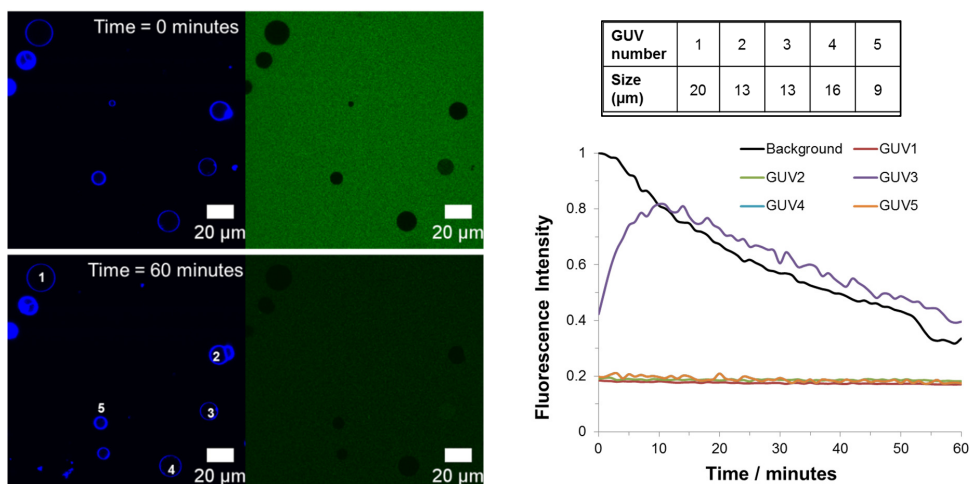


Figure A15. Fluorescence micrographs before (top) and after (bottom) addition of carboxyfluorescein loaded CP_4E_4 liposomes to CP_4K_4 GUVs . GUVs lipid membranes are supplemented with ATTO 633 DOPE (blue) and LUVs are loaded with carboxyfluorescein (green). In the right, normalized fluorescence intensity profiles over time for individual numbered GUVs and average background and in the upper panel the average diameter over time for GUVs .
