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DNA Oligonucleotide-Functionalized Liposomes: Bioconjugate Chemistry, Biointerfaces, and Applications

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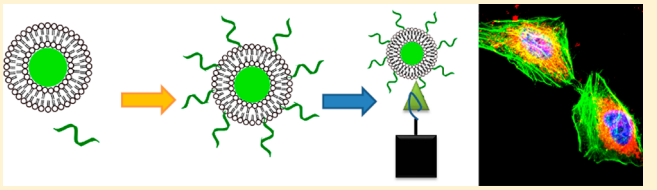
ABSTRACT: Interfacing DNA with liposomes has produced a diverse range of programmable soft materials, devices, and drug delivery vehicles. By simply controlling liposomal composition, bilayer fluidity, lipid domain formation, and surface charge can be systematically varied. Recent development in DNA research has produced not only sophisticated nanostructures but also new functions including ligand binding and catalysis. For noncationic liposomes, a DNA is typically covalently linked to a hydrophobic or lipid moiety that can be inserted into lipid membranes. In this article, we discuss fundamental biointerfaces formed between DNA and noncationic liposomes. The methods to prepare such conjugates and the interactions at the membrane interfaces are also discussed. The effect of DNA lateral diffusion on fluid bilayer membranes and the effect of membrane on DNA assembly are emphasized. DNA hybridization can be programmed to promote fusion of lipid membranes. Representative applications of this conjugate for drug delivery, biosensor development, and directed assembly of materials are briefly described toward the end. Some future research directions are also proposed to further understand this biointerface.



INTRODUCTION

Associating DNAs (DNA) and ribonucleic acids (RNA) with liposomes started a few decades ago, mainly for the purpose of DNA transfection. Cationic liposomes were mixed with anionic plasmid DNA, antisense oligonucleotides, and later siRNA and microRNA, to condense nucleic acids and allow an overall positive charge of the complex to be internalized by cells.[1] With developments in DNA synthesis and the discovery of new functions of DNA, construction of liposome/DNA hybrids has attracted more and more attention. Such hybrids represent an interesting biointerface system for both practical applications and fundamental studies.

Liposomes are attractive for their applications in drug delivery,[2] templated materials synthesis,[3−5] and biosensor development.[6] Liposomes can be made from natural lipids and possess excellent biocompatibility. Indeed, many Food and Drug Administration (FDA) approved drug delivery formulations contain a liposome component.[7] The flexible and fluid nature of lipid membranes also provides versatility for designing new soft materials. Liposomes can encapsulate guest molecules and can also encapsulating inorganic materials forming supported bilayers.[8] The charge, surface chemistry, and fluidity of liposomes can be readily tuned by varying its lipid composition.

DNA, beyond their genetic function, can be programmed into various sophisticated nanostructures.[9−11] In addition, DNA oligonucleotides with catalytic and ligand binding properties have also been reported.[12−15] Functionalizing liposomes with DNA has produced a diverse range of hybrid materials, combining the merits of both components.[16−18]

To control the function of both DNA and liposomes, we must understand their interfaces and interactions, which, in turn, may provide new insights for designing better materials. First, DNA must be stably anchored on the surface of liposomes. At the same time, the function of DNA must be maximally retained or even enhanced by liposomes. It is also important to keep the stability of liposomes to ensure long-term content encapsulation. In this article, we review related work on this topic with an emphasis on fundamental surface interactions.

Both lipids and nucleic acids are basic components of life, and they are believed to be essential for enabling early life on Earth. By encapsulating nucleic acids in liposomes, many protocells were designed to understand the origin of life.[19,20] In those cases, nucleic acids were trapped inside liposomes, and such research is beyond the scope of this review. In addition, using cationic liposomes to deliver DNA is excluded from our discussion as well. DNA nanostructures have been made into nanopores to gate the inside/outside communication of liposomes.[21−24] This specialized topic is not covered by our review either. In this review, we focus on DNA anchored or adsorbed on liposome surface through nonelectrostatic interactions.[25] After discussing the fundamental aspects of this system, a few representative applications are described. Toward the end, future research opportunities are speculated.

BASIC PROPERTIES OF LIPOSOMES

Liposomes are self-assembled lipid bilayer vesicles with sizes ranging from ∼30 nm to tens of micrometers. The general structure scheme of a liposome is shown in Figure 1A. A

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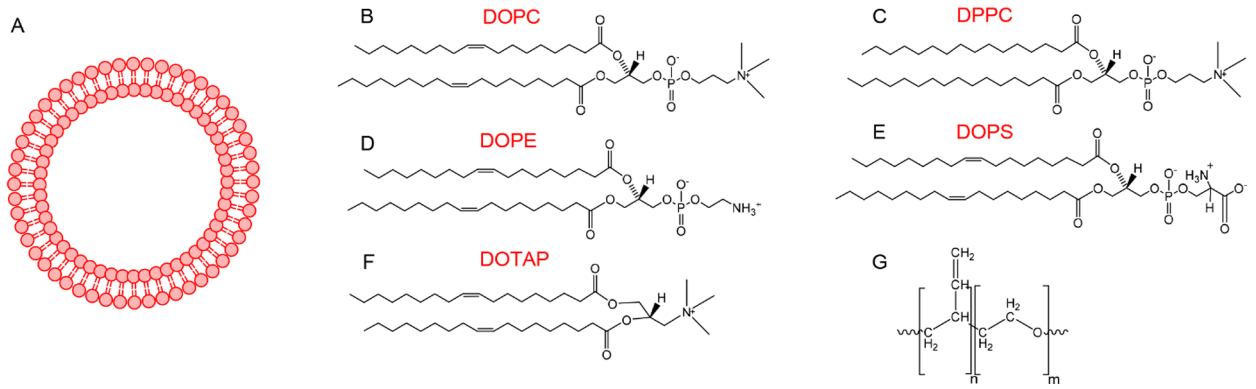

Figure 1. (A) General structure of a liposome. (B–G) Chemical structures of DOPC (panel (B)), DPPC (panel (C)), DOPE (panel (D)), DOPS (panel (E)), and DOTAP lipids (panel (F)), and a PBD-*b*-PEG block copolymer (panel (G)).

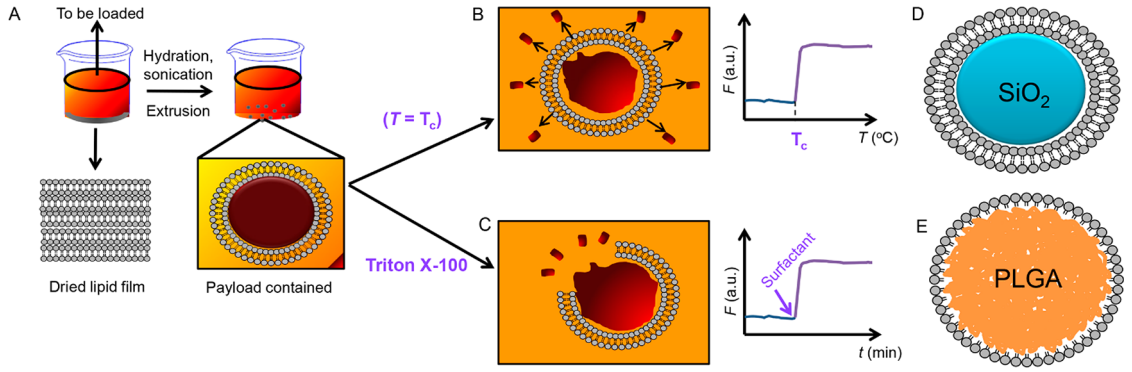

Figure 2. Schemes of (A) encapsulation of molecules in liposomes, and content release of liposomes induced by (B) raising temperature and (C) adding surfactants. Schemes of (D) a supported lipid bilayer on hydrophilic SiO_2 nanoparticles and (E) a supported lipid monolayer on hydrophobic PLGA nanoparticles.

typical lipid molecule has a polar headgroup with two long hydrophobic tails. Through purification of natural membranes and chemical synthesis, hundreds of lipids are commercially available. Here, we briefly discuss a few important properties of liposomes. Since DNA is highly negatively charged, the charge of liposome is quite relevant, in terms of electrostatic interactions. Most lipids in natural biological membranes are either neutral or negatively charged. Dioleoylphosphatidylcholine (DOPC) and dipalmitoylphosphatidylcholine (DPPC) lipids are zwitterionic with a positively charged choline and a negatively charged phosphate (Figures 1B and 1C, respectively). PC lipids are the most abundant lipids in the outer membrane of eukaryotic cells and are highly biocompatible. Dioleoylphosphatidylethanolamine (DOPE), also with an overall neutral charge, has a relatively small headgroup but cannot form liposomes on its own (Figure 1D).[26] Dioleoylphosphatidylserine (DOPS) lipids are anionic, and their presence on the outer cell membrane is an indication of apoptosis (Figure 1E).[27] Cationic lipids have also been synthetically prepared, and, as an example, dioleoyltrimethylammonium-propane (DOTAP) is shown in Figure 1F.

In addition to charge, another interesting aspect of liposomes is their phase transition temperature (T_c). Based on the packing of the hydrophobic tails, a liposome can be in the gel phase where the tails are ordered with a small diffusion coefficient. By increasing the temperature, the gel phase liposome reaches the fluid phase with increased lipid lateral diffusion. For example, a DPPC liposome with all saturated lipid tails is in the gel phase at room temperature. When the temperature is above 41 °C (phase transition temperature, T_c), a phase transition occurs to yield a fluid DPPC membrane. DOPC liposomes, on the other hand, are fluid at room temperature, with a phase-transition temperature of $T_c = -20$ °C, since its unsaturated tails create a kink in packing. When the gel phase and fluid phase lipids are mixed, phase separation may occur, forming domains of one phase distributed in the other.[28]

The size of liposome can also be controlled during synthesis. Extrusion allows the preparation of quite uniform liposomes from ∼50 nm to 200 nm. Beyond this size, more multilamellar structures encapsulating smaller vesicles are formed. The size of such liposomes is often characterized by dynamic light scattering (DLS) and cryo- or negative stain electron microscopy. The larger liposomes reaching tens of micrometers in size are called "giant liposomes". These are often prepared by swelling lipid films in the presence an AC electric field,[29] and they can be observed with optical microscopy.

Certain amphiphilic block copolymers can also form vesicular structures called polymersomes.[30] A hydrophilic–hydrophobic–hydrophilic triblock polymer may form a vesicle with just a single layer of the polymer, while a diblock copolymer forms the typical bilayer structure. A representative polymer (polybutadiene-*b*-polyethylene glycol, PBD-*b*-PEG) for this purpose is shown in Figure 1G. Polymersomes are more stable than typical liposomes and some examples of their DNA conjugates are also elaborated in this review.

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CONTENT ENCAPSULATION

Encapsulation of guest molecules is a key property of liposomes. Encapsulation of small molecules or polymers can be achieved by adding them at a high concentration during hydration of dried lipid films (see Figure 2A). However, the efficiency of encapsulation is quite low, generally because of the small volume fraction of the liposomes. Some specialized techniques such as freeze−thaw cycling might help increase encapsulation efficiency of certain types of guest molecules. Large hydrophilic molecules can normally be stably encapsulated inside. Leakage of the content happens the fastest at the T_c of the liposome (Figure 2B). Alternatively, liposomes can be ruptured by adding a surfactant such as Triton X-100 to fully release the content (Figure 2C).

A special form of encapsulation involves wrapping lipid bilayers around inorganic nanoparticles forming supported lipid bilayers (Figure 2D). In this case, the inorganic core can define the size of the bilayer and increase the stability of the lipid membrane, compared to the free liposomes. The most commonly used core material is silica, which can support PC bilayers after a simple mixing with fluid liposomes.[31,32] Other surfaces sometimes require more stringent conditions.[33] For example, on a hydrogel surface, electrostatic attraction is important,[34−36] while on TiO_2, low pH is critical to support PC bilayers.[37] By tuning liposome composition, many other oxides can also form supported bilayers.[38−40]

If the core material is hydrophobic, lipids are more likely to form supported lipid monolayers (Figure 2E). A good example is poly(lactic-*co*-glycolic) acid (PLGA), which is a biodegradable polymer commonly used for delivery of hydrophobic drugs.[41] Coating a lipid layer on PLGA has been used to facilitate its dispersion in aqueous solutions and for bioconjugation of targeting and antifouling ligands.[42,43]

DNA AND FUNCTIONAL DNA

DNA has a relatively simple structure with a phosphate backbone and four types of nucleobases (Figure 3A). The simple Watson−Crick base pairing rule has made DNA a highly predictable and programmable polymer, and many impressive DNA nanostructures have been reported.[10,44] Chemically, DNA is known for its function to hybridize to complementary nucleic acids, which is the basis for nucleic acid detection.[45] In addition, DNA aptamers have been selected to bind to many other molecules,[13,46] such as small molecules, proteins, metal ions, and even cells.[47] Figure 3B shows a guanine-rich aptamer called AS1411 that can bind to several cancer cells.[48] This particular aptamer dimerizes through the formation of a G-quadruplex structure before binding to its target, nucleolin, on the cell surface. The catalytic function of DNA has also been reported, and these are called DNAzymes.[14,15,49,50] Unlike ribozymes, however, DNAzymes have not yet been found in nature.

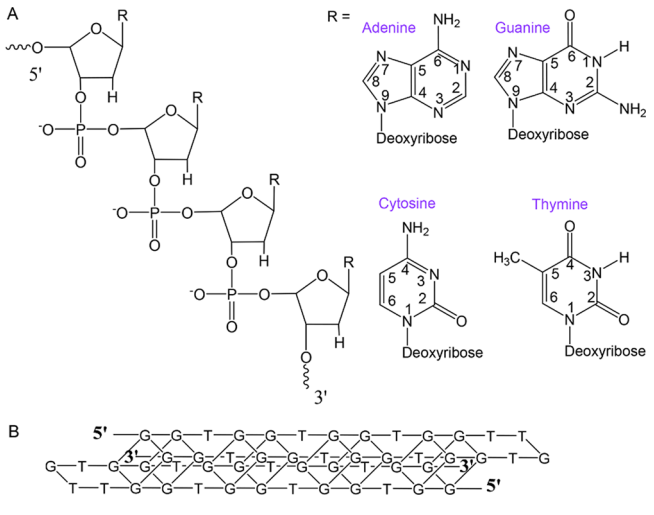

Figure 3. (A) Basic structure of a 4-mer DNA oligonucleotide consisting of phosphodiester linkages and four types of bases. The ring atoms in the nucleobases are numbered. (B) The AS1411 DNA aptamer in its dimeric form, forming a G-quadruplex motif.

BIOCONJUGATE CHEMISTRY

DNA is a highly negatively charged and polar molecule. Typically, DNA oligonucleotides cannot associate with lipid membranes except through electrostatic attractions using cationic liposomes. For noncharged or negatively charged liposomes, DNA conjugation is achieved through a covalent modification as discussed below.

Cholesterol-Modified DNA. Cholesterol is a popular anchoring group for DNA due to its commercial availability (Figure 4A, top structure). Cholesterol is widely found in animal cells contributing to membrane stability. Its bulky hydrophobic region can insert itself in the membranes. Covalent modification of DNA with cholesterol occurs through the polar hydroxyl group. While the insertion of cholesterol-modified DNA into lipid bilayers is spontaneous, it has been reported to be somewhat unstable, as simple rinsing can remove much of the anchored DNA.[51] To overcome this, bivalent cholesterol has been used to increase the stability (Figure 4B).[52] In one example, a duplex DNA was composed of a cholesterol-modified 15-mer DNA, which was complementary to the first 15 bases in a cholesterol-modified 30-mer DNA. The resulting duplex contained two terminal cholesterol units, which anchored more strongly into the lipid bilayer. In the literature, both monovalent and bivalent cholesterol anchors have been used, and single cholesterol anchors were more popular.[53,54] Cholesterol anchored DNAs have also been used for biosensing,[55] DNA origami,[56] drug delivery,[57] and stimuli-responsive materials.[58]

Cholesterol anchors have noticeable effects on membrane stability. Bunge et al. compared the effect of cholesterol and tetra ethylene glycol (TEG)-modified cholesterol on 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes using ^{31}P and ^{2}H NMR spectroscopy.[53] The structure of a cholesterol-TEG is shown in Figure 4A (middle structure). The cholesterol anchor disrupted the bilayer packing order, causing an increase in membrane thickness. The reason for this was attributed to the so-called "condensation effect": the formation of a lipid-cholesterol complex that provides long-range order within the membrane, increasing the overall thickness.[59] On the other hand, very little difference was found in the packing order and overall stability of the membrane when a TEG-cholesterol anchor was used. This was consistent with previous simulation data when a larger hydrophilic moiety was added to the cholesterol headgroup.[59] Essentially, the larger hydrophilic group changes the depth of insertion so that the lipid-cholesterol complexes do not form, leaving the liposome membrane unaffected.

Lipid-Modified DNA. In addition to cholesterol, long hydrophobic tails typically seen in lipids have been linked to

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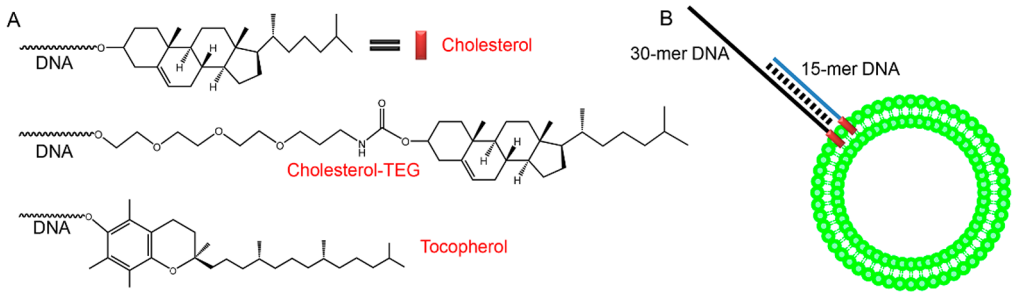

Figure 4. (A) Chemical structure of cholesterol (top), cholesterol-TEG (middle), and tocopherol (bottom) used as an anchor for DNA. (B) A bivalent cholesterol to provide a higher stability to DNA-liposome conjugates.

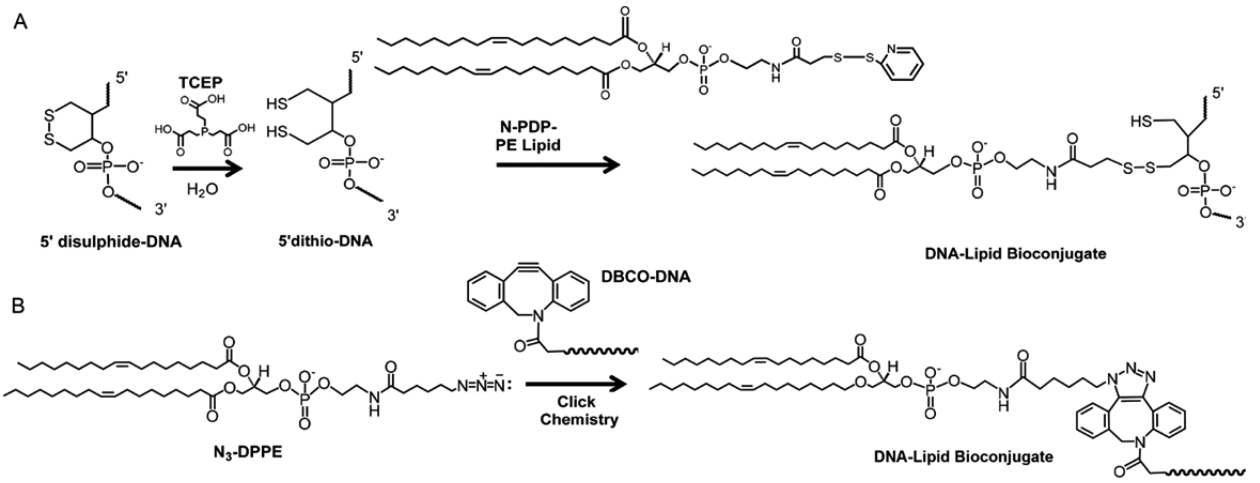

Figure 5. Schemes of bioconjugation methods: (A) a disulfide exchange reaction, where a 5′-disulfide DNA is reduced by TCEP to produce free thiol groups, which exchanges with the thio-pyridyl group of the N−PDP-PE lipid to form a disulfide linkage; and (B) a click chemistry reaction, where an azide-terminated DPPE lipid reacts with the alkyne group of a DBCO-terminated DNA.

DNA during the solid-phase synthesis of DNA.[25,60,61] These lipid-modified DNAs form more stable conjugates than those with single-cholesterol anchors and have seen significant use in recent years. For example, Vogel and co-workers conjugated both ends of a single-stranded DNA with a palmitoyl lipid for DNA detection.[62] Using the same palmitoyl moiety, Cogoi et al. inserted a G4-decoy oligonucleotide into a POPC liposome for delivery into pancreatic cancer cells.[63] They reported a loading density of ca. 100 DNA strands per liposome. Recently, a tocopherol molecule was attached to DNA for drug delivery applications.[18] Tocopherol was chosen because of its low cost and nontoxicity (Figure 4A, bottom).

Bioconjugate Reactions. A lipid-modified DNA can insert into preformed liposomes, or it can be used together with free lipids to prepare liposomes. In the latter case, the DNA is displayed in both the inner and outer leaflets. Another method is to add a small fraction (usually 0.5−5%) of reactive lipids to the lipid formulation. Following this, a modified DNA can be conjugated to the liposome. For example, the disulfide exchange approach used by Yosihina-Ishii et al. (Figure 5A) required both the DNA and the lipid to be terminated by thiol groups.[64] In the first step, the disulfide bond in the DNA was reduced by TCEP to produce free thiol. These thiols then reacted with N−PDP-PE (a custom disulfide-terminated lipid based on DOPE) to form the lipid−DNA bioconjugate with thio-pyridyl as the leaving group. With 0.5 mol % of the reactive lipid, however, only 1−2 DNA molecules were conjugated to each liposome. Typical liposomes contain tens of thousands of lipids and significant reactive lipids per liposome, even at 0.5% doping. As such, in this approach by Yosihina-Ishii et al., the efficiency of conjugation was likely to be very low.

Highly efficient conjugation was achieved with the maleimide−thiol reaction.[65] An early example of this was shown by Willner and co-workers.[16] DNA modified with a thiol group was reacted with a preformed liposome containing a small fraction of maleimide−phosphatidylethanolamine. A Michael addition reaction then occurred, resulting in a stable thioether linkage between a thiolated DNA and the lipid.[66] Using this method, the authors reported up to 60 DNA strands per liposome.

Finally, click chemistry has been an attractive tool to conjugate DNA to liposomes. The classic click reaction involves an azide and an alkyne to form a substituted triazole (the so-called azide−alkyne cycloaddition). An example was demonstrated by the Mirkin group (Figure 5B).[18,67] They reacted a DBCO (a cyclooctyne) terminated DNA with a DPPE-N_3 lipid overnight, and this uncatalyzed reaction formed the desired bioconjugate. A loading of up to 330 strands of DNA per liposome was achieved, depending on the stoichiometry of the lipids to the DNA. The click method was also employed for polymersomes.[68] In this case, the azide was attached to the hydrophilic polyethylene glycol (PEG) block, while DBCO was attached to the DNA. A DNA loading of ca. 127 strands per polymersome was achieved.

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SURFACE INTERACTIONS

Without the addition of divalent cations, the interaction between DNA and noncationic liposomes is very weak.[69] Such a weak interaction maximally retains the activity of anchored DNA. This is in strong contrast to adsorption of DNA by inorganic surfaces.[70] For example, if a low density of DNA is adsorbed stably on a gold surface, it cannot hybridize with its complementary strand, since DNA binds to gold more strongly than DNA base-pairing interactions.[71,72] For DNA to be functional, the gold surface must be blocked by small thiol molecules to avoid nonspecific DNA/gold interactions.[72] Since the interaction between DNA and lipid surface is weak, direct measuring of their binding cannot be easily performed. Most fundamental studies were performed on DNA with a hydrophobic anchor.

One fundamental study by Banchelli et al. explored the surface characteristics of 35 nm POPC liposomes conjugated with a T-rich 18-mer DNA oligonucleotide via a cholesteryl-TEG anchor.[73] With increasing ratios of oligonucleotide/liposome, an increase in the hydrodynamic size of the liposome-DNA conjugate was observed, using dynamic light scattering (DLS). Banchelli et al. concluded that the conformation of the DNA changed as the loading density increased (Figure 6A). At a low loading density, because of the high ionic strength used, the charge on the DNA strands was quasi-neutral and they adopted a random coil conformation (called a "mushroom") on the liposome surface. As the loading density increased, this conformation changed to a more rigid, stretched structure (called a "brush"), because of repulsion between the overlapping DNA strands. The authors also measured the kinetics of DNA hybridization with loading density. At low density, the diffusion of the cDNA to the liposome surface was fast. If the density was too high, the dense packing of the brush conformation hindered diffusion of the cDNA to the liposome surface, lowering the rate of hybridization.

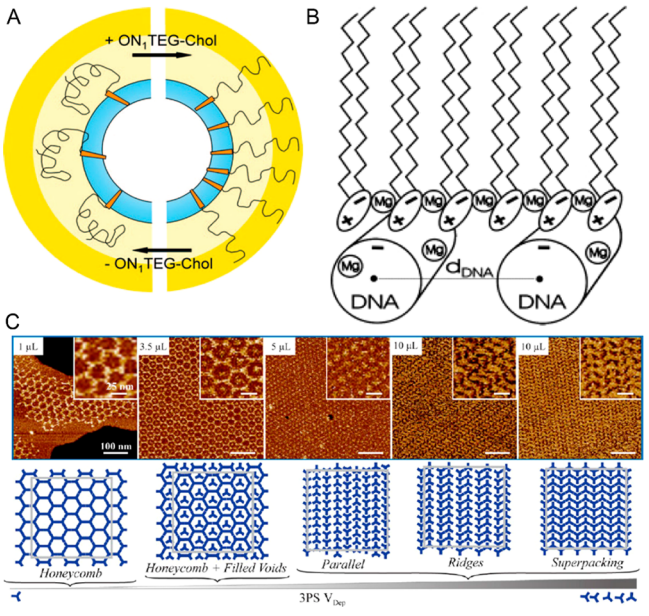

Figure 6. (A) Conformation of DNA oligonucleotides on a liposome surface as a function of loading density. Lower loading density results in a coiled state (the left half), whereas a higher density results in a more rigid, brush state (the right half). [Reproduced with permission from ref 73. Copyright 2008, American Chemical Society, Washington, DC.] (B) Divalent ion-mediated DNA adsorption on zwitterionic lipid surfaces. Mg^{2+} binds to the phosphate groups in the lipid head, effectively neutralizing the negative charge. [Figure reproduced with permission from ref 74. Copyright 2006, American Chemical Society, Washington, DC.] (C) Self-assembly of DNA tiles on the surface of DPPC lipid bilayers (without cholesterol) with increasing loading density. AFM micrographs corresponding to the different modes of self-assembly are also shown. [Figure reproduced with permission from ref 79. Copyright 2017, American Chemical Society, Washington, DC.]

Cholesteryl-TEG-anchored DNA duplexes on POPC liposomes was explored by Bunge et al.[53] Upon hybridization, the anchored duplex adopted a rigid structure similar to the free duplex (i.e., nonanchored), also supporting the lack of lipid/DNA interactions. However, one major difference was the melting temperature (T_m) of DNA. A ca. 28 °C increase in T_m with the anchored duplex DNA was reported, compared to that observed for free duplex DNA. This was attributed to favorable hydrophobic interactions between the cholesterol moieties in close proximity to each other stacking upon the favorable Watson–Crick base pairing. Indeed, the T_m of the free, cholesterol-TEG DNA duplex (no liposome) was very close to that of the liposome sample, indicating that cholesterol-TEG label alone could increase the T_m drastically. DNA anchoring was only present with a high ionic strength (ca. 137 mM NaCl). With low ionic strength, longer-range electrostatic repulsion dominated over the shorter-range hydrophobic attraction of cholesterol to the membrane and hindered anchoring of a high density of DNA.

The above studies were conducted at relatively high concentrations of monovalent salt (NaCl). The interaction of DNA with PC membranes in the presence of divalent ions has also been studied. Early investigations by Budker et al. showed that negatively charged polynucleotides (without a hydrophobic anchoring group) bound relatively strongly to PC bilayers in the presence of a mixture of Na^+ and Mg^{2+}.[69] Furthermore, the DNA structure changed upon adsorption to the lipids, with a corresponding increase in the T_m of the DNA (in the case of duplex studies). This was explored in more depth by McManus et al.[74] and Gromelski and Brezesinski.[75] Both studies concluded a reorientation of the lipid bilayer in the presence of divalent cations. More specifically, the cations were associated with the negatively charged phosphate group within the lipid headgroup, effectively neutralizing the negative charge and making the lipid surface positively charged (see Figure 6B). Therefore, the negatively charged DNA can have an attractive electrostatic interaction with the DOPC/DPPC membranes. Many groups used Mg^{2+} to attach complex DNA architectures on lipid surfaces, and Mg^{2+} was often needed also for stabilizing DNA structures. In most cases, a cholesterol anchor was still used to achieve directional attachment.[76−78]

An interesting example was shown by Sleiman and coworkers, who prepared supported lipid bilayers on a planar silica surface and observed self-assembly of DNA tile superstructures using atomic force microscopy (AFM).[79] On liquid disordered DOPC, self-assembly was only observed with cholesterol anchored DNA (compared to unmodified DNA). On the other hand, long-range ordered structures were found with both unmodified and cholesterol-modified DNA on gel phase DPPC. This effect was attributed to the closer packing of lipids (in the gel phase) and subsequent higher positive charge density (Mg^{2+}-mediated) increasing the rate of DNA diffusion on the lipid surface. Furthermore, the pattern of self-assembled

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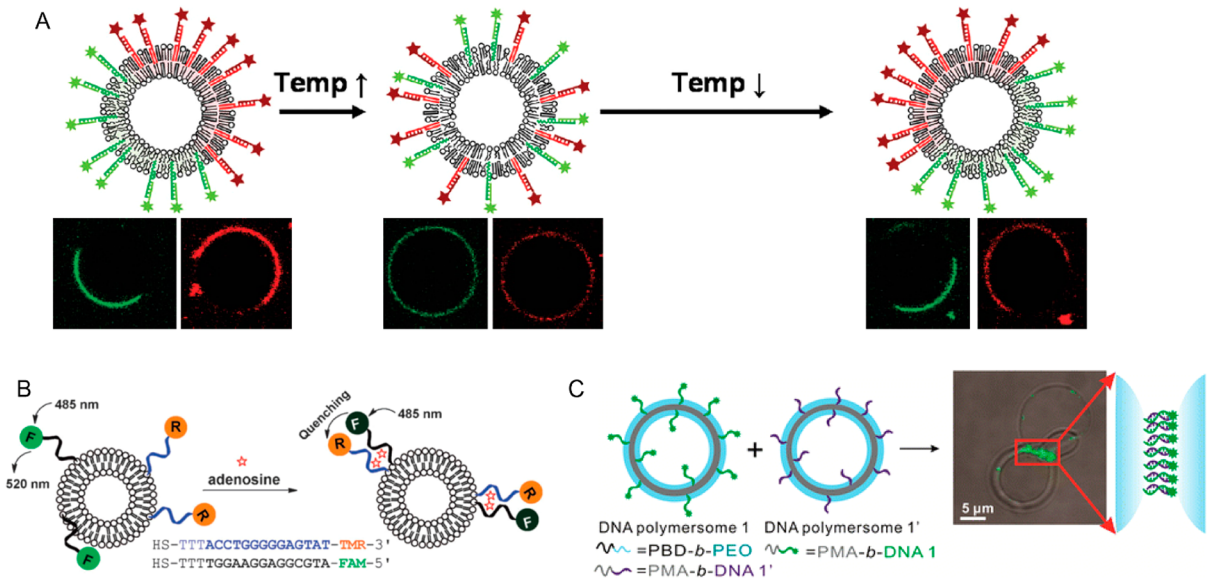

Figure 7. (A) Lateral diffusion of dye-labeled DNA on the surface of liposomes containing liquid-ordered (LO) and liquid-disordered (LD) domains. At low temperature, the domains were separated and DNAs partitions in different domains. At temperatures beyond the phase transition, the liposome membrane was all LD and both DNA strands were evenly spread out, and this process was reversible. [Reproduced with permission from ref 85. Copyright 2010, American Chemical Society, Washington, DC.] (B) Lateral diffusion of the split adenosine aptamer on a liposome in the presence of adenosine to form the full aptamer. [Reproduced with permission from ref 86. Copyright 2012, Royal Society of Chemistry, London.] (C) Confocal microscopy images of polymersome aggregation induced by DNA hybridization. The fluorescent label on the DNA was localized at the interface for hybridization showing lateral DNA diffusion across the membrane. [Reproduced with permission from ref 87. Copyright 2016, American Chemical Society, Washington, DC.]

structures on the DPPC surface was dependent on whether a cholesterol modification was present, as well as the concentration of DNA used (Figure 6C).

LIPOSOME FLUIDITY AND DNA LATERAL DIFFUSION

Lipids can diffuse laterally on liposomes, and this is a major difference between liposomes and inorganic surfaces. Such lateral diffusion is important for cell signaling and has been studied for many years.[80] Initial investigations showed that certain lipid domains in cell membranes did not diffuse as freely as other ones. In the late 1990s, it was found that domains consisting of cholesterol/sphingolipid complexes with contained proteins formed so-called "rafts" on cell surfaces.[81,82] These rafts could be mobile or static (depending on the constituents) and provide an interface for interesting biochemical reactions such as dimerization of certain proteins upon ligand binding.[83]

Using this concept of lateral diffusion, Brinker and co-workers proposed dynamic ligand binding for targeting cancer cells.[84] They conjugated peptides on different cargo-loaded supported lipid bilayers, which would bind to receptors on the cell surface, leading to endocytosis. Interestingly, only a small degree of conjugation was needed for effective internalization. The fluidity of the membrane allowed the targeting peptides to reorganize themselves to bind to the cell receptors. This was achieved more easily for lipids in their fluid state, whereas gel phase DPPC (as an example) needed a higher density of peptides to achieve a similar targeting efficiency.

A few reports also exploited nucleic acid lateral diffusion on lipid membranes. Loew et al. used cholesterol and palmitoyl-anchored peptide nucleic acids (PNAs) to study the effect of temperature on the gel and fluid phases of liposomes.[85] Depending on the anchor they used, the PNA would partition either in the gel or the fluid phase of their liposomes. They then added a dye-labeled cDNA, which hybridized with the PNA and visualized both the gel (red) and fluid (green) phases using confocal microscopy as a function of temperature (Figure 7A). At low temperatures, these domains were clearly separate from each other. When the temperature was increased beyond the gel−liquid transition, the DNA-PNA complexes diffused around the liposome until the distribution was homogeneous. Once the temperature was reduced, the domains were again segregated accordingly.

Our group used lateral diffusion of DNA aptamers for the detection of adenosine.[86] The adenosine aptamer was split into two parts (one with a fluorophore and the other with a quencher), and both were immobilized on a DOPC liposome (Figure 7B). In the presence of adenosine, the fluidity of the DOPC membrane allowed the two halves to bind together (mediated by adenosine), resulting in fluorescence quenching. When this was done using an inorganic silica nanoparticle (i.e., a nonmobile surface), the fluorescence was not quenched likely due to the lack of fluidity of the surface, and the aptamer halves were immobilized very far away from each other.

While the above work focused on liposomes, membrane fluidity was also observed in polymersomes. Luo et al. prepared giant polybutadiene (PBD)-*b*-polyethylene glycol (PEG) polymersomes with a small percentage of dye-labeled polymethyl acrylate (PMA)-*b*-DNA.[87] They then prepared another polymersome with the cDNA strand. Before the polymersomes interacted with each other, fluorescence was homogeneous on the membrane under confocal microscopy. Once the polymersomes were close enough, the fluorescence localized near the zone of interaction (Figure 7C). This corresponded to diffusion of DNA on the membrane for hybridization with its complementary strand.

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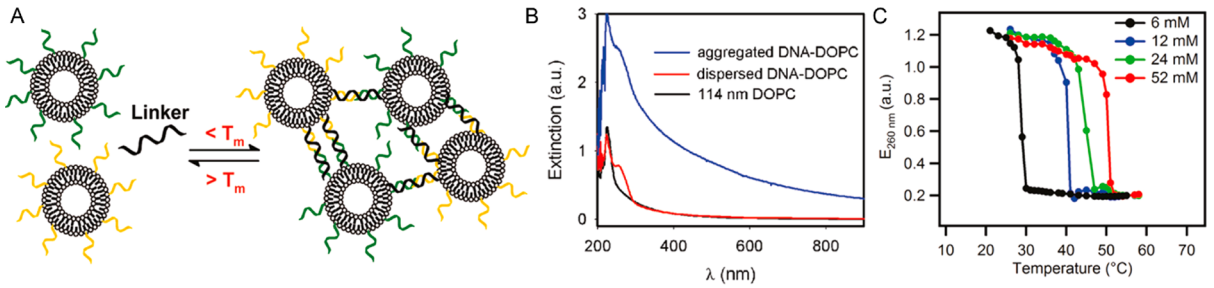

Figure 8. (A) Scheme of DNA-directed assembly of DNA-functionalized liposomes controlled by temperature. (B) UV-vis spectra of free DOPC liposomes, DNA-DOPC liposomes, and after assembly by linker DNA. (C) Sharp melting transitions of DNA-linked liposomes. With more NaCl, the melting transition shifted to higher temperatures. [Reproduced with permission from ref 90. Copyright 2011, American Chemical Society, Washington, DC.]

DNA-DIRECTED ASSEMBLY OF LIPOSOMES

DNA-directed assembly of nanomaterials is an interesting process with excellent programmability.[88] By functionalizing nanoparticles with DNA, each nanoparticle can be arranged like an atom in a molecule,[89] or in a crystal.[10] DNA-assembled liposomes have also been reported. As an early example, Yoshina-Ishii and Boxer exploited DNA hybridization to tether dye-labeled liposomes to a supported lipid bilayer.[49] With only a few DNA strands on each liposome, they still observed diffusion of these liposomes across a 2D space using confocal microscopy. In a follow-up work, they improved DNA conjugation to liposomes,[67] and integrated this system into a microfluidic device.[66] They described DNA docking on a lipid membrane to be governed by two processes: (1) diffusion of the liposome across the lipid membrane and (2) lateral diffusion of DNA to bind with the cDNA on another liposome.

Vogel and co-workers modified both ends of a single-stranded DNA with a lipid anchor.[48,62] Once mixed with nonfunctionalized liposomes, both ends of the DNA were anchored within the same liposome, and the system remained well-dispersed. In the presence of a complementary target DNA, the dual lipid-modified DNA formed a rigid duplex and, thus, could cross-link two liposomes, leading to aggregation. This assembly was reversible with temperature and allowed for single-mismatch discrimination of DNA targets.

We also investigated DNA-directed liposome assembly. The liposomes were functionalized with two types of DNA, respectively, and large aggregates were formed using a linker DNA (Figure 8A). Once aggregated, the products scattered more light (Figure 8B), allowing the (dis)aggregation of the system to be monitored.[90] DNA-linked liposomes also showed a cooperative melting response manifested by a sharp melting transition (Figure 8C), similar to that of DNA-modified gold nanoparticles (AuNPs). This cooperative melting was attributed to multiple DNA linkages formed between neighboring particles and all the linkages must break before dissociation of the particles could occur.[91,92] The T_c value of the lipids, and charge and size of liposomes, did not affect the melting behavior of the conjugates. A follow-up work assembled DNA-conjugated AuNPs with DPPC liposomes and observed increased membrane stability from UV irradiation.[93]

More recent work by van der Meulen and Leunissen assembled SiO_2 supported bilayers with double-stranded DNA anchored via bivalent cholesterol.[94] The assembly was controlled by DNA sticky ends, which could hybridize with a sticky end on another particle. This supported liposome also displayed the previously shown characteristics of temperature-reversible (dis)aggregation. Apart from temperature, Hernández-Ainsa et al. prepared liposomes sensitive to multiple stimuli by using a special anchor containing an azobenzene moiety.[95] Azobenzene switches from a trans to a cis confirmation upon irradiation with UV light, with a corresponding change in the polarity of the molecule. In its cis state, the anchor was more hydrophilic and did not insert as stably into the membrane as when it was in the trans state. Therefore, illumination with UV light disrupted liposome aggregation. Mg^{2+} and temperature also modulated the DNA-liposome aggregation.

DNA-directed assembly was also observed with polymersomes. Liu et al. synthesized $PMOXA_7$-*b*-$PDMS_{42}$-*b*-$PMOXA_7$ polymersomes and anchored DNA using click chemistry with a modified block copolymer.[68] They also prepared cDNA-anchored polymersomes and observed clustering of the polymersomes upon mixing. Interestingly, depending on the size of the polymersomes used, the resulting superstructure was different. If the polymersomes were the same size, then chainlike structures were formed, while with scattered amounts of larger polymersomes, satellite-like structures were observed. In both cases, the membrane was heavily deformed at the area of hybridization, although no rupture of the polymersomes was observed.

DNA-DIRECTED LIPOSOME FUSION

Fusion of lipid membranes is seen in many natural systems, such as during the fertilization of an egg. The mechanism of the fusion process is still under investigation, but it is generally thought to involve the formation of a "stalk" connecting the two membranes at the point of fusion.[96] Proteins are believed to be directly responsible for fusion through a type of pore formation, whereas other studies suggest proteins simply bring membranes closer with the actual fusion being a lipid-driven process.[97] It is now generally accepted that fusion proteins exist (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors or "SNAREs"), and are heavily involved in eukaryotic cell membrane fusion.[96]

Inspired by proteins, DNA structures were also used for directing liposome fusion.[98−101] Höök and co-workers designed bivalent cholesterol anchored DNA of unequal lengths for this purpose.[99] Two sets of DNA motifs were used, forming duplexes with an overhang. One such duplex was anchored on a liposome with a FRET pair in the membrane (not on the DNA), and the other duplex was on a liposome without any fluorophore label (Figure 9A). When the two liposomes were mixed, a rehybridization of the DNA strands

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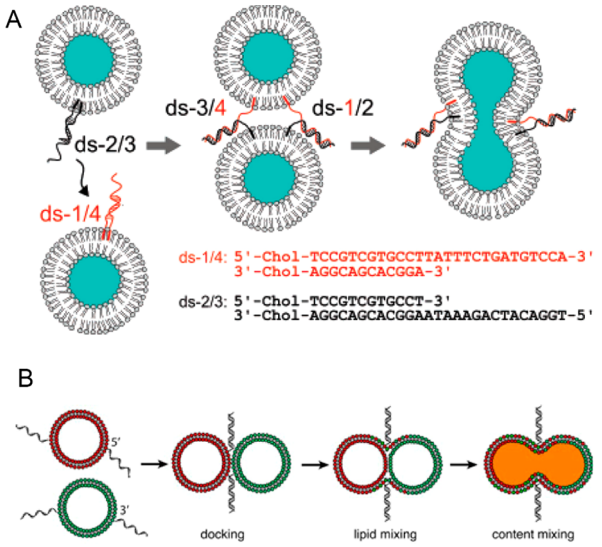

Figure 9. (A) Two DNA-functionalized liposomes were designed such that the initial duplex with an overhang was in favor of rehybridization to form new duplex structures linking the two liposomes together (docking). Finally, lipid and content-mixing occurred, concluding the fusion process. [Reproduced with permission from ref 99. Copyright 2007, American Chemical Society, Washington, DC.] (B) In this design, these DNA strands were single-stranded. Upon mixing, hybridization occurred, followed by lipid and content mixing. [Reproduced from ref 101, which is an open access publication.]

was believed to occur. Similar to SNARE proteins, this brought the liposomes close together. Fusion was indicated by a decreased FRET efficiency, because of dilution of the fluorophore pair to the nonlabeled liposome.

Boxer and co-workers proposed another method to induce liposome fusion.[101] One liposome was anchored with a DNA strand at the 5′ end, and the other liposome was anchored at the 3′ end with the complementary DNA strand (see Figure 9B). Upon hybridization, the liposomes would be close enough for fusion to occur. They observed both lipid and content mixing as a means to confirm fusion, unlike the previous study from Höök's group, where only lipid mixing was observed. There was a general concern from Boxer's group about the initial energy requirement to melt the DNA duplex in Höök's work.

In a follow-up work, Höök's group confirmed the validity of their method by observing both lipid and content mixing.[98] In addition, they saw no difference in the length of the DNA strands used and the fusion rate. Effectively, this meant that the rate of fusion was limited by the mixing of lipids once the liposomes were in close proximity and not as much by the rate of docking of the liposomes (which was previously observed in SNARE proteins). On the other hand, Boxer's group observed slower content mixing, compared to lipid mixing in their system.[100] They concluded that content mixing was the rate-limiting step. With an additional poly-T linker strand, they observed lower fusion rates. This was attributed to a larger distance of separation between liposomes upon docking with the longer DNA strands. Therefore, while the actual fusion

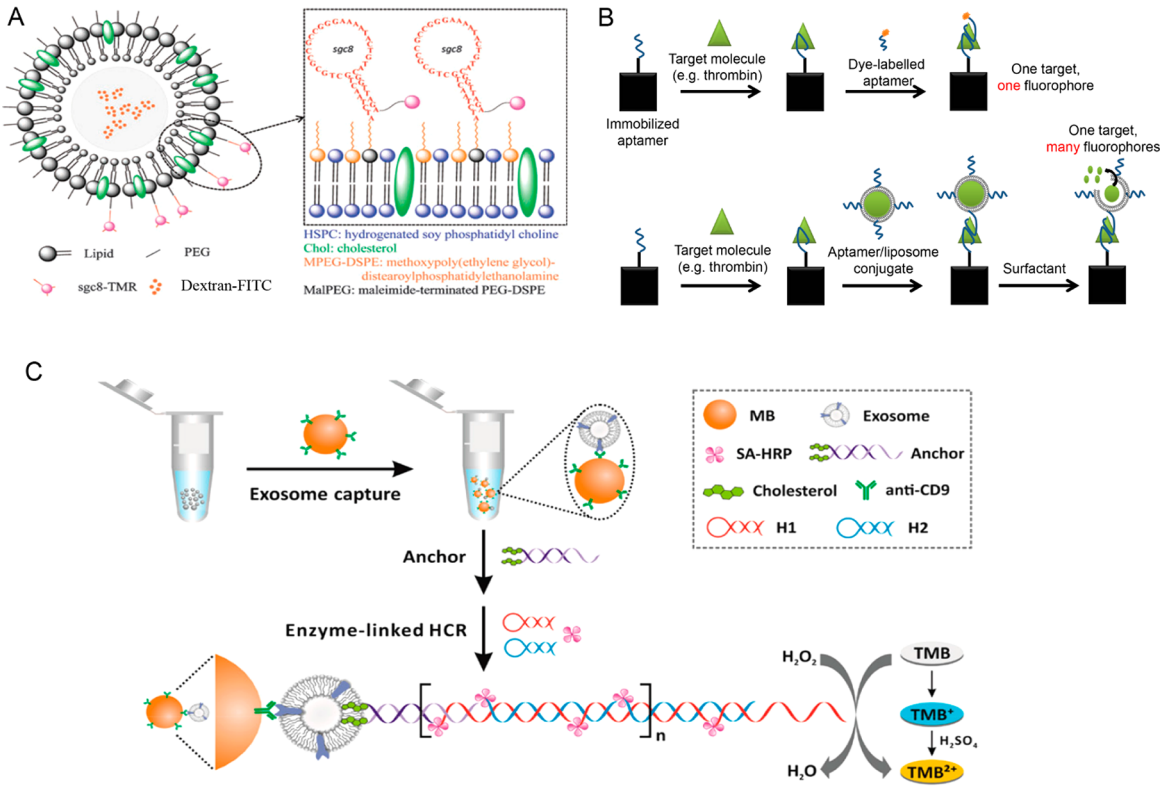

Figure 10. (A) Aptamer-loaded liposomes for drug delivery. A DSPE/HSPE/cholesterol liposome was conjugated to the sgc8c aptamer. Low-molecular-weight FITC-dextran was encapsulated as a model cargo for delivery. [Reproduced with permission from ref 120. Copyright 2010, Royal Society of Chemistry, London.] (B) DNA-based sandwich assays using a conventional fluorescent probes (top row) and fluorophore-encapsulated liposomes for signal amplification (bottom row). (C) Detection of exosomes using enzyme-linked HCR for signal amplification of peroxidase assays. [Reproduced with permission from ref 54. Copyright 2017, American Chemical Society, Washington, DC.]

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step likely did not involve DNA, the docking step was still critical (albeit not rate-limiting) to the probability of liposome fusion. This conclusion was reached by both groups, despite the differences in their systems. Höök's group later used Ca^{2+} to mediate liposome membrane fusion,[102] and, more recently, liposome fusion was used to detect microRNA.[103]

Apart from Höök and Boxer's work, DNA-mediated liposome fusion also was undertaken by Vogel and co-workers.[60,104,105] They reported a liposome fusion cascade effect using lipid conjugated DNA, similar to Boxer's group.[104] To achieve this, they synthesized four different liposome populations with six different lipid-DNA conjugates. Sequentially, they added one liposome population to another, which not only induced fusion, but provided the lipid−DNA conjugate to bind the next liposome population. However, they only observed significant fusion at high temperature, indicating an energy barrier to the cascade effect. Apart from directly using DNA as a SNARE mimic, Xu et al. used a programmable DNA origami platform to organize SNARE for membrane fusion.[106] In this case, SNARE proteins were conjugated onto liposomes and DNA was then used passively to bring the liposomes together for the SNAREs to assist liposome fusion. These DNA strands increased the initial rate of fusion by 10-fold, compared to simply having the SNAREs alone.

■ REPRESENTATIVE APPLICATIONS

The above contents are mainly focused on the fundamental aspects. Given the useful properties of both liposomes and DNA, impressive progress has been made on the application front as well, and a few applications are briefly discussed here. Since the main focus of this work is on the fundamentals, we only highlight representative examples in this section.

Drug Delivery. Liposomes are good drug delivery vehicles.[107] Drug molecules could be contained within the hydrophilic core, or in the hydrophobic membrane. Attaching DNA aptamers may facilitate active targeting to cancer cells (compared to passive targeting). Release of drugs within cells could be achieved by lowering pH, enzymes, or heat. Normally, a combination of lipids were used, rather than just one.[108]

Using the concept of spherical nucleic acids (SNAs), Mirkin and co-workers prepared densely functionalized liposomes.[18] They used a tocepherol moiety to anchor a DNA within the membrane of the liposomes and reported excellent uptake of the DNA-liposome conjugate in cells. It is important to note that the DNA used in this study was not an aptamer sequence. As such, this showed that DNA-functionalized liposomes generally were readily internalized by cells. In addition, the high density of DNA hindered degradation of the conjugate by serum proteins, increasing the stability in biological environments.

With respect to targeted delivery to cancer cells, Lu and co-workers attached the AS1411 aptamer to a liposome, using a cholesterol anchor, and observed high uptake in MCF-7 (breast cancer) cells.[17] They loaded cisplatin (an anticancer drug) within the liposomes and only saw a decrease in cell viability when the aptamer was conjugated. The effect of the aptamer was neutralized by adding its cDNA, forming a duplex. Tan's group used a different aptamer for targeting cancer cells.[120] They directly conjugated the aptamer to the lipid with a PEG spacer (Figure 10A), and uptake was preferred in the target cells. Since then, many studies have been published using this concept, and active targeting was achieved, even in mice.[109−111] PLGA supported lipids have also been conjugated with aptamers for targeting purposes.[112,113]

In addition to serving the targeting purpose, Mirkin and co-workers used their liposomal SNA system to stimulate the immune system through increased cytokine production.[67] They used a CpG-rich DNA sequence, which activated the toll-like receptor 9 (TLR9). After incubation with macrophages, a statistically significant increase in immune response was noted. This increase was higher for lipid-anchored DNA, compared to cholesterol-anchored DNA, suggesting that anchoring stability played a role in its immunomodulatory activity.

Biosensors. An interesting application of liposome/DNA conjugates is biosensor development. A regular sandwich assay correlates a binding event with a single fluorescent probe (Figure 10B, top row). In comparison, using a fluorophore-loaded liposome can correlate this binding event with hundreds or even thousands of fluorophores (Figure 10B, bottom row).[114−116] Bound liposomes are finally lysed using a surfactant to release the fluorescent cargo. Therefore, DNA-liposome conjugates provide a mechanism for signal amplification upon target recognition. This idea has been pursued by Baeumner and co-workers.[114−116] In these systems, the signal amplification was maximally ∼500-fold. This was far below the theoretical limit, assuming that each target DNA is associated with a liposome. Therefore, either multiple DNAs were needed to immobilize each liposome, the encapsulated dye was below the assumed concentration, or nonspecific liposome adsorption occurred. Amplified detection using liposomes was not confined to fluorescence. Willner and co-workers also used this concept for electrochemical detection.[16,117,118] More recently, Lin et al. encapsulated enzymes within liposomes for colorimetric detection of ochratoxin A.[119] This exploited the signal amplification capability of liposomes, as well as enzymes, to achieve highly sensitive detection.

Exosomes are another type of vesicle composed of cellular components and are similar to liposomes in size. Using cholesterol-functionalized DNA, Ye and co-workers designed a magnetic bead (MB)-DNA hybrid for exosome capture (Figure 10C).[54] Essentially, receptor-conjugated MBs were added to the sample containing exosomes to initiate capture by binding to the CD-9 on the exosome surface. Then, a duplex DNA with a sticky end was attached to the exosomes using a bivalent cholesterol anchor. Next, a hybridization chain reaction (HCR) was initiated using biotinylated DNA strands, followed by direct conjugation of streptavidin-horseradish peroxidase (HRP). The HRP catalyzed the oxidation of 3,3′,5,5′- tetramethyl benzidine (TMB) (mediated with H_2O_2), providing a colorimetric signal for the exosome recognition. This cascade signal amplification yielded a detection of 2.2×10^3 exosomes/μL, which was 100-fold better than ELISA-based detection mechanisms at the time.

In addition to using liposomes for signal amplification, we constructed a biomimetic sensor by splitting the adenosine aptamer into two halves.[86] One half of the aptamer was labeled with a FAM (fluorophore) and a cholesterol, and it was anchored into the liposome membrane at a density of 60 strands/liposome. The second half was labeled with TAMRA (quencher) and was anchored at a density of 120 strands/liposome. These two strands were dynamically assembled to the full aptamer in the presence of adenosine to produce fluorescence quenching of the FAM fluorophore, and the system had a detection limit of 60 μM adenosine.

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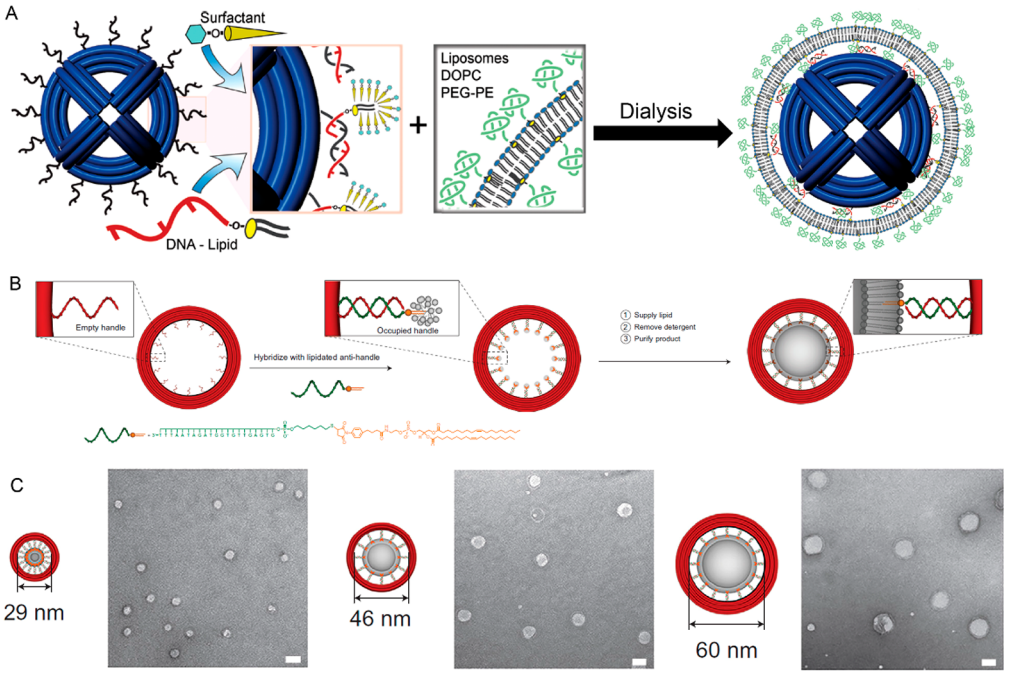

Figure 11. (A) Scheme for DNO encapsulation by liposomes. The DNOs were hybridized with DNA-lipid conjugates, which served as a template for growing a full lipid bilayer. [Reproduced with permission from ref 122. Copyright 2014, Royal Society of Chemistry, London.] (B) Schemes for DNA-origami templates for preparing uniform liposomes of different sizes. A hollow-spherical DNA shell was made with inner-sticky ends which could hybridize with DNA coupled with a lipid. The addition of more lipids directed the self-assembly within the DNA superstructure. (C) TEM micrographs of liposomes made using different sizes of DNA templates. [Reproduced with permission from ref 123. Copyright 2016, Nature Publishing Group, London.]

DNA Nanotechnology and Liposomes. DNA nanotechnology utilizes the structural properties of DNA to produce sophisticated nanostructures.[10,121] In recent years, such DNA nanostructures have been used for synthesizing liposomes. Shih's group used the DNA origami method to make DNA nano-octahedrons (DNOs) encapsulated by liposomes for drug delivery (see Figure 11A).[122] These DNOs were conjugated with lipids, which were stabilized by surfactants. Simple mixing with DOPC liposomes resulted in encapsulation of the DNOs. The liposome coating provided stability to the DNA against nucleases. Furthermore, the liposome-coated DNO was fully distributed in the circulatory system in rats, compared to free DNOs, which were retained in the bladder.

More-sophisticated liposomal structures were synthesized by Lin and co-workers.[123,124] An example of their concept is shown in Figure 11B.[123] They used DNA origami to synthesize a hollow DNA sphere, where the inner core was functionalized with a DNA-lipid conjugate. They then mixed the DNA sphere with extruded liposomes, resulting in the migration of lipids to the core and the subsequent formation of uniform liposomes defined by the DNA core. Optionally, the DNA template was removed, yielding uniform liposomes, as shown by transmission electron microscopy (TEM) (Figure 11C). A more recent work by the same group used rodlike DNA cages to form beads of uniform liposomes confined within the cage.[124]

Sleiman and co-workers showed dynamic behavior of DNA cages anchored on spherically supported lipid bilayers.[76] The cages were made using three 96-base strands and the self-assembly was spontaneous upon annealing from 95 °C to 5 °C. They anchored the cages on liposomes using cholesterol. They then systematically added shorter strands with a fluorescent label and observed hybridization with sides of the cages. The cages retained their structure upon anchoring. Furthermore, after certain modifications in the DNA strands added, dimerization of cages, as well as cage displacement by shorter oligomers was observed.

CONCLUSIONS AND FUTURE DIRECTIONS

In summary, we discussed a few important fundamental aspects of DNA-functionalized liposomes, in terms of bioconjugation, DNA conformation, surface interactions, directed assembly, and fusion. The soft and self-assembled nature of liposomes sets them apart from most inorganic nanomaterials, allowing high biocompatibility, lateral diffusion of DNA ligands, and their dynamic reorganization in response to external stimuli. For noncationic liposomes, their interaction with DNA is generally very weak, and this allows the binding activity of DNA to be maximally retained. Such materials have already found important applications in biosensing, drug delivery, and materials assembly. With further development of DNA bionanotechnology, we expect more intense research on this topic in coming years.

On the fundamental side, many interesting questions remain to be addressed. More quantitative measurement of DNA binding properties on the membrane surface is needed, as a function of salt concentration and lipid composition. Negatively charged DNA can be better screened and, thus, more densely packed at higher salt concentrations. Quantitative measurement of DNA lateral diffusion as a function of lipid composition is also an interesting topic, taking advantage of the ease of changing the length and structure of DNA and availability of many fluorophore labels. In addition, we expect that more modified DNA will be used. Most of the work reviewed in this manuscript used terminally modified DNA and more interesting examples might be produced by also

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internally modified DNA with hydrophobic chains or lipid molecules.[125,126]

On the application side, for biosensing, most work focused on simple DNA hybridization and some work also used aptamer binding. We have not seen many examples of conjugating DNAzymes to liposomes. Given the catalytic activity of DNAzymes, we expect to see interesting examples on this front in the near future. While representative examples have been shown for the signal amplification of sandwich assays using liposomes, potential research can be explored in varying liposome composition and its effect on liposome stability and subsequently, signal amplification of the biosensor. Other signal amplification using liposome surface can also be explored.

Some interesting novel applications are also scattered in the literature, and we may see more of their growth in the near future. For example, membrane-anchored DNA assembly was used for energy harvesting and electron transfer.[127] Using lipid-labeled DNA, it is possible to functionalize cell membranes with DNA, and to program the assembly of cells.[128] This has opened up new tools for studying cell−cell interactions. Liposome-to-micelle transitions have also been realized via DNA hybridization.[129] Finally, DNA nanostructures have also been used to grow interesting liposome structures, including those with nonspherical shapes.[61,123] It is also interesting to learn to dynamically control DNA to realize more-sophisticated functions such as transmembrane communications.

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Notes

The authors declare no competing financial interest.

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