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Efficient Fusion of Liposomes by Nucleobase Quadruple-Anchored DNA

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Abstract

Anchoring DNA *via* hydrophobic units into the membrane of vesicles allows tagging of these nanocontainers with sequence information. Moreover, the hybridization of DNA on the surface of liposomes enables sequence specific functionalization, vesicle aggregation and vesicle fusion. Especially, DNA hybridization-based approaches for 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 for anchoring oligonucleotides on a membrane by lipid-modified nucleobases rather than by attaching hydrophobic units to the 3'- or 5'-termini. The lipid anchors were incorporated into the DNA sequence *via* phosphoramidite nucleotide building blocks during automated solid phase synthesis allowing variation of the number and position of hydrophobic units along the DNA backbone. Single-stranded DNA functionalized with four lipid-modified nucleobases was stably grafted onto the membrane of lipid vesicles. It was found that the orientation of DNA hybridization and the number of anchoring units play a crucial role in liposomal fusion, which in the most efficient system reached remarkable 29% content mixing without notable leakage.

Introduction

Liposomes are a particularly effective class of nanocontainers, being able to encapsulate and protect both small molecules and bio-macromolecules, such as proteins or DNA.^[1-3] 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” vesicle designs allow the release of the encapsulated cargo through physicochemical responses of the liposomal membrane to external stimuli^[4,5] or by incorporation of transport channels.^[6-9] Another strategy by which liposomes can deliver their payload to cells, is *via* membrane fusion,^[10-12] which has previously been demonstrated for drug^[13-16] and gene delivery^[17-20] applications.

In many cellular processes, including exocytosis, endocytosis, and the transfer of membrane proteins between cellular compartments, membrane fusion plays a crucial role.^[21, 22] 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.^[23-26] However, full fusion induced by these systems was only achieved to a limited extent, *i.e.* below 4%,^[25,27] or with a significant degree of content leakage.^[28] 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 or weak mechanical coupling between the anchor and the oligonucleotides may disable further fusion (Figure 1A). Here, we report a powerful new approach for anchoring DNA on a membrane and to achieve vesicle-vesicle fusion by employing DNA that is modified with lipid chains at four nucleobases (Figure 1B, C). This strategy resulted in 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.

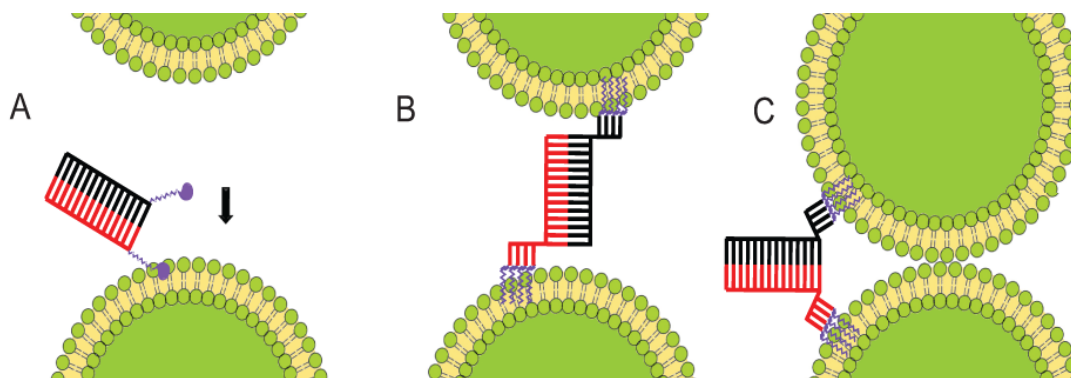


Figure 1. Schematic representation of vesicle fusion using lipid-modified oligonucleotides. An oligonucleotide anchored with a single unit might be pulled out of the membrane after hybridization and aggregation of two vesicles, which hinders full fusion (A). In the strategy presented here, highly efficient 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

After 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 hybridization, as demonstrated by a Fluorescence Resonance Energy Transfer (FRET) assay^[30] (Figure 2). Since ATTO488 and rhodamine dyes show energy transfer when there is a sufficiently short distance between them,^[31] 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 2D, 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 2A), whereas for vesicles containing non-complementary lipid-DNA, CrU4-18, (Figure 2B) or no lipid-DNA at all (Figure 2C), no FRET was observed.

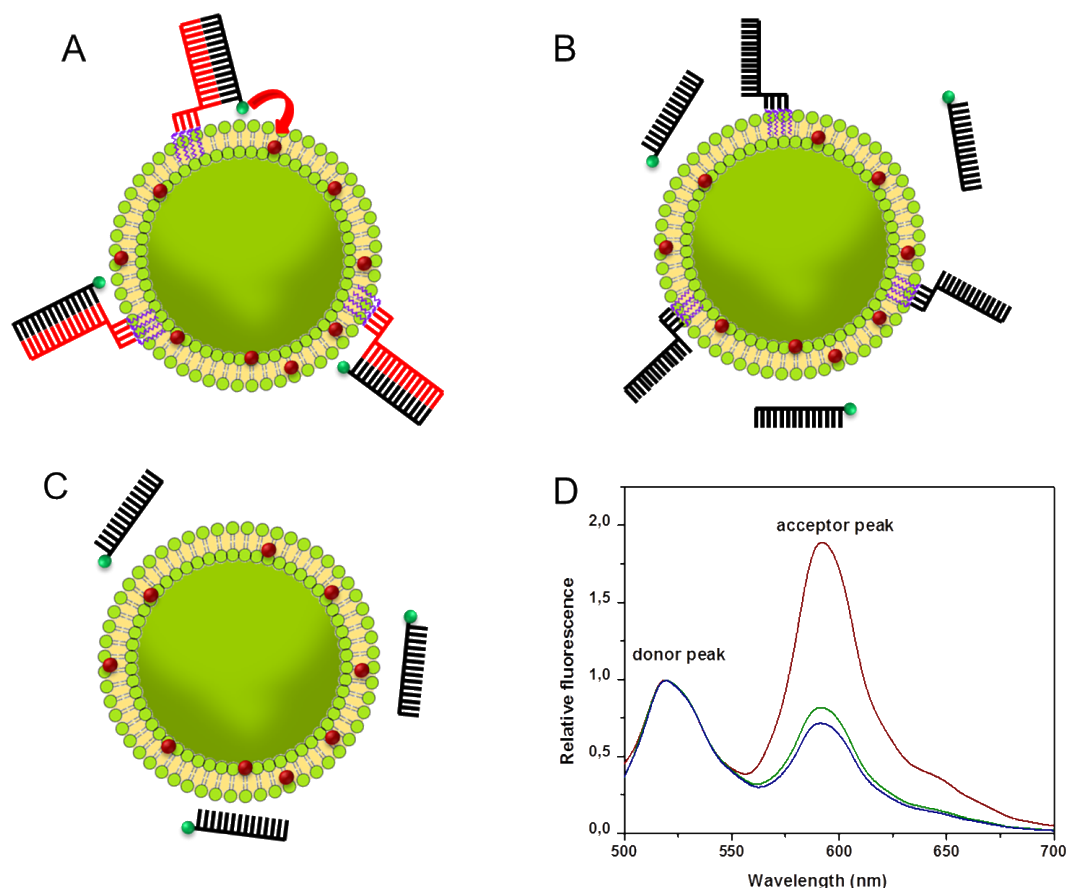


Figure 2. Anchoring of lipid-DNA in the membrane, hybridization on the vesicle surface and Fluorescence Resonance Energy Transfer (FRET) upon hybridization of donor-modified complementary DNA with DNA-functionalized, acceptor-containing vesicles. FRET is achieved when complementary Cr-ATTO488 DNA hybridizes 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, Table S2), 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). The same holds true when a 16mer oligonucleotide with only two nucleobase lipid anchors was employed (data not shown).

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, 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 3). 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.

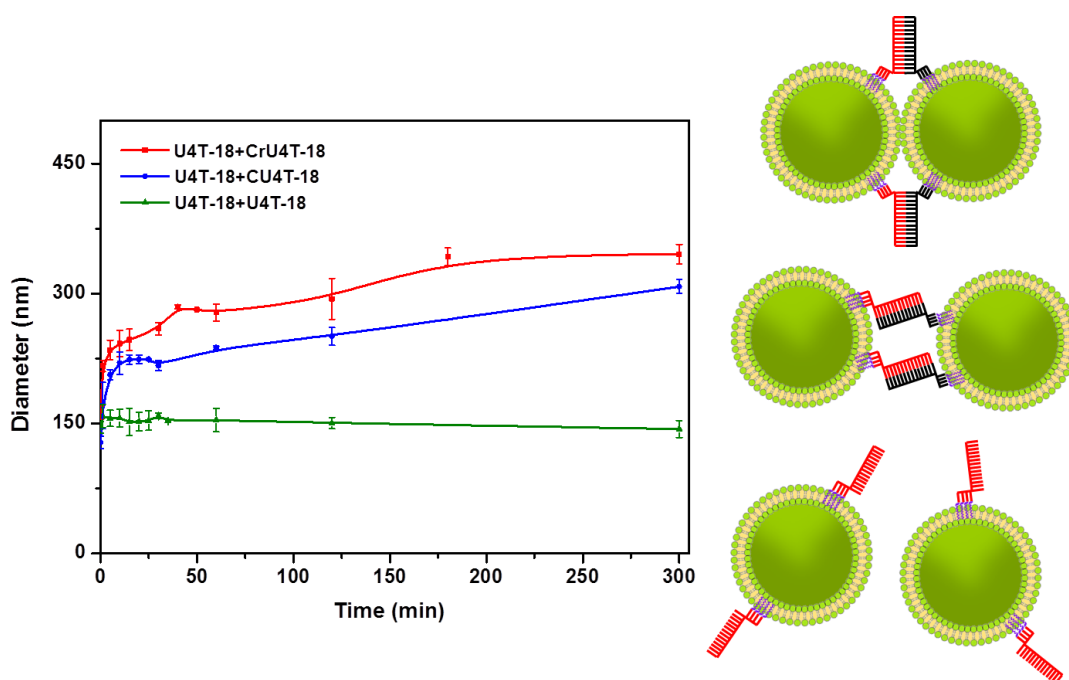


Figure 3. Time evolution of average diameter measured by DLS of vesicles functionalized with DNA. Upon incubation of U4T-18-grafted vesicles (diameter 130 nm) with vesicles of equal size containing complementary DNA sequences, hybridization in either zipper (CrU4T-18, red) or non-zipper (CU4T-18, blue) orientation, resulted in an increase in average diameter of the entire population. For U4T-18-grafted vesicles alone (green), the average diameter remained constant.

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 4A). In contrast, strong aggregation was observed in the mixture of U4T-18 and CU4T-18 decorated liposomes when incubated overnight (Figure 4B), as well as in the mixture of U4T-18 and CrU4T-18 decorated liposomes (Figure 4C, 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 4D). 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.

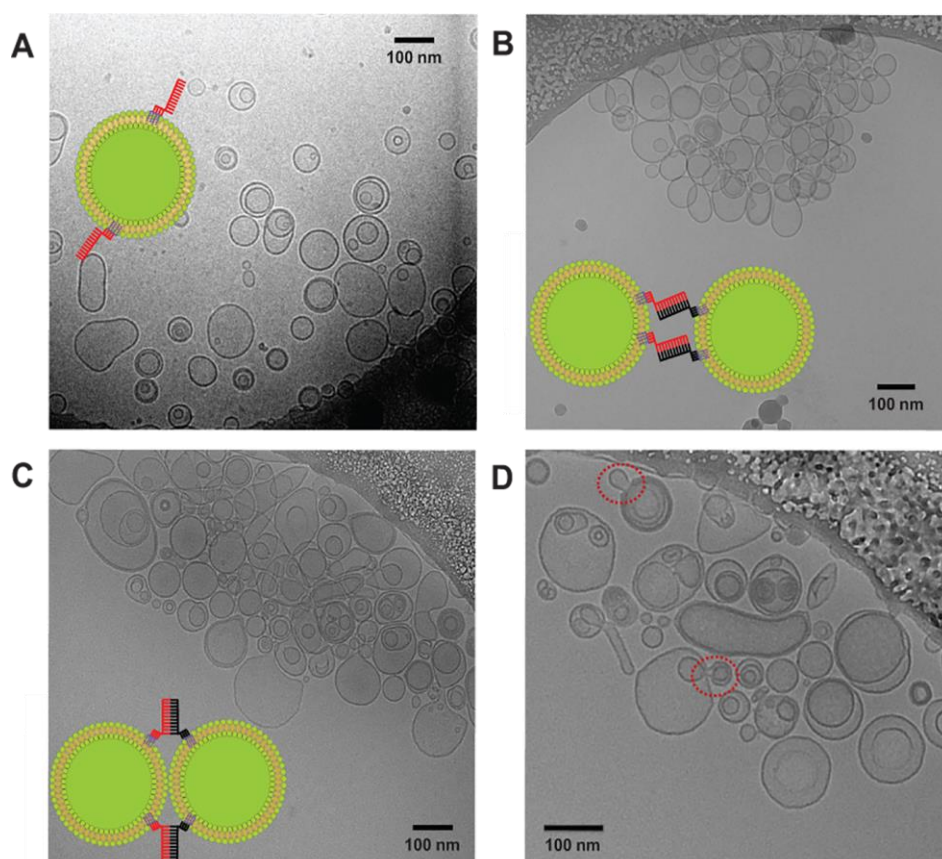


Figure 4. Cryo-TEM images of (A) U4T-18 decorated liposomes, (B) a mixture of U4T-18 and CU4T-18 decorated liposomes, and (C, D) a mixture of U4T-18 and CrU4T-18 decorated liposomes. The red circles in (D) indicate vesicles that are suggestive of hemifusion. (All the samples were incubated at 4°C overnight.)

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.^[32] Similar to a procedure reported previously,^[33] 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 ($\pm 40\%$, Figure 5), suggesting that hemifusion occurs irrespective of the orientation of DNA hybridization.

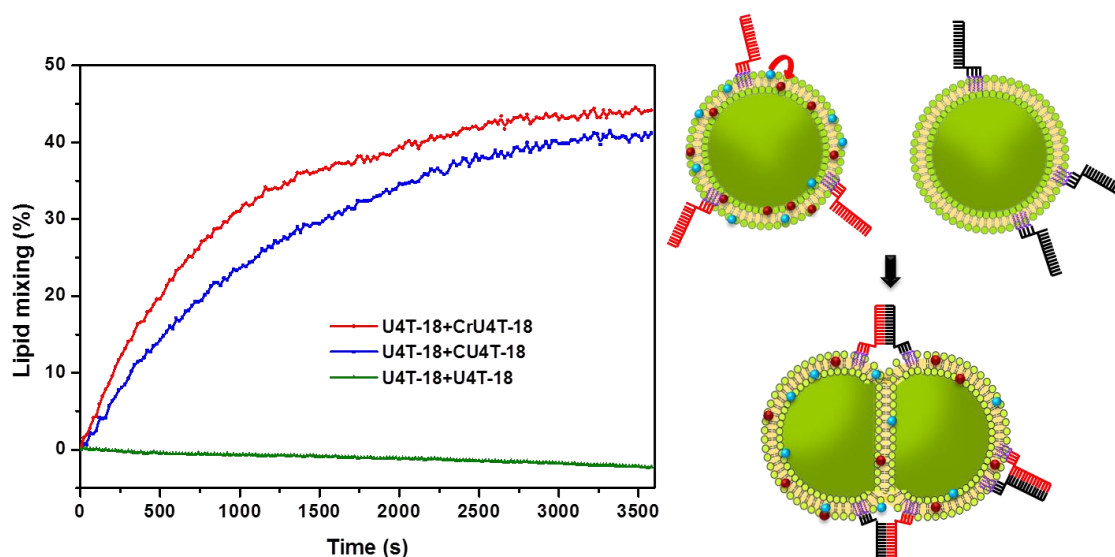


Figure 5. Lipid mixing between U4T-18 grafted vesicles loaded with 0.5 mol% NBD-DHPE and 0.5 mol% Rh-DHPE and CrU4T-18 (zipper, red) or CU4T-18 (non-zipper, blue) decorated vesicles measured by an increase in NBD emission due to a reduction in FRET efficiency. For NBD/DHPE loaded vesicles incubated with unloaded vesicles that contained non-complementary DNA (U4T-18), no reduction in FRET efficiency was observed (green). The NBD emission of vesicles prepared with 0.25 mol% of NBD-DHPE and 0.25% Rh-DHPE was considered full (100%) lipid mixing (These data represent the average of three experiments).

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.^[33] 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 (29%, after 1 hour) than that by DNA hybridized in non-zipper orientation (18%) (Figure 6), while for liposomes grafted with the same, and therefore non-complementary, U4T-18 lipid-DNA, only a negligible amount of dequenching occurred (2%).

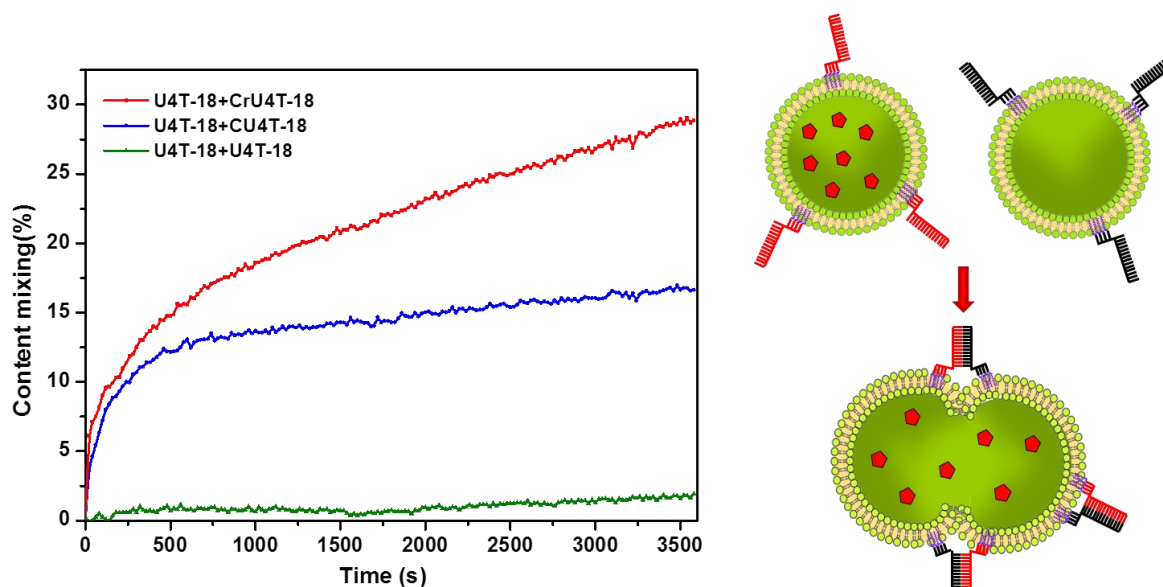


Figure 6. Content mixing between liposomes decorated with U4T-18 and loaded with sulforhodamine B and unloaded liposomes functionalized with CrU4T-18 (zipper, red) or CU4T-18 (non-zipper, blue). Content mixing was measured as an increase in sulforhodamine B emission due to dequenching, suggesting DNA-induced 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 (These data represent the average of three experiments).

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.^[28] 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 fluorescence intensity (Figure S9), demonstrating that full fusion was achieved with minimal leakage. The leakage was calculated to be below 2% for both DNA configurations.

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 S1), 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 7). Moreover, for vesicles that contained single anchored oligonucleotides, that consisted of single-stranded DNA modified with poly(propylene oxide) (PPO)^[34] and cholesterol^[28] anchors at either terminus (Figure S10 and Table S1), full fusion was only achieved to a moderate degree (5%, Figure S11).

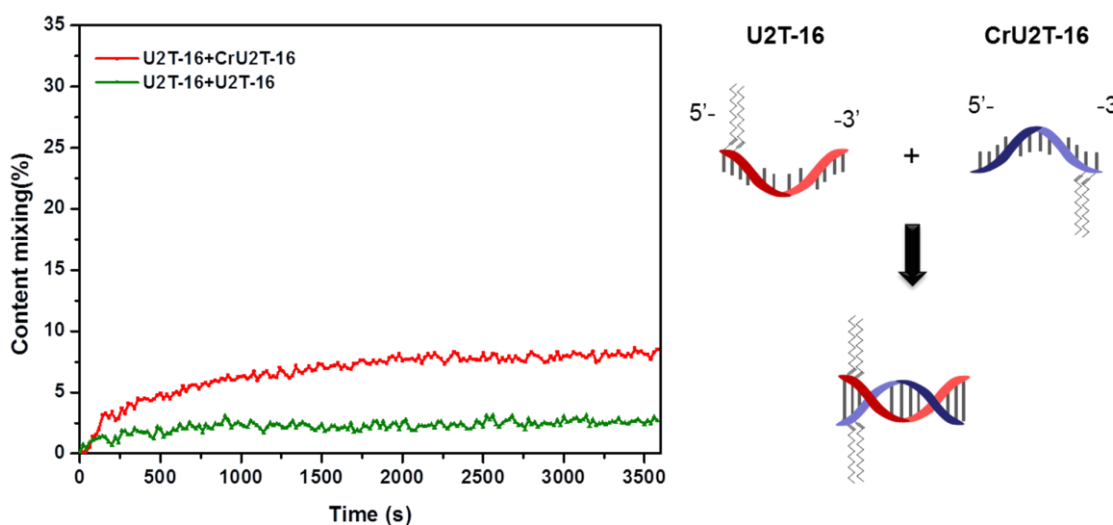


Figure 7. Content mixing between liposomes decorated with U2T-16 and loaded with sulforhodamine B and unloaded liposomes functionalized with CrU2T-16. Content mixing was measured as an increase in sulforhodamine B emission due to dequenching (red), indicating full fusion induced by zipper-oriented hybridization. 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 (These data represent the average of three experiments).

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.^[23] 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.^[35] 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,^[28] 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 terminally double anchored oligonucleotides. Single-stranded complementary DNA modified with a C18 diglyceride at either terminus was used,^[24] 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^[24] and the presence and length of non-hybridizing, linking sequences.^[25] 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.^[24,25] 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 for 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.^[28] Upon obtaining an extent of full fusion that was quite similar to that reported previously by Höök *et al.* (Figure S11B), 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.

Conclusions

In this study, we have established a new anchoring strategy for oligonucleotides in vesicle membranes enabled by attaching a hydrophobic unit to the nucleobase. The membrane anchors are incorporated into the oligonucleotide by automated solid phase synthesis allowing precise control over the position and number of hydrophobic units within a DNA sequence. Therewith, this strategy overcomes structural limitations in the context of terminal labeling with lipid moieties. With a zipper configuration and four anchoring units close to 30% full fusion was achieved, which might be related to the higher affinity of a quadruple lipid anchor to the membrane, as compared to a double or single lipid anchor. We speculate that strong anchoring limits (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-orientated 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.

In the future, we will investigate DNA sequences with nucleobase mediated anchoring of different designs, 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.

Experimental Section

Synthesis and Membrane-Incorporation of Quadruple-Anchored DNA: In the approach to achieve fusion employing novel anchoring units, 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^[29]. Enabled by the previously published phosphoramidite building block and automated DNA synthesis, U4T-18 has been fabricated to contain four modified uracil nucleobases at the 5' position of the 18-mer oligonucleotide (Table S1), 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 1B). 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 (Figure 1C).

Preparation of lipid-DNA liposomes: An appropriate amount of freeze-dried lipid-DNA was mixed with DOPC:DOPE:Cholesterol (50:25:25 mol% in chloroform), to obtain 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. 10 mM sulforhodamine B was encapsulated in U4T-18 decorated liposomes for content mixing. Subsequently, the dispersion was extruded 21 times, using an extruder and 100 nm polycarbonate membranes (Whatman), to obtain unilamellar vesicles. After extrusion, external buffers of each sample were removed by size exclusion chromatography. The column was filled with Sephadex G-75 (GE Healthcare Life Sciences) and equilibrated with buffer (100 mM NaCl, 20 mM Tris, pH 7.5). Lipid-DNA liposomes were used within one day. All liposomal formulations had an average diameter of around 130 nm as determined by DLS (ALV/CGS-3 ALV-Laser Vertriebsgesellschaft mbH, Langen, Germany). The ratio between lipid and U4T-18 was 500:1, unless stated otherwise.

Characterization 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. U4T-18 was incorporated in Rh-DHPE/(DOPC+DOPE) (3:97 molar ratio) liposomes to obtain U4T-18 liposomes with a lipid to U4T-18 ratio of 500:1. Subsequently, an aliquot of these liposomes was mixed with a small amount of Cr-ATTO488 such that $[U4T-18] = [Cr-ATTO488] = 0.906 \mu M$ 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 over a period of 140 min. Afterwards, the emission spectra of Cr-ATTO488/Rh-DHPE pair were measured.

Cryo TEM: Liposomes (total lipid concentration 2 mg/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.

Lipid mixing: Fluorescence measurements were performed on a Tecan Plate Reader Infinite M1000 (Männedorf, Switzerland). NBD emission was measured continuously, at 530 nm for 3500 s, 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 to an equal volume of U4T-18 decorated liposomes at $t=0$. 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, $\%F(t)$. $\%F(t) = (F(t) - F_0) / (F_{100\%} - F_0)$ where $F(t)$ is the fluorescence intensity of NBD measured at time t .

Content mixing: 10 mM 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 3600 s. Measurements were performed on a Tecan Plate Reader Infinite M1000 (Männedorf, Switzerland) at room temperature. F_0 was the fluorescence intensity measured at the time when two liposome populations 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. The fluorescence intensity of U4T-18 decorated Sulforhodamine B liposomes mixed with U4T-18 decorated non-fluorescent liposomes was used as a negative control.

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Keywords: vesicle fusion • liposomes • DNA hybridization • lipid-modified DNA •

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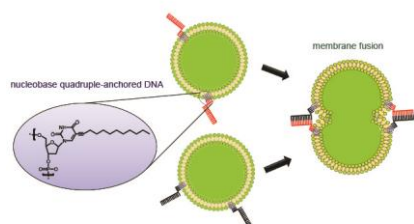
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A powerful new approach is reported to achieve vesicle-vesicle fusion by employing DNA that is modified with four terminally lipid-modified bases to tightly anchor the nucleotide to the bilayer of vesicles. This strategy achieved a highly stable incorporation 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.