Title: A DNA-programmed liposome fusion cascade

Authors: Stefan Vogel, Philipp M. G. Löffler, Oliver Ries, Alexander Rabe, Anders H. Okholm, Rasmus P. Thomsen, and Jørgen Kjems

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: Angew. Chem. Int. Ed. 10.1002/anie.201703243
Angew. Chem. 10.1002/ange.201703243

Link to VoR: http://dx.doi.org/10.1002/anie.201703243
http://dx.doi.org/10.1002/ange.201703243
**A DNA-programmed liposome fusion cascade**


**Abstract:** Chemically engineered and functionalized nanoscale compartments are used in bottom-up synthetic biology to construct compartmentalized chemical processes. Progressively more complex designs demand for spatial and temporal control over entrapped species. Here, we address this demand by a DNA-encoded design for successive fusion of multiple liposome populations. Three individual stages of fusion are induced by orthogonally hybridizing sets of membrane-anchored oligonucleotides. Each fusion event leads to efficient content mixing and transfer of the recognition unit for the subsequent stage. In contrast to fusion protein-dependent eukaryotic vesicle processing, this artifical fusion cascade exploits the versatile encoding-potential of DNA hybridization and is generally applicable to small and giant unilamellar vesicles. Thus, our platform will enable numerous applications in artificial cellular systems and liposome-based synthetic pathways.

The fusion of biological membranes is an ubiquitous and highly regulated mechanism in living cells and is crucial for transport, uptake and release of biomolecules, e.g. in signal transduction, endo- and exocytosis, viral transfections and intracellular trafficking.[1] Membrane fusion in eukaryotic cells is controlled by the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE)[2], SNAREs and SNARE-mimics have been used to study fusion of model membranes,[3] Xu et al. used DNA-tethers to accelerate the fusion process of the natural SNARE complex.[4] Others have shown that SNARE-derived lipopeptides induce fusion through the formation of membrane anchored coiled-coil structures[5] It has also been demonstrated that membrane fusion can be induced by the hybridization of membrane anchored DNA[6] or peptide nucleic acid (PNA).[7] These systems principally allow programmable fusion of liposomes, but were applied in a single independent fusion event only. The amounts of content mixing (CM, 15-25%)[6a, 7b, 8] and/or leakage (L, 15-20%)[7b, 8] in these reports makes multi-step fusion challenging. We contribute a platform that provides high effective content mixing in a single stage (>90% CM, ≤ 5% L) and implement it in a DNA-programmed three-stage fusion cascade (50-80% CM and 5-15% L per stage) which, to the best of our knowledge, is unprecedented in literature.[6-9]. Four liposome populations were functionalized with six different lipid-DNA conjugates ( LiNAs) designed to induce sequential fusion via three orthogonally hybridizing duplex sequences (Scheme 1): [M0] – loaded with self-quenching SRB – and unlabeled populations [1], [2] and [3] were functionalized to encode a fusion cascade: the monitored population [M0] can fuse with [1] that also carries the LiNA for binding [2]. Product population [M1] can then fuse with [2], which delivers the LiNA that allows [M2] to fuse with population [3]. Liposome populations functionalized with complementary ss(LiNA) strands assemble and fuse upon DNA duplex formation. The cooperative action of LiNA duplexes deforms docked liposomes, while increasing the double-bilayer contact area.[10] As Brownian motion rarely results in interliposomal distances below 5 - 10 nm[11], geometry suggests that short LiNA duplexes (e.g. 17 bp) hybridize from the membrane-distal ends towards membrane-proximal ends – in a zipper-like manner – forcing the membranes into closer proximity. This strongly increases the probability of formation of a fusion stalk, pore-formation and fusion which finally relaxes the membrane.[11] Consequently, entrapped contents are mixed and all LiNAs now reside on the same surface.

![Scheme 1](https://example.com/scheme1.png)

**Scheme 1.** A three-stage liposome fusion cascade encoded by three sets of complementary membrane-anchored recognition sequences (AA', BB', CC'). Fusion is observable via dilution of entrapped self-quenching Sulforhodamine B upon content mixing (M0 through M3, magenta color). Box: Illustration of the fusion-mediating interliposomal tethering via hybridization of LiNA-zippers. Membrane anchor (X) and triethylene glycol spacer (P3) are incorporated within the phosphodiester backbone during DNA synthesis, either near the 5'- or the 3'-end to achieve a zipper-type LiNA. See the Supporting Information (SI), Table S1 for B, B', C and C' sequences.

For liposome assembly and fusion, two lipid moieties are necessary for stable anchoring of LiNAs into phospholipid bilayer membranes.[12] Herein, the anchor building block (X) carries two C16-alkyl chains and is linked to a non-repeating 17 nt DNA sequence (Tm >55 °C, Table S2), directly or via a triethylene glycol linker (P3) as shown in Scheme 1. We tested the anchoring

---

[a] P. M. G. Löffler[†], O. Ries [*], A. Rabe, Prof. S. Vogel*
University of Southern Denmark
Department of Physics, Chemistry and Pharmacy
Campusvej 55, 6230 Odense (Denmark).
E-mail: snv@sdu.dk

[b] R. Thomsen, A. Okholm, Prof. J. Kjems
University of Aarhus
Nanoscience Center (iNANO)
Gustav Vieds Vej 14, B000 Aarhus C

* Authors contributed equally to this work

Supporting information is for this article is available on the WWW under http://
COMMUNICATION

of LiNAs into liposomes by agarose gel electrophoresis, where stably anchored LiNAs cannot migrate. (at 50 °C, see SI, Figures S1-S3). To benchmark our system with existing data in literature we applied a well-established lipid mixing FRET assay[13] and experimental conditions from earlier reports including a cholesterol-anchored reference LiNA-system from literature (see SI, Figure S4).[8-9] Content mixing (CM) of LiNA functionalized DOPC/DOPE/Cholesterol (CH) (2:1:1, molecular ratio) liposomes was monitored by a fluorescence assay. Liposomes entrapping self-quenching Sulforhodamine B (SRB, 20 mM) were mixed with unlabelled ones as described.[5,7a] Content mixing leads to SRB dilution (de-quenching) and fluorescence increase, but likewise does leakage. Thus, we defined the signal from this experiment as “apparent CM” which is always accompanied by a leakage measurement to be able to correct for the leakage contribution to the signal: using self-quenching SRB in both populations, fluorescence increase can only occur via leakage (Figure 1, top). Control measurements in absence of LiNAs and with non-complementary LiNAs were made to test the hybridization-dependence of fusion and CM (see SI, Figure S5).

Based on a standard curve of different entrapped SRB concentrations (see SI, Figure S6), the effective dilution of entrapped SRB was calculated giving a measure for an average number of fusion events for liposomes of the SRB-labeled population [M]. Changes in the liposome size distribution were measured by nanoparticle tracking analysis (see SI). Results showed that fusion strongly depends on temperature, especially content mixing increased remarkably at elevated temperatures. The presence of spacer P3 (TXP3- vs. TX-LiNAs, Figure 1A) influenced fusion significantly and the reduced fusion efficiency of the TX-LiNA pair is presumably caused by release of the 3'-TX-[A] strand from the liposomes at higher temperatures as shown by gel electrophoresis (see SI, Figure S1).

If anchoring is compromised, hybridization to the complementary LiNAs occurs, thereby reducing the number of LiNAs available for liposome docking and fusion. Leakage during fusion at all temperatures was lower than for non-functionalized liposomes, as previously observed by others,[14] likely due to electrostatic repulsion by anchored nucleic acids, giving a negative surface potential.[10a] This was even more pronounced in controls with non-complementary LiNA strands, which showed very low signal increase, verifying that fusion was indeed initiated by LiNA hybridization. A time-course of leakage corrected content mixing (CM-L, Figure 2A) is shown, illustrating the steep initial kinetics where 90 % of the content mixing occurs within the first 5 min. The overlaid curve (encapsulated [SRB], Figure 2A) underlines that indeed multiple fusion events occur to dilute [SRB] close to three-fold. The average number of fusion events per liposome are summarized in Figure 2B. Labeled populations encoded to fuse multiple times with a complementary population (1:3 ratio) showed an average of 2.8 fusion events for the XP3 setup (93% fusion yield) at 50 °C. At 37 °C approx. 0.7 fusion events were observed, i.e. 70% of the labeled population had fused. For measurements with the multi-step fusion systems, all four populations involved are pre-encoded with cascade-programming LiNAs (~40 strands per liposome) and mixed in a 1:1:1:1 ratio. While monitoring SRB fluorescence (583 nm) of populations [M], downstream populations [1], [2] and [3], were added at t = 0 min, 10 min and 20 min, respectively (Figure 3A, red arrows).

Figure 2. A) Time-course of net content mixing and [SRB] dilution for XP3 at 50 °C (single stage). B) Summary for calculated effective fusion at different temperatures. Ratio labeled/unlabeled liposomes 1:3, LiNA-concentration (~195 and 65 LiNA/liposome, respectively). Control (-), without LiNA. Avg. values after 30 min, error bars: standard error.

Figure 3. A) Liposome fusion cascade programmed by orthogonal XP3-LiNAs at 50 °C. Red arrows indicate addition of a new liposome population. B) Fusion yields for each stage of the cascade. Ratio M2/1/2/3 1:1:1:1, LiNA-concentration (~ 40 LiNA/liposome). For T, values of the orthogonal set of LiNAs (non-modified) see SI, Table S2. error bars: standard error. Apparent CM was repeated 4 times, leakage twice.
The time offset allowed us to observe the fast initial increase of [SRB] fluorescence due to content mixing and the later slope that approaches the one of the leakage curve (Figure 3A). The dilution effect measured in our SRB assay decreases for each subsequent fusion event (see SI, Table S3), but characteristic initial incline of the signal was observed upon addition for each additional LiNA encoded liposome population. Over three rounds, the leakage signal remained significantly below the apparent CM signal (~20% thereof). Thus, a high net content mixing (leakage corrected) at 50 °C was observed (50%-80% fusion yield per stage, Figure 3B). Content mixing for the first step in a 1:1 [SRB]/unlabeled liposomes setup, gave I/I₀ lower than obtained for the respective XP3-LiNA single stage experiment (1:3 ratio, 50 °C). At the end of the fusion cascade, the corrected I/I₀ was ~1.8, compared to ~2.1 for the single stage, which was likely due to lower LiNA concentration (~40 vs. ~195 strands per M₀ liposome, respectively) for the initiation of fusion. For apparent CM data for the cascade at 50 °C and 37 °C and controls with non-complementary LiNAs are shown in the SI, Figure S12. Furthermore, "knock out" of one or two encoding LiNAs in the cascade resulted in a stalling of the fusion, showing that indeed transfer of the recognition units was needed to proceed in the complete cascade (Figure S13).

We realize that the SRB assay is not suitable for monitoring fusion beyond our three-step cascade. However, by using a well-established assay with a good signal-to-noise ratio, we intended to provide a proof of concept for programmed multistep fusion. In ongoing efforts, we focus on applying LiNA-mediated fusion to compartmentalized chemical reactions, where product yields will be a measure the fusion efficiency.

In conclusion, LiNA-induced fusion provides a platform for applications in the bottom-up construction and operation of nanoreactors harboring chemical and heat-tolerant biochemical reactions. The reported fusion cascade at 50 °C underlines the potential of DNA-mediated membrane fusion for studies of fundamental chemical relevance in aqueous environments, with tunable water activity inside confined lipid compartments. Efficient programmable fusion will also facilitate new designs and construction techniques for bottom-up synthetic biology, e.g. for multi-component artificial cellular systems. [16] At 37 °C the single-stage setup still provided significant fusion, presenting a tool to study reactions with compartmentalized (multi-)enzyme complexes under crowded and non-equilibrium conditions. Further, we envision applications in targeted drug and gene delivery by site-specific fusion of liposomes with target cell membranes (e.g. using LiNA-targeting vector complexes). [17]

**Acknowledgements**

The authors gratefully acknowledge funding by the Biomolecular Nanoscale Engineering Center (BioNEC), a Centre of Excellence funded by THE VILLUM FOUNDATION, grant no. VKR022710.

**Keywords:** self-assembly • vesicle fusion • liposomes • GUVs • nanoparticles


Hot fusion: Membrane fusion mediated by the hybridization of lipid-oligonucleotide conjugates (LiNAs) allows rapid content mixing with low leakage for small liposomes and giant unilamellar vesicles at elevated temperatures of 37 °C and 50 °C. A three-stage fusion sequence controlled by orthogonal sets of complementary LiNAs was demonstrated at 50 °C.