

# Covalent Attachment of Lipid Vesicles to a Fluid-Supported Bilayer Allows Observation of DNA-Mediated Vesicle Interactions

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Specific membrane interactions such as lipid vesicle docking and fusion can be mediated by synthetic DNA–lipid conjugates as a model for the protein-driven processes that are ubiquitous in biological systems. Here we present a method of tethering vesicles to a supported lipid bilayer that allows the simultaneous deposition of cognate vesicle partners displaying complementary DNA, resulting in well-mixed populations of tethered vesicles that are laterally mobile. Vesicles are covalently attached to a supporting lipid bilayer using a DNA-templated click reaction; then DNA-mediated interactions between tethered vesicles are triggered by spiking the salt concentration. These interactions, such as docking and fusion, can then be observed for individual vesicles as they collide on the surface. The architecture of this new system also permits control over the number of lipid anchors that tether the vesicle to the supporting bilayer. The diffusion coefficient of tethered vesicles anchored by two lipids is approximately 1.6-fold slower than that of vesicles anchored only with a single lipid, consistent with a simple physical model.

## Introduction

To study the dynamics of and interactions between individual lipid vesicles, we have developed a tethered membrane strategy that uses DNA–lipid conjugates, shown in Figure 1 (molecule i), first to tether vesicles to a fluid-supporting lipid bilayer and then to mediate their interactions (Figure 2).<sup>1–3</sup> Labeled individual vesicles are readily visualized by fluorescence microscopy as they diffuse randomly in the plane parallel to the supporting bilayer.<sup>1,4</sup> Collisions between vesicles are observed, but unless specific recognition components are displayed on their surfaces, these collisions are reversible, with no evidence of lipid or content mixing. However, if vesicle populations displaying sense sequences are initially tethered to a supporting bilayer approximately 100  $\mu\text{m}$  from where those displaying antisense sequences are tethered (to avoid interactions in the bulk solution during the tethering process), then over time the two populations will mix by diffusion and collisions can lead to vesicle docking as illustrated in Figure 2. The sequence, length, and number-density dependence of the docking probability have been studied in some depth when the DNA is anchored to the vesicle membrane at the 5' end in both populations.<sup>3</sup> Especially when relatively low number densities of docking DNAs are present, most collisions do not lead to docking so it is very time-consuming to collect satisfactory statistics. This limitation is intrinsic to single-object measurements: the objects, vesicles in this case, must be very dilute in order to be individually observed, yet their concentration must be high enough to obtain useful numbers of collisions before photobleaching.

The DNA docking strategy has been further extended by incorporating DNA–lipids anchored at the 5' end in one population and at the 3' end in the other, which causes the membrane

surfaces to approach each other during hybridization. In this case, docking is followed by lipid and to a lesser extent content mixing, suggesting that membrane fusion has occurred, though thus far this has been demonstrated only in 3D mixing assays.<sup>5–7</sup> As the events become more rare (and more interesting), even larger numbers of collisions must be sampled to extract meaningful information on the steps that lead to fusion.

In this article, we develop an alternative to the remote and dilute deposition strategy illustrated in Figure 2 by making the tethering step chemically orthogonal to the docking/fusion steps. We still require that the tethered vesicles be mobile so that individual collisions can be observed. This can be accomplished by using a DNA-templated “click” reaction<sup>8–10</sup> to introduce a covalent link between the DNA and lipids, as illustrated in Figure 3, which uses the new molecules shown in Figure 1 (molecules ii–iv). Vesicles displaying sense and antisense DNA on their surfaces but each protected by their respective antisense and sense strands in the presence of high salt concentration are covalently linked to the supporting bilayer at high density (Figure 3, step 1). When the salt concentration is lowered, the protecting strands are released (step 2) but the vesicles remain attached to the supporting bilayer by their covalent linkage; this is the key difference with respect to the earlier approach (Figure 2) in which lowering the salt concentration would also release the tethered vesicles from the surface because they are tethered by DNA hybridization. At this point, vesicles do not dock or fuse because DNA hybridization does not occur at low salt concentrations. The salt concentration is then rapidly increased, vesicle collisions

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(1) Yoshina-Ishii, C.; Boxer, S. G. *J. Am. Chem. Soc.* **2003**, *125*, 3696–3697.  
(2) Yoshina-Ishii, C.; Miller, G. P.; Kraft, M. L.; Kool, E. T.; Boxer, S. G. *J. Am. Chem. Soc.* **2005**, *127*, 1356–1357.  
(3) Chan, Y.-H. M.; Lenz, P.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 18913–18918.  
(4) Yoshina-Ishii, C.; Chan, Y.-H. M.; Johnson, J. M.; Kung, L. A.; Lenz, P.; Boxer, S. G. *Langmuir* **2006**, *22*, 5682–5689.

(5) Chan, Y.-H. M.; van Lengerich, B.; Boxer, S. G. *Biointerphases* **2008**, *3*, FA17–FA21.

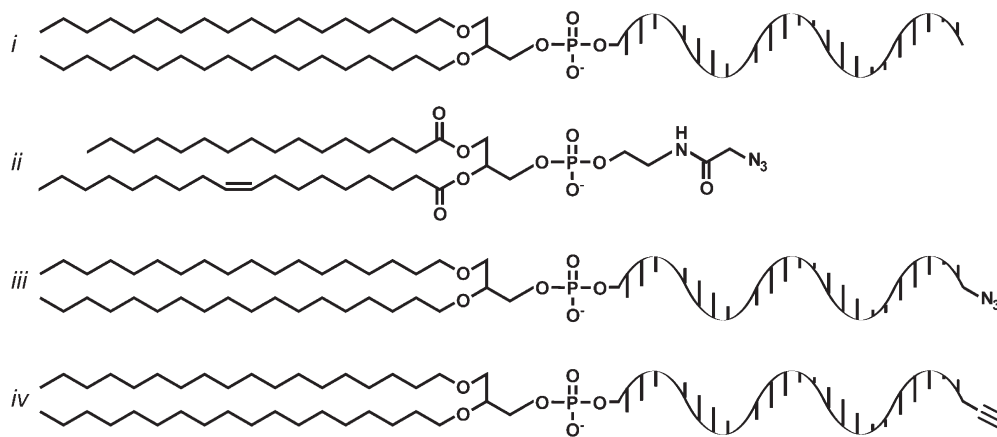
(6) Chan, Y.-H. M.; van Lengerich, B.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 979–984.

(7) Stengel, G.; Zahn, R.; Höök, F. *J. Am. Chem. Soc.* **2007**, *129*, 9584–9585.

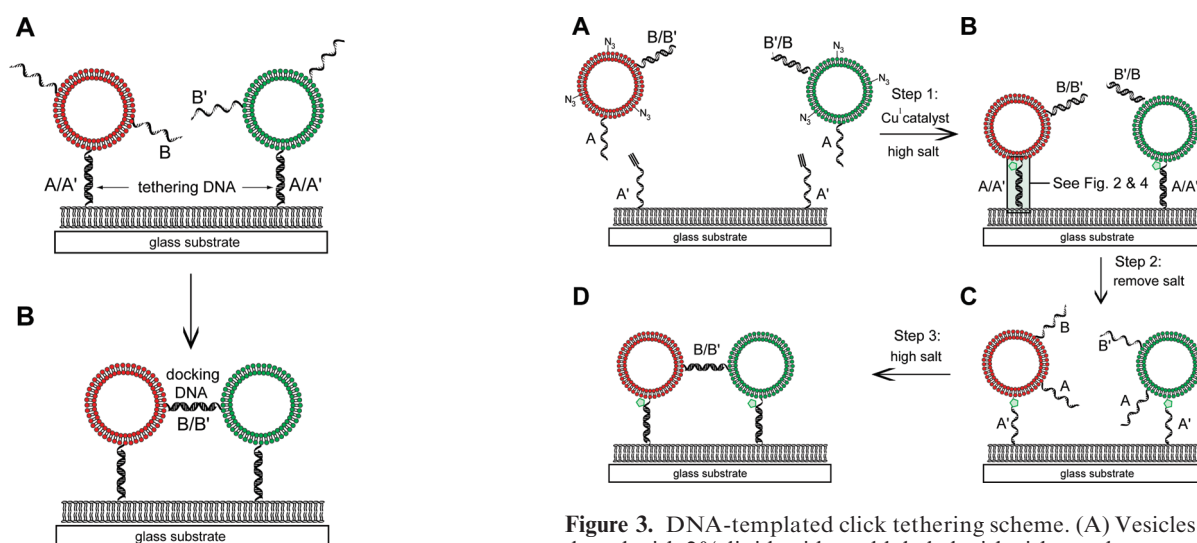
(8) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599.

(9) Kanan, M. W.; Rozenman, M. M.; Sakurai, K.; Snyder, T. M.; Liu, D. R. *Nature* **2004**, *431*, 545–549.

(10) Kumar, R.; El-Sagheer, A.; Trumpane, J.; Lincoln, P.; Wilhelmsson, L. M.; Brown, T. *J. Am. Chem. Soc.* **2007**, *129*, 6859–6864.



**Figure 1.** Structures of lipids functionalized with DNA, azide, or alkyne. (i) 24mer DNA attached to lipid used for templating and docking; (ii) POPE-N<sub>3</sub>; (iii) 24mer DNA–lipid functionalized with azide or (iv) alkyne.



**Figure 2.** DNA-mediated docking between tethered vesicles displaying complementary DNA. (A) Red and green fluorescently labeled vesicles tethered with complementary sequence A/A' and displaying complementary docking DNA sequences B and B' (see Supporting Information for sequences) diffuse freely in the plane parallel to the supporting bilayer. (B) Hybridization of B and B' leads to a docked pair, observed as the co-localization of the red and green vesicles, which diffuse in tandem but do not exchange lipid or content. If B is anchored at the 5' and B' at the 3' end, then docking can bring the membrane surfaces into close proximity to each other and cause lipid and content mixing (i.e., vesicle fusion<sup>5</sup>). To avoid docking and/or fusion between vesicle pairs in the bulk solution before tethering, the two populations in A are initially tethered far from each other on the supporting bilayer and then randomly diffuse, collide, and dock and/or fuse.

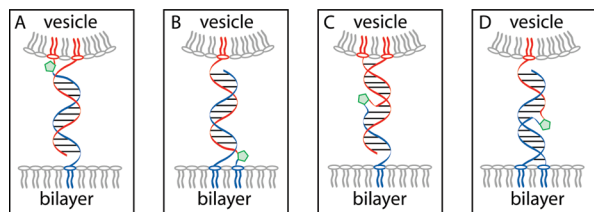
occur, and in some cases DNA hybridization leading to docking and potentially fusion (depending upon the orientation of the DNA–lipid linkage) can be observed. Some preliminary results are presented, and a detailed analysis of this will be the subject of future work. Additionally, different locations of the covalent linkage on the strand can be readily achieved, as shown in Figure 4, and the orientation can be controlled so that either a single lipid molecule anchors the tethered vesicle to the supporting bilayer (Figure 4A,C) or the anchor consists of two lipid molecules (Figure 4B,D). Because tethered vesicle diffusion can be measured directly by single-vesicle tracking, we can observe the effects of anchor size on lateral diffusion.

**Figure 3.** DNA-templated click tethering scheme. (A) Vesicles, doped with 2% lipid azide and labeled with either red or green lipid fluorophores and displaying DNA–lipids A and B or B' (protected with their respective free DNA complements B' or B), are added in the presence of salt and copper catalyst to a glass-supported lipid bilayer displaying alkyne-functionalized strand A'. (B) Step 1: Vesicles tether by DNA hybridization and azide and alkyne react to form a triazole (green pentagon, the click reaction). (C) Step 2: Upon being rinsed with deionized water, both the protecting DNA and the templating DNA–lipid unhybridize. Templating DNA–lipid strand A can diffuse away but remains anchored in the vesicle;<sup>14</sup> the protecting strands are permanently washed away. (D) Step 3: Addition of salt to the system allows templating strand A to rehybridize with covalently linked A'. Vesicles diffuse randomly and eventually collide and dock via the hybridization of B with B'.

## Experimental Section

**Materials.** Palmitoyl-oleoyl phosphatidylethanolamine (POPE), dioleoyl phosphatidylserine (DOPS), and egg PC were obtained from Avanti. Texas red dihexanoyl phosphatidylethanolamine (DHPE) and Oregon green dihexanoyl phosphatidylethanolamine (DHPE) were obtained from Invitrogen. (Triethyl-2,2',2''-(4,4',4''-nitrotris(methylene)tris(1*H*-1,2,3-triazole-4,1-diyl)-triacetate (TTMA) was generously donated by the Chidsey laboratory at Stanford University. Azidobutyrate *N*-hydroxysuccinimide (NHS) ester was obtained from Glen Research. Propargyl dPEG 1-NHS ester was obtained from Quanta Biodesign.

**Lipids and Lipid–DNA Conjugates.** POPE-N<sub>3</sub> (Figure 1ii) was synthesized from POPE and azidoacetic acid (prepared as



**Figure 4.** Illustration of different locations of the covalent linkages obtained by employing the DNA-templated click reactions, resulting in singly or doubly anchored vesicles. (A) The tethered vesicle contains the lipid azide (red lipid) as well as the templating strand (red strand), and the bilayer displays DNA-alkyne (blue strand). Upon covalent coupling of red lipid azide to blue DNA alkyne, a double-lipid anchor (red) is formed in the tethered vesicle and the vesicle remains singly anchored in the supporting bilayer. (B) Flipped scenario of A, where the bilayer contains lipid azide (blue lipid) and templating DNA-lipid (blue strand). Here the double-lipid anchor is formed in the supporting bilayer (blue lipids), thus the vesicle is doubly anchored to the bilayer. (C) A longer (48mer) red DNA-lipid, which is anchored to the tethered vesicle, templates the covalent coupling between two shorter 24mer lipid-DNAs (shorter red and blue strands, one attached in either membrane). This results in a vesicle singly anchored to the supporting bilayer. (D) Flipped scenario of C, where the longer-templating DNA-lipid (blue) is now anchored to the supporting bilayer; covalent linkage forms a doubly anchored vesicle.

in ref 11). POPE and azidoacetic acid in dichloromethane were stirred at 0 °C in a dry flask, and then 2 equiv of dicyclohexylcarbodiimide and 2 equiv of triethylamine were added and the reaction was stirred and allowed to warm to room temperature overnight. The reaction was then concentrated on a rotovap and purified by silica gel chromatography (9:1 dichloromethane/methanol). POPE-N<sub>3</sub> was characterized by <sup>1</sup>H NMR and LC/MS (MH<sup>+</sup> = 799). Lipid-DNA conjugates were synthesized as described in ref 5 with nonrepeating sequences of 24 or 48 nucleobases (Supporting Information). For azide- and alkyne-functionalized strands, an amine linker was incorporated before the first nucleobase. Following lipid phosphoramidite addition after the last nucleobase,<sup>5</sup> the lipid-oligonucleotide conjugate was cleaved from resin and simultaneously deprotected in 30% ammonium hydroxide at 55 °C overnight and then purified by preparative HPLC. The free amine was then reacted with either azidobutyrate NHS ester or propargyl-2-PEG-1-NHS ester to form azide- or alkyne-functionalized lipid-DNA and then purified again by HPLC and characterized by MALDI.

**Vesicles.** Lipid mixtures were prepared in chloroform and dried under a stream of nitrogen, followed by additional drying in a vacuum desiccator for at least 8 h. Typical bilayer mixtures consisted of 2 mol % DOPS and 98% egg PC (chicken); tethered vesicles consisted of 0.5% Texas red DHPE or Oregon green DHPE, 2% DOPS, and 97.5% egg PC. For vesicles containing POPE-N<sub>3</sub>, DOPS was replaced with 2% POPE-N<sub>3</sub>. This keeps the charge ratio constant between the bilayer and tethered vesicles because POPE-N<sub>3</sub> has one negative charge at pH 7.5. Dried lipid films were then rehydrated with buffer containing 10 mM Tris (tris(hydroxyl)amine) and 100 mM sodium chloride at pH 7.5. These aqueous suspensions were vortex mixed for 1 min to create multilamellar vesicles, which were then extruded through 50 nm pore membranes using an Avanti miniextruder for vesicles destined to form the supporting bilayer or 100 nm pore membranes for vesicles destined to be tethered. Lipid-DNA was added from 20 μM stock solutions to vesicles to a final concentration of 0.04 mol % DNA for both tethering and templating strands and was incubated for at least 4 h at 4 °C. This corresponds to an average number density of 3 DNA molecules per vesicle for the 50 nm supporting bilayer vesicles and 12 DNA molecules per vesicle for the 100 nm tethering vesicles. For docking

experiments, an additional 24mer strand of DNA-lipid (sequence B) was incubated with the vesicles. After at least 4 h, a 5-fold excess of the free DNA complement (sequence B') was added to these vesicles and allowed to incubate an additional hour at 4 °C. Final lipid concentrations for this incubation step were 1 mg/mL for vesicles to be tethered and 4 mg/mL for vesicles to be used for supporting bilayer formation. It proved essential to use vesicles within about 2 days of extrusion to obtain optimal results because vesicle aging caused a decreased mobility of tethered vesicles and larger numbers of nonspecifically bound vesicles. (See Supporting Information for further details.)

**DNA-Templated Tethered Vesicles.** Glass coverslips were boiled in 7× detergent (MP Biomedicals) diluted 1:7 with deionized water until the solution became clear (about 20 min), rinsed with copious amounts of deionized water, and then baked for 4 h at 400 °C. Immediately before use, the coverslips were plasma cleaned under slight air pressure for 30 min (Harrick Plasma Cleaner). Then, 50 μL of a 0.4 mg/mL solution of supporting bilayer vesicles in 7.5 mM Tris + 575 mM sodium chloride buffer was added immediately to a Coverwell perfusion chamber gasket (Invitrogen) affixed to the cleaned glass slide. After 30 min, the sample was washed extensively with 10 mM Tris + 100 mM sodium chloride buffer. Subsequently, 5 μL of freshly prepared 10 mM sodium ascorbate in water, 5 μL of a solution containing 0.5 mM CuSO<sub>4</sub> and 1 mM TTMA in water, and 1 to 2 μL of Texas red-labeled vesicles at 1 mg/mL displaying DNA-alkyne (Figure liii), DNA-azide (Figure liv), or 2% lipid azide (Figure lii) were added (step 1 in Figure 3). After approximately 1 h, samples were washed with 10 mM Tris + 100 mM NaCl buffer and then extensively with deionized water (step 2 in Figure 3) to remove noncovalently attached vesicles and then with 10 mM Tris + 100 mM NaCl buffer again (step 3 in Figure 3) and then imaged on an inverted fluorescence microscope (Nikon TE300). The density of covalently attached vesicles remaining after the water and buffer rinses could be controlled by varying the reaction time or by changing the added vesicle concentration.

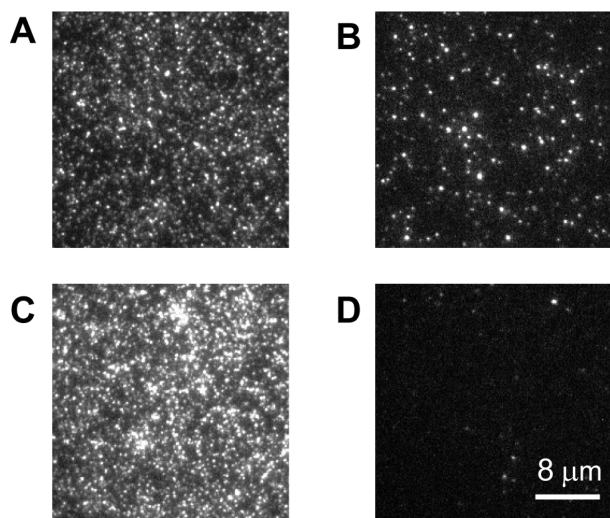
## Results and Discussion

To observe DNA-mediated interactions such as docking and fusion between individual tethered vesicles, the cognate vesicle partners must be prevented from interacting in the bulk solution during the tethering process. Although previously we achieved this by spatially separating the two vesicle populations during the tethering process, here we achieve this by using chemically independent steps for the tethering and docking/fusion reactions. Vesicles were covalently tethered to the supported lipid bilayer by a click reaction, and docking was mediated by the hybridization of complementary DNA. We found that it was necessary to template the click reaction using DNA to obtain reasonable yields, but the templating strand of DNA can be released after the covalent connection. This covalent tethering strategy enables us to create well-mixed populations of vesicles tethered to the supported bilayer, which is essential to obtaining adequate statistics during the real-time observation of docking and fusion reactions between randomly diffusing tethered vesicles.

We present two slightly different covalent tethering methods that result in the triazole formed near the membrane or near the midpoint of the DNA duplex. Both of these methods allow the orientation of the duplex to be flipped, which controls the number of lipids anchoring the vesicle to the bilayer. The first method is briefly described above and is shown in Figure 3 and in detail in Figure 4A. Vesicles displaying lipid-azide and templating 24mer strand A were covalently attached to a supporting lipid bilayer displaying the alkyne-functionalized complementary strand, A'-alkyne (step 1 in Figure 3). After the covalent connection was formed, salt was removed from the system by washing the sample with deionized water, releasing any vesicles that are not covalently linked (step 2 in Figure 3). Upon addition of salt back into

(11) Laughlin, S. T.; Bertozzi, C. R. *Nat. Protoc.* **2007**, *2*, 2930–2944.





**Figure 5.** Fluorescence microscopy images of vesicles tethered using the strategy shown in Figure 3. The bright spots in images A and C are vesicles tethered before removing salt, and B and D are after removing salt by washing the sample with deionized water. The lower two panels (C and D) represent a control experiment where everything was kept identical to the above experiment except that the tethering DNA (strand A' in Figure 3) was not functionalized with an alkyne so no covalent linkage was formed. Only a few nonspecifically bound vesicles remain in D when the salt concentration is lowered.

the sample<sup>12</sup> (step 3 in Figure 3), a templating strand (red strand in Figure 4A) rehybridizes with the now covalently linked strand (blue strand in Figure 4A). In this way, the double-stranded DNA tether is anchored to the supporting bilayer by one lipid, shown in blue in Figure 4A. The orientation of the templating reaction can be flipped (Figure 4B) simply by incorporating the lipid azide and the templating strand into the supporting bilayer rather than in the tethered vesicle; DNA-templated tethering of these vesicles results in a vesicle that is doubly anchored to the supporting bilayer.

In control experiments (Figure 5), vesicles were tethered in the absence of the copper catalyst or using a DNA strand that was not functionalized with an alkyne. In the presence of the high-concentration salt buffer, these vesicles were tethered as expected by DNA hybridization but most were released from the surface upon rinsing the sample with deionized water, indicating that the vesicles were not covalently linked. Typically, a small number of nonspecifically attached vesicles are not removed after extensive washing, presumably because of bilayer or substrate defects, but this number could be minimized by using fresh lipids and properly cleaned slides (Supporting Information). The yield of the click reaction could be improved by increasing the reaction time, but for the purpose of obtaining collision-free conditions for single-particle tracking, the reaction time was purposefully shortened to obtain low yields. It is possible that the yield was not always quantitative because the alkyne and the TTMA ligand are non-polar and may not be able to easily access the zwitterionic azide near the polar headgroup region of the bilayer.

(12) To test the integrity of the vesicles during these washes, which cause an osmotic imbalance across the membrane, vesicles containing a highly self-quenched concentration (50 mM) of carboxyfluorescein were tethered using this strategy. If the vesicles were to leak or rupture, then the average fluorescence intensity of vesicles would either increase due to dequenching or completely decrease.<sup>15</sup> However, no significant change in average vesicle fluorescence intensity or approximate surface density was observed after washing the sample with deionized water, implying that the vesicles had not leaked or ruptured.

(13) Johnson, J. M.; Ha, T.; Chu, S.; Boxer, S. G. *Biophys. J.* **2002**, *83*, 3371–3379.

The second method used to achieve DNA-templated covalent tethering, shown in Figure 4C,D, forms the triazole toward the midpoint of the DNA duplex. Here, both the azide and the alkyne are attached to the end of different DNA–lipids (Figure 1, sequences C and C' in the Supporting Information), and the reaction is templated by a DNA–lipid conjugate with a longer DNA strand, with half of its length being complementary to the lipid–DNA–azide and the other half being complementary to the lipid–DNA–alkyne. This brings the azide and alkyne into close proximity to each other and allows the click reaction to take place in the presence of copper catalyst. The shorter strands are both fully overlapping sequence 24mers, and the templating strand is the complementary 48mer strand. Longer strands (a total of 48 bases as compared to 24) were chosen for this architecture than for the first case in order to prevent the detachment of noncovalently linked vesicles due to the low melting temperatures of shorter strands (e.g., 12mers). This method allows the possibility to template the click reaction with free DNA instead of a lipid–DNA conjugate (i.e., the complementary 48mer does not need to be lipid-anchored in either bilayer). It also requires less overall dopant lipid to tether than in the first strategy, where 2% POPE-N<sub>3</sub> must be incorporated to obtain appreciable yields of the click reaction. Lower concentrations of reactants can be used in this second strategy because both lipid–DNA–azide and lipid–DNA–alkyne hybridize onto the 48mer template and their functional ends are constrained to be in close proximity to each other, whereas in the first strategy the lipid azide molecules are free to diffuse in and out of close proximity to the DNA–alkyne.

**Vesicle Mobility.** Although a number of laboratories have fixed vesicles via tethers to surfaces,<sup>14</sup> (e.g., as small isolated vessels for single-molecule measurements<sup>15</sup>), we are interested in vesicles that diffuse in the plane parallel to the supporting bilayer so that vesicle–vesicle interactions can be observed. Vesicles tethered using the strategies described here are observed using fluorescence microscopy in the presence and absence of salt. In the presence of salt, the vesicles appear to be diffraction-limited spots that are mobile in two dimensions; that is, they remain in the z-focal plane. Upon washing the sample exhaustively with deionized water, vesicles not covalently linked detach (Figure 5). Those that remain generally become immobile, but upon the readdition of salt, they regain mobility.<sup>16</sup> To understand the origin of this observation, we consider two possibilities: either the vesicles become immobile in the absence of salt because they are tethered (covalently) by only a single strand (the templating DNA–lipid, still anchored in the bilayer, is expected to be liberated and diffuse away under low salt conditions<sup>17</sup>) or there

(14) Christensen, S. M.; Stamou, D. *Soft Matter* **2007**, *3*, 828–836.

(15) Boukobza, E.; Sonnenfeld, A.; Haran, G. *J. Phys. Chem. B* **2001**, *105*, 12165–12170.

(16) This has the advantage that the locations of tethered vesicles in low salt concentration can be determined before initiating DNA-mediated docking and/or fusion by spiking the salt concentration.

(17) As a control and model for this, supported bilayers were formed which displayed sense-antisense 3'- and 5'-anchored DNA–lipid conjugates with Cy3 or Cy5, respectively, at the membrane distal end. These dyes constitute a FRET pair when DNA hybridization occurs. The ratio of Cy3-labeled DNA–lipid conjugate (donor) to Cy5-labeled DNA–lipid conjugate (acceptor) in the bilayer was 1:2 to ensure the maximal FRET signal, and the total concentration of the labeled DNA–lipid conjugate was approximately 1 DNA/200 nm<sup>2</sup> area (0.3 mol %). At high salt concentrations, FRET from Cy3 to Cy5 was observed, but when the salt concentration was lowered, FRET was greatly reduced, consistent with dehybridization. This process was reversible upon raising the salt concentration again. We note that binding-constant data for DNA hybridization is well documented for oligonucleotides in solution and, to a lesser extent, for hybridization when one partner is anchored on a surface as in DNA arrays, but we are not aware of precise binding measurements when both partners are anchored to a fluid surface. Similar effects arise for membrane protein clustering or assembly.<sup>18</sup>

is some nonspecific effect of salt on the tethered vesicle mobility. These possibilities can be distinguished by an electrophoretic separation experiment as follows.

Upon application of an electric field parallel to a supporting bilayer, negatively charged, membrane-anchored molecules such as the DNA–lipid conjugate move by electrophoresis toward the positive electrode whereas mobile tethered vesicles move by electroosmosis in the direction of the field, toward the negative electrode.<sup>19</sup> Vesicles were first covalently attached to a supporting bilayer as described for the above experiments using the double-lipid anchor configuration shown in Figure 4B. The sample was then rinsed with deionized water, immobilizing the covalently tethered vesicles and liberating<sup>17</sup> the supporting membrane-anchored, templating DNA strand from the now covalently attached DNA tether. Because these electrokinetic experiments are simpler to perform in confined regions, barriers were formed at this stage by scratching the surface of the prepared sample with sharp tweezers to form approximately  $100 \times 100 \mu\text{m}$  corrals.<sup>20</sup> The liberated templating DNA–lipids in the supporting bilayer were then swept by electrophoresis toward the positive electrode by an electric field of 10 V/cm; the covalently tethered vesicles remained immobile. The templating DNA–lipid formed a sharp concentration gradient against the side of the corral closest to the positive electrode; this was monitored by incorporating a Cy3-labeled DNA–lipid of equal length but different sequence into the bilayer. The electric field was then turned off, and a concentrated solution of salt was immediately spiked into the sample. We observed that the mobility of vesicles recovered uniformly over the entire area of the corral, despite the fact that nearly all of the templating DNA–lipid was still concentrated at the side of the corral (it slowly diffuses back over the entire corral on a much longer time scale). This implies that a double-stranded DNA tether is not necessary for vesicle mobility and that mobility instead requires a minimal salt concentration. The diffusion coefficient of these singly anchored, single-stranded tethered vesicles in the presence of salt was approximately  $0.13 \mu\text{m}^2/\text{s}$  ( $N = 33$ ), which is slower than for singly anchored, double-stranded tethered vesicles (discussed below), implying that the flexibility of the single-stranded tether may allow more interactions with the supporting lipid bilayer, slowing the vesicle diffusion. A greater number of ssDNA vesicles would need to be analyzed and other experiments would need to be performed to validate this interpretation, and these ssDNA tethered vesicles were not pursued fully.

The conclusion that salt, but not a double-stranded DNA tether, is required for mobility was confirmed by another experiment using the second templating method (similar to Figure 4C, D), where the templating DNA could be incorporated without a lipid anchor and thus could be permanently washed out of the system by deionized water after the click reaction. This leaves vesicles covalently attached to the bilayer with a single-stranded DNA tether and without any templating strand present in the sample. In the absence of salt, these vesicles were immobile but recovered mobility upon adding salt back to the sample, again indicating that salt is required for the mobility of these vesicles.

In the absence of salt, electrostatic repulsion should dominate the interaction between the negatively charged vesicles and the negatively charged supporting bilayer as a result of the increased Debye length. Because we would expect that this repulsion should allow vesicles to retain their mobility, our observation that vesicles become stuck in the absence of salt implies that this effect is not due to electrostatics. There could be other effects, such as

the depletion effect<sup>21</sup> or osmotic tension,<sup>22</sup> that may lead to increased attraction between the vesicle and the supporting bilayer and therefore may cause the vesicles to become immobilized on the surface, but we have no conclusive evidence of a particular mechanism that describes the observed phenomenon.

Note that even though our experiments suggest that salt, rather than whether the DNA tether is double- or single-stranded, appears to control vesicle mobility, we expect that rehybridization will readily occur between the templating and tethering strands upon the readdition of salt to a sample, as long as the liberated templating strands<sup>17</sup> are not segregated from the immobilized tethered vesicles, as in the electrophoresis experiments above. The mobility of doubly versus singly anchored vesicles not subjected to electrophoresis (described below) indicates that a templating strand does hybridize with the covalently linked strand within the timescale of mixing the high-concentration salt buffer back into the sample. This is not surprising because we estimate that the average surface density of the freely diffusing templating strands is approximately  $380 \text{ strands}/\mu\text{m}^2$  and so each covalently tethered vesicle should quickly encounter a templating strand and rehybridize upon the addition of salt back into the sample.<sup>17</sup>

**Single-Particle Tracking of Covalently Tethered Vesicles.** Although DNA-tethered vesicles are drawn with a single DNA duplex tether in Figures 2–4 rather than multiple tethers, direct evidence of this is difficult to obtain. In earlier work,<sup>4</sup> we observed that vesicles prepared to have on average 0.1 tethering DNA–lipid per vesicle and those with on average 50 DNA–lipids per vesicle exhibited similar diffusion coefficients when tethered. In the case of vesicles prepared with 0.1 DNA–lipid per vesicle on average, it is expected that most vesicles do not contain any DNA–lipid at all and that the probability that any given vesicle would have more than one DNA–lipid is quite small. However, for vesicles prepared to have on average 50 DNA–lipids per vesicle, multiple tethers would certainly be possible. Because similar diffusion coefficients were observed for vesicles prepared in either manner and because we expect that more membrane anchors should slow vesicle diffusion, we inferred that at most one tether is formed between a vesicle and the supporting bilayer. Note that this discussion applies only to small,  $\sim 100 \text{ nm}$  unilamellar vesicles; if giant unilamellar vesicles (GUVs) are tethered to a supporting bilayer, then multiple tethers are indeed possible and can lead to GUV rupture and tethered patch formation.<sup>23</sup>

Although we expect that more membrane anchors will slow diffusion, there is only limited experimental data on the effects of multiple anchors on diffusion in membranes,<sup>24,25</sup> and in the absence of this data, diffusion is an indirect way to infer the stoichiometry of a molecular assembly. The different linkage strategies described in Figure 4 offer an opportunity to test the effect of multiple anchors on membrane diffusion because vesicle populations with either one or two lipid anchors in the supporting bilayer can easily be constructed.

For each of the four orientations described in Figure 4, diffusion coefficients of the mobile, covalently tethered vesicles were measured by single-particle tracking under dilute, collision-free conditions and calculated as previously described.<sup>4</sup> The values and distributions of diffusion coefficients (Figure 6) resemble those of (noncovalently attached) DNA-tethered vesicles characterized

(21) Asakura, S.; Oosawa, F. *J. Chem. Phys.* **1954**, *22*, 1255–1256.

(22) Bailey, S. M.; Chiruvolu, S.; Israelachvili, J. N.; Zasadzinski, J. A. N. *Langmuir* **1990**, *6*, 1326–1329.

(23) Chung, M. S.; Lowe, R. D.; Chan, Y-H. M.; Ganesan, P. V.; Boxer, S. G. *J. Struct. Biol.* **2009**, *168*, 190–199.

(24) Benkoski, J. J.; Höök, F. *J. Phys. Chem. B* **2005**, *109*, 9773–9779.

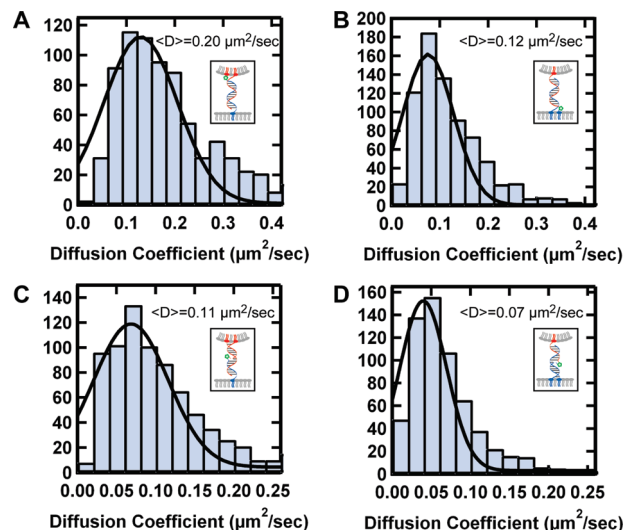
(25) Knight, J. D.; Falke, J. J. *Biophys. J.* **2009**, *96*, 566–582.

(18) Zhou, F. X.; Cocco, M. J.; Russ, W. P.; Brunger, A. T.; Engelman, D. M. *Nat. Struct. Biol.* **2000**, *7*, 154–160.

(19) Yoshina-Ishii, C.; Boxer, S. G. *Langmuir* **2006**, *22*, 2384–2391.

(20) Groves, J. T.; Boxer, S. G. *Biophys. J.* **1995**, *69*, 1972–1975.





**Figure 6.** Histograms of diffusion coefficients of tethered vesicles anchored by a single (A, C) or double (B, D) lipid anchor. Histograms A and B are from vesicles tethered as shown in Figure 4A,B respectively, and histograms C and D, as shown in Figure 4C,D. Black traces are Gaussian fits to the histograms. The mean  $\pm 1$  SD is (A)  $0.20 \pm 0.12 \mu\text{m}^2/\text{s}$  ( $N = 802$ ), (B)  $0.12 \pm 0.078 \mu\text{m}^2/\text{s}$  ( $N = 766$ ), (C)  $0.11 \pm 0.078 \mu\text{m}^2/\text{s}$  ( $N = 786$ ), and (D)  $0.070 \pm 0.051 \mu\text{m}^2/\text{s}$  ( $N = 629$ ). Bin widths are 0.03 for A and B and 0.02 for C and D. The average diffusion coefficients for singly and doubly anchored vesicles were statistically different ( $p < 0.0001$ ) in both cases.

previously.<sup>4</sup> Distributions generally tailed toward larger diffusion coefficients, also consistent with previous measurements, and this is indicated by Gaussian fits centered at values below the arithmetic mean of the data set. The standard deviation of each distribution of diffusion coefficients was larger than the theoretical standard deviation,<sup>26,27</sup> which varies from 0.02 to  $0.05 \mu\text{m}^2/\text{s}$  for our experiments and depends on the average diffusion coefficient, the number of steps in the random walk measured (in our case typically 100), and the number of time intervals used (typically 10) to fit the mean-squared displacements. These larger standard deviations may have occurred because of variations in individual vesicle diffusion due to the presence of surface defects or other systematic or nonrandom errors introduced by the tracking algorithm or stage movement as discussed in detail in ref 4. However, the addition of more data points to each data set did not decrease the standard deviation significantly, indicating that the practical limit of the standard deviation was reached.

In the orientations shown in Figure 4A,C, a templating strand (red strands in Figure 4A,C) rehybridizes with the covalently linked strand (blue strands in Figure 4A,C) when the salt concentration is raised such that the vesicle is anchored to the supporting bilayer by one lipid (shown in blue in Figure 4). In the flipped orientation (Figure 4B,D), rehybridization<sup>17</sup> results in a doubly anchored tethered vesicle. As seen in Figure 6, for the

covalent tethering methods presented here, the average diffusion coefficient of the singly anchored vesicles was approximately 1.6 times larger than that of the doubly anchored vesicles, consistent with the expectation that multiple anchors will decrease the diffusion coefficient.<sup>28,29</sup>

To predict how the diffusion coefficient depends on the number of lipid anchors, we use a simplified model of a cylinder translating laterally in a viscous medium. The drag coefficient  $\gamma$  experienced by a cylinder of length  $L$  and radius  $r$  is given by<sup>30,31</sup>

$$\gamma = \frac{4\eta_0\pi L}{\ln\left(\frac{L}{2r}\right) + \alpha}$$

where  $\eta_0$  is the viscosity of the membrane region and  $\alpha$  is an end-effect correction that depends on  $(L/2r)$  and can be reliably estimated using the cylindrical shell model developed by Tirado and Garcia de la Torre.<sup>30,31</sup> Using this model, we can compare the drag coefficient calculated for a single lipid anchor with that calculated for a linked double anchor, treated in the simplest model as a larger cylinder with the same surface area in contact with the hydrophobic membrane core as two single anchors.<sup>32</sup> Using reasonable estimates for both  $L$  and  $r$  for a typical single lipid anchor (2.25 and 0.45 nm, respectively), we find that the larger cylinder representing the linked double anchor (length = 2.25 nm and radius = 0.84 nm) has a 1.5-fold higher drag coefficient than the single lipid anchor. Because the drag coefficient is inversely proportional to the diffusion coefficient by the Einstein relation, we find that this simple model is roughly consistent with the 1.6-fold decrease in the average diffusion coefficient that we observed between singly and doubly anchored tethered vesicles.

Referring to the original inference that equivalent tethered vesicle diffusion implies equivalent numbers of anchors, we now have stronger evidence to support the claim that 100 nm vesicles are tethered to a supporting bilayer by a single DNA hybrid, irrespective of the number of tethering strands available on the vesicle. It should be noted that this conclusion was arrived at by comparing vesicles tethered by a double-stranded DNA that is linked to either one or two lipid anchors and that this situation is different from that in which vesicles are tethered by either one or multiple independent strands of DNA (i.e., in which the lipid anchors on the independent tethers are not linked to each other via DNA hybridization). We expect that having multiple DNA tethers would decrease the diffusion coefficient even further, beyond what would be expected by increasing the drag coefficient by having more lipid anchors, because vesicles tethered by unlinked and independently mobile anchors would be expected to diffuse more slowly than if the anchors were linked. We also note that our observation that vesicles are tethered by a single DNA hybrid is in contrast to previous reports that have suggested that there are multiple tethers between vesicles tethered to each other or to a supporting bilayer using cholesterol–DNA conjugates.<sup>24,33,34</sup> The results from these reports, however, are

(26) Qian, H.; Sheetz, M. P.; Elson, E. L. *Biophys. J.* **1991**, *60*, 910–921.

(27) Saxton, M. J. *Biophys. J.* **1997**, *72*, 1744–1753.

(28) Although we expect that the diffusion coefficient of tethered vesicles is influenced by the number of lipids anchoring the vesicle to the supporting bilayer (i.e., the number of blue lipids in Figure 4), we do not expect that the motion of the tethered vesicle should be affected by whether the tethered vesicle membrane contains one or two lipid anchors (i.e., the number of red lipids shown in Figure 4).

(29) Although it is difficult to interpret absolute diffusion coefficients, it is notable that the architecture where the triazole is formed at the midpoint of the DNA template results in a smaller average diffusion coefficient than if the triazole is formed near the bilayer or vesicle, possibly because of a kinked duplex structure for the former case resulting in more frictional interaction between the vesicle and the supporting bilayer. For this reason, this covalent tethering strategy will be least useful for monitoring vesicle–vesicle interactions between mobile tethered vesicles.

(30) Tirado, M. M.; Garcia de la Torre, J. *J. Chem. Phys.* **1979**, *71*, 2581–2587.

(31) Garcia de la Torre, J. G.; Bloomfield, V. A. *Q. Rev. Biophys.* **1981**, *14*, 81–139.

(32) We note that the double-lipid anchor in Figure 6B is composed of lipids with different tails (palmitoyl-oleoyl and stearyl) whereas in Figure 6D the double-lipid anchor is composed of two lipids with identical tails (stearyl), but in the model, we estimate that these have approximately the same radius when compared to the single anchor (stearyl for both Figure 6A,C). Because the difference in the diffusion coefficient is the same for both tethering strategies, the slight differences in the size and shape of the anchor due to the tails do not appear to be significant.

(33) Beales, P. A.; Vanderlick, T. K. *J. Phys. Chem. A* **2007**, *111*, 12372–12380.

(34) Beales, P. A.; Vanderlick, T. K. *Biophys. J.* **2009**, *96*, 1554–1565.

difficult to compare directly with our system because the binding mechanisms, tracking methods, and anchors are different.

**Docking.** We briefly conclude with a demonstration that the covalent tethering strategy we describe will be useful for studying DNA-mediated vesicle docking and fusion (as schematically shown in Figure 3). A movie of a docking event between a red and green vesicle tethered to the supporting bilayer using this new strategy is provided in the Supporting Information. The docking event is mediated by the hybridization of complementary DNA, and each vesicle population was prepared to have an average of 50 DNA–lipids per vesicle of a fully overlapping 24mer sequence (strand B or B' in Figure 3). Evidence of DNA-mediated fusion between vesicles has also been observed using this strategy, as determined by lipid mixing (not shown), and a more detailed analysis of docking and fusion events using this new covalent tethering strategy will be described in future work.

### Conclusions

We have presented a successful method for covalently tethering lipid vesicles to a supported lipid bilayer using a DNA-templated click reaction. These covalently attached vesicles are mobile in the plane parallel to the lipid bilayer, and their diffusion coefficients were measured by single-particle tracking. This method of tethering is orthogonal to DNA-mediated docking or fusion reactions between vesicles and allows us to observe these reactions on the single-event level under conditions where many vesicles are

tethered to the surface and where the vesicles displaying sense and antisense docking strands are well mixed on the surface, resulting in many collisions between docking pairs that will provide ample sampling of the efficiency of docking even when the probability of docking per collision is low. We have presented a preliminary observation of DNA-mediated vesicle–vesicle docking between vesicles displaying complementary DNA using this approach. Because our method allows us to control the number of lipid anchors attaching the tethered vesicles to the bilayer, we have also shown that average diffusion coefficients of vesicles with a single lipid anchor are approximately 1.6 times higher than those of doubly anchored vesicles.

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**Supporting Information Available:** Fluorescence microscopy video of the DNA-mediated docking of tethered vesicles as depicted in the final step in Figure 3. Further description of the Experimental Section detailing nonspecific vesicle adhesion and DNA sequences. This material is available free of charge via the Internet at <http://pubs.acs.org>.