Oligonucleotide delivery: a patent review (2010-2013)

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Abstract

Introduction

The use of aptamers, antisense technology and RNA interference has allowed nucleic acids to be considered promising alternatives to classical drugs. However, nucleic acids face several obstacles and pitfalls in the creation of effective nucleic acid drugs. The development of these approaches has increased the pipeline toward clinical trials.

Areas covered

This review covers research and patent literature of the last three years focused on the development of safe and effective non-viral drug delivery systems for the treatment of diseases such as cancer or genetic disorders by using oligonucleotides.

Expert opinion

The therapeutic applications of oligonucleotides have undergone multiple obstacles especially in biodistribution and cellular internalization. Cationic lipids are the most used vehicles for the preparation of novel formulations. Combinatorial libraries of these compounds and the use of solid lipid nanoparticles carrying these synthetic cationic lipids, cholesterol and polyethyleneglycol have emerged to enhance cellular uptake and biocompatibility of nucleic acids. Besides this extensive use, synthesis of oligonucleotide covalently linked to lipids has also emerged as a promising alternative

to formulations. The use of peptides alone or in combination with lipids is also an expanding field for oligonucleotide delivery. Polymeric platforms are also good candidates as they showed improved cellular uptake, biodegradability, biocompatibility, and the possibility to incorporate several components such as ligands for receptor-mediated endocytosis and molecules to facilitate endosomal escape. Finally, nanomaterials may also play an important role in the future. The last developments showed improved *in vivo* efficacy, thus gaining a foothold in therapeutics.

Keywords

Antisense, aptamers, cell penetration peptides, drug delivery, liposomes, nanoparticles, oligonucleotides, polymers, RNA interference, siRNA

1. Introduction

The search of novel drugs in medicinal chemistry is based on the use of small molecules derived from the screening thousands of compounds along with computational studies that directly interact with target molecules, mainly proteins.

Nucleic acids, in contrast to conventional small drugs, are macromolecules that target specifically messenger RNAs (mRNAs) inhibiting the expression of a known gene sequence. This mode of action, that allows the specific inhibition of a target protein, covers a range of biomedical applications that are not treatable with current drugs [1].

Oligonucleotides face multiple obstacles in order to be considered clinically acceptable therapeutic drugs. They are susceptible to degradation in the presence of nucleases and, because of their size and charge, oligonucleotides do not cross cell membranes by passive diffusion. Chemical biology of nucleic acids has accelerated the process of discovery of modified nucleic acids with an enhanced stability hence protecting them from degradation. Furthermore, these chemical modifications have also allowed the synthesis of novel nucleic acids with more drug-like properties, target affinity and specificity without losing biologic activities of natural nucleic acids [2].

According to Figure 1, we can find at least three potential sites in order to modify chemically oligonucleotides: (a) the sugar ring, (b) the phosphate backbone and (c) the nucleobases. Chemical modifications on the sugar ring affect the stability of the DNA:RNA duplex ~~and~~ therefore improving, in most ~~of~~ the cases, protein binding and resistance to degradation in serum. These 2ô-modifications mainly comprise 2ô-fluoro, 2ô-*O*-methyl, 2ô-*O*-methoxyethyl substituents and bicyclic sugars locked nucleic acids (LNAs), which have the effect to fix the sugar conformations hence increasing the stability of duplexes.

Modifications carried out directly on the phosphate group comprise phosphorothioate (PS) backbones, which is the most common modification in antisense oligonucleotides (ASOs) and siRNAs. Modified ASOs and siRNAs containing PS moieties have shown better stability profiles in serum facilitating intravenous and subcutaneous administrations. In a more radical change, the ribose phosphate backbone has been replaced with morpholino (PMO) moieties, a six-membered ring that has been successfully introduced into antisense oligonucleotides, showing low toxicities and promising activities. Peptide nucleic acids (PNAs) represent other kind of variation comprising the introduction of (2-aminoethyl)-glycine peptide backbones and replacing the corresponding ribose or deoxyribose rings giving strong interactions with RNA molecules. Because PNA are uncharged and have been shown excellent nuclease-resistant molecules, they have been mainly evaluated in many antisense studies [3].

Considerable progresses made by researchers to boost drug-like properties of nucleic acids have allowed the launch of multiple clinical trials [4] for several ASOs and siRNAs (Table 1). To date, there are only a few nucleic acid-based drugs approved by the FDA: Macugen, a RNA aptamer [5] designed for the treatment of age-related macular degeneration; Mipomersen [6], a fully synthetic antisense oligonucleotide against hypercholesterolemia and Vitravene, an antisense oligonucleotide drug to treat cytomegalovirus retinitis in AIDS patients was launched in 1998 [7]. However, despite the increased number of potentially therapeutic nucleic acids, there are only few examples in which non-viral delivery agents have been employed. For instance, the use of either transferrin receptor-targeted nanoparticles (CALAA-01), cationic liposome-based strategies (ALN-TTR, ALN-PCS02, Atu027, TKM-080301) or the use of polymeric nanoparticles (CRLX-101) are in progress. The rest of the modified siRNAs and ASOs that are under active investigation are directly administered in their natural

unmodified form without using any delivery system, being ocular, electroporation, parenteral, intramuscular or systemic the most preferred administration routes.

The ability of oligonucleotides to cross cell membranes is very poor. The use of viral carriers, like retrovirus or adeno-associated virus (AAV) increases the transfection efficiencies of nucleic acids. However, several concerns about the immunogenicity and possible recombination of oncogenes have been expressed [8]. It is for this reason that non-viral carriers have emerged in the last years as safer alternatives inducing less toxicity and immunogenicity although their transfection efficiencies are lower compared to viral vectors [9]. The development of biocompatible materials (Figure 2) such as cationic lipids [10], liposomes [11], stable nucleic acid lipid particles (SNALPs) [12], polymers [13] or cell-penetrating peptides [14] has allowed nucleic acids to bind to non-viral carriers taking advantage of the electrostatic interactions.

The development of covalent strategies has also allowed the synthesis of more stable conjugates containing lipids [15], polymers [16] or dendrimers [17] with oligonucleotides. The use of neutral lipids like cholesterol is one of the preferred strategies for the chemical modification of nucleic acids. In particular, cholesterol-siRNA constructs have considerably improved the cellular uptake properties of siRNA molecules hence facilitating their intravenous administration for silencing ApoB expression with good efficiencies [18]. Nonetheless, despite these promising results, the use of covalent strategies remains scarcely exploited compared to electrostatic formulations, which are being used in the majority of clinical trials in both antisense and RNA interference therapies.

2. Strategies using short nucleic acids as drugs

2.1 Antisense technology and RNA interference

Antisense technology and RNA interference (RNAi) mechanisms are the major processes to control gene expression. In both cases, the expression of disease-causing proteins is down-regulated at the level of mRNA. In the case of antisense technology (Figure 3A), single-stranded ASO molecules are able to interact with target mRNA through Watson-Crick base-pair interactions within hybridization-accessible sites. Depending on the design and the chemistry, the corresponding ASO:RNA hydrid may result in degradation by the action of RNAseH endonuclease that hydrolyzes the duplex, therefore inhibiting the synthesis of proteins. This particular strategy also includes miRNA-based constructs such as antagomirs, locked nucleic acids (LNA) anti-miR, microRNA sponges and miR-masks that have shown good results in gene inhibition.

RNAi mechanism (Figure 3B) is the other approach to control the gene activity. This mechanism occurs in the cytoplasm and is based on the recognition of double-stranded small interference RNA (siRNA) by a multi-protein complex called RNA-induced silencing complex (RISC). First, long double-stranded RNAs (dsRNA) are cleaved by the action of the RNAse endonuclease Dicer producing siRNAs. After siRNAs are loaded into the RISC complex, Argonaute-2 protein (Ago-2), which is one of the components of RISC, cleaves and releases the passenger strand of siRNA, leading to an activated form of RISC that contains a guide strand of RNA. The guide strand binds to target mRNA by intermolecular base pairing and mRNA is cleaved thereby inhibiting protein synthesis.

Synthetic miRNA mimics to restore tumor suppressor miRNA-expressionBesides cellular uptake limitations, there are two additional hurdles to overcome in RNAi. Synthetic siRNAs in mammalian cells could interfere with the effect produced by

micro-RNAs (miRNAs), which control the efficiency of mRNA translations giving undesirable off-target effects [19]. Moreover, siRNAs can activate the immune response by activating Toll-like receptors leading to unwanted side effects.

2.2 Aptamers

Aptamers are nucleic acids with high affinity to a particular protein. These molecules are initially selected by means of PCR-based techniques, starting from random libraries of short nucleotide segments (SELEX). Interesting applications of aptamers in the biosensing field are related to the analysis of chemical or biological pollutants but ~~also~~ they can also be used as potential drugs [20]. The first aptamer-based drug was approved by the FDA. Pegaptanib or macugen binds the isoform of VEGF165 and inhibits its interaction with the VEGF receptor [21] being used in the treatment of age-related macular degeneration.

3. Evaluation of patents

Patent literature in this review is divided into several areas depending on the nature of the compounds used for enhancing cellular uptake.

3.1 Formulations containing cationic lipids

Cationic lipids constitute the main non-viral approach to deliver nucleic acids inside cells. Although there are some limitations associated to their use, such as low transfection efficiencies, toxicity and stability in the presence of serum [22], to date, the design and synthesis of novel cationic lipid libraries and lipid-like molecules has

prompted promising improvements in the delivery of siRNA and ASO molecules in both *in vitro* and *in vivo* experiments [23].

Solid lipid nanoparticles (SLNP) have emerged as an alternative to liposomes. These drug delivery vehicles combine the advantages of lipid emulsions and polymeric nanoparticles and overcome some issues like the stability of the particles *in vivo* [24]. They are composed of cationic lipids to provide higher efficiency in neutralizing the negative charge of nucleic acids by forming tighter complexes; cationic or fusogenic lipids and, finally, hydrophilic molecules such as polyethylene glycol (PEG) derivatives, which generally improve the plasma stability and biodistribution by altering the surface properties of SLNPs.

The therapeutic effects of some SLNPs in RNAi or antisense technology have been reported in multiple *in vivo* experiments and clinical trials for hypercholesterolemia, transthyretin (TTR), solid tumors or cancer treatments (see Table 1) becoming systemic administrations as the most preferred application for these delivery platforms [25, 26].

PEGylated lipids, also known as shielding lipids, are one of the most usual components of SLNPs along with cationic lipids. In general, the role of PEGylated lipids in formulations is mainly based on regulating fusogenicity, shielding surface charges, reducing the particle size and avoiding aggregation during storage [27]. Furthermore, the presence of these shielding lipids in SLNPs normally causes better tolerability *in vivo* hence improving the pharmacokinetic profiles of the particles. In order to increase the efficacy of such SLNPs, some groups have optimized the chemical space by synthesizing combinatorial libraries of novel PEGylated and cationic lipids evaluating their efficiencies in both *in vitro* and *in vivo* studies. In particular, a series of novel PEGylated derivatives have been synthesized and combined with various synthetic

cationic lipids to form the SLNPs formulations. For instance, Alnylam Pharmaceuticals [28, 29] and Abbott Laboratories [30] published several patent applications in which synthetic approaches to obtain a variety of shielding lipids were described. Some of them are displayed in Figure 4A. The strategy followed by Alnylam consisted of fixing the hydrophobic part with a long hydrocarbonated alkyl chain and subsequently varying the hydrophilic part by introducing several series of ethylene glycol derivatives (PEG-C-DMSO, PEG-C-DMSA, **3** and **4**). In the case of the method described by Abbott Laboratories, the hydrophilic part was maintained fixed varying the length of hydrocarbonated alkyl chains (lipid-PEG derivatives **5** and **6**). In both cases, these lipid-based particles were prepared by using preformed vesicle methods. A first screening containing PEG-C-DMSA in the formulation revealed better silencing activities than using PEG-C-DSMO in subcutaneous Hep3B-luc tumors in mice (58 versus 30%, respectively). The optimal formulations containing PEG-C-DMSA were also studied *in vitro* and *in vivo* in order to silence murine clotting factor VII mRNA, which is a prominent protein in the coagulation cascade [29]. The preparation of several SLNPs containing PEGylated lipids **5** and **6** along with other PEGylated lipids, such as PEG-Chol, was carried out to determine the transfection efficiency both *in vitro* and in several tumor models of siRNAs targeting the expression of luciferase [30].

Cationic lipids are the other major components of SLNPs. Several companies have carried out an exhaustive study to elucidate the structure-activity relationship (SAR) of these lipids. As a general rule, these cationic lipids forming SLNPs often consist of amino lipids with ionizable amine headgroups and one hydrophobic part containing generally a double-hydrocarbonated alkyl chain. Designing and synthesizing combinatorial libraries of modified cationic lipids has become the main objectives of research groups in order to improve the potency of formulations in RNA interference

and antisense technology [31]. One of the main properties of SLNPs to take into account is the dissociation constant (pK_a) of ionizable lipids since it can play an important role in both the ability to encapsulate nucleic acids and the promotion of *in vitro* and/or *in vivo* deliveries. By optimizing the design of SLNPs including the number of lipid-chain unsaturations, cationic head and linker chemistry, it could be estimated which dissociation constants would be optimal for nucleic acid delivery [32, 33].

The general structure for cationic lipids proposed by Alnylam Pharmaceuticals and Merck is shown in Figure 4B [34, 35]. These lipids are formed by a cationic head group, a linker, two hydrophobic alkyl chains and, finally, two bio-cleavable moieties containing hydrophobic chains as well. Representative cationic lipid derivatives synthesized by Alnylam are displayed in Figure 4B (compounds **7** - **10**). Test formulations containing **7** ï **10** along with a mixture of 1,2-distearoyl-sn-glycerol-3-phosphocholine (DSPC), cholesterol (Chol) and PEG-lipid at their optimal molar ratios were able to encapsulate efficiently siRNA molecules in well-established protocols. The efficacy of SLNPs to impart cellular uptake and silence FVII mRNA were determined by a simple and plate-based colorimetric assay. The use of these formulations *in vivo* containing lipids (**7** ï **10**) showed high levels of effectiveness compared to control animals with values of ED_{50} << 0.1 mg/kg.

Other cationic lipids were published [36-38]. The synthesis of cationic lipids libraries containing two hydrocarbonated alkyl chains; in which the first one contained a long unsaturated hydrophobic moiety with double bonds and the second one was formed by a short saturated alkyl chain was reported. Biodegradable groups like esters (saturated and unsaturated) and different cationic heads [36-38] were successfully introduced. A selection of cationic lipid derivatives is displayed in Figure 4B (compounds **11** ï **14**).

SiRNAs were encapsulated by impinging jet processes and the corresponding test formulations were evaluated to reduce ApoB levels *in vivo*.

The alkylation reaction with epiclorhidrin and subsequent epoxide opening either with Grignard additions or alcohol residues afforded a novel family of cationic lipids containing both ether and diether-based alkyl residues, respectively. Representative compounds are displayed in Figure 4B (compounds **15** and **16**) [39, 40]. Additionally, one of the cationic lipid alkyl chains could also be further functionalized with cholesterol. This modification allowed the preparation of amino derivatives containing different cationic heads and cholesterol residues (compounds **17** and **18**) [41]. SLNPs containing cationic lipids **17** and **18** were evaluated for *in vivo* efficacy in a luciferase mouse model in order to silence ApoB mRNA *in vivo*.

Other cationic lipids have been recently synthesized by Merck [42, 43]. The most representative ones are the cyclic amine **19** and **20**, respectively (Figure 4C). After studying luciferase activity *in vivo*, a greater efficacy was observed in both cases reducing the luciferase expression in a dose-response manner. The evaluation of the toxicity and the efficacy of these two formulations to reduce *in vivo* ApoB was also accomplished obtaining an improved tolerability in rats compared to other formulation controls.

Besides using ionizable lipids, guanidinium-based cholesterol derivatives (**21** and **22**, Figure 4C) described to deliver siRNA molecules [44]. It is well-known that having guanidinium groups (pK between 13 and 14) helps to compact zwitter ionic structures with negatively charged phosphate groups of nucleic acids. Thus, cationic lipids **21** and **22** containing cholesterol and arginine moieties were encapsulated into SLNPs and evaluated in both *in vitro* and *in vivo* targeting human ErbB3 mRNA in cancer cells and

evaluating the down-regulation in human colon cancer in xenografted mice models, respectively.

Cholesterol has also been introduced into other kinds of constructs. The synthesis and applications of a cationic carbamate series containing several cholesterol and hydrocarbonated alkyl residues covalently conjugated to N^6-tetrakis-(3ô-aminopropyl)-1,3-propanediamine unit as a core structure has been published [45]. Some cationic carbamates are displayed in Figure 4C. Optimal formulations were produced by mixing cationic lipids (**23** ï **26**) with phospholipids, polyethylenglycol derivatives and a sterol, preferably cholesterol. Two optimal formulations containing the cationic carbamate **26** were used for liver siRNA delivery which reduced ApoB levels *in vivo* at a dosage of 0.5-4 mg siRNA/Kg and lung siRNA delivery with promising results as well.

Finally, Dicerna pharmaceuticals [46] developed a formulation strategy that combines polypeptide **27** containing Gly-$(Arg\text{-}His)_5$ residues with several palmitoyl derivatives such as l-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (AzPC) and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (GIPC) cationic lipids (Figure 4C). The synthetic strategy based on the conjugation of lysophosphatidyl choline with the two selected dicarboxylic acids of different length allowed the incorporation of the cationic polypeptide sequence separated by two ethylene glycol moieties obtaining either cleavable (**28**) or stable (**29**) constructs. The efficiency of these formulations containing these novel cationic lipids was demonstrated both *in vitro* and *in vivo* by reducing the expression of a target gene in an orthotopic animal tumor model.

3.2 Lipid-oligonucleotide conjugates

As an alternative to the use of formulations obtained through electrostatic interactions, the introduction of covalent bonds between neutral or cationic lipids and nucleic acids represents an attractive approach to improve both the cellular uptake and the stability properties of nucleic acids.

The introduction of cholesterol in the 3ô-termini of a siRNA was the first example of this approach to silence apoB in liver and jejunum [47]. This approach has allowed the publication of patent applications and research articles covering covalent strategies to introduce lipophilic moieties at polynucleotides. In all cases, modifications containing two hydroxyl groups clearly differentiated, along with spacers of different length, are the preferred strategies to obtain nucleic acids modified with lipophilic moieties in a covalent manner.

The synthesis of several oligonucleotide cholesterol type conjugates introduced at the 5ô- and/or 3ô-end through several hydrocarbon or ether-linked moieties comprising 6 and 10 carbons of length (Table 2) has been recently described by miRagen [48]. These conjugates were designed to mimic or target miR-208, which is a family of miRNA precursors that are specifically expressed in cardiac tissues obtaining good gene knockdown activities of miR-208.

Other lipophilic moieties have been effectively introduced to modify siRNA molecules at both 3ô- and 5ô-termini [49, 50]. Sylentis SAU introduced sphingosine modifying siRNA molecules with **30** and **31**. They observed that siRNA-sphingosine conjugates (Figure 5A) were able to silence EGFP expression showing an improved stability without affecting the efficacy of naked siRNAs *in vitro* [49]. Furthermore, a series of siRNA conjugates (**33a-e**) containing neutral and cationic lipids covalently linked at their 3ô- and 5ô-termini has also been described [50]. The authors observed that lipid

modifications did not affect the RISC machinery when lipid-siRNA conjugates were transfected in the presence of lipofectamine thereby obtaining similar inhibition activities than unmodified siRNA targeting TNF-Ŭ gene. Promising results were achieved when the lipid-siRNA conjugate **35**, modified with two hydrocarbonated alkyl chains, efficiently silenced TNF-Ŭ expression in around 50% in the absence of commercially available cationic lipids [50].

Other strategies used for the delivery of siRNA molecules have been published [51]. These constructs are based on a cholesterol molecule covalently linked through amide bonds to alkyl or pegylated spacers and a dipeptide having phenylalanine (Phe) and lysine (Lys) residues. Additionally, the general structure can contain a cleavable *p*-aminobenzylcarbamate spacer and a siRNA binding site, which has a *p*-nitrophenyl activated carbonated moiety (**36**, Figure 5C). Therefore, modified amino siRNAs can be efficiently conjugated to the cholesterol-di-peptide structure by ~~the~~ nucleophilic displacement of *p*-nitrophenyl moiety thereby obtaining the corresponding carbamate molecule **37**. When these kinds of cholesterol-siRNA conjugates are taken up by cells, an expected 1,6-elimination reaction is produced, thus inducing the release of the siRNAs by lysosomal degradation inhibiting gene expression of a target mRNA. This patent application describes the ability of these siRNA conjugates containing Phe-Lys dipeptides to impart cellular uptake reducing ApoB levels in both *in vitro* and *in vivo* assays. SiRNA conjugates containing cleavable linkers showed better gene knockdown efficiencies than non-cleavable cholesterol siRNA conjugates. The authors also confirmed that the original Phe-Lys motifs present in cholesterol di-peptide siRNA conjugates were strictly necessary to decrease the production of ApoB so that the same siRNA conjugates containing other dipeptide motifs obtained less activity efficiencies in silencing ApoB. Finally, Phe-Lys dipeptide-siRNA conjugates **38** containing both

cholesterol and *N*-acetyl-*D*-galactosamine ligands were covalently linked. Carbohydrate ligands increased the efficacy of these conjugates in cellular uptake processes through receptor-mediated endocytosis due to their high binding affinities for the asialoglycoprotein receptor (ASGPR).

3.3 Peptides.

Peptides have emerged as a new class of non-viral vectors allowing the delivery of various drugs including nucleic acids through a mechanism of internalization that is still controversial and depends on the nature of the peptide. Cell penetrating peptides (CPP) also named protein transduction domains, comprise short and usually basic amino-rich peptides originating from proteins able to cross cellular membranes. Several groups have published patent applications covering delivery molecules formed by different polypeptides that are bound to nucleic acids by conjugation or by a non-covalent approach via electrostatic interactions. Polypeptide delivery systems are different peptide combinations containing one or several peptides related to extracellular receptor proteins of targeted cells, peptides that enhance the delivery or interact with nucleic acids. These systems form stable complexes with nucleic acids in the presence of serum and sometimes are able to escape from endosomal-lysosomal compartment.

3.3.1 Non covalent approach. Chimera cell delivery peptides comprising a positively charged peptide and a targeting-delivery peptide are capable of highly efficient delivery of nucleic acids into cardiac and skeletal muscle cells. Therefore the constructs may be used for treatment of a cardiac or skeletal muscle diseases or muscular dystrophy [52]. The targeting-delivery peptide is selected from MSP (muscle-specific protein) or from reported peptides to transfect antisense PNA (peptide nucleic acid) or PMO

(phosphorodiamidate morpholino oligonucleotide) to skeletal muscle with high efficiency.

In a similar system, the carrier is composed of three main peptide segments, a unit designed to bind to specific target cells, a unit designed to mediate penetration into the interior of target cells and a unit that can interact with nucleic acids designed to mediate RNAi. The complexes (peptide/ siRNA) are formed by combining the 'Her' domain for binding heregulin receptors of breast cancer cells and peptides derived from recombinant adenovirus (Ad) capsid protein. Finally, binding and transport of nucleic acids can be mediated by the polylysine, 'KlO' domain [53].These carriers also protects the siRNA from serum nucleases.

Synthetic peptides including apolipoprotein E (ApoE) which are believed to bind with various proteins (albumin and/or immunoglobulins) that are presumably recognized by the low-density lipoprotein receptor (LDLR) pathway are able to cross the blood-brain barrier (BBB) through transcytosis [54]. Peptide constructs to deliver nucleic acids comprise a mixed sequence of hydrophilic amino acids and a blood-brain barrier agent derived from the receptor binding domain of an apolipoprotein.

Another interesting invention relates the use of combination of anti-cadherin oligonucleotides to one or more other anti-cadherin agents including peptides that bind cadherin extracellular domains useful in the treatment of acute, delayed healing and chronic wound [55]. Similarly, chimeric RNA linked to a polypeptide moiety is able to reduce DNA methylation. The RNA oligonucleotide also includes a therapeutic agent selected from a demethylating agent (e.g., 5-azacytidine (azacitidine), 5-aza-2'-deoxycytidine (decitabine)) to enhance binding to DNA methyltransferases (DNMT) [56].

Antimicrobial compositions including a CPP is combined with or conjugated to a functional nucleic acid (including morpholino oligonucleotides), such as an external guide sequence (EGS) which can target and reduce expression of essential microbial genes or genes than impart resistance to antimicrobial drugs [57]. Likewise, different polypeptides moieties containing at least one of thymosin, any interferon or pegylated derivative, myrcludex B or any antiviral cytokine or pegylated derivative have been used to enhance the delivery of antiviral oligonucleotide chelates [58].

Another strategy for improving the delivery and stability of therapeutic oligonucleotides, named NickFect, relates to chemically modified new branched CPP with fatty acids attached to a targeting moiety that is capable of reaching specific cells or tissues of interest. The targeting moiety may be an aptamer or a targeting peptide, such as a homing peptide or a receptor ligand [59].

3.3.2 Covalent approach. The main drawbacks of using CPP not covalently linked to nucleic acids are their potential toxicity and the endosomal entrapment [60]. Linking CCP to oligonucleotides leads to well-defined, single-component systems that use only equimolar ratios of the delivery material and nucleic acid.

Several groups describe different constructs based on directed attachment to nucleic acids. One strategy is the preparation of amino functionalized oligonucleotides that are transformed to maleimido-oligonucleotides. These are able to react with modified cysteine forming a maleimido-thiol covalent bonds [61]. These therapeutic conjugates contain a peptide that specifically homes to endothelial cells in the tumour for the efficient delivery of sequence-specific antisense to cells of a selected type.

By linking a cell-penetrating peptide to a substantially uncharged antisense nucleic acid analogue via a glycine or proline amino acid, the resulting conjugates decreased the toxicity and/or enhanced cell delivery, potency, and/or tissue distribution [62].

Another approach consists of preparing peptide segments that are useful for facilitating the uptake across cell membranes, the segments are separated by aminohexanoic spacer residues and may also include non-natural amino acids [63]. Both PMO and PNA antisense are conjugated at the C-terminus of the peptide and are able to modulate dystrophin pre- mRNA splicing, thereby specifically restoring the dystrophin reading frame and generating a truncated but semi-functional dystrophin protein.

Modified and derivatised CPP with fatty acid conjugated to nucleic acids have been used to enhance transfection with less toxicity for splice correction as well as siRNA delivery [64]. Additionally, the system comprises irreversibly chloroquine-coupled compounds for endosomal escape.

Maurocalcine is the first demonstrated example of an animal toxin peptide with efficient cell penetration properties. The toxin is a 33-amino acid peptide and the invention relates to small CPPs derived from this toxin and to their use as vectors for the intracellular delivery of various drugs and agents [65]. The peptide is derivatized and coupled to oligonucleotides via a disulphide bond, thioether, thiol-maleimide or amide linkage. Other methods of linking the peptide to the oligonucleotide include the use of a C-terminal aldehyde to form an oxime, the use of a click reaction or the formation of a morpholino linkage with a basic amino acid on the peptide.

Formulations containing phase changing charge-trapped peptides and nucleic acids have been optimized to increase cellular uptake and cytoplasmatic delivery of oligonucleotides [66]. Some of these changes includes pH change induced

protonation/deprotonation, disulphide reduction, hydrolysis and proteolysis. The process involves designing candidate-phase changing, charge-trapped peptides, including designing amino acid consensus sequences with phase changing charge-trapped residues, providing a library of such peptides and screening the peptides for their efficacy in knocking down the cellular expression of a target gene.

Another invention relates to the use of a carrier peptide system that is designed to harness and/or exploit fully natural pathways for initial cell targeting and internalization, followed by retrograde transport through membranous compartments to the endoplasmic reticulum (ER) and retro-translocation from the ER to the cytosol via the ER-associated degradation pathway (ERAD) [67]. Specifically, the delivery system comprises several modules linked by cysteine side chain as branch point. One module mediates cell targeting and facilitates cellular uptake, whereas the other facilitates transport to the ER. The last one mediates translocation from the ER to the cytosol.

3.4 Small-molecule ligands

3.4.1 Small-molecule ligands for receptor mediated uptake. A number of promising small organic ligands have been developed to improve the delivery of nucleic acids by direct conjugation. These bioactive molecules have very diverse structures and are linked to nucleic acids through different bonds.

Vitamins have been conjugated to nucleic acids. In this context, Ŭ-tocopherol has been linked to nucleic acids such as siRNA providing a method for suppressing a target gene expression (apoB) *in vivo* more safely and efficiently [68]. Alternatively, Ŭ-tocopherol-

bound nucleic acids were mixed with extracted chylomiclon, and then administered. Multivitamin B12 conjugates for RNAi therapeutics can assist in one or more ~~of~~ cell targeting, and/or the release the oligonucleotide or oligonucleotide conjugate into the cell cytoplasm [69]. The above conjugate can be formulated to provide some protection from degradation or denaturing of the oligonucleotide. The delivery systems also comprise nanoparticles which may assist cell targeting. Folic acid has been also conjugated to nucleic acids. Folate conjugates can enter cells by receptor- mediated endocytosis. Folate siRNA using different linkers targets tumors or cells of the immune system overexpressing folate receptors and causing cancer, or inflammatory diseases, respectively [70].

Other small molecules such as selected alkyl or heteroaromatic [71] or minor groove binders [72] have been attached to siRNA in order to improve cell penetration and biodistribution properties.

In addition, small organic ligands for a membrane-bound protein are covalently coupled to an oligonucleotide [73]. The ligand is coupled through a linking group that comprises a phosphate group covalently coupled to an aliphatic or an aliphatic oxide group. Ligands are designed for membrane-bound proteins, such as G-protein, or several receptors such as ion-channel receptors, tyrosine kinase-linked receptors, receptor tyrosine kinases, cytokine receptors, and receptors with intrinsic enzymatic activity.

One of the most clinically advanced conjugate is the *N*-acetylgalactosamine (GalNAc) conjugate by Alnylam Pharmaceutical. A triantennary system with optimized spacer-linked GalNAc molecules is designed to achieve targeted delivery of RNAi therapeutics to hepatocytes through uptake by the asialoglycoprotein receptor with high affinity. These constructs have effective delivery efficiency, as well as efficient cellular uptake

and/or endosomal escape properties. Improved knockdown of the target gene was obtained by subcutaneous administration. No evidence of cytokine induction, complement activation or other adverse effects was observed using a wide therapeutic window. *N*-acetylgalactosamine-conjugated melittin-like peptide platform with potent cholesterol-siRNA targeting hepatitis B virus resulted in a good in vivo therapeutic treatment without changes in cytokines [74].

A siRNA conjugate targeting the Transthyretin (TTR) gene was described for the treatment of TTR-mediated amyloidosis [75]. Similarly, a modular composition comprising the tetraGalNAc ligands attached to the passenger strand of siRNA at different 2'-positions of the ribose rings and/or at different terminal 3' and/or 5'-positions were described. These systems allow the addition of other targeting ligands such as solubilizing agents, pharmacokinetics enhancing agents, lipids, and/or masking agents [76]. Asialoglycoprotein and folate receptors were also targeted by a galactose cluster-pharmacokinetic modulator moiety (siRNA-conjugate) with in vivo circulating and targeting properties compared to the targeting ligand alone. The pharmacokinetic modulator comprises a hydrophobic group. The delivery system also comprises a polyamine polymer not conjugated to siRNA [77].

The incorporation of a non-ionic, low-osmolar contrast agent to antisense oligonucleotides is described for treating a neurodegenerative disease [78]. These contrast agents such as iobitridol, iohexol, iomeprol, iopamidol, iopentol, iopromide, ioversol or ioxilan are able to cross the blood-brain-barrier and to be delivered to any tissue throughout the body, particularly the entire nervous system; this includes both the and the peripheral nervous system. Antisense oligonucleotides can bind to a survival motor neuron (SMN) gene or other related genes. In addition, morpholino antisense

oligonucleotides can bind CAG repeats, CTG repeats, CCTG repeats, or GGGCC repeats.

Targeted delivery of siRNA is accomplished by the the conjugation of 5'-thio-siRNA with selective small molecule alpha-V-beta-3 [79] or LFA-1 [80] VLA-4 [81] integrin antagonists containing appropriate linkers (e.g. ethyleneglycol) and functional groups (e.g. maleimido). Moreover, the conjugation of oligonucleotides with intracellular retrograde transport inhibitors (retro compound) such as an imine derivative or a benzodiazepine derivative increases the delivery and/or the activity of the oligonucleotide in the cell [82].

3.4.2 Multiplex structures. The delivery system called QFect for intracellular cargo delivery comprise a multicomponent system containing quinoline or naphthalene compound, an aliphatic linear or branched hydrophobic chain and an optional targeting moiety, such as homing peptide or an aptamer [83].

Another multiplexed structure invention to enhance oligonucleotide delivery comprises a modular composition. Specifically, an oligonucleotide (siRNA) with one or more linkers attached at any 3' and/or 5' end and one or more peptides attached to the linkers and optionally lipids, solubilizing groups and/or targeting ligands [84].

3.4.3 Aptamers. Targeted aptamers can be used as vehicles to deliver specific agents to particular sites. Alternatively, targeted aptamers can also be used along detection techniques to determine either the presence or the absence of specific targets in heterogeneous backgrounds.

Chimeric molecules such as aptamer-oligonucleotide conjugate have been described to improve the delivery and inhibition of the conjugated to specific cells. Specifically, a prostate-specific membrane antigen (PSMA) aptamer is conjugated to siRNA in order to inhibit nonsense-mediated mRNA decay (NMD), a surveillance mechanism which prevents the expression of mRNAs containing a premature termination codon [85].

New aptamers are developed to target tumor-associated antigen (CEA) [86]. Aptamers have been also used to release a specific drug. This is the case of an aptamer/insulin conjugate complex from which insulin may be released by an innocuous, orally administrable trigger, such as quinine [87].

3.5 Polysaccarides and other natural polymers

Chitosans, glucan, dextrans and glucosamines are usually used as a delivery vehicle of therapeutic nucleic acids. These compounds may provide some advantages, including efficient transfection, bio-delivery, and availability, buffering ability or serum stability. Modified chitosan with one secondary or tertiary amine are able to introduce imidazole groups [88]. Other approaches include chitosan-arginine, chitosan-lysine and chitosan-histidine modifications [89].

Additionaly, hydrophobic modified polynucleotide complexed with a glucan-containing particle have significantly reduced toxicity, are better recognized by phagocytic cells, and have a more efficient payload release and overall efficacy [90].

Several nanoparticle compositions containing chitosan have been described to improve nucleic acids delivery. A composition of a water-soluble chitosan, a thiamine pyrophosphate, a protamine, and a neutral or anionic phospholipid exhibits biostable

nanoparticle properties. This improved chitosan composition prevents aggregation of the chitosan with serum proteins [91]. Similarly, modified nanoparticle chitosan with a biocompatible layer that comprises poly(L-histidine) or a chelating agent moiety has been used for a divalent metal transporter of an olfactory nerve terminal. This method comprises nucleic acid delivery to the central nervous system [92].

Cyclodextrins are a group of cyclic polysaccharides comprising six to eight naturally occurring D(+)-glucopyranose units in alpha-(1,4) linkage. Modified forms of cyclodextrin with cationic arms covalently bound form well defined stoichiometric complexes with nucleic acids (siRNA) [93].

3.6 Synthetic polymers

A large number of polymeric systems have been developed for drug delivery [94]. Most of the polymeric systems have been developed for the delivery of small therapeutic molecules decreasing toxicity by slow degradation of polymer and / or release of the drugs in cellular conditions.

Cationic polymers such as polyethyleneimine (PEI) were one of the first polymers used for oligonucleotide delivery, since the acidic pH of the endosomes trigger the liberation of the cargo in the cytoplasm. Recently, new PEI derivatives carrying several ethylcarbamoylphenyl side chains have been proposed for siRNA delivery to decrease undesired toxicity [95]. Moreover, polypropyleneimine (PPI) has been described to deliver siRNA [96].

In a recent development a large number of polymers and conjugates have been described for delivering nucleic acids [97]. These systems include polyacrylates,

polylactides, polyesters and polyvinyl derivatives, as well as hydrophilic-hydrophobic copolymers such as polylactic-glycolic acid (PLGA), polyethyleneglycol-PLGA and others. Surprisingly, negatively charged polymers as well as amphiphilic polymers have demonstrated improved silencing efficiency and reduction of cell toxicity when used to deliver morpholino (PMO) and PNA oligomers [98]. PLGA microparticles have been described for the encapsulation of triplex-forming PNAs [99].

Specifically biodegradable polymeric made of poly(lactic) copolymerized with several hydrophilic-hydrophobic polymers such as polymetacrylic acid or poly(ethyleneglycol), or polystyrene, or poly(vinylpyridine) and more have been developed [100]. The main effect of the copolymers is the possibility of tuning up different degradabilities of the polymers depending on the amount of copolymer without compromising the low toxicity of poly(lactic) polymer [101].

Polyamides derived from diaminopropane derivatives [102], ornithine [103] or ornithine-phenylalanine [104] carrying protonable amine groups as side chains have been described as membrane lytic polymers for delivery of siRNA. Oligonucleotides as well as polyethyleneglycol and *N*-acetylgalactosamine molecules for cell targeting are conjugated to amino side chains.

Dynamic polyconjugates (DPC) made of polyvinylether copolymers with several side chains for controlling functionalization and biodegradability are probably one of the most exciting solutions for nucleic acid delivery [105]. The use of reversible linkages connecting the different components of the delivery system provides physiologically modulation of the release of the components [105]. Modified polyacetal derivatives resulting from the oxidation of polydextranes have been efficiently loaded with nucleic acids and several targeting groups with good biodegradability properties [106]

The combination of graft copolymers of polyacrylic acid with cationic lipids has been described [107]. Electrostatic interaction between polyacrylic polymers direct the binding of cationic lipids and this combination can be used for the delivery of plasmid DNA, antisense oligonucleotides and siRNA [107]. The use of ionic monomers such as dimethyloctylammonium acrylate derivatives in the polymerization of methyl methacrylate has been described for the preparation of core-shell polymers complexes with oligonucleotides designed for restoring the expression of the dystrophin protein by exon skipping mechanism [108]. Moreover, functionalization of polymers derived from dimethylacrylic acid with siRNA and ligands to facilitate endosomal escape has been developed [109].

3.7 Nanomaterials

The rapidly expanding field of nanotechnology has allowed the synthesis of well defined nanoparticles (NPs) of diverse materials. These NPs can be functionalized either with positive charges to condense DNA or RNA molecules or with therapeutic oligonucleotide sequences such as antisense, siRNA or aptamers. The functionalized biocompatible nanomaterials, such as gold, silica, iron oxide, carbon nanotubes or graphene oxide, have been used for the delivery of oligonucleotides [4].

The ability of oligonucleotide-NP conjugates, especially gold NPs, to penetrate a wide variety of cell types has been used to provide new delivery systems for therapeutic agents such as alkylating agents, DNA intercalating agents, antimetabolites or proteins of interest [110-112]. In order to avoid the large uptake of gold NPs from the reticuloendothelial system, the addition of an embolic agent such as lipid emulsions or biodegradable microspheres has been proposed [113].

Recently, gold nanoparticles have been used as a template for the preparation of hollow silica particles that can be functionalized with therapeutic oligonucleotide sequences with high load and low toxicity [114]. The coating of mesoporous silica NPs with cationic polymers has also been described as a suitable carrier for siRNA transfection [115].

Carbon nanotubes have also been developed for drug delivery. In a recent description single wall carbon nanotubes functionalized with morpholino oligonucleotides are prepared in order to form two-component self-assembling units that allow the efficient delivery of the active morpholino oligonucleotides inside of tumor cells [116]. Carbon nanotubes can be functionalized with cationic compounds such as polylysine, cationic lipids, dendrimers for targeted delivery of therapeutic oligonucleotides to specific cell types to reduce cellular toxicity [117].

4. Expert opinion

Cellular delivery of nucleic acids requires the combination of two factors: cellular penetration and escape from endosomal compartments. Much progress has been made in the last years in understanding these issues, which are crucial for the efficacy of therapeutic oligonucleotides. Cationic lipids are the most used vehicles for the preparation of novel formulations as they are the simplest and the fastest way to bind non-viral vectors with nucleic acids taking advantage of the electrostatic interactions. Solid lipid nanoparticles carrying synthetic cationic lipids, cholesterol and polyethyleneglycol have emerged as a winning alternative to liposomes. Combinatorial libraries of cationic lipids have been used for the screening and development of cationic lipids with enhanced cellular uptake and biocompatibility. Significant developments

from Alnylam Pharmaceuticals, Merck Sharp & Dohme, Dicerna pharmaceuticals and Agave Pharma in searching new cationic lipids have led to the successful delivery of oligonucleotides to liver and lung tissues.

The synthesis of an oligonucleotide covalently-linked to lipids has emerged as a promising alternative to the use of formulations. The introduction of covalent links to neutral lipids such as cholesterol (miRagen Therapeutics), sphingosine (Sylentis) or cationic lipids into nucleic acids represents an attractive approach to improve both the cellular uptake and stability properties of nucleic acids. The use of peptides as cleavable spacers between cholesterol and the oligonucleotide moieties (Hoffmann-LaRoche) adds a very interesting novelty for lysosomal degradation that generates better silencing efficiencies.

The use of peptides as non-viral vectors instead or in combination with lipids is also an expanding field for oligonucleotide delivery. This strategy is certainly advantageous for delivering non-charged nucleic acids derivatives such as PNA and PMO derivatives showing interesting results in the treatment of muscular dystrophy by exon skipping. The covalent linking of peptides to oligonucleotides seems promising given to the use of several peptides with different functions but in fact it did not provide until now interesting results until now, except in the already mentioned delivery of PNA and PMO derivatives.

The introduction of small molecule ligands into oligonucleotides in order to increase cellular uptake by receptor-mediated endocytosis has been also pursued with relatively good results in the case of the use of *N*-acetylgalactosamine conjugates that provide good uptake to hepatocites by the asialoglycoprotein receptor. These conjugates are currently under clinical evaluation.

Synthetic and natural polymers may also play an important role in the forthcoming years. The large development in the last years directed to the delivery of small therapeutic drugs has provided good technological polymeric platforms in terms of nice cellular uptake, good biodegradability, good biocompatibility, and the possibility to incorporate several components such as ligands for receptor mediated endocytosis and molecules to facilitate endosomal escape. Low functionalization, toxicity and batch-to-batch reproducibility are still the potential negative outcomes that will be solved in the coming years with the most advanced polymers chitosans, dextrins, PLA, PLGA and PEI derivatives.

Finally, the use of nanomaterials has opened a new avenue for oligonucleotide delivery with similar advantages and disadvantages described for polymeric delivery systems. In our opinion, silica and gold nanoparticles have the best options to provide efficient functionalization and good cellular uptake properties. Recent results with graphene oxide functionalized with cationic molecules may also be an interesting alternative.

The use of nucleic acids for inhibiting the expression of target genes at the level of mRNA has obtained, in most cases, excellent results *in vitro*. However, the therapeutic applications of oligonucleotides *in vivo* have undergone multiple obstacles, such as stability and cellular internalization. Immunogenicity and cytotoxicity are other issues to take into account. These hurdles clearly reduce the possibilities of success for these therapies although some proof-of-principle studies have demonstrated promising silencing activities without secondary effects. The development of safe and efficient delivery systems continues to be challenging, but the last developments showed improved *in vivo* efficacy, thus gaining a foothold in therapeutics.

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Figure 1. Chemical modifications carried out to improve the stability of oligonucleotides

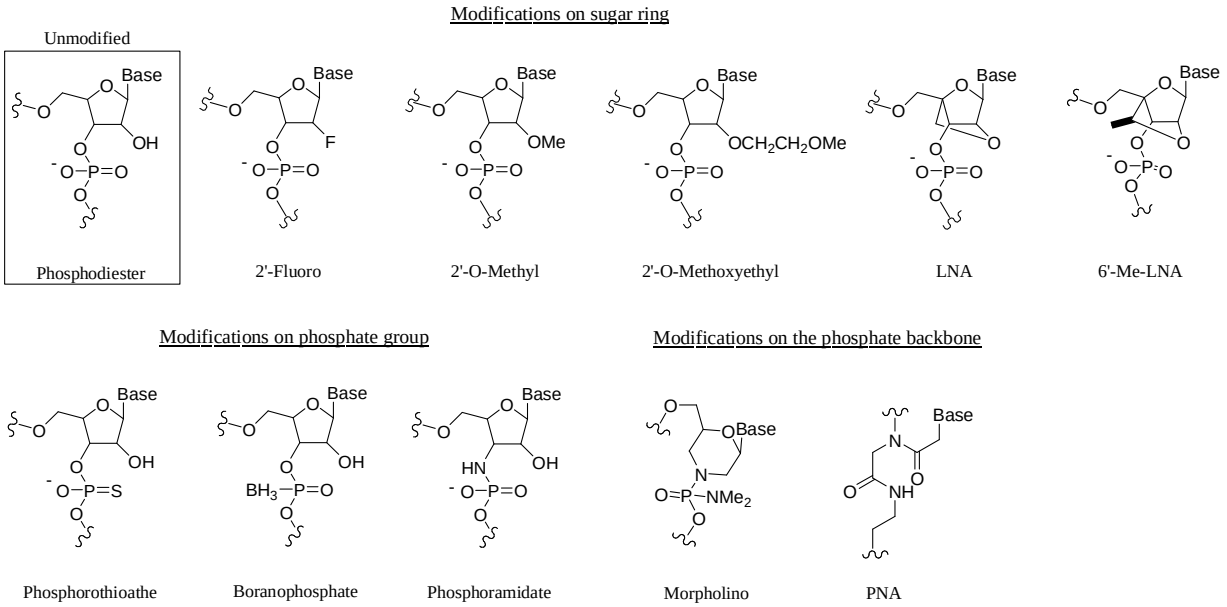


Table 1. Antisense and siRNA oligonucleotides in the clinical pipeline

Drug	Carrier	Target	Disease	Phase	Company	Status*
Macugen	RNA aptamer	VEGF	Age-related macular degeneration	III	Pfizer	approved
Mipomersen	ASO	ApoB	Hypercholesterolaemia	III	ISIS	approved
Oblimersen	ASO	Bcl2	cancer	II	Genta	completed (NCT00064259)
Custirsen	ASO	Clusterin	Prostate cancer	III	OncoGenex	ongoing (NCT01578655)
IMO-2055	ASO	TLR9	Renal cell carcinoma	II	Idera Pharmaceuticals	completed (NCT00729053)
PF04523655	siRNA	RTP801/REDD1	Wet AMD and DME	II	Quark Pharmaceuticals	completed (NCT01445899)
Excellair	siRNA	SYK kinase	Asthma	II	ZaBeCor	ongoing
TD101	siRNA	Keratin 6a (N171 mutation)	Pachyonychia Congenita	Ib	TransDerm	completed (NCT00716014)
AGN211745	siRNA	VEGFRI	Age-related macular degeneration	II	Allergan	completed (NCT00395057)
ISIS-STAT3Rx	ASO	STAT3	DLBCL limphoma	I/II	Isis Pharmaceuticals	ongoing (NCT01563302)
ISIS-PTP1BRx	ASO	PTP-1B	Type 2 Diabetes	II		ongoing (NCT01918865)
Eteplirsen (AVI-4658)	PMO	Exon 5	Duchenne muscular dystrophy	II	Sarepta Therapeutics	completed (NCT01396239)
Bevasiranib	siRNA	VEGF	AMD	III	Opko Health, Inc	completed (NCT00499590)
ALN-VSP02	siRNA		Solid tumors	I	Alnylam Pharmaceuticals	completed (NCT01158079)
Sirna-027	siRNA	AMD	Age-related macular degeneration	I/II	Allergan/Sirna Therapeutics	completed (NCT00363714)
SYL040012	siRNA	ADRB2	Ocular hypertension	I/II	Sylentis	completed (NCT01227291)
SYL1001	siRNA	TRPV1	Dry eye	I/II		ongoing (NCT01776658)
CALAA-01	siRNA	RRM2	Solid tumor cancers	I	Calando Pharmaceuticals	completed (NCT00689065)
ALN-TTRSC	siRNA	TTR	TTR cardiac amyloidosis	II	Alnylam Pharmaceuticals	ongoing (NCT01981837)
ALN-TTR02	siRNA			II		ongoing (NCT01617967)
ALN-RSV01	siRNA	RSV	RSV infection	IIb		completed (NCT01065935)
ALN-PCS02	siRNA	PCSK9	Hypercholesterolemia	I		completed (NCT01437059)
TKM-080301	siRNA	PLK1	Advanced solid tumors	II	Tekmira Pharmaceuticals	ongoing (NCT01262235)
SPC2996	LNA PS	Bcl2	Chronic lymphocytic leukemia	I/II	Santaris Pharma	completed (NCT00285103)
Atu027-I-02	siRNA	PKN3	Metastatic pancreatic cancer	I/II	Silence Therapeutics	ongoing (NCT01808638)
RXI-109	siRNA	CTGF	Hypertrophic scar	II	RXi Pharmaceuticals	ongoing (NCT02030275)
CRLX-101	siRNA	Topoisomerase I	Rectal cancer	II	Cerulean Pharma	ongoing (NCT02010567)
QPI-1002	siRNA	P53	ischemia-reperfusion-related injuries	I/II	Quark Pharmaceuticals	ongoing (NCT00802347)

*http://www.clinicaltrials.gov

Figure 2. Strategies to deliver nucleic acids (cargo) by using non-viral carriers: A. Formulation and B. Covalent approaches.

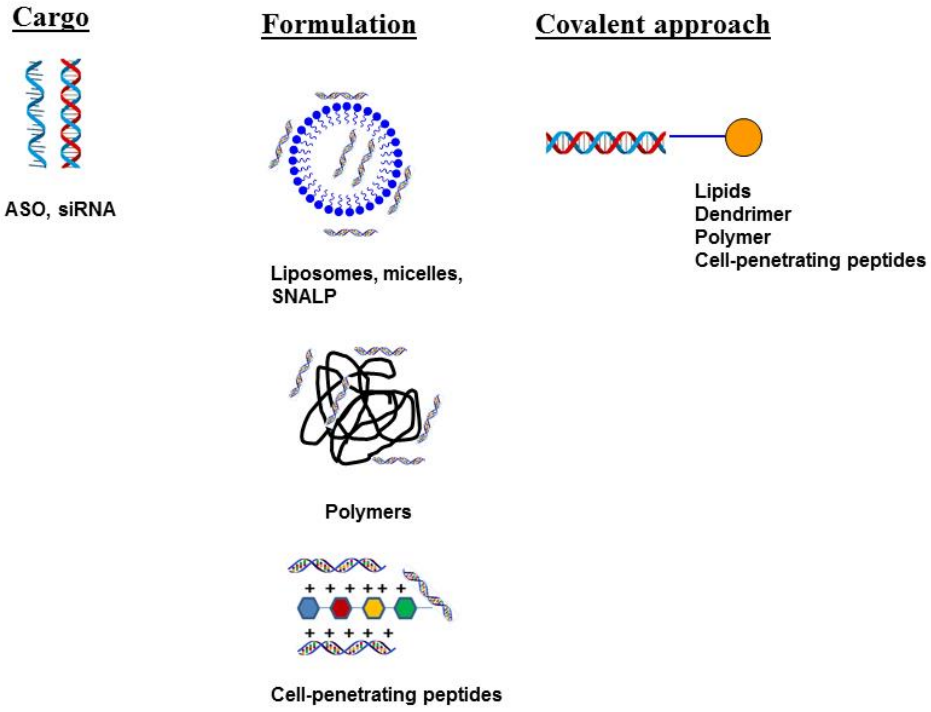


Figure 3. Some of mechanisms used for the inhibition of gene expression implies antisense inhibition with the intervention of RNase H (A), RNA interference mechanism (B) or steric-blocking of mRNA (C).

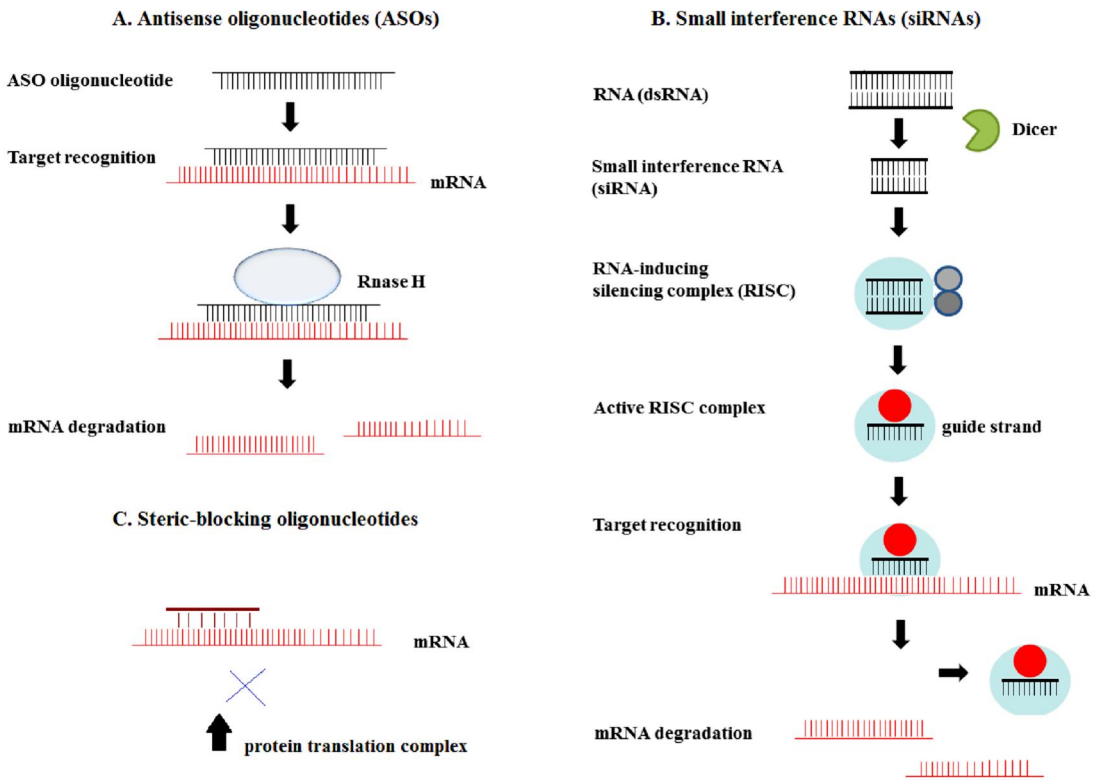
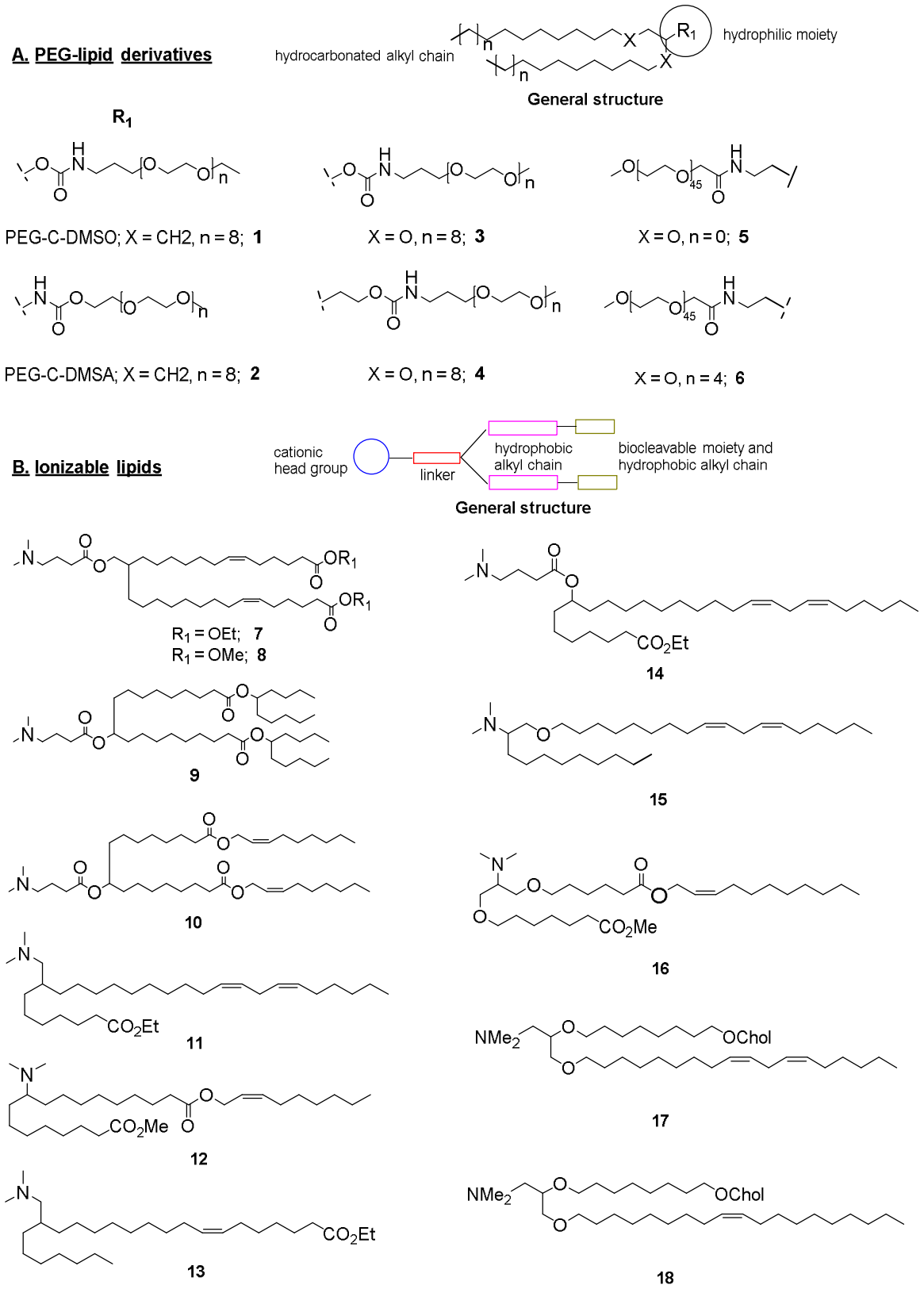
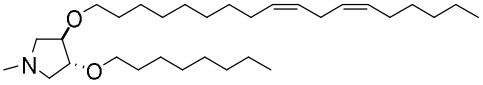


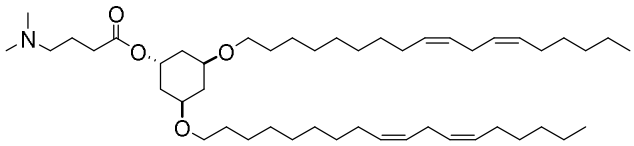
Figure 4. General structures of modified PEGylated lipids (A), representative ionizable cationic lipids containing biodegradable moieties (B) and other constructs (C) used in formulations.



C. Other constructs



19



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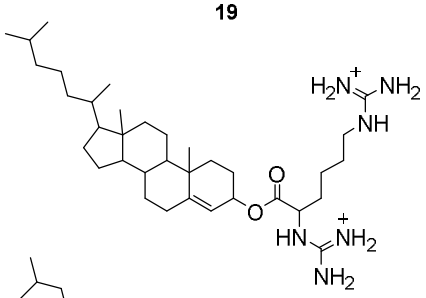

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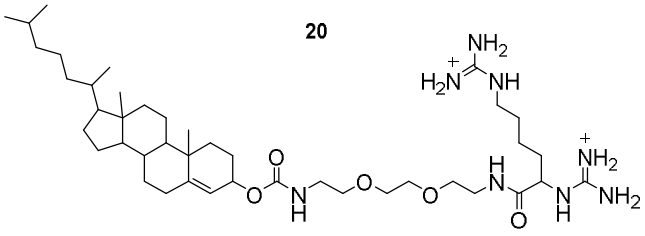

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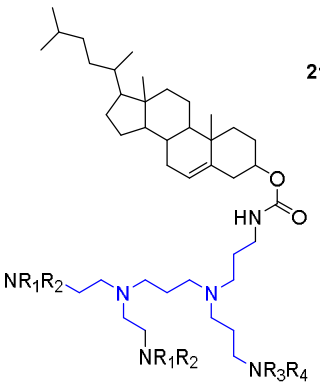


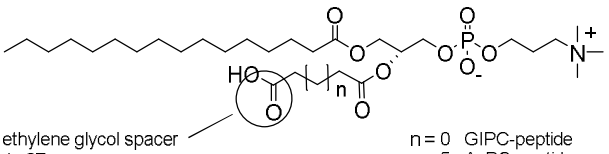
R_1, R_2, R_3 and R_4 = Chol **23**

R_1, R_2, R_3 and R_4 = Gua **24**

R_1, R_2 = Gua; R_3, R_4 = Chol **25**

R_1, R_2, R_3 and R_4 = H (preferred) **26**

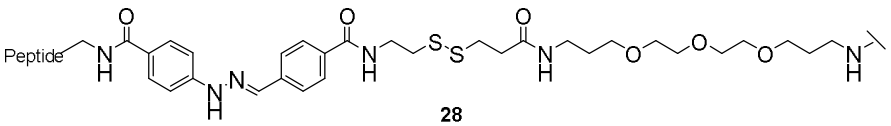
General structure



R: potential site to introduce ethylene glycol spacer and (Arg-His)5-Gly polypeptide **27**

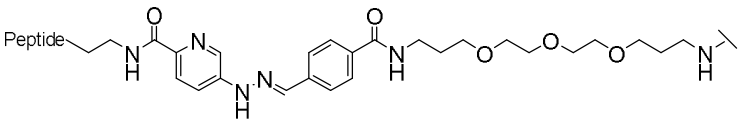
n = 0 GIPC-peptide
n = 5 AzPC-peptide

R



cleveable (disulfide) peptide

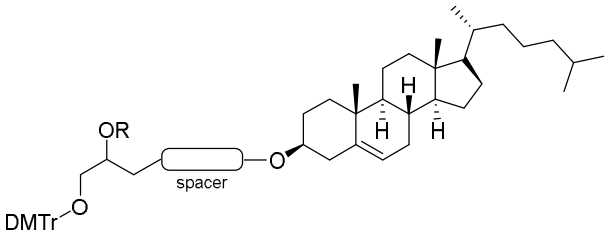
28

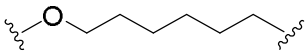
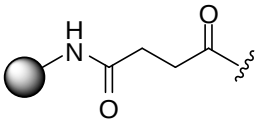
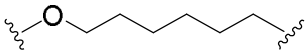
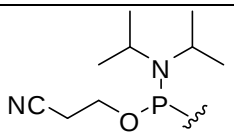
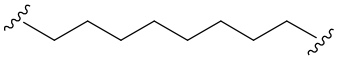
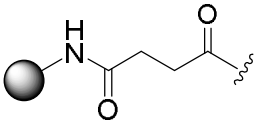


stable peptide

29

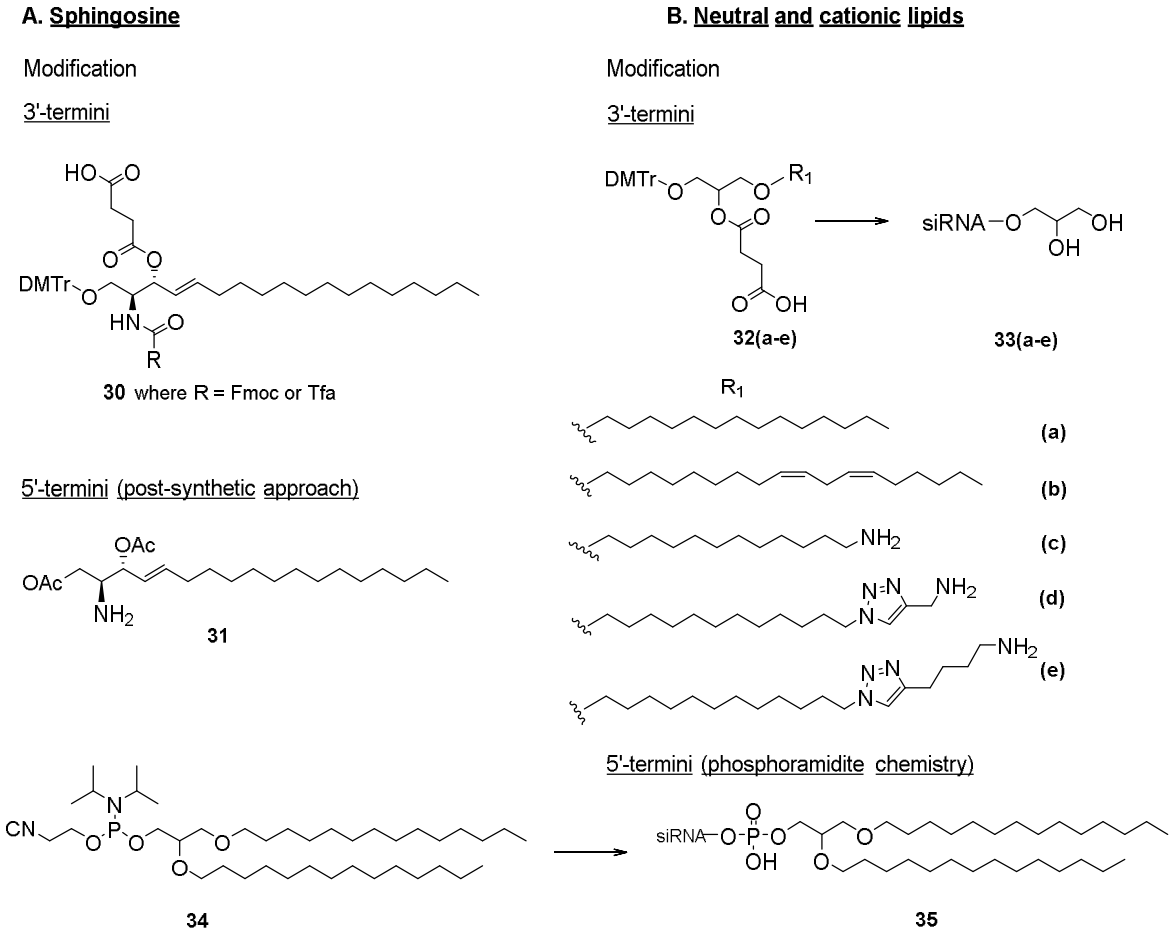
Table 2. Potential sites of conjugation with cholesterol modifying mir-208 molecules at both 3ô- and 5ô-termini



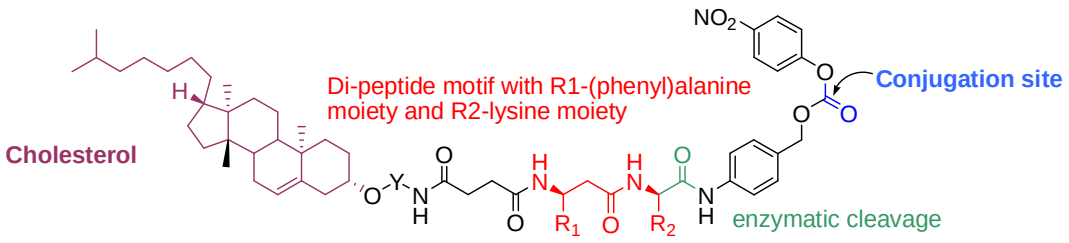
Entry	Spacer	R	Site of conjugation
1			3ô
2			5ô
3			3ô

 = Solid support

Figure 5. Synthetic strategy to introduce sphingosine (A), neutral and cationic lipids (B) at both 3ô- and 5ô-termini and other constructs (C)

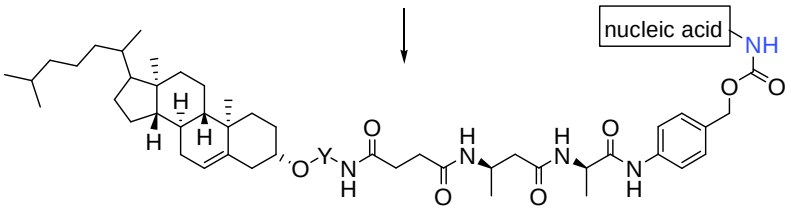


C. <u>Other constructs</u>



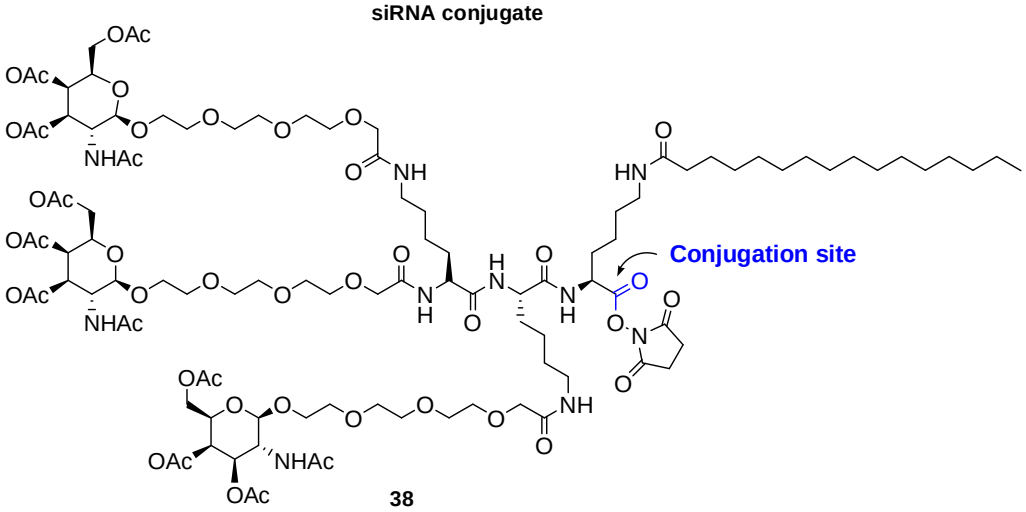
36

where Y = $-(CH_2)_3-$ or $C(O)N-(CH_2-CH_2-O)_n-CH_2-CH_2-$



37

siRNA conjugate



38