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Functionalization of Fatty Acid Vesicles through Newly Synthesized Bolaamphiphile–DNA Conjugates

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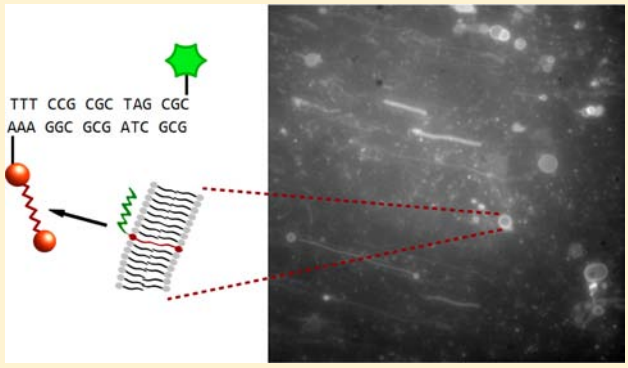
Supporting Information

ABSTRACT: The surface functionalization of fatty acid vesicles will allow their use as nanoreactors for complex chemistry. In this report, the tethering of several DNA conjugates to decanoic acid vesicles for molecular recognition and synthetic purposes was explored. Due to the highly dynamic nature of these structures, only one novel bola-amphiphile DNA conjugate could interact efficiently with or spontaneously pierce into the vesicle bilayers without jeopardizing their self-assembly or stability. This molecule was synthesized via a Cu(I)-catalyzed [3 + 2] azide–alkyne cycloaddition (click reaction), and consists of a single hydrocarbon chain of 20 carbons having on one end a triazole group linked to the 5′-phosphate of the nucleic acid and on the other side a hydroxyl-group. Its insertion was so effective that a fluorescent label on the DNA complementary to the conjugate could be used to visualize fatty acid structures.



INTRODUCTION

The functionalization of self-assembled soft matter structures with molecules that are able to undergo specific recognition is being intensively pursued to develop applications in medicine, material science, and synthetic biology.[1−9] Tight interactions between supramolecular assemblies and functional molecular agents can be realized either by means of physical association (noncovalent interactions) or by chemical derivatization of the molecular agents with an amphiphilic moiety, such as phospholipids,[10] block copolymers,[11] and cholesterol.[12] The latter approach is usually preferred[12−15] as it leads to specific and durable interactions. However, most of the examples in biosensing or medicine are dealing with phospholipid liposomes, e.g., composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).

Our interest is to achieve functionalization of fatty acid nanostructures with polyelectrolytes (nucleic acids, peptides or sugars). Fatty acids are simple single-chain amphiphiles with an acid headgroup and are capable of forming vesicular structures.[16,17] These vesicles can be used in synthetic chemistry (as nanoreactors)[18,19] or in medical applications for targeted drug delivery.[20] Fatty acid vesicles have also been studied as models for primitive membranes,[18,21−25] due to their simple chemical structure and their availability in meteoritic organic compounds.[26] In every application, functionalization of the vesicle surface can improve their functionality, for example, by allowing the assembly of a complex catalyst or by tethering two nanoreactors together.[14,27]

Fatty acid vesicles are stabilized by hydrogen bonds between protonated and ionized carboxylic acid headgroups, and are stable around a pH close to the pK_a of the acid, e.g., from pH 7.0 to 7.2 for decanoic acid (DA).[28] They are also characterized by higher critical vesicle concentrations (CVC) and higher exchange rates of molecules between assemblies and solution compared to similar phospholipid vesicles. Both parameters increase with decreasing hydrocarbon chain length.[22] These properties lead to a higher sensitivity of fatty acid vesicles to fluctuations in pH, ionic strength, and temperature, as well as the presence of solutes in the suspension, all of which can induce transient vesicle permeabilization or in extreme cases complete structure disruption. The more dynamic bilayer membranes, however, allow for morphological studies of the vesicle alterations resulting from uptake of additional amphiphile molecules[23] or the in situ production of new amphiphiles, as during photocatalysis.[19]

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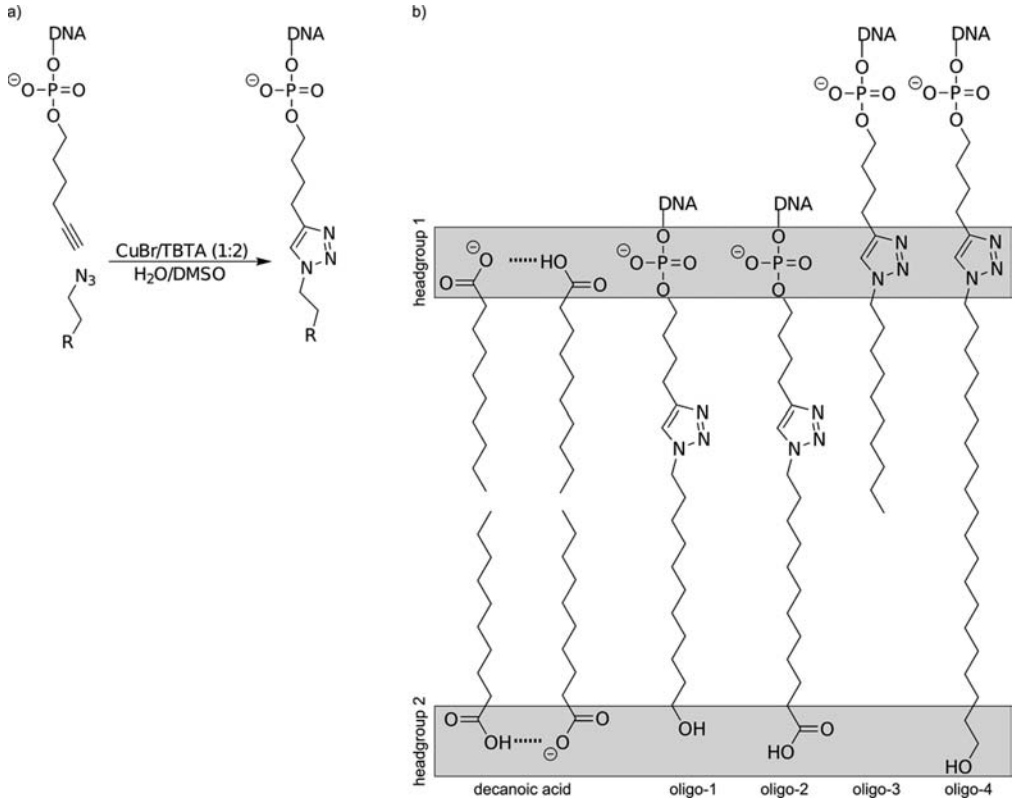

Figure 1. (a) Synthetic scheme of amphiphile fabrication employing azide units and a 5′-hexynyl substituted DNA 15-mer that are connected by click chemistry. (b) Overview of bolaamphiphile structures and related derivatives studied in this project. DA structure added for comparison. Gray rectangles denote regions of expected hydrophilic headgroup placement. Note that the transmembrane arrangement of oligo-1, 2, and 4 is postulated; oligo-4 is still under investigation as it might adopt a folded conformation and reside in one monolayer only.

In earlier work, we have designed a self-replicating chemical system, a protocell, which is based on the functionalization of DA fatty acid membranes to carry out photochemical reactions.[29] For efficient catalysis to occur, anchoring of various components in the membrane was essential. Metal-catalyzed, photochemical conversion of DA precursors was demonstrated on the surface of the vesicles using simple aliphatic hydrocarbon chains (10 carbons long). Such a simple hydrophobic moiety was sufficient for almost quantitative anchoring the various functional components of the photocatalytic unit, i.e., the metal complex photosensitizer, ruthenium(II) *tris*-(bipyridine), and its relay donor, an 8-oxoguanine nucleobase.[30] According to the protocell concept,[29] its reaction network should be controlled by replicable information molecules. That is, molecules containing an information sequence that can be chemically copied, e.g., nucleic acid oligomers instead of a single nucleobase (8-oxoguanine). To further develop our model, our catalytic system therefore requires the tethering of nucleic acids onto the membrane surface to allow for sequence-specific functionalization of the electron relay. The stable tethering of DNA oligomers, which are replicable and can support the photochemistry in our system, would permit the realization of a complete protocell.[29] Furthermore, it should find applications in molecular targeting of nanocompartments by bioaffinity as the DNA sequence would direct a specific interaction with complementary strands, or even in the direct delivery of chemicals encapsulated in the aqueous lumen of vesicles[8] or that of the tethered oligomers themselves.

The tethering of nucleic acids onto the negatively charged fatty acid vesicles posed a more complex challenge than the previously derivatized molecules. Indeed, short nucleic acids are relatively well solubilized in an aqueous medium and their amphiphilic derivatives can self-assemble on their own, e.g., into layers at the interface between water and air[31] or into micelles.[32] Furthermore, repulsive electrostatic interactions between the negatively charged vesicle surfaces and the amphiphilic nucleic acids could negatively impact their insertion in the membranes.

To achieve the insertion of DNA in DA bilayers, two possible types of anchoring moieties were considered (i) prototypical DNA amphiphiles with multiple linear anchoring moieties (i.e., DNA with 2 single hydrocarbon chains incorporated on two nucleobases[31,32]) and (ii) novel nucleic acid conjugates consisting of an ssDNA and a bolaamphiphile anchoring moiety. The nature of the bolaamphiphile tether could lead to a better retention of the anchored DNA, because such an amphiphilic molecule has a hydrophilic group at both ends of its hydrophobic core.[33] Thus, it could potentially interact with the fatty acid headgroups on both sides of the bilayer (transmembrane anchoring), provided the length of the hydrophobic core was comparable to the thickness of the bilayer: In the case of decanoic acid, estimates of the bilayer thickness in the literature range from 22−26 Å[34] to 28 Å.[18,35]

To assess more rapidly the importance of various parameters in the insertion process, a synthetic route via click chemistry (Figure 1a) was chosen. This synthetic approach enabled us to prepare different amphiphile and bolaamphiphile DNA conjugates in a modular fashion by only employing one DNA strand and various other organic moieties that can serve as an anchor (Figure 1b). As the metal catalyst in this Cu(I)-

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catalyzed [3 + 2] azide−alkyne cycloaddition (CuAAC)[36−38] can be harmful to DNA,[39] the copper(I) stabilizing ligand *tris*-(benzyltriazolylmethyl)amine (TBTA)[40] was utilized to protect the DNA from degradation during synthesis.

The incorporation of the chosen and synthesized DNAs derivatized with hydrophobic moieties into DA vesicles was assessed using two different methods for the insertion: post-vesiculation insertion (classical method), where the conjugates have to spontaneously insert into the bilayer themselves (i.e., pierce the bilayers), and insertion during vesiculation aimed at promoting their association with the bilayers during self-assembly. The insertion efficiency was gauged by using a fluorescently labeled DNA with complementary sequence to that of the bolaamphiphile DNA conjugates and determining whether its hybridization could enable the direct visualization of the DA membranes.

RESULTS AND DISCUSSION

Incorporation of Prototypical Lipid-DNA into Fatty Acid and POPC Vesicles. The aim of this study was to tether single- and double-stranded DNA to decanoic acid vesicle membranes and gain insight into the parameters that influence the stability of the newly formed complexes. To this end, we first tested the incorporation of lipid-DNA, a prototypical DNA amphiphile. In this system, hydrophobic units, dodec-1-yne chains, were introduced at the 5-position of uracil. The resulting modified nucleoside (Figure 2) could be incorporated into oligonucleotides by standard solid phase synthesis procedures and the hydrophobic residue did not impair the ability of the DNA amphiphiles to undergo sequence specific base pairing.[32] As other DNA amphiphiles, lipid-DNAs self-assemble into micelles in buffer medium[32] or form layered structures at the air/water interface.[31] Two lipid DNA molecules were investigated regarding their incorporation into fatty acid vesicles. The first one was a 20mer DNA sequence with two hydrophobically modified deoxyuridines (dU) placed in the center of the DNA strand (**dU20**), which forms a hairpin. The second molecule was a 11mer with two hydrophobic Us at its 5′-terminus (**dU11**).

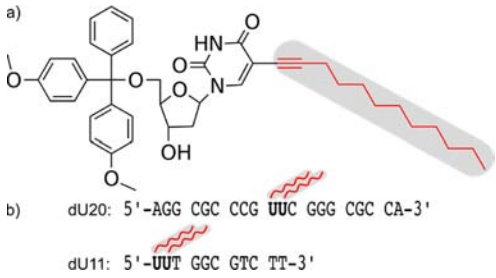


Figure 2. Prototypical DNA intended to associate with the exterior of lipid containers through hydrophobic anchors (hydrocarbon chains, in red over a gray background). (a) Structure of the precursor of modified dU amidite. (b) DNA sequences of the two prototypical DNA-lipids: the derivatized U residues are in bold with their hydrocarbon chains schematically represented.

Insertion experiments were first carried out with DA vesicles (30 mM) and the DNA amphiphiles, but no significant insertion yields could be detected. Moreover, a precipitate was observed upon DNA addition, indicating that some DA vesicles were disrupted by the conjugates (data not shown). By contrast, for the oleic acid vesicles the short **dU11** could be tethered and a higher DNA fraction was associated with the vesicles (Table 1) compared to nonamphiphilic salmon sperm DNA (unspecific association). A relatively effective, specific association of **dU11** and **dU20** with POPC vesicles was also detected.

Table 1. Insertion Rate (%) of Prototypical Lipid DNA Conjugates dU11 and dU20 and Unspecific Interaction of Salmon Sperm DNA[a]

	lipid-DNA amphiphiles (%)		
vesicle type	**dU11**	**dU20**	DNA (salmon sperm) (%)
Oleic acid	19.5	5.5	10.5
POPC	50	37	11.0

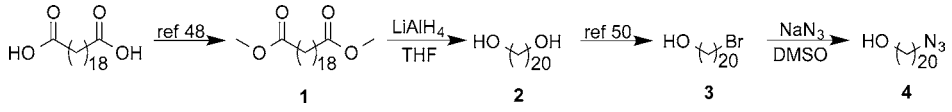
[a]In all cases, 0.5 mg/mL DNA was used. Considering the amphiphile concentrations, molar ratios of amphiphile to Lipid-DNA amphiphiles were as follows: For **dU11**, 130:1 and 36:1 with oleic acid (17.9 mM) and POPC (5 mM), respectively. For **dU20**, 230:1 and 130:1. However, POPC and oleic acid concentrations (1:3) ensured comparable vesicle surfaces (considering headgroup areas and CVCs). Standard deviations: max 5% units.

Thus, the hydrocarbon chain anchors of **dU11** and **dU20** did not facilitate the insertion of the negatively charged DNA sequence into the targeted DA fatty acid membranes, and even led to the disruption of some vesicles. The insertion of the amphiphilic DNA within the fatty acid structures depends on a balance between the energy cost of solubilization in the buffer, in particular, for the hydrophobic moiety of the conjugates (dodec-1-yne chains), and the entropy gained by the systems when these hydrophobic chains reside in the hydrophobic core of the DA bilayers (the hydrophobic effect). The repulsive interactions between the negatively charged vesicle surface and the oligomer polyelectrolyte backbone could, however, have canceled out the entropic gain. The absence of functional groups in the hydrophobic moiety might have also contributed to the result, as it would preclude additional stabilizing interactions between the fatty acid headgroups and the anchoring moiety. The vesicle disruption, and to some extent the precipitation, could then be consistent with the formation of mixed "micelles", which have already been reported for these two DNA conjugates.[32]

These orienting experiments clearly underlined the need for a novel, better-suited anchoring moiety in the case of fatty acid vesicles. We therefore designed and synthesized four different DNA conjugates (oligo-1, -2, -3, and -4; Figure 1b), using click chemistry, which was recently reviewed in the context of DNA conjugation strategies.[41] The bolaamphiphile chemical structures of oligo-1, oligo-2, and oligo-4 were chosen to match the reported thickness of the DA bilayer (between 22 and 28 Å),[34,35] which equals 18 carbons between the headgroups. They, however, differed in the nature of their hydrophobic core, as well as in the chemical functionality of their potential headgroups (Figure 1b), which could all potentially form hydrogen bonds with the acid headgroups on both sides of the bilayer.

Assuming that the resulting bolaamphiphile anchor spans the whole DA bilayer, the polar triazole ring, which results from the conjugation step, could either be buried in the hydrophobic core of the DA bilayer (oligo-1 and oligo-2) or act as a headgroup (oligo-3 and oligo-4) (Figure 1). The structure of oligo-3 resembles that of both oligo-1 and oligo-2 in terms of

Scheme 1. Synthesis Scheme for the Preparation of Azide 4



the hydrocarbon chain length, but lacks a second hydrophilic group. This design aimed at investigating the influence of the "bola"-nature, i.e., the necessity of a second hydrophilic group, on insertion and retention of the conjugates in the bilayer. Additionally, it could also shed light on the influence of interactions between anchors and headgroups when compared to the lipid–DNA conjugates. In fact, it could use either the 5′-phosphate or the triazole ring as headgroup, although the former arrangement would force the triazole ring inside the hydrophobic core of the membrane.

Synthesis of the Bolaamphiphile–DNA Conjugates. Hexyn-5-yn-1-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphor-amidite was synthesized in moderate yields according to El-Sagheer et al.[42] and was attached at the 5′-end of a 15-mer DNA oligonucleotide (Figure 1a) by use of an automatic DNA synthesizer using standard conditions. The coupling yield corresponded to that of common DNA amidites.

12-Azidododecanoic acid[43] was synthesized from 12-bromododecanoic acid in 93% yield using a procedure reported by Diaz et al.,[44] while 11-azido-1-undecanol[45,46] and 1-azidodecane[47] were synthesized from 11-bromo-1-undecanol and 1-bromodecane, respectively, according to literature procedures. Yields were 78% and 98%, respectively.

20-Azidoicosane-1-ol (**4**) was synthesized in four steps from eicosanedioic acid (Scheme 1). First, eicosanedioic acid was converted to its dimethyl diester derivative **1** according to literature procedures.[48] **1** was then reduced to 1,20-icosanediol (**2**)[48,49] using $LiAlH_4$ in THF in moderate yields (59%). The diol **2** was converted to 20-bromoicosan-1-ol (**3**) using aqueous HBr (48% in H_2O) in petroleum ether (bp. 100–140 °C) according to a literature procedure (yield = 36%).[50] Finally, 20-azidoicosane-1-ol (**4**) was synthesized by azidification of **3** with NaN_3 in DMF in 72% yield.

CuAAC click chemistry was carried out as described in the "click chemistry manual" by BaseClick[51] using CuBr/TBTA in the ratio 1:2. This is an optimized method[52−54] which also has been proven successful for other oligonucleotides. The synthesis yields for each conjugate are reported according to Optical Density equivalents (OD) in Table 2. Analysis by RP-HPLC and MALDI-TOF mass spectrometry confirmed the successful 1,3-dipolar Huisgen cycloaddition between alkyne and azide (see Supporting Information, Table SI-1 and spectra).

Table 2. Synthesis Yields for Oligos -1 to -4[a]

	starting DNA amount (OD)	recovered amount of DNA conjugate (OD)	%
oligo-1	15	9.7	65%
oligo-2	15	11.7	78%
oligo-3	15	12.4	83%
oligo-4	15	12.2	81%

[a]The recovered amounts for each DNA conjugate were determined after the desalting procedure.

Incorporation of Bolaamphiphile–DNA Conjugates into Decanoic Acid Vesicles. Three types of experiments were conducted to assess the validity of the bolaamphiphile anchoring approach, two aiming at the investigation of the preparation methods and their influence on the incorporation. The third type aimed at gauging the extent and strength of the insertion into vesicle bilayers over time, as well as other parameters such as the feasibility of hybridization of a single stranded DNA to its complementary sequence once anchored in the vesicles.

To detect the insertion and the retention of the derivatized DNAs, two single stranded DNA sequences were labeled with an Alexa-488 fluorescent dye at their 5′-end: master-1 oligomer was a 15-mer ssDNA with complementary sequence to the bolaamphiphile-DNA conjugate sequence, while the master-2 oligomer consisted of a noncomplementary sequence.

In each type of experiment, the presence of DA vesicles (total concentration 27 mM DA, i.e., 5 mM amphiphiles forming structures if the CVC of 22 mM is considered[18]) was first confirmed in all investigated samples using Nile red as staining reagent. The insertion of the DNA conjugates was then evaluated using the fluorescence of the Alexa-488 on the master oligomers. A representative set of micrographs is shown in Figure 3.

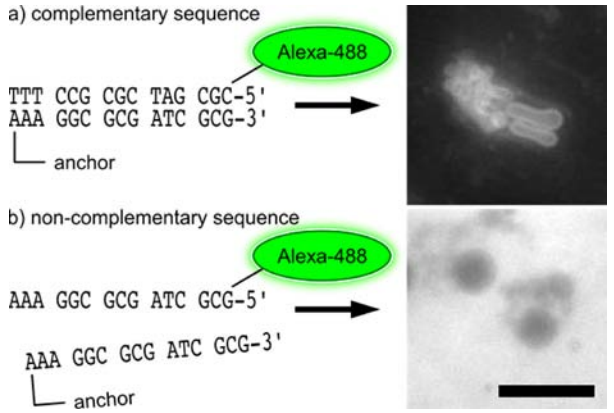


Figure 3. Chemical systems for the detection of the anchoring of DNA to DA vesicles: (a) master-1 oligomer is complementary to the anchored DNA chain (vesicular structures are observed after hybridization); (b) master-2 is not complementary and does not hybridize with the anchored oligomer (dark "shadow" vesicles are observed due to the contrast with fluorescent master-2 present in solution). Scale bar 10 μm).

The localization of the fluorescence in the micrographs (Figure 3) permitted a qualitative assessment of the DNA conjugate insertion in the DA vesicles. In the case of oligo-4/master-1 mixtures, the fluorescence was mostly associated with DA bilayers compared to a darker background. The negative control with master-2 was successful, as the fluorescence remained localized in the buffer, without any significant accumulation on the surface of the structures. In fact, the structures could only be observed because the master-2 was added in the external medium and did not diffuse into the structure's aqueous lumina. When comparing the micrographs

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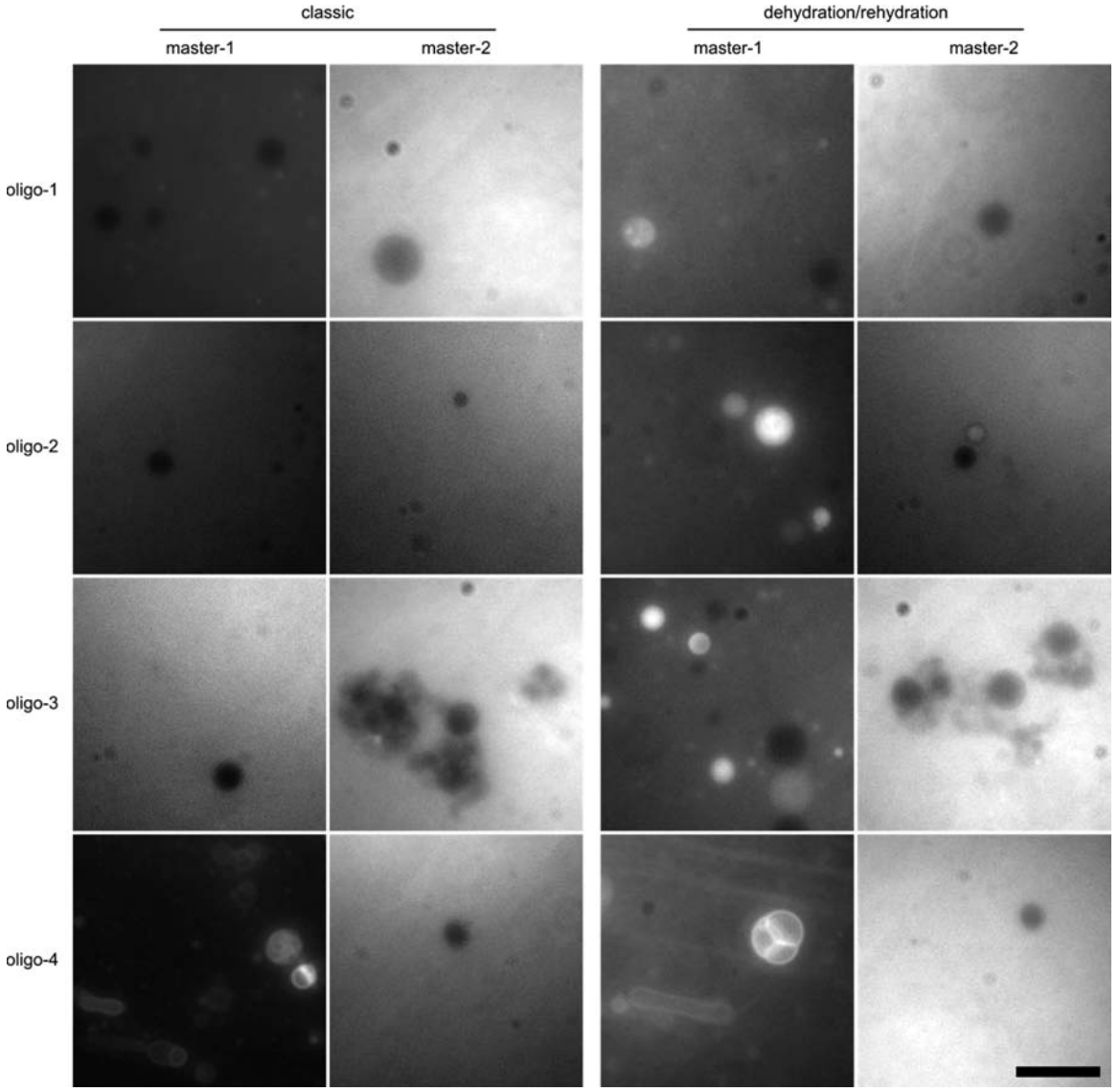

Figure 4. Representative images obtained with the incubation of DA vesicles with different bolaamphiphile DNA anchors (oligo-1, 2, 3, and 4) and different reporter oligomers (master-1 and 2). Two different methods of preparation, "classical" and "dehydration−rehydration", are presented. Scale bar 10 μm.

of DA vesicle sample incubated with only one of the master sequences, in the absence of DNA conjugates, the same overall characteristics were observed (data not shown). That is, unspecific interactions between the bilayers and DNA, which were previously observed with the salmon sperm sample, could be excluded here.

Thus, the label location ensured the possibility of detecting only a positive bolaamphiphile insertion that does not compromise the structure stability. Furthermore, these results indicate that this method can be applied to gauge insertion yields, as a good insertion would lead to a fluorescent highlighting of the self-assembled structures. To better understand the insertion dynamics of the DNA conjugates, two preparation methods were investigated: classic and dehydration−rehydration.

Post-Vesiculation Insertion−Classic Method. The incorporation of bolaamphiphile−DNA conjugates into the DA vesicle membrane was first studied post-vesiculation. DA vesicles prepared by pH vesiculation were incubated with a conjugate solution that consisted of the prehybridized DNA conjugate and reporter oligomer. The microscopic investigation of the samples revealed that oligo-1, oligo-2, and oligo-3 did not show any incorporation into DA vesicles, as only dark circular "shadows", the aqueous lumen of the vesicles, could be observed in a fluorescent background (Figure 4). By contrast, membranous structures were highlighted by fluorescent labeling in samples containing oligo-4 as DNA membrane anchor. As expected, the incubation of oligo-1, oligo-2, oligo-3, and oligo-4 with master-2 (noncomplementary sequence) did not result in the fluorescent labeling of the membranous structures.

The different behavior of the various amphiphile anchors could have been caused by different insertion rates in the bilayer, due to both the nature of the hydrophobic cores or that of the headgroups (acid versus hydroxyl group). Studies of the flip−flop rates of fatty acid molecules in bilayers have established that only neutral species (protonated[55,56] or associated with cations[57]) are able to cross the hydrophobic cores of bilayers. Thus, by analogy, protonation of the oligo-2 acid group should in principle improve its spontaneous insertion into the bilayers. As an equilibrium between the

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deprotonated and protonated species existed at the experimental pH, a prolonged incubation period could have improved the insertion. However, measuring after 24 and 96 h incubation periods, the only noticeable change was the progressive disappearance of the shadow vesicles, as DA vesicles fragmented and reformed and the labeled oligo-2 diffused into the vesicular aqueous lumina.[22] Only the oligo-4 system still showed clearly outlined membranes on a dark background (Figure 5).

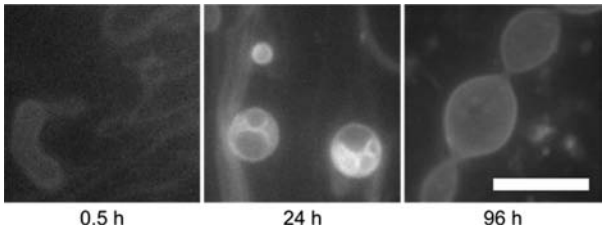

Figure 5. DA vesicles containing oligo-4 and master-1 visualized over different periods of time. Scale bar 10 μm.

Upon addition of Nile red to the suspension containing oligo-1, vesicles and tubes were revealed (Figure 6) that were invisible using the master-1 oligomer as sole fluorescent marker.

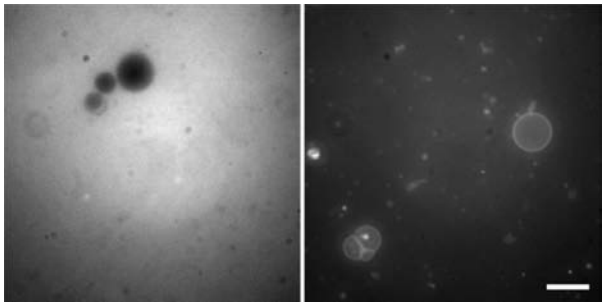

Figure 6. DA vesicles containing oligo-1 and master-1. Left: in green light sensitive channel showing "shadow" vesicles. Right: in red light sensitive channel (with addition of Nile red). Scale bar 25 μm.

Insertion during Vesiculation—Dehydration–Rehydration Method. The "dehydration/rehydration" preparation method[58] was employed to force the association of the bolaamphiphile–DNA conjugate with DA bilayers. Because of the bilayer fragmentation that occurs during the procedure, the association of the conjugates with charged headgroups should have been facilitated and their retention improved during the rehydration procedure, thereby leading to higher insertion yields.

The results of this preparation procedure confirmed the extensive insertion of oligo-4 into the DA vesicles (Figure 4). The other DNA conjugates (oligo-1, oligo-2, oligo-3) employed during the procedure yielded interesting results. Contrary to the post-vesiculation method, bright structures, whose sizes and shapes could have corresponded to vesicles (Figure 4), were observed in all cases. However, an unambiguous observation of the vesicle bilayer structure (bright circumferences of the vesicle with a darker internal lumen) cannot be reported. That is, the presence of these DNA conjugates likely led to the formation of new structures, such as oil droplets, micelles, or even crystals.

The results obtained with both preparation methods can be summarized as follows: oligo-4 could spontaneously insert in the bilayers of preformed vesicles and be stably incorporated during vesiculation. By contrast, the other investigated anchoring systems were not incorporated to such an extent that bilayers could be visualized by the fluorescence on the tethered DNA system.

From the oligo-4 chemical structure, its extensive incorporation can be explained by the presence of a long hydrocarbon chain that fits well in the hydrocarbon core of the DA bilayer as expected from their similar number of carbons. As a result, the triazole ring and hydroxyl functions were correctly positioned to interact with the acid headgroups of the DA bilayer, probably leading to additional stabilization (Figure 1b). The hydroxyl headgroup was expected to be advantageous for the spontaneous insertion, as it is not charged at any pH (unlike the acid). In addition, it should have a constructive interaction with the fatty acid headgroups, as the stabilization of fatty acid vesicles by fatty alcohols has been previously reported.[59−61]

The different behavior of the other anchors can be explained with similar arguments. The shorter hydrocarbon chain of the azido-moiety, used as starting material in the click synthesis of oligo-1 and -2, likely resulted in positioning of the triazole group in the hydrophobic core of the bilayers. The charged 5′-phosphate of the DNA and the hydroxyl or acid headgroup should then be able to interact with the DA headgroups. In additional experiments where the bolaamphiphile–DNA conjugates and master-1 were added at two different stages of the preparation, i.e., the conjugate during dehydration/rehydration procedure and the master sequence after the vesicle formation, the results with oligo-1 and oligo-2 indicated that a slightly better stabilization could be achieved with an acid headgroup (data not shown). Assuming that the anchors experienced a similar increase of their pK_a within the structures as fatty acids do, a large fraction of their acid functions should be deprotonated at the pH used in all experiments (pH 7.2). The charged headgroup would then have slowed down the dissociation from the bilayers. However, it would also have decreased the rate of insertion during spontaneous insertion experiments.

As any stable insertion into the bilayers relies on the correct balance between the stabilization of the hydrophobic moiety of the amphiphile and the cost of its solvation in the aqueous medium, it seems that the triazole function within the bilayer weakened the hydrophobic interactions and led to the dissociation of the bolaamphiphile–DNA conjugate from the bilayers. Thus, the disruption of the packing of bilayer membranes could not or only partially be counterbalanced by the stabilization due to headgroup interactions. The hydrophobic incompatibility (mismatch) of triazole inside the hydrophobic core of the membrane was already reported for phospholipids by Smith's group.[62] Furthermore, recent investigations[18,63,64] of the insertion of polycylic aromatic hydrocarbons in fatty acid vesicles have established that only minute amounts of these molecules can be inserted into these bilayers, before the molecules in excess are expelled into the medium. Finally, the correct positioning of these anchors (i.e., the triazole embedded within the bilayer hydrophobic core and the 5′-DNA phosphate interacting with the acid group of DA) would have brought the negatively charged phosphates of DNA backbones into closer proximity to the negatively charged vesicle surfaces, thereby further weakening the anchor insertion.

Oligo-3 lacked a second headgroup and could not insert into the membrane even though it could have in principle interacted through either its phosphate or its triazole group with bilayer hydrophilic headgroups (Figure 1b). That is, the improvement of the electrostatic interactions with the hydrophilic interface of the bilayers (compared to the lipid-DNAs **dU11** and **dU20**) was not sufficient to shift the association/dissociation equilibrium of the conjugate. This result underlined the importance of anchor hydrophobic length in relation to the bilayer thickness (i.e., the importance of the hydrophobic effect and van der Waals interactions): The hydrophobic chain of C_{10} was too short to stabilize the insertion of such a 15mer ds DNA polyelectrolyte system in the highly dynamic DA vesicle membrane.

Our data however do not exclude the possibility of the formation of micelle-like structures or even large oil-like systems around the amphiphile anchors as the results of the dehydration−rehydration methods with oligo-1 to 3. This formation is even more plausible if the results of experiments to separate nonassociated bolaamphiphile−DNA conjugates from vesicles are taken into consideration (data not show). Indeed, the free DNA conjugates could not even pass through 30 kDa pores.

Hybridization of Oligonucleotides to DNA Amphiphiles Inserted into DA Vesicles. Oligo-4 showed good insertion into DA vesicles. The microscopic images of this system clearly depicted membranous structures of various shapes and sizes (Figures 4 and 5, Supporting Information video 1) showing that hybridization of oligo-4 easily integrated into fatty acid bilayers.

In the next step, experiments elucidating the ability of the fluorescently labeled DNA to hybridize to the anchored complementary DNA were performed. When master-1 solution was added to preformed DA vesicles containing oligo-4 molecules, we initially observed dark "shadow" vesicles. However, with time, master-1 molecules migrated to vesicle surfaces and hybridized to the complementary oligo-4 sequence, giving rise to good microscopic images of vesicular structures. This process is illustrated in Supporting Information video 2. The prolonged incubation also highlighted the retention of anchors within the vesicle bilayers (Figure 5). Such a system could be used to observe the hybridization process in real time.

In this study, the incorporation of two types of amphiphilic DNA conjugates into single hydrocarbon chain amphiphile bilayer structures was investigated. The anchoring of 15mer ds DNA into decanoic acid vesicles was achieved even though these structures are extremely dynamic systems. From the 6 candidates (2 prototypical lipid-DNA and 4 DNA conjugates whose synthesis by click chemistry is reported here), only one, oligo-4, showed strong association with the bilayers. The anchoring was strong enough to visualize the vesicle structures using the fluorescent label attached to the complementary DNA strand. The presence of two headgroups on oligo-4, a triazole and a hydroxyl group, flanking a hydrophobic core whose length was adjusted to match the thickness of the fatty acid bilayer, was clearly crucial for its stable incorporation into the bilayer. As such our modular synthetic strategy has been shown to be easily adaptable to other bilayer types, because the hydrophobic part of the amphiphile can be easily adjusted to its counterpart in the membrane.

The demonstrated spontaneous insertion of polyelectrolytes into fatty acid vesicles (i.e., the modification of the vesicular containers after their formation) and, in the case of nucleic acids, the preservation of the hybridization capabilities will be important. Functionalized vesicle surfaces will allow for molecular recognition. Thus, such nanoreactors should find application in synthesis of chemicals and/or their delivery, as well as in protocell models.

EXPERIMENTAL PROCEDURES

Syntheses. *General Materials and Methods in Synthesis.* All reactions were monitored by analytical thin layer chromatography (TLC) using precoated TLC plates DC-Alufolien 60 F_{254} from Merck. UV-light or a stain of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O/Ce_2(SO_4)_3$ (50:1) in 5% sulfuric acid was used for visualization. Column chromatography was performed using Merck Kieselgel 60 (0.040−0.060 mm). Anhydrous THF was distilled over sodium benzophenone immediately prior to use. CH_2Cl_2 was distilled prior to use. Anhydrous pyridine and DMF were dried over molecular sieves (4 Å), while anhydrous MeCN was dried over 3 Å sieves. All other reagents and solvents came from commercial sources and were used as received. Melting points (mp) were determined on a Büchi 535 melting point apparatus and are uncorrected. High resolution electrospray ionization (ESI) mass spectra were recorded on an Applied Biosystems/MDS Sciex Q-star pulsar spectrometer. NMR spectra were recorded on a Bruker Avance III instrument using the deuterated solvent as lock. Chemical shifts are reported in ppm relative to tetramethylsilane as internal standard for ^{1}H NMR or the deuterated solvent $CDCl_3$ (δ 77.00) as internal standard for ^{13}C NMR.

Synthesis of the Novel Bolaamphiphile−DNA Conjugates. The synthetic pathway of 20-azidoicosane-1-ol (**4**) is presented in Scheme 1.

Dimethyl icosanedioate (**1**) and 20-bromoicosan-1-ol (**3**) were synthesized according to literature procedures.[48,50]

*Icosane-1,20-diol (**2**).* $LiAlH_4$ (2.05 g, 54.0 mmol, 2 equiv) was suspended in anhydrous THF (100 mL) under N_2 on an ice bath. Dimethyl icosanedioate (**1**, 10.0 g, 27.0 mmol) was dissolved in anhydrous THF (60 mL) and added dropwise. The icebath was removed and the reaction mixture was stirred at rt overnight. The reaction was quenched by carefully adding H_2O (2.0 mL), 15% NaOH solution (2.0 mL), and H_2O (6.0 mL) dropwise, while cooling on an ice bath. The resulting slurry was filtered through a pad of Celite and washed with $CHCl_3$. The organic phase was washed with H_2O and the solvent was removed under reduced pressure to give **2** as a white solid (5.03 g, 16 mmol, 59%). Mp 94−99 °C (lit. 95 °C).[49] NMR spectra in accordance with literature data.[48,49] MS (ESI): m/z = 337.3078 (MH^+).

*20-Azidoicosane-1-ol (**4**).* To a solution of 20-bromoicosan-1-ol (**3**, 0.250 g, 0.66 mmol) in anhydrous DMF (5 mL) was added NaN_3 (0.085 g, 1.30 mmol, 2 equiv). The reaction mixture was stirred at 80 °C for 40 h under N_2. After cooling to rt, water (10 mL) was added and the mixture was extracted with Et_2O (3 × 15 mL). The combined organic phases were evaporated and the residue was taken up in CH_2Cl_2/H_2O (3:2, 25 mL). The phases were separated, and the aqueous phase was extracted with CH_2Cl_2 (2 × 15 mL). The combined organic phases were dried (Na_2SO_4), evaporated under reduced pressure, and dried *in vacuo*. White solid (0.162 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.26−1.36 (m, 32H, 16 × CH_2), 1.64−1.52 (m, 4H, 2 × CH_2), 3.25 (t, 2H, J = 7.0 Hz, CH_2N_3), 3.63 (t, J = 6.6 Hz, 2H, CH_2OH). ^{13}C NMR (101 MHz, $CDCl_3$): δ 25.72, 26.69, 28.81, 29.13, 29.42, 29.46, 29.52, 29.58,

29.60, 29.64, 29.66, 32.79 (12 × CH_2), 51.47 (CH_2N_3), 63.05 (CH_2OH). HRMS (ESI): m/z calcd for $C_{20}H_{41}N_3ONa^+$ (MNa^+): 362.3142, found 362.3140.

Oligonucleotide Synthesis. All modified oligonucleotides were synthesized using standard automated solid-phase phosphoramidite coupling methods on an ÄKTA oligopilot plus (GE Healthcare) DNA synthesizer in 50 μmol scale. All solvents and reagents for oligonucleotide synthesis were purchased from Novabiochem (Merck, UK) and SAFC (Sigma-Aldrich, Netherlands). Solid supports (Primer SupportTM, 200 μmol/g) from GE Healthcare were used for the synthesis of DNA. Oligonucleotides were purified by anion exchange high pressure liquid chromatography (HPLC) using a HiTrap Q HP 5 mL column (GE Healthcare) through custom gradients using elution buffers A: 25 mM Tris-HCl pH 8.0 and B: 25 mM Tris-HCl pH 8.0 and 1.0 M NaCl. Fractions were desalted using centrifugal dialysis membranes (MWCO 3000, Sartorius Stedim). Afterward the oligonucleotides were characterized by MALDI-TOF mass spectrometry (MS) using a 3-hydroxypicolinic acid matrix. Spectra were recorded on an ABI Voyager DE-PRO MALDI TOF (delayed extraction reflector) Biospectrometry Workstation mass spectrometer. The concentrations of the DNA were measured on a SpectraMax M2 spectrophotometer (Molecular Devices, USA) using 1 cm light-path quartz cuvette.

Post-Oligo Synthesis. Click chemistry on oligonucleotides was carried out according to the "Click chemistry manual" by BaseClick GmbH, Germany.[51]

The "click solution" (0.1 M CuBr/0.1 M TBTA (1:2) in DMSO/*t*-BuOH (3:1)) was prepared by mixing the following two freshly prepared solutions: CuBr (1 mg) in 70 μL DMSO/*t*-BuOH (3:1) and 140 μL of the TBTA-solution (54 mg in 1 mL DMSO/*t*-BuOH (3:1)).

To the freshly prepared "click solution" (30 μL) a 2 mM DNA solution (0.1 μmol in 50 μL H_2O) was added followed by the azide (12-azidododecanoic acid, 11-azido-1-undecanol, 1-azidodecane or **4**; 1.0 μmol in 50 μL DMSO). The mixture was thoroughly mixed and shaken at rt for 4 h using an Eppendorf Mixmate. The reaction was subsequently quenched with 3 M NaOAc (100 μL) and the DNA was precipitated from cold EtOH (1 mL) in a freezer overnight. The vial was centrifuged, the supernatant was removed, and the pellet was washed with cold EtOH (2 × 1 mL). The pellet was dried in a centrifugal evaporator, Speedvac, (RVC-18 CD *plus*, Martin Christ Gefriertrocknungsanlagen GmbH, Germany with a chemically resistant membrane pump *vacuubrand* MZ 2C NT, from Vacuubrand GmbH, Germany), redissolved in pure water (1 mL), and desalted on a NAP-10 resin column from GE Healthcare. After drying in a speedvac, the oligo was redissolved in double deionized water. The identities of click-modified oligonucleotides were confirmed by MALDI-TOF MS on a Bruker Daltonics Microflex LT using a 3-hydroxy picolinic acid matrix and by analytical RP-HPLC carried out on a Waters 600 system with a Waters 600 controller, a Waters 2996 PDA detector, and a Waters 717 autosampler on an XBridge OST C18 2.5 μm, 19 × 100 mm column with an XBridge Prep C18 5 μm, 10 × 10 precolumn using a 38 min linear gradient of 0–53% MeCN in 0.05 M triethylammonium acetate (pH 7.4) at a flow rate of 2.50 mL/min.

*Synthesis of the Prototypical Lipid–DNA Conjugates (**dU11** and **dU20**).* Lipid-DNA was prepared as described earlier.[31,32] The sequences of the two DNA amphiphiles with hydrophobic units attached to the uracil base and the corresponding nucleoside building block are depicted in Figure 2. Two amphiphilic DNA strands were synthesized; one was a 20mer sequence with two central lipid-modified nucleotides (**dU20**) and another 11mer with two terminal hydrophobic modifications (**dU11**).

Assessment of the Incorporation of Amphiphile–DNA Conjugates into Membranes. *General Materials and Methods for Preparation of Vesicles.* Sodium hydroxide solution (1 M), hydrochloride solution (1 M), decanoic (capric) acid (DA), cholic acid, oleic acid, and Nile red were purchased from Fluka, Switzerland. Phosphate buffer (Na_2HPO_4) was obtained from Sigma, USA. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Inc. (USA).

Vesicles were prepared by pH vesiculation[16] for the fatty acids (DA in 100 mM phosphate buffer, pH 7.2, and oleic acid in 100 mM Trizma buffer, pH 9.0) or film hydration for POPC (100 mM Trizma buffer, pH 9.0). For studies of prototypical lipid-DNA insertion into fatty acid and POPC vesicles, the vesiculation was followed by size homogenization by extrusion (hand-held mini-extruder from Avanti Polar Inc., USA, sequential use of 400 nm polycarbonate filters from Whatman plc, UK).

In brief, to prepare 1 mL DA (30 mM) vesicle suspension in phosphate buffer (100 mM), 3.81 mg DA was weighed and suspended in 500 μL phosphate buffer (200 mM, pH 7.0). 1 M NaOH solution was used to reach pH above 9.5, and the resulting sample was briefly heated to 43 °C. Finally, the sample was acidified with 1 M HCl to a pH 7.2 and Milli-Q water was added to obtain the final suspension volume of 1 mL.

Microscopy. All coverslips and glass slides were treated with dimethyldichlorosilane (PlusOne Repel-Silane ES, GE Healthcare Life Sciences) in order to improve the hydrophobicity of the surface. A Nikon Eclipse TE2000-S inverted fluorescence microscope (Nikon, Japan), with a mercury lamp as the light source and a filter set, as well as a 100× oil-immersion fluorescence APOCHROMAT objective coupled with a multiplication factor of 1.6× on the camera was used. The open-source software package μManager was used.[65] Movies were obtained using the Multi-Dimensional Acquisition window, each comprising 100 images taken at 50 ms intervals. The stage position list window was used if the same coverslip position had to be found more than once.

Microscopy assessment with Nile red (excitation and emission filters: 540–585/600–650 nm) was used to confirm the presence of DA vesicles, especially prior to further experiments with a vesicle sample. 5 μL vesicle solution was pipetted onto a glass slide and 0.5 μL Nile red was applied to a coverslip.

*Spontaneous Membrane Association of **dU11**, **dU20**, and Unspecific DNA.* The prototypical DNA amphiphiles **dU11** and **dU20** or sonicated DNA (sonicated salmon sperm DNA resulting in on average 500 nt long fragments) were incubated (final concentration: 0.5 mg/mL) with the vesicle suspension (final concentrations: 30 mM DA, 17.9 mM oleic acid or 5.0 mM POPC) at 23 °C for 24 h, before being subjected to a gel separation column (Bio-Gel A-1.5m from Bio-Rad Laboratories, Inc., USA) to remove the free DNA from the suspension. The vesicles were destroyed by addition of 1.8 molar excess of cholate (150 mM stock solution) or by increasing the pH to 11 for the POPC and fatty acid systems, respectively. UV measurements were corrected for residual scattering.

Spontaneous Membrane Association of Bolaamphiphile–DNA Conjugates. To evaluate the association of the bolaamphiphile–DNA conjugates (oligo-X), master-1 and master-2 oligonucleotides were purchased from Invitrogen, USA. The sequence of master-1 was complementary to the incorporated 15mer and was 5′-CGC GAT CGC GCC TTT-3′ with fluorochrome Alexa-488 (ex 495/em 519) attached to the 5′ end to qualitatively assess the association by fluorescence microscopy (excitation and emission filters: 455–490/499–540 nm). The sequence of master-2 was noncomplementary to the incorporated 15mer and was 5′-GCG CTA GCG CGG AAA-3′ with fluorochrome Alexa-488 attached to the 5′ end.

Post-Vesiculation Insertion—Classic Method. 9 μL of the 30 mM DA vesicle suspension was incubated for half an hour at a slightly elevated temperature (30 °C) with the mixture of two oligomers: 1.8 μL (57 mM) bolaamphiphile–DNA conjugate (oligo-1, -2, -3, or -4) and 1.7 μL (57 mM) detection oligomer (master-1 or 2). This procedure was carried out to ensure enough time for the association process with the membranes. The final concentrations were 8.9 mM of a bolaamphiphile–DNA conjugate, 8.4 mM master-1, and 22 mM decanoic acid. This solution was then used directly in microscopic studies.

Alternatively, oligo-X and detection oligomer were first incubated in an Eppendorf tube for 30 min at 30 °C in order to facilitate hybridization. Subsequently, 9 μL 30 mM DA were added to the Eppendorf tube, followed by incubation for 30 min at 30 °C. The final concentrations in all cases were 8.9 mM oligo-1, 8.4 mM master-1, and 22 mM decanoic acid. This solution was then used directly in microscopic studies.

Dehydration–Rehydration Method.[58] Samples were prepared similarly to the post-vesiculation method, using 0.5 mL Eppendorf tubes. Nitrogen gas was then gently applied through the tubes for about half an hour, until all liquid had evaporated. Milli-Q water was then added to reach the former volume. Finally, the samples were sonicated for 5 min, vortexed, centrifuged, and left for half an hour for equilibration at 30 °C. This solution was then used directly in microscopic studies.

Time-Resolved DNA Hybridization on Functionalized Decanoic Acid Vesicles. The solution of DA vesicles was prepared as described above, but only with oligo-4 bolaamphiphile DNA without reporter DNA oligo (master). 3 μL of vesicle solution was added to the glass slide and 0.4 μL master-1 solution (57 mM) was applied to the coverslip. The final concentrations were ∼8 mM oligo-4, ∼7 mM master-1, and ∼22 mM DA. This setup was then observed under a microscope in the green channel (master-1 emission) as described above. Within the first 2 min the first visible structures were observed and recorded as time point 0. Subsequently, the sample was observed for the total duration of 40 min and micrographs taken with few minutes time intervals.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR and ^{13}C NMR spectra of compound **4**, MALDI-TOF spectra for **oligos-1** to **4**, analytical RP-HPLC spectra for **oligos-1** and **2** and for the 15-mer ssDNA before the click chemistry reaction. Movie showing short composition of images taken in the oligo-4/master-1 "classical" system (video 1). Movie showing progressive hybridization of master-1 on oligo-4 containing vesicles (video 2). In both movies the scale bar denotes 10 μm. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

dd water, double deionized water; dsDNA, double stranded DNA; C10, alkane hydrocarbon chain with the length of 10 carbon atoms; CuAAC, Copper-catalyzed azide–alkyne cycloaddition; CVC, critical vesicle concentration; DA, decanoic acid; DMF, dimethylformamide; EtOH, ethanol; OD, optical density equivalents; MALDI-TOF, matrix assisted laser desorption/ionization–time-of-flight; MeCN, acetonitrile; MeOH, methanol; pK_a, logarithmic acid dissociation constant; RP-HPLC, reverse phase high-performance liquid chromatography; ssDNA, single stranded DNA; TBTA, *tris*-(benzyltriazolylmethyl)amine; *t*-BuOH, *tert*-butyl alcohol

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