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Label-free observation of DNA-encoded liposome fusion by surface plasmon resonance.

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Supporting Information

Contents

Materials and methods

Materials and general methods. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids in highest available purity. Cholesterol (Chol) was purchased from Riedel-de Haën in extra pure quality. All other reagents were purchased from standard suppliers in cell culture grade. High purity water (MilliQ, Merck Millipore Advantage A10 system) was used for all samples and experiments. The liposome fusion and assembly experiments, both for UV-vis and SPR monitoring carried out in a HEPES buffered saline (HBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mM NaCl in water, adjusted to pH 7.0 with a NaOH solution ([Na+] = 110 mM).

Synthesis and characterization of lipid-nucleic acid conjugates. Standard solid-phase automated DNA synthesis was used to synthesize LiNAs, incl. the DMT-protected phosphoramidite anchor building blocks (0.05 M in DCE/ACN, 2:1, 400 µl, 20 min reaction time) either near the 5'- or 3'-end as previously described.[1] All LiNAs were purified using RP-HPLC after final DMT-cleavage (Table S1) and verified by MALDI-TOF mass spectrometry. LiNA stock solutions (50% acetonitrile/water) were kept at 4 °C. Prior to fusion experiments, LiNAs were diluted to 1 µM in HBS (Abs. at 260 nm and calculated extinction coefficients) stored at RT and used within 48 h.

General liposome preparation. To produce liposomes (DOPC/DOPE/Chol; 2:1:1, molar ratio), appropriate volumes of lipid stock solutions in chloroform were mixed and lipid films formed by solvent removal using a gentle stream of N2 followed by high vacuum (> 2h). After resuspension in HBS (30 min, RT, the suspensions were placed in a bath-type sonicator at 50 °C (10 min) and extruded 21x through a 100 nm polycarbonate membrane (Whatman Nucleopore Track-Etched Membranes) using a handheld extrusion device (Avanti Polar Lipids) (stored at 4 °C and used within one week).

Nanoparticle Tracking Analysis (NTA). Size distributions. The size distributions of freshly extruded liposomes (DOPC, DOPE, Cholesterol (Chol); 2:1:1, molar ratio) and samples from fusion experiments were analyzed using a NanoSight (Wiltshire, UK) instrument, equipped with the NanoSight LM14 flow-cell and laser assembly (405 nm diode laser), a 20× objective and CCD camera. Data was recorded and analyzed using the NTA v. 2.3 software (recording: 5 × 30 s with shutter 387 (25 fps), gain 200-350, histogram: 520 - 16383); analysis: detection threshold 10, blur 'auto', min. track length 10, expected size 'auto'). Liposome preparations were measured at 2 µM total lipid concentration ([lipid]).

The nanoparticle tracking analysis (NTA) system allows nanoparticles to be individually visualized (but not imaged) in liquids and from which high resolution particle size distribution profiles can be obtained compared to other light scattering techniques (e.g. DLS). Sample pretreatment is minimal requiring only dilution with a suitable solvent (buffer) to an acceptable particle concentration range, between 10^8 to 10^{10} particles per ml depending on sample type. Accurate and reproducible analyses can be obtained from video and the results allow particle number concentration to be recovered. Given the close to real-time nature of the technique, particle-particle interactions are accessible as is information about sample aggregation and dissemination. All particle types can be measured and in any solvent type providing that the particles scatter sufficient light to be visible. The minimum detectable size measurable depends on particle refractive index but is around 40 nm for low refractive index materials such as liposomes whereas particles with high refractive index can be detected to sizes down to 10 nm (colloidal metals).[3, 4] A video for the field of view (120 µm x 90 µm x 10 µm) is recorded during a peridod of 120 s to ensure a sufficient statistical data set. Movement of individual particles is followed through the video sequence and the mean squared displacement determined for each particle for as long as it is visible. The video images of the particle's movement under Brownian motion can be analysed by a single particle tracking programme (Nanoparticle Tracking Analysis - NTA software 2.1). For the nanoparticle range (10-600 nm) to which the NanoSight system is best suited, the particles act as point scatterers whose dimensions are below the Rayleigh or Abbé limit, only above which can structural information and shape be resolved. The user can further select for trajectories whose lifetimes are sufficiently long to ensure statistically accurate results, ignoring those which are so short (e.g. below 5 or 10 video frames) that the estimation of diffusion coefficient is statistically inaccurate. This feature is employed under automatic control. The possible occurrence of measurement of trajectories which cross is automatically eliminated thus minimising errors in particle tracking. From simultaneous measurement of the mean squared displacement of each particle tracked, the particle diffusion coefficient (Dt) and hence sphere-equivalent, hydrodynamic radius (r) can be determined using the Stokes-Einstein equation:

$$Dt = \frac{K_b\, T}{6\pi\eta r_n}$$

K_B is Boltzmann's constant, T is temperature and η is solvent viscosity.

Engraftment of liposomes with LiNAs. Liposomes were engrafted with LiNAs in two populations (**A**, 100 µM [lipid] with 0.1 µM LiNA-**1**; **B,** 100 µM [**l**ipid] with 0.1 µM LiNA-**2** or LiNA-**3**, lipid/DNA ratio 1000:1, approx. 80 strands per 100 nm liposome, 15 min incubation at room temperature).

Thermal denaturation of DNA/LiNAs duplexes. The thermal denaturation experiments were performed using solutions of 0.5 µM DNA oligomers in HBS buffer (HEPES 10 mM, Na+ 110 mM, Cl- 108 mM, pH 7.0) in a total volume of 1 mL using a Varian Cary 100. Thermal denaturation temperatures (T_m) were determined from first derivatives of melting curves. The oligos underwent an annealing cycle prior to the measurement by a fast heating to 80°C followed by cooling to 20°C. Absorbance was recorded at 260 nm as a function of temperature at a rate of 0.5°C/min. Samples started with a temperature of 20°C and were heated to 80°C and went through 3 heating and cooling cycles. All melting temperatures are determined from multiple experiments and thus reported with an uncertainty of ±0.5°C.

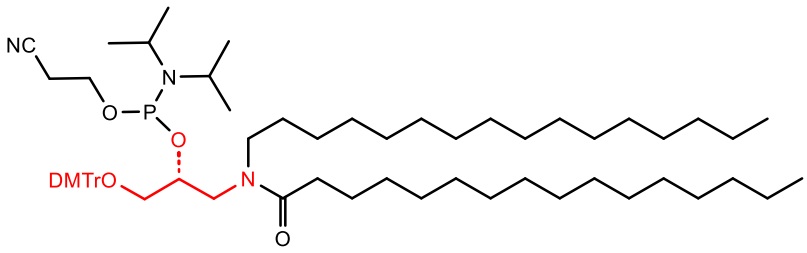
Thermal denaturation of assembled liposomes. DOPC/DOPE/Chol liposomes (250 µM) were divided in aliquots, each mixed with a specific DNA strand (250 nM) and incubated at room temperature for 15 minutes to ensure that the DNA is anchored in the membrane. The aliquots were mixed in pairs with a 1:1 ratio in a UV/Vis cuvette (1 mL total volume). All concentrations are given as final concentration after mixing in the cuvette. The measurements were carried out between 20 and 70°C, using the same method as the previously described for thermal denaturation of LiNAs.

Assembly and fusion monitored by time-resolved UV-absorbance. Starting with LiNA engrafted liposomes (0.1 mol%), but in asymmetric volumes to enable t_0 measurement (1st population in 800 µl, 2nd in 200 µl). Samples were monitored at 37 °C at 260 nm at a final concentration of 250 µM total lipid, 0.25 µM total LiNA, at a 1:1 ratio between the two populations, pairing either LiNA-**1** and LiNA-**2A** (assembly), LiNA-**1** and -**2F** (fusion) or LiNA-**1** on both populations (non-complementary control). After approx. 1.5 h, 100 µl of 550 U/ml DNAse I in 10X DNAse I reaction buffer was added (final activity 50 U/mL).

Surface Plasmon Resonance (SPR) Assay. SPR sensorgrams were recorded on a Biacore™ X100 system using L1 sensor chip (GE Lifesciences, now Cytiva) at 37°C. The L1 chip was conditioned via four injections of iPrOH / 50 mM NaOH 4:6 (120 s, flow rate: 10 µL/min). In both channels: after capture of liposomes carrying LiNA-**1** (DOPC, DOPE, Chol, 2:1:1, molar ratio, avg. diameter 124 nm, PDI 0.095, 1 mM, 900 s, flow rate: 1 µL/min). Any weakly associated material was removed by increasing the flow rate of the running buffer to 10 µL/min for 300 s to achieve a stable baseline (treatment with dilute alkaline was avoided due to the presence of ionizable DOPE). Then a secondary population was injected carrying either LiNA-**1** (non-complementary control) LiNA-**2** (fusion), LiNA-**3** (assembly), or running buffer (reference sensorgram); 900 s, flow rate: 1 µL/min), followed by a flow of running buffer to observe dissociation (900 s, flow rate: 1 µL/min). Subsequently, a 50 U/ml solution of DNAse I in DNAse I-buffer (both Sigma-Aldrich) was injected in all cases (900 s, flow rate: 1 µL/min), after another 900s at 1 µl/min the L1 chip was regenerated by two injections of 50 mM NaOH in 50% v/v isopropanol/water (20 µL, flow rate: 10 µL/min). The data was aligned and exported using the BiaEvaluation software (ver. 2.1) and injection positions further synchronized in Origin to allow reference subtraction.

DNA oligonucleotides

Characterization of lipidated nucleic acids (LiNAs) and structure of modification "X" as phosphoramidate building block



"X"

The LiNAs were synthesized according to our previously described procedure.[2]

Table S1: *List of LiNA oligonucleotide sequences and their properties.*

Entry	Sequence (5'-3')	R_t [min][a]	Mass (calcd.) [g/mol]	Mass (found) [m/z]
LiNA-1	T**X**TGTGGAAGAAGTTGGTG	10.1[b]	6265.7	6267.3
LiNA-2F	CACCAACTTCTTCCACA**X**T	15.2[c]	6264.9	6267.6
LiNA-2A	T**X**CACCAACTTCTTCCACA	17.8	5953.8	5951.1
LiNA-3F	T**XP3**TGTGGAAGAAGTTGGTG	13.8[d]	6476.9	6481.9
LiNA-3A	TGTGGAAGAAGTTGGTG**P3X**T	13.5[e]	6476.9	6488.0
LiNA-4	CACCAACTTCTTCCACA**P3X**T	13.5[e]	6165.8	6173.2

[a] HPLC methods: Solvent A = 0,05 M TEAA, pH = 7, solvent B = 0,05 M TEAA / ACN (1:3, v,v), pH = 7. [b] Flow = 1 mL/min, starting conditions are 10% B, gradient: 0 → 1, 10% B; 1 → 10, 100% B; 10 → 13, 100%. [c] Flow = 1 mL/min, starting conditions are 10% B, gradient: 0 → 4, 10% B; 4 → 8, 50% B; 8 → 16, 100% B; 16 → 19, 100% B. [d] Flow = 1,4 mL/min, starting conditions are 32% B, gradient: 0 → 1, 32% B; 1 → 20, 100% B; 20 → 25, 100% B; 25 → 27, 32% B; 27 → 30, 32% B. [e] Flow = 1,4 mL/min, starting conditions are 32% B, gradient: 0 → 1, 32% B; 1 → 16, 100% B; 16 → 19, 100% B; 19 → 20, 32% B; 20 → 23,5, 32% B.

T_m of LiNAs

Table S2: *List of T_m values for the LiNA oligonucleotides and the corresponding unmodified sequences.*

Entry	Sequence	T_m [°C]	ΔT_m [°C][a]
5'-A	TGTGGAAGAAGTTGGTG	57.7	-
3'-A'	ACACCTTCTTCAACCAC		
5'-LiNA-3F	T**XP3**TGTGGAAGAAGTTGGTG	78.8	+21.1
3'-LiNA-4	T**XP3**ACACCTTCTTCAACCAC		
5'-LiNA-4	CACCAACTTCTTCCACA**P3X**T	60.4	+2.7
5'-LiNA-3A	TGTGGAAGAAGTTGGTG**P3X**T		
5'-LiNA-1	T**X**TGTGGAAGAAGTTGGTG	77.1	+19.4
3'-LiNA-2F	T**X**ACACCTTCTTCAACCAC		
5'-LiNA-1	T**X**TGTGGAAGAAGTTGGTG	59.6	+1.9
5'-LiNA-2A	ACACCTTCTTCAACCAC**X**T		

[a] The difference in T_m is calculated between the stated pair and the unmodified duplex.

Nanoparticle Tracking Analysis data

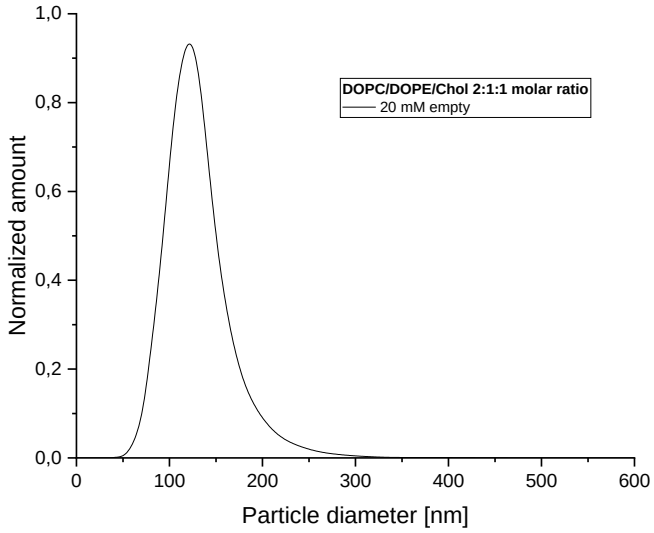


Figure S1: Mean diameter for liposomes used for UV measurements was determined to be 131±1 nm by NTA.

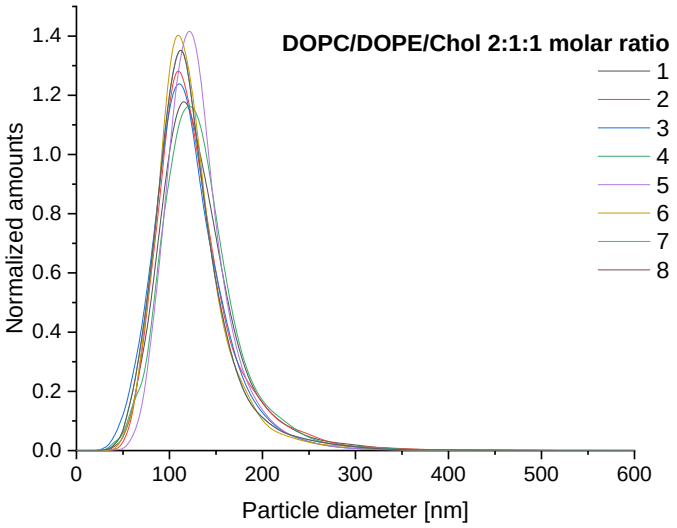


Figure S2: Size distribution of the different batches of liposomes used for SPR measurements. The mean diameter is determined to be between 123 nm and 134 nm by NTA for all batches.

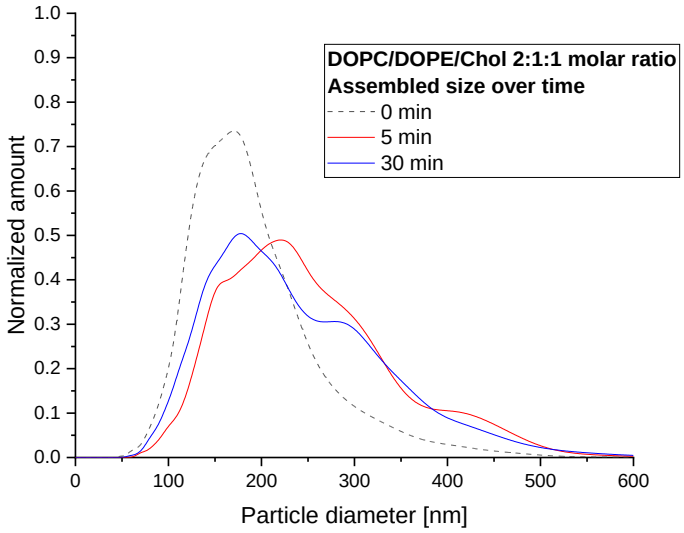


Figure S3: Size distribution of assembled liposomes with anchored LiNAs at 50°C before mixing, 5 minutes after mixing and 30 minutes after mixing the two populations. The data shows the increase in particle size over time from the initial mixing of complementary LiNA labeled liposome populations during fusion starting at time point “0”, measured at the next time point after 5 min and an additional third time point after 30 min showing a size increase towards a plateau average size for the fused population.

UV-vis thermal denaturation profiles

LiNA duplexes in solution

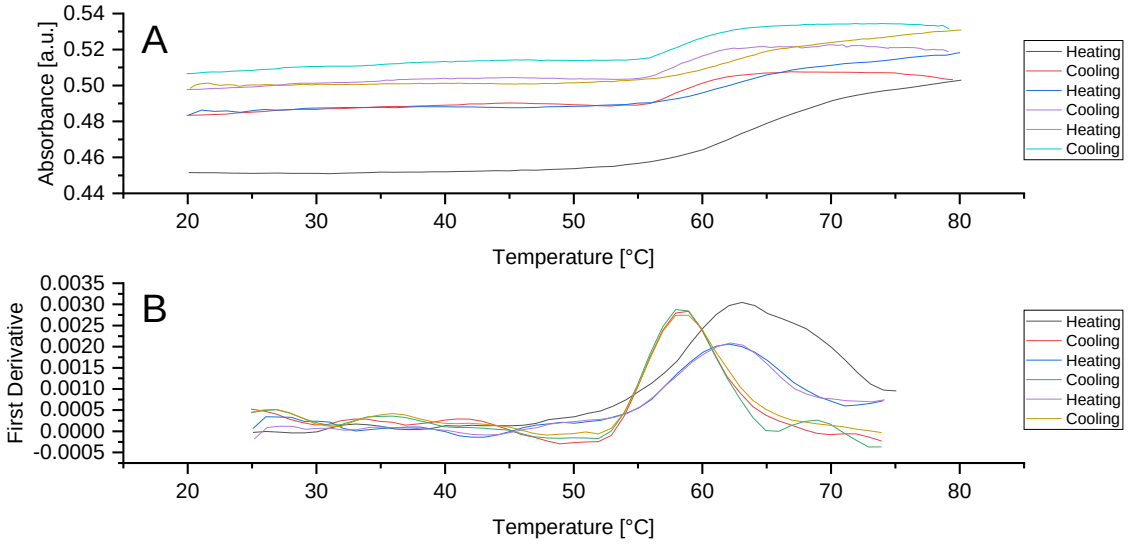


Figure S4: A) Thermal denaturation curves for the duplex LiNA-3A and LiNA-4. **B)** The first derivative calculated from the thermal denaturation curve. All absorption measurements have been recorded at UV 260 nm wavelength.

Table S3: *List of T_m values for the assembled liposomes (Ls) using different LiNA oligonucleotide pairs and their hysteresis between melting (heating cycle) and reassembly (cooling cycle).*

Entry	Cycle	T_m [°C]	ΔT_m [°C]
Ls-LiNA-1	Heating	62.6	2.3
Ls-LiNA-2A	Cooling	60.3	
Ls-LiNA-3A	Heating	65.2	2.4
Ls-LiNA-4	Cooling	63.6	

Thermal denaturation of liposomes functionalized with assembly-type LiNAs

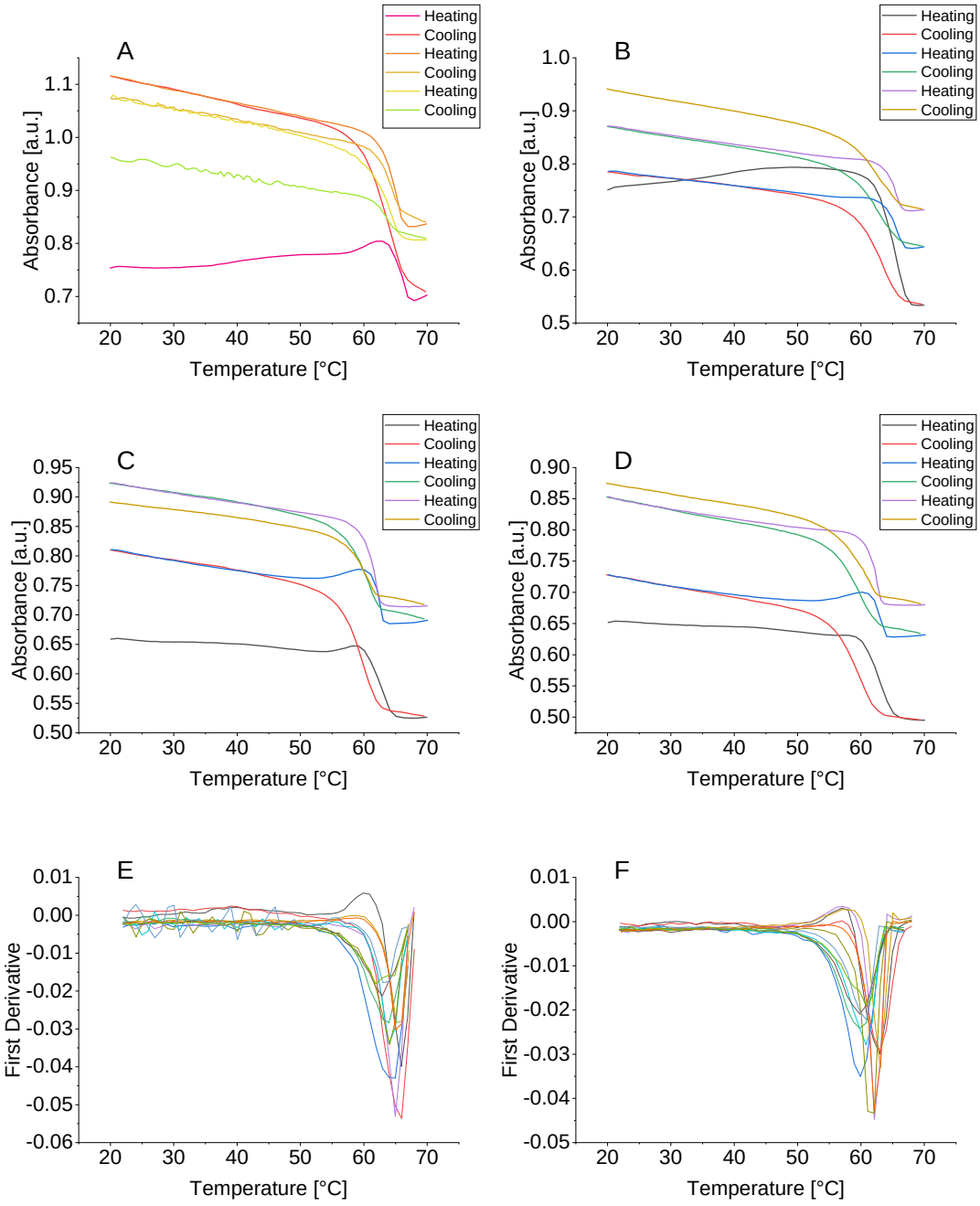


Figure S5: Thermal denaturation curves for liposome assembly. **A)** Thermal denaturation curves for liposomes with LiNA4 and LiNA-3A. **B)** Thermal denaturation curves for liposomes with LiNA-4 and LiNA-3A. **C)** Thermal denaturation curves for liposomes with LiNA-1 and LiNA-2A. **D)** Thermal denaturation curves for liposomes with LiNA-1 and LiNA-2A. **E)** The first derivate calculated from the thermal denaturation of LiNA-4 and LiNA-3A. **F)** The first derivate calculated from the thermal denaturation of LiNA-4 and LiNA-3A. All absorption measurements have been recorded at UV 260 nm wavelength.

Additional UV-vis spectrometry time-course graphs for assembly and fusion

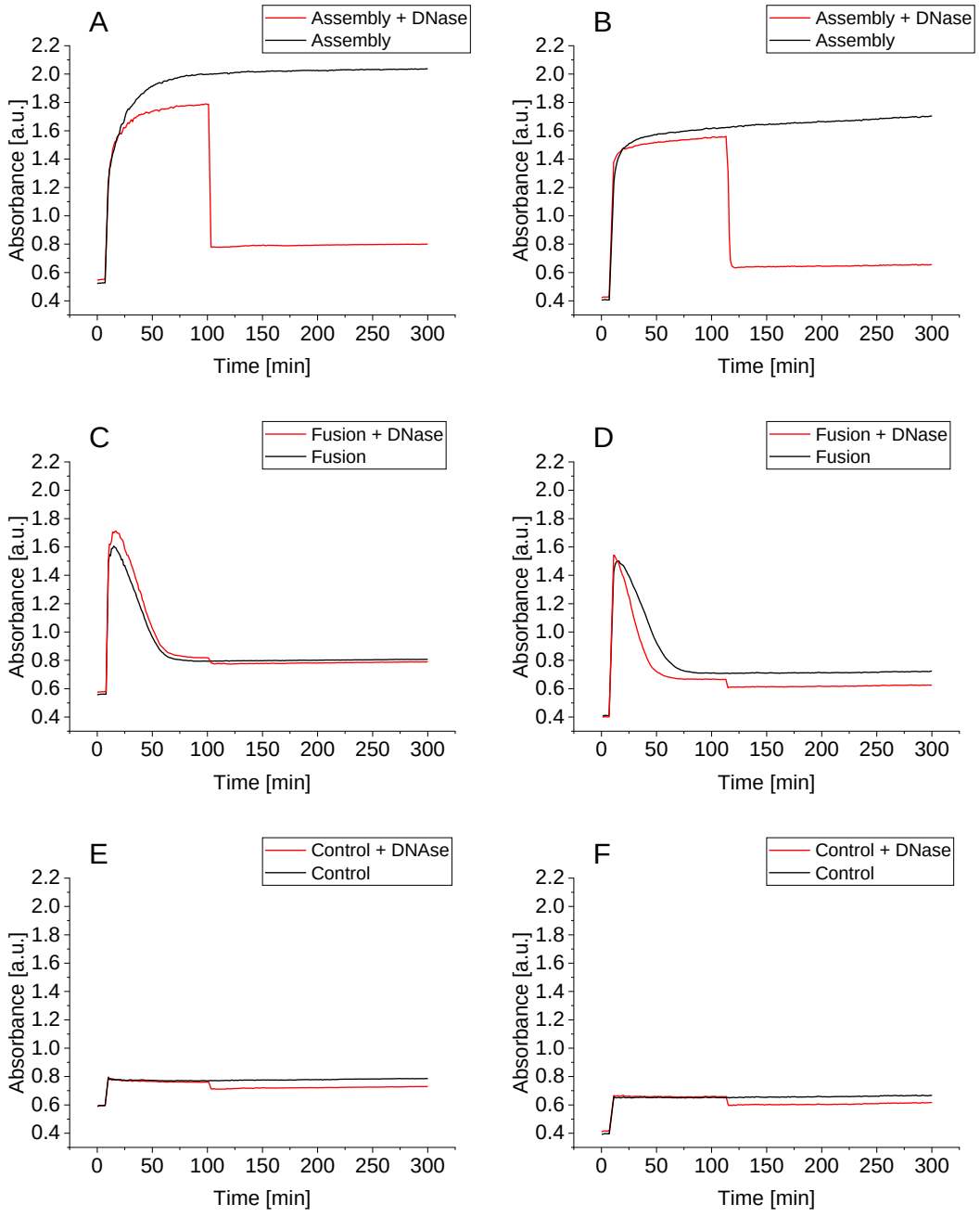


Figure S6: The time-course experiments of liposome fusion and assembly all measured at 37°C. **A)** Assembly of liposomes using LiNA-1 and LiNA-2A and the influence of DNase. **B)** Assembly of liposomes using LiNA-1 and LiNA-2A and the influence of DNase. **C)** Fusion of liposomes using LiNA-1 and LiNA-2F and the influence of DNase. **D)** Fusion of liposomes using LiNA-1 and LiNA-2F and the influence of DNase. **E)** Liposomes without LiNAs measured in the presence of DNase. **F)** Liposomes without LiNAs measured in the presence of DNase. All absorption measurements have been recorded at UV 260 nm wavelength.

Additional SPR data

Raw data for X-LiNAs (LiNA-1/2A and LiNA 1/2F)

Full SPR experiment cycle

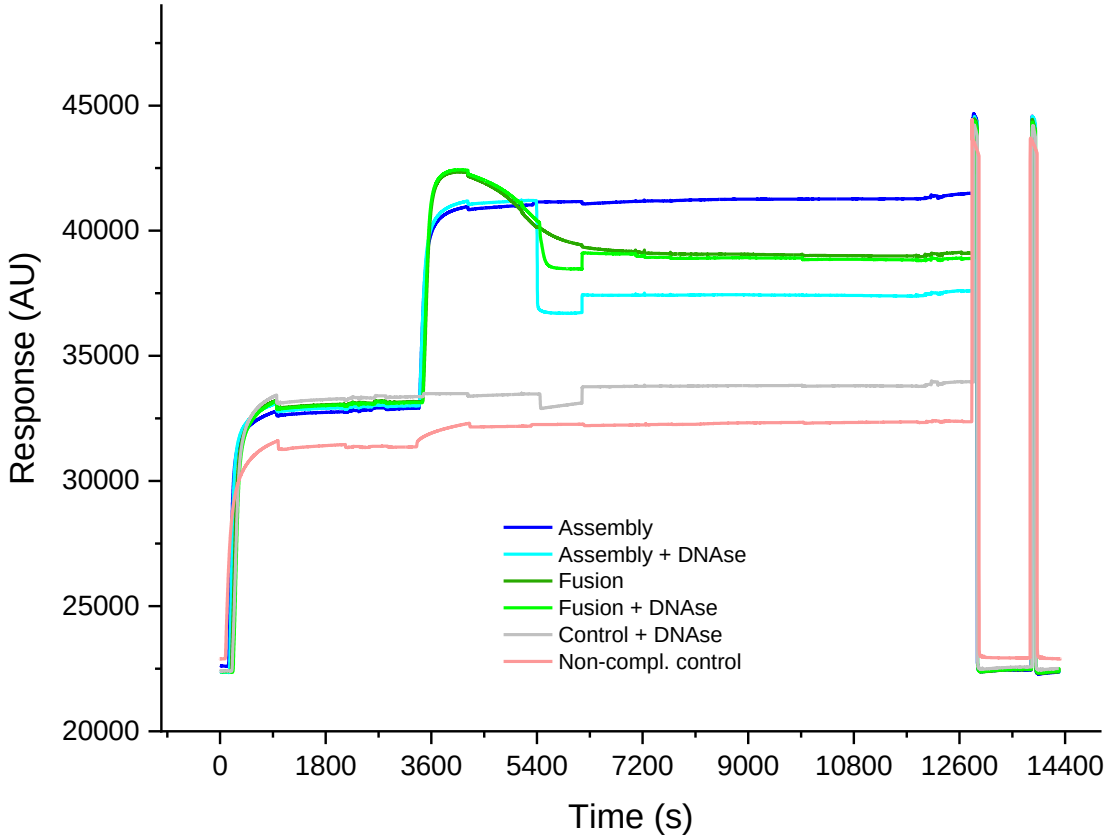
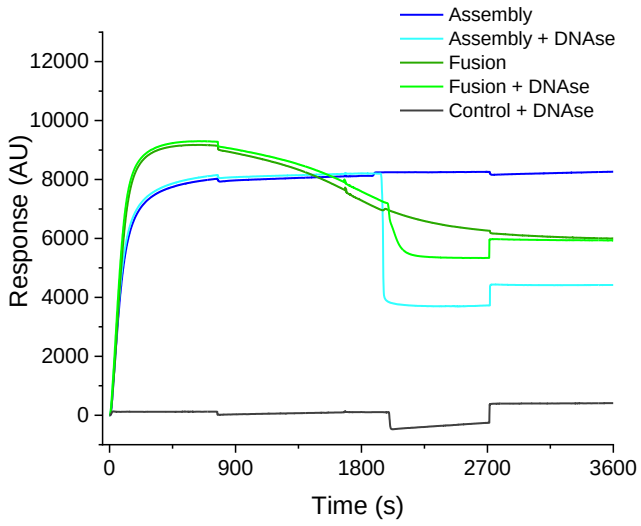


Figure S7: SPR raw data of the full-time course including primary liposome capture (0-900 s), enhancement with high-flow buffer flushing, stabilization at low-flow running buffer (900-3400 s) secondary liposome binding (3400-4100 s), dissociation (4100-5000 s) and addition of DNAse I (5100-6000 s) and Biacore™ L1 chip regeneration (12600-14400 s).

Aligned graphs (LiNA-1/2A and LiNA 1/2F)

Experiment #

1T



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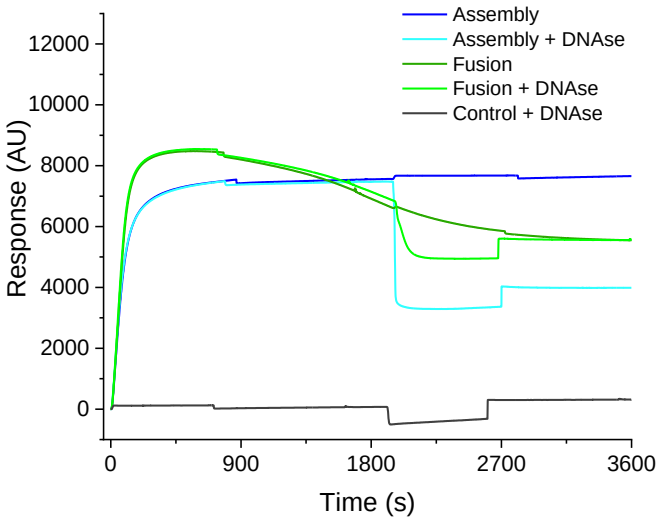

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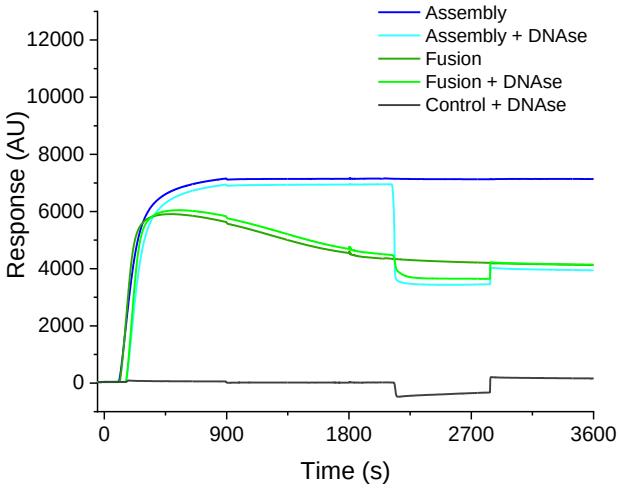


Figure S8: Triplicate experiments with X-LiNAs for assembly LiNA-1/2A, fusion (LiNA 1/2F) and running buffer injection control. Curves aligned at injection of the secondary LiNA-functionalized liposomes.

Raw data for XP3-LiNAs (LiNA-3/4A and LiNA 3/4F)

Full SPR experiment cycle

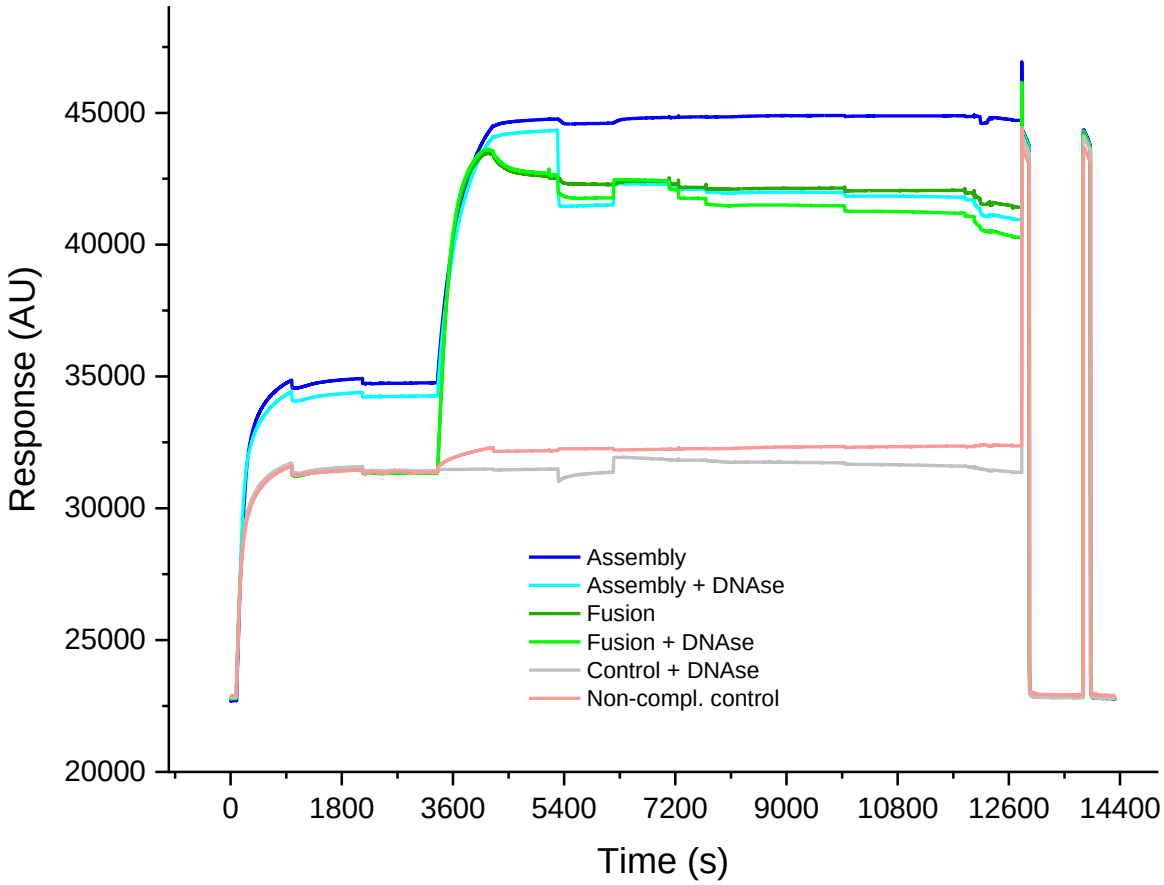
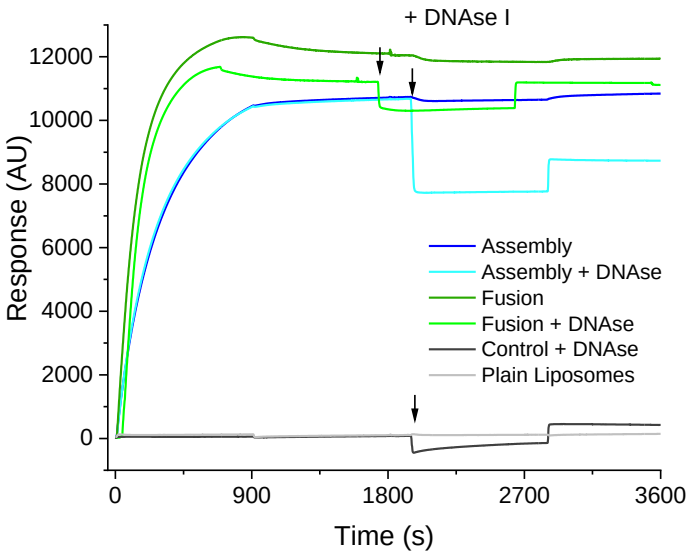


Figure S9: SPR raw data of the full-time course including primary liposome capture (0-900 s), secondary liposome binding (3600-4200 s) and addition of DNAse I (6000-6300 s) and Biacore™ L1 chip regeneration (12600-14400 s).

Aligned graphs

Experiment # 1



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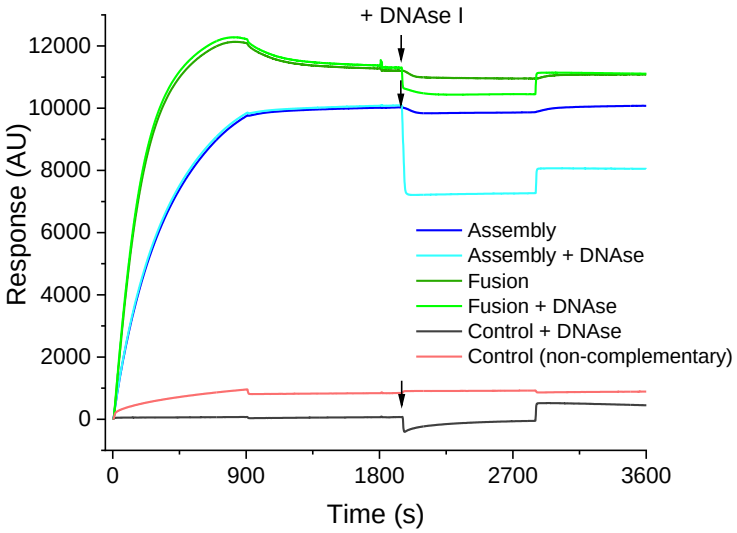

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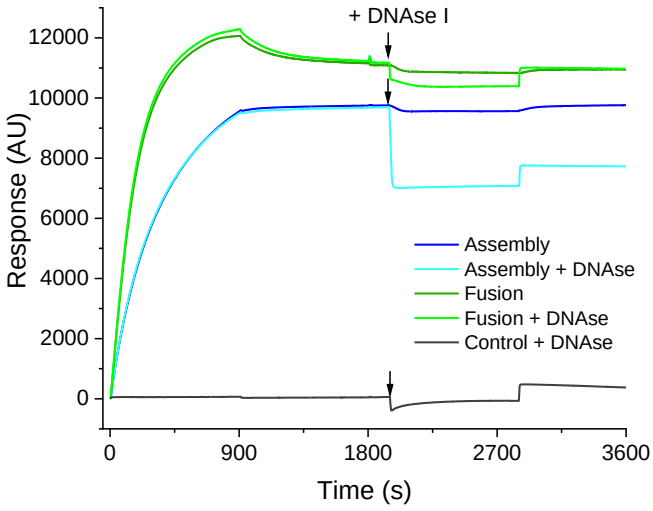

Figure S10: Triplicate experiments with X-LiNAs for assembly LiNA-1/2A, fusion (LiNA 1/2F) and running buffer injection control. Curves aligned at injection of the secondary LiNA-functionalized liposomes. Arrows mark the injection of DNAse I.

References

1. U. Jakobsen, A. C. Simonsen and S. Vogel, *Journal of the American Chemical Society*, 2008, **130**, 10462-10463.
2. O. Ries, P. M. G. Löffler and S. Vogel, *Organic & Biomolecular Chemistry*, 2015, **13**, 9673-9680.
3. Carr, B.; Malloy A.; Nanoparticle Tracking Analysis – The NANOSIGHT system. White paper. Nanosight Ltd. **2006**.
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