Lipid-anchored DNA mediates vesicle fusion as observed by lipid and content mixing

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A general method for synthesizing 5′- and 3′-coupled DNA-lipid conjugates has been developed and employed in DNA-mediated vesicle fusion. Vesicles presenting complementary DNA fuse, resulting in both outer and inner leaflet mixing as well as content mixing. Fusion is maximized using 5′- and 3′-coupled DNA on opposite vesicle partners, rather than only 5′-coupled DNA, showing the importance of DNA orientation to the process. Lipid and content mixing assays show a dependence of fusion kinetics on the sequence and average number of DNA per vesicle. Vesicles without DNA or presenting noncomplementary sequences also appear to undergo some degree of lipid mixing or exchange, but no content mixing. Total lipid mixing appears to occur more efficiently than inner leaflet mixing and content mixing, and this may be explained by the observed nonspecific lipid mixing and/or the rise of a hemifused intermediate. The ability to control DNA sequence and the relative experimental simplicity of this system make it highly attractive to probe fundamental questions of membrane fusion using both ensemble and single vesicle assays. © *2008 American Vacuum Society.* [DOI: 10.1116/1.2889062]

I. INTRODUCTION

The fusion of membranes plays a fundamental role in many biological processes including neurotransmission, exo- and endocytosis, and viral infection. Several strategies have been used to achieve fusion between lipid vesicles *in vitro*, which can be broadly categorized as involving either nonspecific or specific interactions. Examples of nonspecific interactions are chelation and depletion effects arising from the presence of divalent cations[1] and poly(ethylene glycol),[2] respectively. Specific interactions are mediated by recognition between molecules presented on the vesicle surface, such as small molecules[3] or membrane proteins.[4,5] These studies suggest that bringing vesicles into close apposition can lead to subsequent hemi- and full-fusion events, which are typically signified by lipid and/or content mixing. In the case of neuronal vesicle fusion, it has been suggested that the SNARE proteins are the minimal machinery needed for recognition and to drive the fusion reaction.[4] Even though the mechanism of this process is not fully understood, the SNARE proteins are widely thought to form a four-helix bundle in a "zippering" process, which begins at the membrane distal ends of the proteins and proceeds toward the membrane proximal ends [Fig. 1(A)].[6,7]

Recently, DNA-lipid conjugates have been utilized to tether lipid vesicles to supported lipid bilayers.[8–10] These tethered vesicles are observed to diffuse parallel to the supported bilayer surface and collide, but not interact. The kinetics of DNA-mediated binding between tethered vesicles were characterized as a reaction analogous to the docking step in the protein-mediated fusion reaction.[11] In these studies, where DNA was coupled to the lipid anchor at the 5′ end, lipid mixing was not observed between tethered vesicles and the supporting bilayer or between docked vesicles. This observation suggests vesicle fusion occurs minimally, which is likely a consequence of the resulting duplex DNA acting as a spacer between the membrane surfaces, thus inhibiting the contact necessary for further reaction. As illustrated in Fig. 1(B), docking between two vesicles presenting 5′- and 3′-coupled DNA should result in a geometry that more closely resembles what is thought to occur in SNARE-mediated fusion. Indeed, Stengel *et al.* have demonstrated lipid mixing between vesicles using a cholesterol-anchored DNA construct and this was interpreted as evidence for vesicle fusion.[12] Here, we report a general and simple method to synthesize both 5′- and 3′-coupled DNA-lipid conjugates and demonstrate that vesicle pairs displaying complementary DNA can mediate vesicle fusion as demonstrated by both lipid and content mixing.

II. EXPERIMENT

A. Reagents

1,2-O-dioctadecyl-rac-glycerol was obtained from Chem-Impex. 2-cyanoethyl N, N-diisopropylchlorophosphoramidite, terbium (III) chloride, and dipicolinic acid (DPA) were obtained from Sigma. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2–1,3-benzoxydiazol-4-yl) (NBD-DOPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(Lissamine Rhodamine *B* Sulfonyl) (Rh-DPPE), and cholesterol (Chol) were obtained from Avanti Polar Lipids. Columns and amidites for reverse DNA synthesis were purchased from Glen Research and Chem Genes, respectively. All materials were used as received.

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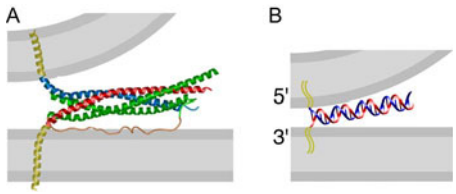

FIG. 1. Schematic illustration of the binding of a vesicle to a planar membrane mediated by (A) SNARE proteins (Ref. 19) and (B) 5′- and 3′-coupled complementary DNA.

TABLE I. Sequences and coupling orientations of nonrepeating (*NR*/*NR*′) or Poly *A*/*T* (*A*/*T*) DNA oligonucleotides coupled to lipids as shown in Fig. 2 and used to mediate vesicle fusion.

Name	Sequence	Coupling
NR	TCG ACA CGG AAA TGT TGA ATA CTA	5′
NR′	TAG TAT TCA ACA TTT CCG TGT CGA	3′
NR′	Same as above	5′
T	TTT TTT TTT TTT TTT TTT TTT TTT	5′
A	AAA AAA AAA AAA AAA AAA AAA AAA	3′

B. Synthesis of lipid phosphoramidite and DNA-lipid synthesis

Figure 2 shows the general synthetic scheme to make the lipid phosphoramidite, which is based on similar reactions described previously.[13,14] Here, 0.5 g of 1,2-O-dioctadecyl-rac-glycerol (0.83 mmol) and 0.32 mL diisopropylethylamine (1.8 mmol) were dissolved in 10 mL anhydrous dichloromethane at 0 °C under a nitrogen atmosphere. Then, 0.25 g 2-cyanoethyl N, N-diisopropylchlorophosphoramidite (1.3 mmol) was added dropwise to the reaction, which was stirred at 0 °C for 15 min, then at room temperature for 2 h. The crude product solution was washed with a 0.5 M solution of sodium bicarbonate, and the solvent evaporated. Purification by silica gel chromatography (90:9:1 hexanes:ethyl acetate:triethylamine) yielded 330 mg (50% yield) of product, and purity was confirmed by ^{1}H and ^{31}P NMR. The synthesized lipid-phosphoramidite was dissolved in dichloromethane to be used as the last "base" to be coupled onto the DNA oligomer on a conventional DNA synthesizer. Reverse-phase high performance liquid chromatography (HPLC) purification and mass-spectrometric identification of the DNA-lipid was performed as described.[9]

C. Preparation of DNA-labeled vesicles

Lipid vesicles were formed by the extrusion method[8] and typically contained a 2:1:1 molar ratio of DOPC, DOPE, and Chol. For lipid mixing experiments, 1.5 mol % NBD-DOPE and 1.5 mol % Rh-DPPE were included. Lipid stocks were combined in chloroform and dried first under a stream of nitrogen and then under vacuum. Lipid films were resuspended in 10 mM Tris buffer, *p*H 7.5, containing 100 mM NaCl. For content mixing assays, lipids were suspended in buffer containing either 8 mM $TbCl_3$ and 60 mM sodium citrate or 80 mM DPA, and 10 mM Tris (*p*H 7.5) in both cases. The lipid suspensions were then extruded through a 0.05 μm polycarbonate filter (Whatman) to form vesicles of nominal 50 nm diameter. Characterization of vesicle size by dynamic light scattering gave average diameters of 120 nm. Lipid stocks were used within 1 month of being received from the manufacturer as we observed substantial increases in the efficiency of fusion with older material.

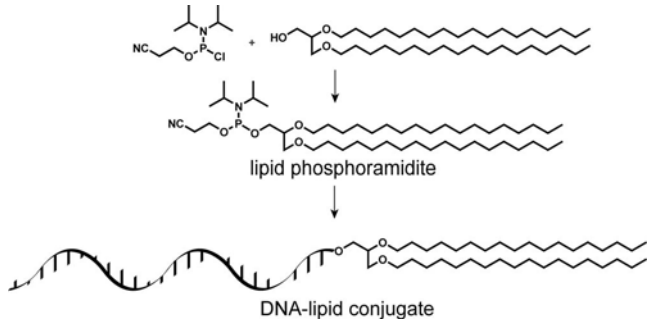

FIG. 2. Synthetic scheme to produce the lipid phosphoramidite for use on a DNA synthesizer.

Stocks of DNA-lipid conjugates were dissolved in a 1:1 mixture of water and acetonitrile. An appropriate volume of this solution was added to lipid vesicle stocks to achieve the desired average DNA-to-vesicle ratio (calculated assuming a vesicle diameter of 50 nm), then incubated at 4 °C overnight. For inner leaflet mixing experiments, NBD on the outer leaflet was bleached by adding sodium dithionite to the vesicle suspension to a concentration of 40 mM for 1 min immediately before column purification. After incorporating the DNA-lipids, the vesicles were passed through a CL-4B matrix size-exclusion column (Sigma) equilibrated with 10 mM Tris buffer, *p*H 7.5, containing 100 mM NaCl to separate any unincorporated DNA-lipids and external dithionite, $TbCl_3$, and DPA from the content-labeled vesicles. The concentrations of the resulting vesicle stocks were determined using an enzymatic assay for choline.[15] Sequences and coupling orientation for all DNA-lipids used in this study are presented in Table I. It is important to note that these DNA-lipid conjugates partition essentially quantitatively into vesicles with a simple, single lipid anchor, and that the 3′-coupled DNA behave similarly to 5′-coupled DNA in tethering studies (data not shown).

D. Fusion assays

The standard fusion assays shown schematically in Fig. 3 were used. Pairs of DNA-lipid vesicles were mixed at a 1:1 ratio to a final lipid concentration of 0.05 mg/mL (74 μM) in 10 mM Tris buffer containing 100 mM NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA). All experiments were performed at room temperature (22 °C). For lipid mixing assays, the fluorescence spectra at different times were taken on a SPEX Fluorolog-3 spectrofluorometer using 465 nm excitation. The Förster resonant

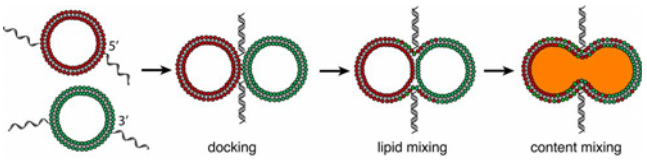

FIG. 3. Schematic illustration of possible states in the DNA-mediated vesicle fusion reaction. Mixing of vesicles presenting 5′- and 3′-coupled DNA leads to docking, then to lipid mixing (shown here as a possible hemi-fusion intermediate), and last to content mixing (shown here as a possible result of full fusion of the vesicles).

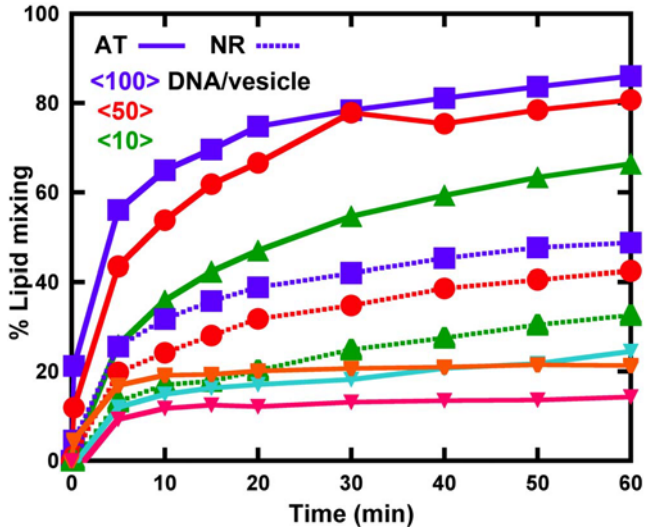

FIG. 4. Reaction time courses of DNA-mediated lipid mixing. Vesicles presenting either an average of 100 (blue squares), 50 (red circles), or 10 (green triangles) copies of Poly A/T (AT, solid lines) or nonrepeating (NR, dashed lines) sequences of DNA were mixed and the FRET ratio monitored over 60 min. Time courses for the following are also shown: complementary 5′-coupled DNA (cyan), noncomplementary 5′- and 3′-coupled DNA (magenta), and no DNA (orange).

energy transfer (FRET) ratio was defined as the ratio of the emission intensity at 590 nm to that at 525 nm. To determine the extent of lipid mixing, the FRET ratio at each time point was compared to a standard curve measured for vesicles with known composition incorporating equimolar amounts of NBD-DOPE and Rh-DPPE ranging from 0.5 to 1.5 mol %. Percent lipid mixing is calculated such that 100% corresponds to the FRET signal if the labels are diluted to half of their original concentration in the membrane. Thus, 0% and 100% lipid mixing corresponds to the FRET measured for vesicles containing 1.5 and 0.75 mol %, respectively, of each of the lipid dyes. For inner leaflet mixing experiments, the standard curve was taken from vesicles after treatment with dithionite.

The content mixing assay was based on the formation of a fluorescent $Tb(DPA)_3$ complex as described by Wilshut and Papahadjopoulos.[16] The emission intensity at 545 nm upon excitation at 278 nm was measured at each time point. Leakage of contents into the extravesicular solution did not lead to a signal increase as added 1 mM EDTA quenches 99% of the $Tb(DPA)_3$ fluorescence. Thus, only content exchange between vesicles is recorded as fusion. The percent content mixing is estimated based on comparison of the fluorescence intensities to that of standard samples assuming that 100% mixing leads to a twofold dilution of content.

III. RESULTS

Synthesis of the DNA-lipids using the technique described gives yields similar to those using previous methods.[8,9] Since the lipid-phosphoramidite is compatible with direct use on conventional DNA-synthesizers, this method is able to create both 5′- and 3′-coupled DNA-lipids using the appropriate synthesis columns and reagents. For this study, two 24-bp, 3′-coupled strands were made that are complementary to two 5′-coupled sequences made previously—one nonrepeating (NR) and one Poly A/T (see Table I for full sequences). Vesicles presenting the following combinations of DNA were mixed: no DNA; ⟨50⟩ copies of 5′- and 3′-coupled noncomplementary sequences ($5'-NR'/3'-NR'$); ⟨50⟩ copies of 5′- and 5′-coupled complementary sequences ($5'-NR/5'-NR'$); and ⟨10⟩, ⟨50⟩, or ⟨100⟩ copies of 5′- and 3′-coupled complementary sequences ($5'-NR/3'-NR'$ or $5'-A/3'-T$).

Lipid mixing was observed on a fluorometer by labeling one population of vesicles with a pair of fluorescent leaflet labels that can undergo FRET. For all experiments, vesicles were mixed over roughly 5 s to 74 μM lipid concentration. As leaflet mixing occurs, the dye-labeled lipids are diluted, increasing the average distance between the NBD donor and Rh acceptor and decreasing the FRET efficiency. Figure 4 shows representative kinetic time-courses for all DNA labeling schemes studied. All DNA combinations studied show a change in FRET ratio that is consistent with dilution of the fluorescent labels, surprisingly even when vesicles with no DNA are mixed. It is unclear whether the vesicles without DNA are undergoing fusion (content mixing is not observed, see below) or some other exchange process that reduces the FRET ratio. Vesicles labeled with noncomplementary DNA ($5'-NR'/3'-NR'$) show the lowest extent of lipid mixing, and those with 5′-coupled complementary DNA ($5'-NR/5'-NR'$) show similar behavior to that of vesicles without DNA. Most vesicle combinations with 5′- and 3′-coupled complementary DNA ($5'-NR/3'-NR'$ and $5'-A/3'-T$) show rates and extents of lipid mixing that are above those of the controls. For both sequences, the reaction efficiency increases as the average copy number of DNA per vesicle is increased. Furthermore, vesicles presenting the Poly A/T sequence generally show faster rates and greater extents of lipid mixing than for vesicles presenting the same average number of the nonrepeating sequence. Inner leaflet mixing was measured using vesicles for which NBD in the outer leaflet had been chemically quenched by reaction with sodium dithionite. As seen in Fig. 5, inner leaflet mixing proceeds much less efficiently than total lipid mixing when controlling for DNA number and sequence.

For content mixing, vesicles containing DPA displayed the 5′-DNA lipid conjugate and vesicles containing Tb^{3+} dis-

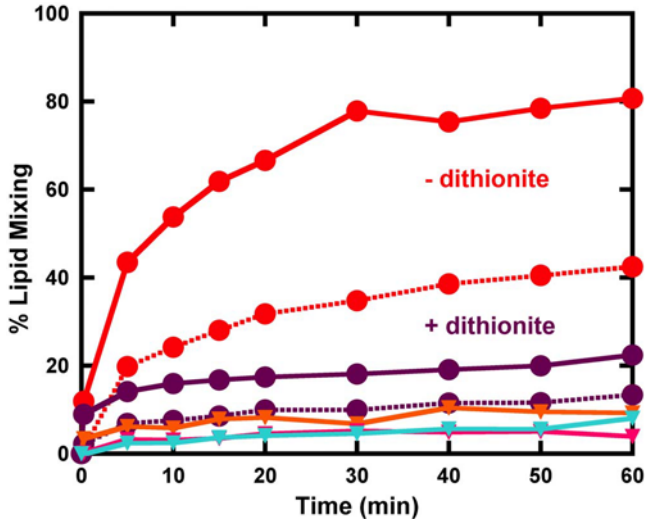

FIG. 5. Reaction time courses of DNA-mediated inner leaflet lipid mixing. Red traces are the same as in Fig. 4. Purple traces are taken for vesicles treated with dithionite to bleach the NBD in the outer leaflet and presenting an average of 50 copies of Poly A/T (solid lines) or nonrepeating DNA (dashed lines). Time courses for the following are also shown: complementary 5′-coupled DNA (cyan), noncomplementary 5′- and 3′-coupled DNA (magenta), and no DNA (orange).

played the 3′-DNA lipid conjugate. Figure 6 shows that vesicles displaying complementary DNA ($5'-NR/3'-NR'$ and $5'-A/3'-T$) exchange contents, resulting in a rise in fluorescence with sequence and copy number dependence similar to that observed for lipid mixing. The reaction generally proceeds faster and to a greater extent with more DNA

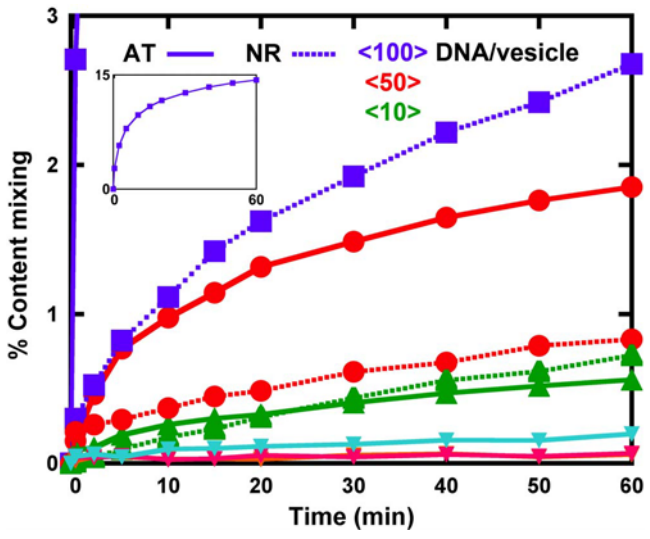

FIG. 6. Reaction time courses of DNA-mediated content mixing. Vesicles presenting either an average of 100 (blue squares), 50 (red circles), or 10 (green triangles) copies of Poly A/T (AT, solid lines) or nonrepeating (NR, dashed lines) sequences of DNA were mixed and the emission of Tb^{3+}-DPA monitored over 60 min. The inset shows the full time course for $\langle 100 \rangle$ AT. Time courses for the following are also shown: complementary 5′-coupled DNA (cyan), noncomplementary 5′- and 3′-coupled DNA (magenta, close to baseline), and no DNA (orange, obscured by magenta).

per vesicle and for the Poly A/T sequence over the nonrepeating sequence. The sequence dependence of content mixing is less pronounced at lower copy numbers of DNA. Controls using vesicles presenting no DNA, noncomplementary DNA, or only 5′-coupled DNA were performed for content mixing assays as for lipid mixing assays. These showed little or no rise in fluorescence, indicating minimal content mixing unless complementary, oppositely oriented DNA are used. Content leakage during the fusion reaction was demonstrated by reacting vesicles containing both Tb^{3+} and DPA and presenting $\langle 100 \rangle$ $5'-NR/3'-NR'$ with EDTA in the external solution. A decrease of $\sim$10% in the fluorescence intensity was observed over 1 h due to sequestration of leaked Tb^{3+}.

IV. DISCUSSION

Membrane fusion is generally thought to proceed through several stages. First, docking brings two membrane surfaces into proximity. Then, outer leaflet merger may result in stalk formation and a hemi-fused intermediate. Last, content mixing is achieved either through transient pore formation or complete merger of the two membranes. The lipid and content mixing experiments provide strong evidence that DNA-mediated vesicle fusion is occurring. Fusion is most efficient when the DNA on the reacting vesicles are complementary and in the opposite orientation. Both assays indicate that the fusion reaction is more efficient when using greater average copy numbers of DNA per vesicle as well as when using the repeating Poly A/T sequence rather than the nonrepeating sequence. These trends are consistent with those found for the kinetics of DNA-mediated docking of tethered vesicles.[11] The Poly A/T strands are able to hybridize with only partial overlap of the DNA, relaxing geometric constraints in comparison to the nonrepeating sequence. The differences in kinetics seem to be most pronounced at shorter times. At longer time scales, the reaction likely becomes very complicated with multiple docking and fusion events probable. To address questions of reaction mechanism and initial rates, concentration dependence and rapid mixing measurements are in progress along with direct measurements on individual tethered vesicles following the methods introduced in Ref. 11.

For all copy number and sequence combinations studied, lipid mixing occurs more quickly and to a greater extent than content mixing. The controls run indicate that a significant basal change in FRET occurs even in the absence of DNA, suggesting that the percentages of lipid mixing from these experiments may be artificially high. We are unsure about the reason underlying this observation, though some nonspecific exchange or mixing mechanism may be responsible. Nevertheless, correcting for this phenomenon is a nontrivial issue that must be considered for any lipid-mixing assay based on fluorescence. Another possible explanation is that content mixing does occur more slowly than lipid mixing, perhaps due to the nature of pore formation or bilayer merger in this system. Inner leaflet mixing also occurs more slowly and to a lesser extent than mixing of both outer and inner leaflets. Since the membrane anchor spans only one leaflet of the

bilayer, a stable hemi-fusion intermediate may be produced,[17] thus allowing mixing of the outer leaflet lipids while content and inner leaflet mixing are restricted.

Since fusion is maximized using vesicle pairs displaying complementary 5′- and 3′-coupled DNA over noncomplementary or only 5′-coupled DNA, the orientation of the DNA clearly plays an important role in driving the reaction beyond docking. From the data obtained so far, it is not yet possible to tell whether fusion is initiated simply by bringing the two membranes within some critical distance, or if the energetics of the hybridization reaction contribute overcoming the activation barrier to leaflet mixing. These and other mechanistic questions can be explored in a straightforward fashion by controlling the DNA sequences to modify the geometric or thermodynamic parameters of hybridization. For instance, the question of how close membranes must be brought to allow fusion can be addressed by inserting spacer sequences to position the complementary DNA at varying distances from the membrane surface. These measurements are in progress.

We conclude with a comparison of our system to that of Stengel *et al.*, who demonstrated lipid but not content mixing for vesicles using DNA with a cholesterol anchor.[12] Due to the transient partitioning of single-cholesterol anchored DNA,[18] these authors used doubly anchored, sticky-ended DNA duplexes (12 bases double-stranded proximal to the vesicle, 15 bases single-stranded distal to the vesicle) to mediate fusion. They observed lipid mixing for vesicles displaying complementary DNA, but minimal mixing for vesicles displaying noncomplementary DNA or no DNA. It was hypothesized that the duplex dissociates to allow full "zippering" that would bring the vesicles into closest proximity. It is unclear how the membrane anchors and restricted geometry affect strand displacement, how or if more than one duplex would achieve this sequence of events in a coordinated fashion, and, if it occurs, what happens to the required intermediate in which the hybridizing DNA strands on the two membranes are anchored by single cholesterols and therefore should rapidly dissociate from the vesicles. In contrast, the lipid-DNA conjugate reported here and in our earlier work[9–11] requires only one lipid anchor displaying a single-stranded DNA, avoiding the complexity of the cholesterol-anchored system. Future results from the two quite different systems should shed light on the effect of the anchor on the fusion reaction.

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