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Lipid Conjugated Oligonucleotides: A Useful Strategy for Delivery

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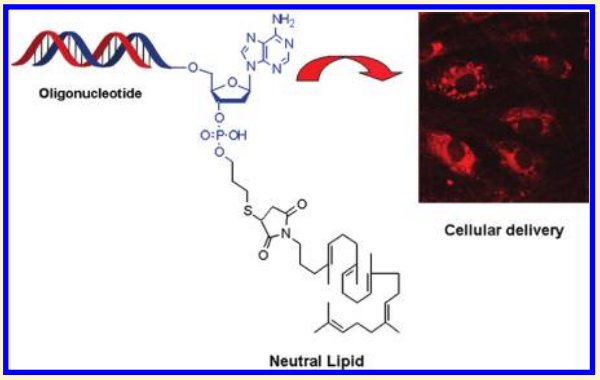
ABSTRACT: Oligonucleotides, including antisense oligonucleotides and siRNA, are promising therapeutic agents against a variety of diseases. Effective delivery of these molecules is critical in view of their clinical application. Therefore, cation-based nanoplexes have been developed to improve the stability as well as the intracellular penetration of these short fragments of nucleic acids. However, this approach is clearly limited by the strong interaction with proteins after administration and by the inherent toxicity of these positively charged transfection materials. Neutral lipid−oligonucleotide conjugates have become a subject of considerable interest to improve the safe delivery of oligonucleotides. These molecules have been chemically conjugated to hydrophobic moieties such as cholesterol, squalene, or fatty acids to enhance their pharmacokinetic behavior and trans-membrane delivery. The present review gives an account of the main synthetic methods available to conjugate lipids to oligonucleotides and will discuss the pharmacological efficacy of this approach.



INTRODUCTION

The therapeutic use of oligonucleotides (ONs) (either antisense oligonucleotides AS-ON or siRNA) has gained a lot of attention. Their high specificity covering a wide range of biomedical applications allows the inhibition of target proteins that are not easily accessed and modulated by conventional small molecular weight or protein drugs.[1−8] As a prelude, it is important to recall that ONs do not cross the intact cell membranes to any significant degree via simple diffusion. This is primarily due to the highly hydrophilic and anionic character of these molecules which have poor affinity for the negatively charged cell membranes. Thus, development of efficient delivery systems is one of the most challenging hurdles to turn ONs into clinically acceptable therapeutic drugs. So far, a wide variety of approaches including viral vectors as well as nonviral delivery systems, such as liposomes, nanoparticles, micelles, and polyplexes[9−15] have been investigated to enhance target cells uptake and silencing potency in vivo. However, the safety of viral vectors is questionable due to immunogenicity and possible recombination of oncogenes. On the other hand, most of the nonviral vectors are designed using polycations, either polymers or lipids, whose (cyto)toxicity is now well-documented.[16,17] That is the reason the conjugation of nucleic acids with neutral lipids offers an interesting alternative, since it has been reported that this approach could (i) significantly enhance cellular uptake of AS-ONs and siRNA, (ii) prolong the half-life of these molecules in plasma, and (iii) increase the efficiency of siRNA-induced gene silencing in vivo.[13,18−20] Despite the richness of the different synthetic strategies developed for the modification of the ONs,[21] the synthesis of lipid−ON conjugates (LOC) is anything but trivial and requires extensive expertise in organic chemistry and solid-phase synthesis. These covalently attached LOCs can be synthesized either by postsynthetic coupling of prepurified lipids and ONs or by stepwise solid-phase synthesis without intermediate purification. This short review attempts to provide an overview of the vast repertoire of chemical protocols developed so far for the chemical synthesis of LOCs.

BACKGROUND

Antisense and RNAi Mechanisms. Antisense oligonucleotides (AS-ONs) and RNA interference (RNAi) are the two major approaches for therapeutic gene expression. Although the activity of both types of molecules is due to their base-pairing capacity with complementary cellular nucleic acids, the method by which they inhibit gene expression is different. An understanding of the mechanisms of action of AS-ON and RNAi is a prerequisite for the design of efficient lipid conjugates of these molecules.

AS-ON. AS-ONs are single-stranded DNA sequences of 13−25 bases, which can highly selectively inhibit the expression of target genes by hybridizing with their specific "sense" sequences in mRNA (mRNA) or DNA molecules.[22,23] Ribonuclease H

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(RNase H)-mediated degradation of complementary mRNA is the major mode of action of AS-ONs.[24] This enzyme specifically cleaves the RNA strand of RNA-DNA heteroduplexes, leading to the release of the AS-ON which can further bind to a new mRNA strand; this mechanism is therefore catalytic.[24]

RNAi. Suppression by double-stranded RNA (dsRNA) is an important endogenous mechanism of gene regulation.[25] Briefly, the "Dicer" enzyme and its cofactors cleave dsRNA into 21- to 23-mer segments called short interfering RNAs (siRNAs) and assist its loading onto the Argonaute 2 (Ago 2)-containing "RISC" proteic complex. In the RISC assembly process, Ago2 cleaves the passenger strand, thereby liberating the guide strand from the siRNA duplex and producing active RISC capable of cleaving target mRNA.[26,27] The guide strand serves as a template for the recognition of homologous mRNA, which upon binding to RISC is cleaved by the catalytic activity of Ago2 between bases 10 and 11 relative to the 5′ end of the guide siRNA strand;[5,28−31] therefore, the RNA is degraded and the translation is arrested. Noteworthy, the template siRNA is not affected by this reaction, so the RISC can undergo numerous cycles of mRNA cleavage that explain the high efficiency of RNAi and its use as a therapeutic target.

Major Challenges for Oligonucleotides and siRNA Delivery in Vivo. As polyanionic macromolecules, ONs face multiple obstacles including plasma membrane, cytoplasm, and nuclear membrane to reach their intracellular site of action. Moreover, naked AS-ONs and siRNA are relatively unstable in the blood due to nonspecific uptake by the reticulo-endothelial system (RES) and aggregation with serum proteins and are rapidly cleared from the body by rapid renal excretion owing to degradation by nucleases (half-life in serum is few minutes).[32] Issues of stability have been solved in part by the introduction of chemical modifications that provide greater metabolic stability or improved bioavailability. There have been several recent reviews that provide a good overview of chemical modification strategies for siRNA[33,34] and AS-ONs.[35,36] Nonetheless, ON delivery still faces many challenges. As illustrated in Figure 1, ONs have to overcome several obstacles to reach their intracellular site. Then, an effective delivery strategy must take account the need to (i) protect the ON from the enzymatic digestion, (ii) improve the pharmacokinetics by avoiding uptake via the RES and rapid renal filtration, (iii) allow better translocation through the endothelium, (iv) facilitate the diffusion through the extracellular matrix, and (v) enhance cellular uptake and (vi) intracellular endolysosomal escape. Such delivery systems would also minimize potential ON-induced toxicities.[37]

Mechanisms of Endocytosis and Intracellular Trafficking. Endocytosis covers multiple distinct uptake pathways that are subdivided into three major classes: (i) phagocytosis (that mainly takes place in "professional phagocytes" such as macrophages and granulocytes); (ii) macropinocytosis (in which macromolecules being internalized as simply dissolved in the ambient medium); and (iii) receptor mediated endocytosis (that often involve a cell surface receptor). The last class comprises three diverse uptake pathways: (i) the "classic" clathrin-coated pit pathway, (ii) the caveolar pathway, and (iii) one or more noncaveolar, clathrin-independent pathways (CLIC pathways).[38]

It is generally believed that ONs are internalized into the cytoplasm by cell surface receptors via receptor-mediated endocytosis[39,40] which includes three major steps. The first step consists of the ligand−receptor binding. In fact, the complex enters vesicles that bud from the cytosolic face of the plasma membrane. In the second step, the initial uptake is followed by sequential intracellular trafficking into a variety of low pH endomembrane compartments, including early/sorting endosomes, late endosomes/multivesicular bodies, and lysosomes. The last step leads to the endosomal escape of ON to reach the cell cytoplasm (or nucleus) and to exert pharmacological activity. The general aspects of the cellular uptake of ONs have been studied and extensively reviewed.[35,40−42]

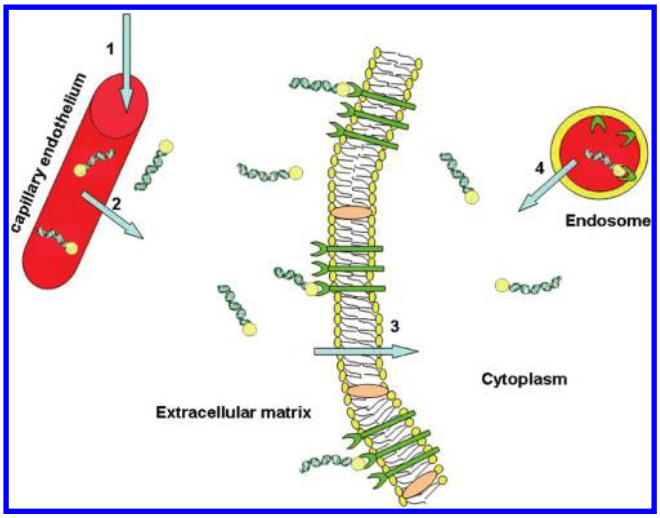

Figure 1. In vivo barriers for the effective delivery of antisense and siRNA oligonucleotides. There are four major barriers for oligonucleotides to gain access to cells and take effect on their intracellular targets. After administration into the blood circulation, the oligonucleotide must avoid rapid degradation by serum nucleases, clearance by the reticulo-endothelial system, and rapid excretion by renal filtration.[1] Further, the oligonucleotide must gain access to the target cells by crossing the capillary endothelium and diffusing through the extracellular matrix.[2] The oligonucleotide has to be further taken up by the target cells, typically through a receptor-mediated endocytosis process.[3] Inside the cells, the oligonucleotide must be released from the endosomes to reach their intracellular targets.[4]

Approaches to Deliver Oligonucleotides. Two major approaches, based on either chemistry or formulation, were adopted to overcome the above-mentioned limitations concerning ONs administration. The chemical approach consists either of synthesizing nucleic acids with variations in their natural structure (in order to improve resistance toward degradation) and/or of attaching a lipid, peptide, or polymer moiety associated or not with a cell targeting ligand. The potential advantages of the chemical strategy are as follows: (i) ON preferential targeting toward specific tissues and cells which express specific receptors, (ii) delivery of ON to appropriate intracellular compartments, i.e., cytoplasm or nucleus, and (iii) better ON away from the blood and widespread distribution in tissues due to the small size of the chemical conjugates in comparison to the nanoformulations mentioned below.

The formulation approach generally uses cationic lipids or polymers which can interact with ONs through strong electrostatic interactions, leading to the so-called "nanoplexes" with size between ten and a few hundred nanometers. The expected advantages of the nanoformulation approach are better stability in biological fluids and also improved cell penetration and tissue biodistribution. This strategy has already

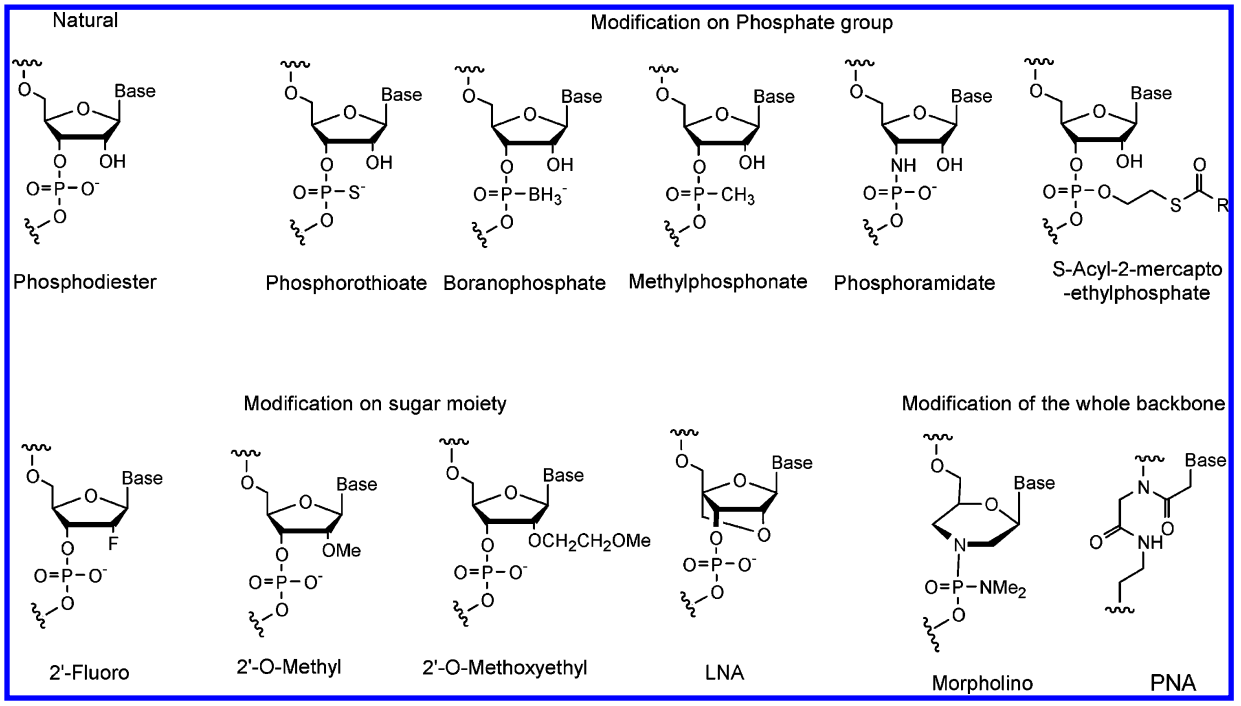

Figure 2. Chemical modifications: structures of the various chemically modified oligonucleotide residues.

achieved significant success in both cellular and animal studies.[43−45] However, the toxicity of cationic transfectants either lipidic or polymeric may raise major issues for clinical utilization.[16] Indeed, creating a positive surface charge promotes serum protein binding, so that cationic components are typically toxic even when biodegradable linkages are employed.[46] For instance, concerning cationic liposomes, significant toxicity including cell contraction, mitotic inhibition, formation of aggregates in blood, and the tendency to induce inflammatory response has been reported.[47,48] Similarly, polymers like PEI have shown marked cytotoxicity due to induction of cell death (necrosis and apoptosis).[49] This explains why, although hundreds of compounds have been in the discovery phase, only fourteen entered clinical trials, among which four are designed for ocular administration or inhalant administration and only five for systemic administration (QPI-1002 by Quark Pharm., ALN-TTR01, TKM-ApoB, ALN-VSP by Alnylam Pharm, TKM-PLK1 by Protiva Biotherapeutics and CALAA-01 by Calando Pharm) (www.clinicaltrials.gov). Since these clinical trials are, to our knowledge, only in Phase I, further data concerning toxicity and efficacy are not yet available. In this context, the use of non-ionic lipids for ON delivery represents an attractive methodology, since many lipids are safe, nontoxic, and biocompatible, with some of them being of natural origin. However, the association of ONs through physical interactions (i.e., adsorption or encapsulation) with neutral lipid nanoparticles or liposomes suffers from lower ON loading and encapsulation yield as compared to cationic transfectants.[50−52] Thus, it is our opinion that the chemical linkage of nucleic acids with neutral lipids has been insufficiently exploited, although it may combine biocompatibility and important drug loading capacity, therefore representing an interesting delivery strategy.

It is noteworthy that an additional strategy to maintain intracellular penetration without using cationic lipids is the design of ligand-decorated nanocarriers allowing transport of ON into cells (also called "active targeting"). However, important issues have to be mentioned about active drug targeting: as an example, the use of human transferrin as targeting ligand for cyclodextrin-based siRNA delivery (i.e., CALAA-01) raises the question of the difficulty realizing success on a commercial scale due to difficulty of manufacturing, conjugation, processing, and storage as reported by Xu and Anchordoquy.[46]

CHEMICAL STRATEGIES FOR THE SYNTHESIS OF LIPID−OLIGONUCLEOTIDE CONJUGATES

Below are discussed the main synthetic methods for performing covalent conjugation of lipids to ONs. In many cases, changes in pharmacokinetic and pharmacodynamic properties were observed.

Site of Conjugation and Chemical Modification. Conjugation of lipids to ONs generally occurs at the 5′- or 3′-ends.[53,54] In siRNA, there are four terminal ends for potential conjugation sites, since siRNA is a hybridized product of two complementary strands (sense and antisense). The antisense (guide) siRNA strand needs to be incorporated into the RISC complex to initiate the RNAi mechanism as previously described. Therefore, for steric reasons this strand should not be chemically modified. On the contrary, the presence of the inactive sense strand makes it an ideal site for chemical conjugation.

Argonaute2 is a key component of the RISC complex and responsible for mRNA cleavage in the RNAi pathway as discussed before.[55] It is composed of PAZ, Mid, and PIWI domains. The X-ray structural analysis of a cocrystal between a PIWI protein and an siRNA reveals that the 5′-phosphate of the siRNA guide strand is recognized by the PIWI domain and the 5′-phosphate interacts with the carboxyl group of a C-terminal residue of the domain through a divalent metal ion.[56,57] Previous observations showed that the modification of the 5′-hydroxyl groups of the siRNA guide strand by methoxy groups completely hampered the siRNA function in *Drosophila* embryo lysate and HeLa cell extract.[58,59] Thus, these observations have

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suggested that the 5′-end of a guide strand is not suitable for modification by lipophilic groups. On the contrary, the 3′- and 5′-ends of the sense strand are primarily considered as potential sites for conjugation with minimal influence on RNAi activity.[60−63]

Although duplex RNA is more resistant to nuclease attack than single-stranded ON, unmodified siRNAs are nevertheless rapidly degraded when administered intravenously in mammals. Therefore, before conjugation of ONs to lipids, it is of prime importance to protect them from degradation. Thus, a number of chemically modified ONs have been developed to enhance stability and to confer other desirable properties in single-stranded AS-ONs and siRNA. Chemical modifications may occur at three different sites: (i) at phosphate groups, (ii) on the sugar moiety, and/or (iii) on the entire backbone structure of the ON.

The most common stabilizing modification for both antisense and siRNA is substitution at the phosphorus atom of sulfur for oxygen (S → O) to form phosphorothioate ONs. Several phosphorothioate oligoribonucleotides are now in various clinical trials, often in combination with other anticancer drugs. It is worth mentioning that a phase II clinical trial combining Oblimersen (Bcl2 antisense) and rituximab (monoclonal antibody against CD20) for the treatment of recurrent non-Hodgkin's lymphoma has been just completed. Moreover, a phosphorothioate oligodeoxynucleotide agonist of Toll-like Receptor 9 (IMO 2055) is now being tested in Phase Ib trial in combination with cetuximab and FOLFIRI (folinic acid, 5-FU, irinotecan) in patients with colorectal cancer who have progressed following chemotherapy for advanced or metastatic diseases (http://www.clinicaltrials.gov/). However, extensive phosphorothioate modification can lead to unwanted side effects due to a variety of nonspecific cellular protein bindings.[64] So, it may be preferable to limit the use of this modification to terminal positions (or 3′ overhangs) where nuclease resistance is probably most important. Nevertheless, this modification can be safely used to improve the stability of siRNA.[65−68] It can also be mentioned that the replacement of a nonbridging oxygen with a boron (boranophosphate), nitrogen (phosphoramidate), or methyl (methylphosphonate) group will provide nuclease resistance and has been used to help stabilize single-stranded AS-ONs. Figure 2 shows various such modifications that improve nuclease stability and can also be employed in siRNAs. Boranophosphate-modified DNA or RNA resistant to nuclease degradation and the boron modification appears to be compatible with siRNA function. However, boranophosphates are not easily prepared using chemical synthesis.[69,70] In contrast, PS modification is easier and has been extensively used to improve nuclease stability of both antisense ONs and siRNAs. Phosphoramidate and methylphosphonate derivatives were extensively explored for antisense applications but were found to significantly alter the interaction between the nucleic acid and cellular enzymes such as RNase H.[66,71−74] Hydrophobic moieties can also be used for the same protective purpose by masking the internucleosidic phosphodiester bonds of an AS-ON with biodegradable or thermolabile protecting groups. *S*-Acyl-2-mercaptoethyl and alkoxymethyl-protected AS-ONs are the most representative example of this approach.[75,76]

A further important class of modified ONs is represented by derivatives with substitutions at the 2′ position of the sugar. *O*-Methyl group (2′-O-Me), 2-methoxyethyl (2′-O-MOE) group, and fluoro (2′-F) group substitutions have been made. These modifications increase the nuclease resistance and the stability of the complexes formed with native RNA. However, these modifications also have consequences for ON activity. This is not the case for 2′-*O*-methyl RNA, which is a naturally occurring base present in mammalian rRNA. A number of reports have described patterns of 2′-*O*-methyl incorporation that retain full potency of the siRNA compared with unmodified RNA and are stable in serum.[61,65,77] According to Layzer et al. (2004), fluoro-substituted siRNA did not show increased in vivo activity, despite better stability in plasma.[61,66] However, in another study *O*-methoxyethyl derivatives were found to have the same activity as the unmodified sequence.[78] Another recent approach has shown that the ribose moiety can also be used to produce locked nucleic acid (LNA), in which a covalent bridge is formed between the 2′ oxygen and the 4′ carbon of the ribose, fixing it in the 3′-endo configuration. These constructs, referred to as inaccessible RNA, are extremely stable in biological medium, able to activate RNase H and form tight hybrids with complementary RNA and DNA.[79,80] LNAs can be incorporated into siRNAs; however, location of the modified ribose to achieve the best effect is more restricted than for 2′-*O*-methyl or 2′-F modified bases.[81]

Other highly improved ON chemistries consist of modification of the whole backbone structure. For example, the phosphodiester backbone can be completely replaced by a peptide backbone consisting of (2-aminoethyl)-glycine residues. These are known as peptide nucleic acids.[82] They have the advantages of strong hybridization, low toxicity, and good biological stability with respect to nucleases, but also the disadvantages of low solubility in water, poor intracellular penetration, and a lack of ability to activate RNase H. Another completely modified structure is the morpholino AS-ON, in which the ribose is mimicked by a morpholino ring while phosphoramidate intersubunit linkages are used as a substitute for phosphodiester bonds. They have low toxicity and show affinity for their targets similar to that of unmodified AS-ON.[83,84] A Phase II clinical trial for morpholino-oligomer directed against exon 5 (AVI-4658 PMO) for Duchenne muscular dystrophy treatment has just been completed in April, 2011. Another example of phosphorodiamidate morpholino-antisense oligomer already in Phase I clinical trial is AVI-6002, by AVI BioPharma, for the treatment of Ebola hemorrhagic fever. The final data collection started in November, 2011.

Synthetic Approaches. The synthetic approaches employed for LOC synthesis fall into two major categories: the postsynthetic conjugation approach (or solution-phase approach) and the presynthetic conjugation approach (or stepwise solid-phase synthesis approach). In the postsynthetic conjugation approach, the ON is equipped with an appropriate functionality, to which the lipid moiety is coupled in solution after release and purification of the ON. When using the stepwise solid-phase approach, the LOC is prepared on a single support by assembling the conjugate group (i.e., lipophilic group) by a stepwise process prior to or after the ON synthesis and using suitable protecting groups or/and functional moieties which minimize side reactions. The advantage of the solid-supported approach compared to conjugation in solution is a less laborious purification method. To achieve high coupling efficiency, the conjugate group is indeed generally used in considerable excess which complicates the purification when the conjugation is carried out in solution. By contrast, on a solid support, the unreacted conjugated group and the possible side products may be removed by simple washing, which markedly

Table 1. Summary of Strategies Used for the Synthesis of Lipid−Oligonucleotide Conjugates

ON conjugate	site of conjugation on the ON	linker and/or functional group	covalent linkage	conjugate chemistry	target siRNA	ref
Cholesterol-siRNA	3′-end of sense strand	cholesterol-aminocaproic acid-4-hydroxyproline linker	not specified	solid-phase synthesis (phosphoramidite)	Apo-B-1 siRNA	13
Cholesterol-siRNA	5′-end of sense strand	cholesterol phosphoramidite coupled to (CH_2)−S-S-$(CH_2)_6$ linker	reducible (disulfide)	solid-phase synthesis (phosphoramidite)	p38 MAP kinase siRNA	88
Squalene-siRNA	3′-end of sense strand	maleimide modified squalenic acid	thioether	postsynthesis conjugation (Michael addition)	RET/PTC1 siRNA	85
α-tocopherol-siRNA	5′-end of anti sense strand	R-tocopherol phosphoramidite	phosphodiester cleavage (site of Dicer	solid-phase synthesis (phosphoramidite)	Apo-B-1 siRNA	86
Lipid- and steroid-siRNA	3′-end	not specified	trans-4-hydroxyprolinol-aminohexanoic linker	solid-phase synthesis phosphoramidite)	Apo-B-1 siRNA	18
Lipid- and cholesteryl siRNA	3′-end of sense strand	not specified	glycerol scaffold with 2-hydroxy-ethoxycarbonylamino-butyl-carbamic acid linker	solid-phase synthesis (phosphoramidite)	Apo-B-1 siRNA	19

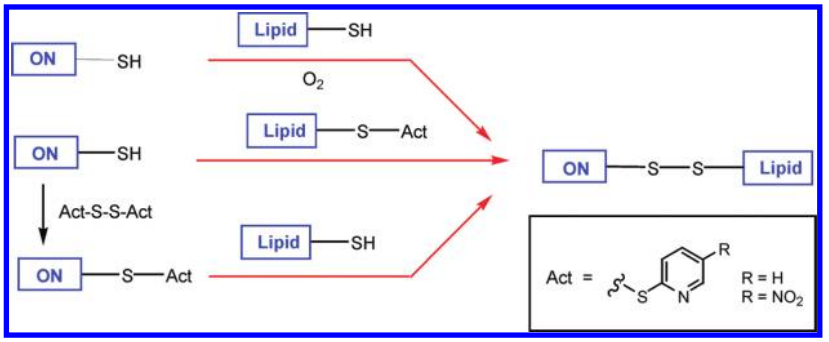


Figure 3. Routes for formation of disulfide-linked conjugates.

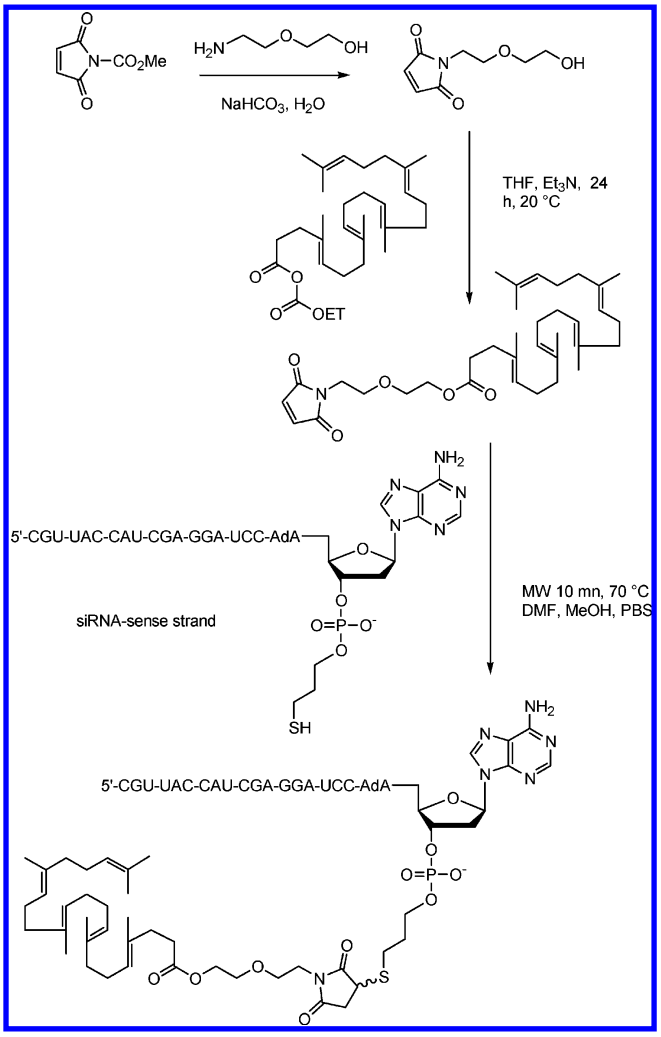
facilitates the chromatographic purification after the conjugate is released from the support. The use of a solid support may also help to avoid the problems otherwise arising from the limited solubility of one of the reactants. These approaches can be further categorized into different categories depending on the type of covalent bond generated between the ON and the lipid. The covalent linkages frequently used for connecting lipids to the termini of ONs are as follows: thioether,[85] disulfide,[117] phosphate,[86] or phosphoramidate.[87,88] In order to avoid eventual steric hindrance, a simple alkyl chain[88] is often used as a spacer arm and may be introduced between the ON and the lipid, whereas aminocaproic−pyrrolidine linker,[13] ether linker,[85] and non-scissile triazole linker[89] are other spacers used for LOC synthesis. Some conjugation strategies of LOCs are summarized in Table 1.

Post-Synthetic approach. It is noteworthy that stepwise solid-phase synthesis of LOC is difficult and expensive due to the conflicting chemistries and different solubility profiles of ONs and lipids. One of the simplest approaches to overcome this issue is to prepare separately both functionalized components, i.e., by exploiting standard lipid and ON synthetic methodologies before proceeding toward the final conjugation of the ON and the lipid. Of course, prior to the conjugation, it is required to ensure a unique and specific linkage between the lipids and ONs when attaching certain reactive functionalities to both molecules. Recently, Raouane and co-workers established a new, efficient method to synthesize the lipid conjugates of ONs via a thioether linkage.[85] However, at the beginning, these authors have chosen the covalent linkage of the thiolated siRNA sense strand (with sequence toward the RET/PTC1 fusion oncogene) to squalene derivative (SQ) through a disulfide linkage, taking advantage of the 3-mercaptopropyl phosphate group introduced at the 3′-terminal of the ON during its manufacture on solid support, since thiol groups have a specific reactivity and afford rapid conjugation with a wide variety of substrates. The disulfide linkage can be formed either by direct oxidation of two thiols[90] or by preliminary activation of one thiol with pyridyldisulfanyl or 5-nitropyridyldisulfanyl groups followed by nucleophilic substitution[91] as illustrated in Figure 3. Attempts to couple thiol-containing ON with thiopyridyl-activated SQ via disulfide linkage met with repeated failure even after activation of both substrates.[85] Thus, the challenging squalenoylation was more efficiently achieved by the use of the maleimide group as thiol acceptor as previously described by Weber et al.[92] It was described (see Scheme 1) that thiolated siRNA 1 could selectively react with maleimide-derivatized SQ 2 possessing an ether linker to form the nonreducible thioether bond.[85] This chemical construct was found to self-organize as nanoparticles in water and was found to inhibit tumor growth in a mice xenografted experimental model of the thyroid papillary carcinoma.[85]

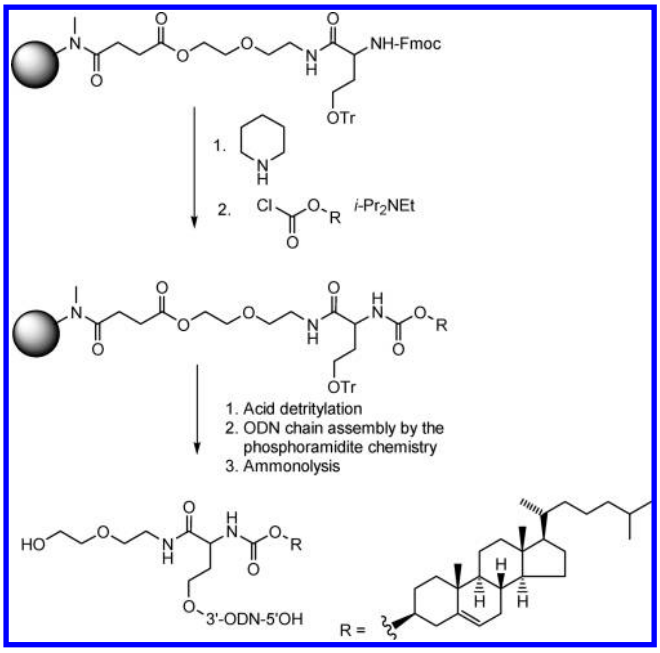
Presynthesic Approach. The strategy of modifying the ON during solid-phase synthesis is by far the most widely used approach to add hydrophobic moieties to ONs. Due to the elongation of the ON chain in the 3′-5′ direction during solid-phase synthesis, the modification is more conveniently incorporated at the 5′-end as compared to the 3′-end, which requires substantial additional synthetic efforts.[93,94]

The LOCs are usually obtained by one of the three following solid-phase approaches: either (i) 3′-conjugates by chain assembly on a support bearing the desired hydrophobic group or (ii) 5′-conjugates by introducing the conjugate group as a phosphoramidite reagent; (iii) intrachain conjugates

Scheme 1. Postsynthetic Strategy for the Direct Hydrophobization of siRNA by Squalene Conjugation



Scheme 2. Solid-Phase Synthetic Strategy Developed by Stetsenko for Preparation of 3′-ODN Conjugate



may also be obtained by making use of prefabricated nucleosidic building blocks or their congeners incorporating the conjugate group.

Preparation of Oligonucleotide 3′-Lipid Conjugates. For the synthesis of lipid 3′-conjugates ONs, an accessible solid-phase method has been described by Stetsenko et al. (2001). This method involves the use of a branched alcohol bearing orthogonally protected amino and hydroxyl groups which is attached to the support via a succinyl linker as illustrated in Scheme 2. The conjugate group (i.e., cholesterol) bearing a hydroxyl function is first attached to the deprotected amino group of a homoserine scaffold as a chloroformate ester and the ON is then assembled on the deprotected hydroxyl function.[95] A similar amino-alcohol supported CPG−resin allowing stepwise immobilization of cholesterol and 3′-ON had previously been proposed.[96] Related supports have more often been prepared by immobilization of a prefabricated linker−cholesterol conjugate.[19,97−102] Supports derived from *trans*-L-4-hydroxyprolinol have additionally been used for the preparation of ON conjugates of many other steroids and diacylated glycerols,[103] 2′-*O*-[94] and 3′-*O*-succinylated uridine.[104]

Interestingly, instead of conventional succinyl linker, an aminoalkyl support was acylated with 3-chloro-4-hydroxyphenylacetic acid and the 3′-terminal nucleoside was coupled to the hydroxyl function by the normal phosphoramidite chemistry.[105] The 2-cyanoethyl protection was then removed with triethylamine in pyridine and the lipid group, e.g., cholesterol was coupled to the exposed phosphodiester linkage by the phosphotriester chemistry. The ON was then assembled using standard phosphoramidite protocols and the LOC was obtained after normal ammonolysis.

Soutschek et al. (2004)[13] used 4-hydroxyprolinol as a linker to connect RNAs and lipophilic groups, while Ueno et al. (2008)[106] selected glycerol which was more flexible than hydroxyprolinol as a linker to join lipophilic groups at the 3′-terminus of RNAs (Scheme 3). It is worth noting that a report in 2004 by Soutschek and co-workers using a cholesterol−siRNA conjugate provided the first mechanistic in vivo proof of concept for RNAi. Chemical conjugation of siRNA to cholesterol has indeed been shown to facilitate intracellular siRNA uptake in vitro[107] and in vivo[13] In the latter case, intravenous administration of cholesterol conjugated siRNA oriented toward apolipoprotein B (ApoB) was shown to silence endogenous ApoB gene expression by 55% in the liver and by 70% in the jejunum, the two principal sites for ApoB expression, resulting in decreased plasma levels of ApoB protein and consequently to a remarkable reduction in the cholesterol levels by 35−40%.[13] Cholesterol conjugation imparted critical pharmacokinetic and cellular uptake properties to the duplex as evidenced by the fact that the unconjugated ApoB siRNA was rapidly cleared after administration and unable to induce any mRNA silencing.[13] Radiolabeled cholesterol conjugates of siRNA displayed an elimination half-life of 95 min, whereas unconjugated siRNAs had a half-life of only 6 min. The prolonged half-life of the cholesterol conjugates relative to naked siRNA was explained, at least in part, by an enhanced binding to serum proteins. The mechanism of improved biodistribution and cellular uptake of siRNA through cholesterol conjugation has been further investigated in a recent study:[18] cholesterol−siRNA conjugates were found to incorporate into circulating lipoproteins, especially in LDL, allowing liver targeting, i.e., improved

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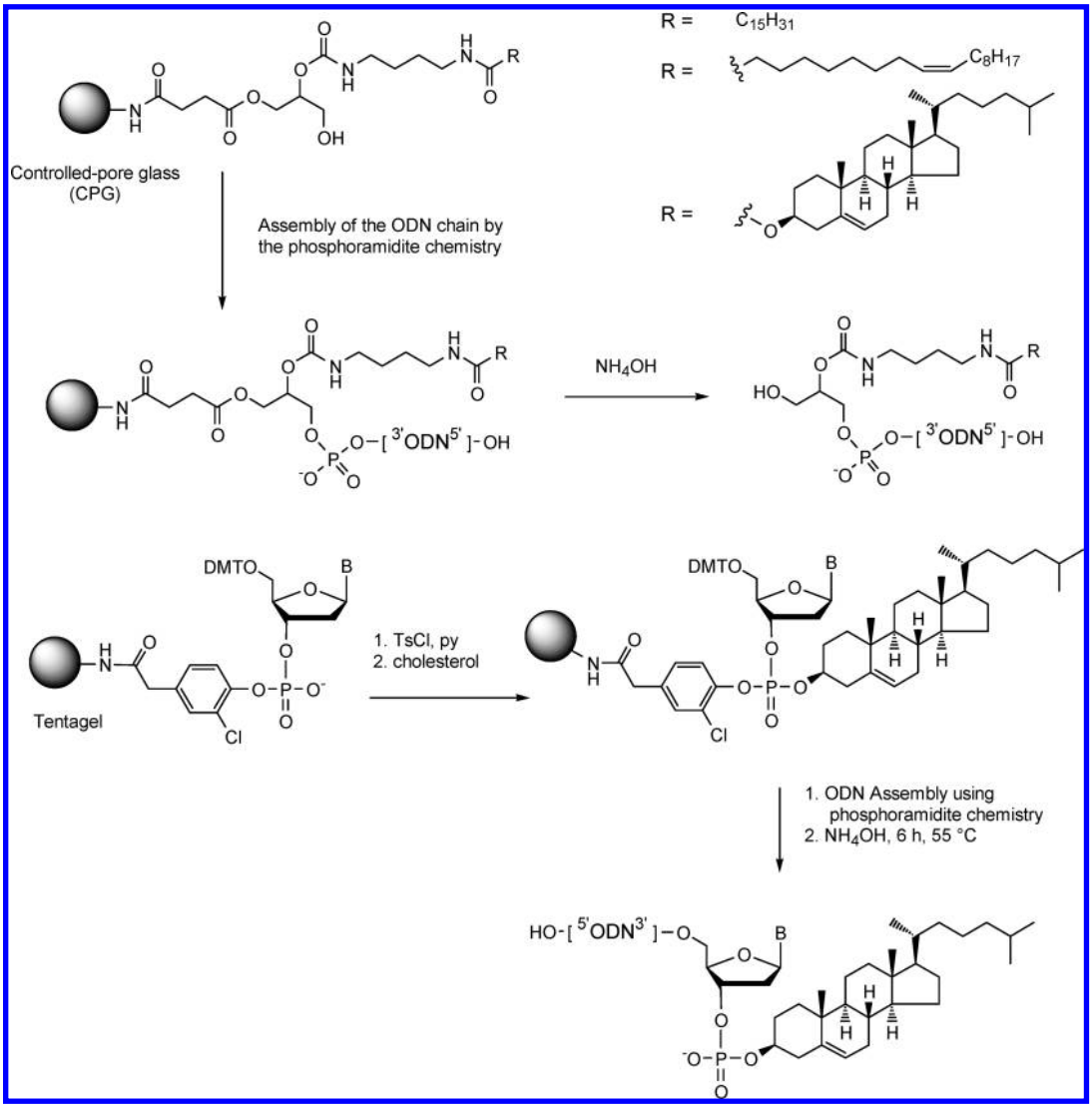
Scheme 3. Synthesis of 3′-Lipid-Conjugated Oligonucleotides According to Ueno et al. and D'Onofrio et al.



distribution in vivo, whereas efficient hepatocyte internalization occurred via receptor-mediated processes (i.e., efficient cellular uptake).[18] Besides, preloading of cholesterol−siRNA bioconjugates to natural lipoparticles dramatically improved silencing efficacy in mice, whereas the nanoparticle type was found to affect cholesterol−siRNA conjugate distribution in various tissues especially in favor of the liver as explained before (see mechanisms of endocytosis and intracellular trafficking).[18] Wolfrum et al. have elegantly extended the in vivo efficacy seen with cholesterol to other conjugates such as bile acids and long-chain fatty acids. It has been shown that conjugation to bile acids and long-chain fatty acids mediated siRNA uptake into cells and gene silencing in vivo and that efficient and selective uptake of these siRNA conjugates not only depended on interactions with lipoprotein particles, lipoprotein receptors, and transmembrane proteins, but was also affected by the nature of the conjugated lipid. Thus, a variety of lipophilic siRNA conjugates were synthesized using cholesterol or fatty acids of different chain lengths as lipid moieties. The fatty acid conjugates with longer saturated alkyl chains such as stearoyl and docosanyl, but not those with shorter chains, reduced target gene expression in mouse livers.[18] The efficacy of the conjugation of nucleic acids with cholesterol has been further confirmed in another study, showing enhanced cellular uptake in cell culture as well as hepatic deposition after systemic administration.[108] Finally, other authors have demonstrated that the conjugation with cholesterol may enhance siRNA uptake not only via receptor-mediated endocytosis, but also by increased membrane permeability of the otherwise negatively charged RNA.

Using a transgenic mouse model for Huntington's disease, it was also established that a single intrastriatal injection of cholesterol−siRNA conjugate allowed significant inhibition of a mutant Huntingtin gene, attenuating neuronal pathology as well as delaying the abnormal behavioral phenotype.[109]

Preparation of Oligonucleotide 5′-Lipid Conjugates. Apart from 3′ linkage, there are also some LOCs which have been prepared carrying lipids at the 5′-end of the ONs. Figure 4 illustrates different LOCs bearing lipids at the 5′-end of the ONs. Many phosphoramidite and H-phosphonate reagents, both non-nucleosidic and nucleosidic, were designed to introduce hydrophobic conjugate groups into the 5′-terminus of ONs.[89,93,101−104] An archtypical one is the phosphoramidite reagent derived from the allyl ester of 3-α-(2-hydroxyethoxy)-cholic acid obtained by selective phosphitylation of the primary hydroxyl group with 2-cyanoethyl-*N*,*N*-diisopropylphosphoro-

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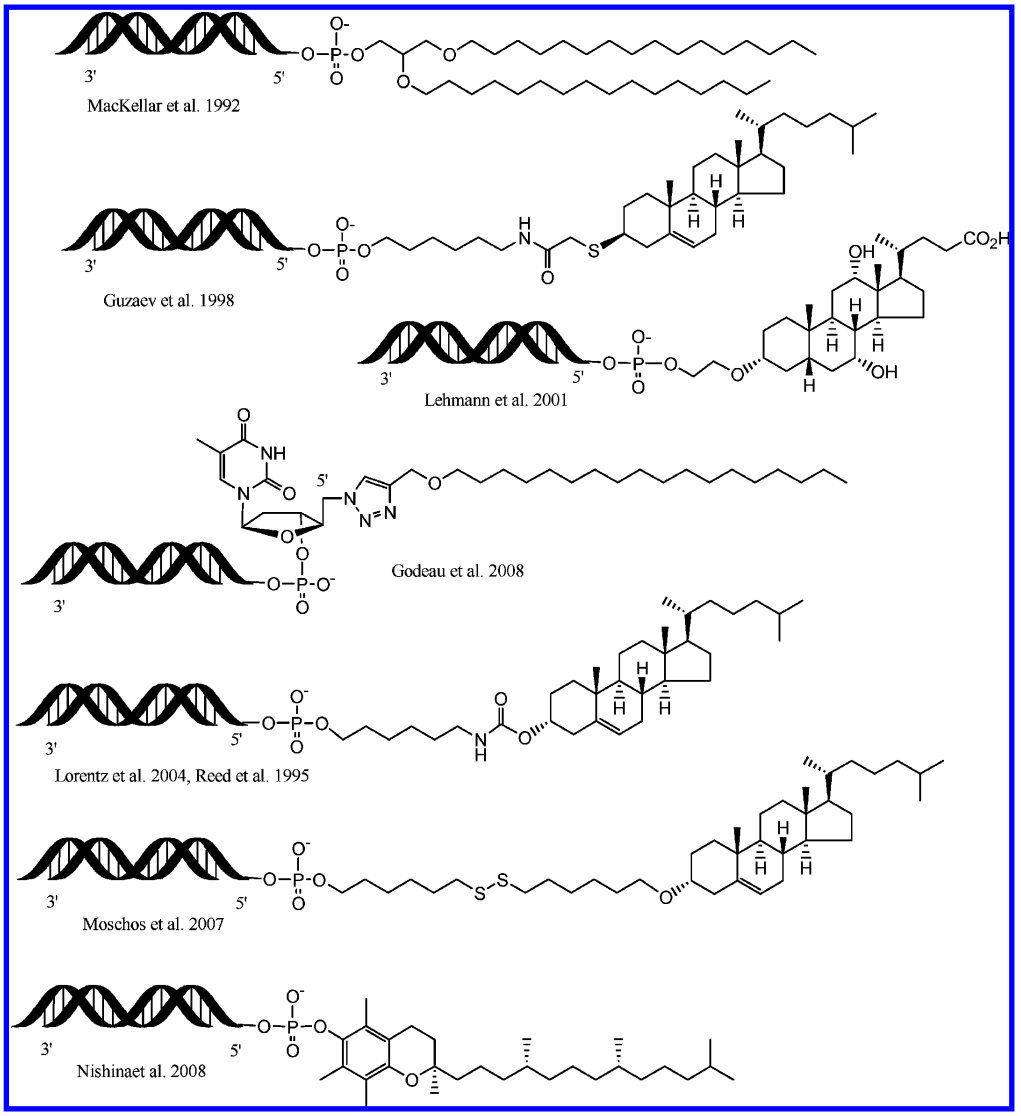


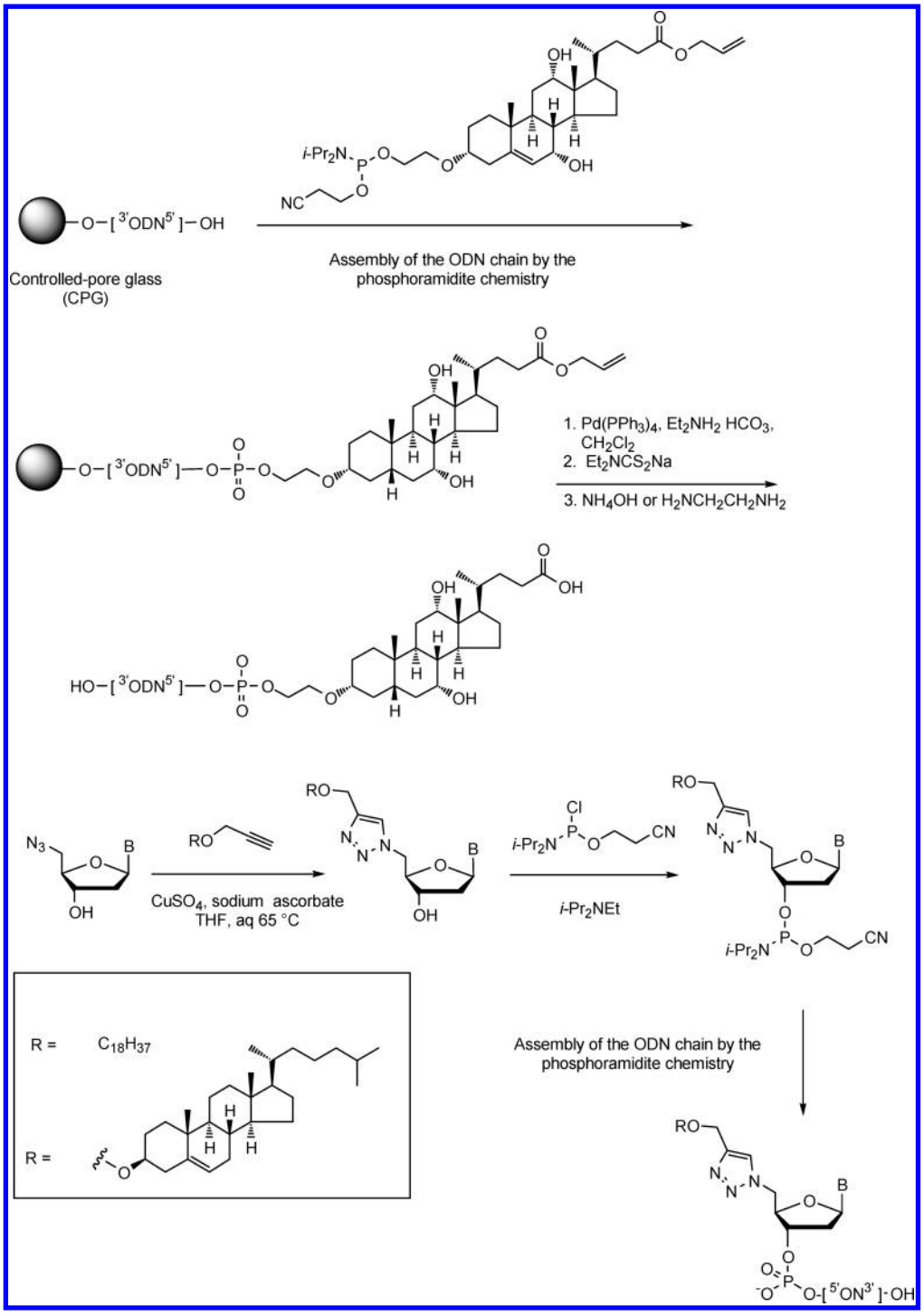
Figure 4. Selected lipid–oligonucleotide conjugates bearing lipids at 5′-ends of the oligonucleotide.

chloridite without interference of the secondary hydroxyl functions.[110] The allyl protection may then be removed on-support using $Pd[PPh_3]_4$-catalyzed deallylation.

Another straightforward approach for the synthesis of 5′-lipid ON conjugates has been developed for ON conjugation to fatty acids.[111] In this approach, 5′-palmitoyl ONs have been obtained by using base-labile *t*-butylphenoxyacetyl protecting groups for base moiety amino functions and oxalyl-CPG anchor group. These modifications to the standard phosphoramidite strategy allowed mild deprotection with ethanolamine, and hence, the 5′-ester linkage remained intact.[111]

In addition, the method mentioned below, described by Letsinger, had been used by Krieg to conjugate cholesteryl moiety at the 5′-end of AS-ON targeted against an mRNA encoding immunosuppressive protein.[112] It was described that a cholesteryl–AS-ON conjugate increased cellular association (by enhance binding to LDL) and improved efficacy.[112] Conjugation of lipids to ONs by 1,3-dipolar cycloaddition reaction involves reaction between alkyne and azide to form 1,2,3-triazoles. This so-called "click chemistry" reaction has been added to the repertoire of ON modifying methods, because the 1,3-dipolar cycloaddition chemistry is very chemoselective, only occurring between terminal alkynyl and azido functional groups with high yield. In addition, the resulting 1,2,3-triazoles are stable under aqueous conditions and high temperature. The use of the Huisgen 1,3-dipolar cyclo-addition for postsynthetic modification of ONs has been the subject of an interesting recent minireview.[113] Practically, the "click chemistry" reaction has been employed to synthesize LOC in three steps starting from alkyne-modified lipids derived from cholesterol and octadecanol.[89] The resulting 1,3-dipolar cycloaddition reaction provided the expected 1,2,3-triazole intermediates, which were next converted into phosphoramidites in one step. The phosphoramidites were further coupled to the 17-mer 2′-*O*-methylribonucleotide antisense chain of the hepatitis C virus (HCV) RNA, using a classical solid support synthesis, in which the ON was elongated in the 3′-5′ direction. This reaction could be carried out in either water or organic medium and be employed postsynthetically on purified units modified with alkynyl and azido functionalities without additional functional group protecting strategies. Godeau et al. (2008) reported that the incorporation of a lipid moiety via a nonscissile triazole linker noticeably increased the lipophilicity of the ONs, which had a positive impact on their intracellular delivery (Scheme 4). ONs conjugated with cholesterol or octadecanol moiety induced a dose-dependent reduction of

Scheme 4. Synthesis of 5′-cholic Acid-Conjugated Oligonucleotides According to Lehmann et al. and Conjugation of Lipid Moiety via "Click Chemistry" According to Godeau et al.



HCV-internal ribosome entry site (IRES)-dependent translation in the Huh7 cell line. More importantly, the toxicity of the LOCs was negligible and biological activity of the LOCs was not affected by the presence of serum.[89]

Lorenz et al. (2004) conjugated the siRNA β-galactosidase to cholesterol at the 5′ end of the sense strand. The cellular uptake of siRNA β-galactosidase in human liver cells was increased when it was conjugated to cholesterol. In fact, incubation of cells with 50 nM of siRNA β-galactosidase during 4 h with a modified sense strand down-regulated β-galactosidase expression to a higher extent than siRNAs with a modified antisense strand or two modified strands.[107] Cholesterol-conjugated siRNA has also been administered directly into the lung via intratracheal instillation. In this case, the target was p38 MAP kinase mRNA. The cholesterol-conjugated sense strand RNA was synthesized using a cholesterol phosphoramidite coupled to a C6-SS-C6 linker at the 5′-end of the RNA. The so-obtained cholesterol−siRNA conjugate was shown to have a target-specific RNAi effect in mice. The escalation of lipophilic siRNA doses improved the duration of the RNAi effect.[88]

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Evidence of the utility of cholesterol for oligonucleotide delivery was provided in a recent report showing cholesterol-mediated delivery of 2′-*O*-methyl and phosphorothioate-modified single-stranded RNA for the knockdown of microRNA expression in various tissues, e.g., liver, lung, kidney, and heart, following intravenous injection.[114]

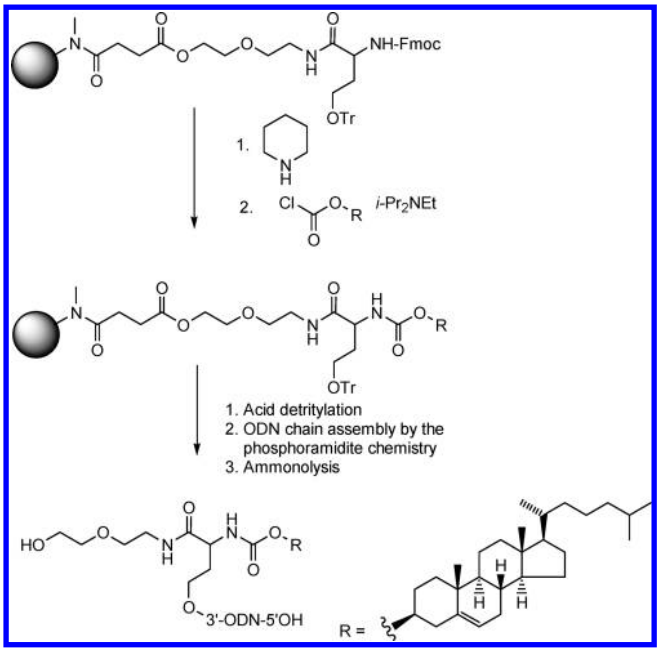
Another interesting LOC, α-tocopherol (vitamin E)-siRNA, was introduced for systemic siRNA delivery to the liver.[86] Lipophilic vitamin E was covalently conjugated to the 5′-terminus of the antisense strand of 27/29-mer siRNA and was partially modified with 2′-*O*-methylated ribose and phosphorothioate linkage. After intracellular delivery, the 27/29-mer siRNA was processed by the action of Dicer to generate 21/21-mer siRNA, which caused the simultaneous release of the vitamin E moiety. The intravenous administration of the conjugate achieved a significant reduction of the target protein ApoB in the liver without any induction of inflammatory interferons, i.e., interferon-α or -β.[86] Such as with cholesterol, conjugation with α-tocopherol promoted siRNA delivery via binding to serum proteins and lipoproteins. α-Tocopherol was also incorporated into intrachain positions of the siRNA as described below.

Dodecylamine, octadecanethiol, and thiocholesterol have been conjugated to the 5′-terminus of a support-bound ODN by elongation of the chain with 6-chloroacetamidohexanol phosphoramidite and displacing the chlorosubstituent with nitrogen sulfur nucleophiles in dioxane in the presence of DBU, and normal ammonolysis then released the conjugate.[115]

Several lipophilic alcohols including cholesterol, borneol, menthol, and heptadecanol have been converted to 2-alkoxy-2-thiono-1,3,2-oxathiaphospholane and reacted in DCM in the presence of DBU with the 5′-hydroxy group of a support-bound ODN. Again, a normal ammonolysis was finally carried out.[116] However, those constructions have been tested neither in vitro nor in vivo.

Lipid Incorporation into Intrachain Positions of the Oligonucleotide Chain. The incorporation of hydrophobic groups into intrachain positions of the oligonucleotide chain is usually performed with the aid of prefabricated nucleosidic building blocks bearing a lipid moiety either on the base or on the 2′-position of the ribose. Alternatively, the phosphate linker has been replaced by a phosphoramidate group allowing tethering of the hydrophobic chain on the internucleoside phosphorus linker. The exploited nucleosidic building blocks include the phosphoramidites of C5-derivatized pyrimidine 2′-deoxyribonucleosides,[117,118] 2′-*O*-derivatized uridine, and a thymine amino-LNA monomer.[119] Several lipophilic moieties such as cholesterol,[117,119] α-tocopherol,[118] adamantanylacetic acid,[94] long-chain fatty acids,[94] and diacylglycerol[94] have been introduced in this manner. In fact, the first solid-phase synthesis of a cholesterol conjugate is a nice example of such an approach involving oxidative phosphoramidation of a support-bound dinucleoside-3′,5′-(*H*-phosphonate) with cholester-3-yloxycarbonylaminoethylamine and subsequent assembly of the ODN chain.[87] This elegant strategy to functionalize the 3′-end and 5′-end internucleoside phosphorus was developed by Letsinger, from the corresponding *H*-phosphonate manually attached beforehand to the solid support (Scheme 5). The ODN chain is then assembled by phosphoramidite chemistry, and upon ammonolysis, only the ODN bearing the conjugate group is released from the support, since the phosphodiester and phophoramidate linkages are not cleaved by ammonolysis. The advantages of this method are as follows: first, there is an ability

Scheme 5. Strategy Developed by Letsinger to Modify the 3′-End of Oligonucleotide



to attach more than one lipid to ONs at defined sites and the freedom to attach additional moieties, i.e., fluorescent groups at both the 3′- and 5′-ends of the ON; second, linkage by this process did not interfere with ON-to-target hybridization. Thus, Letsinger et al. (1989) showed that the antiviral activity of a 20-mer cholesteryl−phosphorothioate derivative was significantly increased. A complete inhibition of the human immunodeficiency virus type 1 replication, as judged by inhibition of syncytia formation and expression of viral proteins p17, p24 in Molt-3 cells, was obtained with an oligomer concentration of 0.2 μM.[87]

Non-nucleosidic hydrophobic building blocks have been incorporated at either the 3′ or 5′ terminus of ODNs using a phosphoramidite reagent derived from dioctyl 2,2-bis-(hydroxymethyl)malonate.[120] Again, a prolonged coupling time of 10 min is required for high coupling yield (>98%), and the subsequent detritylation has to be carried out with TFA. Interestingly, the ester linkages at internucleosidic positions withstand normal ammonolysis. Besides exploitation of prefabricated solid supports and phosphoramidite reagents bearing the conjugate group, several on-support conjugation procedures have been reported.

■ SUPRAMOLECULAR ORGANIZATION

There are only a few studies which have investigated in deep detail the structure of LOCs in water, although these molecules have a natural tendency to self-assemble and to form supramolecular organizations, due to their strong amphiphilic character.[121,122] For example, Wu et al.[123] have described the formation of DNA aptamer micelles by linking a simple lipid tail phospholipid with diacyl chains onto the end of an aptamer inserted with PEG linker. This amphiphilic unit self-assembled spontaneously in water into a spherical micelle structure of 68 nm. In a similar way, the linkage of squalene to RET/PTC1 siRNA led to the formation of spherical nanoassemblies with a size of around 170 nm. Transmission electron microscopy examinations have revealed the formation of nanoparticles

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rather than micelles.[85] In another study, a series of charge-reversal amphiphiles with different spacers separating the headgroup from the hydrophobic chains are described for the delivery of DNA and siRNA.[124] Given their polar headgroup and long hydrophobic alkyl chains, these amphiphiles self-assembled to form bilayer vesicles in aqueous solution. X-ray diffraction investigation has shown that the DNA is located between the adjacent lipid bilayers.

CONCLUSION

The literature is full of studies using cationic polymers or lipids to promote the delivery of antisense oligonucleotides or siRNA.[10] If the strong ionic interactions of these transfectants may appear as an advantage for the delivery of small fragments of nucleic acids, their (cyto)toxicity remains the major toxicological issue even if few cationic nanoformulations are currently in early clinical trial. Surprisingly, the use of neutral, safe, and biocompatible lipids has not drawn so much attention for nucleic acids administration, probably because loading these hydrophilic molecules onto neutral lipid based nanocarriers raises difficulties from a formulation point of view. Thus, chemical conjugation of oligonucleotides/siRNA to neutral lipids is an interesting option, which is insufficiently exploited today. In our opinion, this approach should avoid the toxicological issue inherent to (poly)cations, even if the conjugation of oligonucleotides/siRNA with a neutral lipid is less obvious than the design of ion pairs of nucleic acids with polycations. Additionally, the chemical strategies used for LOC synthesis need to use a hydrolyzable spacer able to release, in a controlled manner, these short fragments of nucleic acids both in vitro and in vivo. The relevance of LOCs for the treatment of human diseases has, however, never been demonstrated until now in clinical trials, probably because additional preclinical studies are still needed. Thus, the aim of the present review was to give account of the availability of the many chemical conjugation methodologies that allow construction of lipid nucleic acids capable of in vivo gene silencing.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS:

AS-ON, antisense oligonucleotide; ApoB, apolipo protein B; Ago 2, Argonaute 2; DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene; dsRNA, double-stranded RNA; EPR, enhanced permeability and retention; ECM, extracellular matrix; GFP, green fluorescent protein; HCV, hepatitis C virus; HDL, high density lipoprotein; IRES, internal ribosome entry site; LOC, lipid oligonucleotide conjugate; LNA, locked nucleic acid; LDL, low density lipoprotein; mRNA, mRNA; miRNA, microRNA; PNA, peptide nucleic acid; ODN, oligodeoxyribonucleotide; ON, oligonucleotide; RES, reticulo-endothelial system; RET/PTC1, rearranged during transfection/papillary thyroid carcinoma type 1; RNase H, Ribonuclease H; RISC, RNA-induced silencing complex; RNAi, RNA interference; SR-BI, scavenger receptor; siRNA, small interfering RNA; SQ, squalene.

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