On the mechanism of cationic lipid-mediated delivery of oligonucleotides
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Chapter 2

Make sense of antisense oligonucleotides

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Submitted
Abstract

For more than two decades antisense oligonucleotides (ODNs) have been used to modulate gene expression for the purpose of applications in cell biology, and for development of novel sophisticated medical therapeutics. Conceptually, the antisense approach represents an elegant strategy, involving the targeting to and association of an ODN sequence with a specific mRNA via base-pairing. As a result, the translation of the gene into protein will be impaired, thereby potentially revealing its functional and/or harmful effect in normal and diseased cells/tissue, respectively. It is still poorly resolved how to design the most efficient antisense oligonucleotides, but chemical modification of their structure can improve their efficiency of action, in part due to an enhanced intracellular stability. Appropriate carriers have been developed to allow efficient entry of ODNs into cells in vitro, and the mechanisms of delivery, both in terms of biophysical requirements for the carrier and cell biological features of uptake, are gradually becoming apparent. Remarkably, for some tissues such as liver and brain, it has been shown that ODNs may acquire intracellular access and delivery into the nucleus, a step needed for accomplishing the antisense effect, without the need of packaging into a delivery vehicle. This suggests differences in entry mechanisms, knowledge that is imperative to further appreciate and bolster antisense technology in its broadest sense. In this context, proper consideration will be given here to antisense design and chemistry, and the challenge of extra- and intracellular barriers to be overcome.
Introduction

In 1978, synthetic oligonucleotides (ODNs) complementary to Rous sarcoma virus mRNA were shown to inhibit virus replication (1). This important observation represents one of the hallmarks in the initiation of the development of antisense technology for therapeutic and cell biological purposes. In fact, the principle of the approach reflects a naturally occurring physiological event since endogenously expressed antisense molecules exist which are able to regulate endogenous gene expression and to defend both prokaryotes and eukaryotes from viral invasion (2, 3).

Over the last two decades, numerous studies have been carried out to obtain insight into the mechanism of the action of antisense ODNs, including issues concerning optimal design, chemical modification, specificity and pharmacology (4, 5). In principle, antisense technology has a wide perspective of potential applications. At present, prompted by the rapid developments in genomics, most of these applications are in fundamental research and are largely focussed on an inhibition of gene expression, which provides crucial insight into the function and potential regulation of novel genes (6-8). However, there is a growing interest in developing drugs based upon an antisense protocol, mostly aimed at interfering with viral infections and cancer (9, 10). In addition, the antisense approach may be a good alternative to traditional agents for diagnosis and treatment in nuclear medicine, although relatively few attempts have been made thus far to evaluate this option (11-13).

Irrespective of the goal of application, a major challenge in antisense technology represents the design of an antisense sequence, which is target-specific, effective and nontoxic (14-19). In spite of drawbacks, significant progress has also been made in recent years in this area, which has resulted in the commercial production of antisense therapeutics such as Vitravene, used in the treatment of CMV retinitis in HIV infections (20, 21) and the undertaking of several promising phase III trails (22-24).

As with drug development in numerous areas, a detailed understanding of antisense mechanism and pharmacology are essential for successful application and further improvement of its effectiveness. Here, we will discuss therefore the understanding thus far of molecular obstacles and intracellular and extracellular barriers in antisense action and application.

Principle features of the mechanism of antisense action

Crucial toward a prosperous development and widely applied antisense technology is the need for a detailed understanding of the mechanism of antisense action per se (Fig. 1). Conceptually, the ability of antisense molecules to bind to complementary mRNA through Watson-Crick base
pairing may sound simple. However, insight into issues as where and how antisense reaches its target(s) are still largely obscure, especially in case of in vivo studies. Moreover, no evidence is available which demonstrates the direct binding of ODNs within the cell to the targeted sequence(s). It is generally believed that antisense either sterically blocks mRNA’s function (Fig. 1A) or promotes enzyme-mediated mRNA degradation (Fig. 1B). As an example of the former possibility, in a cell-free translation system it has been shown that antisense ODNs, such as peptide nucleic acids (PNAs), which are unable to activate RNase H, can be targeted to mRNA coding sites. As a result, an interference with polypeptide chain elongation occurs, resulting in the biosynthesis of truncated protein fragments (Fig. 1A; 4, 25, 26). Similarly, steric occupancy of the 5’cap region with 2’-MOE ODNs interferes with the assembly of the 80S translation initiation complex, and with the elevation of target mRNA (27). Steric interference of antisense molecules on distinct RNA regions may also cause an inhibition of pre-mRNA splicing (Fig. 1C) or polyadenylation editing (Fig. 1D), and/or induce mutations of the encoding gene (28, 29).

Antisense-mediated down-regulation of mRNA as a result of the latter’s degradation by RNase H action, may occur following binding of phosphodiester- or thioate-linked ODNs, as revealed in cell lysates (30-33). Consequently, the expression of the encoded protein will become reduced, an effect that will be apparent only after significant turnover of the pre-existing endogenous protein pool. When the targeted ODN sequences are within the 5’or 3’ non-encoding regions (Fig. 1D), the antisense induced cleavage at these non-translation sites accelerates the degradation of the entire target mRNA. Nevertheless, progress in carefully defining the intracellular mechanism(s) of processing and action of antisense ODN has been particularly frustrated by the technical inability to verify the localization of the relatively small amounts of antisense that gain access within the cells, and the limited recovery of targeted mRNA or protein fragments (Fig. 1).

As noted, in the antisense field, RNase H is thought to play a pivotal role. However, it is essential to take into account that only phosphodiester, phosphothioate and chimeric ODNs (cf. Fig. 3) can activate RNase H. Any (chemical) modification of the nucleotide and/or changes in the backbone will reduce or eliminate RNaseH recognition of the DNA-RNA duplex (31, 32). Given the potentially effective role of RNases in antisense technology, further insight into the presence of other intracellular RNases may prove worthwhile in fully exploiting the potency of this technology (34). For example, the 2’, 5’oligoadenylates bind to and activate the latent RNase L, cleaving viral and cellular RNAs at the 3’side of UpNp sequences, thus leading to an inhibition of protein synthesis (35). In fact any RNase, which recognizes the double stranded RNAs or RNA/DNA duplexes could play a key role in the overall action of antisense ODN. In
both plants and animals, RNAi is present which consists of about 22 nucleotides in length that is homologous to the gene that is being suppressed. These 22-nucleotide sequences serve as a guide sequence that instructs a multicomponent nuclease, RNA-induced silencing complex (RISC), to destroy specific messenger RNAs (36-38). The RISC contains a helicase activity that unwinds the double RNA strand, thereby allowing the antisense to bind to the targeted RNA sequence and causing the ensuing activation of an endonuclease activity that hydrolyzes the targeted RNA at the site where the antisense strand is bound. Recently, RNase III, which is involved in the maturation of prokaryotic and eukaryotic RNA, has emerged as a key player in this new and exciting biological field of RNA silencing or RNA interference (39). In fact RNase III appears to contain very high affinity RNA binding sites, which readily interact with the double-stranded RNA binding domain (dsRBD), thus initiating rapid and efficient cleavage.

Figure 1. Major sites of the actions of antisense oligonucleotides. A. Translational arrest. Antisense oligonucleotides bound to complementary mRNA inhibit polypeptides elongation. B. RNase H activation. Hybridized mRNA is degraded by RNase H that is activated upon the binding of antisense oligonucleotide on the mRNA. C. Inhibition of the splicing of pre-mRNA. Antisense oligonucleotides bound on the splicing site of pre-mRNA interfere with the maturation of mRNA by preventing the binding of spliceosome on pre-mRNA. D. Pre-mRNA destabilization. Antisense oligonucleotides bound on encoding or non-encoding regions on pre-mRNA can accelerate the RNase-mediated degradation of the mRNA, or interfere with polyadenylation or cap formation.
Protocols for designing proper antisense sequences

Obviously, the proper selection of an appropriate antisense sequence is a crucial step and remains a major challenge in the successful application of antisense technology (Fig. 2). Knowledge of the RNA secondary structure, antisense affinity and antisense chemistry are key factors in antisense design. mRNA is not a single stranded random coil but displays a secondary or tertiary structure, which plays a significant role in determining the efficacy of ODN antisense activity (40-47). Usually, a stretch of 10-30 nucleotides on the mRNA is selected as a potential target for the antisense. This preselected mRNA fragment may engage in intramolecular base pairing, thus constituting stable secondary and tertiary structures, which may render large parts of the mRNA inaccessible towards interaction with the matching antisense ODNs. The number of potential conformational states grows exponentially with the chain length. For example, the number of hairpin conformations increases from 138 for a 10-nucleotide chain to 24,666 for a 16-nucleotide chain. RNA hairpins are stabilized predominantly by base-stacking interactions (48).

mRNA folding and stem loops can be predicted by certain computer programs (49, 50), such as mfold (51-52). However, precise prediction of these structures remains difficult. In addition, cellular factors may influence the mRNA structures as well. For example, the association of AUF1, one of A + U-rich binding factors, with RNA substrates induces the formation of condensed RNA structures (53). The spliceosome (cf. Fig.1C) removes introns from pre-messenger RNAs by a mechanism that entails extensive remodeling of the RNA structure. The most conspicuous rearrangement involves disruption of 24 base pairs between U4 and U6 small nuclear RNAs (snRNAs). In this case, the yeast RNA binding protein Prp24 has been shown to re-anneal these snRNAs (54). Thus ODN ‘walks’, i.e., spacing ODNs of a given length at intervals along the RNA and choosing the one with the highest activity, still play an important role in selecting appropriate antisense molecules. In fact, currently a variety of approaches are applied to design the most appropriate antisense sequence (Fig.2). Also sequences that are targeted to initial coding regions are frequently applied since these regions lack secondary structure. However, although an attractive target, such an approach has been shown to be of little value in general, since ODNs targeted to these regions have been shown to often generate a poor downregulation of mRNA content (14). Furthermore, translation initiation sites may display a shared homology in both related and non-related genes, since ODNs targeted to these sites displayed both antisense specific effects toward the targeted gene, and non-specific effects on cell proliferation.
Figure 2. Schematic representation of antisense design. A. mRNA walking. Oligonucleotides of a given length, complementary to sequences along the RNA sequence, are synthesized and screened for antisense activity. B. Computer folding of mRNA. By prediction of mRNA structure, oligonucleotides are designed to accessible sites on the mRNA. C. Oligonucleotide array. In the scanning array, all oligonucleotides complementary to the target mRNA sites are synthesized and hybridized with mRNA transcripts. Antisense sequences are chosen by selecting the ones with high affinity to the mRNA on the array. D. RNase H mapping. RNase H is used to cleave mRNA that hybridizes to a random oligonucleotide library. Appropriate antisense oligonucleotides are selected based on identifying RNase H cleavage sites of target mRNA.

Overall, four distinct approaches have been developed which are currently actively pursued in the design of antisense molecules, though selection of active antisense molecules still remains largely a matter of trial and error (Fig. 2). First, mRNA walking or sequence walking (Fig.2A), which relies on synthesizing a variety of sequences which are targeted to distinct regions on a given sequence. Usually some 100 different sequences will be tested in such an approach. The outcome is that often only a few sequences turn out to be effective (55, 56). Obviously, the advantage of this approach is that given the number of sequences applied an appropriate and effective sequence may be recovered, but the procedure is costly, time-consuming and laborious. However, such an approach may suit large-scale pharmaceutical purposes for screening of optimal antisense drugs. A second procedure relies on computer facilitated screening (Fig.2B), based on the predicted folding of mRNA in order to identify accessible sites (57-58). As
discussed above, the precise prediction still suffers from structural uncertainties. On the other hand, this is an inexpensive, fast and easy approach involving the screening of a few sequences. With this approach usually an effective sequence can be obtained, even though it may not be the most effective one. On the other hand, it should also be realized that it is not always necessary to completely inhibit gene expression, particularly in case of functional studies.

In recent years, ODN arrays (Fig.2C) and RNase H susceptibility (Fig.2D) emerged as important approaches to select antisense molecules. In the scanning array approach, all ODNs complementary to the target mRNA sites are synthesized, and hybridization is performed with mRNA transcripts in vitro, i.e., under non-physiological conditions (45, 59). Since these studies are done in vitro, relevant cellular factors are not present in the system. Nevertheless, with this approach, a good correlation has been obtained between the eventual antisense effect in cells, and the binding affinity of antisense ODNs to the mRNA in vitro. With the progress in automation and miniaturisation of DNA chips, this approach is quite promising and can be readily adapted to a routine screening in common research laboratories.

Finally, appropriate antisense ODNs can be selected based upon the principle of RNase H-mediated cleavage of target mRNA after binding of a random ODN library, followed by analyzing accessible ODN binding sites on the mRNA by gel electrophoresis (60-62). The advantage of this method is that the random ODN library is easy to synthesize and suitable to any target mRNA, although the challenge remains to identify the precise cleavage sites, given that a multifold of such sites may exist whereas the resolution, as obtained by gel electrophoresis, is usually limited. Accordingly, further improvement is needed to adapt this procedure as a generally applicable approach.

On improving Antisense Chemistry

Chemical modification of antisense ODNs (Fig. 3) is aimed at (i) improving stability and affinity of ODNs to target mRNA, (ii) facilitating recruitment of intracellular enzymes (mostly RNase H) to efficiently cleave targeted mRNA, and (iii) reducing/eliminating the toxicity of ODNs as such. Early during development, antisense ODNs contained phosphorodiester (PO-ODNs) linkages. PO-ODNs are capable to recruit and activate RNase H to ODN/mRNA hybrids. The PO-ODNs per se are sensitive to nucleases and show relatively short half-lives, both intracellularly and in circulation. Although still in use, currently most applications rely on the employment of chemically modified ODNs. The most frequently used chemically-modified antisense compounds are phosphorothioate-linked ODNs (PS-ODNs), in which unbridged oxygen is replaced by sulfur. In fact PS-ODNs harbor more advantages than only their ability to
resist nuclease-mediated degradation. PS-ODNs not only exhibit a long half-life in vitro and in vivo, but the modified compound retains the ability to recruit RNase H in order to degrade PS-ODN/mRNA hybrids (63, 64). Importantly, when applied systemically, PS-ODNs may spontaneously enter tissues, mostly liver, kidney, spleen, intestine and lung. A disadvantage of the PS linkage is its affinity for proteins, which on the one hand prolongs the circulation time of ODNs in vivo by protecting them from removal by filtration, but on the other hand frustrates the interpretation of the antisense effect since non-sequence specific inhibition of cell proliferation may occur (65, 66). For example, PS-ODNs may bind in a length and to some extent in a sequence-dependent manner to heparin-binding proteins, such as fibroblast growth factors, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and its receptor, as well as to the epidermal growth factor receptor (EGFR) (17, 67, 68).

Figure 3. Oligonucleotide analogues and chemical modifications.
Morpholino ODNs are nonionic DNA analogues, which, compared to PO-ODNs, show less affinity to proteins, but similar affinity to mRNA. However, they do not recruit RNase H, and whether these compounds are active as appropriate antisense molecules in vivo, remains to be determined (69-71). In PNA, the uncharged N-(2-aminoethyl)-glycine linkage replaces the phosphate deoxyribose backbone. Nonionic PNA binds with a relatively enhanced affinity to target mRNA, reduces the nonspecific binding to protein, but like morpholino ODNs, does not recruit RNase H (72-75). It is rapidly cleared from the circulation with an elimination half-life of about 17 min, in contrast to PS-ODNs, which show a half life of approx. 1 h (76-79). Locked nucleic acid (LNA) bases are RNA analogues that contain a methylene bridge connecting the 2’-oxygen of the ribose with the 4’-carbon. This modification renders outstanding affinity of this nucleotide to complementary mRNA, but reduces RNase H cleavage on the mRNA due to modification of the sugar. To improve the binding affinity and activation of RNase H, chimeras of LNA and PO-ODNs have been developed (80, 81). The in vivo activity of such chimeras has been demonstrated recently (82). 2’-O-methoxyethyl ODNs(2’-MOE) modification conveys nuclease resistance, high affinity to the target RNA, similar pharmacokinetics as PS linked ODNs, and importantly, causes activation of RNaseH (83-88).

Although some comparative work on the biostability, the antisense efficiency and in vivo pharmacokinetics of the various chemically modified ODNs has been done, there is no clear-cut picture as to a single best design for an antisense structure. For example, it has been reported that the antisense efficacy of neutral morpholino derivatives and cationic PNA were much higher than that of negatively charged 2’-O-Me and 2’-O-MOE congeners in a cell culture model (89). However, the same laboratory also reported that 2’-O-methoxyethyl (2’-O-MOE)-phosphorothioate and PNA-4K oligomers (peptide nucleic acid with four lysines linked at the C terminus) exhibited sequence-specific antisense activity in a number of mouse organs. Morpholino oligomers were less effective, whereas PNA oligomers with only one lysine (PNA-1K) were completely inactive (90). LNA-DNA ODNs were found to be the most efficient single-stranded antisense ODN, when compared to PS-ODNs and 2’-ME ODNs, the former in turn being more active than the latter (91). Claims have also been made that the efficiency of an ODN in supporting RNase H cleavage correlates with its affinity for the target RNA. One such a comparative study established an order of efficiency of LNA > 2’-O-methyl > DNA > phosphorothioate (81). In summary, no conclusive data have been presented which would support the general superiority and versatility of a specifically preferred chemically-modified antisense construct. Rather, data currently available suggest that apart from some obvious parameters such as biostability, the preferred antisense structure for efficient down-regulation of
a given mRNA target can only be revealed by direct comparison in a given experimental model system, and no prior prediction can be made.

**Overcoming cellular barriers in ODN delivery**

*Crossing the plasma membrane.*

When naked ODNs are added to cells, they usually do not permeate across plasma membrane. A small fraction can be endocytosised via adsorption (Fig. 4A). Yet, this fraction is usually very low since adsorption of the negatively charged ODNs to the net negatively charged plasma membranes is rather poor. Hence, attempts have been made to chemically modify ODNs to eliminate the negative charge in order to promote adsorption. However, an enhanced adsorption does not necessarily improve the antisense effect, because endosomal escape (Fig. 4B) poses as a next barrier in ODN delivery into the cytosol, a minimal requirement for accomplishing an antisense effect. In some studies, some antisense activity has been reported upon addition of free antisense ODNs, provided a relatively high concentration was applied, i.e., 10-20 µM. Often, significant escape of oligonucleotides from the endosomal compartments was not observed in these studies, implying that arrival of ODNs at the nucleus was not detectable. Yet, substantial accumulation of administered naked ODNs into the nucleus has been reported to occur in cultured bovine adrenal cells (92). Whether a cell type-dependent ODN translocation mechanism may exist is at present unclear. One such a possibility could be a cell-type dependent expression of a nuclei acid transporting channel, as identified on rat renal brush border membranes (Fig. 4A2, 93, 94).

Bypassing the plasma membrane can be accomplished experimentally by microinjection, electroporation or membrane permeabilization with chemical agents (Fig. 4A2). Microinjection has generated valuable insight into the understanding of intracellular antisense processing and the potential correlation with efficiency. For example, both microinjection and permeabilization lead to nuclear localization of antisense ODNs (95-98), a localization now thought to be crucial in order to acquire a potential antisense effect. For practical purposes however, these procedures are too laborious (microinjection) or harmful to cells (permeabilization and electroporation), requiring careful adjustment for each cell type employed.

Vector-facilitated delivery of ODNs, relying on the use of liposomes, polymers and peptides, appears an appropriate means to overcome the plasma membrane barrier in an efficient manner. In essence, liposomes and polymers are capable of efficiently complexing ODNs and enhancing the adsorption of complexed ODNs to the plasma membrane. Together, both features promote a strong enhancement in the intracellular concentration of ODNs as accomplished by endocytosis
Some plasma membrane proteins have been claimed to be involved in liposome-mediated delivery of oligonucleotides, which trigger endocytosis (99, 100). The exact function of these membrane proteins still needs to be clarified, but the evidence to support a specific role of distinct proteins is scanty. More likely, these complexes enter cells by non-specifically exploiting the endocytic mechanism, presumably mainly involving clathrin-mediated endocytosis, as has been well-defined for complexes of plasmids with cationic lipids (lipoplexes; 101, 102) and polymers (polyplexes; 102, 103).

Figure 4. Cellular entry and subcellular trafficking of oligonucleotides. A. Oligonucleotides associate with the cell membrane via absorption or charge attraction. Following their binding, the ODN complexes are internalized by endocytosis (A1); alternatively, oligonucleotides may become translocated across cell membranes via nucleic acid channels, fusion or pore formation on the plasma membrane (A2); via coupling of specific ligands to the complex, targeted internalization may be accomplished by receptor-mediated endocytosis (A3). B. Following endocytosis, oligonucleotides are either released from endosomes and/or are transported to lysosomes. C. After endosomal release, oligonucleotides are transferred into the cytosol, and acquire access to the nucleus and eventually to mRNA. See text for details.

Distinct peptides, which have the ability to penetrate into the cell membrane, thereby forming membrane-localized channels, may mediate ODN delivery via a non-endocytic pathway (Fig. 4 A2). Thus the uptake of ODNs complexed with the antennapedia homeodomain peptide and Tat protein showed a temperature-, energy- and receptor-independent pathway of internalization, characteristics which are typical of a non-endocytic mode of uptake (104-106). Also, proteins
and ligands, which are known to be processed by receptor-mediated endocytosis, such as transferrin or certain growth factors, may serve the purpose of ODN delivery (Fig.4 A3). Indeed, intracellular uptake of antisense ODNs linked to transferrin and folic acid was more effective than addition of unmodified antisense (107, 108). Similarly, antisense ODNs targeted to cancer cells via the epidermal growth factor receptor or to macrophages via the mannose receptor were found to be taken up more efficiently than naked ODNs (109, 110). However, as noted, once the plasma membrane barrier is overcome by exploiting the endocytic entry path, the next intracellular barrier constitutes the endosomal membrane (Fig.4B), which can be considered the crucial limiting step in the overall pathway that eventually elicits the antisense effect. This conclusion may be inferred from the notion that cytosol-localized ODNs, as readily accomplished by microinjection, rapidly accumulate in the nucleus.

**Release from endosomes.**

Naked ODNs are not able to permeate endosomal membranes. Accordingly, a perturbation of the endosomal membrane stability is a necessary step in the translocation process, and it has been concluded that cationic liposomes (but also polymers) are able to accomplish such a destabilization. Cationic liposomes readily accommodate DNA via electrostatic interactions in a complex structure, known as lipoplexes (111, 112). Cationic liposomes not only enhance the uptake of ODNs over 1000-fold, but also promote their translocation across the endosomal membrane. As a result, the antisense activity as mediated by a cationic lipid vector can already be revealed in the nanomolar concentration range of the ODN (113-115). However, the exact mechanism of this translocation is not clear, although some key factors have been revealed, which interfere with this process. For plasmid release, following lipoplex entry, it has been proposed that after the cationic lipid/DNA complexes are internalized into cells by endocytosis, lipid flip-flop occurs at the level of the endosomal membrane. Thus anionic phospholipids, in particular phosphatidylserine (PS), are thought to translocate from the cytoplasm-facing endosomal monolayer to the inner monolayer, from where the lipid may laterally diffuse into the complex and form a charge neutral ion pair with the cationic lipids. The potential ability of the negatively charged lipid to transfer into the lipoplex may be triggered and/or facilitated by the non-lamellar phase properties of (part of) the membrane phase in the lipoplex. In addition, the translocation and mixing with PS may further promote such a non-lamellar structure of the lipoplex, factors that are likely further potentiating the process of membrane destabilization (116-118). Moreover, the presence of PS will also cause displacement of the DNA from the cationic lipids and release of the DNA into cytoplasm (119-120), a phenomenon that can be
readily simulated in vitro (121). An unresolved question in this context is the issue why the release of DNA (largely, if not exclusively) occurs at the endosomal membrane, and not at the plasma membrane, particularly since the composition of its outer monolayer is thought to be reminiscent of that of the inner endosomal membrane.

From observations that several parameters interfere with ODN transport from the endosome into the nucleus, lipoplex-mediated transfection or release of DNA or ODN from cationic lipid complexes in in vitro model systems, insight into a potential mechanism of the release of both (plasmid-) DNA and ODNs from the endosomal compartment has been generated. Endosomal release of DNA is relatively inhibited when the lipoplexes are consisting of cationic lipids and dioleoylphosphatidylcholine (DOPC), rather than DOPE. While DOPE displays polymorphic properties and readily adopts a nonlamellar, i.e. hexagonal H\(_{II}\) organization in isolation, DOPC is a lamellar membrane bilayer-stabilizing lipid, which moreover is much more strongly hydrated than DOPE (117, 122, 123). The latter parameter will preclude opposing membranes from tight intermembrane interactions, which are needed in ODN (or plasmid translocation). Indeed, when including a membrane spacer such as PEGylated lipids, the bulky and extended polyethylene glycol headgroup separating opposed membranes, ODN delivery from endosome to nucleus is strongly inhibited and only becomes apparent when the PEG-lipid has diffused from the complex, the latter being accomplished by incorporating exchangeable PEG-lipids in the complex (124). Furthermore, structural studies of ODN cationic lipid complexes as well as lipoplexes, relying on small angle X-ray scattering and cryo-electronmicroscopy have elucidated that the complex’ ability to adopt a non-lamellar, i.e., hexagonal phase, is crucial for obtaining efficient plasmid or ODN delivery, respectively. Close intermembrane interactions between complex and target membrane are instrumental in this event, which is promoted by parameters such as the nature of helper lipids, as noted above, and the ionic environment. The latter may modulate the state of hydration of the interphase and thereby govern head group repulsion within the lateral plane of the bilayer, close head group- head group interactions facilitating nonlamellar transitions in bilayer organization. PEGylated lipids and strongly hydrated lipids, such DOPC, may oppose such transitions by maintaining a distinct distance between and within (laterally) membranes due to steric interference or by means of hydration repulsion and bilayer stabilization, respectively (117, 123-126). Indeed, protruding PEG-spacers strongly inhibit endosomal release of ODN (124, 127). Remarkably, PEGylated lipids, incorporated at mole ratios as low as 1mol% of the total lipid completely eliminated endosomal release of ODNs, without significantly interfering with the internalization of the complexes. As noted, upon
diffusion-mediated removal of polyethylene glycol analogues from the complexes, a time-dependent release of ODNs was observed in the cells, as reflected by their nuclear accumulation.

Apart from the overall structural and physical considerations that apply to accomplishing optimal ODN delivery, endosomal release of ODNs can also be affected by the presence of serum proteins. When such proteins absorb and/or penetrate into the complexes, the ODNs also remain trapped in the endosomal compartments, and significant ODN release does not occur (115, 128). In such cases, serum proteins might stabilize the complex membrane by penetration or via surface adsorption, and/or may cause a steric interference which precludes the intimate interaction between complex and endosomal membranes in order to induce the release of ODNs into the cytosol, as described above.

**Passage across the nuclear membrane.**

Upon microinjection into the cytosol or following their escape from endosomes, ODNs rapidly accumulate into the nucleus by passive diffusion through the nuclear membranes, whereas other organelar membranes pose as an effective barrier. A recent study suggested that the diffusion rate of ODNs is dependent on their length (129). A fragment consisting of 100 bp appears fully mobile in the cytoplasm, displaying a diffusion rate compatible to that of similarly sized FITC dextran, and only 5 times slower than that obtained in water. The diffusion of larger fragments is remarkably slowed down, with little or no diffusion for nucleotides with a length beyond 2000bp. Small ODNs, up to several hundreds of base pairs, readily acquire access into the nucleus. However, although ODNs of 500 bp diffuse relatively rapidly through the cytoplasm, their avid crossing of the nuclear membrane was not observed. In passing, such observations are also highly relevant in elucidating the mechanism by which (much larger sized) plasmids are supposed to acquire access towards the nuclear machinery.

Within the nucleus, nucleic acid fragments of all sizes were nearly immobile on a distance scale of 1 micron over a time interval of several minutes. Interestingly, similarly sized FITC dextrans i.e., up to 580 kDa, diffused freely in the nucleus. Accordingly, it was speculated that once entering the nucleus, the ODNs are rapidly recruited and immobilized upon their interaction with nuclear components, including the positively charged histones (Lukacs,-G-L,2000). Other studies have shown that intranuclear ODNs may also extensively bind to the nuclear RNA matrix (130, 131). In deed, some controversy may exist concerning the (fractional) nuclear mobility of ODNs. In fact, it has been shown, that at least a substantial fraction of the ODNs may shuttle between the cytoplasm and the nucleus (132). Thus, when ODNs were microinjected into one nucleus of a binuclear cell, they were found to appear readily in the other nucleus. Presumably,
the extent of shuttling may very much depend on the nature of the ODN (length) and whether or not appropriate target sites are reached. However, at steady state, fluorescently tagged ODNs are usually predominantly found in the nucleus, and a significant pool in the cytosol is not observed.

When cationic lipids are used to deliver ODNs, the overall distribution of the ODNs is similar to that obtained upon microinjection of ODNs in the cytosol, except for a minor fraction that may become entrapped in endocytic compartments. The lipids of the vector itself remain associated with endocytic compartments, and in contrast to polymers (103), cationic lipids have not been observed in association with the nuclear membrane (115, 133). However, when the complexes are released from artificially ruptured endosomes, the dissociation of cationic lipids and ODNs can be seen to occur at the nuclear membranes, the ODNs becoming detectable in the nucleus, while the cationic lipids diffuse into the nuclear membrane (130). As noted, at least some cationic polymers may directly mediate the delivery of ODNs at the nuclear membrane, likely as a result of endosomal rupture. A typical example is polyethylenimine (PEI), which is capable of mediating a cell cycle independent nuclear entry of plasmid DNA (103). Also with ODNs as cargo, PEI arrives at the nuclear membrane, in contrast to cationic liposomes (134).

Nuclear transport of ODNs, following their deposition into the cytosol, occurs by diffusion since it is not affected by depletion of the intracellular ATP pool or temperature; neither is the effect of nuclear association affected by the presence of excess unlabeled oligomers. It has been suggested that nuclear entry can be accomplished by translocation across the nuclear pores, a conclusion that was derived from the observation that entry could be inhibited by wheat germ agglutinin, a nucleoporin inhibitor (135, 136). Although the size-dependent ODN transport as noted above, may also reflects a size-limiting pore-mediated mechanism of nuclear ODN translocation, more specific experiments, for example, by antibody-specific inhibition, would be helpful to firmly establish the role of nuclear pores in entry. Indeed, further work will be needed, as no consensus has been reached yet (99).

Within the nucleus, accumulation of the oligomers involves at least in part their association with a distinct set of proteins, as revealed by crosslinking of photosensitive oligomers (95). These proteins can be extracted in the presence of high salt (0.2 M-0.4 M NaCl). Importantly, all experimental evidence available thus far supports the view that nuclear accumulation of ODNs is important for eventually obtaining an antisense activity. In the following section, we will discuss the potential mechanisms by which antisense activity is acquired.
Intranuclear distribution of ODNs; a correlation with antisense activity.

Once ODNs cross the nuclear membrane, they distribute throughout the nuclear lumen. Examination by light microscopy revealed that ODNs usually do not reach the nucleoli, where the ribosomal RNAs are transcribed from a number of chromosomes and assembled with ribosomal proteins into ribosomal subunits. In fact, ODNs can form condensed spherical foci (150-300nm) in a concentration dependent manner, which are not colocalized with the known nuclear bodies. The function of these oligonucleotide bodies is not known. It has been speculated that the accumulation of ODNs as nuclear bodies is a response of the cell to sequester excess ODNs in order to reduce their toxic effect (137). The exact localization of the predominantly nucleoplasm-localized distribution of ODNs is still largely unresolved. We (130) and others (137) have shown that the diffusely distributed ODNs within the nucleoplasm are resistant to mild removal of loosely bound nuclear proteins and to removal of chromatin with DNase. However, they are susceptible to the removal of RNA, following Rnase treatment (137), and following treatment with high salt (95). These data thus indicate that ODNs are extensively bound with non-chromatin structures in the nucleus, which is usually designated as the nuclear matrix. Among others, these structures are composed of heteronuclear RNA (hnRNA), the fraction of nuclear RNA which contains primary transcripts of the DNA, representing a nuclear precursor fraction that is processed to messenger RNA, and hnRNPs, a large family of proteins, implicating in the processing of nascent mRNA transcripts.

In permeabilized cells, ODNs readily migrate into the nucleus at ambient temperature, at which conditions PS-ODNs, rather than PO-ODNs, give rise to a multitude of large, irregular aggregates (138). High-affinity PS-ODN, but not PO-ODN, presumably reacts with the nuclear lamina. Simultaneously, ODNs cause decompaction of chromatin, the PS-ODN aggregates appearing as compact inclusions in homogeneously dispersed chromatin. After microinjection of S-ODN into intact cells, these effects were not observed, although the nucleic acids rapidly moved into the nucleus and condensed into a large number of well-defined, spherical speckles or longitudinal rodlets (135, 136, 139). A high degree of complex formation between ODNs and nuclear proteins has also been reported, as demonstrated in a gel shift assay (65). However, the nature and number of these proteins is unknown, and whether such interaction may also occur intracellularly, remains to be determined. Even though ODNs presumably bind to nuclear matrix proteins, this will shorten the spatial and temporal distance to target mRNA. Nuclear matrix binding of ODNs is not sequence-dependent, implying that nuclear matrix binding per se does not reflect the efficiency of an antisense effect. Thus it remains to be determined how such
interactions provide insight as to how antisense, but not mismatched sequences, finds their targets.

*Meeting intranuclear targets and identifying the fate of targets.*

Obviously, the target of antisense molecules is the complementary mRNA. In spite of the fact that antisense ODNs can hybridize to in vitro transcribed mRNA or to synthesized complementary RNA or DNA fragments, the evidence that such an event also occurs within cells is scanty. In an effort to gain such evidence, we have delivered radio-labeled or fluorescently-labeled antisense ODNs and mismatched sequences into target cells. Following delivery, both ODNs become extensively bound to the nuclear matrix, similarly as described above. When total RNA was isolated from cells treated with the labeled antisense or mismatched ODNs, the antisense sequence showed the expected high affinity to target mRNA, but not to other mRNAs whereas the mismatched sequence did not display affinity to target mRNA. These data were taken to indicate that antisense ODNs hybridize to target mRNA with high affinity over mismatched control via their initial binding to the nuclear matrix (130). Accordingly, this scenario would be consistent with the notion that upon hybridization of antisense ODNs to target mRNA, its induced degradation occurs, and as a consequence protein synthesis becomes impaired. Importantly, intracellular cleavage of target mRNAs by antisense binding could be revealed in these studies (30, 32, 130).

In order to appreciate the biological effect of antisense treatment, it should be taken into account that the existing pool of a target protein will not be affected other than by its natural turnover. Thus, the antisense can only inhibit or eliminate *de novo* synthesis of target proteins. Hence, the time course of the appearance of an antisense effect will vary according to the half-life of target protein degradation, implying that relatively shortly following treatment, a large preexisting pool of target proteins may obscure a potential biological effect of the antisense in causing effective reduction of newly synthesized targets.

*In vitro versus in vivo; role of extracellular barriers.*

It is becoming apparent that many fundamental issues on the mechanism of nucleic acid delivery can be conveniently and properly investigated *in vitro*, including intracellular processing and intracellular ODN stability. However, it has become equally clear that a direct extrapolation from *in vitro* to *in vivo* should be done but with caution. Apart from factors related to the circulation, the role of the extracellular matrix is still puzzling, and it seems that thus far its role has been inadequately mimicked *in vitro*. One such an example involves studies on the delivery of ODNs to brain tissue. Thus, ODNs can be readily introduced into a variety of
neuronal cell lines, provided that delivery is mediated via an appropriate vector, such as cationic lipids. By contrast, when injected intracerebrally in rats, the ODNs also acquire ready access to neuronal cells, accumulating in cytoplasm and nuclei without the need of such a carrier (Shi et al., unpublished observ.; 140). Up to some 4 hr after injection of the free ODNs into whole brain, the ODNs show a diffuse distribution in the extracellular matrix and in the cells. After 24 hr, the associated pool of ODNs with the extracellular matrix has strongly diminished while concomitantly, the pool of intracellular ODNs greatly increases. Another example represents the uptake of ODNs by liver tissue. Following intravenous administration of naked ODNs, they become associated with the extracellular matrix, which is followed by internalization in all cell types of the liver, including appearance of ODNs, albeit in relatively minor amounts, in the nucleus. Although the mechanism of uptake remains to be determined, it is again apparent that also in this case nuclear access of ODNs in vivo can be accomplished in a vector-independent manner (141).

How these remarkable differences between in vivo and in vitro application in terms of cellular access of ODNs should be accounted for, has not been addressed thus far. Whether the extracellular matrix harbors means to effectuate and/or to facilitate the mechanism of entry, remains to be determined. In the context of in vitro observations it is difficult to envision that if endocytosis would represent a major means of entry in vivo as well how endosome-localized ODNs would readily acquire access to the cytosol in vivo and not in vitro. This raises the issue of the existence of alternative (facilitated) mechanisms of entry in vivo, which should be capable of mediating direct delivery of ODNs into the cytosol, thus allowing rapid nuclear delivery. ODN transport via a plasma membrane-localized nucleic acid channel as recently described to be present in rat renal brush board plasma membranes could fulfill such an alternative (93, 94). It is possible that an enhanced endocytic capacity in vitro and/or a less efficient translocation mechanism at such conditions, possibly depending on extracellular modulators for optimal activity, may obscure the seemingly low delivery to nucleus of added free ODNs in vitro.

Even though naked ODNs can enter cells in certain tissues, they permeated very poorly into others such as brain or tumor after systematic administration. This necessitates the use of appropriate vectors in vivo, particularly in targeted delivery.

*Overcoming the reticuloendothelial system (RES).*

Dictated by pharmacokinetic distribution studies in vivo on drug delivery, organs of the RES, such as liver, spleen, kidney and lungs represent most likely effective clearance sites, following systemic administration of ODN complexes. Even though a wide tissue distribution of ODNs has
been reported, except for the brain and testes, a major fraction, irrespective of the route of administration, appears to end up in RES organs. Accordingly, directing ODNs to non-RES organs remains a challenge in antisense technology.

Free ODNs are cleared from plasma according to a kinetics with two exponentials, showing half-lives of about 20 min and approx. 24 h, respectively, although it may vary with the chemical nature of administered ODNs. Provided that the majority of naked ODNs are cleared in the first few hours, liposomes and other delivery vectors are used to protect and prolong the half-life of ODNs in the circulation in order to improve the interaction frequency with non-RES organs. However, to avoid the RES, additional modification of the delivery vehicle per se is needed, like the inclusion of PEGylated lipids, modulation the surface charge and inclusion of appropriate targeting devices for tissue/cell-specific delivery. Such systems have proved useful, for example, in targeted delivery of DNA into the brain. Thus a PEGylated (DSPE-PEG) DNA lipoplex, consisting of phosphatidylcholine and the cationic lipid didodecylammonium bromide, was conjugated with transferrin receptor antibodies, OX-26. Following systemic administration, such complexes were shown to cross the blood brain barrier by transcytosis via transferrin receptors on brain endothelial cells. The complexes subsequently distributed over a large area in the central nervous system, including neurons, choroid plexus epithelium, and the brain microvasculature (12, 142, 143). A priori, in this manner it should thus be possible to specifically target to defined brain cell populations, following systemic administration. Importantly however, successful application of such an approach will require the dissociation of the PEGylated lipid from the complex, as discussed above. Hence rather than the essentially non-exchangeable DSPE-PEG, this kind of approach requires a controllable release of PEGylated lipid analogues (124, 144, 145).

Reducing the toxicity of ODNs.

In spite of the fact that successful clinical trials have been reported (22-24), scepticism remains as to unambiguous proof for accomplishing a genuine antisense effect. Obviously this will frustrate a more general acceptance and effectuation of antisense therapy. In fact, for any kind of drug application, including the application of antisense technology, potential toxic side effects are a primary concern. In cell culture, the main toxicity is due the inhibition of cell growth as a result of the binding of ODNs to membrane or other intracellular proteins. In vitro cytotoxicity can be usually controlled by adjusting the proper dosage of ODNs or by their packaging in delivery vectors. The in vivo toxicity often correlates with the capture and long-term deposit of ODNs in RES organs, causing harmful side effects as renal tubule
degeneration/necrosis, splenomegaly, thrombocytopenia and elevation of liver transaminases (146, 147). Some of these side effects are similar to those associated with delivery of other non-related polyanions such as dextran sulfate (148). Improved ODN chemistry, for example by reducing the negative charge, may at least in part relieve these problems. The potential toxicity of ODNs with chemically modified linkages has not been addressed thus far. Thus future work should also be aimed at clarifying the metabolic fate of ODNs. However, in general, from the clinical toxicity profile of ODNs directed against viruses and tumors, this new generation of drugs is still more tolerable than conventional chemotherapy and radiotherapy (149-151).

**Conclusion and remarks** In the past 5 years, considerable progress has been made in understanding the mechanism(s) of antisense activity, in developing ODN chemistry and in setting up antisense-related clinical trails, with as landmark the production of the first commercial antisense drug against CMV retinitis. Evidently, the activities leading to these prosperous developments have raised a wealth of insight into many fundamental cellular processes ranging from events related to the regulation of gene function to signal transduction pathways. Thus therapeutic progress is closely related to that of fundamental development and the spin off for the latter is far beyond the actual purpose of ODN research as such. In terms of therapeutic application of antisense technology, the window could be extended beyond treatment of primarily cancers and viral infections, which may be accomplished by improving therapeutic index, better specifying the antisense effect and lowering the costs.

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**References:**


