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## Lipid-Oligonucleotide Conjugates for Simple and Efficient Cell Membrane Engineering and Bioanalysis

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### Abstract

Cell membrane modification is important for tissue engineering, cell-based therapies, and cell biology studies. Recently, oligonucleotides have attracted considerable attention to remodel and functionalize live cell membranes. In particular, a type of amphiphilic lipid-oligonucleotide conjugates have been rationally designed and synthesized for this purpose. These conjugates have enabled a rapid, straightforward and efficient cell membrane modification. Taking advantage of the highly precise and programmable self-assembly of DNAs and RNAs, lipid-oligonucleotide conjugates have been used for membrane bioanalysis, therapeutics, building artificial membrane structures, and regulating cell–surface and cell–cell interactions. In this review, we have summarized the current knowledge in the design, synthesis, and regulating membrane properties of lipid-oligonucleotide conjugates. In addition, their state-of-the-art applications in cell membrane engineering and bioanalysis have been illustrated.

### Keywords

Lipid-oligonucleotide conjugate; DNA; cell membrane modification; membrane analysis; cell regulation

### Introduction

Composed of a protein-and-carbohydrate-embedded lipid bilayer, cell membranes serve as borders for biological organization and protect the cell from its surroundings. The membrane structures also play important roles in cellular communication and signaling. There has been an ever-increasing interest in synthetically modifying cell membranes. Such engineered membranes can be broadly useful from studying cell biology to tissue engineering and cell-based therapies [1–3].

DNA oligonucleotides have emerged as the material of choice to functionalize and remodel cell membranes [4,5]. Cell membrane-anchored DNA oligonucleotides have been used for

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Conflicts of interest

There are no conflicts of interest to declare.

membrane bioanalysis and regulation, drug deliveries, and controlling cell–surface and cell–cell interactions. The most attractive feature of these oligonucleotides is their highly precise and tunable self-assembly. Various structures and nanodevices can be rationally designed with molecular-level precision and programmable performance [6,7].

Several strategies have been developed for modifying DNAs onto cell membranes, including *in situ* chemical interaction with membrane functional groups [8–10] and non-covalent anchoring through antibodies, aptamers, or hydrophobic moieties [4,5,11,12]. Among these, lipid-oligonucleotide conjugates have attracted particular attention [4,5,7]. By chemically conjugating a hydrophobic lipid moiety with hydrophilic oligonucleotide, these amphiphilic conjugates enable fast, easy, and highly efficient DNA modification on live cell membranes. In this contribution, we will discuss the design, synthesis, and membrane properties of lipid-oligonucleotide conjugates, as well as their recent applications for cell membrane analysis and engineering.

## Design of lipid-oligonucleotide conjugates

Lipid-oligonucleotide conjugate typically comprises three moieties: lipid, linker, and nucleic acid (Figure 1a). To construct cell membrane nanodevices, DNA is the most commonly used nucleic acid moiety. DNA can be cost-effectively synthesized and easily conjugated with lipids and other functional groups, such as fluorophores, drugs, and cross-linkers [13]. Modified oligonucleotides including PNA, LNA, 2'-F, and 2'-OMe-modified oligomers and siRNA analogs, have been conjugated with lipids for membrane modification and biomedical deliveries [14]. These modified nucleic acids can improve the cellular and *in vivo* biostability of lipid-oligonucleotide conjugates. Instead of the direct linkage, synthetically challenging conjugates, such as those containing miRNA, siRNA, and mRNA, can also be indirectly prepared based on sequence-specific hybridization [15–17]. The precise self-assembly of nucleic acids have allowed the conjugation of lipids with various nanostructures as well, ranging from duplexes, three-way junctions to DNA/RNA origamis [7,14].

A linker is often added between lipid and oligonucleotide. Polyethylene glycol (PEG) is a popular linker of moderate hydrophobicity and flexibility. DNA oligonucleotides and the head group of lipids can also function as the linker. Some linkers are introduced for easy synthesis, but they can be also useful in adjusting the hydrophilic/hydrophobic ratio of amphiphiles, as well as the morphology and size of aggregations [18]. Linkers may also function as spacers to extend membrane-anchored oligonucleotides, and as a result, to reduce electrostatic interactions between oligonucleotides and the cell surface. These spacers can also increase the membrane availabilities of oligonucleotides for the target binding [19].

Sterols, especially cholesterol, are the most popular lipid moieties due to their commercial availability and high membrane modification efficiency [20–24]. Other natural membrane lipids, such as phospholipids, tocopherols, and lithocholic acids, have also been demonstrated as good candidates [16,24,25]. In addition, natural lipid derivatives, especially synthetic fatty acids of different lengths, numbers of chains, and degrees of saturation, are also useful lipid moieties. These lipids can exhibit tunable hydrophobicity and membrane

interaction patterns [4,26,27]. In general, considering their low cell toxicity and membrane disruption, natural lipid moieties are more favorable than synthetic lipids for cell membrane engineering. While the practical choice of lipids largely depends on their synthetic efficiency, as well as physicochemical and membrane properties.

## Synthesis of lipid-oligonucleotide conjugates

Lipids can be incorporated into oligonucleotide strands through either pre-synthetic or post-synthetic strategies (Figure 1). In pre-synthetic approach, a lipid phosphoramidite (for internal or 5'-modification) or lipid-conjugated controlled-pore glass (CPG, for 3'-modification) is first prepared and used during solid-phase synthesis (SPS) of oligonucleotides. The position, purity, and number of lipid modifications can be precisely controlled. Various phospholipid, cholesterol, tocopherol, and fatty acid-modified oligonucleotides have been synthesized using this strategy [18,20]. Compared with post-synthesis, pre-synthesis exhibits high incorporation efficiency, high accuracy, and short linkage.

Many lipid moieties of limited solubility and stability may not be prepared as phosphoramidite or not tolerate the harsh DNA synthesis condition. Automated DNA synthesizer is also not available in most laboratories. In this case, post-synthesis by conjugating lipids with commercially available oligonucleotides can be a choice. Several functional groups, such as amine, thiol, alkyne, azide, and N-hydroxysuccinimide ester, can be readily synthesized in oligonucleotides. As a result, lipid-oligonucleotide conjugates can be prepared based on interactions such as thiol-maleimide reaction, click chemistry, or amide bond formation [7,13,26]. These post-synthetic approaches can be performed in either solution or solid-phase. In solution-phase synthesis, surfactants are often needed to homogenize the reaction mixture, and extra care is needed to purify the lipid-oligonucleotide conjugates. SPS provides extra benefit in removing excess reactants. The availability of both pre- and post-synthetic strategies have paved the way for potential applications of lipid-oligonucleotide conjugates.

## Physicochemical and membrane properties of lipid-oligonucleotide conjugates

The chemical and biomedical behaviors of lipid-oligonucleotide conjugates are largely determined by their assembly status in solution and cell membranes (Figure 2). Various interactions exist in these amphiphilic conjugates including hydrogen bonding, hydrophobic and electrostatic interactions. In solution, lipid-oligonucleotide can be self-assembled into micelles, liposomes, bilayers, and other nano-/microstructures. One important parameter is the critical aggregation concentration, above which amphiphilic monomers stay in the aggregated form. Our recent results indicate that monomeric, rather than aggregated form, of lipid-oligonucleotide conjugates regulates the kinetics and efficiency of cell membrane modification [27]. Considering the competition between aggregates formation and the cell membrane insertion of lipid-oligonucleotides, the conjugate assembly pattern in solution will indeed also influence their cell membrane modifications.

The cell membrane insertion kinetics, efficiency, persistence, and transmembrane efficiency of lipid-oligonucleotide conjugates are important factors determining their biomedical and analytical applications. In general, fast insertion (seconds to minutes) and long persistence (hours to days) is desirable for cell membrane engineering. While on the other hand, the efficient cell membrane anchoring may also reduce the transmembrane efficiency of lipid-oligonucleotide for potential biomedical applications. High-density cell membrane modification may facilitate applications in regulating cell–surface or cell–cell interactions. While low-efficiency membrane modification, with less influence on natural membrane functions, can be beneficial in membrane bioanalysis.

The hydrophobicity of lipid-oligonucleotide conjugates is critical for their rapid and efficient membrane insertion. Several studies have indicated that lipids with longer or more carbon chains, *i.e.*, more hydrophobic, tend to modify cell membranes with higher efficiency [18,19,21,26]. Our results, however, indicated that there could be an optimal hydrophobicity, above which value more hydrophobic lipids will actually exhibited reduced membrane modification efficiency and kinetics [27]. Those highly hydrophobic conjugates can form tight aggregation in solution, which will reduce their chance in the monomeric form to enter cell membranes.

The net membrane modification efficiency can be viewed as the result of a dynamic equilibrium between membrane insertion, distribution, and release (Figure 2). In addition to the hydrophobicity, the size and shape of oligonucleotide, concentration of conjugates, temperature, and composition of cell membranes and culture medium will also influence the membrane modification of lipid-oligonucleotide conjugates [19,21,28]. It is still challenging to predict membrane modification efficiency of a lipid-oligonucleotide structure under a particular experimental condition.

Low membrane persistence is another challenge in applying lipid-oligonucleotide for long-term cell membrane engineering. Membrane-anchored oligonucleotides normally disappears within 1–4 h [19,23,24]. These lipid-oligonucleotides are subject to either endocytosis or releasing to solution [19,27,29]. Reducing the temperature appears to prolong the membrane retention of lipid-oligonucleotides [30]. Our recent study revealed that by adding an endocytosis inhibitor, filipin, membrane-modified oligonucleotides could exist for over 24 h [27]. Another strategy to improve membrane persistence is by forming large membrane network of these lipid-oligonucleotides [31,32]. For example, based on a hybridization chain reaction, in the presence of polyelectrolytes, lipid-oligonucleotide can be retained on cell membranes for over two weeks [31]. Still, more studies are needed to understand how to fine-tune both the membrane persistence and transmembrane efficiency of these conjugates.

Another important property of lipid-oligonucleotides is their ability to hybridize with strands of complementary sequences. Cis-interacting oligonucleotides (on the same cell membrane) can be useful in forming membrane nanostructures and functional nanodevices [4,5,33]. While trans-interaction of oligonucleotides on different membranes can facilitate their applications in the analysis and regulation of cell–surface or cell–cell interactions [34,35]. It is thus critical to ensure the availability and hybridization specificity of the membrane-anchored oligonucleotides.

More recently, the membrane selectivity of lipid-oligonucleotides begins to be studied. Selective membrane modification can be useful for the targeted therapeutic delivery, membrane barcoding and cell profiling. However, most previous studies indicated that lipid-oligonucleotide would non-selectively insert onto different cell membranes. Interestingly, a recent report indicated that cell-specific membrane proteins, such as alkaline phosphatase, could be used to *in situ* modify the hydrophobicity of lipid-oligonucleotide [28]. As a result, efficient membrane modification could only occur on target cells expressing these membrane proteins. Other similar approaches may show up later to further fine-tune the membrane selectivity of lipid-oligonucleotides.

## Applications in cell membrane engineering and analysis

The high membrane insertion efficiency, generalizability towards various cell types, and noninvasive and simple procedure have made lipid-oligonucleotide a great tool for cell membrane regulation and analysis. Based on different structures, interactions, and switching properties of oligonucleotides, several interesting membrane applications have been recently achieved (Figure 3).

First, functional oligonucleotides have been modified onto live-cell membranes for bioanalysis. For example, using a diacyllipid-DNAzyme conjugate, Qiu *et al.*, were able to monitor metal ion levels nearby the cell surfaces [36]. Similarly, a tocopherol-aptamer conjugate was used to real-time image the release of adenine in brain astrocytes [25]. More recently, cellular extrusion of SO<sub>2</sub> and NO was detected by a membrane-anchored G-quadruplex structure, together with a stimuli-reactive cofactor (Figure 3a) [22]. For all these membrane-anchored oligonucleotide sensors, one key feature is that they specifically measure the local dynamics of targets in the membrane microenvironment. Such information can be potentially important in understanding cellular communication and signaling pathways.

These membrane-anchored oligonucleotides can also be used in therapeutics. For example, by anchoring cancer-targeting DNA aptamers onto immune cell surfaces, Xiong *et al.*, developed a cell-based therapeutic approach to target and kill cancer cells (Figure 3b) [35]. As another interesting example, by conjugating therapeutic nucleic acids with lipid moieties, the anti-tumor efficacy can be dramatically improved [18,37]. These conjugates have enhanced stability during circulating and exhibited enriched local concentration at the tumor site. The cell membrane binding of lipid-oligonucleotide contributed to their enhanced therapeutic effects [18,38].

In addition, based on the sequence-specific DNA hybridization, different types of nanostructures can be built on the cell membranes. For example, membrane-anchored DNA nanopores have been used for controlled target transportation [39–41]. With precisely controlled shapes and sizes, these DNA nanostructures can potentially mimic and extend the function of natural membrane proteins. As another example, a lipid-modified DNA duplex was recently used to quantify cell membrane potentials (Figure 3c) [42]. Here, the stoichiometric DNA hybridization allows the ratiometric quantification with a voltage-sensitive dye and an internal reference.

The specific cell membrane DNA hybridization can also be used to regulate cell–surface and cell–cell interactions. In addition to their potential applications in tissue engineering, we have recently demonstrated a lipid-DNA hairpin probe to visualize and measure mechanical forces at cell–cell junctions (Figure 3d) [23]. Upon experiencing tensile forces generated by neighboring cells, unfolding of the DNA hairpin led to a dramatic increase in the fluorescence signal. In another recent study, lipid-DNA pyramidal probe was developed to control cell–cell interactions [43]. Interestingly, by incorporation three cholesterols, the membrane-anchoring efficiency of these DNA pyramids can be dramatically improved.

Other membrane biophysical events can also be measured based on the dynamic hybridization of oligonucleotides. For example, we recently engineered a DNA probe to monitor transient membrane lipid–lipid interactions (Figure 4) [24,44]. Here, a proximity-based strand displacement reaction was used to convert transient membrane interactions into detectable fluorescence signals. As a result, previously undetectable membrane events can now be studied with the help of lipid-oligonucleotide conjugates.

## Conclusion and outlook

Lipid-oligonucleotide conjugates are becoming popular for cell membrane engineering, regulation, and analysis. Recent progress in bioconjugate chemistry and synthetic biology has further promoted the fast growing of this field. Taking advantage of these lipid-oligonucleotide conjugates, different functional moieties can be efficiently engineered onto live cell membranes, without affecting natural cellular behavior. Various previously inaccessible membrane events can now be exploited to enhance our understanding of cell membrane structures, interactions, and functions.

These lipid-oligonucleotide conjugates exhibit several attractive features for membrane applications, including (1) spontaneous, rapid, and efficient membrane insertion after simple incubation; (2) universality towards different cell types; (3) precise hybridization and dynamic switching of oligonucleotides; and (4) versatile functionality thanks to the simple DNA conjugation chemistry.

Even though lipid-oligonucleotide conjugates have demonstrated great potential and unique membrane applications, they are still emerging tools for cell membrane engineering. There are several questions and challenges. (1) Even though the lipid moieties are either naturally existing or structurally similar to those in cell membranes, the biological influence of these synthetic membrane anchors still need to be carefully assessed. (2) Membrane persistence of many lipid-oligonucleotides needs to be improved. Tunable membrane insertion efficiency and persistence will be beneficial for different applications that may need either long-term membrane modification or fast transmembrane delivery. (3) Another interesting question is how to achieve selective anchoring of lipid-oligonucleotide onto the membrane of particular cell type, which may provide some new routes for targeted therapy or cell profiling.

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## Abbreviations

<b>PNA</b>	peptide nucleic acid
<b>LNA</b>	locked nucleic acid
<b>2'-F</b>	2'-fluoro
<b>2'-OMe</b>	2'-O-methyl
<b>CPG</b>	controlled-pore glass
<b>DNA</b>	deoxyribonucleic acid
<b>RNA</b>	ribonucleic acid
<b>miRNA</b>	microRNA
<b>mRNA</b>	messenger RNA
<b>siRNA</b>	small interfering RNA
<b>NO</b>	nitric oxide
<b>PEG</b>	polyethylene glycol
<b>SO<sub>2</sub></b>	sulfur dioxide
<b>SPS</b>	solid-phase synthesis
<b>FRET</b>	fluorescence resonance energy transfer

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\* Papers of special interest

\*\* Papers of outstanding interest

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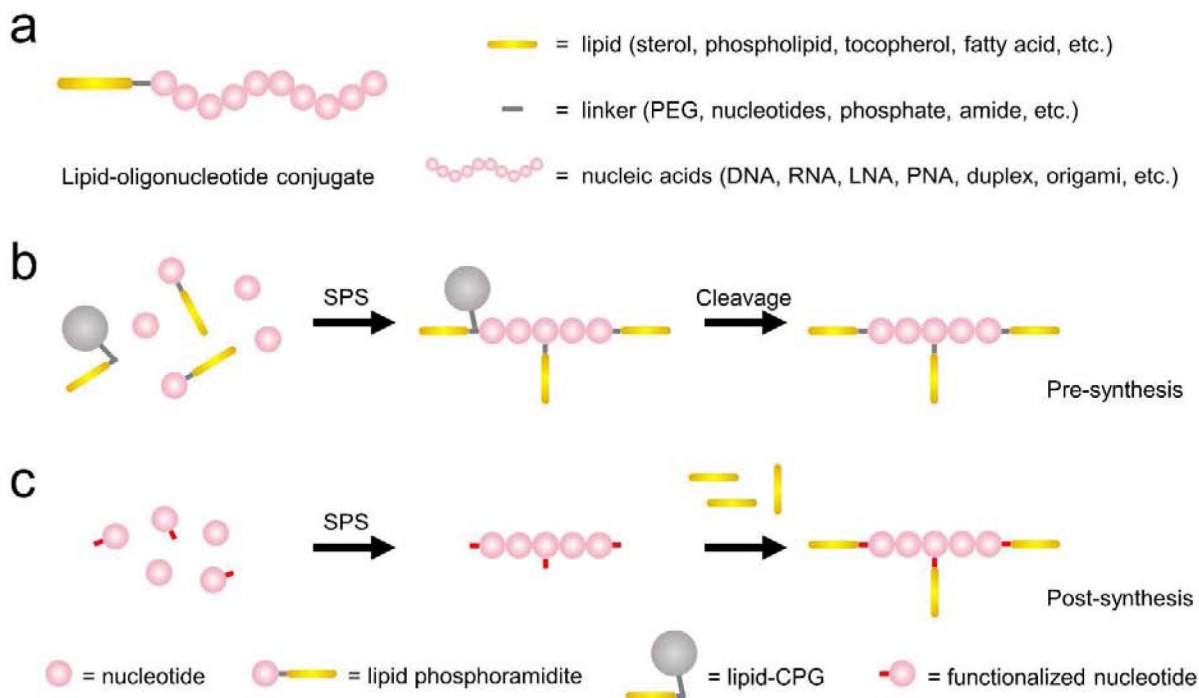
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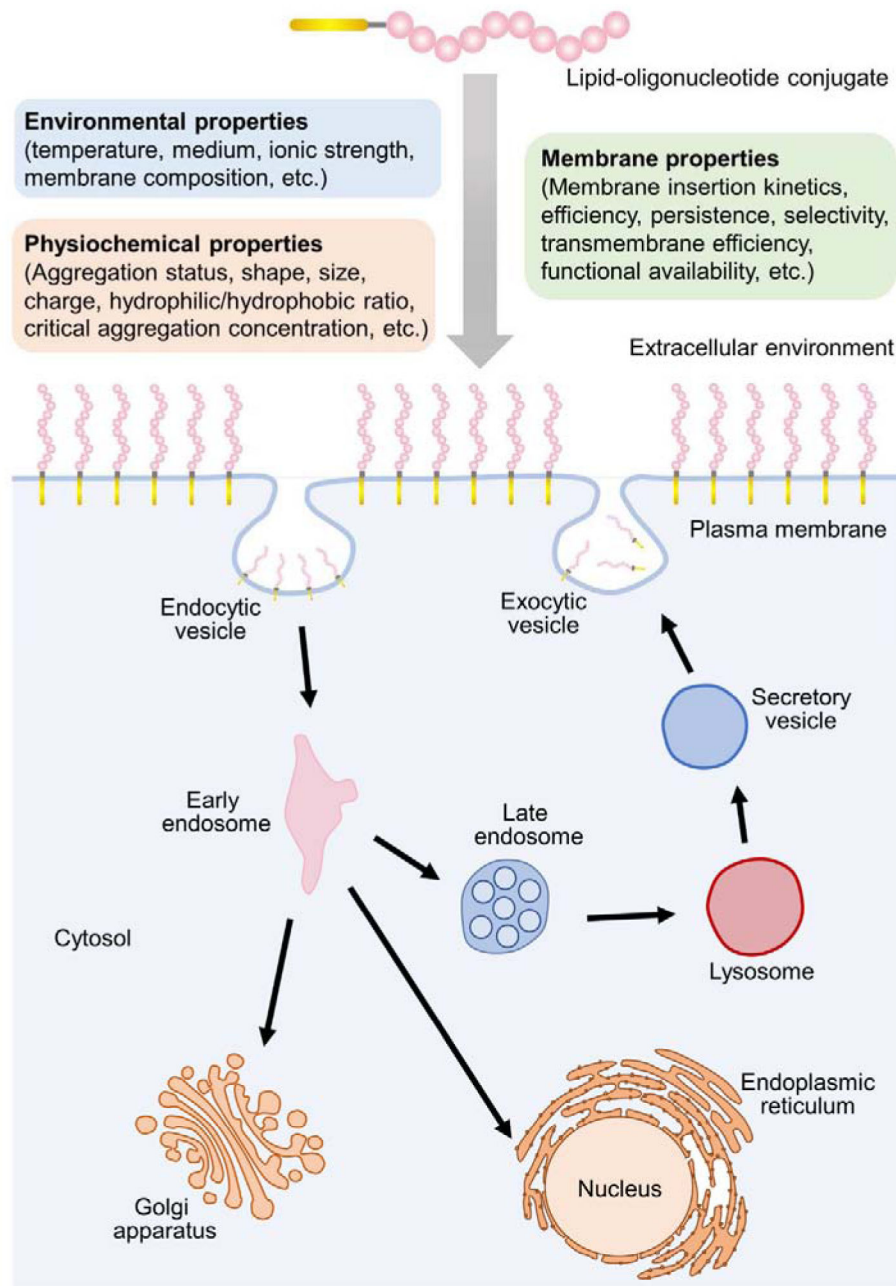
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### Highlights

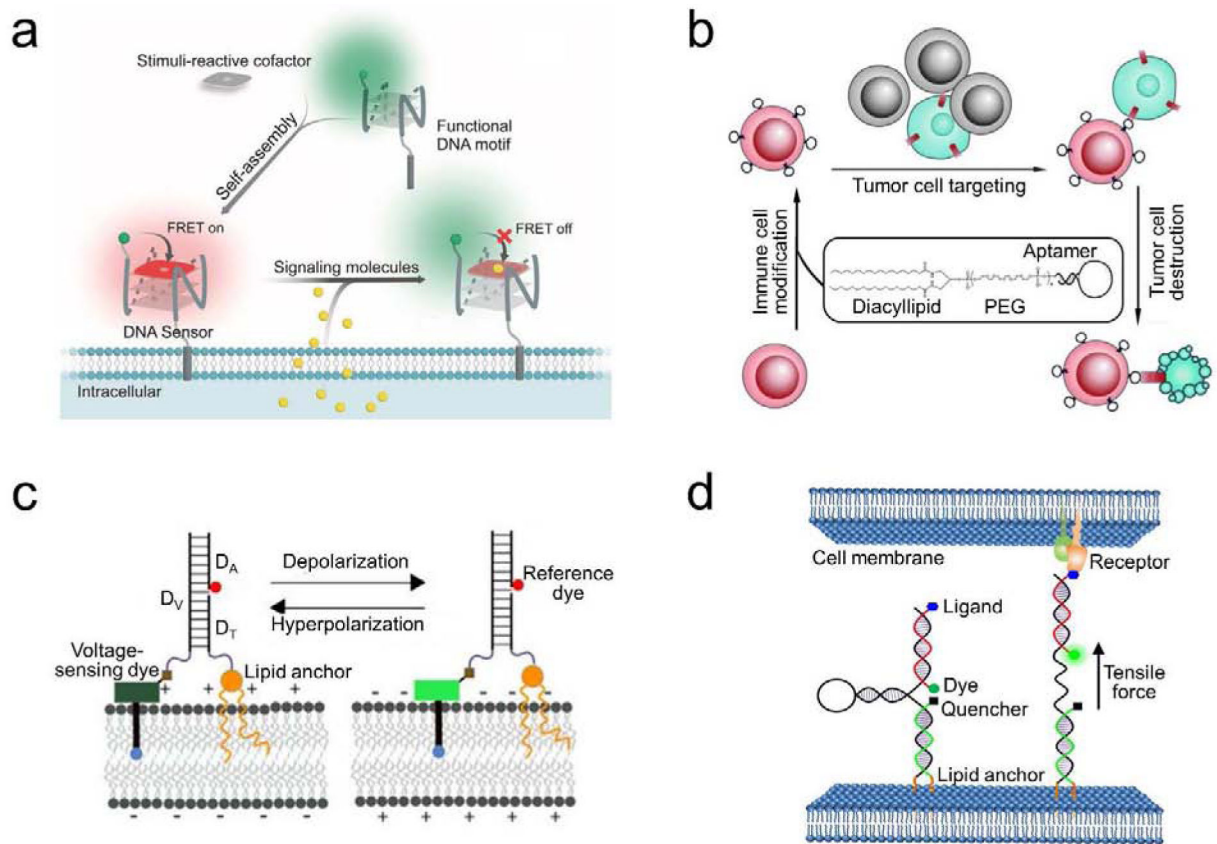
1. Lipid-oligonucleotide conjugate is a new tool for the rapid, straightforward and efficient modification of live cell membranes.
2. These amphiphilic conjugates can be rationally designed and synthesized.
3. Cell membrane insertion kinetics, efficiency, persistence, and transmembrane property of lipid-oligonucleotide conjugates can be tuned for various biomedical and analytical applications.
4. Several previously inaccessible membrane events can now be exploited using lipid-oligonucleotide conjugates.



**Figure 1.** Design and synthesis of lipid-oligonucleotide conjugates. (a) The conjugate typically comprises three moieties: lipid, linker, and nucleic acid. (b) Schematic of the pre-synthetic approach for the synthesis of lipid-oligonucleotide conjugates. Lipid phosphoramidite or lipid-conjugated controlled-pore glass (CPG) was conjugated together with other nucleotides during the solid-phase synthesis (SPS) of oligonucleotides. After cleavage from CPG, lipids were precisely modified at different positions of oligonucleotides. (c) Schematic of the post-synthetic approach for the synthesis of lipid-oligonucleotide conjugates. After solid-phase synthesis (SPS) of oligonucleotides containing some functional groups, lipids were then chemically conjugated through reaction with these functional groups.



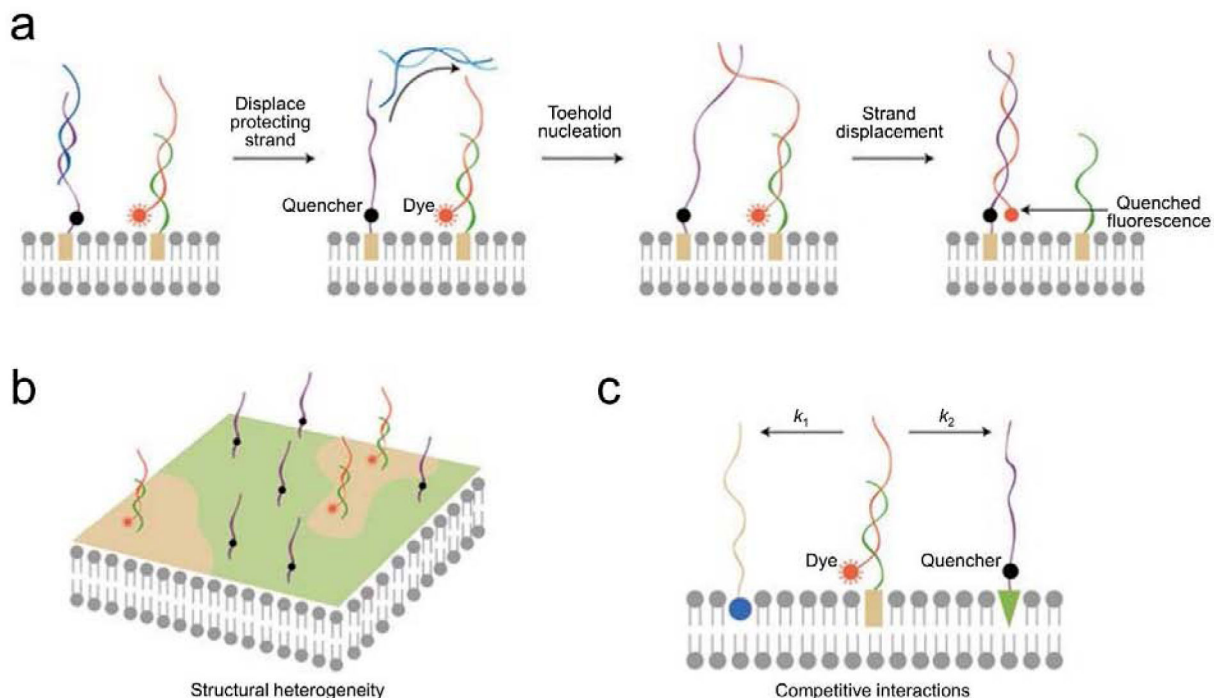
**Figure 2.** Physicochemical and membrane properties of lipid-oligonucleotide conjugates. Various environmental properties and physicochemical properties of the conjugates will influence their membrane properties. Shown are the dynamic process of lipid-DNA probe modification on the cell membrane and internalization into the cells through endocytosis pathway.



**Figure 3.**

(a) Schematic of a membrane-anchored DNA G-quadruplex sensor for the real-time imaging of signaling molecules [22]. Target-induced changes in the fluorescence resonance energy transfer (FRET) efficiency between the DNA-modified fluorophore and a stimuli-reactive cofactor was used for the detection. (b) Schematic of a lipid-aptamer conjugate for the regulation of cell-cell interactions and cell-based therapy [35]. With the help of diacyllipid, cancer cell-targeting DNA aptamers were first modified onto the surface of immune cells. The anchored aptamers then facilitated the binding and killing of cancer cells by these membrane-engineered immune cells. (c) Schematic of a lipid-modified DNA voltmeter for the membrane potential detection [42]. The precise self-assembly of three DNA strands,  $D_V$ ,  $D_A$ , and  $D_T$ , provided a scaffold for the ratiometric measurement between a voltage-sensing fluorophore and a reference dye. The membrane potential could thus be accurately imaged and quantified. (d) Schematic of a DNA-based tension probe for visualizing intercellular tensile forces [23]. The design was based on the self-assembly of three oligonucleotides with a pair of cholesterol anchors at one end to insert onto cell membranes. Upon experiencing an intercellular tensile force exceeding the threshold force to unfold the DNA hairpin, the dye separated from the quencher and resulted in an increased fluorescence signal. Figures are adapted and modified with permission from references [22], [23], [35], and [42].





**Figure 4.** (a) Schematic of using DNA probes for monitoring transient lipid–lipid encounters on live cell membranes [24]. The addition of an initiator strand (cyan) hybridized and removed the block strand (blue). As a result, once there was a membrane lipid–lipid encounter, the translocation of the DNA probe (red) from one anchor site (green) to another (purple) induced the quenching of fluorescence. (b) Lipid-DNA probes can be used to study membrane heterogeneity. Different lipids preferred to insert into different membrane domains. Lipids of different domains tended to encounter less with each other and resulted in a slow fluorescence quenching. (c) Lipid-DNA probes can be used to measure the competitive interactions among different membrane lipids. Dye-labeled lipid (orange rectangle) was given two possible destinations, unlabeled lipid 1 (blue ball) or quencher-labelled lipid 2 (green triangle). The relative interaction rate ( $k_1$  and  $k_2$ ) could be determined based on the rate of fluorescence quenching. Figures are adapted and modified with permission from reference [44].