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Oligonucleotide delivery in cancer therapy

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Importance of the field: Cancer is frequently caused by altered protein expression. Oligonucleotides (ONs) are short synthetic nucleic acid fragments, able to selectively correct protein expression into cells by different mechanisms. However, biological barriers hamper the therapeutic use of ONs without suitable delivery strategies.

Areas covered in this review: This review summarizes the most meaningful non-viral strategies for ON delivery, including the chemical modifications of the ON backbone and non-viral delivery systems.

What the reader will gain: The reader will gain an update of the main strategies for ON delivery in cancer. Advantages and limits of each approach are underlined. Emphasis is given to the delivery strategies that contributed to bringing ONs into clinical trials.

Take home message: In the long story of ONs for cancer therapy, the development of delivery strategies has led, in the last few years, to different opportunities to use the high therapeutic potential of these molecules in humans.

Keywords: cancer, cationic liposome, cationic polymer, oligonucleotide, oligonucleotide delivery

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1. Introduction

Cancer is a leading cause of death in industrialized countries [1]. Although mortality rates have declined in recent years owing to earlier detection and more options in treatment, most cancers remain incurable. Mutations and epigenetic alterations of cancer genes promote the malignant transformation of cancer progenitor cells by disrupting key processes involved in normal growth control and tissue homeostasis [2]. In addition, tumor development and progression are also dependent on the microenvironment surrounding the malignant cell [3].

Conventional chemotherapy for cancer utilizes cytotoxic agents that elicit their therapeutic effect partly through apoptosis induction. Moreover, overexpression of anti-apoptotic proteins in cancer cells can inhibit programmed cell death and engender chemoresistance [4]. Therefore, chemotherapeutic interventions fail to determine complete health in patients. Conversely, drugs developed more recently, known as 'targeted therapy', may show less unwanted toxicity, although they are generally cytostatic. Thus, there is an urgent need to develop new effective drugs.

An emerging strategy for combating disease processes consists of targeting the transcriptional or translational process by using oligonucleotides (ONs). This method involves an intervention in the normal transfer of genetic information in the cell from DNA to messenger RNA (mRNA) to protein. Knowing the sequence of bases provides the selectivity of a target, as genes differ from each other precisely

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Article highlights.

- Oligonucleotides offer a new concept of cancer therapy.
- There are several types of oligonucleotide, each one with a different mechanism of action.
- Different biological barriers hamper the oligonucleotides' use in cancer therapy.
- Chemical modifications on the oligonucleotide backbone can improve pharmacokinetics and/or pharmacodynamics.
- Several non-viral strategies for oligonucleotide delivery are known, with some of them in clinical trials.

This box summarizes key points contained in the article.

in their base sequences, leading to the opportunity to interfere directly with either transcription or translation [5].

2. Therapeutic ON mechanisms of action and potential application in cancer therapy

RNA has long been recognized as playing a pivotal role in the central dogma of molecular biology, that is, mRNA, transfer RNA (tRNA) and ribosomal RNA (rRNA). Surprisingly, a family of small RNA, small nuclear RNA (snRNA), was found to play key roles in the splicing process by binding to the splice donor and acceptor sites. A new class of small RNA, microRNA (miRNA), which controls translation of targeted mRNA, acting as naturally occurring antisense ONs (ASOs), has been identified [6]. Thus, the roles of RNA in translating information from the genetic code to protein products have greatly expanded. Also, each new class of small RNA and a diverse group of large non-coding RNA are poorly characterized [7]. Recent estimates suggest that as much as 90% of the genome is transcribed, resulting in production of an extremely diverse population of cellular RNA, including overlapping transcripts in both the sense and antisense orientation, highlighting that there is still much to be learnt about RNA [7].

2.1 Antisense oligonucleotides

RNA function can be modulated by ONs (8 – 50 nucleotides in length), which are capable of binding RNA through Watson–Crick base pairing by specific hydrogen bonding interactions between bases on the drug and the target RNA strand as well as hydrophobic interactions resulting from base shape complementarity and coaxial base stacking, generating a secondary structure. Imperfections in the duplex structure lead to unique shapes that can interact with other portions of RNA to form more complex tertiary structures, similar to protein folding. Antisense strategy includes a wide variety of ON designs modulating RNA through a diverse set of post-binding mechanisms. The first one involves binding to the RNA and interference with its function without promoting RNA degradation; the second one is based on antisense promoting degradation of the RNA either through endogenous enzymes, such as RNase H, or Argonaute 2 (RNA interference), as well as cleavage mechanisms designed into the ONs [8]. Interestingly, ASOs have the exquisite capability of distinguishing a single nucleotide mismatch [9]. The first ASO for which proof-of-principle of an effect in human tumors was demonstrated was oblimersen (G3139, Genasense), directed against the anti-apoptotic Bcl-2 mRNA. Preclinical and clinical evidence suggest that oblimersen synergizes with conventional chemotherapeutics against a variety of cancers [10]. Clinical trials have reported oblimersen administration in several cancers and clarified pharmacokinetic issues as well as drug adverse effects. Other ASOs such as OGX-011 (Teva Pharmaceutical Industries Ltd, USA) targeting clusterin, LY2181308 (Eli Lilly and Company, Indiana, USA) against survivin, ISIS-EIF4ERx (LY2275796, Eli Lilly and Company and the Wood Hudson Cancer Research Laboratory) targeting eIF-4E and OGX-427 (OncoGenex) against Hsp27 have been introduced in clinical trials [11].

2.2 RNA interference

RNA interference (RNAi) was discovered a decade ago and has since become a standard for various types of laboratory research. RNAi relies on complementarity between the RNA and its target mRNA to bring about destruction of the target. Long stretches of double-stranded (dsRNA) can interact with the DICER endoribonuclease to be cleaved into short (21 – 23 nt) dsRNA with 30 overhangs *in vivo* [12]. Then, the endogenous or synthetic short stretches of dsRNA enter the multinuclease-containing RNA-induced silencing complex (RISC), and these enzymes lead to specific cleavage of complementary targets [12]. Whereas short (< 23 nt) segments of RNA are generally considered optimal for gene silencing, longer (< 30 nt) sequences can lead to efficient, and even more potent, gene silencing [13], though they are supposed to induce immune responses [14]. Actually known are short-interfering RNA (siRNA), short-hairpin RNA (shRNA) and miRNA, all of which can inhibit expression of the target gene product. The siRNA and shRNA were designed to overcome issues with immune system stimulation and complete translation arrest observed when longer RNA sequences were used for RNAi, optimizing the silencing effects [13]. Despite the recent discovery of RNAi, several siRNA molecules have already been evaluated in human clinical trials [15]. Interestingly, miRNA, a naturally occurring mammalian post-transcriptional gene regulatory system, also mediates regulation of genes in disease states, including cancer [16]. Thus, endogenous and exogenous miRNAs represent therapeutic strategies [17]. The introduction of shRNA into a larger miRNA context that could be expressed from RNA polymerase II promoters and the discovery that these miRNAs could be placed in the 3′ untranslated regions of transgenes allowed the coupling of reporter and RNAi expression [18]. Clinical trials on siRNAs are now emerging and can be consulted by visiting the database of continuing and completed clinical trials given in [11].

2.3 Ribozymes

Ribozymes are based on catalytic RNA originally found in the protozoan *Tetrahymena* [19]. Although the naturally occurring ribozymes are self-splicing, modifications have yielded catalytic ONs that can cleave a targeted RNA sequence or revise the mRNA to generate correct sequences that can be translated into normal proteins [19]. Ribozymes can be targeted to a variety of molecules, and have been developed as experimental therapeutics for cancer and other human diseases [20]. Most clinical data about ribozymes are on HIV infection ribozymes as potential anti-HIV-1 therapeutic agents [21]. However, a few studies reported the synthetic ribozyme RPI.4610 against VEGF receptor mRNA [11].

2.4 Aptamers

Aptamers are short stretches of RNA or DNA with specific three-dimensional structures forming complexes and inhibiting target proteins. Therefore, aptamers can be considered chemical antibodies [21], used to target extracellular and cytoplasmic proteins. The only aptamer in a Phase II clinical trial is AS1411, formerly developed as AGRO100 by Aptamera (Louisville, KY, USA). AS1411 is an unmodified guanosine rich 26-mer, which anneals to form a bimolecular structure and whose anticancer properties were discovered *via* serendipity [22]. AS1411 showed antitumor activity *in mice* bearing human tumor xenografts without significant toxicity. Recently, AS1411 was inserted in a multimodal cancer-targeted imaging system *in vivo* [23].

2.5 Immunoactive CpG oligonucleotides

In the mid-to-late 1990s it was discovered that certain motifs within the nucleotide sequences, containing either unmethylated CG or GGGG motifs, were capable of stimulating an immune response [24]. ONs containing an immunostimulatory sequence have been developed to stimulate the immune system. The CpG ONs are now being used in clinical trials for several diseases, including cancer [24]. It has been shown that intratumoral injections of CpG ONs activate dendritic cells, leading to tumor regression in both mouse colon adenocarcinoma and melanoma [25,26], enhance the immune response and show a robust antitumor therapeutic activity, and elicit antitumor immune memory in a model of colon carcinoma [27]. Administration of CpG ONs with the LL-37 antimicrobial peptide generated significantly better therapeutic antitumor effects and enhanced survival in ovarian tumor-bearing mice [28]. Recently, a combined approach based on the preparation of bifunctional siRNAs with both pro-inflammatory and specific silencing activities has been described [29].

2.6 Anti-gene oligonucleotides

Since ~ 20 years ago, the potential of anti-gene ONs has been fully understood. These ONs bind in the major groove of the DNA duplex to form a triple-helical structure thanks to specific hydrogen bonding interactions between the anti-gene and an oligopurine strand of the duplex. These short ONs could be used to induce a DNA cleavage at a specific site on DNA through triplex formation [30]. Interestingly, triple-helix target sites are over-represented at promoter regions of human genes, probably playing a pivotal role for correct positioning of transcription factors [31]. Anti-gene ONs constitute an interesting DNA sequence-specific tool for many applications, as they are very specific DNA binders and easy to synthesize. Actually, there are only a very few *in vitro* studies reporting their applications in cancer therapy. Anti-gene ONs against c-myc and p53 in ovarian cancer cells lines exerted both antiproliferative and stimulatory activity at concentrations achievable *in vivo* [32].

2.7 Decoy oligonucleotides

Decoy ONs are considered ideal tools to prevent the binding of a transcription factor to target gene promoter regions. Occupation of the transcription factor DNA-binding site by the decoy renders the protein incapable of subsequently binding to the promoter regions of target genes. Decoy ONs have been used as a tool for investigating the role of transcription factor in cells [33]. An innovative decoy ON technology has been proposed, termed complex decoy ON (cdON), in which multiple *cis* elements are engineered to attack NF-κB, E2F and STAT3 concomitantly, mimicking the drug cocktail approach and inhibiting tumor growth in nude mice [34]. Decoy ONs can be used to inhibit and upregulate the expression of genes transactivated or suppressed by the binding of a transcription factor. NF-κB decoy ON transfection has been proved to inhibit both adenocarcinoma-induced cachexia [35] and hepatic metastasis [36] in mice. STAT3 decoy ONs significantly suppressed lung cancer cells proliferation *in vivo* [37].

3. Biological barriers to using oligonucleotides in cancer therapy

The therapeutic use of phosphorodiester ONs is seriously hampered by pharmacokinetic issues. Upon intravenous administration, ONs are rapidly cleared form the blood with a rapid degradation to nucleotides with a half-life of ~ 5 min [38]. Indeed, ONs are the substrate of endo- and exonucleases present in the cells as well as in the extracellular space. Moreover, ONs cannot diffuse through the cell membrane because of their macromolecular and polyanionic nature. It has been found that ONs can be actively transported across the plasma membrane in a temperature-dependent, saturable and structurally specific manner by means of an endocytic process, involving specific receptor proteins [39]. In particular, at moderate and high concentrations, ONs predominantly enter cells by fluid-phase endocytosis, whereas at low ON concentrations an absorptive endocytosis plays the major role [39]. Once in the endolysosomes, the ON concentration is expected to fall dramatically owing to lysosomal enzymes, with only a small percentage of the ONs being able to gain access to the cytoplasm. It is

worth noting that, following ON accumulation in the cytoplasm, ONs can enter the nucleus, probably by diffusion through pores of the nuclear membrane [40].

In light of these considerations, the development of therapies based on phosphodiester ONs should need high doses, and frequent, local administrations. Chemical and/or technological approaches have been developed to increase ONs' enzymatic stability and improve ONs' entry into cells, without altering the interaction with the intracellular molecular target. Moreover, in the last few years delivery systems able to target tumor tissues or cancer cells have been investigated. This review is an updated overview of the more successful non-viral strategies to deliver ONs in cancer therapy. In particular, the main chemical modifications of the ONs' backbone, together with their pharmacokinetics and pharmacodynamics, are reported. Then, the most successful non-viral delivery systems for ONs are described and discussed.

4. Essentials of medicinal chemistry of therapeutic oligonucleotides

The aim of most of the chemical modifications applied to therapeutic ONs is to overcome the pharmacokinetic and pharmacodynamic drawbacks. Further chemical modifications described in this review have been applied specifically to the several types of therapeutic ONs. Most of the chemical modifications conceived for ASOs [8], with the aim of enhancing the nuclease stability and increasing survival in a biological environment, can be applied to the other types of therapeutic ONs, although with severe limitations in several cases.

Taking into account the previous observations, the ONs' backbone presents an obvious first target in the chemical modification strategies aimed at improving the properties of ASOs. Accordingly, much research has been devoted to finding backbone modifications able to increase nuclease resistance and maintain or improve affinity and specificity to the target RNA, which are crucial aspects for several types of therapeutic ONs, including ASOs, siRNAs, miRNAs, and also ribozymes, to some extent. In **Figure 1**, the main chemical modifications used for ONs are illustrated. Phosphorothioate ONs (PS-ONs) contain one of the earliest and remain one of the most widely used backbone modifications for antisense drugs as well as for other therapeutic ONs. In PS-containing ONs one of the non-bridging phosphate oxygen atoms is replaced by a sulfur atom, conferring several properties onto ONs that are crucial for their use as systemic antisense drugs. First, the PS linkage greatly increases stability to degradation by nucleases, such that PS-ONs possess sufficient stability in plasma, tissues and cells to avoid metabolism before reaching the target RNA. Second, PS-ONs are able to elicit efficiently RNase H cleavage of the target RNA, which is critical in the mechanism of action. Furthermore, this modification confers a substantial pharmacokinetic benefit by increasing the binding to plasma proteins, preventing rapid renal excretion and facilitating binding to other acceptor sites that facilitate uptake to tissues. Other backbone modifications of ONs have been less successful, mainly because they do not support RNase H activity, even if most of them preserve a good affinity to the RNA target. Among these, peptide-nucleic acid (PNA) is noteworthy owing to its unusual peptide backbone showing high affinity when paired with RNA and DNA. Apart from the backbone, the heterocycle bases and the sugar can also be usefully modified. The C5 position has been a common place for chemical manipulation on pyrimidine heterocycles. However, modifications to the 2′-position of the sugar moiety have provided most value in enhancing the drug-like properties of ASOs. Unfortunately, essentially all 2′-modifications greatly reduce or completely inhibit the ability of RNase H to cleave the RNA strand opposite the modification. This limitation has been minimized by using a gapmer strategy, where regions of 2′-modified residues flank a central DNA region of the ONs. The 2′-modified wings thus increase affinity and nuclease resistance, whereas the central gap region allows RNase H-mediated cleavage of the target RNA. The 2′-fluoro modification imparts the highest binding affinity for the target RNA among the 2′ modifications. 2′-*O*-alkyl groups improve binding affinity to a lesser degree than do the 2′-fluoro nucleosides but impart a substantial degree of nuclease resistance to the resulting ONs. Among the 2′-modified series, the 2′-*O*-methoxyethyl (MOE) modification is at present the most advanced and has entered clinical trials for multiple indications. However, the sugar modification showing the largest known improvement in binding affinity is a bicyclic system with the 4′-carbon tethered to the 2′-hydroxyl group through a methylene bridge (LNA: locked nucleic acid). LNA shows dramatically improved hybridization properties relative to a DNA–RNA duplex and improves nuclease resistance. LNA-modified ONs have been exploited for numerous antisense mechanisms. However, as uniform LNA ONs do not support RNase H, a gapmer strategy must also be used with LNA-modified ONs to support the RNase H mechanism.

The use of siRNA-based therapeutic ONs [41] shows further difficulties compared with ASOs. When introducing chemical modifications into a siRNA it is important to recall that the RNA must interact with several different cellular proteins, many or all of which may be sensitive to changes in RNA structure caused by the modifying group. The most used modified backbone for siRNAs is the PS one. Restricting placement of PS bonds to the end of ONs will provide resistance to exonucleases while minimizing the overall PS content, thereby limiting unwanted side effects. 2′-*O*-methyl (2′OMe) RNA is non-toxic and can be placed within either the sense (S) or the antisense (AS) strands of a siRNA. However, heavy modification with 2′OMe RNA can reduce potency or completely inactivate a siRNA. Incorporation of 2′-F at pyrimidine positions maintains siRNA activity *in vitro* and *in vivo*. The combined use of 2′-F pyrimidines with 2′OMe purines can result in RNA duplexes with extreme

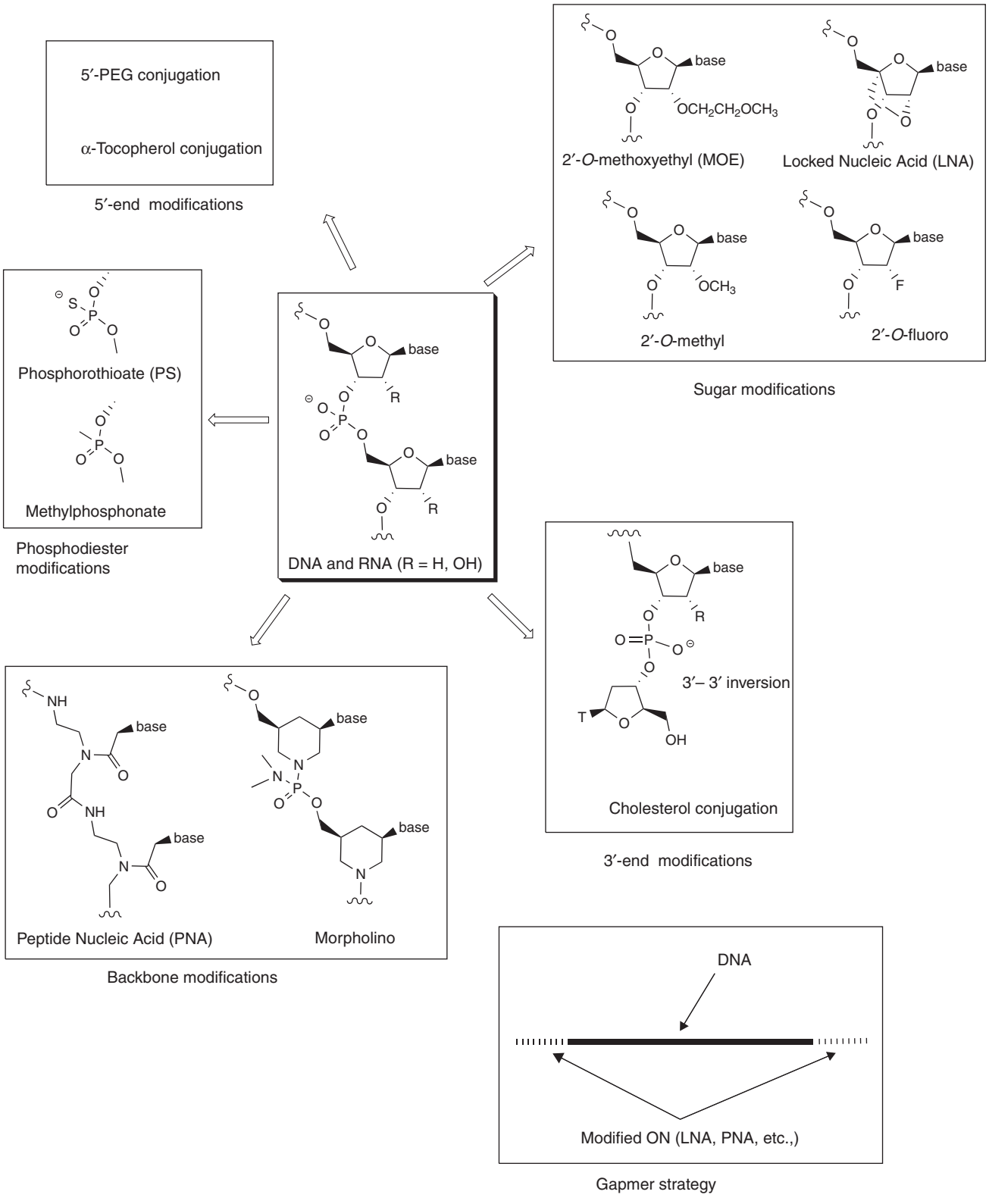


Figure 1. Scheme of the most important chemical modifications used for therapeutic oligonucleotides.

stability in serum and improved *in vivo* performance. Extensive modification of a siRNA with LNA bases generally results in decreased activity; however, siRNAs with limited incorporation retain functionality and offer significant nuclease stabilization. Steroids and other hydrophobic lipid groups can be attached to siRNAs that alter protein binding in serum, extending circulation time, and can facilitate direct cellular uptake. Direct conjugation of Vitamin E and polyethylene glycol (PEG) moieties to a siRNA have also been demonstrated to mediate delivery of siRNAs.

Concerning the aptamers, useful modifications can be incorporated into starting pools or incorporated into the aptamers post-systematic evolution of ligands by an exponential enrichment (SELEX) [42]. For the first case, it is important that the modifications are also compatible with the molecular biology steps required to execute the SELEX process. Modifications that have been incorporated successfully into starting pools include replacement of the 2′H (DNA) or 2′OH (RNA) of the ribose ring with a 2′F or 2′OMe group by using mutated forms of RNA polymerase that can incorporate these modified nucleotides into the selection library. The same modification can be applied post-SELEX; however, the chemically modified aptamers have to be assayed for the desired activity. The introduction of several modified nucleosides allows a structure–activity map of the molecule to be created that identifies positions at which a given substitution increases, has no impact on, or decreases binding affinity and/or functional activity. Beneficial or tolerated substitutions identified in scans can be combined to build composite molecules whose binding and stability properties are fully optimized. Further useful modifications include introduction of a 3′–3′ inversion of polarity sites (stabilizing against exonucleases) and 5′-conjugation with PEG, which increases the molecular mass of the molecule and extends its elimination half-life by slowing renal filtration and distribution from the central compartment.

The anti-gene strategy [43] is based on the formation of a triple helix (triplex) between the triplex-forming ONs (TFOs) and the duplex DNA target. As the triple helix is usually less stable than the duplex, most of the proposed modifications are aimed at improving the triplex stability. In this frame a successful modification is the introduction of positively charged amino functions on the sugar and/or base unit, designed to make extra binding contacts between phosphodiester residues of the target Watson–Crick duplex besides the base–base interactions. Several other modifications have been successful at increasing the triplex stability; however, among these, the PNA backbone has shown the best properties. PNA is able to form both very stable triplexes DNA–DNA–PNA and PNA–PNA–DNA.

Potential chemical modification concerning ribozymes [44] may involve both the RNA-recognition domain and the catalytic domain. They include the presence of PS groups, 2′-OMe and/or 2′-F nucleosides and a 3′–3′ inversion of polarity site. However, it should be taken into account that most of the chemical modifications, particularly in the catalytic domain, could severely affect the catalytic activity and should be carefully validated.

As to the decoy [45] and the immune-stimulatory [23] CpG ONs, their requirement to interact with several proteins limits the potential modifications of their chemical structures. As for other therapeutic ONs, the most used modification is the introduction of PS groups.

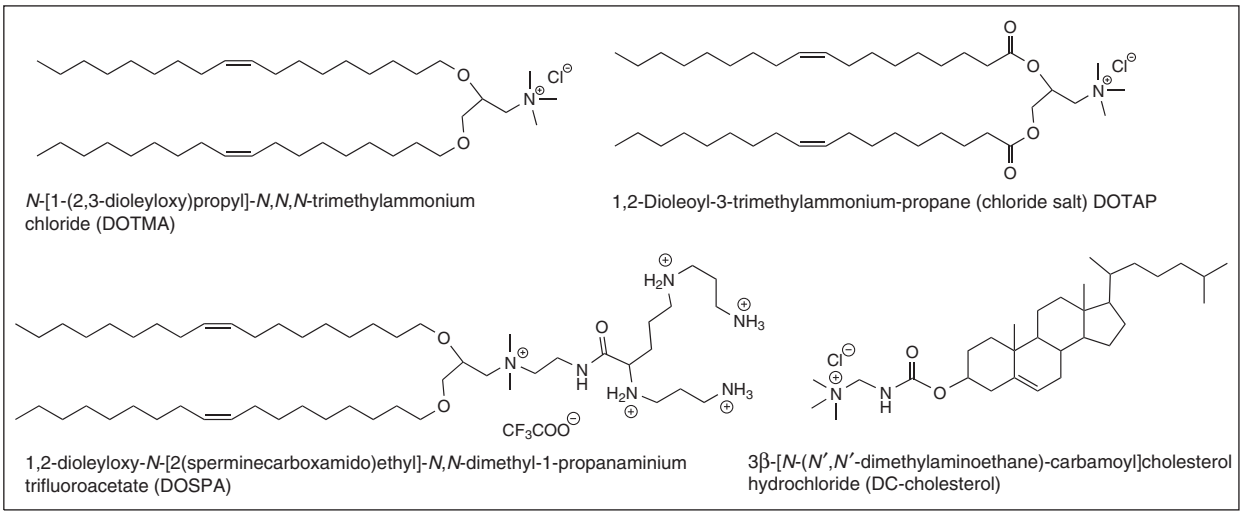
The biological instability of DNA and RNA precludes the use of unmodified nucleic acids as drugs because they are degraded by ubiquitous nucleases that cleave the phosphodiester linkage before they reach their target. Furthermore, the pharmacokinetics of ONs make them unacceptable systemic therapeutics because they are weakly bound to plasma proteins and rapidly filtered by the kidney and excreted into the urine. The aim of most of the chemical modifications applied to therapeutic ONs is to overcome these drawbacks. Further chemical modifications to be described in this review have been applied specifically to the several types of therapeutic ON. Most of the chemical modifications conceived for ASOs (A1) with the aim of enhancing the nuclease stability and increasing survival in a biological environment can be applied to the other types of therapeutic ONs, although with severe limitations in several cases.

5. Delivery systems for oligonucleotides

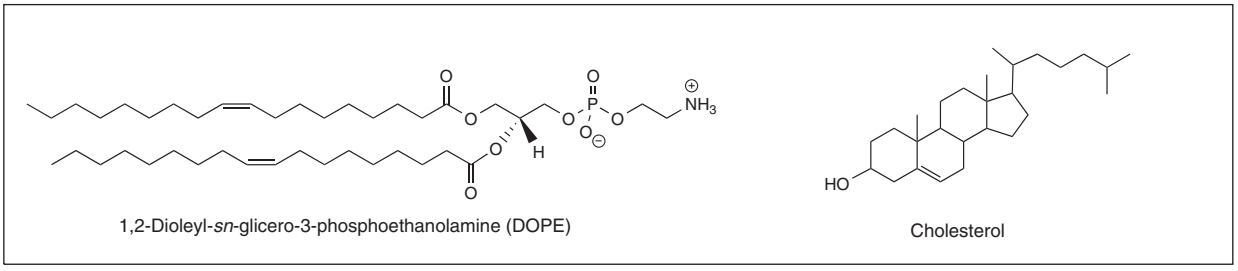
The use of delivery systems has been proposed as an alternative strategy to the chemical modifications of ONs, although frequently the two approaches have been combined. Several types of delivery system are on the market today and can be used as transfection agents for *in vitro* experiments. In the following, the most promising non-viral strategies for delivering ONs in cancer are summarized.

5.1 Lipid-based delivery systems

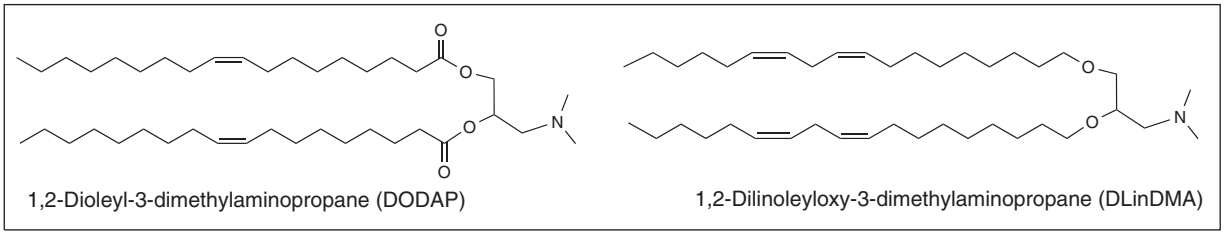
Liposomes based on cationic lipids are the most investigated delivery system for ON delivery. Cationic lipids (**Figure 2**) used for transfection purposes generally consist of two aliphatic chains or a cholesterol unit (hydrophobic portion) linked to a hydrophilic portion with one or more cationic charges. Optimal transfection efficiency for ONs can be achieved by selecting the type and length of the hydrophobic chains, as well as the type of polar head [46]. In **Figure 2**, some of the most frequently used lipids are reported. Cationic liposomes interact with negatively charged nucleic acids forming complexes, also termed lipoplexes, able to enhance the potency of ONs at least 1000-fold on cultured cells [47]. When complexed with cationic liposomes, ONs are efficiently protected against enzymatic degradation [48]. Then, the cationic charge of liposomes favors the interaction with the cell membrane [49], while lipoplex crossing of cell membrane occurs by clathrin-mediated endocytosis. ON escape from the endosomes has been explained by Zelphati and Szoka, with destabilization of endosomal membrane occurring for a 'flip-flop' mechanism, after which ONs can diffuse into the



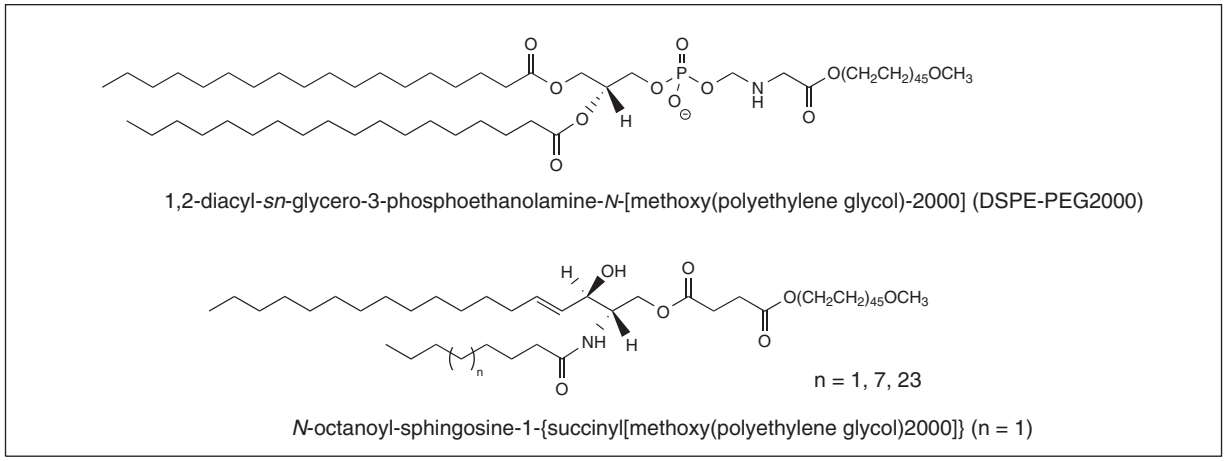
Cationic lipids



Helper lipids



Ionizable lipids



PEGylated lipids

Figure 2. Examples of lipids used in the formulation of lipid-based delivery systems for oligonucleotides.

cytoplasm [50]. The addition of the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE) (**Figure 2**) to the formulation can favor ON escape from endosomes, thus improving the efficiency of ON delivery into cells [51]. The experimental conditions in which lipoplexes are prepared, and in particular the incubation medium, the +/- charge ratio, can be controlled in order to optimize ON transfection efficiency [52]. The surfactant activity of cationic lipids, as well as their ionic interaction with intracellular components, could justify the cytotoxicity of lipoplexes.

The usefulness of cationic liposomes for delivering ONs by intratumoral administration has been demonstrated [53,54], although this approach remains difficult to propose in a clinical setting. On the contrary, the intravenous administration of lipoplexes is certainly more clinically feasible, but hampered by biological drawbacks. Once in the bloodstream, lipoplexes rapidly aggregate, with initial accumulation in the pulmonary capillaries, and following distribution into non-parenchymal cells of the liver [55]. Moreover, the systemic administration of lipoplexes does not result in a significant increase of intratumoral ON levels, probably because of the rapid clearance of the complexes. However, the observed reduction of tumor growth was ascribed to a different ON distribution in the tumor [55].

The inclusion of a PEG shell on the liposome surface (**Figure 3**) increases the stability of ON-containing lipoplex in serum, mainly for the sterical hindrance of long-chain PEG molecules, rather than shielding of positive charge [56]. The presence of PEG-lipids (**Figure 2**) has a minimal influence on the lipoplex binding to the cell membrane and on the following endocytosis, whereas it strongly limits ON escape from endosomes by hampering lipoplex mixing with anionic membranes and stabilizing the lamellar (L_α) phase of the lipoplex bilayer. For this reason, the molar ratio of PEG modification has to be evaluated carefully in order to maintain transfection efficiency [56]. Finally, the higher stability in serum results in an increased circulation time of lipoplexes, which accumulate in organs characterized by fenestrated capillaries (i.e., liver) or in solid tumor, owing to the increased permeability of the vessels associated to a reduced lymphatic drainage, the so-called enhanced permeability and retention (EPR) effect [57]. It is worth noting that the use of a PEG-lipid with a smaller hydrophobic moiety facilitates its transfer from the complex, thus enabling the adaptation from the L_α to the inverted hexagonal (H_{II}) phase [58].

At the moment, a siRNA (Atu027) against protein kinase N3 formulated with liposomal cationic lipids (namely siRNA-lipoplex/AtuPLEX) and containing neutral fusogenic and PEG-modified lipid components, which successfully inhibited cancer progression in preclinical studies [59], is in Phase I clinical trial for gastric, lung and other solid cancers (see website of Silence Therapeutics, Berlin, Germany at www.silence-therapeutics.com).

The combination of lipoplexes with cell-penetrating peptides (CPPs) allows the lipoplex transfection efficiency to be enhanced, whereas the use of specific ligands (transferrin, folate, etc.) or monoclonal antibodies improves the specificity of the interaction with cancer cells. With this approach, inhibition of tumor growth and prolonged mouse survival were obtained on different experimental tumor models [60,61].

A significant contribution to the field has been the development of carrier-encapsulating ONs based on ionizable lipids (**Figure 2**). These lipid-based carriers are also known as stabilized antisense lipid particles (SALPs) or stable nucleic acid lipid particles (SNALPs). The presence of a positively charged lipid assures high ON encapsulation efficiency, while the net positive charge of the particles can be neutralized after preparation [62]. Indeed, SNALPs have a minimal positive charge density in the blood, while, once in the acidic environment of the endosome, the protonation of the ionizable lipid activates the membrane-destabilization properties of the carrier. The degree of saturation as well as the type of linkage into the ionizable lipid (alkoxy or ester bonds) strongly affect transfection efficiency *in vivo* [63], and the use of a more lipophilic PEG-lipid assures a longer half-life [62]. However, the use of a rapidly exchangeable PEG-lipid abrogates the immune response [64]. Optimized SNALP formulations containing different siRNAs were well tolerated in both rodent and non-human primates, without changes in animal appearance or behavior and significant alteration in key serum chemistry and hematology parameters [63]. This technology has been used by Alnylam Pharmaceuticals, Inc. (Maryland, USA) with ALN-VSP, a siRNA targeting *VEGF* receptor as well as *KSP*, two key genes involved in the disease pathway of liver cancer. In preclinical studies, ALN-VSP formulated as a SNALP demonstrated the ability to silence the expression of both *KSP* and *VEGF* in the tumor, to reduce the growth and number of significant tumor masses in the liver, with a statistically significant increase in survival in animals. ALN-VSP is actually in Phase I clinical trial for the treatment of liver cancer.

An alternative strategy to systemically deliver ONs by using cationic lipids consists of ONs complexed with cationic liposomes in the presence of protamine, to form a virus-like structure in which nucleic acids are coated by two cationic lipid bilayers [65]. These nanoparticles, also called lipid-polycation-DNA or LPD nanoparticles, displayed neutral surface charge and minimal protein binding when mixed with serum, resulting in little reticuloendothelial system uptake and high tumor accumulation [66]. Targeting moieties, such as anisamide or hyaluronic acid, have also been bound to LPD nanoparticles to improve the delivery efficiency of different siRNAs into experimental tumors [67,68].

5.2 Polymer-based carriers for oligonucleotide delivery

The use of polymeric micro- and nanostructured platforms has also been largely investigated for ON delivery. However, the selection of a suitable polymeric platform depends on the specific requirements of the therapeutic protocol. In the

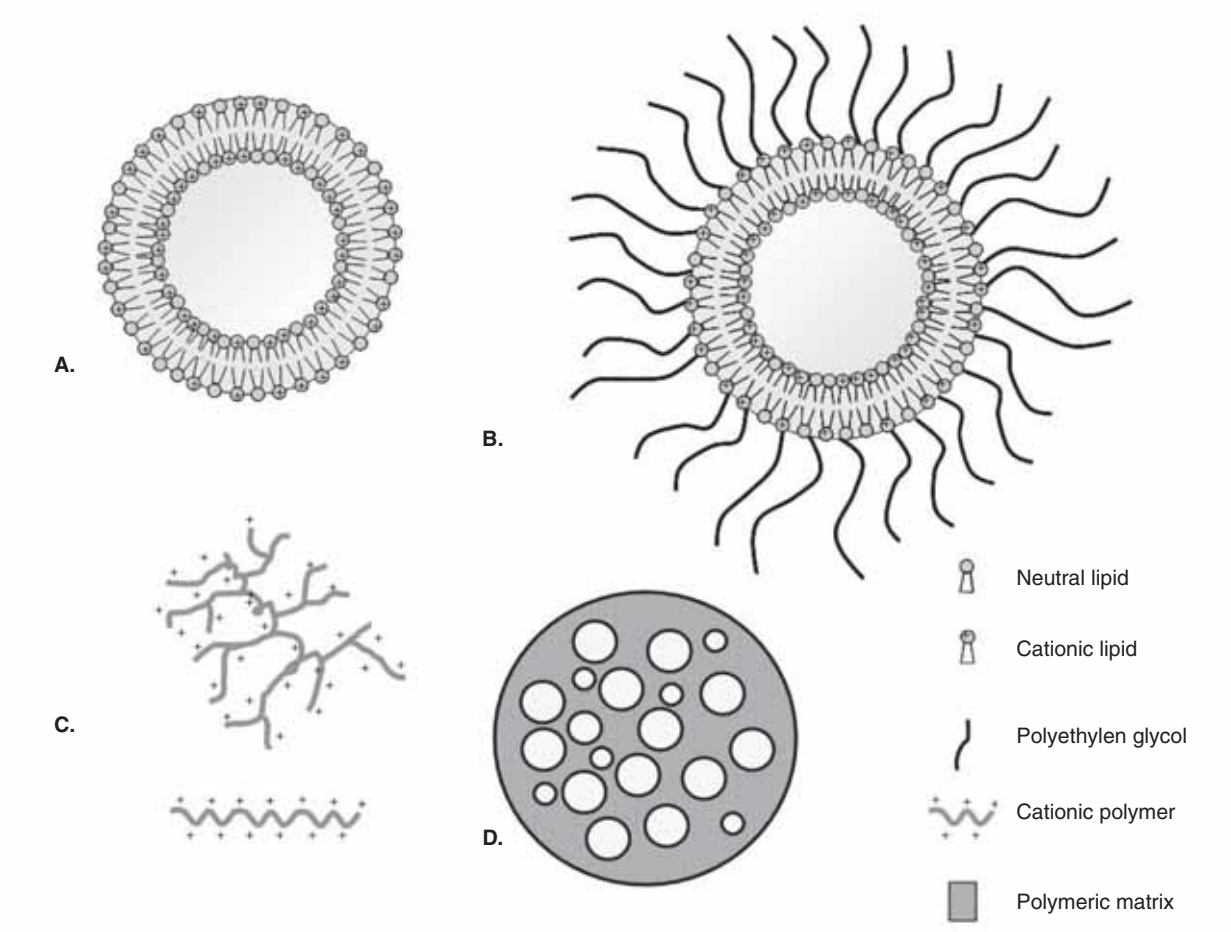
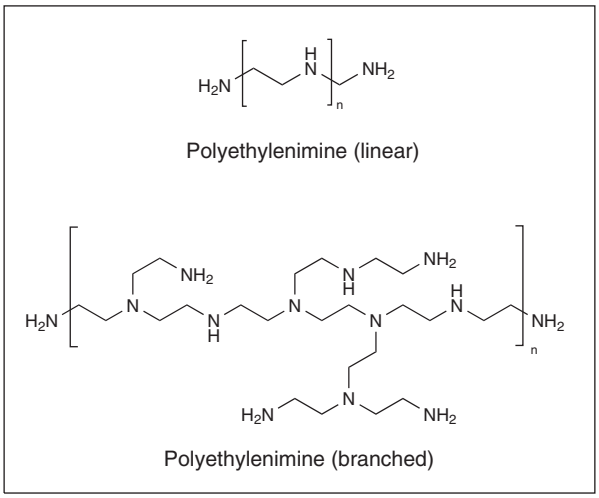


Figure 3. Schematic representation of some nano- or microcarriers used in oligonucleotide delivery. A. Cationic liposome. **B.** PEGylated cationic liposome. **C.** Cationic polymer. **D.** Polymeric nano- or microparticle.

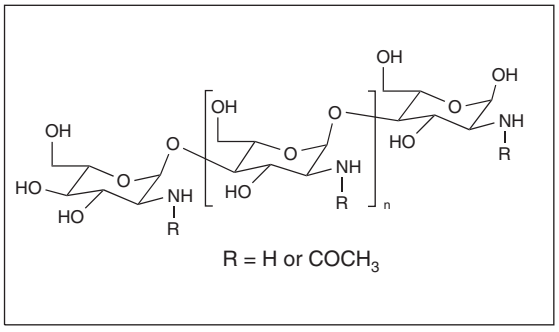
following paragraph, the most meaningful polymer-based systems for ONs are summarized, with the aim of elucidating the opportunities and peculiarities of each one, together with their own limits.

Polyethylenimine (PEI) is a positively charged polymer (**Figure 3**) available on the market in different molecular masses and in linear or branched form (**Figure 4**). The transfection property of PEI was first observed by Boussif *et al.* [69], who observed avid endocytosis of PEI/nucleic acid complexes, also defined polyplexes, in different cell lines. The high transfection efficiency of PEI has been justified with the so-called 'proton sponge effect'. Briefly, once in the endolysosomes PEI induces proton entry together with an influx of Cl^-; the higher osmolarity leads to water uptake into endolysosomes with consequent burst and ON diffusion into the cytoplasm [70]. In addition, ON complexation with PEI prevents degradation by nucleases, but this effect can be slightly different depending on the PEI molecular mass and conjugation with PEG [71]. Optimized transfection can be achieved by changing the type of PEI, the incubation medium and the N/P ratio of complex preparation [72]. PEI cytotoxicity remains a critical issue, but it can be significantly reduced by PEGylation [73].

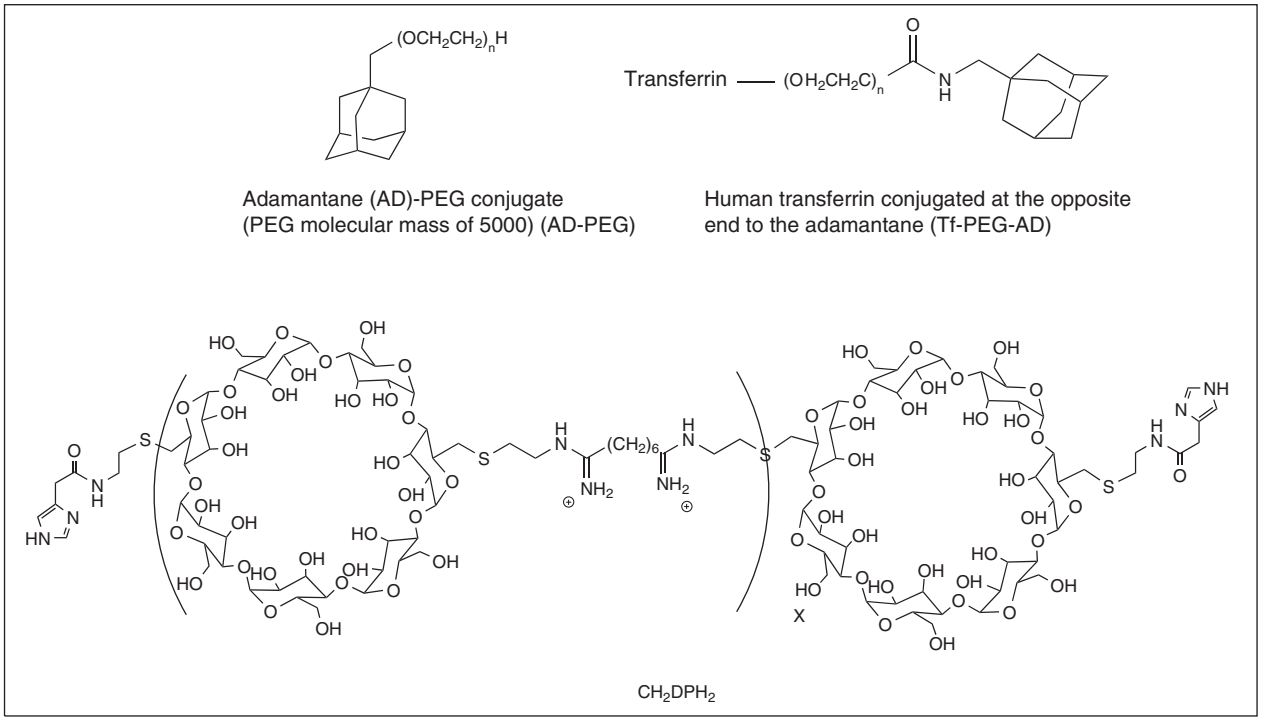
Following intravenous injection of PEI/ON complexes, ON half-life increases significantly with ON accumulation in liver and spleen [74]. Instead, when using low-molecular-mass PEI or PEI-PEG conjugates, polyplex disintegration occurs a few minutes after intravenous injection, leading to biodistribution similar to that observed with free ONs. These findings have been justified with the weaker binding between nucleic acid and PEG-PEI, which forms less compact complexes with a less efficient protection against nucleases [75]. For the same reasons, ON/PEG-PEI complexes dissociate on liver passage, suggesting that this system might not be suitable for systemic administration, but may be useful when the first-pass effect is circumvented [74]. Efficient ON delivery has been shown on intratumoral administration of PEI/ON complexes [76]. In an interesting study, ON/PEI complexes were administered by aerosol, with a decreased lung weight and tumor index in mice showing metastasis [77].



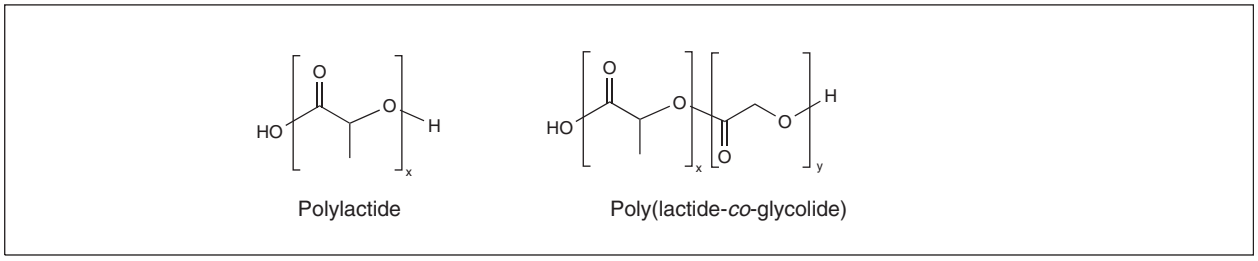
Polyethylenimines



Chitosan



Components of the self-assembled nanoparticles based on CDP



Polyesthers

Figure 4. Materials used in the formulation of polymer-based delivery systems for oligonucleotides.

Improved transfection efficiency has also been achieved by PEI conjugation with targeting moieties [78].

The only natural polymer of interest in ON delivery is the chitosan, a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine (**Figure 4**). This is a non-toxic biodegradable polycationic polymer with low immunogenicity [79]. Owing to its positive charge, chitosan can interact with negatively charged macromolecules, such as nucleic acids, in an aqueous environment. Chitosan-based colloidal particles for drug delivery can be prepared by different mechanisms, including desolvation, ionic crosslinking and complexation. When encapsulating ONs, nanoparticle properties depend on the molecular mass, degree of deacetylation and concentration of the polymer, the ratio between chitosan and crosslinker, pH, salts and loading method [80]. Independently from the N/P ratio, ON complexation with chitosan resulted in efficient nucleic acid protection from the enzymatic degradation [81]. However, an optimization of nanoparticle preparation (polymer molecular mass, degree of deacetylation, N/P ratio, preparation method) is required for optimal transfection efficiency [82], while limited cytotoxicity can be obtained by using low-molecular-mass chitosan [83]. Intravenous administration of ON-encapsulating chitosan nanoparticles resulted in extended circulatory half-life of siRNAs, increased siRNA stability and very pronounced accumulation in the kidney at 24 h [84]. This accumulation could reflect the mucoadhesion of chitosan to the mucosal epithelium lining the kidney and could be exploited for ON-based renal tumor treatments. The potential of chitosan nanoparticles for ON delivery on systemic administration has been demonstrated in different animal models, following intratumoral or intravenous administration [85,86]. The possibility successfully to combine CPPs with chitosan for improved ON delivery in tumor has also been reported [87].

About 10 years ago, a new class of cyclodextrin (CD)-based polymers (**Figure 4**) was proposed for the delivery of nucleic acids [88]. The choice of CDs was a result of their low toxicity and lack of immunogenicity. Different CD-containing polymers (CDPs), varying in distance between the CD and the charge center and its nature, were investigated to achieve the lowest toxicity and minimal complement activation, together with the best delivery efficacy. The inclusion of the imidazole termini into CDPs did provide an enhanced nucleic acid delivery, owing to the buffering of endocytic vesicles in living cells [89]. CDPs mixing with ONs resulted in self-assembled nanoparticles with mean diameter > 100 nm, when increasing nucleic acid concentration. However, the association of CDP with adamantine (AD) conjugated with PEG (PEG-AD) (**Figure 4**) resulted in nanoparticles with a mean diameter 60 – 80 nm, independent of ON concentration [89]. The use of ligands, that is, galactose and transferrin (Tf), at the free extremity of the PEG-AD (**Figure 3**) allowed targeting to tumor cells to be improved. Interestingly, only the formulation containing Tf showed an antitumor effect, following intravenous administration. This formulation was used in a disseminated murine model of Ewing's sarcoma, and its potency safety was demonstrated in non-human primates [90]. The interesting results induced Calando Pharmaceuticals (California, USA) in 2008 to use these nanoparticles to deliver a siRNA against RRM2 (CALAA-01) in human patients with metastatic melanoma refractory to standard-of-care therapies. The results of this study have been published recently [91]. The authors showed that CPD nanoparticles encapsulating a siRNA efficiently entered tumor cells, resulting in a successful silencing effect on the target mRNA. This is the first direct evidence reporting an RNAi mechanism of action in humans.

Most of the delivery systems described above were designed mainly to improve ON uptake into cells and have often been used by systemic or local administration. However, intratumoral administration requires a clinical setting and skilled healthcare personnel, certainly disadvantageous for long-term therapy with frequent administrations. Delivery systems based on biodegradable polymers, such as polyesters, can be used to release the ONs in a sustained manner, thus reducing the number of required administrations. Among biodegradable polymers, polylactide (PLA) and its copolymers with glycolic acid, poly(lactide-*co*-glycolide) (PLGA) (**Figure 4**), are materials that have been widely investigated in drug delivery and are available on the market in several forms, with a different biodegradation rate. The safety of their use is testified by different pharmaceutical products that have already been on the market for several years. Moreover, the possibility of formulating these polymers in the form of microspheres (**Figure 3**) allows one to inject the delivery system directly into the administration site without the need for surgical implantation. ON encapsulation in PLGA microspheres results in efficient protection against serum nucleases, while optimum experimental conditions allow sustained release of the ONs to be obtained [92,93]. PLA/PLGA microspheres with a large mean diameter (> 5 μm) can act as an extracellular depot for sustained ON release. This latter case has also shown an enhanced ON uptake into the cells [94] and a prolonged ON effect *in vivo* [95]. With microspheres formulated with a mean diameter < 4 – 5 μm, particle uptake has also been observed [96,97]. The usefulness of these systems has recently been shown in an experimental animal model of cancer [98]. A further improvement of ON uptake after release from microspheres can be achieved by co-encapsulation of a carrier, such as PEI [99,100].

6. Expert opinion

Despite the number of studies on the therapeutic potential of ONs, for many years the only ON-based product on the market was Vitravene™ (fomivirsen, Novartis Ophtalmics AG, USA). The low compliance of the required administration, as well as the poor demand for this product, led the holder of the marketing authorization to withdraw the product from the market in the European Union in 2002. This event

contributed to spreading a feeling of distrust in the scientific community concerning the possibility of using ONs for therapeutic purposes. The discovery of RNA interference contributed to giving an important impulse to the research of strategies for ON delivery, especially cancer. In these last 2 years the use of delivery systems for ONs has begun to become a reality, entering Phase I of clinical studies. Some of these studies have been carried out on cancer.

The success of some delivery systems for ONs is the result of the huge number of publications in the field, which have contributed to overcoming different obstacles to their use in therapy. In the case of cationic liposomes, many works have addressed overcoming the physical instability in the presence of serum. The use of PEG has allowed sufficient stabilization of cationic liposomes in the blood, and with this approach Silence Therapeutics has begun a clinical trial with a siRNA. Among lipid-based carriers for ONs, SNALPs certainly represent a milestone. This is because SNALPs summarize all the qualities needed for optimal transfection *in vivo*, namely, encapsulation of ONs into the vesicles with high ON loading and high protection against enzymes, and neutral charge on the surface of the particle, which, together with PEG, assure high stability in the blood and good transfection efficiency. A clinical trial of Phase I is now underway for the treatment of liver cancer with a siRNA encapsulated in SNALPs.

PEI is certainly the most investigated polymer for ON delivery. However, despite the high transfection efficiency, this polymer has never overcome the preclinical phase. Different chemical modifications of PEI, especially PEG conjugation, have been carried out to improve the physical stability of polyplexes in serum. However, higher physical stability of polyplexes often resulted in rapid decomplexation of the complexes in the blood or in the liver, resulting in reduced intratumoral delivery efficiency. Moreover, the risk of PEI accumulation in the body has to be taken into account, before moving from the bench to clinical studies.

Interesting results have also been obtained with chitosan, although the chemical variability resulting from its natural origin could represent a serious drawback in the large-scale use of this polymer. Nanoparticles based on CDPs, after a first phase of optimization of the system, rapidly berthed to humans. This delivery system can be easily prepared by mixing the contents of three vials before use.

Minor attention has been paid to biodegradable polyester-based micro- or nanospheres, probably because of their limited transfection properties. However, when intratumoral injection is possible, the sustained release of ONs should avoid the need of frequent administrations, assuring therapeutic levels of ONs for a prolonged time.

In general, the use of moieties such as transferrin, monoclonal antibody, and so on, allowed targeting of cancer cells to be obtained. However, apart from the case of intratumoral administration, the colloidal carriers lack tissue specificity. Indeed, it is not a coincidence that clinical trials with SNALPs are aimed at treating liver cancer; this organ remains the main site of nanocarrier accumulation, after a first and transient localization into the lungs. In the case of the central nervous system, for example, different strategies allow the blood–brain barrier to be crossed, but only a negligible percentage of the total amount of administered drug reaches the target tissue. Thus, further studies are needed to control better the biodistribution of the ONs when systemically administered into the body.

Safety data on the long-term use of ON delivery agents are not available owing to the few and only recent clinical trials. Thus, only hypotheses can be made on this issue. From a general point of view, only materials that are biodegradable and give rise to non-toxic metabolites should be suitable for repeated administration. This is especially true when systemic administration of the ON is required. Thus, the use of non-biodegradable materials, such as PEI, is certainly unsuitable for long therapy in humans and hence should be avoided. On the contrary, PLGA, although never used in humans for ON delivery, has been largely used for other applications, demonstrating its safety on long time use.

In conclusion, in view of the recent success of non-viral delivery systems, ON-based therapies for cancer in humans could be not very far away. This could lead to very selective and efficient therapy, with limited risk of side effects. Although the safety of non-viral delivery systems is still under investigation, advantages such as cheapness, easy preparation and manipulation, limited toxicity and risks of immune response make the non-viral approach certainly preferable to the use of viral vectors.

Declaration of interest

The authors declare no conflict of interest.

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