In vitro and in vivo delivery of functionalized nanoparticles via coiled-coil interactions
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Chapter 5

Efficient Fusion of Liposomes by Quadruple-Anchored DNA

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Chapter 5 has been submitted for publication.
ABSTRACT

Hybridization of membrane-grafted DNA strands represents a powerful strategy for achieving controllable fusion of vesicles, by exploiting the affinity and specificity of complementary oligonucleotide base pairing. So far, DNA hybridization-based approaches for vesicle fusion employing oligonucleotides terminally modified with one or two anchoring units were hindered by a limited degree of full fusion or by significant leakage during fusion.

The current work deals with a new strategy that uses single-stranded DNA functionalized with four lipid-modified nucleobases to achieve stable grafting of DNA onto the membrane of lipid vesicles. While the orientation of DNA hybridization played a significant role in the efficacy of full fusion of DNA-grafted vesicles, the number of anchoring units was found to be a crucial factor as well. As compared to vesicles functionalized with single anchored or double anchored DNA, liposomes containing quadruple anchored oligonucleotides were found to be highly fusogenic, achieving considerable full fusion of up to 30% without notable leakage. This study demonstrates the importance of the DNA-anchoring strategy in hybridization induced vesicle fusion, as not only the structural properties of the unit itself, but also the number of anchoring units determine its favorable fusion-inducing properties.
INTRODUCTION

Liposomes are a particularly effective class of nanocontainers, being able to encapsulate and protect both small molecules and larger bio-macromolecules, such as proteins or DNA. The engineering of liposomes has advanced to a level that enables the manipulation of their surfaces with specific ligands in order to improve their functionality. For instance, proteins, carbohydrates and vitamins have been used as targeting units to improve the cellular specificity of these nanocontainers. Moreover, some “smart” liposome designs are able to release their cargo through physicochemical responses of the liposomal membrane to external stimuli or through incorporation of transport channels. Another strategy by which liposomes can deliver their payload to cells, is by membrane fusion, which has previously been demonstrated for drug and gene delivery applications.

In many cellular processes, including exocytosis, endocytosis and the transfer of membrane proteins between cellular compartments. Most membrane fusion events follow a similar order: docking, hemifusion and full fusion. As part of the docking process, membranes are brought into close proximity, which can cause the outer layers to merge while the inner layers stay separated, resulting in hemifusion. Full fusion is achieved when the outside and inside layers of both membranes merge and content mixing occurs. Recently, several groups have reported hemifusion and full fusion of liposomes by exploiting Watson-Crick base pairing of complementary membrane-anchored oligonucleotides. In these studies, DNA was grafted onto the liposomal surface using cholesterol- or fatty acid-derivatives conjugated at the 5’- or 3’-end of the DNA oligomers. However, full fusion induced by these systems was only achieved to a limited extent, i.e. below 4%, or with a certain degree of leakage. These limitations may be related to DNA duplex formation and/or linkers separating the two membrane surfaces, thereby inhibiting further membrane contact and preventing full fusion. However, the design of the hydrophobic anchor employed to graft the DNA into the lipid membrane could play a crucial role as well. Once two vesicles are brought close enough for full fusion, insufficient affinity of the hydrophobic domain of the DNA-conjugate for the bilayer would cause the anchor to be “pulled out” of the membrane, disabling further fusion. Here, we report of a powerful new approach to achieve vesicle-vesicle fusion by employing DNA that is modified with lipid chains at four nucleobases to tightly anchor the nucleotide to the lipid membrane. This strategy achieved a highly stable incorporation of DNA into the liposomal bilayer, thereby limiting dissociation and keeping the base-pairing nucleotides close to the surface and allowing for a markedly more efficient full fusion as compared to other, previously reported, anchoring strategies.
Figure 1. Schematic representation of vesicle fusion using lipid-modified oligonucleotides. An oligonucleotide anchored with a single unit might be pulled out of the membrane after hybridization and aggregation of two vesicles, which hinders full fusion (A). In the strategy presented here, highly efficient vesicle fusion was induced by DNAs that were modified at the nucleobases, enabling stable grafting of quadruple anchored oligonucleotides capable of non-zipper-oriented (B) and zipper-oriented hybridization of complementary strands (C).

RESULTS AND DISCUSSION

Synthesis and Embrane-Incorporation of Quadruple-Anchored DNA

In the approach presented here, complementary oligonucleotides containing four uracil (U) bases modified with dodec-1-yne (C$_{12}$H$_{22}$) at 3’ or 5’ position of DNA oligomers were employed: U4T-18 has been designed to contain four modified uracil nucleobases at the 5’ position of the 18-mer oligonucleotide (Figure 2A, Table S3), whereas CU4T-18 is complementary to U4T-18 with the lipid anchor at the same terminus (i.e. the 5’ position) as U4T-18. Upon hybridization, the lipid functionalities are oriented in the DNA double helix in a so-called ‘non-zipper’-like arrangement (Figure 2B). In contrast, CrU4T-18, which is also complementary to U4T-18, was prepared with the lipid anchor on the opposite terminus (i.e. the 3’ position) and therefore allows for a ‘zipper’-like orientated hybridization.
Table 1. Sequences and modifying orientations of lipid-DNA

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>U4T-18</td>
<td>UUUUGCGGATTCGTCTGC</td>
</tr>
<tr>
<td>CU4T-18</td>
<td>UUUUGCAGACGAATCCGC</td>
</tr>
<tr>
<td>CrU4T-18</td>
<td>GCAGACGAATCCGCUUUU</td>
</tr>
<tr>
<td>noU4T-18</td>
<td>TTTTCGGATTCGTCTGC</td>
</tr>
<tr>
<td>Cr-ATTO488</td>
<td>GCAGACGAATCCGC-ATTO488</td>
</tr>
</tbody>
</table>

*: U represents the modified uracil base.

![Chemical structure of the lipid-modified uracil nucleobase](image)

![Schematic representations of lipid-modified DNA hybrids](image)

**Figure 2.** Chemical structure of the lipid-modified uracil nucleobase (a) and schematic representations of lipid-modified DNA hybrids in non-zipper and zipper like arrangements (b).
Upon synthesis of the nucleobase-modified DNA hybrids, the lipid DNAs could be stably anchored into the membrane of DOPC:DOPE:cholesterol lipid vesicles, while the oligonucleotides remained available for complementary hybridization, as demonstrated by a Fluorescence Resonance Energy Transfer (FRET) assay.\(^{28}\) (Figure 3). Since ATTO488 and rhodamine dyes show energy transfer when there is a sufficiently short distance between them,\(^{29}\) ATTO488 was covalently attached to the 3’ end of a 14-mer DNA complementary to U4T-18 (Cr-ATTO488) to act as a donor, and in parallel, rhodamine-functionalized phospholipid (Rh-DHPE) was incorporated in the liposomal bilayer to function as an acceptor. As demonstrated by the increase in the maximum intensity ratio \(I_{592}/I_{520}\) (acceptor/donor peak) (Figure 3D, Figure S5), hybridization only occurred upon mixing of Cr-ATTO488 with U4T-18-grafted Rh-DHPE-containing vesicles, positioning both dyes sufficiently close to each other to achieve FRET (Figure 3A), whereas for vesicles containing non-complementary lipid-DNA, CrU4-18, (Figure 3B) or no lipid-DNA at all (Figure 3C), no FRET was observed.

Figure 3. Anchoring of lipid-DNA in the membrane and hybridization on the vesicle surface and Fluorescence Resonance Energy Transfer (FRET) upon hybridization of donor-modified complementary DNA with DNA-functionalized, acceptor-containing vesicles. FRET is achieved when complementary Cr-ATTO488 DNA hybridizes with U4T-18 and brings the donor close to the acceptor, rhodamine, positioned in the membrane (A). If hybridization is not possible, either due to mismatch of the two DNA strands (B) or the absence of membrane-grafted DNA (C) FRET does not occur. Fluorescence spectra (D) of systems capable of FRET (red) and non-FRET controls, either due to DNA mismatch (blue) or absence of membrane-grafted DNA (green).
Moreover, the $I_{592}/I_{520}$ ratio increased markedly with higher U4T-18 densities in the membrane (Figure S6), and disruption of vesicles by addition of Triton X-100 to a final concentration of 0.3% (v/v) resulted in a drop in FRET in the U4T-18 vesicles hybridized with Cr-ATTO488 (Figure S7), confirming that FRET was indeed caused by bringing the donor in close vicinity to the acceptor dye located in the liposomal membrane. Finally, the lipid–DNA remained stably anchored in the liposomes for at least 24 hours (Figure S8).

**Docking of Liposomes Grafted with Quadruple-Anchored DNA**

After establishing that lipid-modified oligonucleotides remained stably incorporated into phospholipid bilayers for extended period of times, their functionality for hybridization-induced vesicle-vesicle interaction was explored. The fusion of lipid bilayers is a three-step process: docking, hemifusion and full fusion. DNA hybridization allows docking of vesicles by overcoming the repulsive hydration forces between the lipid-headgroups, i.e. bringing the lipid bilayers of the liposomes functionalized with complementary DNA into close proximity to each other. Liposomal docking was observed when U4T-18 vesicles were incubated in a 1:1 ratio with vesicles decorated with the complementary DNA sequence (CrU4T-18 or CU4T-18), each formulation with an average diameter of around 130 nm.

After 5 hours, the average liposomal diameter, as determined by dynamic light scattering (DLS), increased from 130 nm to around 350 nm and 300 nm, for the zipper and non-zipper orientated hybridization, respectively, while the diameter of the U4T-18 vesicles alone did not change notably (Figure 4). This indicates that DNA hybridization and vesicle aggregation has taken place in both binding modes, although zipper orientation hybridization resulted in on average slightly larger objects.

The docking of U4T-18 liposomes was also investigated with cryogenic transmission electron microscopy (cryo-TEM), and no apparent aggregation was observed in the absence of complementary DNA-functionalized liposomes (Figure 5A). In contrast, strong aggregation was observed in the mixture of U4T-18 and CU4T-18 decorated liposomes when incubated overnight (Figure 5B), as well as in the mixture of U4T-18 and CrU4T-18 decorated liposomes (Figure 5C, D). Moreover, signs of liposomal fusion were present in the U4T-18/CrU4T-18 zipper-like arrangement sample, such as bridging membranes and the presence of large vesicles (red circles, Figure 5D). The molar ratio between phospholipids and lipid-DNA was optimized to be 500:1 (around 140 DNA strands per vesicle, data not shown), unless stated otherwise.
Figure 4. Time evolution of average diameter measured by DLS of vesicles functionalized with DNA. Upon incubation of U4T-18-grafted vesicles (diameter 130 nm) with vesicles of equal size containing complementary DNA sequences, hybridization in either zipper (CrU4T-18, red) or non-zipper (CU4T-18, blue) orientation, resulted in an increase in average diameter of the entire population. For U4T-18-grafted vesicles alone (green), the average diameter remained constant.

Figure 5. Cryo-TEM images of (a) U4T-18 decorated liposomes, (b) a mixture of U4T-18 and CU4T-18 decorated liposomes, and (c, d) a mixture of U4T-18 and CrU4T-18 decorated liposomes. The red circles in (D) indicate vesicles that are suggestive of hemifusion. (All the samples were incubated at 4°C overnight.)
Hemifusion of Liposomes Grafted with Quadruple-Anchored DNA

To investigate the second step of vesicle fusion, i.e. hemifusion, a lipid mixing assay based on FRET was conducted. Similar to a procedure reported previously, the membranes of liposomes decorated with U4T-18 were stained with 0.5 mol% NBD-DHPE (donor) and 0.5 mol% Rh-DHPE (acceptor) (FRET liposomes), while complementary DNA-functionalized vesicles, grafted with CrU4T-18 or CU4T-18, were prepared without fluorescently-labeled lipids (non-fluorescent liposomes). Lipid mixing between FRET and non-fluorescent liposomes would increase the average distance between donor and acceptor dyes, thereby attenuating FRET and consequently increasing donor emission. Both zipper orientated and non-zipper orientated hybridization were able to induce lipid mixing to a similar extent (± 40%, Figure 6), suggesting that hemifusion occurs irrespective of the orientation of DNA hybridization.

![Figure 6. Lipid mixing](image)

Full Fusion of Liposomes Grafted with Quadruple-Anchored DNA

The concluding step of vesicle fusion consists of content mixing, i.e. the merging of the aqueous compartments of both liposomes. This process was evaluated by a content mixing assay, employing a protocol as reported previously. In short, the fluorescent dye sulforhodamine B was encapsulated at a self-quenching
concentration (10 mM) into U4T-18 functionalized liposomes, while CrU4T-18 or CU4T-18 functionalized liposomes were prepared without any dye. Full fusion of the U4T-18 vesicle with its complementary counterpart would lead to content mixing and Sulforhodamine B dilution, thereby dequenching its fluorescence resulting in an increase in emission.

Upon exposure of U4T-18-decorated Sulforhodamine B-containing liposomes to complementary DNA-decorated unloaded liposomes, there was a prominent increase of sulforhodamine B emission. The mixing induced by DNA hybridization in the zipper orientation was markedly higher (30%, after 1 hour) than that by DNA hybridized in non-zipper orientation (18%) (Figure 7), while for liposomes grafted with the same, and therefore non-complementary, U4T-18 lipid-DNA, only a negligible amount of dequenching occurred (2%).

![Figure 7. Content mixing between liposomes decorated with U4T-18 and loaded with sulforhodamine B and unloaded liposomes functionalized with CrU4T-18 (zipper, red) or CU4T-18 (non-zipper, blue). Content mixing was measured as an increase in sulforhodamine B emission due to dequenching, suggesting DNA-induced full fusion. U4T-18-grafted sulforhodamine B-loaded liposomes mixed with unloaded U4T-18 decorated liposomes, which could not hybridize, were used as a control (green). The fluorescence intensity upon maximal dequenching of sulforhodamine B by disruption of liposomes in 0.3% (w/v) Triton X-100 was considered 100% content mixing.]

Leakage of the aqueous content of vesicles into the surrounding medium during the fusion process, possibly due to pore formation, has shown to be a significant hurdle in DNA-induced vesicle fusion. To distinguish clean fusion from leaky fusion in the dye dequenching-based content mixing assay employed here, U4T-18-grafted vesicles incubated with either CU4-18- or CrU4T-18-grafted vesicles were precipitated using an ultracentrifuge and the fluorescence intensity of the supernatants analyzed. Supernatants of liposomes fused in either orientation, as well as that of U4T-18 before fusion,
displayed a very similar fluorescent intensity (Figure S9), demonstrating that full fusion was achieved with minimal leakage.

Influence of Number of Anchoring Units on Efficacy of DNA-Induced Full Fusion

To evaluate whether the strategy by which the DNA is anchored into the lipid bilayer, and specifically the number of anchoring units, is a determining factor in hybridization-induced vesicle fusion, double anchored variants of U4T-18 comprising the same (complementary) sequence for hybridization, but modified with only two, rather than four, lipid-modified uracil nucleobases (U2T-16, CrU2T-16, Table S3), were synthesized and evaluated. As compared to the quadruple-anchored DNAs, incubation of vesicles functionalized with complementary U2T-16 oligonucleotides resulted in markedly lower full fusion efficacy (8%, Figure 8). Moreover, for vesicles that contained single anchored oligonucleotides, that consisted of single-stranded DNA modified with poly(propylene oxide) (PPO) and cholesterol anchors at either terminus (Figure S10 and Table S3), full fusion was only achieved to a moderate degree (5%, Figure S11).

![Content mixing between liposomes decorated with U2T-16 and loaded with sulforhodamine B and unloaded liposomes functionalized with CrU2T-16.](image)

**Figure 8.** Content mixing between liposomes decorated with U2T-16 and loaded with sulforhodamine B and unloaded liposomes functionalized with CrU2T-16. Content mixing was measured as an increase in sulforhodamine B emission due to dequenching (red), indicating full fusion induced by zipper-oriented hybridization. U2T-16-grafted sulforhodamine B-loaded liposomes mixed with unloaded U2T-16 decorated liposomes, which could not hybridize, were used as a control (green). The fluorescence intensity upon maximal dequenching of sulforhodamine B by disruption of liposomes in 0.3% (w/v) Triton X-100 was considered 100% content mixing.
Chapter 5

Discussion

These results demonstrate that, besides zipper or non-zipper orientation of hybridization, the extent of full fusion in DNA hybridization-induced vesicle fusion is highly dependent on the anchoring strategy of the hybridizing nucleotides. Previously, other research groups have studied vesicle fusion using lipid-anchored DNA. Höök et al. were the first to exploit the unique properties of polynucleotides to induce controllable vesicle fusion via complementary hybridization. In their approach, sticky-ended, double-stranded DNA constructs were used, which were grafted into the liposomal bilayer by means of two cholesterol anchors, each conjugated via a PEG-linker to the termini of the double-stranded DNA anchors. Upon the rehybridization of complementary sticky-ended duplexes into energetically more favorable blunt-ended duplexes, up to 15% content mixing of vesicles grafted with zipper-oriented sequences could be achieved. The double-stranded, bivalent cholesterol-anchored DNA was much more efficient in inducing vesicle fusion than single-stranded, monovalent cholesterol-anchored DNA, which only resulted in around 5% content mixing after 1 hour, indicating insufficient grafting stability of a monovalent anchor to withstand the strain during DNA hybridization and bilayer reorganization. Bivalent single-stranded oligonucleotides, i.e. two cholesterol moieties conjugated to a single DNA, were evaluated as well and although only the efficiency regarding hemifusion, rather than full fusion, was reported, hemifusion of vesicles grafted with complementary single-stranded, bivalent cholesterol-anchored DNA was similarly effective as that of their bivalent double-stranded counterparts.

A second DNA-mediated vesicle fusion strategy, reported by Boxer et al., also utilized double anchored oligonucleotides. Single-stranded complementary DNA modified with a C18 diglyceride at either terminus was used, which, besides a longer chain length, are structurally relatively similar to the U2T-16 lipid-DNAs used in the current study. The hemifusion of vesicles functionalized with complementary diglyceride-anchored DNA was highly efficient, illustrated by lipid mixing ratios of up to 80%, depending on number of DNAs per vesicle and the presence and length of non-hybridizing, linking sequences. Remarkably, however, full fusion of vesicles grafted with the double anchored diglyceride-modified DNA remained quite limited, with content mixing of around 2-3% for non-repeating DNA sequences. Also taking into account the markedly reduced full fusion achieved with the double anchored U2T-modified DNAs as compared to the quadruple-anchored U4T-modified oligonucleotides, it is conceivable that the number of anchoring moieties, is an important factor in the design of lipid-DNAs and that a multivalent anchor is an important prerequisite or efficient vesicle fusion.

Variations in experimental setup commonly obscure any comparison of results produced in different studies, in particular of those performed in different research groups. In order to bring the results of the current study into context with previously reported data, cholesterol-anchored DNAs used by Höök et al. were synthesized and
evaluated in vesicles using the content mixing assay that was also used for the U4T-18-grafted vesicles. Upon obtaining an extent of full fusion that was quite similar to that reported previously by Höök et al., it could be concluded that U4T-anchored DNA indeed possesses highly favorable fusogenic properties when incorporated into liposomal membranes, and that its remarkable efficiency was not merely related to experimental factors.

It is feasible that the high affinity of a quadruple lipid anchor to the membrane, as compared to a double or single lipid anchor, limits its (partial) dissociation during fusion, thereby preventing leakage due to pore formation, keeping the double-stranded DNA close to the vesicle surface, and consequently bringing docked vesicles in close proximity to enhance full fusion. This ‘proximity effect’ is further supported by the observation that zipper-orientated hybridization is more efficient than non-zipper-oriented hybridization. In addition, a conformational change of the lipid-modified DNA during hybridization could induce a reorientation of the lipid anchors, disrupting the arrangement of lipids around the lipid-modified nucleobases, and thereby facilitating membrane fusion.

Finally, an important appealing feature of the nucleobase-anchoring strategy presented here, concerns its versatility: not only can the number of anchors be varied, but they can be positioned at any place within the polynucleotide as well. This widens the range of possible configurations, such as multiple anchoring regions within a single strand, allowing to further improve the efficacy of the DNA-induced vesicle fusion. These studies will be the subject of a forthcoming publication.

ACKNOWLEDGMENTS

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EXPERIMENTAL PROCEDURES

1. Materials

Cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, USA) (Scheme 1a-c, purity >99%) and used without further purification. Headgroup-labeled phospholipid, Lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (triethylammonium salt) (Rh-DHPE) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (triethylammonium salt) (NBD-DHPE) were purchased from Invitrogen (Amsterdam, Netherlands), and used as received (Scheme 1d-e). The DNA-dye conjugate Cr-ATTO488 ([5’-GCAGACGAATCCGC-3’] –[ATTO488]) was purchased from Biomers.net GmbH (Ulm, Germany). Trition X-100 (10% in water), Sulforhodamin B (Scheme 1f) Tris/HCl buffer were purchased from Sigma-Aldrich (St. Louis, United States). Anhydrous CHCl₃ was purchased from Acros Organics (Geel, Belgium) and stored over molecular sieves. Preparation of liposomes was performed in double deionized water (Super Q Millipore system).
Efficient Fusion of Liposomes by Quadruple-anchored DNA

Figure S1. Structures of lipids: DOPC(a), DOPE(b), Chol(c), fluorescent lipids: Rh-DH-PE(d), NBD-PEI and fluorescent dye Sulforhodamine B(f).

Figure S2. Synthesis of 5-(dode-1-cynyl) deoxyuracil 2 and 5-(dode-1-cynyl) deoxyuracil phosphoramidite 3. Condition of a): C_{12}H_{22}, Tetrakis(triphenylphosphine) palladium(0), CuI, DIPA, DMF, RT; b): 2-Cyanoethyl N, N-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, RT.

2. Synthesis and Characterization of Amphiphilic Oligonucleotides

The synthesis of 5-(dode-1-cynyl) deoxyuracil and 5-(dode-1-cynyl) deoxyuracil phosphoramidite were reported previously\textsuperscript{34-35} (Scheme S2). In short, the modified uracil phosphoramidite was dissolved in CH_3CN to a final concentration of 0.15 M, in the presence of 3 Å molecular sieves, and the prepared solution was directly connected to a DNA synthesizer (ÄKTA oligopilot plus, GE Healthcare (Uppsala, Sweden). Oligonucleotides were synthesized on a 10 μmol scale using standard β-cyanoethylphosphoramidite coupling chemistry. Deprotection and cleavage from the PS support was carried out by incubation in concentrated aqueous ammonium hydroxide solution for 5 h at 55 °C. Following deprotection, the oligonucleotides
were purified by using reverse-phase chromatography, using a C15 RESOURCE RPCTM 3 mL reverse phase column (GE Healthcare) through a custom gradients elution (A: 100 mM triethylammonium acetate (TEAAc) and 2.5% acetonitrile, B: 100 mM TEAAc and 65% acetonitrile). Fractions were desalted using centrifugal dialysis membranes (MWCO 3000, Sartorius Stedim). Oligonucleotide concentrations were determined by UV absorbance using extinction coefficients. Finally, the identity and purity of the oligonucleotides was confirmed by RPC-HPLC and MALDI-TOF mass spectrometry.

Figure S3. MALDI-TOF spectra of lipid-DNAs. a) U4T-18; b) CU4T-18; c) CrU4T-18.
3.1. Preparation and Characterization Lipid-DNA Liposomes

3.1.1. Preparation

An appropriate amount of freeze-dried lipid-DNA was mixed with DOPC:DOPE:Cholesterol (50:25:25 mol% in chloroform), to reach the required lipid:lipid-DNA ratio. For lipid mixing experiments, 0.5 mol% NBD-DHPE and 0.5 mol% Rh-DHPE were included. Afterwards, chloroform was removed by evaporation under an air stream and then under vacuum overnight. An aqueous buffer (100 mM NaCl, 20 mM Tris, pH 7.5) was added to the flask and the solution was vortexed and freeze-thawed 5 times. 10mM Sulforhodamine B was encapsulated in U4T-18 decorated liposomes for content mixing. Subsequently, the dispersion of extruded 21 times, using a extruder and 100 nm polycarbonate membranes (Whatman), to obtain large unilamellar vesicles (LUVs). Lipid-DNA liposomes were used within one day. All liposomal formulations had an average diameter of around 120 nm as determined.
by DLS (ALV[CGS-3], ALV-Laser Vertriebsgesellschaft m-b.H., Langen, Germany). Unless stated otherwise, the ratio between lipid and U4T-18 was 500:1.

3.1.2. Lipid-DNA/liposome Ratio Determination

The amount of lipid-DNAs per liposome was calculated using the equation:

\[
\frac{\text{Lipid-DNA/ liposome}}{\text{lipid/ Lipid-DNA}} = \frac{\Phi}{a}
\]

where \( \Phi \) is the number of lipids per liposome which can be calculated from geometrical considerations:

\[
\Phi = \frac{S_{\text{outer}} + S_{\text{inner}}}{a} = \frac{4\pi R_{\text{outer}}^2 + 4\pi (R_{\text{outer}} - 5)^2}{a}
\]

where \( S_{\text{outer}} \) and \( S_{\text{inner}} \) are the outer and inner surface area of the spherical liposomes. Assuming the thickness of the lipid bilayer is 5 nm,\(^{36-38}\) \( \alpha \) is the average cross-sectional area of the lipid head-groups, which is assumed to be \((2\times80+65)/3=75 \text{ Å}\) for DOPC:DOPE(2:1 molar ratio).\(^{39}\) \( R_{\text{outer}} \) is the averaged radius of spherical liposomes, which could be determined by DLS.

3.2 Stability of Lipid-DNA Incorporation in Liposomes Measured by Fluorescence Resonance Energy Transfer (FRET) assay

Fluorescence emission spectra of Cr-ATTO488 (donor) and Rh-DHPE (acceptor) in the 500–700 nm region were recorded with excitation at 470 nm using a SPECTRAMAX M2 (Molecular Devices) fluorescence spectrophotometer. Measurements were carried out at constant temperature of 25.0 °C, using a 100 mM NaCl, 20 mM Tris, pH 7.5 buffer.
3.2.1 FRET Assay via DNA Hybridization

U4T-18 was incorporated in Rh-DHPE/(DOPC+DOPE) (3:97 molar ratio) liposomes to obtain U4T-18 liposomes with a ratio lipid to U4T-18 of 500:1. Subsequently, an aliquot of these liposomes was mixed with a small amount of Cr-ATTO488 such that \([\text{U4T-18}]=\text{[Cr-ATTO488]} = 0.906 \, \mu\text{M}\) and with a final lipid (DOPC+DOPE) concentration of 0.45 mM. Then, U4T-18 and Cr-ATTO488 were hybridized using an Eppendorf Mastercycler (Germany). The protocol consisted of heating the mixture 15 min to 40 °C and slowly cooling to 4 °C for 140 min. Afterwards the emission spectra of Cr-ATTO488/Rh-DHPE pair were measured.

3.2.2 Dynamic Change of Donor and Acceptor Emission

To observe the dynamic emission changes of donor and acceptor after adding Cr-ATTO488, fluorescence emission spectra of Cr-ATTO488 (donor, 520nm) and Rh-DHPE (acceptor, 595nm) were evaluated after excitation at 470 nm. Figure S5 shows the emission of the donor and acceptor over 30 min. The fluorescence of donor significantly decreased by adding Cr-ATTO488 and the fluorescence of acceptor slightly increased at the same time, illustrating that efficient FRET is induced by DNA hybridization.

![Figure S5. Oligonucleotides anchored into liposomal bilayers via lipid-DNA remain available for hybridization.](image-url)

To observe the dynamic emission changes of donor and acceptor after adding Cr-ATTO488, fluorescence emission spectra after excitation at 470 nm of Cr-ATTO488 (donor, emission maximum 520 nm) and Rh-DHPE (acceptor, emission maximum 595 nm) were recorded over 30 min. The fluorescence of donor significantly decreased by adding Cr-ATTO488 and the fluorescence of acceptor slightly increased at the same time, illustrating that FRET is induced by DNA hybridization.
3.2.3 Spatial Stability of Lipid DNA Incorporation in The Liposomal Bilayer

The efficiency of lipid-DNA incorporation in the lipid membrane was also checked by FRET. A Rh-DHPE/lipid-mixture was mixed with U4T-18 at different v/v ratios (5000, 1000, 100, 62.5). The final concentration of Cr-ATTO488 and lipid-mixture (DOPC+DOPE) were kept at 7.32uM and 0.45mM in all the FRET experiments. Table S2 contains the values of intensity ratio \( \frac{I_{592}}{I_{520}} \), acceptor/donor peak, from the spectra shown in Figure S6. The figure shows that the value \( \frac{I_{592}}{I_{520}} \) increases markedly with a higher U4T-18 concentration in the lipid membrane, which further confirm that the lipid anchor in our system has a high efficiency of incorporation.

![Figure S6. U4T-18/Rh-DHPE fluorescence spectra of FRET liposomes mixed with Cr-ATTO488 at different lipid/U4T-18 ratio. The inset shows a zoom-in of the acceptor Rh-DHPE peak. Solid lines and dashed lines represent the spectra of the FRET system before and after adding Triton X-100, respectively. Lipids were mixed with U4T-18 at different molar ratios (5000, 1000, 100, 62.5). The final concentration of Cr-ATTO488 and lipid-mixture(DOPC+DOPE) were kept at 7.32 µM and 0.45 mM, respectively, in all FRET experiments.](image)

To further investigate the engraftment of the lipid-DNA hybrids into the membrane, the FRET liposomes were disrupted with Triton X-100 at a final concentration of 0.3% (v/v). Decreased acceptor emission and FRET efficiency were observed due to an extension of the donor–acceptor distance (Figure S7a).
Table S2. The acceptor/donor fluorescence intensity ratios (I590/I520) at different lipid/U4T-18 ratios.

<table>
<thead>
<tr>
<th>Lipid : U4T-18 ratio</th>
<th>U4T-18:liposome ratio</th>
<th>I590/I520 FRET system</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000</td>
<td>8</td>
<td>0.22</td>
</tr>
<tr>
<td>1000</td>
<td>38</td>
<td>0.24</td>
</tr>
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<td>100</td>
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</tr>
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<td>62.5</td>
<td>608</td>
<td>0.44</td>
</tr>
</tbody>
</table>

As expected, in the two control non-FRET systems in which DNA hybridization could not occur on the liposomal surface, thereby preventing energy transfer from donor to acceptor, a similar spectrum was observed before and after liposomal disruption (Figure S7b, S7c).

Figure S7. To further investigate the engraftment of the lipid-DNA hybrids into the membrane, FRET liposomes were disrupted with Triton X-100 at a final concentration of 0.3 % (v/v). Fluorescence spectra of FRET liposomes before and after adding Triton X-100 (A). Decreased acceptor emission and FRET efficiency were observed due to an extension of the donor–acceptor distance. As expected, in two control non-FRET systems in which DNA hybridization could not occur, either due to absence of DNA on the membrane (B) or the presence of non-complementary DNA (C), thereby preventing energy transfer from donor to acceptor. Similar spectra were observed before and after liposomal disruption.
3.2.4 Temporal stability of the lipid-DNA in the liposomal membrane FRET liposomes consisting of U4T-18/Cr-ATTO/Rh-DHPE were mixed with no-FRET liposomes at different ratios (v/v): 1:1, 1:5 and 1:10, respectively (Scheme S8a). The final lipid concentration was kept constant at [DOPC+DPOE] = 0.45 mM in the three resulting samples. The Cr-ATTO/Rh-DHPE emission spectra of each ratio were monitored over 24 h after mixing.

Figure S8. Stability of lipid-DNA in liposomes over time. FRET (U4T-18/Cr-ATTO488/Rh-DHPE) liposomes were incubated with non-FRET (NF) liposomes (A) at different ratios (1:1, 1:5 and 1:10), and there relative Rh-DHPE/ATTO488 (IA/ID) emission intensity of each ratio was monitored over 24 h after mixing (B). Fluorescence spectra of Cr-ATTO488/Rh-DHPE pair in FRET liposomes mixed with NF liposomes at different ratio(v/v): 1:1(red line), 1:5(blue line), 1:10(green line) (C). If lipid-DNA redistributes from FRET liposomes to NF liposomes, a decrease in relative fluorescence of acceptor peak would be observed. After 24 h, some of the acceptor intensity had dropped, but the relative fluorescence of the mixture remained at a similar value as that during the initial measurement before non-FRET liposomes were added. The results demonstrate that the lipid–DNA is stably anchored in the liposomes over at last 24 hours. Solid and dashed lines represent the spectra of the mixed systems before and after adding Triton X-100, respectively.

3.3 Cryo TEM

Liposomes (total lipid concentration 2MG/mL) were deposited on a glow-discharged holey carbon-coated grid (Quantifoil 3.5/1, QUANTIFOIL Micro Tools GmbH). The
excess of solution was blotted off with a filter paper. The grid was vitrified in liquid ethane using a Vitrobot (FEI) and stored in liquid nitrogen before being transferred to a Philips CM 120 cryo-electron microscope equipped with a Gatan model 626 cryo-stage, operating at 120 kV. Images were taken in low-dose mode using slow-scan CCD camera.

3.4 Lipid mixing

Fluorescence measurements were performed on a Tecan Plate Reader Infinite M1000. NBD emission was measured continuously, at 530 nm for 3500s, upon mixing fluorescent U4T-18 decorated liposomes with non-fluorescent CU4T-18 or CrU4T-18 decorated liposomes. The 0% value ($F_0$) was determined by measuring NBD emission of U4T-18 decorated liposomes, which were added with an equal volume of Tris-buffer. The 100% value of lipid mixing ($F_{100\%}$) was determined by measuring NBD emission of liposomes which contained 0.25mol% NBD-DHPE and 0.25% Rh-DHPE. The percentage of lipid mixing was determined by the fluorescence (NBD) increase, $\%F(t) = (F(t) - F_0) / (F_{100\%} - F_0)$ where $F(t)$ is the fluorescence intensity of NBD measured at time $t$.

3.5 Content mixing

10mM sulforhodamine B was encapsulated into liposomes decorated with U4T-18, CU4T-18 or CrU4T-18 was grafted onto non-fluorescent liposomes. Liposomes with encapsulated Sulforhodamine B were separated from non-encapsulated dye using Sephadex G-75 size exclusion columns equilibrated with 100 mM NaCl, 20 mM Tris buffer, pH 7.5. After mixing two liposome formulations, the percentage of content mixing was determined by the increase in emission of the sulforhodamine B, $\%F(t) = (F(t) - F_0) / (F_{100\%} - F_0)$ where $F(t)$ is the fluorescence intensity of Sulforhodamine B measured at time $t$. The fluorescence intensity at 580 nm was monitored in a continuous fashion for 3600s, measurements were performed on a Tecan Plate Reader Infinite M1000 (Männedorf, Switzerland). $F_0$ was the fluorescence intensity measured at the time when the two liposomes were mixed together. The 100% value ($F_{100\%}$) was the fluorescence intensity measured after disruption of liposomes in 0.3%(w/v) Triton X-100 to obtain 100% release. Control experiment was determined by measuring the fluorescence intensity of U4T-18 decorated Sulforhodamine B liposomes mixed with U4T-18 decorated non-fluorescent liposomes.
Figure. S9. Investigation of leaching of content during fusion by measuring fluorescence spectra of the incubated DNA-functionalized vesicles. Before centrifugation (A) differences in fluorescent intensity of sulforhodamine B-loaded U4T-18 liposomes incubated with either unloaded CrU4T-18 liposomes (red line) or unloaded CU4T-18 liposomes (blue), as compared to sulforhodamine B-loaded U4T-18 liposomes alone (black), suggests vesicle fusion due to dequenching of the fluorescent dye. In case vesicle fusion is accompanied by content leakage (leaky fusion), the fluorescence intensity of the supernatants of the fusing vesicles would be higher than that of control, non-fusing vesicles. The bery similar fluorescence intensities of the supernatants of each sample, including control, upon ultracentrifugation at 80,000g (B) confirmed that dequenching occurred within the vesicles as a result of clean fusion, rather than leakage of the contents into the aqueous environment.

To further prove the intensity increase was due to content mixing after vesicle fusion, rather than leakage, U4T-18 decorated sulforhodamine B liposomes were mixed with either CrU4T-18 DNA alone, undecorated liposomes, CrU4T-18 decorated liposomes blocked with the DNA sequence of U4T-18 without lipid anchor (1:2, mole ratio), and U4T-18 vesicles without sulforhodamine B, respectively (Scheme S9).
4.3 Influence of DNA Anchor

Figure S10. Illustration of modified DNA. Chemical structure of (A) PPO-DNA and (B) Chol-DNA. (C) Schematic representation of ss, ds PPO-DNA and Chol-DNA.

Figure S10 shows the evolution of fluorescence intensity in the three negative control experiments over 1 hour, which in all cases remains below 5%.
Figure. S11. Content mixing between liposomes decorated with 22PPO/Cr22PPO (A) and 14Chol/Cr14Chol (B). Content mixing was measured by increase in sulforhodamine B emission due to de-quenching.

Table S3. Sequences of DNA modified with lipid-nucleobases, poly(propylene oxide) and cholesterol.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′→ 3′)*</th>
</tr>
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<tbody>
<tr>
<td>U4T-18</td>
<td>UUUUGCGGATTCGTTCGC</td>
</tr>
<tr>
<td>CU4T-18</td>
<td>UUUUGCAGACGAATCCGC</td>
</tr>
<tr>
<td>CrU4T-18</td>
<td>GCAGACGAATCCGCUUU</td>
</tr>
<tr>
<td>Cr-ATTO488</td>
<td>GCAGACGAATCCGC-ATTO488</td>
</tr>
<tr>
<td>U2T-16</td>
<td>UUGCGGATTCGTTCGC</td>
</tr>
<tr>
<td>CrU2T-16</td>
<td>GCAGACGAATCCGCUU</td>
</tr>
<tr>
<td>22PPO</td>
<td>poly(propylene oxide)-5′-CCTCGCTCTGCTAATCCTGT TA-3′</td>
</tr>
<tr>
<td>Cr22PPO</td>
<td>5′-TAACAGGATTAGCAGAGCGAG-3′-poly(propylene oxide)</td>
</tr>
<tr>
<td>14Chol</td>
<td>Cholesterol-5′-GCGGATTCGTCTGC-3′</td>
</tr>
<tr>
<td>Cr14Chol</td>
<td>5′-GCAGACGAATCCGC-3′-Cholesterol</td>
</tr>
</tbody>
</table>

*: U represents the lipid-modified uracil base.
REFERENCES LIST


25. van Lengerich, B.; Rawle, R. J.; Bendix, P. M.; Boxer, S. G., Individual vesicle fusion events mediated by lipid-anchored DNA. *Biophys. J.* 2013, 105, 409-419.


Efficient Fusion of Liposomes by Quadruple-Anchored DNA


