

# Supporting Information

## Programmable fusion of liposomes mediated by lipidated PNA

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### Abbreviations.

A, adenine; aeg, *N*-(2-aminoethyl)glycine; C, cytosine; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; Fmoc, Fluorenylmethyloxycarbonyl; G, guanine; HATU, 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-

yl]ethanesulfonic acid; HOBt, hydroxybenzotriazole; LiP, lipidated peptide nucleic acid; MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight; NMM, *N*-methylmorpholine; NMP, *N*-methyl-2-pyrrolidone; PEG, polyethylene glycol; PNA, peptide nucleic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) sodium salt; RAM, Rink amide; RP-HPLC, reverse-phase high performance liquid chromatography; SRB, sulforhodamine B; T, thymine; TBE, Tris-borate-EDTA; TEAA, triethylammonium acetate; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol.

## Materials.

Organic solvents for reactions were supplied by *VWR* or *Sigma-Aldrich* in HPLC grade. Ultrapure water with MilliQ® water quality for HPLC and buffer preparation was purified with a MilliQ Advantage A10 water purification system from *MilliPore*.

Lipids for extrusion of liposomes were supplied by *Avanti Polar Lipids Inc.* The TentaGel XV RAM resin was purchased from *Rapp Polymere GmbH*. HBTU, the Fmoc-Gly-preloaded and Fmoc-Glu(OtBu)-preloaded Wang resin were purchased from *Merck Millipore*. PNA monomers (Fmoc-A(Bhoc)-aeg-OH, Fmoc-C(Bhoc)-aeg-OH, Fmoc-G(Bhoc)-aeg-OH and Fmoc-T-aeg-OH) were purchased from *LINK Technologies Ltd.* and *ASM Research Chemicals*. Fmoc-NH-PEG<sub>12</sub>-CH<sub>2</sub>CH<sub>2</sub>COOH was purchased from *ChemPep Inc.* Fmoc-L-Glu(OtBu)-OH and HATU were supplied by *Fluorochem Ltd.* and *N<sup>α</sup>*-Fmoc-*N<sup>ε</sup>*-palmitoyl-L-lysine by *Chem-Impex International, Inc.* Reagents for DNA synthesis were purchased from *Sigma-Aldrich* and *BioAutomation*. Unmodified DNA and RNA were purchased from *Sigma-Aldrich*. All other chemicals were purchased from *Sigma-Aldrich* as well.

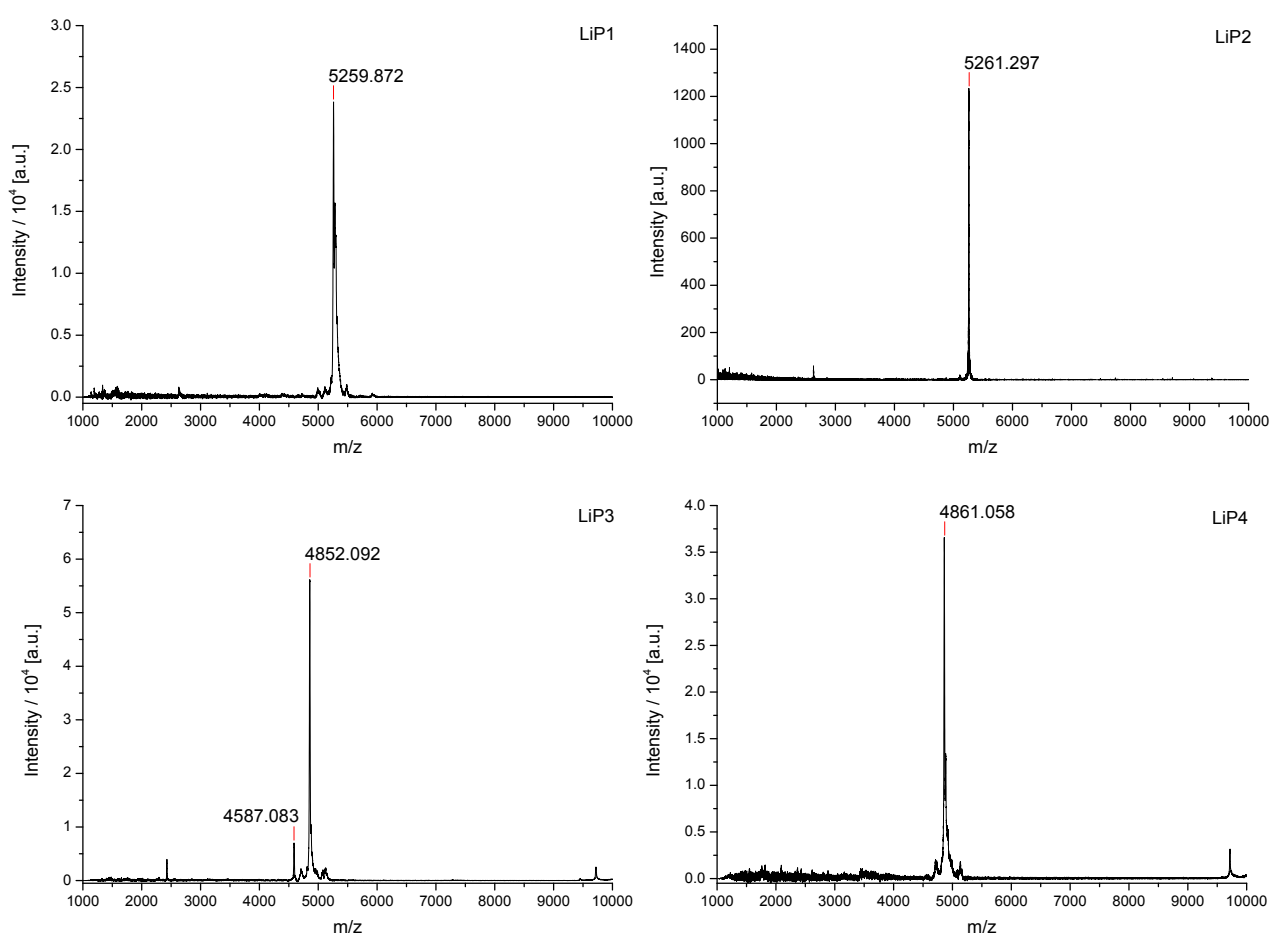
The following buffers were used for the biophysical experiments: SRB buffer (20 mM SRB, 10 mM HEPES-NaOH, 100 mM NaCl, pH 7), HEPES buffer (10 mM HEPES-NaOH, 100 mM NaCl, pH 7), TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3).

## MALDI-TOF mass spectrometry.

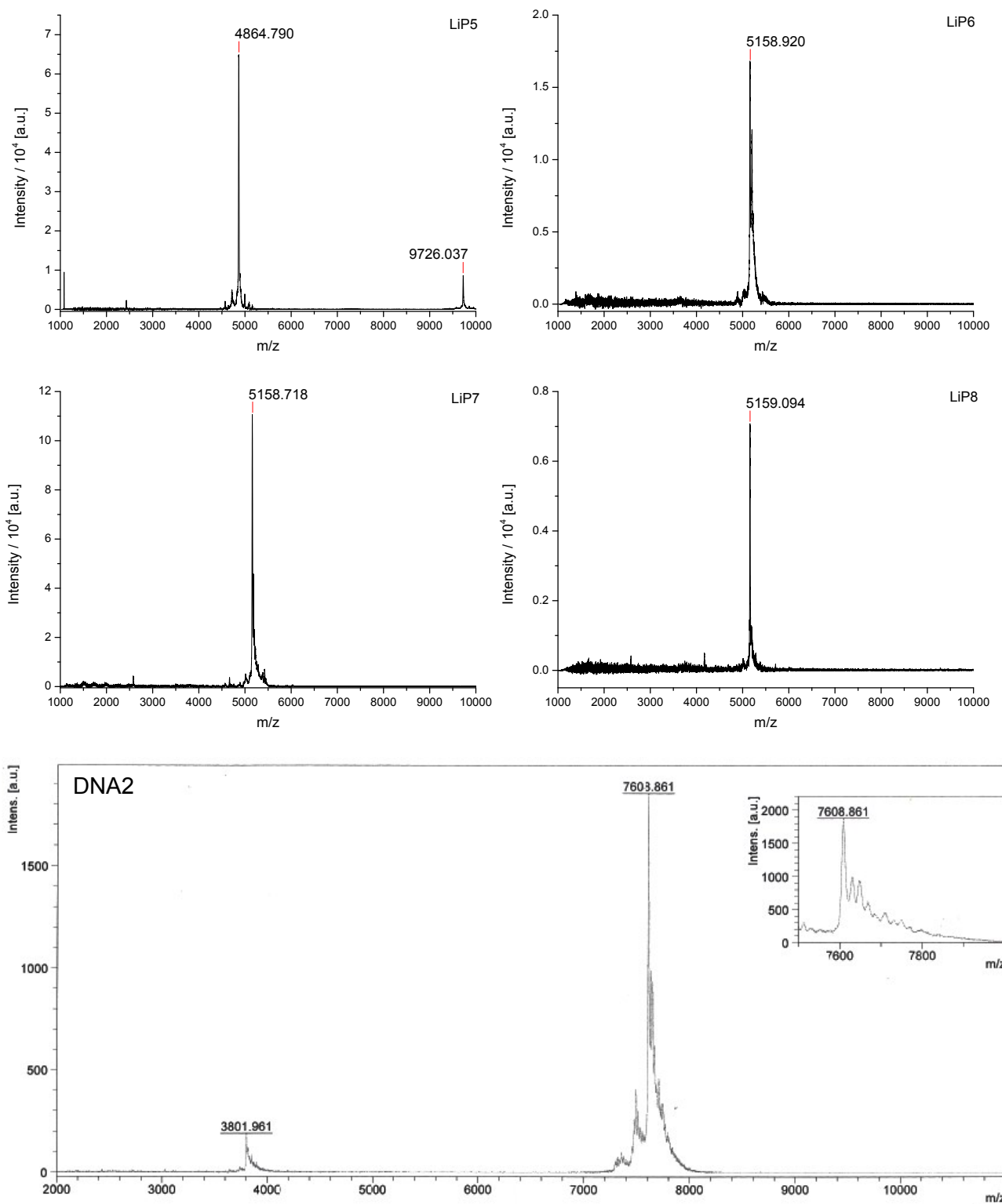
MALDI-TOF mass spectrometry was performed on a Bruker Daltonics Autoflex III Smartbeam or Bruker Daltonics Microflex LT spectrometer. A saturated solution of sinapinic acid in a mixture of water/MeCN (2:1) and 0.1% trifluoroacetic acid was used as matrix solution for MALDI-TOF of liPs. A solution of diammonium hydrogen citrate (4.4 mM) and 3-hydroxypicolinic acid (72 mM) in water was used as matrix solution for MALDI-TOF of DNA2.

**Table S1.** Calculated and found masses of LiPs and DNA2.

sample	M	calc. m/z [M+H] <sup>+</sup>	found m/z [M+H] <sup>+</sup>
LiP1	C <sub>230</sub> H <sub>355</sub> N <sub>75</sub> O <sub>68</sub>	5260	5260
LiP2	C <sub>230</sub> H <sub>355</sub> N <sub>75</sub> O <sub>68</sub>	5260	5261
LiP3	C <sub>211</sub> H <sub>313</sub> N <sub>75</sub> O <sub>60</sub>	4861	4852
LiP4	C <sub>211</sub> H <sub>313</sub> N <sub>75</sub> O <sub>60</sub>	4861	4861
LiP5	C <sub>209</sub> H <sub>311</sub> N <sub>77</sub> O <sub>60</sub>	4863	4865
LiP6	C <sub>223</sub> H <sub>330</sub> N <sub>78</sub> O <sub>66</sub>	5160	5159
LiP7	C <sub>223</sub> H <sub>330</sub> N <sub>78</sub> O <sub>66</sub>	5160	5159
LiP8	C <sub>221</sub> H <sub>328</sub> N <sub>80</sub> O <sub>66</sub>	5162	5159
DNA2	C <sub>242</sub> H <sub>312</sub> N <sub>90</sub> O <sub>149</sub> P <sub>24</sub>	7610	7609



**Figure S1.** MALDI-TOF spectra of LiP1-4.



**Figure S2.** MALDI-TOF spectra of Lip5-8 and DNA2.

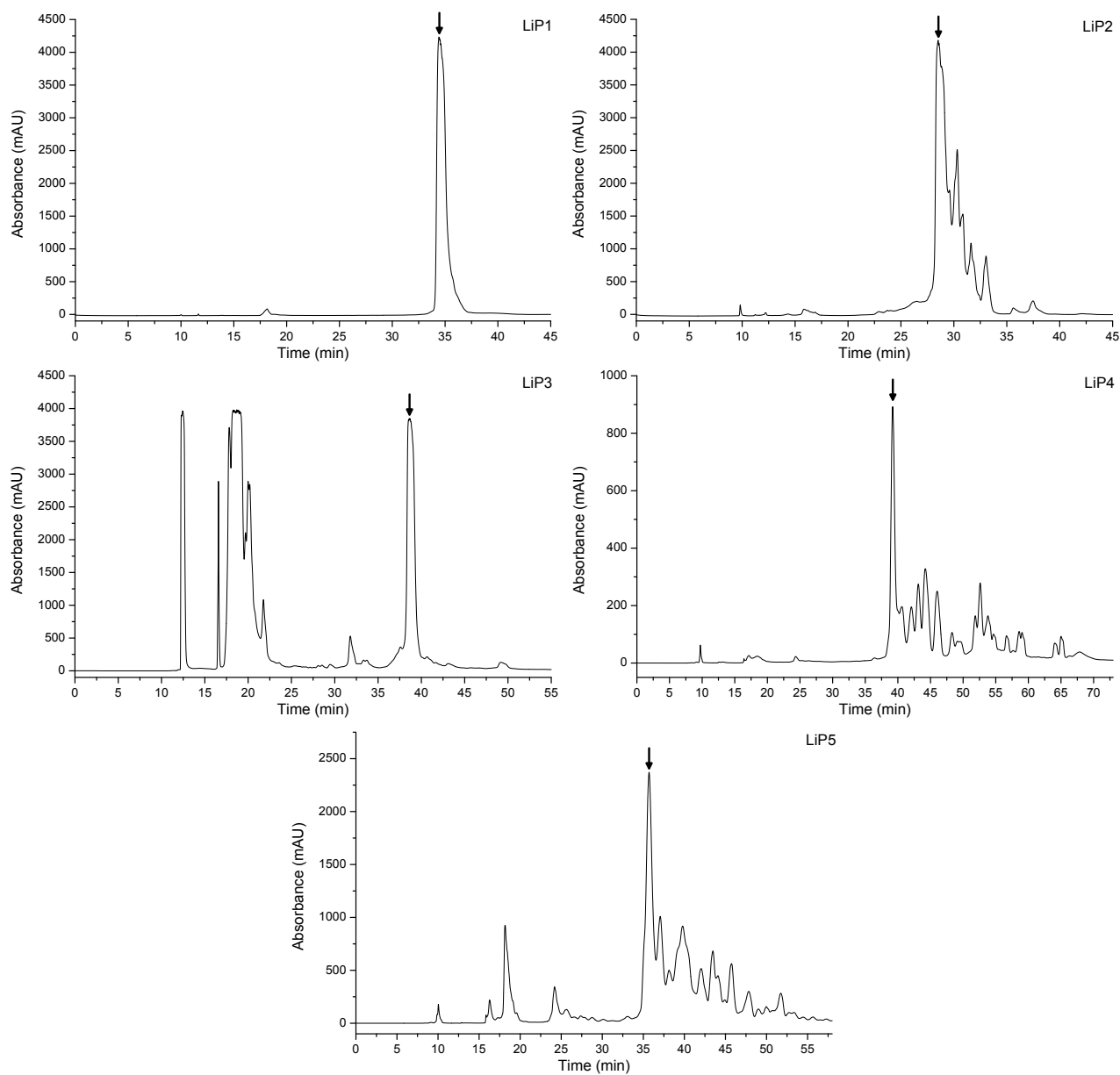
## HPLC purification.

The PNA purification was performed using a Dionex UltiMate® 3000 HPLC system, equipped with an UltiMate® 3000 Photodiode Array Detector. The analytical and preparative separations were done using a reverse phase Waters XBridge™ C<sub>18</sub> or C<sub>8</sub> column (150 x 10 mm, 5 µm), using an UV detector at 290, 254, 214 and 280 nm wavelengths. The eluents were water with 0.1% TFA (eluent A) and an acetonitrile/water mixture (9:1) with 0.1% TFA (eluent B). The crude samples were dissolved in water/acetonitrile/TFA 50:50:0.1 and centrifuged (5000 rpm) prior to injection.

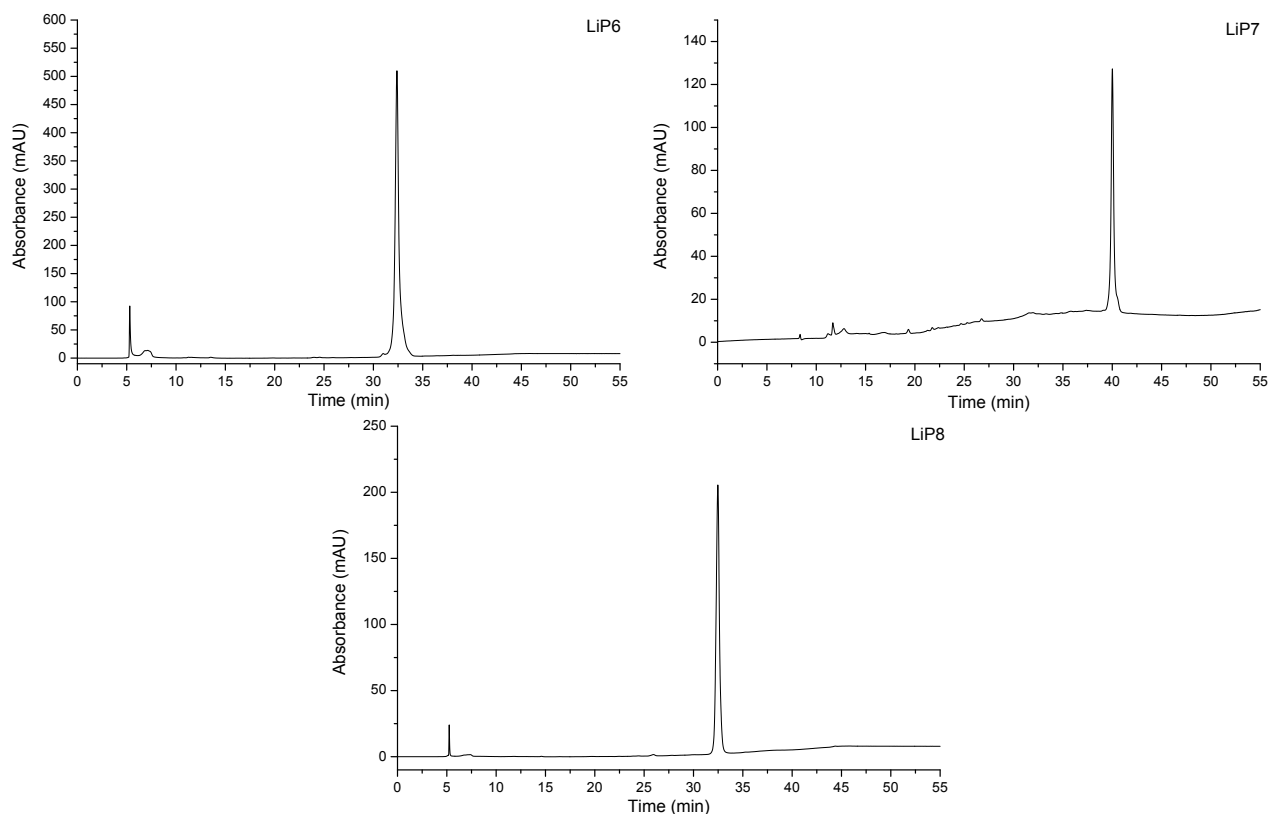
**Table S2.** RP-HPLC purification of LiPs.

sample	Column	flow rate (mL/min)	temperature (°C)	gradient	R <sub>f</sub> (min)
LiP1	C <sub>8</sub>	1	55	0% B (0 min) → 100% B (35 min) → 100% B (40 min)	34.46
LiP2	C <sub>8</sub>	1	55	0% B (0 min) → 100% B (25 min) → 100% B (39 min)	28.54
LiP3	C <sub>8</sub>	1	55	0% B (3 min) → 75% B (30 min) → 90% B (52.5 min)	38.64
LiP4	C <sub>8</sub>	1	55	0% B (3 min) → 60% B (15 min) → 85% B (57 min)	39.20
LiP5	C <sub>8</sub>	1	55	0% B (3 min) → 60% B (15 min) → 84% B (40 min)	35.70
LiP6	C <sub>18</sub>	2	36	0% B (2 min) → 100% B (40 min) → 100% B (50 min)	39.76
LiP7	C <sub>18</sub>	2	55	2% B (2 min) → 70% B (20 min) → 100% B (50 min)	32.88
LiP8	C <sub>18</sub>	2	55	2% B (2 min) → 70% B (20 min) → 100% B (50 min)	33.30

DNA2 was purified on a Thermo Scientific Ultimate 3000 UHPLC system. Analytical and preparative separation was done using a Merck Millipore Chromolith® Performance RP-18e column (100 × 4.6 mm, 2 µm) with UV detection at 260, 280 and 295 nm wavelengths. The eluents were 0.05 M TEAA in water (eluent A) and 0.05 M TEAA in ACN/water (3:1, eluent B). The flow rate was 1.25 mL/min, the temperature was 35 °C and the gradient was 4% B (1 min) → 40% B (17 min) → 100% B (18 min). The retention time of DNA2 was R<sub>f</sub> = 13.25 min.



**Figure S3.** HPLC chromatograms of the purification of LiP1-5. In the case of LiP1 it is the second purification step. The methods are noted in **Table S2**. Fractions containing only the product were combined.



**Figure S4.** The HPLC chromatograms of LiP6-8 were made from the purified compounds with a different method than noted in Table S2. LiP6 and LiP8: reverse phase Waters XBridge™ C<sub>8</sub> column (150 x 10 mm, 5 μm), 36 °C, 2 mL/min, 2% B (2 min) → 100% B (40 min) → 100% B (55 min). LiP7: reverse phase Waters XBridge™ C<sub>18</sub> column (150 x 19 mm, 5 μm), 55 °C, 4 mL/min, 2% B (2 min) → 70% B (20 min) → 100% B (50 min) → 100% B (55 min).

## DNA synthesis.

DNA2 was synthesized with an Expedite 8909 DNA synthesizer (Applied Biosystems) on a 0.2 μmol scale using standard phosphoramidite chemistry as described previously.<sup>[1]</sup> Activator42 was used as the activator. Deprotection and cleavage from the solid support were done with conc. ammonia at 55 °C overnight. After removal of the solvent, the residue was extracted two times with 75 μL acetonitrile/water (1:1) and purified on a UHPLC system. Fractions were concentrated to dryness, dissolved in acetonitrile/water (1:1, v/v) and analysed by MALDI-TOF-MS.

## PNA synthesis.

The synthesis of PNA strands was just introduced in our lab and was improved over time in terms of invested time and efficiency. Therefore, different variations of the synthesis are given. It is likely, that all variations would have been suitable for all LiPs.

## Manual solid-phase PNA synthesis.

The manual solid-phase peptide syntheses of LiP1 to LiP5 were done in a 0.015 mmol or a 0.03 mmol scale on a Fmoc-Gly-preloaded or Fmoc-Glu(OtBu)-preloaded Wang resin, which was swollen 30 min in NMP prior to synthesis. The synthesis was performed either in a syringe, equipped with a frit, or on a Biotage® Initiator Peptide Workstation, enabling microwave-assisted synthesis.

### Washing

Washing of the resin was performed under shaking in NMP, DCM and again in NMP (each 3 x 4 mL).

### Deprotection

The Fmoc-protected peptidyl resin was shaken at room temperature in a solution of 20% piperidine in NMP (2 x 4 mL, each 10 min).

### Coupling

**Variation A** (0.015 mmol scale, LiP1): The particular amino acid (4.00 eq.) and HATU (3.90 eq.) were dissolved in NMP (0.24 mL) before 2,6-lutidine (4.88 eq.) and DIPEA (3.25 eq.) were added. The solution of the activated amino acid was added to the deprotected resin and shaken for 60 min at room temperature. This step was repeated. The coupling conditions were inspired by a method of GUHA *et al.*<sup>[2]</sup>

**Variation B** (0.03 mmol scale, LiP2): The particular amino acid (4.00 eq.) and HATU (3.90 eq.) were dissolved in NMP (0.98 mL) before 2,6-lutidine (4.88 eq.) and DIPEA (3.25 eq.) were added. The solution of the activated amino acid was added to the deprotected resin and heated under microwaves at 60 °C for 20 min.

**Variation C** (0.03 mmol scale, LiP3 to LiP4): The particular amino acid (2.50 eq.), HBTU (2.50 eq.) and HOBt (2.50 eq.) were dissolved in a solution of 40% pyridine in NMP (0.92 mL) before NMM (5.00 eq.) was added. The solution of the activated amino acid was added to the deprotected resin and heated under microwaves at 60 °C for 20 min. The coupling conditions were inspired by a method of AVITABILE *et al.*<sup>[3]</sup>

### Capping

The resin was shaken in a solution of 10% acetic anhydride and 20% 2,6-lutidine in NMP (2 x 4 mL, each 5 min). The resin was washed with 5% DIPEA in NMP (3 x 4 mL) prior the regular washing.

Deprotection, coupling and capping was performed for each building block. After each step, the resin was washed. In case of interruption after the capping step and regular washing, the resin was washed with DCM (10 x 4 mL) under shaking and dried under vacuum. In the end of the synthesis, the final Fmoc group was removed and the deprotected resin was washed with DMF, DCM, diethyl ether and methanol (each 5 x 4 mL).

## Automated solid-phase PNA synthesis.

The syntheses of LiP6 to LiP8 were done on a *Liberty1* automatic microwave peptide synthesizer from CEM, equipped with a *Discovery* microwave unit on a TentaGel XV Rink amide resin (loading = 0.26 mmol/g) in a 0.025-mmol scale.

The resin was swollen for 30 min in DMF. The Fmoc deprotection was performed twice in 7 mL of 20% (v/v) piperidine in DMF (10 min, 20 W, 25 °C), followed by a coupling reaction (20 min, 18 W, 65 °C) in a mixture of 1 mL activator solution, containing HBTU (0.08 M, 3.1 eq.) and HOBt (0.08 M, 3.1 eq.) in DMF, 1.5 mL



activator base solution, which is NMM (0.10 M, 6.2 eq.) in pyridine, and 1.25 mL monomer solution (0.06 M, 3.1 eq.). The resin was washed once in the beginning of a cycle, once between the deprotection steps, 4 times before and 3 times after the coupling reaction with DMF (7 mL).

After the final Fmoc deprotection under the same conditions like before, the N-terminus was capped in 7 mL of a 10% (v/v) acetic anhydride solution in DMF (2 min, 40 W, 65 °C). The resin was washed once before and once between the final deprotection steps, 4 times before and 3 times after the N-terminal capping.

The resin was washed with DMF, DCM, diethyl ether and methanol (each 5 x 4 mL) in a syringe, equipped with a frit, and dried under vacuum.

## Cleavage, sidechain deprotection and work up of the PNA.

The cleavage and deprotection of the side chains was done for 2 h at room temperature under shaking in a cleavage cocktail of TFA/*m*-cresol/TIPS (92.5:5.0:2.5). The crude LiP was precipitated in cold diethyl ether, washed twice with diethyl ether and dried under vacuum. The crude was purified by RP-HPLC and the combined fractions were freeze-dried for storage at -20 °C.

## T<sub>m</sub> measurements.

T<sub>m</sub> measurements of 1 μM oligonucleotide solutions in HEPES buffer were performed in four cycles between 20 and 85 °C at 260 nm wavelengths on a *Cary 100* or *Cary 5000* UV/Vis spectrophotometer in order to prove that the LiP strands are able to hybridize. The melting temperatures are listed in **Table S3**. It was not possible to determine the T<sub>m</sub> of the LiP6/LiP7 duplex. Apart from the fact that PNA/PNA duplexes have in general a higher T<sub>m</sub> than the corresponding PNA/RNA and PNA/DNA duplexes,<sup>[4-5]</sup> the stabilization of the PNA/PNA duplex via hydrophobic interactions of the alkyl chains in close proximity likely increased the T<sub>m</sub> to > 90 °C, in agreement with previous observations for duplexes from lipidated DNAs with the same zipper-like configuration (ΔT<sub>m</sub> approx.. 20 °C).<sup>[6]</sup>

The T<sub>m</sub> of the double-zippers, on the other hand, should not be affected by alkyl interactions, because the bridging oligonucleotide is not alkylated. However, the double-zipper design is similar to a nicked DNA duplex, where configurations with stacked and unstacked nucleobases are in equilibrium.<sup>[7-8]</sup> The interacting alkyl chains of the two neighbouring LiPs might stabilize the conformation with stacked nucleobases, which would explain why the two bridged duplexes dissociate simultaneously and only one transition temperature could be observed for the double-zipper designs, even though the transition temperatures of the individual two-strand duplexes differ from each other.

An indirect determination of the T<sub>m</sub> for membrane-bound double-zippers was obtained with a bridging DNA allowing only assembly (no fusion) but having the same individual binding sequences. The liposome assembly/disassembly experiments are described below (page 13).

**Table S3.** Selected duplexes and their melting temperatures.

Sample	LiP and DNA sequences	T <sub>m</sub> (°C)
LiP6 DNA3	Ac-GluGlu <b>XX (PEG)<sub>12</sub></b> ata gtt aca tgc GluGlu-CONH <sub>2</sub> 5'-GCA TGT AAC TAT	36
LiP7 DNA4	Ac-GluGlu gca tgt aac tat <b>(PEG)<sub>12</sub> XX</b> GluGlu-CONH <sub>2</sub> 5'-ATA GTT ACA TGC	37
LiP8 DNA5	Ac-GluGlu tac gtg cca gcg <b>(PEG)<sub>12</sub> XX</b> GluGlu-CONH <sub>2</sub> 5'-CGC TGG CAC GTA	56
RNA1	5'-GCA UGU AAC UAU CGC UGG CAC GUA	38
LiP6 RNA1	Ac-GluGlu <b>XX (PEG)<sub>12</sub></b> ata gtt aca tgc GluGlu-CONH <sub>2</sub> 5'-GCA UGU AAC UAU CGC UGG CAC GUA	45
LiP8 RNA1	Ac-GluGlu tac gtg cca gcg <b>(PEG)<sub>12</sub> XX</b> GluGlu-CONH <sub>2</sub> 5'-GCA UGU AAC UAU CGC UGG CAC GUA	73
LiP6 LiP8 RNA1	Ac-GluGlu <b>XX (PEG)<sub>12</sub></b> ata gtt aca tgc GluGlu-CONH <sub>2</sub> Ac-GluGlu tac gtg cca gcg <b>(PEG)<sub>12</sub> XX</b> GluGlu-CONH <sub>2</sub> 5'-GCA UGU AAC UAU CGC UGG CAC GUA	71 <sup>a</sup>
LiP6 LiP8 DNA1	Ac-GluGlu <b>XX (PEG)<sub>12</sub></b> ata gtt aca tgc GluGlu-CONH <sub>2</sub> Ac-GluGlu tac gtg cca gcg <b>(PEG)<sub>12</sub> XX</b> GluGlu-CONH <sub>2</sub> 5'-GCA TGT AAC TAT CGC TGG CAC GTA	63 <sup>b,c</sup>
LiP6 LiP8 DNA2	Ac-GluGlu <b>XX (PEG)<sub>12</sub></b> ata gtt aca tgc GluGlu-CONH <sub>2</sub> Ac-GluGlu tac gtg cca gcg <b>(PEG)<sub>12</sub> XX</b> GluGlu-CONH <sub>2</sub> 5'-GCA TGT AAC TAT <b>(PEG)<sub>4</sub></b> CGC TGG CAC GTA	60 <sup>c</sup>

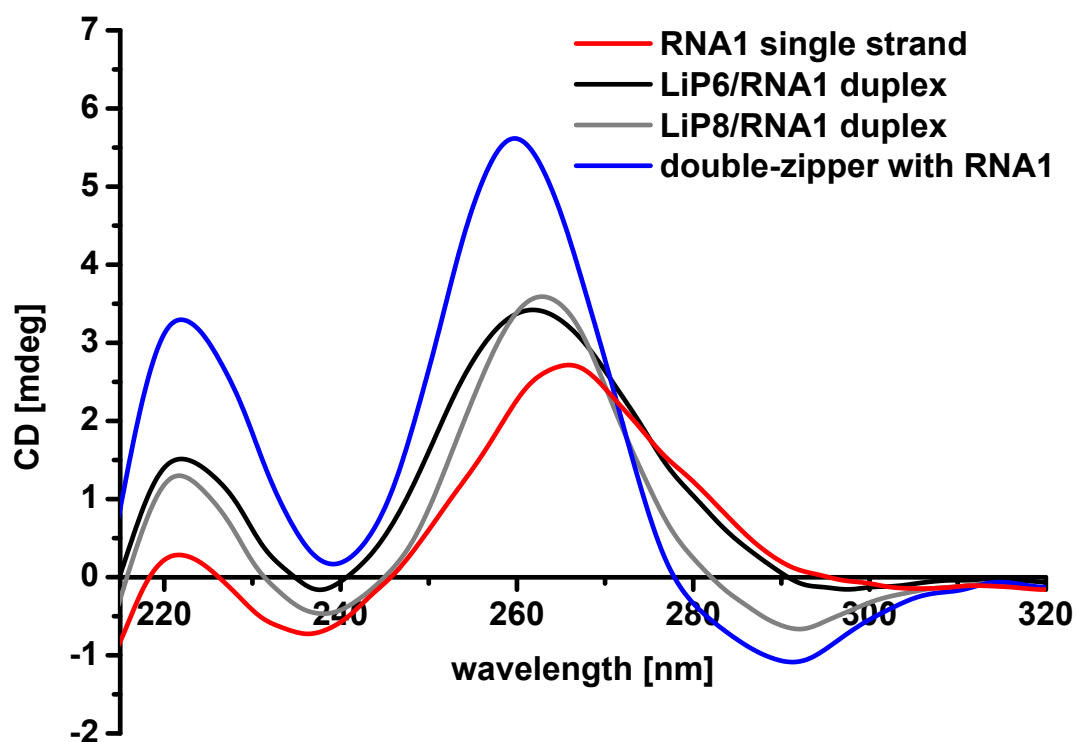
a) The melting temperature corresponds to the denaturation of the PNA/RNA duplex.

b) A broad transition was observed at T < 20 °C, which is probably related to unspecific hydrophobic lipid interactions and was observed for lipidated DNA before.<sup>[9]</sup>

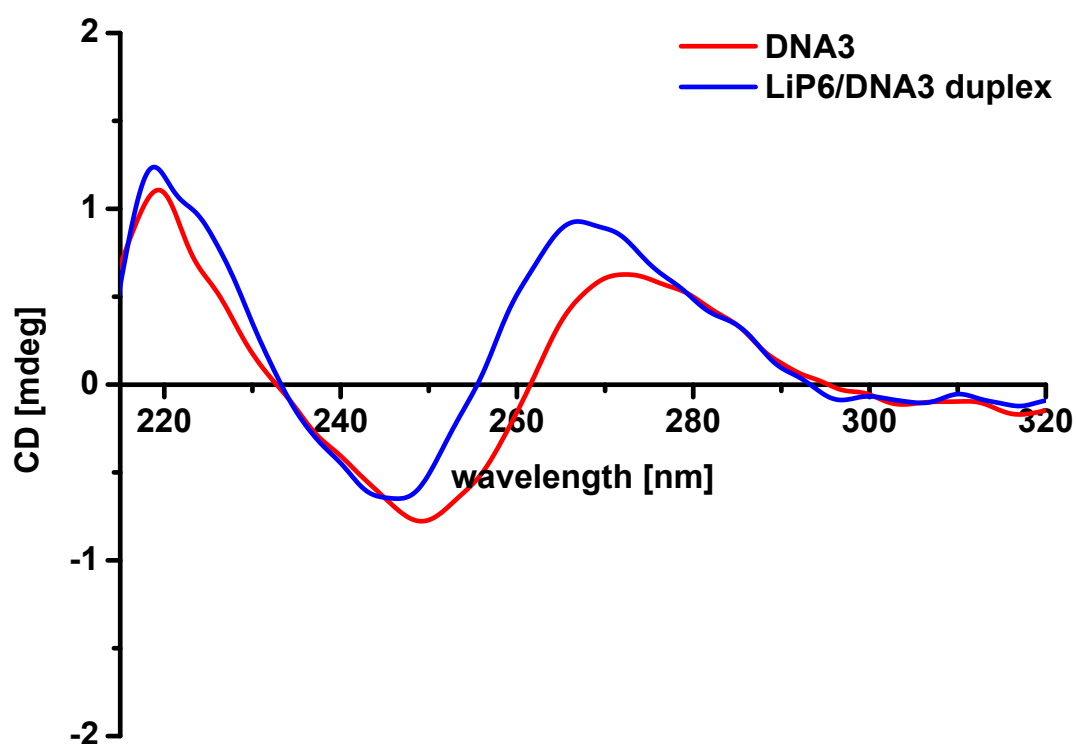
c) The melting temperature corresponds to the denaturation of the PNA/DNA duplex.

## Circular dichroism (CD).

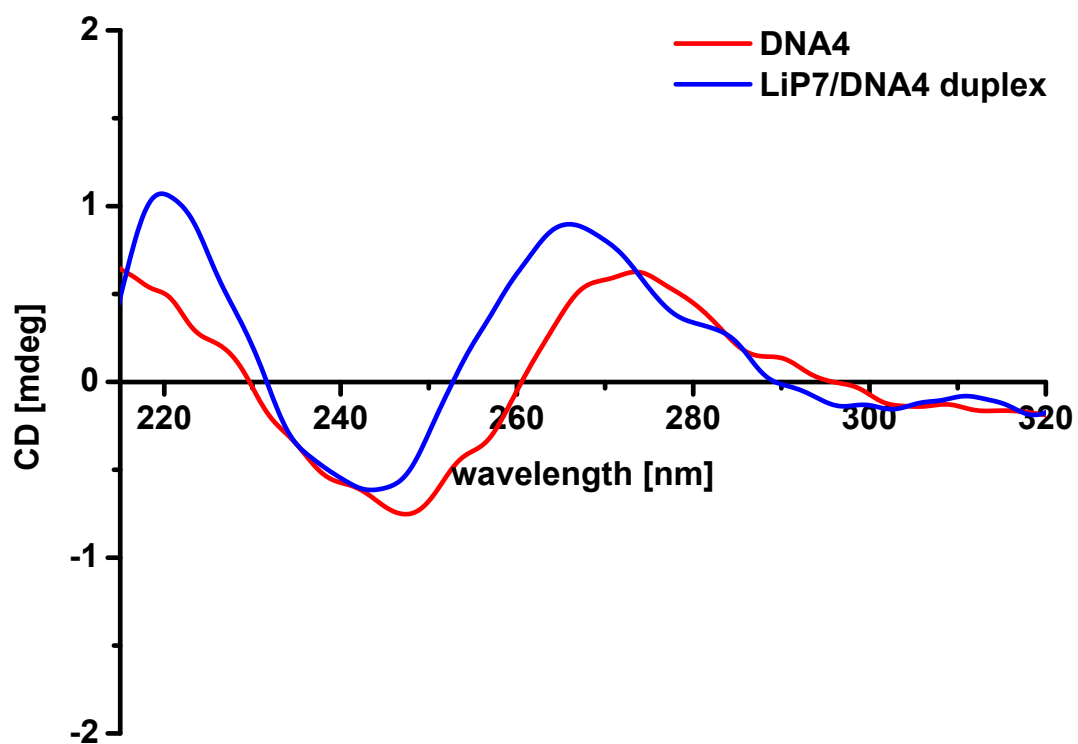
CD measurements of 1 μM oligonucleotide solutions in HEPES buffer were performed on a *Jasco J-815* CD spectrophotometer at room temperature (**Figure S5-S8**). The LiP6/LiP7 zipper design did not show optical activity, due to the achiral nature of PNA. Only the CD spectra of LiP/DNA or LiP/RNA duplexes are shown.



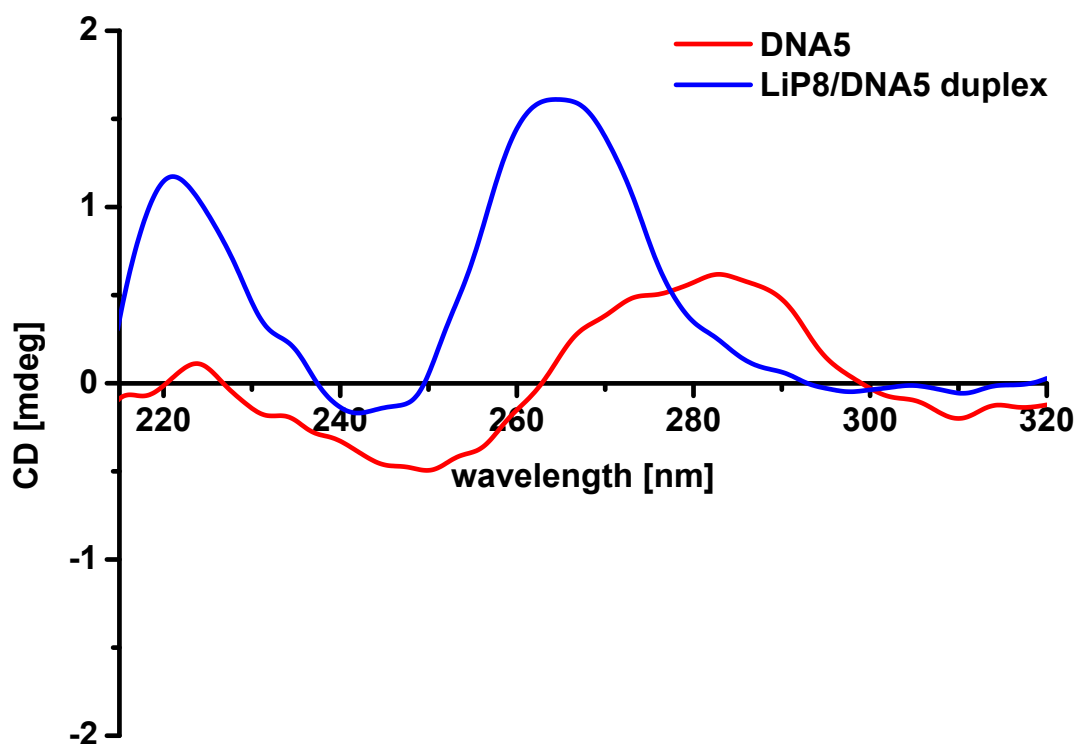
**Figure S5.** CD spectrum of the double-zipper with bridging RNA1 and the LiP6/RNA1 duplex, the LiP8/RNA1 duplex and the RNA1 single strand in comparison.



**Figure S6.** CD spectrum of LiP6 in a duplex with the unmodified complementary DNA3 and DNA3 as single strand.



**Figure S7.** CD spectrum of LiP7 in a duplex with the unmodified complementary DNA4 and DNA4 as single strand.



**Figure S8.** CD spectrum of LiP8 in a duplex with the unmodified complementary DNA5 and DNA5 as single strand.

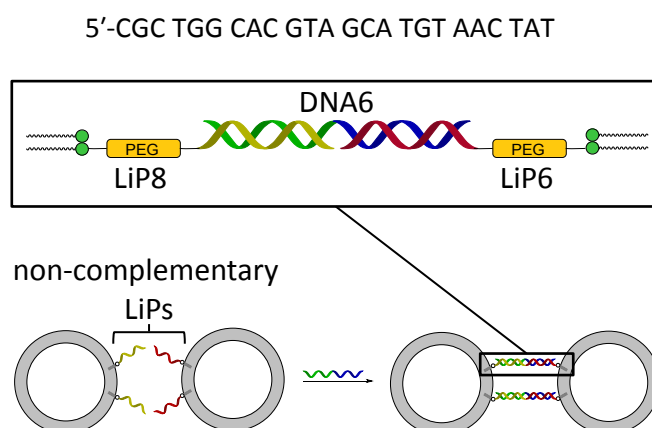
## Preparation of liposomes.

A lipid mixture in chloroform or methanol was evaporated to form a lipid film, which was dried under vacuum. Multilamellar vesicles (MLVs) were prepared by incubation of the lipid film in buffer for 30 min at room temperature, vortexing for 5 min, incubation at 50 °C in an ultrasound bath and vortexing for further 5 min. In order to obtain small unilamellar vesicles (LUVs, nominal diameter 100 nm) for fusion assays, the MLVs were extruded 21 times with an *Avanti* Mini Extruder, equipped with a polycarbonate Nuclepore Track-Etched Membrane from *Whatman*® with a pore size of 100 nm. Before usage, the Mini Extruder was rinsed with buffer and air, in order to minimize the dead volume. The extrusion of POPC liposomes (nominal diameter 100 nm) for the agarose gel electrophoresis experiments was done on a *Lipex* 10 mL barrel extruder, equipped with two polycarbonate membranes of the same kind.

## Assembly/disassembly of liposomes

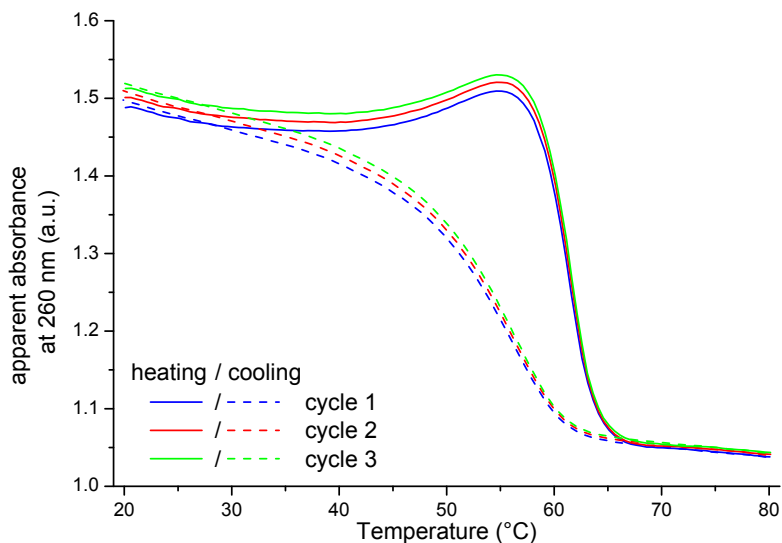
Assembly/disassembly of liposomes was observed to determine indirectly the  $T_m$  of LiP/DNA duplexes when incorporated in liposomes. Assembled liposomes scatter light in a higher degree than disassembled liposomes and show therefore a decrease in apparent absorbance, *i.e.* a decrease in turbidity, during thermal dissociation. The double zipper or zipper fusion design could not be measured on this way, because fusion of liposomes would make the interpretation of the thermal curves difficult. Therefore an assembly system was used similar to the double zipper fusion system. The DNA bridging strand DNA6 was designed in a way that the anchor moieties of LiP6 and LiP8 were on opposite sides of the formed LiP6/LiP8/DNA6 duplex (**Figure S9**). On that way, the liposomes were assembled but not forced into a distance, which promotes fusion.

Sequence of DNA6:



**Figure S9.** Assembly system to determine the  $T_m$  of the LiP6/LiP8/DNA6 duplex when incorporated into lipid bilayers.

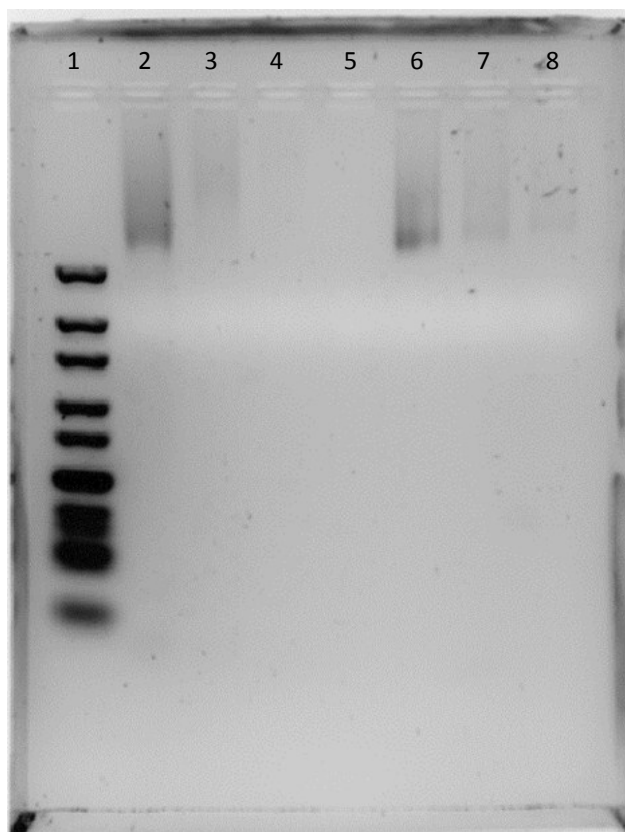
A LiP6-decorated and a LiP8-decorated POPC liposome population were mixed in a 1:1 ratio with a total lipid concentration of 500  $\mu\text{M}$  and a lipid/liP ratio of 1000:1. The bridging ON DNA6 was added in a concentration of 0.5  $\mu\text{M}$  (1.0 eq.). The apparent absorbance was measured at 260 nm wavelength in three cycles of heating and cooling between 20 and 80 °C (0.5 °C/min) after an initial annealing cycle (**Figure S10**). The thermal dissociation temperature of the membrane-incorporated LiP6/LiP8/DNA6 duplex was determined to be 61 °C.



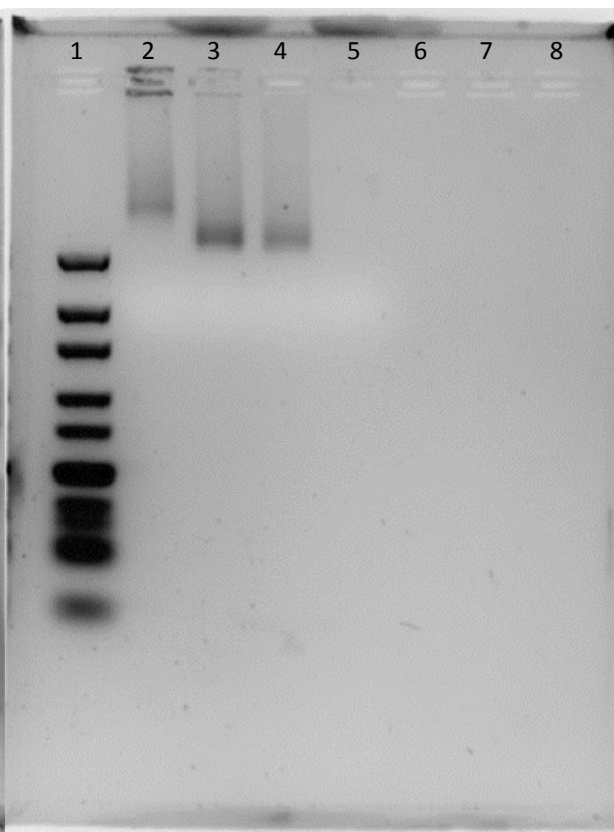
**Figure S10.** Assembly/disassembly of liposomes, linked via the LiP6/LiP8/DNA6 duplex. The assembly/disassembly of liposomes was observed by an apparent absorbance change of the liposome solution, which is caused by a higher degree of light scattering of assembled liposomes. Therefore, it appears as an inverse  $T_m$  curve with a larger offset. The transition temperature of the disassembly of liposomes was determined to be 61 °C.

### Agarose gel electrophoresis.

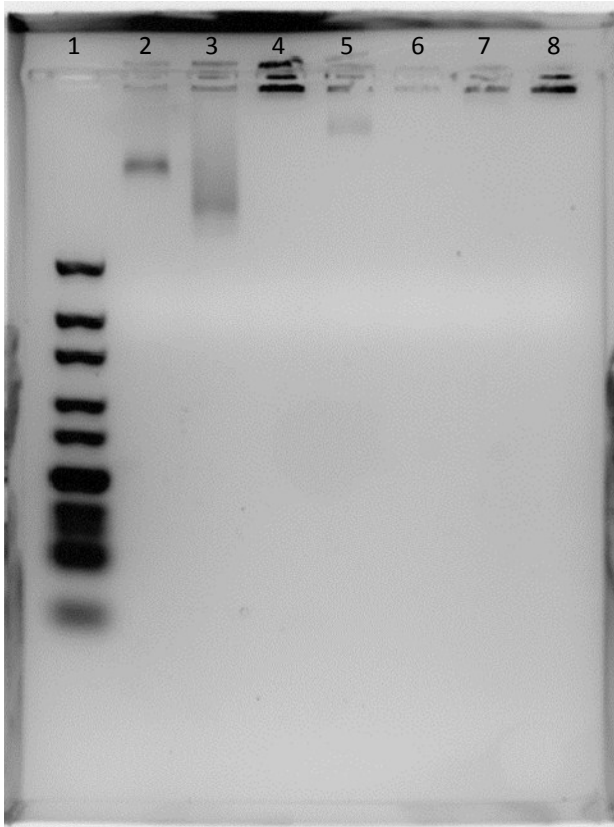
250 pmol of the LiP was added to 163.7 nmol POPC liposomes (nominal diameter 100 nm) in HEPES buffer (20  $\mu$ L) and incubated under different conditions. 4  $\mu$ L of a loading dye (10 mM Tris-HCl, 60 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, pH 7.6) was added to each sample. After incubation the samples were loaded immediately on 3% agarose dissolved in TBE buffer and 0.13% GelRed 10,000x solution. An ultra-low range DNA ladder (10-300 bp) was sampled as well. The agarose gel electrophoresis was carried out in a horizontal electrophoresis apparatus at 50 mA, 300 W and 100 V until the loading dye reached the outmost part of the gel. All agarose gels are shown in **Figure S11-S14**. **Figure S15** shows the summary of the agarose gels.



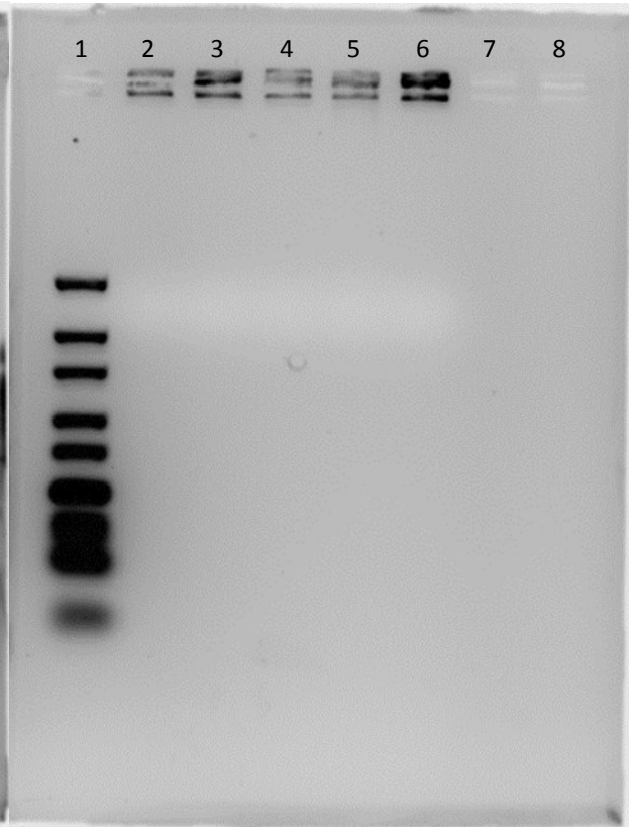
**Figure S11.** 250 pmol (2), 100 pmol (3), 50 pmol (4) and 10 pmol (5) of LiP3 and the complementary DNA as duplex as well as 250 pmol (6), 100 pmol (7) and 50 pmol (8) of the single stranded LiP3 were added to the wells of an agarose gel (3%) to test differences between the staining of a duplex and a PNA single strand with GelRed™ and to test the needed amounts. Lane 1 shows an ultra-low range DNA ladder (10-300 bp). The gel shows that single stranded PNA was stained sufficiently and 250 pmol were necessary to obtain a well visible spot.



**Figure S12.** Electrophoresis in an agarose gel (3%) to test incorporation of LiP3 into the membrane of 100 nm POPC liposomes. The wells were loaded with an ultra-low range (10-300 bp) DNA ladder (1), a mixture of LiP3 (250 pmol) and POPC liposomes (164 nmol) after incubation overnight at room temperature (2), a mixture of LiP3 (250 pmol) and POPC liposomes (164 nmol) after incubation for 15 min at room temperature (3), LiP3 (250 pmol) only (4) and POPC liposomes (164 nmol) only (5). Wells 6 to 8 were empty. It is visible that liposomes are not stained by GelRed but they should stay in the well of the gel. The gel shows also that more PNA remained in the well after overnight incubation in comparison to incubation for 15 min. Therefore, more PNA is incorporated in the membrane overnight than after 15 min. Because there is still mobile PNA visible, it shows that not all PNA is incorporated.

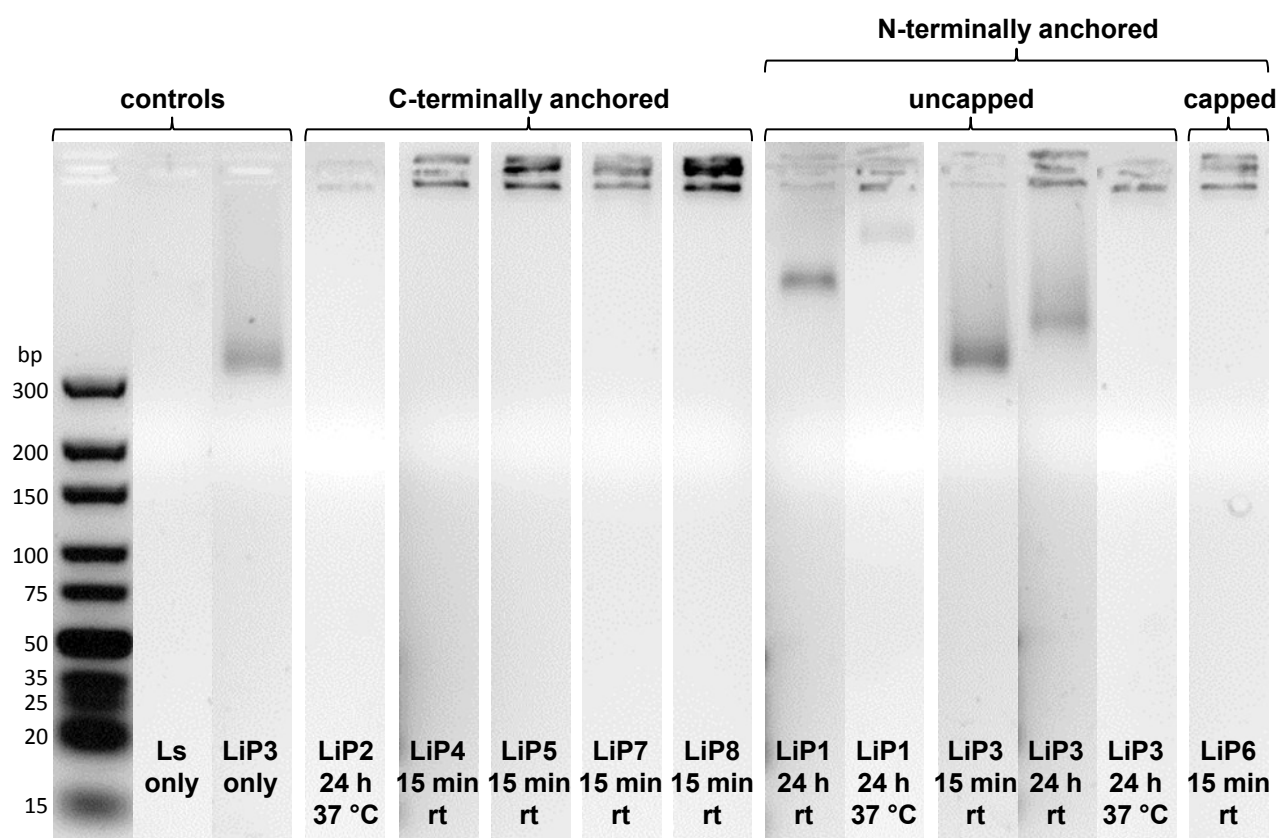


**Figure S13.** Electrophoresis in an agarose gel (3%) with different PNA samples (250 pmol) in a mixture with 100 nm POPC liposomes (164 nmol) after previous incubation overnight at room temperature or 37° C. Well 1: Ultra-low range DNA ladder (10-300 bp); Well 2: LiP1, room temperature; Well 3: LiP3, room temperature; Well 4: LiP5, room temperature; Well 5: LiP1, 37 °C; Well 6: LiP2, 37 °C; Well 7: LiP3, 37 °C; Well 8: LiP5, 37 °C.



**Figure S14.** Electrophoresis in an agarose gel (3%) with different PNA samples (250 pmol) in a mixture with 100 nm POPC liposomes (164 nmol) after previous incubation for 15 min at room temperature. The wells contained liposomes in a mixture with LiP4 (2), LiP5 (3), LiP6 (4), LiP7 (5) and LiP8 (6). Well 1 contained an ultra-low range DNA ladder (10-300 bp) and 7 and 8 were empty. All samples with a C-terminal anchoring as well as LiP6 with a capped N-terminus seemed to incorporate well into the membrane of liposomes.





**Figure S15.** Agarose gels (3%) of mixtures of liposomes and LiPs (lipid/LiP 655:1) after incubation at different conditions. POPC liposomes (Ls) only and LiP3 only were sampled as controls. Lane 1 shows the ultra-low range DNA ladder (10-300 bp). Sample names, incubation times and temperatures are indicated for each lane. Intensity differences were assumed to originate from different intercalation affinities of GelRed™ towards the different PNA single strands. GelRed™ does not stain liposomes. The different mobility of the strands could be due to different aggregations, GelRed™-LiP interactions or detergent effects, depending on concentration of free LiPs.

## Encapsulation of sulforhodamine B (SRB).

In order to encapsulate sulforhodamine B (SRB) into liposomes, MLVs were prepared in SRB buffer, where the lipid concentration was 16 mM. MLVs were extruded as described above. To remove the SRB in the outer media of liposomes, a size exclusion spin column (Illustra Microspin S-200 HR columns from GE Healthcare) was washed with HEPES buffer (3 x 200  $\mu$ L, 2700 rpm, 1 min), the liposome solution (50  $\mu$ L) was added and the spin column was centrifuged (2700 rpm, 1 min) and the eluate collected. Further HEPES buffer (50  $\mu$ L) was added to the spin column and centrifuged (2700 rpm, 1 min) in order to elute the remaining liposomes.

## SRB fusion assays (content mixing and leakage).

It has previously been claimed that even inner leaflet lipid mixing does not guarantee content mixing<sup>[10]</sup> and we thus used content mixing data as our main criterion for fusion efficiency. To measure content mixing, SRB filled liposomes and HEPES buffer filled liposomes (DOPC/DOPE/cholesterol/POPG 2:1:1:0.06) were prepared 24 h ahead of the experiment. In order to prepare a 1  $\mu$ M stock solution of LiPs in HEPES buffer, the LiP was dissolved in HEPES buffer for 30 min in an ultrasounds bath at 60 °C shortly before the experiment. SRB filled

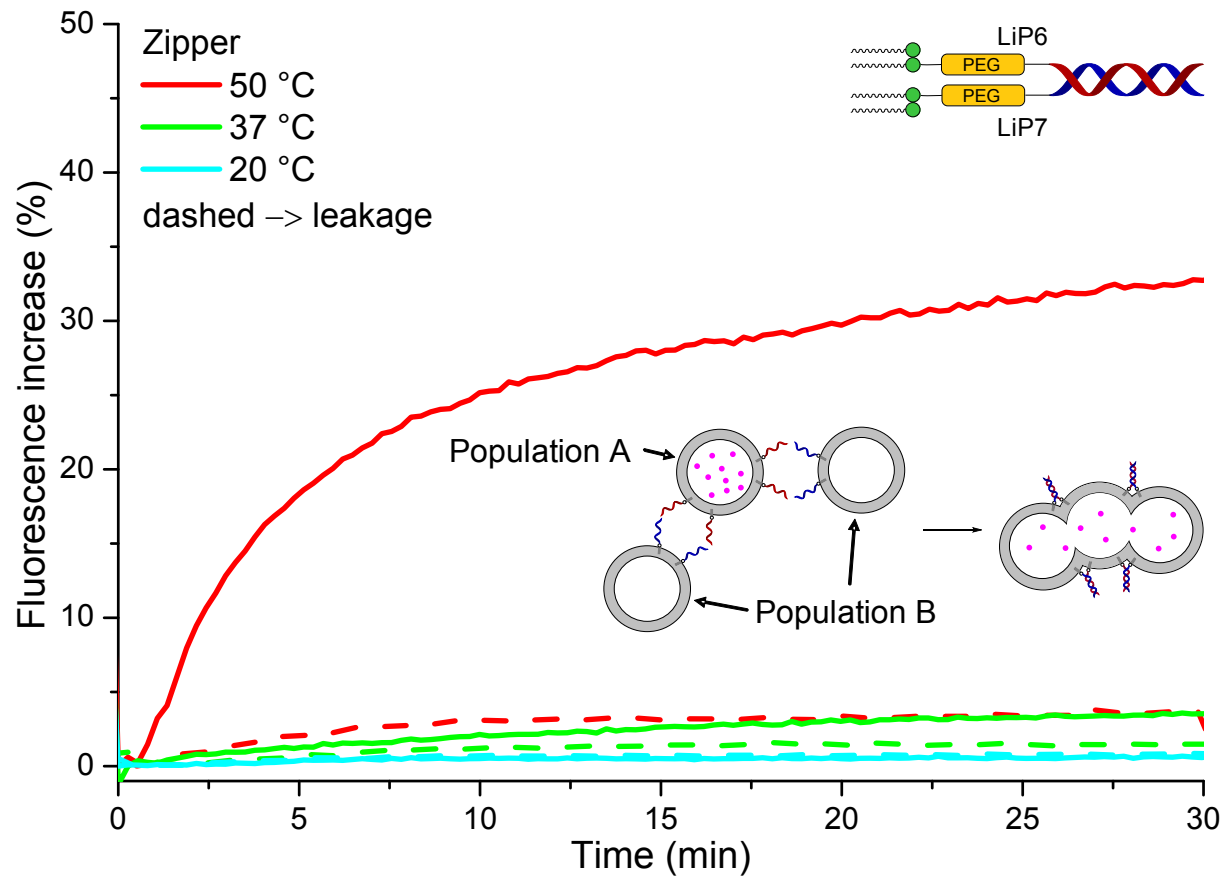
liposomes were incubated 15 min at room temperature with LiP6 (population A) and SRB (leakage) or HEPES buffer (content mixing) filled liposomes with LiP7 or LiP8 (population B), respectively. The lipid to LiP ratio was for population A 655:1 and for population B 1310:1. Both populations as well as in case of the double-zipper a solution of the bridging strand (1.5 eq. relative to LiPs) in HEPES buffer were mixed to get a final lipid concentration of 103.1  $\mu\text{M}$ . The fluorescence intensity was measured ( $\lambda_{\text{ex.}} = 545 \text{ nm}$ ,  $\lambda_{\text{em.}} = 583 \text{ nm}$ ) for 30 min (measuring every 15 s) at different temperatures. As a control, both liposome populations were decorated with LiP6. The fluorescence increase was calculated as follows:

$$F(\%) = \frac{F_t - F_{\min}}{a F_{\text{total}} - F_{\min}} \cdot 100$$

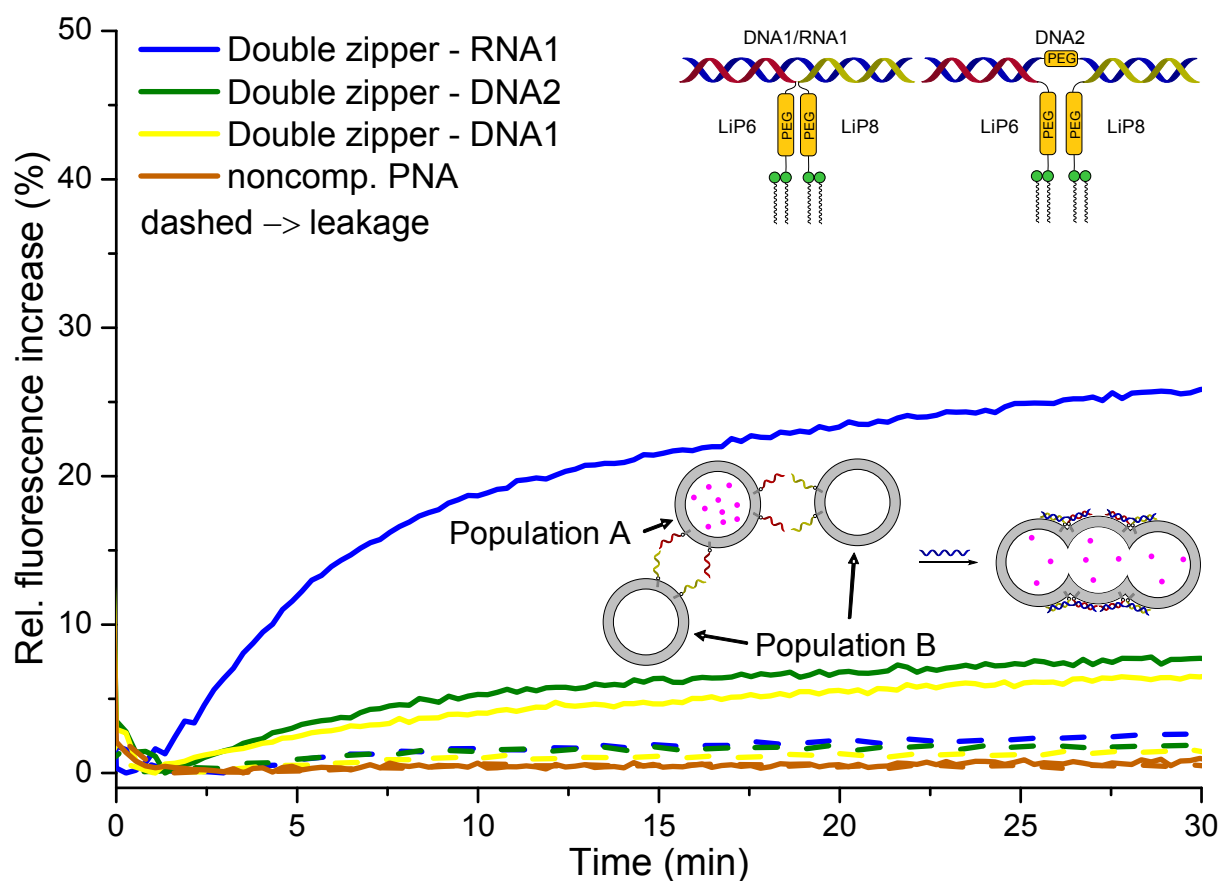
$F_t$  is the fluorescence intensity at the time  $t$ . The given content mixing and leakage values were calculated with  $t = 30 \text{ min}$ .  $F_{\min}$  is the minimum fluorescence intensity after mixing the liposome populations.  $F_{\text{total}}$  is the fluorescence intensity after the lysis of liposomes in 1% Triton X-100.  $a$  is a dilution factor, depending on the volume of the sample  $V_{\text{sample}}$  and the volume of the added Triton X-100 solution  $V_{\text{triton}}$ :

$$a = \frac{V_{\text{sample}} + V_{\text{triton}}}{V_{\text{sample}}}$$

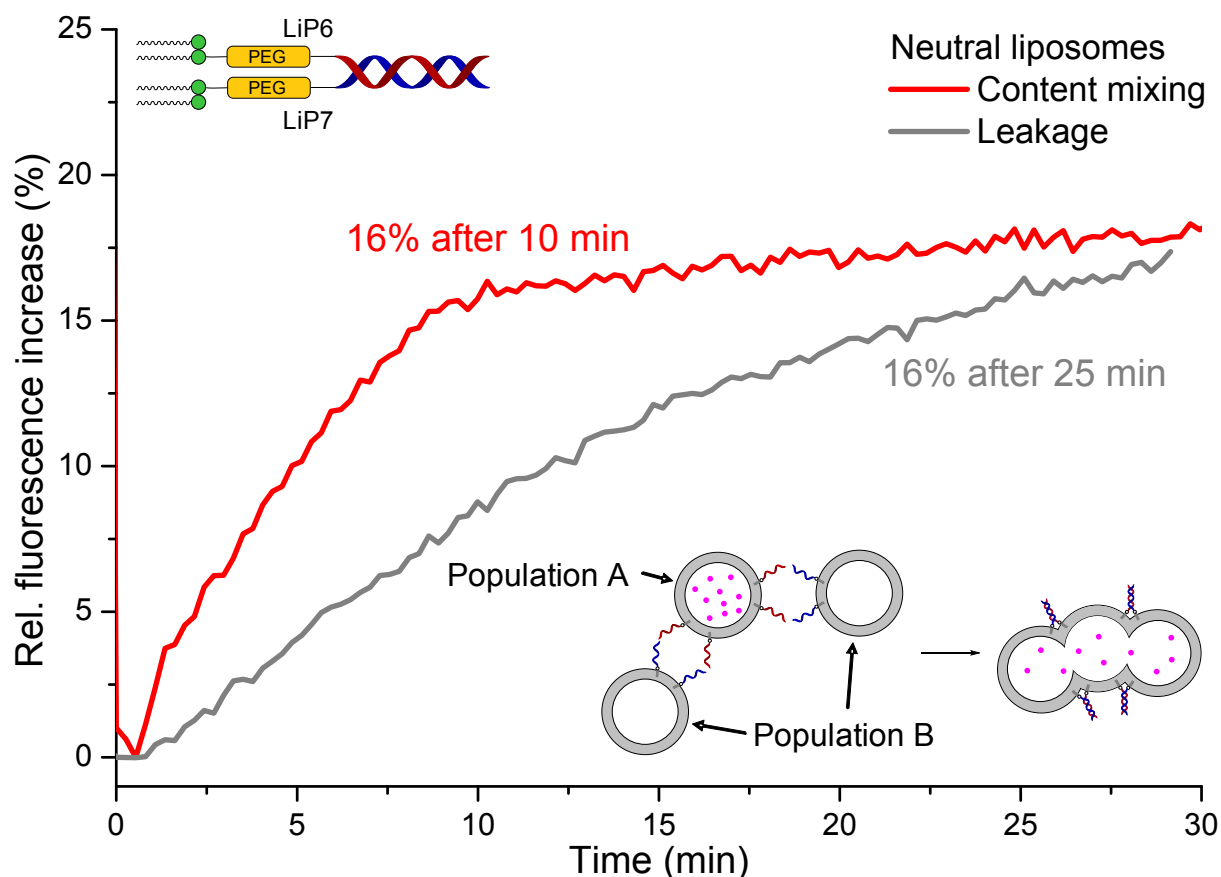
The content mixing and leakage vs. time is shown in **Figure S16** and **Figure S17**.



**Figure S16.** Content mixing (solid lines) and leakage (dashed lines) during fusion of two populations of 100 nm liposomes (137.5  $\mu$ M, DOPC/DOPE/cholesterol/POPG 2:1:1:0.06) in buffer (10 mM HEPES-NaOH, 100 mM NaCl, adjusted to pH 7) mediated by LiP6 and LiP7 (105 nm final concentration of each strand) at different temperatures (red 50 °C, green 37 °C, blue 20 °C). One population (content mixing assay) or both populations (leakage assay) of liposomes were filled with a self-quenching SRB concentration (20 mM in HEPES buffer), which led to a fluorescence increase due to dilution during mixing or leaking of content. The 100% value of fluorescence increase was determined by lysis of liposomes with 1% Triton X-100.



**Figure S17.** Content mixing (solid lines) and leakage (dashed lines) during fusion of two populations of 100 nm liposomes (137.5  $\mu\text{M}$ , DOPC/DOPE/cholesterol/POPG 2:1:1:0.06) in buffer (10 mM HEPES-NaOH, 100 mM NaCl, adjusted to pH 7) mediated by LiP (105 nm final concentration of each strand). Each liposome population was decorated with either one LiP from a complementary couple (zipper, black) or from a noncomplementary couple (double zipper). The double zipper was bridged by an unmodified RNA (blue), a DNA with a PEG<sub>4</sub> spacer between the two sequences (green) or an unmodified DNA (yellow). The bridging ON was added in 1.5 eq. to the anchored PNAs. One population (content mixing assay) or both populations (leakage) of liposomes were filled with a self-quenching concentration of sulforhodamine B (20 mM in HEPES buffer), which resulted in a fluorescence increase due to dilution. The 100% value for fluorescence increase was determined by lysis of liposomes with 1% Triton X-100. A scheme of the double zipper design and the content mixing assay are also illustrated.



**Figure S18.** Content mixing (red) and leakage (grey) during fusion of two populations of neutral 100 nm liposomes (137.5  $\mu\text{M}$ , DOPC/DOPE/cholesterol 2:1:1) in buffer (10 mM HEPES-NaOH, 100 mM NaCl, adjusted to pH 7) mediated by LiP6 and LiP7 (105 nm final concentration of each strand) at 50  $^{\circ}\text{C}$ . One population (content mixing assay) or both populations (leakage assay) of liposomes were filled with a self-quenching SRB concentration (20 mM in HEPES buffer), which led to a fluorescence increase due to dilution during mixing or leaking of content. The 100% value of fluorescence increase was determined by lysis of liposomes with 1% Triton X-100. A scheme of the zipper design and the content mixing assay are also illustrated.

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