Received: 2 March 2021 | Revised: 22 March 2021 | Accepted: 22 March 2021

DOI: 10.1002/psc.3327

RESEARCH ARTICLE

Journal of PeptideScience WILEY

Membrane fusion mediated by peptidic SNARE protein analogues: Evaluation of FRET-based bulk leaflet mixing assays

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: SFB 803 (project B05)

Peptide-mediated membrane fusion is frequently studied with in vitro bulk leaflet mixing assays based on Förster resonance energy transfer (FRET). In these, customized liposomes with fusogenic peptides are equipped with lipids which are labeled with fluorophores that form a FRET pair. Since FRET is dependent on distance and membrane fusion comes along with lipid mixing, the assays allow for conclusions on the membrane fusion process. The experimental outcome of these assays, however, greatly depends on the applied parameters. In the present study, the influence of the peptides, the size of liposomes, their lipid composition and the liposome stoichiometry on the fusogenicity of liposomes are evaluated. As fusogenic peptides, soluble *N*-ethylmaleimide-sensitive-factor attachment receptor (SNARE) protein analogues featuring artificial recognition units attached to the native SNARE transmembrane domains are used. The work shows that it is important to control these parameters in order to be able to properly investigate the fusion process and to prevent undesired effects of aggregation.

KEYWORDS
leaflet mixing assays, membrane fusion, proteoliposome aggregation, SNARE mimetics

1 | INTRODUCTION

The fusion of lipid membranes can be brought along by the specific interaction of artificial fusion peptides. These peptides resemble SNARE proteins, which are part of the native eukaryotic fusion machinery. However, they have a simpler structure by only maintaining key functional elements. Liposomes with reconstituted peptides or peptidomimetics (proteoliposomes) represent a frequently used experimental system to mimic protein-mediated fusion in a simplified in vitro setting. The liposomes contain lipids labeled with two different fluorophores that constitute a Förster resonance energy transfer (FRET) pair. Membrane fusion involves lipid mixing

Abbreviations: **DIC**, *N*,*N*′-diisopropylcarbodiimide; **DIPEA**, *N*,*N*-diisopropylethylamine; **DOPC**, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; **DOPE**, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; **DOPS**, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; **DTT**, dithiothreitol; **E3**, H-G (EIAALEK)$_3$-OH; **EDTA**, ethylenediaminetetraacetic acid; **FRET**, Förster resonance energy transfer; **HATU**, 1-[bis (dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; **HEPES**, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; **HFIP**, hexafluoroisopropanol; **HOAt**, 1-hydroxy-7-azabenzotriazole; **K3**, H-WWG (KIAALKE)$_3$-OH; **LD**, linker domain; **NBD**, 7-nitro-2-1,3-benzoxadiazole; **NMP**, *N*-methyl-2-pyrrolidone; **Oxyma**, 2-cyano-2-(hydroxyimino)acetate; **P/L**, peptide to lipid; **PNA**, peptide nucleic acid; **Rh**, lissamine rhodamine B; **SDS-PAGE**, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; **SNARE**, soluble *N*-ethylmaleimide-sensitive-factor attachment receptor; **Sx**, syntaxin-1A; **Syb**, synaptobrevin-2; **TMD**, transmembrane domain.

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so that mixing of labeled liposomes with non-labeled ones reduces the FRET efficiency because of an increased average distance between the fluorophores (fluorophore dilution mode, Figure 1A).[1] When donor and acceptor fluorophores are initially located in different liposome populations, lipid mixing is detected by an increase in FRET upon liposome fusion (fluorophore mixing mode, Figure 1B).[2] Both approaches can be used in liposome fusion assays to detect membrane merging.

Non-specific aggregation processes between liposomes, that is, the clustering of liposomes not related to specific peptide–peptide interactions, may arise from various events, such as peptide adsorption at the membrane surface, membrane destabilization, electrostatic attraction or peptide homodimerization (Figure S1). They falsify the outcome of fusion assays: Non-specifically aggregated liposomes will have a lower tendency to fuse, for example because peptides are less accessible for specific interactions. In case the unspecific aggregation leads to peptide-independent liposome fusion, the resulting fused liposomes will have a lower curvature, and thus will less likely undergo fusion.[3,4]

To obtain reliable results in bulk leaflet mixing assays, it is therefore necessary to avoid unspecific aggregation so that the observed changes in FRET can be related to specific peptidomimetic-mediated membrane fusion processes.

In this study, we show how parameters like the concentration and the quality of the peptidomimetics, the size of the liposomes and the measuring mode of the assays determine the outcome of bulk leaflet mixing assays and how adverse aggregation processes can be prevented. Strategies for obtaining reasonable experimental data are presented.

2 | MATERIALS AND METHODS

A detailed description of each procedure as well as analytical data of all peptidomimetics can be found in the supporting information.

2.1 | Synthesis of peptidomimetics

Peptides were synthesized via Fmoc-based solid-phase peptide synthesis by using low-loaded Rink amide or Wang resins. The SNARE linker and transmembrane domains as well as the coiled-coil recognition units were assembled automatically on a Liberty Blue synthesizer (CEM, USA) in a 0.05 or 0.1 mmol scale. Deprotection was carried out using a solution of piperidine in DMF (20 vol%), and the amino acids (5.0 eq) were activated with a mixture of Oxyma (5.0 eq) and DIC (5.0 eq) prior to coupling. Microwave irradiation was applied during deprotection and coupling steps. PNA building blocks (5.45 eq) were assembled manually at room temperature without microwave irradiation in a 5 μmol scale, using HATU/HOAt (5.3 eq/5.45 eq) and DIPEA/lutidine (5.45 eq/8.2 eq) as activators. Double coupling (2 × 1 hr) was performed for each building block. Capping was performed with a mixture of Ac_2O/lutidine/NMP (1:2:7 vol%). After the synthesis, the peptidomimetics were cleaved off the resin by adding a TFA-based mixture (rt, 2 hr), precipitated with ice-cold diethyl ether and dried *i. vac*.

2.2 | HPLC purification

Peptides were dissolved in hexafluoroisopropanol and loaded onto an RP-C18 column. Elution was performed with a linear gradient of

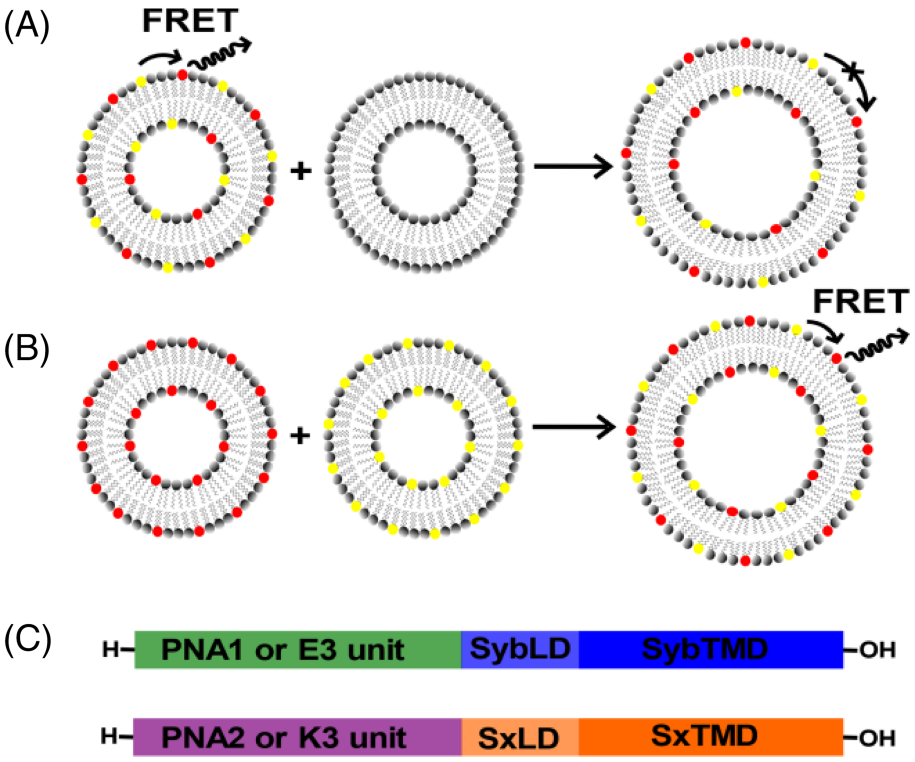

FIGURE 1 Illustration of FRET-based leaflet mixing assays to investigate the process of membrane fusion induced by model peptides. Depending on the position of the fluorophores, leaflet mixing assays can either be performed in the fluorophore dilution mode (A) or in the fluorophore mixing mode (B). The model peptides used in this study are derived from the neuronal SNARE proteins synaptobrevin-2 (Syb) and syntaxin-1A (Sx) (C). They feature an artificial recognition unit made of peptide nucleic acid (PNA) or E3/K3-coiled coil motifs attached to the native linker domain (LD) and transmembrane domain (TMD)

methanol or methanol/1-propanol (4:1) against water at elevated temperatures. All solvents contained 0.1% TFA. Detection was performed based on UV absorbance of the peptides.

2.3 | Preparation of liposomes

Appropriate volumes of stock solutions of lipids in $CHCl_3$ and peptides in TFE were mixed on ice. Usually, a molar ratio of DOPC/DOPE/cholesterol = 2:1:1 mol% was applied. The peptide-to-lipid ratio was either 1:200 or 1:1000. For labeled liposomes to be used in experiments in the fluorophore dilution mode, 1.5 mol% of DOPE labeled with 7-nitro-2-1,3-benzoxadiazole (NBD) and 1.5 mol% of DOPE labeled with Lissamine Rhodamine B (Rh) was added and the content of non-labeled DOPE was reduced accordingly. For experiments in the fluorophore mixing mode, one liposome population carried NBD-DOPE and the other one carried Rh-DOPE. The organic solvents were removed at 50°C and after drying for ~12 hr at reduced pressure, the resulting lipid film was rehydrated in HEPES buffer (20 mM HEPES, 100 mM KCl, 1 mM EDTA, 1 mM DTT, pH = 7.4). After swelling for >2 hr, the mixture was extruded at least 21 times through a polycarbonate membrane (pore diameter 100 nm) using a LiposoFast mini-extruder (Avestin, Canada). Only freshly prepared liposomes were used for the experiments. The concentration of the liposomes was determined with phosphate tests based on a protocol described by Rouser et al.[5]

2.4 | Peptide insertion tests

For tests based on ultracentrifugation, proteoliposome suspensions were mixed with Histodenz medium (80% in buffer) in a 1:1 ratio. The samples were centrifuged at 4°C and 50,000 rpm × *g* for 1.5 hr. After that, aliquots were taken and analyzed via SDS-PAGE. For tests based on fluorescent probes, liposomes containing E3-Syb peptides were mixed with soluble NBD-labeled K3 and incubated for 1 hr. To remove unbound NBD-K3, samples were dialyzed against HEPES buffer in Slide-A-Lyzer mini dialysis chambers (MWCO = 20 kDa, Thermo Scientific, USA). Calibration curves were determined with non-dialyzed samples and with NBD-K3 in HEPES buffer. After dialysis, the emission of NBD at 535 nm was utilized to calculate the concentration of remaining NBD-K3, and with this of inserted E3-Syb peptides.

2.5 | Leaflet mixing experiments

Time-resolved bulk leaflet mixing experiments were recorded on a spectrofluorometer (JASCO, Japan) at 25°C. For experiments in the fluorophore dilution mode, an excitation wavelength of 460 nm and a detection wavelength of 530 nm were applied. Labeled and non-labeled liposomes, each carrying one peptide of a pair of peptides with complementary recognition units, were mixed in a 1:4 ratio (volume-based) in HEPES buffer and leaflet mixing was detected for 1200 s. Data were normalized by considering a 100% value, which was obtained after lysing the liposomes with Triton-X 100. For experiments in the fluorophore mixing mode, liposomes were mixed in a 1:1 ratio and the change in the acceptor emission was detected at 585 nm (excitation wavelength 460 nm). Detergent was not added. To compare the results with those from the fluorophore dilution mode, the obtained fusion curves after background subtraction were scaled arbitrarily. For control experiments, either no peptides were applied or just one liposome population carried a peptide.

2.6 | Dynamic light scattering

DLS was performed on a Zetasizer Nano S device (Malvern Panalytical GmbH, Germany). Measurements were carried out at room temperature, the scattering detection angle was 173°, and the laser power and the number of cycles per measurement were determined automatically by the software. For time-resolved measurements, liposomes were mixed in a separate cuvette and samples were taken out every few minutes.

3 | RESULTS AND DISCUSSION

In the present study, different conditions for bulk leaflet mixing assays performed with established SNARE-like model peptides are inspected and evaluated. The model peptides either contain peptide nucleic acid (PNA)[6] or coiled-coil forming peptide segments (E3/K3)[7] as recognition units (Table 1). Both structural motifs have already been used in earlier studies to explore membrane fusion.[8,9] The recognition units are each attached to the linker and transmembrane domain (TMD) sequences of two native SNAREs, synaptobrevin-2 (Syb) and syntaxin-1A (Sx), to ensure proper anchoring to the lipid membrane. While SNARE model peptides with E3/K3 recognition units represent a purely peptidic system mimicking the *coiled-coil*-type recognition found in native SNAREs, those with PNA recognition units are more

TABLE 1 SNARE-model peptides used throughout this study

Model peptide	Sequence
PNA2-Sx (**1**)	**H-**agtga-KYQSKARRKK-IMIIICCVILGIIIASTIGGIFG-**OH**
PNA1-Syb (**2**)	**H-**tcact-KRKYWWKNLK-MMIILGVICAIILIIIIVYFST-**OH**
K3-Sx (**3**)	**H-WWG-**(KIAALKE)$_3$ **-**QSKARRKK-IMIIICCVILGIIIASTIGGIFG**-OH**
E3-Syb (**4**)	**H-G-**(EIAALEK)$_3$ **-**RKYWWKNLK-MMIILGVICAIILIIIIVYFST**-OH**

Note: The color code used for the sequence segments is the same as in Figure 1c. Capital letters indicate standard α-amino acids, small letters indicate PNA monomers with the respective nucleobases (a = adenine, c = cytosine, g = guanine, t = thymine).

artificial constructs with a topology break between recognition unit and anchoring unit, but still found to induce liposome fusion by forming stable duplexes upon interaction.

The preparation of liposomes as well as the bulk leaflet mixing assays were performed as described earlier.[6,7] In brief, solutions of the respective lipids and peptides were mixed and the organic solvents were evaporated to obtain lipid films. The lipid films were rehydrated in buffer and proteoliposomes were formed by extrusion. As soon as liposomes containing peptides with complementary recognition units had been combined in the fusion buffer, recording of the fluorescence was started and the fluorescence was monitored over a defined time period (typically 20 min).

3.1 | Concentration of peptidomimetics

A commonly used ratio for proteoliposome populations with both Syb- and Sx-based model peptides is P/L = 1:200.[7,10] This ratio reflects the native Syb-to-lipid ratio in synaptic vesicles, but not the native Sx-to-lipid ratio of 1:3000 that is found in presynaptic plasma membranes.[11,12] In order to examine the influence of a lower concentration of peptidomimetics, a reduced P/L ratio of 1:1000 was tested. As expected,[9] a lower extent of leaflet mixing was determined for P/L = 1:1000, compared to the generally employed P/L ratio of 1:200 (Figure 2).

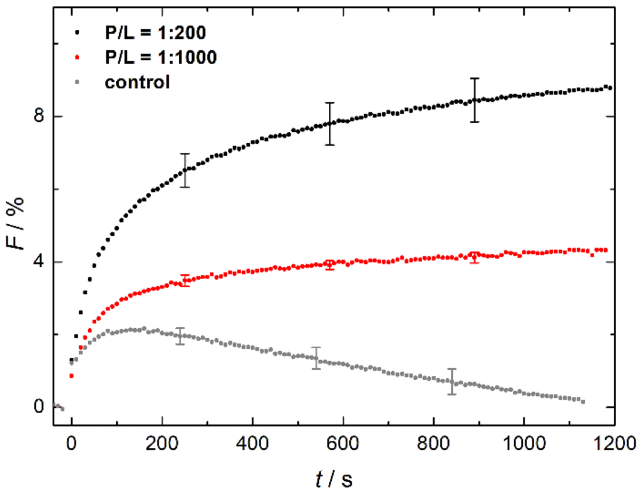

FIGURE 2 Leaflet mixing assays in the fluorophore dilution mode using proteoliposomes decorated with PNA-based SNARE mimetics PNA2-Sx (**1**) and PNA1-Syb (**2**). The P/L ratio was set to a high value (1:200, black) or to a low value (1:1000, red). Control measurements (gray) were performed by using labeled proteoliposomes and non-labeled liposomes. Peptides were purified by HPLC prior to the experiments; n = 4 (black), n = 3 (red, gray). Liposome composition was DOPC/DOPE/cholesterol = 2:1:1 mol% for liposomes carrying peptide **1** or no peptide and DOPC/DOPE/cholesterol/Rh-DOPE/NBD-DOPE = 50:22:25:1.5:1.5 mol% for liposomes carrying peptide **2**. The total amount of lipids per lipid film was 0.625 μmol for each liposome population. Error bars represent the standard deviation of the mean (applies to all presented data in this study)

We observed that proteoliposome suspensions quickly became turbid when they contained Sx-based peptides **1** or **3** in a high concentration (P/L = 1:200). In contrast, liposomes with peptides in a low concentration (P/L = 1:1000) or without any peptides stayed clear for days or even weeks. To quantify this, the transmittance of liposome suspensions was measured over 2 hr at λ = 650 nm, starting immediately after the liposome preparation (Figure 3, top row). The transmittance of liposomes with peptide **1** incorporated at P/L = 1:200 significantly decreased over time, indicating increased turbidity, which was also clearly visible by the naked eye (Figure S2). The liposome stock solutions featuring a P/L ratio of 1:1000 stayed clear and no precipitate was formed, and the transmittance was found to barely change within 2 hr after extrusion. The same was true for liposomes without any peptides ("empty liposomes"). Lipids with fluorophores ("labeled liposomes") did not influence the stability of liposomes.

Dynamic light scattering (DLS) measurements revealed that liposomes without peptides have a narrow size distribution and a small polydispersity index, even months after extrusion. Proteoliposomes, however, showed a broader size distribution, especially at a longer time period after extrusion (see Figure 3, bottom row).

By performing phosphate tests, it was found that lipid material was lost during extrusion of liposomes in particular for proteoliposomes (Table S2). When the liposomes contained no peptidomimetics, the loss was found to be considerably lower (5%–15%) compared to liposomes carrying peptidomimetics (15%–50%). In particular, extrusion of liposomes with K3-SxTMD peptides involved high losses (**3**, 30%–50% on average). Next to phosphate tests, the emission of rhodamine-labeled lipids in proteoliposomes was used to quantify material loss (Table S3). We reason that the loss can be ascribed to the formation of aggregation taking place before extrusion. In this case, aggregates will be retained by the filter of the extruder, resulting in a lower concentration of the final liposome solution.

Taken together, it is likely that peptidomimetics induce aggregation of liposomes. The extent of aggregation is dependent on their concentration. They may induce liposome aggregation and unspecific fusion by perturbing the membrane continuity, which is not only possible when they are integrally inserted but particularly when they are peripherally adsorbed to the membrane surface due to an incorrect incorporation (see also Figure S1).[13,14] By applying density gradient centrifugation,[15] we found that the peptidomimetics are associated with the liposomes (see Figure S3). In an attempt to estimate the recognition units that are available for fusion, a soluble fluorescent counterpart to the recognition unit was added to proteoliposomes. As an example, liposomes with E3-based peptidomimetics were treated with soluble NBD-labeled K3 recognition units (NBD-K3). The results suggested that only a fraction of all recognition units might be available for fusion (see Supporting Information for more details). It has to be noted, however, that the soluble recognition unit might also interact with non-inserted peptides or even the liposomal membrane. The results of this test can therefore only be treated as a rough estimation. Effects like interaction of the recognition unit with the membrane, self-aggregation or incorrect peptide insertion may lead to a

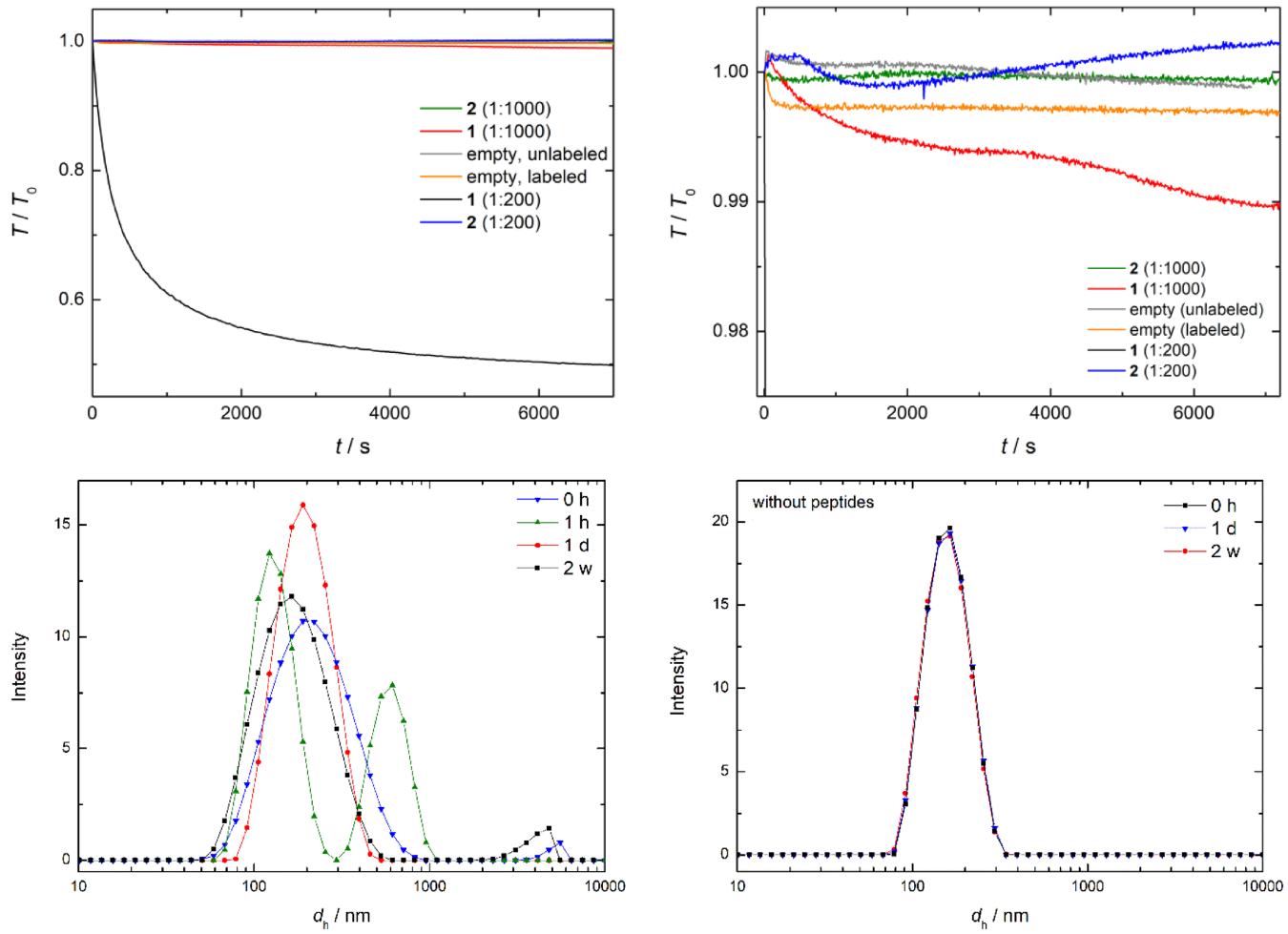

FIGURE 3 Top row: Transmittance T of suspensions of liposomes containing peptides **1** or **2** at different concentrations or no peptides ("empty"), left: Full view, right: Zoom in between T/T_0 = 0.98 and 1. Bottom row: Size distribution of liposomes with and without peptide **1** at different time points after liposome preparation; liposome composition was DOPC/DOPE/cholesterol = 2:1:1 mol% (0.625 μmol total amount of lipids per lipid film, P/L ratio = 1:200)

decreased number of available recognition units. It is easily conceivable that especially the polybasic linker domains of the SNARE mimetics initiate membrane disrupting processes, similar to those proposed for cationic antimicrobial membrane peptides.[13,14,16] In particular, the recognition motif K3 has been shown before to interact with the liposomal membrane.[17] This causes the peptides to be stuck to the membrane, thereby destabilizing the membrane and leading to the formation of larger aggregates.

On a side note, we found that a high concentration of lipids can lead to induce aggregation as well. This is supported by the observation that solutions with a high amount of lipids per lipid film (2.5 μmol) quickly became turbid after extrusion, eventually leading to the formation of a cloudy precipitate. Stock solutions of liposomes prepared from a lipid film with a total lipid amount of 2.5 μmol show a significantly lower transmittance after extrusion than those of liposomes prepared from a lipid film with a total lipid amount of 0.625 μmol (Figure S4a). DLS measurements revealed that shortly after extrusion, liposome stock solutions with a high amount of lipids are largely polydisperse (Figure S5). When performing bulk leaflet mixing assays, liposomes prepared from lipid films with 2.5 μmol lipids show a slightly lower fusion efficiency compared to assays with liposomes prepared from 0.625 μmol lipid stock solutions, even though they are four times higher concentrated (see Figure S6). Therefore, the observed turbidity of the solutions likely is a result of liposomes forming larger aggregates. The formation of aggregates, however, reduces the amount of fusogenic liposomes in the solution, which is why a lower extent of lipid mixing is observed.

In total, we recommend to apply low lipid and peptide concentrations, such as a total lipid amount of 0.625 μmol per lipid film and a peptide-to-lipid ratio of 1:1000, to only use freshly prepared liposomes, and to perform the measurements directly after extrusion. These measures will help reduce the amount of aggregated liposomes in the assay and ensure better comparability of the results.

3.2 | Purity of peptidomimetics

The purification of SNARE mimetics featuring a hydrophobic transmembrane domain is challenging due to their special structural features (i.e., a very hydrophobic transmembrane domain combined with a rather hydrophilic recognition unit) leading to an intrinsic aggregation propensity and a poor solubility especially in aqueous solvents. We found that dissolving the crude peptides in a fluorinated alcohol, such as hexafluoroisopropanol (HFIP), allowed purification via HPLC. By using a binary gradient of methanol/water at elevated temperatures, the crude peptide mixture could be separated on a C18 reversed-phase silica column yielding chromatograms with an acceptable resolution of the peaks (see Figures S7 and S8). HFIP is known to stabilize secondary peptide structures and decrease hydrophobic peptide aggregation, presumably due to the formation of clusters[18,19] and it has been widely used to de-aggregate peptide and protein samples.[20,21]

The purity of the fusogenic peptides has an influence on the extent of the emission intensity detected in bulk leaflet mixing assays. As can be seen in Figure 4, the leaflet mixing efficiency of liposomes containing crude E3/K3-based peptidomimetics is higher than that of proteoliposomes containing the same peptidomimetics purified by HPLC. The same trend was found for PNA-based model peptides (see Figure S9). Impurities, such as deletion sequences, thus seem to enhance the fusogenicity. We hypothesize that this is due to unspecific leaflet mixing evoked by peptide fragments which perturb the bilayer continuity, and therefore, destabilize the membranes and make them more prone to leaflet mixing events. It is important to note, however, that conclusions drawn from experiments with unpurified peptides are valid nonetheless. Control measurements showed that the general tendencies still hold true, regardless of purified or unpurified peptides being used in the lipid mixing assays (Figure S10).

3.3 | Lipid composition

Two lipid mixtures commonly applied in liposome fusion assays with or without 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) were compared. The extent of lipid mixing was drastically reduced in the presence of DOPS (Figure 5). The Coulomb repulsion between the negatively charged serine headgroups and increased dehydration energies for the interstices of the opposing proteoliposome membranes may impair the fusion process.[22–25] In addition, it is likely that these effects avoid aggregation and stabilize the proteoliposomes. This is supported by the observation that proteoliposomes containing DOPS are stable even weeks after extrusion. No turbidity or precipitation could be observed in stock solutions of liposomes that contained DOPS, as shown by measuring the transmission of the solution over 2 hr after extrusion (Figure S4b,c). On top, DLS measurements showed a size distribution that barely changed over 2 weeks when DOPS is present (Figure S11). Addition of DOPS reduces the lipid mixing efficiency, but at the same time it increases the stability of the proteoliposomes, thus helping avoid aggregation and achieve more reliable data.

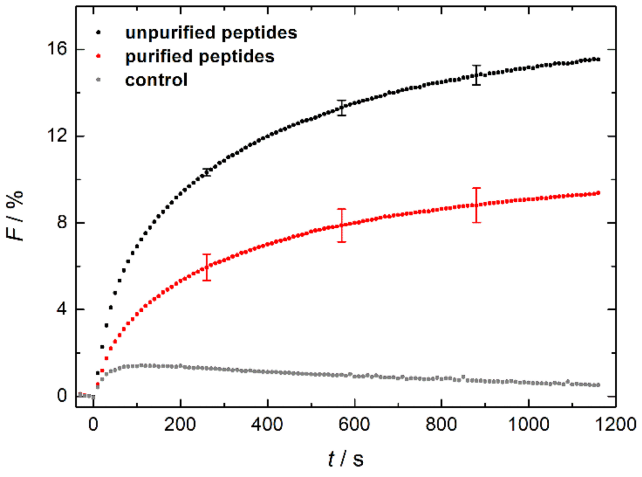

FIGURE 4 Leaflet mixing assays in the fluorophore dilution mode using proteoliposomes decorated with E3/K3-based SNARE mimetics **3** and **4**. Peptidomimetics were used as unpurified (black) or as samples purified by HPLC (red). Control measurements (gray) were performed by using labeled proteoliposomes containing purified **4** and non-labeled liposomes without peptidomimetics. P/L = 1:200, *n* = 3. Liposome composition was DOPC/DOPE/cholesterol = 2:1:1 mol% for liposomes carrying peptide **3** or no peptide and DOPC/DOPE/cholesterol/Rh-DOPE/NBD-DOPE = 50:22:25:1.5:1.5 mol% for liposomes carrying peptide **4**. The total amount of lipids per lipid film was 0.625 μmol for each liposome population

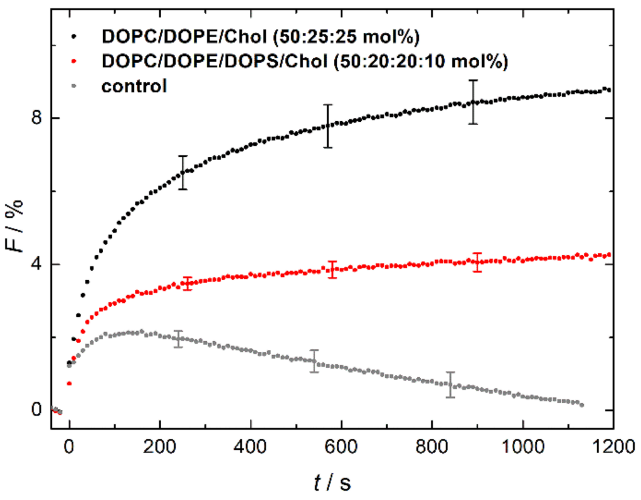

FIGURE 5 Leaflet mixing assays in the fluorophore dilution mode using proteoliposomes decorated with PNA-based SNARE mimetics **1** and **2**. The lipid composition included either no DOPS (black) or 10% DOPS (red). In case of labeled liposomes (carrying peptide **2**) the amount of DOPE was reduced by 3 mol% and Rh-DOPE (1.5 mol%) and NBD-DOPE (1.5 mol%) were added, respectively. Control measurements (gray) were performed by using labeled proteoliposomes and non-labeled liposomes without DOPS. The P/L ratio was 1:200. Peptides were purified by HPLC prior to the experiments; *n* = 4 (black), *n* = 3 (red, gray)

3.4 | Size of liposomes

Typically, proteoliposomes exhibit a hydrodynamic diameter between 100 nm and 200 nm after extrusion through polycarbonate membranes with a pore diameter of 100 nm. It was found that changing the size of proteoliposomes beyond this range had a great influence on the efficiency of peptidomimetic-induced membrane fusion. Combining small and large unilamellar vesicles (both 30–150 nm in diameter on average) decorated with E3/K3-based SNARE mimetics led to a significantly higher extent of leaflet mixing than combining large (240 nm) with giant unilamellar vesicles (>1000 nm, Figure S12). Thus, the larger the liposomes the less fusogenic they are. This is in accordance with observations made previously with large unilamellar vesicles added to flat pore-spanning membranes, where only docking was detected.[26] Larger liposomes exhibit a less pronounced membrane curvature and thus membrane fusion is hampered.[3,4] Hence, adjusting the size of proteoliposomes is a convenient method to control the extent of leaflet mixing.

Monitoring the size distribution over time by using dynamic light scattering (DLS) can be applied as a further method to detect peptide induced liposome fusion[27,28] and was performed for PNA-based SNARE analogues (Figure 6, for underlying size distributions please refer to Figure S13). A significant increase in size was observed after the proteoliposome samples had been mixed. DLS cannot be used to distinguish between docked and fused liposomes straightforwardly; however, as the complementary bulk leaflet mixing assays unambiguously indicated leaflet mixing (Figure S14), the increase in size likely arises from fusion of liposomes. Since DLS provides information on the size that is not accessible with bulk leaflet mixing assays, it is a very useful supportive method to assess the fusogenicity of peptidomimetics.

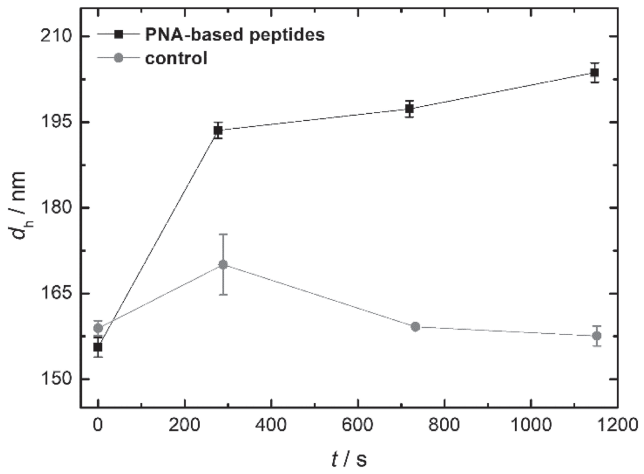

FIGURE 6 Detection of proteoliposome docking/fusion with time-resolved dynamic light scattering. Liposomes contained PNA-based peptidomimetics **1** and **2** (black). The same conditions as in the bulk leaflet mixing assays were applied. Control measurements were performed with one liposome population without peptidomimetics (gray). The P/L ratio was 1:200. Liposome composition was DOPC/DOPE/cholesterol = 2:1:1 mol% for liposomes carrying peptide **1** or no peptide and DOPC/DOPE/cholesterol/Rh-DOPE/NBD-DOPE = 50:22:25:1.5:1.5 mol% for liposomes carrying peptide **2**. The total amount of lipids per lipid film was 0.625 μmol for each liposome population

3.5 | Mode of lipid mixing assays

Depending on the position of the donor and acceptor fluorophores, the experimental outcome of bulk leaflet mixing assays may differ. In the fluorophore dilution mode, typically a steady increase in donor emission was observable, even on long time scales (Figure S15). In the fluorophore mixing mode, by contrast, the emission barely changed after around 600 s (Figure 7).

The increase observed in the fluorophore dilution mode may arise from various processes that are not related to leaflet mixing resulting from specific peptidomimetic recognition, such as liposome rupture or single lipid exchange.[29] The probability of the loss of single lipids from the lipid bilayer depends on the shape of the lipids and is significantly

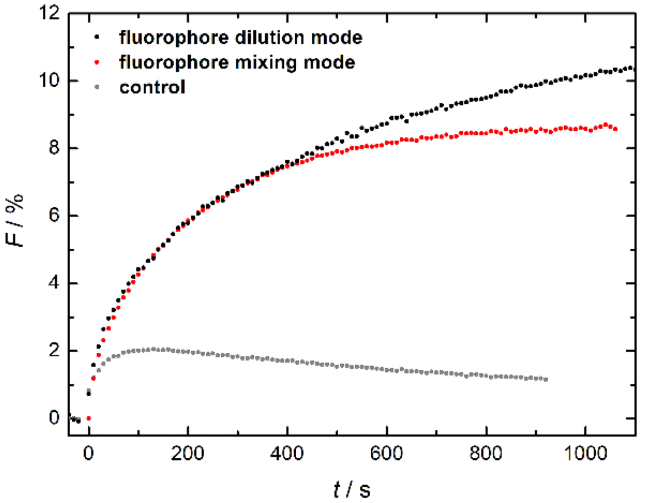

FIGURE 7 Leaflet mixing assays using proteoliposomes decorated with PNA-based SNARE mimetics **1** and **2**. The assays were performed in the fluorophore dilution mode (black) or in the fluorophore mixing mode (red). The control measurement (gray) was performed in the dilution mode with labeled proteoliposomes and non-labeled liposomes containing no peptidomimetics. The fusion curve obtained from the experiments in the fluorophore mixing mode has been scaled arbitrarily to illustrate the different shapes of the curves. The P/L ratio was 1:200. Liposome composition in the fluorophore dilution mode was DOPC/DOPE/cholesterol = 2:1:1 mol % for liposomes carrying peptide **1** or no peptide and DOPC/DOPE/cholesterol/Rh-DOPE/NBD-DOPE = 50:22:25:1.5:1.5 mol% for liposomes carrying peptide **2**. Liposome composition in the fluorophore mixing mode was DOPC/DOPE/cholesterol/NBD-DOPE = 50:23.5:25:1.5 mol% for liposomes carrying peptide **1** and DOPC/DOPE/cholesterol/Rh-DOPE = 50:23.5:25:1.5 mol% for liposomes carrying peptide **2**. The total amount of lipids per lipid film was 0.625 μmol for each liposome population

lower for lipids with a labeled head group.[30,31] Assays performed in the fluorophore mixing mode are less sensitive to these processes and thus represent a more realistic picture of the kinetics. Besides, they offer the possibility to easily adjust the amount of liposomes prior to the experiment via measuring the emission of the fluorophores, which are present in both liposome populations. However, they may detect docking without fusion of proteoliposomes, if in this way donor and acceptor fluorophores come into close proximity.[29] On top, they do not allow estimating the absolute percentage of fused proteoliposomes. This is only possible in assays performed in the fluorophore dilution mode. To determine the absolute number in these assays, either mock-fused liposomes can be used or the liposomes are treated with a detergent to lyse liposomes and thereby maximize the distance between the FRET pair.[1,2] Since both fluorophore dilution and mixing mode are not without any drawbacks it is best to combine both techniques to obtain a full picture of the peptidomimetics' fusogenicity.

4 | CONCLUSION

The detection of membrane fusion through leaflet mixing assays involving SNARE-like model peptides with a natural TMD sequence often suffers from aggregation processes. The analysis of different parameters in this study showed that aggregation already occurring prior to performing these assays can be avoided by lowering the lipid concentration for the proteoliposome stock solutions, by applying a reduced peptide-to-lipid ratio or a lipid composition including phosphatidylserines. Furthermore, the size of the proteoliposomes and the stoichiometry of the mixed proteoliposome populations influence the experimental outcome of the leaflet mixing assays. In addition, it was shown that impurities of the peptide probes and failed insertion of the SNARE mimetics affect leaflet mixing as well. Considering the here presented measures in future proteoliposome fusion assays will minimize the risk of aggregation and will help obtain reliable results.

ACKNOWLEDGEMENT

Generous support from the Deutsche Forschungsgemeinschaft (DFG) in the context of SFB803 (project B05 "Functionality controlled by organization in and between membranes") is gratefully acknowledged.

Open Access funding enabled and organized by Projekt DEAL.

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How to cite this article: Hubrich BE, Wehland J-D, Groth MC, et al. Membrane fusion mediated by peptidic SNARE protein analogues: Evaluation of FRET-based bulk leaflet mixing assays. *J Pep Sci*. 2021;27:e3327. https://doi.org/10.1002/psc.3327