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Review

State of the art and perspectives for the delivery of antisense oligonucleotides and siRNA by polymeric nanocarriers

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ABSTRACT

Knocking down gene expression using either antisense oligonucleotides (AS-ODNs) or small interfering RNA (siRNAs) has raised a lot of interest in designing new pathways for therapeutics. Despite their potentialities, these negatively charged and hydrophilic molecules request chemical modifications or a carrier that allows cell recognition, cell internalization and moreover subcellular penetration. Although chemical modifications were brought to the basic AS-ODNs and siRNAs, their sensitivity to degradation and poor intracellular penetration is still hampering their clinical applications. We present here the potentialities of polymeric carriers or the use of alternative administration route such as oral, ocular and skin delivery to improve their delivery and to circumvent the hurdles for their clinical applications.

Contents

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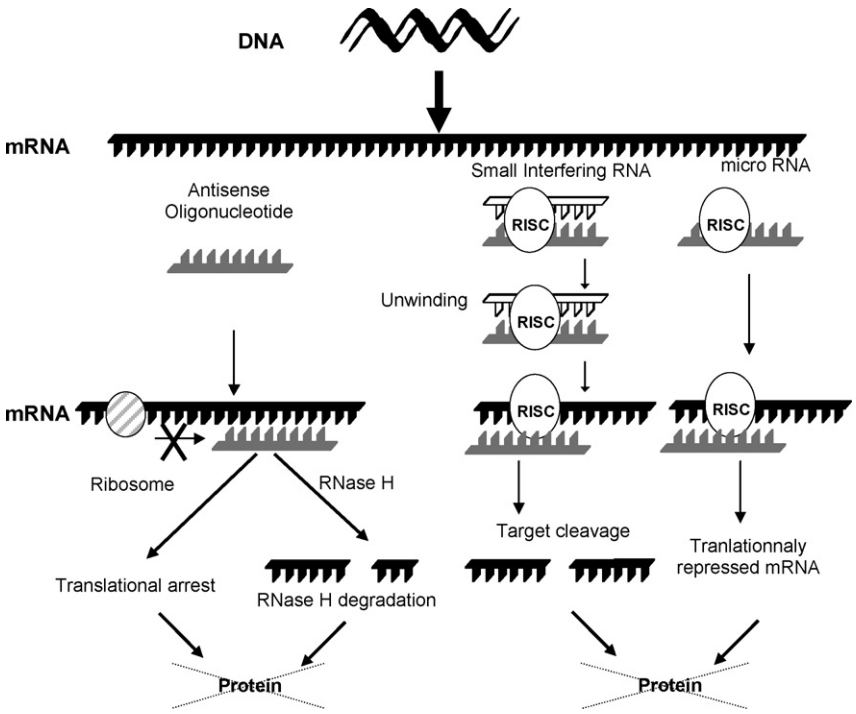

Fig. 1. Mechanism of action of AS-ODNs and siRNAs.

1. Introduction

The discovery of antisense oligonucleotides (AS-ODNs) and more recently siRNA has opened wide perspectives in therapeutics for the treatment of cancer, infectious and inflammatory diseases or to block cell proliferation and diseases caused thereby. However, these molecules are unstable in biological fluids and display a poor intracellular penetration. What are therefore the chances to turn them into drugs? In most cases, research groups have applied the nanotechnology approach to improve their delivery. These studies have allowed the design of new means to overcome most of the barriers that hampered the development of AS-ODN and siRNA therapeutics. These barriers can be summarized as (i) protein and enzyme interactions with the carrier or its contents and (ii) tissular, cellular and subcellular targeting. Besides intracellular targeting, many authors have attempted to use the oral route employing absorption enhancers for improving bioavailability. Finally, local delivery, particularly ocular but also skin delivery, was investigated. Intraocular delivery is the only route of administration that has permitted to market two nucleic acid-based drugs: Vitravene® and Macugen®. This review article will try to give the state of the art of all these delivery approaches but also to put into perspectives the hurdles to jump over to achieve a large success for these therapeutic agents.

2. Basic concept and mechanism of antisense oligonucleotides

AS-ODNs are synthetic single-stranded DNA fragments that bind to specific intracellular messenger RNA strands (mRNA) forming a short double helix. They consist of short sequences, composed of 13 to about 25 nucleotides, which are complementary to mRNA strands in a region of a coding sequence designed as sense strand. By binding to the mRNA molecules, AS-ODNs were shown to stop translation of the mRNA, and hence protein synthesis expressed by the targeted gene (Loke et al., 1989). The ability of an AS-ODN to form a hybrid depends on its binding affinity and sequence specificity. Binding affinity is function of the number of hydrogen bonds formed between the AS-ODN and the targeted sequence. The affinity can be measured by determining the melting temperature (T_m) at which 50% of the double-stranded material is dissociated into single strands. T_m depends on the concentration of AS-ODNs, the nature of the base pairs and the ionic strength of the solvent in which hybridization occurs (Breslauer et al., 1986). Among several recognized mechanisms, one commonly described is the so-called translational arrest. In this mechanism, an AS-ODN binds to the single strand mRNA by Watson-Crick base pairing forming a double-helix hybrid and blocks sterically the translation of this transcript into a protein (Baker et al., 1997). The hybridized mRNA, at the site where translation starts, prevents the binding of factors that initiate or modulate the translation (Fig. 1). In addition, the hybrid formation may block the movement of ribosomes along the mRNA, their progress is therefore delayed (Dias et al., 1999). Another mechanism that was widely described involves RNase-H mediated cleavage of the target mRNA. RNase-H is a ribonuclease that recognizes RNA–DNA duplexes and selectively cleaves the RNA strand (Fig. 1). This mechanism is catalytic: once a RNA molecule is cleaved, the AS-ODN dissociates from the duplex and becomes available to bind a second target mRNA molecule (Walder and Walder, 1988). However, this recognition by RNase-H is restricted to a few compounds since it does not occur with most of the chemically modified As-ODNs.

DNA targeting is also possible by using oligonucleotides. These single-stranded oligonucleotides can form triple helices with DNA. The third strand binds in the major groove of the double helix when one strand of the double helix is composed of purines and therefore the complementary strand of pyrimidines. The bases of the third strand bind to the purines via Hoogsteen bonds or reverse Hoogsteen bonds. The third strand can be made of purines and binds at neutral pH and of pyrimidines and binds at acidic pH (Moser and Dervan, 1987).

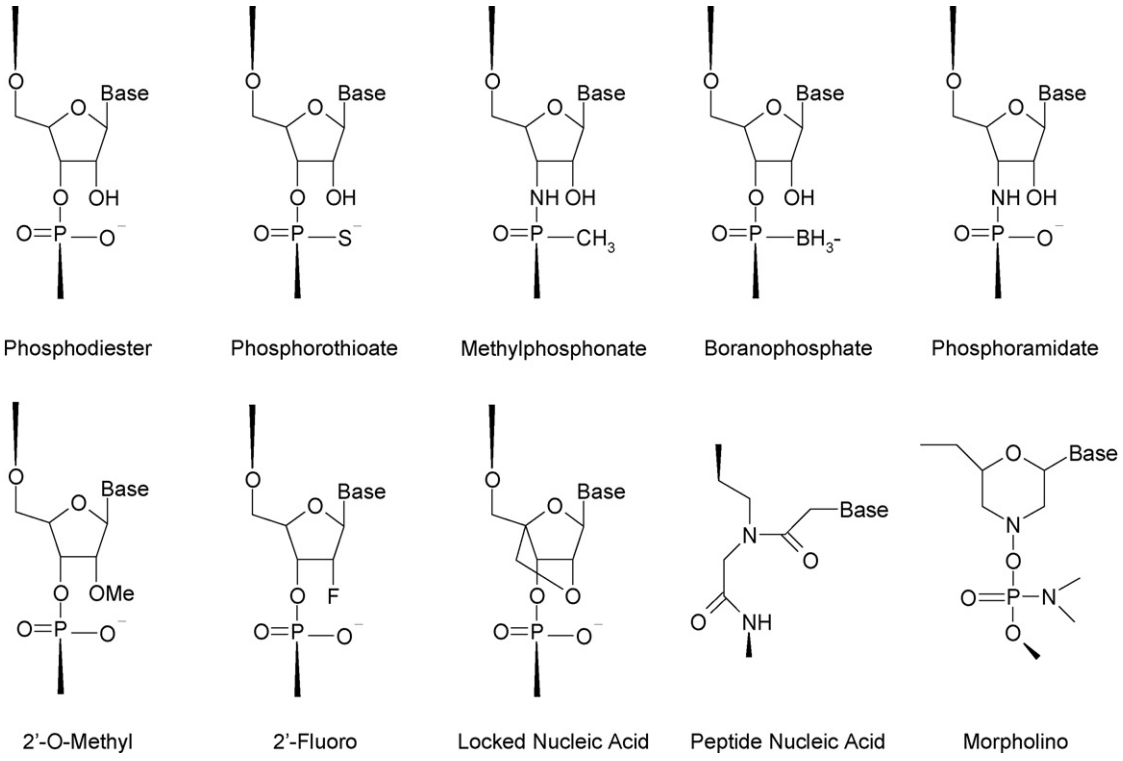

Fig. 2. Chemical modifications of AS-ODNs and siRNAs.

3. Basic concept and mechanism of small interfering RNA (siRNA)

SiRNA were discovered by showing that the introduction of long double-stranded RNA (dsRNA) into a variety of hosts could induce post transcriptional silencing of all homologous host genes and/or transgenes (Fire et al., 1998; Elbashir et al., 2001; Vaucheret and Fagard, 2001; Kennerdell et al., 2002). Within the intracellular compartment, the long dsRNA molecules are metabolized to small 21–23 nucleotide interfering RNAs by the action of an endogenous ribonuclease: dsRNA-specific RNase III enzyme Dicer (Timmons and Fire, 1998; Elbashir et al., 2001; Ketting et al., 2001). The siRNA molecules then assemble to a multiprotein complex, termed RNA-induced silencing complex (RISC) (Fig. 1). Functional RISC contains four different subunits including helicase, exonuclease, endonuclease, and homology searching domains. In case siRNA binds to RISC, the duplex siRNA is unwound by helicase, resulting in two single strands (Fig. 1) (Nykanen et al., 2001) allowing the antisense strand to bind to the targeted RNA molecule (Vaucheret and Fagard, 2001). The endonucleases hydrolyze the target mRNA homologous at the site where the antisense strand is bound. RNA interference has an antisense mechanism of action, as ultimately a single strand RNA molecule binds to the target RNA molecule by Watson-Crick base pairing rules and recruits a ribonuclease that degrades the target RNA (Fig. 1) (Zamore et al., 2000). This mechanism makes feasible the use of small double-stranded siRNA in therapeutics instead of AS-ODNs. When siRNA mediated silencing occurs, the products are cleaved released and degraded, allowing RISC complex to interact with other molecules from the mRNA pool (Leung and Whittaker, 2005). It was also shown that small RNAs (called miRNAs for microRNAs) causes gene silencing in humans as well as in *Caenorhabditis elegans*, *Drosophila melanogaster*, and plants (Lee et al., 1993; Hutvagner et al., 2001). miRNAs are processed from extended RNA hairpins, whereas siRNAs are produced from a range of RNA precursors, such as viral and transposon RNAs and transgenes (Jana et al., 2004). The mechanisms of siRNAs and miRNAs for RNAi have many similarities, for example, the synthesis of both of them is related to the activity of Dicer, though there are many differences between them such as siRNAs cleaving mRNA (Fig. 1), whereas miRNAs translationally repressing mRNA (Fig. 1) (Sontheimer and Carthew, 2005).

Although there are many similarities between AS-ODNs and siRNA, compared to AS-ODNs, very few molecules of siRNA are needed to inhibit gene expression (Bertrand et al., 2002). As suggested by Corey (2007), the reason might be that, unlike siRNA, there is no evolved mechanism for promoting antisense strand recognition and it is likely that AS-ODNs must find their target unassisted (Corey, 2007).

4. Chemical modifications of antisense oligonucleotides and siRNA

AS-ODNs and siRNAs are natural phosphodiester compounds (Fig. 2). However, critical drawbacks such as their poor stability versus nuclease activity *in vitro* and *in vivo* and their low intracellular penetration and low bioavailability have limited their use in therapeutics (Opalinska and Gewirtz, 2002; Dykxhoorn and Lieberman, 2006). As a result, clinical applications of both types of nucleic acids have required chemical modifications with the aim of retaining their capacity to knock down protein expression while increasing stability and cellular penetration.

Replacement of the non-bridging oxygen of the phosphodiester backbone by sulfur resulted in the synthesis of phosphorothioate AS-ODNs or phosphorothioate siRNAs with enhanced stability to enzymatic degradation (Fig. 2). For AS-ODNs, although the duplex formed with the target RNA has a lower melting temperature (T_m) (*i.e.* lower affinity) than the phosphodiester compound, it remains a substrate for RNase-H. With siRNAs, such a modification did not significantly affect silencing efficiency (Harborth et al., 2003). However, the main disadvantage of the phosphorothioate modi-

fication is their binding to certain proteins, inducing undesirable effects (Stein, 1996; Amarzguioui et al., 2003). The importance of phosphorothioate modification might therefore be overestimated. Moreover, some authors have shown that these linkages do not significantly enhance siRNA nuclease stability and reduces the melting temperatures of the duplexes as compared with unmodified RNA (Braasch et al., 2003). In another study, a phosphorothioate modification was shown to clearly reduce siRNA activity (Chiu and Rana, 2003).

Other chemical approaches tested on AS-ODNs has been the replacement of the non-bridging oxygen by a methyl group which results in a methylphosphonate AS-ODN (Fig. 2) and the replacement of the same oxygen by a borane in siRNA resulting in boranophosphate AS-ODNs (Fig. 2). Methylphosphonate AS-ODNs have a greater hydrophobicity due to the loss of the negative charge. However, in this case, RNase-H activation is also reduced (Agrawal, 1999). Boranophosphate modifications were only applied to siRNAs and were shown to be more effective than natural or phosphorothioate siRNAs mostly because they are at least 10 times more nuclease resistant than unmodified siRNAs (Hall et al., 2004, 2006). Other modifications made on the phosphate group have led to the synthesis of phosphoramidates (Fig. 2). These molecules consist in the substitution of every 3′-oxygen by 3′-amino group. This creates a highly nuclease resistant molecule with an ability to form very stable duplexes with RNA by Watson-Click base pairing. In contrast to phosphodiesters, the phosphoramidates also form stable triplexes with double-stranded DNA under near physiological conditions. Although the ability of phosphoramidates to activate RNase-H is weak, they do effectively block translation because of the high stability of the DNA/RNA hybrids formed (Gryaznov et al., 1995, 1996).

AS-ODNs or siRNAs sugar moiety can be modified at the 2′ position of the ribose by linkage of *O*-methyl (2′-OMe), *O*-methoxy-ethyl (2′-OMeEt), fluoro (2′-F) and locked nucleic acid (LNA) (Fig. 2). 2′-*O*-methyl and 2′-*O*-methoxy-ethyl AS-ODNs have raised a large interest. The RNA/RNA duplex formed with these AS-ODNs is highly stable as indicated by the elevated T_m. However, none of the 2′-*O*-alkyl AS-ODNs derivative can induce RNase-H cleavage of target RNA. For this reason, their antisense effect can only be due to a physical blockage reducing their potency (Kurreck, 2003; Urban and Noe, 2003). Nevertheless, it was shown in AS-ODNS that eight phosphorothioate nucleotides are needed in gapmers with 2′-*O*-methyl AS-ODNs to fully activate RNase-H (Kurreck et al., 2002). The siRNA motif consisting of 2′-OMe and 2′-F has enhanced plasma stability and increased *in vivo* potency as well (Allerson et al., 2005). This modification has also been shown to increase the nuclease resistance of siRNA duplexes (Chiu and Rana, 2003). Nevertheless, this high potency is being discussed by several authors. Indeed, it was recently shown that siRNA duplexes containing full 2′-OMe-modified sense strands display comparable activity to the unmodified analog of similar sequence (Kraynack and Baker, 2006). Another interesting study has shown that even though the 2′-F-modified siRNAs greatly improve resistance to nuclease degradation in plasma, this increase in stability did not translate into enhanced or prolonged inhibitory activity of target gene reduction in mice following tail vein injection (Layzer et al., 2004).

LNA, also referred to as inaccessible RNA, is a family of conformationally locked nucleotide analogs that displays unprecedented hybridization affinity toward complementary DNA and RNA (Wahlestedt et al., 2000; Vester and Wengel, 2004). Commonly used LNA contains a methylene bridge connecting the 2′ oxygen with the 4′ carbon of the ribose ring (Fig. 2). This bridge locks the ribose ring in the 3′-endo conformation characteristic of RNA (Bondensgaard et al., 2000; Braasch and Corey, 2001). LNA AS-ODNs possess a high stability in blood serum and cell extracts and are able to activate RNAse-H (Wahlestedt et al., 2000). LNA AS-ODNs have been shown to inhibit tumour growth in a murine xenograft model (Jepsen et al., 2004). However, they also displayed profound hepatotoxicity as measured by serum transaminases, organ weights and body weights (Swayze et al., 2007). LNA were also compatible with siRNA intracellular machinery, preserving molecule integrity while offering several improvements that are relevant to the development of siRNA technology (Braasch et al., 2003; Elmen et al., 2005).

Chemical modifications have also led to the synthesis of an important class which is the peptide nucleic acids (PNAs) (Hanvey et al., 1992) (Fig. 2). In this case, the phosphodiester linkage is completely replaced by a polyamide (peptide) backbone composed of (2-aminoethyl) glycine units. These AS-ODNs have high hybridization properties and a good biological stability versus *endo*- and *exo*-nucleases. Nevertheless, they present some drawbacks such as their poor aqueous solubility and low cellular uptake. However, their aqueous solubility might be improved by conjugation to lysine (Kuhn et al., 1998, 1999). In addition, they do not induce target RNA cleavage by RNase-H. PNAs seem to be non-toxic as they are uncharged molecules with a low affinity for extracellular proteins. Morpholino AS-ODNs were also synthesized (Fig. 2). They consist in non-ionic DNA analogs, in which the ribose is replaced by a morpholino moiety and phosphoroamidate intersubunit linkages are used as a substitute of phosphodiester bonds. These AS-ODNs are interesting because their affinity for their target is similar to that of natural AS-ODNs and they display a low toxicity (Summerton and Weller, 1997; Summerton, 1999).

5. Cellular uptake of antisense oligonucleotides and siRNA

In order to down-regulate gene expression, AS-ODNs and siRNAs must penetrate into the targeted cells and reach the cytoplasm. The exact mechanisms involved in AS-ODNs penetration are so far unclear. Uptake occurs through active transport, which in turn depends on temperature (Yakubov et al., 1989), on the structure, the concentration of AS-ODNs (Vlassov et al., 1994a) and of course on the cell type. At present, it is believed that adsorptive endocytosis and fluid phase pinocytosis are the major mechanisms of AS-ODNs internalization, with the relative proportions of internalized material depending on AS-ODNs concentration. At relatively low AS-ODNs concentration, it is likely that internalization occurs via interaction with a membrane-bound receptor (Yakubov et al., 1989). The mechanism of internalization of exogenous siRNAs is unclear. It is known that they are readily taken up by invertebrate cells such as *C. elegans*. However, they are not taken up by most mammalian cells in a way that preserves their activity. Like AS-ODNs, siRNAs do not readily cross the cellular membrane because of their negative charge and size (Aagaard and Rossi, 2007) and therefore the simple addition of naked, unmodified siRNAs to the culture media that overlies mammalian cells does not result in effective knockdown of the target gene (Dykxhoorn and Lieberman, 2006).

6. Pharmacokinetic and biodistribution of intravenously administered antisense oligonucleotides and siRNA

The first pharmacokinetics studies were conducted with a 25-mer natural phosphodiester AS-ODNs that was injected intravenously in monkeys. Analysis of blood sample by polyacrylamide gel electrophoresis or capillary gel electrophoresis showed that after 15 min all the AS-ODNs was degraded (Agrawal et al., 1995). Blood half-life of AS-ODNs was estimated to be about 5 min (Agrawal et al., 1995). Similar results were reported in rats for a

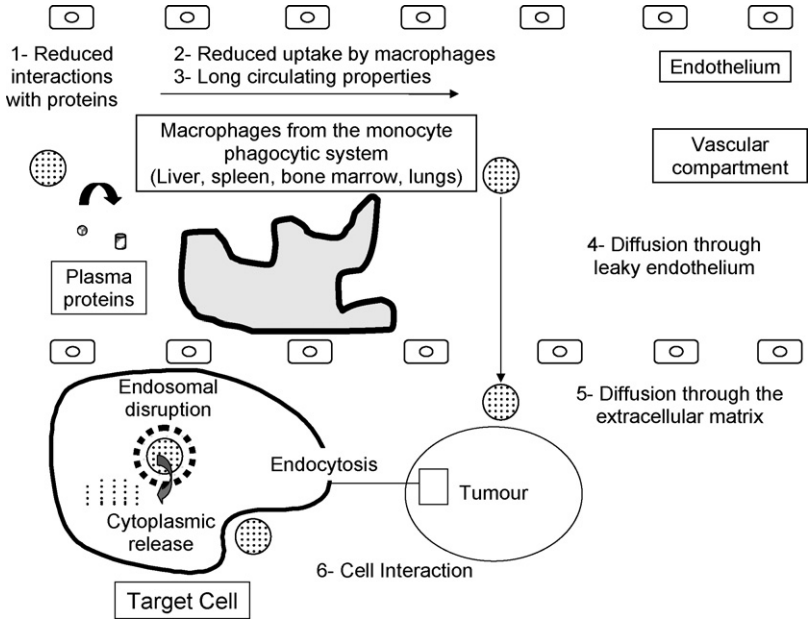

Fig. 3. Properties of an ideal carrier for the delivery of AS-ODNs and siRNA.

20-mer phosphodiester AS-ODN (Sands et al., 1994). The chemically modified AS-ODNs can however improve pharmacokinetics parameters. Indeed in rodent models (rats and mice), phosphorothioate AS-ODNs showed distribution half-lives ranging from 30 to 60 min (Agrawal et al., 1991; Cossum et al., 1993; Zhang et al., 1995; Geary et al., 2001b) For 2′-*O*-methyl derivatives, the pharmacokinetics properties were close to those of phosphorothioates (Geary et al., 2001b; Sewell et al., 2002) while the PNAs did not display any increase in the distribution half-life (McMahon et al., 2002). The plasma clearance depended in some cases on the animal model. In mice, it was at least twofold more rapid than in monkeys at equivalent doses of phosphorothioates AS-ODNs (Yu et al., 2001a). Plasma clearance was largely due to distribution to peripheral tissues. AS-ODNs were mainly distributed to the liver, kidney, spleen, lymph nodes and bone marrow with no measurable distribution to the brain (Agrawal et al., 1991, 1995; Cossum et al., 1993; Geary et al., 1997). The biodistribution pattern depended on the nature of the oligonucleotide backbone. The phosphodiester AS-ODNs accumulated highly in the liver, whereas high kidney uptake dominated the phosphorothioates, 2′-*O*-methyl phosphodiester (Lendvai et al., 2005) and PNA patterns (McMahon et al., 2002). AS-ODNs do not only distribute to tissues but also accumulate within cells in tissues (Butler et al., 1997; Graham et al., 1998; Yu et al., 2001b). At early time points after injection, phosphorothioate AS-ODNs appear to be associated with extracellular matrix and within cells. However, by 24 h, almost all of the AS-ODNs are found within cells in tissues (Butler et al., 1997). The mechanism(s) by which AS-ODNs accumulate within the cells following parenteral administration is currently unknown (Butler et al., 2000). Regarding siRNAs, their half-life *in vivo* is as short as for AS-ODNs (seconds to minutes) (Soutschek et al., 2004). This is predominately due to their rapid elimination by kidney filtration because of their small size (~7 kDa) (Soutschek et al., 2004). Like AS-ODNs, they can also be degraded by endogenous serum RNases with a serum half-life of 60 min. The biodistribution of radiolabeled siRNA in mice showed an accumulation primarily in the liver and kidneys (Braasch et al., 2004). They were also detected in the heart, spleen and lung. These patterns closely resemble the characteristics of AS-ODNs.

7. Barriers and needs for the delivery of antisense oligonucleotides and siRNA

Barriers for nucleic acid delivery were nicely summarized in a recent paper by Li and Szoka (2007). They have defined the different approaches to combine in order to circumvent the barriers. At first, the delivery systems given through the intravenous administration should avoid both interactions with plasma proteins and uptake by the macrophages of the monocyte phagocytic system (MPS) (Fig. 3). This is really critical for cationic delivery systems because these interactions result in the formation of aggregates which are either entrapped in the lung endothelial capillary bed or taken up by the MPS (Opanasopit et al., 2002). Moreover, biocompatibility problems could arise such as complement activation (Plank et al., 1996). Similarly to conventional colloidal drug carriers, delivery systems for nucleic acids should cross permeable endothelium such as in neovascularised tumours or inflammation (Fig. 3). In this case, size is an important matter, since only small particles will be able to pass through the fenestrated barriers. This effect is called EPR for enhanced permeability and retention. This latest properties are due to a low lymphatic drainage in the tumour site (Hashizume et al., 2000). Before reaching the cellular level, the delivery system should be able to cross the tight network made by the extracellular matrix composed of a variety of polysaccharides and proteins over the surface of cells that produce them (Li and Szoka, 2007). Having attained the tumour cells, AS-ODNs and siRNAs will meet the problem of their poor intracellular penetration and subcellular localization that were discussed above. All these barriers need to be part of the rationale to develop new delivery carriers.

8. Polymeric nanoparticles for the delivery of antisense oligonucleotides and siRNA

Despite the continuous synthesis of new entities of AS-ODNs or more recently siRNA, a few issues remain to be solved to optimize their delivery. It is very important to meet all the critical parameters corresponding to an optimal activity: improved stability, increased blood half-life, tissue and cellular targeting, improve cellular

penetration, release the nucleic acids in the right intracellular compartment. Number of research groups have focused on the design of polymeric nanotechnologies that could meet all the requirements mentioned above for an optimal delivery of nucleic acids.

8.1. Association of antisense oligonucleotides and siRNA to cationic polymer systems by complexation

Several polymer nanoparticles systems have been developed to enhance the intracellular delivery of AS-ODN. AS-ODNs which are negatively charged at physiological pH can electrostatically interact with cationic charges to form complexes simply by mixing the components in optimal conditions. The positive charges can either come from soluble cationic polymers or cationic nanoparticles. As the complex possesses generally a global positive surface charge, they get easily attached to negatively charged cell surface with subsequent endocytosis.

8.1.1. Polyethylenimine (PEI)

The main polymer that was used is PEI, a branched polymer with high cationic potential. Cytoplasmic delivery of nucleic acids by PEI can be attributed to the buffering effect or the "proton sponge effect" of the polymer caused by the presence of amino groups in the molecule. The strong buffering effect of the polymer helps in rapid endosomal escape (Boussif et al., 1996). Cytotoxicity and transfection efficiency of PEI are directly proportional to its molecular weight (Godbey and Mikos, 2001). Efforts to reduce the toxicity or improve stability by synthesis of PEI with graft copolymers such as linear poly(ethylene glycol) (Vinogradov et al., 1998; Petersen et al., 2002), incorporation of low molecular weight PEI, and PEI glycosylation (Leclercq et al., 2000) have been tested. *In vitro* efficacy of PEI to deliver AS-ODNs and siRNAs was clearly demonstrated on different cell populations including non-dividing cells (Boussif et al., 1995; Dheur et al., 1999; Gomes dos Santos et al., 2006b). Successful siRNA delivery was also observed within a very narrow window of conditions and only with the 25 kDa branched PEI (Grayson et al., 2006). It was shown that complexation of chemically unmodified siRNAs with PEI leads to the formation of complexes that condense and completely cover siRNAs as determined by atomic force microscopy (Grzelinski et al., 2006). More importantly, upon PEI complexation, siRNAs were efficiently protected against nucleolytic degradation both *in vitro* in the presence of RNase and *in vivo* in the presence of serum nucleases (Urban-Klein et al., 2005). In tissue culture, PEI–siRNA complexes are internalized by tumour cells within a few hours leading to the intracellular release of siRNA molecules which display full bioactivity. The ability of PEI to improve *in vitro* and *in vivo* the efficacy siRNA has been tested for the delivery of a siRNA against the growth factor pleiotrophin (Grzelinski et al., 2006). The intraperitoneal injection of PEI complexes targeting the HER-2 receptor also resulted in a marked reduction of tumour growth through siRNA-mediated HER-2 downregulation (Urban-Klein et al., 2005). Ligand-targeted PEI has been used to improve the efficiency of siRNA delivery. Self-assembling nanoparticles with siRNA were constructed with PEI that was pegylated with an Arg-Gly-Asp (RGD) peptide ligand attached at the distal end of the PEG. The complex was able to target tumour neovasculature expressing integrins and deliver siRNA inhibiting vascular endothelial growth factor (VEGF) receptor-2 expression and to block tumour angiogenesis (Schiffelers et al., 2004).

The effect of PEGylation on the stability of siRNA–PEI complexes was tested by Mao et al. (2006). Stability and size of PEI–siRNA complexes were clearly influenced by PEI-PEG structure, and high degrees of substitution resulted in large (300–400 nm), diffuse complexes which showed condensation behaviour only at high nitrogen to phosphorus ratios (N/P). Stability of siRNA polyplexes against heparin displacement and RNase digestion could be improved by pegylation. In knockdown experiments using NIH/3T3 fibroblasts stably expressing beta-galactosidase, it was shown that PEG chain length had a significant influence on biological activity of siRNA. Pegylated polyplexes with siRNA yielded knockdown efficiencies of around 70% higher than with PEI alone. These results were explained by the fact that the high siRNA condensation of PEI might prevent its release in the cytosol whereas the condensation was lower in the case of pegylated PEI (Mao et al., 2006).

8.1.2. Dendrimers

Other polymers have been used such as starburst polyamidoamine (PAMAM) dendrimers which are synthetic polymer characterized by a branched spherical shape and a high-density surface charge. Transfections of AS-ODNs using dendrimers into clones generated from D5 mouse melanoma and Rat2 embryonal fibroblast cell lines expressing luciferase cDNA resulted in a specific and dose dependent inhibition of luciferase expression of about 25–50% (Bielinska et al., 1996). These results were confirmed by others (Yoo et al., 1999). Helin et al. (1999) studied the intracellular distribution of a fluorescein isothiocyanate-labeled ODN vectorized by PAMAM and found that intracellular AS-ODN distribution was dependent on the phase of the cell cycle, with a nuclear localization predominantly in the G2/M phase. Dendrimers were shown to condense siRNA into nanoscale particles, to protect them from enzymatic degradation and achieve substantial release over an extended period of time for efficient gene silencing (Zhou et al., 2006). The ability of siRNA–dendrimer complexes to deliver siRNA to cells was tested using a model of endogenous gene knockdown constituted by A549Luc cells stably expressing the GL3 luciferase gene. Efficient gene silencing was observed with the specific GL3Luc siRNA–dendrimer complex while neither the naked siRNA nor the non-specific GL2Luc siRNA–G7 complex showed any gene silencing effect (Zhou et al., 2006). For targeting purposes, PAMAM dendrimer was conjugated to Tat peptide, a cell penetrating peptide that should increase cellular delivery. Delivery of both AS-ODNs and siRNAs designed to inhibit MDR1 gene expression in NIH/3T3 cell line was achieved (Kang et al., 2005). MDR1 gene expression was partially inhibited by the antisense complex and weakly inhibited by the siRNA complex when both were tested at non-toxic levels of dendrimer. Conjugation with Tat peptide did not improve the delivery efficiency of the dendrimer (Kang et al., 2005).

8.1.3. Cationic nanospheres

Nanospheres (nanoparticles consisting of spherical polymer matrix) can be the support for complexation of nucleic acids. In this case, the use of cationic nanospheres, made of cationic polymers or nanospheres that are rendered positive through the adsorption of a cationic surfactant is required. In this latest case, nanospheres are composed of synthetic biodegradable polymers such as poly(alkylcyanoacrylate) (PACA) (Fattal et al., 1998) or poly(lactic acid) (PLA) (Singh et al., 2003). Since AS-ODNs have no affinity for the polymeric matrix, association with nanospheres has been achieved by ion pairing with a cationic surfactant, cetyltrimethylammonium bromide (CTAB) adsorbed onto the nanosphere surface (Fattal et al., 1998). AS-ODNs bound to PACA nanosphere in this way were protected from nucleases *in vitro* (Chavany et al., 1992) and their intracellular uptake was increased (Chavany et al., 1994). In addition, nanospheres were able to concentrate intact AS-ODNs in the liver and in the spleen (Nakada et al., 1996). Using such a formulation, AS-ODNs were able to specifically inhibit mutated Ha-*ras*-mediated cell proliferation and tumourigenicity in nude mice (Schwab et al., 1994). More recently, a novel nanoparticulate system proposed recently by de Martimprey et al. (2008) consists of a biodegradable core

of poly(isobutylcyanoacrylate) with a shell of chitosan considered less cytotoxic than CTAB. SiRNA complementary sequence to the Ret/PTC1 junction oncogene was adsorbed to such nanospheres (de Martimprey et al., 2008). These nanospheres were tested in papillary thyroid carcinoma cells injected subcutaneously into the right flank of nude mice. They were shown to inhibit the expression of ret/PTC1 as well as to reduce tumour size compared with free siRNA (de Martimprey et al., 2008).

8.2. Encapsulation of antisense oligonucleotides and siRNA within polymer nanocapsules

For optimal activity and reduced toxicity related to the cationic charges, AS-ODNs were entrapped within nanocapsules instead of being adsorbed. The most appropriate method of encapsulation was the one developed by Lambert et al. (2000b) for the design polyisobutylcyanoacrylate (PIBCA) nanocapsules containing an aqueous core. AS-ODNs encapsulation into the aqueous core of nanocapsules improved their stability against enzymatic degradation (Lambert et al., 2000b). Their half-life in serum was increased from 3 to 6 h compared to naked AS-ODNs and was higher than when AS-ODNs was adsorbed onto PIBCA nanospheres (Lambert et al., 2000b). Encapsulation into PIBCA nanocapsules importantly increased cell uptake of both AS-ODN and siRNA. Toub et al. (2005) have investigated the subcellular distribution of AS-ODNs when incubated under naked or encapsulated form. Incubation of naked As-ODNs with cultured vascular smooth muscle cells resulted in membrane-bound species higher than the internalized fraction, while the encapsulation showed an inverted profile with an internalized fraction 10 times higher as compared to naked ODN. These authors (Toub et al., 2005) confirmed that the intracellular fraction of naked AS-ODNs was mainly found in vesicular fraction. AS-ODNs delivered by PIBCA nanocapsules drastically changed the subcellular partition: less than 40% of ODN remained entrapped into vesicles, whereas about 50% was found in the nuclear fraction. This suggests that PIBCA nanocapsules were able to deliver a substantial amount of its cargo to the cytosolic compartment with further diffusion of the AS-ODNs to the nucleus. Similar AS-ODNs distribution into cells was observed using either fluorescence or radioactivity measurements, confirming the integrity of the internalized compounds (Toub et al., 2005). Finally, AS-ODNs accumulation into the nucleus was found to increase as a function of time, as showed in confocal microscopy experiments performed at different time intervals, ranging from 2 to 15 h (Toub et al., 2006a). The mechanism by which encapsulated AS-ODNs can escape from endosomes is not yet fully understood. However, detergents used in their formulation such as sorbitan monooleate may play a role in endosomal membrane destabilization (Torchilin et al., 1993).

In vivo, AS-ODNs-containing nanocapsules were mainly tested on a murine model of Ewing sarcoma-related tumours targeting EWS Fli-1, a fusion gene resulting from a translocation that is found in 90% of both Ewing sarcoma and primitive neuroectodermal tumour (Lambert et al., 2000a). At a cumulative ODN dose of 14.4 nmol, only AS-ODNs encapsulated within nanocapsules led to a significant inhibition of tumour growth, while a slight effect was observed with naked AS-ODNs at the same dose. Tanaka et al. (1997) observed a significant reduction of tumour growth with injection of 500 nmol naked AS-ODNs. Thus, the use of nanocapsules allowed a 35-fold reduction of the AS-ODN administered dose. In the same experimental model, the efficiency of AS-ODN-containing nanocapsules was compared with that of the same AS-ODNs adsorbed onto poly(isohexylcyanoacrylate) (PIHCA) nanosphere through interactions with preadsorbed CTAB (Maksimenko et al., 2003). In this case, a completely phosphorothioate AS-ODNs and a chimeric phosphorothioate/phosphodiester derivative were tested. Both nanoparticulate systems led to an efficient inhibition of the tumour volume from about 66 to 82%. The antisense effect of the delivered AS-ODN was demonstrated by down-regulation of EWS-Fli-1 mRNA. The authors speculate that in the case of the nanospheres, CTAB can permeate the endosomal membrane, thus inducing a more efficient cytoplasm delivery of the AS-ODN. Using again the same *in vivo* experimental model, Toub et al. (2006b) showed that PIBCA nanocapsules were efficient to deliver siRNAs into tumour. A dramatic inhibition of tumour growth was observed, especially when a higher cumulative dose was employed. In particular, a reduction of the tumour volume from about 43 to about 80% with a cumulative dose of 0.8 and 1.1 mg/kg, respectively, were observed allowing with a lower daily and cumulative dose to obtain a better efficacy than for encapsulated AS-ODNs. For both the doses, naked siRNA had no inhibitory effect on tumour growth.

9. Advantages and limits of polymeric carriers

As discussed above, polymeric carriers have a great versatility since many modifications can be made to these polymers such as changing the molecular weight, the geometry (linear versus branched) and introducing a ligand by covalent linkage. They also present a few advantages over lipid systems such as a relatively small size and narrow distribution, a higher protection against nucleases and an easy control of physical factors (e.g. hydrophilicity and charge). Nevertheless, the limits are numerous and permanent questions are still raised by their potential toxicity. Toxicity of PEI is high although high molecular weight branched polymers are considered more toxic than low molecular weight (Fischer et al., 2003, 2004) or linear polymers (Chemin et al., 1998). Moreover, it is a non-biodegradable polymer that can accumulate within the cells interfering with vital intracellular process. According to Godbey et al. (1999), there are two types of cytotoxicities related to PEI-mediated cell transfection. An immediate toxicity associated with free PEI and another which is a delayed toxicity associated to cellular processing of PEI/DNA complexes (Godbey et al., 2001). When free PEI interacts with negatively charged serum proteins (such as albumin) and red blood cell, it forms large clusters that adheres to the cells surface (Fischer et al., 1999) destabilizing the plasma membrane and inducing the immediate toxicity. This immediate toxicity can be overcome by the addition of PEG. The delayed toxicity by PEI/DNA complex is related to the intracellular dissociation of DNA from PEI leading to the release of free PEI which interacts with cellular components and inhibits normal cellular process (Godbey et al., 2001). It causes several changes to cells, which include cell shrinking, reduced number of mitoses and vacuolization of the cytoplasm (Godbey et al., 2001). Moreover, as observed by Lambert et al. (1998) using nanoparticles, polymeric cationic systems can induce a perturbation in the intracellular metabolic activity despite the absence of toxicity visible with the current cytotoxicity tests (Lambert et al., 1998). Finally, it is important to cite the experiment of Kawakami et al. (2006) who compared the production of proinflammatory cytokines produced after administration of either polyplexes or lipoplexes. They demonstrated that the amount of NF-κB activated by the linear PEI polyplexes was comparable with the control (untreated group) and was significantly lower than the amount activated by lipoplexes (Kawakami et al., 2006). However, this experiment is not sufficient to conclude that polyplexes are less toxic than lipoplexes and should be extended to several *in vitro* and *in vivo* models.

10. Alternative routes for antisense oligonucleotides and siRNA

Alternative routes were employed for the administration of siRNA, antisense oligonucleotides or other type of nucleic acids. As

for other types of macromolecules, these routes of administration have been used either for obtaining systemic absorption (e.g. oral) or to produce a local effect (e.g. ocular and skin).

10.1. Oral delivery

The discovery of new chemical entities of AS-ODNs has raised strong hopes for their administration through the oral route. Indeed, 2′-*O*-methoxy-ethyl AS-ODNs are highly stable with a prolonged half-life allowing achieving and maintaining therapeutic levels for systemic indications (Khatsenko et al., 2000). Moreover, 2′-*O*-methoxy-ethyl AS-ODNs have an increased resistance to nuclease metabolism which enhances both gut stability (Geary et al., 2001a) and tissue accumulation up to eightfold higher than the accumulation of a typical phosphorothioate AS-ODNs (Geary et al., 2003). Thus, with increased tissue accumulation, a bioavailability of 10% or greater should be adequate to achieve systemic therapy. The work of Raoof et al. (2002, 2004) has demonstrated in pig and dog that the use of permeation enhancers, particularly of caproic acid, represents an attractive strategy to improve the oral bioavailability.

10.2. Ocular delivery

Ocular delivery of nucleic acids has raised a lot of interest (for review see Fattal and Bochot, 2006). The first antisense drug approved in November 1998 by the FDA has been Vitravene®, an oligonucleotide discovered by ISIS for the treatment of cytomegalovirus retinitis in persons with AIDS (Perry and Balfour, 1999). Vitravene® was marketed by Novartis Ophthalmics and came on the market just as powerful AIDS medications. Although, it changed the course of the disease, patients today rarely develop cytomegalovirus and Vitravene® sales have been extremely small. Many other antisense molecules are under trial for ocular administration among which an antisense inhibitor of c-Raf kinase, an enzyme important in the signal transduction pathway triggered by the VEGF and other important growth factors. In preclinical studies, antisense inhibition of c-Raf kinase was associated with a reduction in the formation of new blood vessels in the eye involved in both age-related macular degeneration (AMD) and diabetic retinopathy (Danis et al., 2003). Although aptamers were not mentioned before in this review, these molecules are DNA or RNA molecules that have been selected from random pools based on their ability to bind other molecules. Aptamers can bind nucleic acids, proteins, small organic compounds, and even entire organisms (Nimjee et al., 2005; Pestourie et al., 2005; Proske et al., 2005). These novel molecules have shown many potential in the treatment of ocular diseases. The FDA has recently approved Macugen® (pegaptanib sodium injection) discovered by Eyetech Pharmaceuticals, Inc. Macugen® consists in an aptamer with a modified RNA derived from a 2′-fluoro pyrimidine aptamer and also contains 2′-*O*-methyl purine modifications to enhance stability against endonucleases (Ruckman et al., 1998). The aptamer is further modified with a 5′-polyethylene glycol moiety and a 3′-dT attached via a 3′–3′ linkage to confer favourable pharmacokinetic properties and for protection against exonucleases (Ruckman et al., 1998). It is designed to treat wet AMD (Partner Pfizer Inc. is marketing the drug) through binding to VEGF165, the major inducer of abnormal blood vessel growth and leakage in this particular form of AMD (Ng et al., 2006). Chemically modified short siRNA targeting VEGF receptor-1 (VEGFR-1) was also shown to inhibit neovascularisation in several validated preclinical models (Reich et al., 2003; Kim et al., 2004; Tolentino et al., 2004; Murata et al., 2006).

Nevertheless, in most of these diseases, the target site is the posterior segment of the eye. Indeed, due to their physicochemical characteristics free nucleic acids do not penetrate the cornea and remain confined to the superficial epithelial layer (Bochot et al., 1998). However, considering the negative charge of these molecules, nucleic acids are potential candidates to be delivered into the eye by iontophoresis. Iontophoresis is a local non-invasive method used clinically to transfer ionized drugs into the tissues notably the skin using an electric field. This technique can also reproducibly deliver therapeutic concentrations of various ophthalmic drugs (corticoids, antibiotics, peptides, proteins) to both segments of the eye (Halhal et al., 2004; Myles et al., 2005). Transscleral iontophoresis presents advantages compared to transcorneal delivery, including (i) the large surface area of the sclera as compared to the cornea, (ii) enhanced transfer of drugs to the posterior segment, and (iii) less possibilities of systemic absorption. Application can be repeated as many times as necessary to achieve the needed continuous therapeutic levels without any damage to the eye or unwarranted side effects (Myles et al., 2005).

AS-ODNs against VEGFR-2 penetrated into all corneal layers and were even detected in the iris when iontophoresis using a corneal applicator was applied on rat cornea (Berdugo et al., 2003). In a neovascularisation model, AS-ODNs were absorbed by the cornea and were particularly localized within the vascular endothelial cells of the stroma. AS-ODNs extracted from the tissues were intact after 24 h. In the same way, iontophoresis allowed the diffusion of intact carboxyfluorescein-labeled phosphorothioate oligonucleotides into rabbit eyes in the anterior chamber (5 min), the vitreous (10 min) and the retina (20 min) (Asahara et al., 2001). Moreover, oligonucleotide concentrations within ocular tissues increased with time. The transparency of all corneas was maintained and no abnormal findings were observed. Anti-nitric oxide synthase II oligonucleotides down-regulated the expression of NOSII mRNA in iris/ciliary body in endotoxin-induced uveitis rats receiving iontophoresis compared to controls (Voigt et al., 2002). These results clearly indicate that the use of a physical technique is efficient to deliver AS-ODNs or siRNAs into the posterior segment of the eye.

Another strategy consists in injecting directly nucleic acids into the vitreous. Intravitreally injected in rat eyes, anti-TGF-β2 AS-ODN/PEI nanocomplexes migrate along the retinal Müller glial cells and distribute homogenously in all retinal layers 24 h after their injection. 72 h post injection, AS-ODNs seem to preferentially target RMG cells without inducing any detectable toxicity. The fluorescence concentrated in nuclei of the superficial retinal layer and in the inner nuclear layer is in favour of the release of free AS-ODNs in the targeted cells (Gomes dos Santos et al., 2006b). These results tend to demonstrate that the vitreous which is rich in components negatively charged is not a true barrier for the diffusion of polymeric nanocomplexes contrary to what is observed with lipoplexes.

In order to provide a sustained release of AS-ODN for extended periods of time, a so-called “Trojan delivery system” consisting of PLGA microparticles releasing AS-ODN/PEI nanocomplexes was evaluated *in vivo* (Gomes dos Santos et al., 2006a). The anti-TGF-β2 AS-ODN was tested for wound healing after filtration surgery in an animal model of glaucoma. The cryosection of rabbit conjunctiva showed that after 1 day from the subconjunctival injection of microspheres containing AS-ODN/PEI complexes, the AS-ODN was located in the conjunctival stroma. Six days post injection, AS-ODN was located in conjunctival cells, with accumulation into the nucleus. No conjunctival hyperaemia was observed for 6 weeks after the injection. Rabbit treatment with PLGA microspheres releasing anti-TGF-β2 AS-ODN prolong bleb survival for 28 days (50% of the treated eyes) following trabeculectomy compared to control. This effect was significantly higher in the case of PLGA microspheres releasing AS-ODN/PEI complexes with a bleb survival of 42 days on 100% of the treated eyes (Gomes dos Santos et al., 2006a). Such a system combining long-term delivery and enhanced

intracellular penetration might be a good option for intravitreal delivery.

10.3. Skin delivery

Skin delivery of AS-ODNs and siRNA is mainly aiming to achieve local delivery since their systemic absorption is likely to be significantly less than for intravenous administration, due to the relatively low lipophilicity of these molecules. Antisense oligonucleotides as cutaneous drugs will similarly provide major advantages over intravenous antisense oligonucleotides. Cutaneous delivery will allow direct access to target cells in the skin, and will minimise systemic toxicity. However, the stratum corneum acts as the major barrier to penetration of molecules across the skin. Large negatively charged molecules such as antisense oligonucleotides do not easily penetrate the stratum corneum, as they have low lipid solubility. Topical application of simple aqueous solutions of oligonucleotides results in little penetration into the viable epidermis of grafted human skin on athymic mice over a 24-h period (White et al., 1999) although their capacity to be taken up by corneocytes was demonstrated (Brand et al., 1998a). However, properly formulated in delivery vehicles, AS-ODNs have been shown to reach pharmacologically active concentrations at the hair follicles no matter what the chemistry of the AS-ODNs was (Lieb et al., 1997; Dokka et al., 2005).

Iontophoresis has been extensively used to deliver AS-ODNs through the skin. Oligonucleotides have been delivered into a mouse tumour (Vlassov et al., 1994b) across excised hairless mouse skin (Oldenburg et al., 1995; Brand and Iversen, 1996) and across rat skin (Regnier and Preat, 1998) using this method. Transdermal iontophoretic delivery was applied in a rat model to phosphorothioate (Brand et al., 2001) or phosphoramidate AS-ODNs (Arora et al., 2002) targeting cytochrome p450-3A2 (CYP3A2) mRNA translational start sites showing that it could reach concentrations sufficient to induce changes in specific target *in vivo* in the liver and underlying skin. Parameters influencing iontophoresis of phosphorothioate AS-ODNs of different size and sequence were studied systematically and transport through the skin was shown to be influenced by size where for the smaller compounds (less than 20 bases), transport, in general, decreased with increasing size; however, there were several oligonucleotides that did not follow this pattern. These data indicate that factors other than size influence transport and that the impact is greater at shorter lengths (Brand et al., 1998b).

Electroporation (high-intensity electric field pulses that temporarily permeabilise lipid bilayers) has also been shown by confocal microscopy to facilitate nuclear oligonucleotide localization in keratinocytes (Regnier et al., 1998; Regnier and Preat, 1998). Since electroporation not only permeabilises cell membranes, but also the stratum corneum (Bergan et al., 1993), this method may well be appropriate for the topical delivery of AS-ODNs or even siRNAs in the testing of candidates in animal models. However, the widespread clinical use of electroporation appears to be limited at this stage by the practical considerations of using an electroporation device for individual patients.

11. Conclusion

In conclusion, there is a general consensus that AS-ODNs and siRNA are highly potent drugs and there should be more applications in clinics. Although a large number of these nucleic acids are now under clinical trial, improving their delivery should be a major task to achieve. Along with the development of "smart" nanotechnologies able to overcome all the obstacles that these molecules meet once injected, the development of alternative routes of administration should also be investigated more extensively.

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