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European Journal of Pharmaceutics and Biopharmaceutics 50 (2000) 101–119

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Review article

Cellular delivery of antisense oligonucleotides

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Received 3 January 2000; accepted in revised form 28 February 2000

Abstract

Antisense oligonucleotides can be successfully employed to inhibit specifically gene expression. However, many oligonucleotide classes are polyanions and cannot passively transit the cell membrane. Thus, the use of naked oligonucleotides for antisense purposes poses some rather stringent challenges, and it is not a trivial task to appropriately interpret the data derived from experiments in which they have been used. Multiple methods have been developed to improve intracellular, and in particular, intranuclear oligonucleotide delivery, and in doing so, to maximize the performance of the antisense technologies that are currently available. This review discusses the use of cationic lipids, protein and peptide delivery agents, and several novel chemical and viral methods that have recently been explored as delivery vehicles, focussing not only on their strengths, but also on their limitations.

Keywords: Delivery; Antisense oligonucleotides; Cationic lipids; Peptide–oligonucleotide conjugates; Dendrimers; In vivo delivery

1. Introduction

It has long been believed that antisense oligonucleotides can be employed to specifically inhibit gene expression [1,2]. These molecules, usually 18–20 bases in length, can undergo Watson–Crick hybridization to targeted mRNAs, and may, via a variety of mechanisms, inhibit translation of that mRNA into protein [3,4]. Ideally, such an antisense oligonucleotide would be composed chemically of the same backbone as its target, i.e. diester linkages at each phosphorus atom in the chain. This is not practical because phosphodiester oligonucleotides are degraded by ubiquitous nucleases. Furthermore, evidence exists to suggest that the nucleotide–5′-monophosphates released subsequent to nuclease digestion may have base- and cell-type effects on cellular proliferation [5,6]. In order to eliminate the problem of nuclease resistance, phosphorothioate oligomers were designed and synthesized by Stec and colleagues [7,8]. In these molecules, a sulfur atom replaces an oxygen at a non-bridging site at each phosphorus atom of the chain. The substitution retains the charge, and hence, the aqueous solubility characteristics of the parent sequence, and in fact, phosphorothioate oligonucleotides have been employed as therapeutic agents in a number of recent clinical trials [9,10]. Indeed, the FDA has given approval to Vitravene, a phosphorothioate oligonucleotide targeted to a CMV IE mRNA for the intraocular treatment of cytomegalovirus retinitis [11].

However, these phosphorothioate oligonucleotides, and many other oligomer classes that are employed experimentally, are polyanions, and it is very difficult to understand how such molecules can pass through a hydrophobic lipid bilayer. Indeed, it has been demonstrated that even uncharged methylphosphonate oligonucleotides (which substitute a methyl group for a non-bridging oxygen atom at each phosphorus) cannot passively transit the cell membrane [12]. Furthermore, phosphorothioate oligonucleotides can have a very high affinity for proteins, particularly heparin-binding proteins [13,14], which occur commonly on the cell surface membrane. Examples of heparin-binding proteins to which oligonucleotides can bind include the epidermal growth factor (EGF) receptor [15], receptors for vascular endothelial growth factor [15] and Mac-1 [16], an integrin found predominately on polymorphonuclear leukocytes and monocyte-macrophages. In addition, there are a number of poorly characterized cell-surface-binding oligonucleotide-binding proteins that have been discovered over the past decade [17–22]. It is not clear what effects the binding of oligonucleotides to these physiologically important molecules have on the cellular biochemistry, but the effects are probably oligonucleotide length-dependent, and to a lesser extent, sequence-dependent. Interestingly, however, the binding is independent of the sense of chirality at phosphorus (Rp vs. Sp) [23].

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0939-6411/00/$ - see front matter
PII: S0939-6411(00)00088-6

After the binding of an oligonucleotide to a cell-surface protein, internalization into the endocytic compartment occurs. This is an active process [24,25], and despite previous data [26], it now appears that in many cell types, adsorptive endocytosis is the major mechanism by which naked oligonucleotides are internalized. The process of fluid phase endocytosis (pinocytosis) contributes a relatively small amount at a relatively low oligonucleotide concentration, but a cell-type-dependent increasing percentage as the concentration increases. Oligonucleotide classes that adsorb well (e.g. phosphorothioates) to the cell membrane are internalized well and tend to produce 'antisense' effects. Conversely, oligonucleotide classes that do not adsorb well to the cell surface (e.g. morpholino-oligonucleotides, peptide nucleic acids (PNAs), methylphosphonates, and virtually all other uncharged species) are not internalized well when delivered naked and do not produce 'antisense' effects. At this time, the internalization of morpholino-oligonucleotides is perhaps best accomplished by scrape-loading techniques [27]. It may also be possible to internalize these oligomers by cellular electroporation [28] or Streptolysin O permeabilization [29], as has been shown for phosphorothioates. PNAs are also quite difficult to deliver, and interesting ideas, such as covalent modification by a 5′-adamantyl residue to facilitate adsorption, have been proposed [30]. However, at the present time, there are few if any demonstrations of antisense activity with PNAs in tissue culture.

After 'internalization', naked oligonucleotides are localized to a compartment that is topologically still 'outside' of the cell, i.e. a lipid bilayer must still be transited. This compartment is endosomal/lysosomal [31], and seems to represent a dead end for antisense oligonucleotides, because it has been demonstrated that from this location, oligonucleotides may either be released from the cell via exocytosis, or may be partially digested [31]. Regardless of this, the limited data that exists suggests that once oligonucleotides become sequestered in the vesicular compartment, there is no exit to the cytoplasm (J. Tonkinson and C.A. Stein, unpublished observations). However, it is of critical importance that oligonucleotides become localized in the cytoplasm, because once there they are rapidly translocated to the nucleus [32], which appears to be the location of 'antisense' activity [33].

In experiments in which antisense activity is obtained using oligonucleotides delivered with a carrier (e.g. the cationic lipids which are discussed below), the same endpoint (e.g. downregulation of protein expression by Western blot) is rarely, if ever, observed when the identical oligonucleotides are delivered without a carrier. However, there are many experiments in the literature which describe 'antisense' effects after naked oligonucleotide delivery [34,35]. How can this apparent paradox be explained? In some cases it is possible that the rate of spontaneous endosomal/lysosomal rupture is significant enough to produce small, albeit sufficient, intracytoplasmic concentrations of the active oligonucleotide. This possibility might be enhanced if cells were treated with high oligonucleotide concentrations for protracted periods, which would increase the intravesicular concentration by taking maximal advantage of the pinocytotic process. However, the rate of spontaneous endosomal rupture and the conditions under which it occurs may be highly cell-type-specific, and in some cases, may be too slow to support antisense activity in many cell types in the absence of toxic levels of phosphorothioate oligomers. It is also possible that some earlier experiments with naked oligonucleotides do not describe antisense effects, but rather effects that depend on a combination of sequence-specific and non-sequence-specific events, with each event being necessary to produce a given biological endpoint, but neither being sufficient. Another confounding issue has been the utilization for antisense purposes of oligonucleotides containing four contiguous guanosine residues. Four individual oligonucleotide strands containing this sequence motif can associate via a combination of Watson–Crick and Hoogsteen base pair formation at guanosine to form what is known as a G-quartet or G-tetrad [36]. The stacking of four tetrads on each other produces a quadruple-stranded helix, known as a tetraplex. Tetraplexes do not form from all oligonucleotides which contain four contiguous guanosine residues, but when they do form, they appear to have a particularly high affinity for heparin-binding proteins [13]. This may dramatically increase non-sequence specificity vs. the monomeric oligonucleotide species.

Thus, the use of naked phosphorothioate oligonucleotides for antisense purposes poses some rather stringent challenges, and it is not a trivial exercise to appropriately interpret the data derived from their use. An exception to this, perhaps, is when antisense oligonucleotides are employed in the central nervous system of experimental animals [37]. A large number of experiments have demonstrated that these cells appear to easily internalize phosphorothioates, and transport them directly to the cell nucleus. Similarly, it is possible that naked oligonucleotides can be effective (i.e. produce 'antisense' effects) systemically when injected intravenously into immuno-incompetent mice bearing xenografted tumors, whose cells, when in tissue culture, do not respond to naked oligonucleotides [38,39]. This paradox may be more apparent than real, as it is possible that endogenous oligonucleotide carriers exist. For example, respirable phosphorothioate antisense oligonucleotides have been shown to be active against adenosine A1 receptors in the rabbit lung [40], possibly because the surface of that organ is lined with zwitterionic lipid-based surfactant which is cationic at a neutral pH [41]. Nevertheless, in the perhaps more mundane experiments performed in tissue culture, it likely that some form of carrier/delivery system will be a universal requirement. The remainder of this review is devoted to a summary of the methods that have been developed improve intracellular, and in particular, intranuclear oligonucleotide delivery, and by doing so, maximizing the performance of the antisense technology.

2. Liposomes as DNA delivery agents

Liposomes, microscopic spheres composed of one or more closed, concentric phospholipid bilayer membranes surrounding an internal aqueous compartment, have been utilized as drug carriers since the mid-1970s [42]. Highly polar, water-soluble drugs can be entrapped in the internal aqueous space of the liposome, while the lipids form into bilayers. Liposomes are usually characterized by size, the number of concentric bilayers, and the composition and physical properties of the lipids [43]. Techniques for the preparation of various types of liposomes have been extensively reviewed [44].

2.1. Mechanism of cationic lipid-mediated oligonucleotide delivery

Three common types of cationic lipids are currently employed in lipid-based DNA delivery [45]. The first group is represented by two quaternary ammonium salts with long mono-unsaturated aliphatic chains, those being *N*-(2,3-(dioleyloxy)propyl)-*N*,*N*,*N*-trimethyl ammonium chloride (DOTMA, one component of the commercially available transfection agent Lipofectin®), and *N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride (DODAC). 3β-(*N*-(*N*-(dimethylaminoethane)carbamoyl) cholesterol (DC-Chol), a cationic derivative of cholesterol, belongs to the second group. Lipids of a third category are distinguished by the presence of multivalent headgroups, such as dioctadecyldimethylammonium chloride (DOGS), commercially available as Transfectam®.

In general, structure-activity studies indicate that cationic lipids with unsaturated hydrocarbon chains are more effective as delivery agents than saturated lipids, and that unsaturated phosphatidylethanolamines (PEs) are more effective at supporting transfection than other neutral lipids [46,47] with the possible exception of cholesterol [48–50]. A number of studies have attempted to identify specific structural features of a headgroup region that promote gene transfer activity. For DOTMA (type I) lipids, the addition of a hydroxyl moiety appears to enhance transfection. For cationic derivatives of cholesterol and spermine-based lipids, a spacer moiety between the hydrophobic anchor and positive charge has been shown to enhance transfection [51]. Despite various structural details, all these compounds are cationic amphiphiles with hydrophobic aliphatic chains, which orient the molecule in a bilayer with the cationic headgroup at the membrane surface.

Initially, Felgner [46] demonstrated the utility of cationic lipid and unsaturated phosphatidylethanolamine (PE) mixtures for transfecting genes into cells in tissue culture. This process of the transportation of nucleic acid across the cellular membrane into the cytoplasm and nucleus (lipofection or cytofection) is now widely employed for introducing plasmid constructs, as well as oligonucleotides, into cells [51,52]. The term 'liposome' is now widely used to describe the complexes of cationic lipids with oligonucleotides. However, the structures of these complexes may differ from that of the classical liposome [53]. When DNA (or oligonucleotides) are mixed with lipids, complexes form spontaneously due to electrostatic interactions. In most cases, these electrostatic interactions result in condensation of the polyanionic nucleic acid molecule into a tightly packed structure [54]. The size of final product is dependent on the size of the initial lipid vesicles, the positive to negative charge ratio, the ionic strength of the medium, and the concentration of all reagents. Generally, smaller particles are preferred for both in vitro and in vivo applications [45]. However, some recent data of Ross [55] demonstrated that the efficiency of transfection with cationic liposome–DNA complexes (lipoplexes) increased with increasing lipoplex size. Moreover, by using large lipoplexes, the degradative properties of serum may be minimized.

For systemic in vivo gene therapy, it is critical that the integrity of a plasmid or oligonucleotide is maintained in the blood for a sufficient length of time for the nucleic acid to reach tissue sites. When complexed with lipids, DNA is protected from nuclease degradation to various extents [56]. Similar to what has been observed with naked oligonucleotides, the delivery of the complexes of DNA or oligonucleotide by lipids into the cell cytoplasm occurs by the endocytotic–lysosomal pathway, and not by direct delivery through the fusion of lipid–DNA particles with the plasma membrane [53,57,58]. This conclusion is supported by the temperature dependence of the fusion process [47], although at present, it is unclear why endocytosis is required for fusion, since the membrane composition of the plasma membrane and vesicles derived from the membrane are quite similar.

The role of endosome maturation and their fusion with lysosomes in the transfection process is also unclear. Some reports demonstrate that lysosomotropic agents, such as ammonium chloride, chloroquine and monensin, inhibit transfection [51]. However, others show that gene transfer [58] and oligonucleotide delivery [53,59] are enhanced, or even that the presence of lysosomotropic agents does not effect delivery at all.

Lipid mixing between lipid–DNA particles and the endosomal membrane is an important part of the mechanism of endosomal release [47]. Lipids capable of promoting intermembrane lipid exchange (e.g. unsaturated PEs, unsaturated acyl chains and hydroxylated headgroups) will increase the ability of lipids to disrupt the endosomal membrane [60]. Dioleoylphosphatidyl ethanolamine (DOPE) has been particularly useful for cationic liposomes because of its ability to form non-bilayer phases and promote destabilization of the bilayer [52,61].

Zelphati and Szoka [62,63] have proposed a model that describes oligonucleotide release from the endosome (Fig. 1). After the cationic lipid nucleic complex is internalized by endocytosis, it destabilizes the endosomal membrane. This destabilization induces a flip–flop between anionic

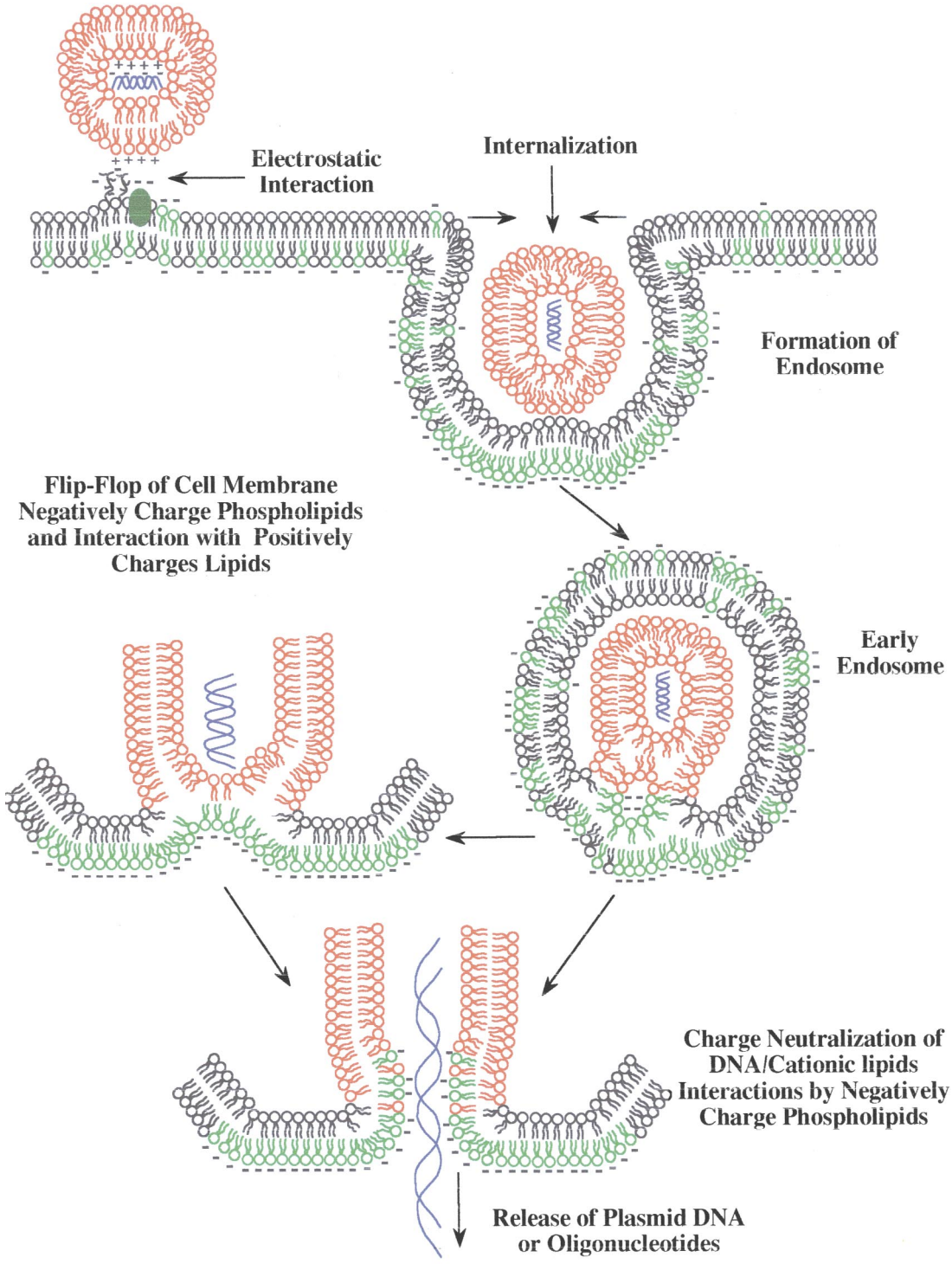

Fig. 1. Proposed mechanism of internalization into cells of cationic lipoplexes and release of DNA in cytoplasm. The cationic lipoplex binds to negatively charged groups on proteins, glycosaminoglycans and sialolipids on the cell surface through electrostatic interactions. This binding cross-links anionic patches on the cell surface and induces an internalization of the lipoplex into a macro'endosome'. Shortly after internalization, the lipoplex induces a membrane destabilization in the endosomal membrane that causes a flip–flop of anionic lipids from the cytoplasmic face of the endosome into the internal face. The anionic lipids form a charge pair with the cationic lipid. This neutralizes the positive charge, and in the process, the anionic nucleic acid is released from the lipoplex. An important aspect of the release process is the entropy of mixing that arises from the mixing of the anionic lipids into the cationic lipid membrane. In the color version of the diagram, the red represents cationic lipids, the black represents zwitterionic lipids, the green represents anionic lipids and the blue represents the nucleic acids. (From [255] with permission).

and cationic lipids. The nucleic acid is displaced from the cationic lipid and diffuses into the cytoplasm of the cell. However, if the DNA/cationic lipid complex is transferred to lysosomes, the nucleic acid may be rapidly degraded by nucleases. Alternatively, full-length oligonucleotides could also be released from endosomes into the extracellular compartment by exocytosis [31,64].

The release from the endosomes, and subsequent 'diffusion' into the nucleus, represents the limiting step for DNA transfection [58]. However, fluorescent measurements of phosphorothioate oligonucleotide delivery to cells demonstrated that 'the diffusion' appears to be very efficient, depending upon the cell type [45]. Nuclear pores allow molecules the size of antisense oligonucleotides (and smaller) to 'diffuse' freely between the nucleus and cytoplasm. Using the quantitative fluorescence technique, it may be possible [45] to determine what proportion of oligonucleotide taken into cells by endocytosis is actually released into the cytoplasm.

Cationic lipid vesicles not only enhance the rate of oligonucleotide uptake into cells, but also markedly change the subcellular distribution of the oligonucleotide. The major difference in the distribution of the oligonucleotide in the presence of cationic lipids [57,65] is the localization of the oligonucleotide in the cell nucleus. The cytoplasmic distribution of the oligonucleotides in the presence of DOTMA also appears to be different, with larger oligonucleotide-accumulating structures seen.

In addition to enhancing uptake and modifying the distribution of the oligonucleotides, cationic lipids may also enhance antisense activity by increasing the rate of oligonucleotide hybridization to its target mRNA [66]. Cationic detergents have also been shown to stabilize the DNA–RNA duplex, with respect to thermal denaturation. Thus, it might be predicted that DOTMA and other cationic lipids, which contain a quaternary amine and long aliphatic groups, should exhibit a similar activity [59].

Great cell-type and lipid-dependent variation has been observed in the uptake of oligonucleotide–lipid complexes [67]. This fact may be related to diverse cell-dependent rates of endocytosis [47,57,58], and to variable interactions of the positively charged cationic lipid/DNA complexes with anionic residues on the cell surface [68].

Liposome technology has been successfully employed for the delivery of different types of antisense oligonucleotides and the subsequent inhibition of gene expression. Efficient downregulation of the intercellular adhesion molecule I (ICAM-1) [69], mutant Ha-ras mRNA [70], A-raf and C-raf kinases [71,72], PKC-α [38], and the antiapoptotic proteins Bcl-2 [73] and Bcl-xL [74,75], has been achieved by phosphorothioate oligonucleotides complexed to commercially available cationic lipids. In several other examples, among the many that can be found in the literature, liposomal delivery of methylphosphonate [76] and phosphorothioate [77] antisense oligodeoxynucleotides also caused an efficient inhibition of the in vitro growth of leukemia cells. Lipid-mediated delivery of PNAs has also been successfully employed for the inhibition of human telomerase [78].

However, cationic lipids as oligonucleotide carriers have several disadvantages. The main disadvantages of cationic lipids are their toxicity and markedly decreased activity in the presence of serum [79]. Newer cationic lipid formulations are available that reportedly exhibit decreased toxicity [59,64,80]. Also, inclusion of a helper lipid (DOPE or cholesterol) reduces the effective charge ratio required to deliver oligonucleotides into cells and permits delivery in the presence of high serum concentrations in the culture medium [79]. The lipid formulation GS 2888 (Cytofectin®) efficiently transfects oligonucleotides and plasmids into many cell types in the presence or absence of 10% serum [80]. Under certain conditions, this formulation may be more than 20-fold more efficient in eliciting antisense effects in the presence of serum when compared with Lipofectin®.

2.2. Modifications of liposomes

Modifications of the cationic lipids have been proposed to maximize oligonucleotide delivery. Complexes with very long circulation times (i.e. serum stable) may be obtained by the inclusion of negatively charged lipids into the surface of the liposomes. These lipids include polyethylene glycol-modified phosphatidyl ethanolamine (PEG-PE) [81], dipalmitoyl–DL-alpha-phosphatidyl-L-serine [82] or spermine derivatives [83]. Such constructs with polyaminolipids increase the permeability of cellular membranes to the oligonucleotides [84,85]. Derivatization of liposome surfaces with hydrophobic polymers, such as polyethylene glycol (PEG) and its derivatives, also permits much longer lifetimes in the circulation and minimizes uptake by the liver and spleen [86–88]. Small liposomes with a long circulation time seem to be able to enter effectively into some types of tumors [89,90] or other extravascular regions [91], thus providing a passive targeting mechanism.

The combination of a viral vector and liposome has produced a novel vesicle named a 'virosome'. After virosome internalization through receptor-mediated endocytosis, cationic virosomes fuse efficiently with the membranes of the endosomal compartment, and encapsulated nucleic acids can be delivered to the cell cytoplasm. Hemagglutinating virus of Japan (HJV) [92] and adenovirus [93] have also been employed to increase the efficiency of DNA transfer. Influenza virus A envelopes were used in cationic virosomes for the delivery antisense L-myc oligonucleotides to small cell lung cancer cells [94]. HJV has been used for virosome construction and antisense oligonucleotide delivery into the myocardium of adult rats [95]. Cationic virosomes delivered oligonucleotide into the cytoplasm more efficiently than cationic liposomes.

Alterations in the lipid composition can render liposomes pH-sensitive [96]. These formulations are capable of enter-

ing cells by endocytosis, and once in the low pH endosome compartment, can then fuse with the endosomal membrane, delivering encapsulated molecules to the cytoplasm [97–99]. Fusogenic liposomes are composed of a non-bilayer-forming lipid, such as PE, and a titratable amphiphile, such as oleic acid (OA) or cholesteryl hemisuccinate (CHEMS). At pH 7, the titratable amphiphile retains the lipid mix in a bilayer form, while at pH 6 or less, a non-bilayer phase is formed, which then promotes membrane fusion. Since transfer of oligonucleotides from the endosomal compartment to the cytosol is a critical issue in antisense pharmacology, pH-sensitive liposomes may have an important role as delivery systems for oligonucleotides. Duzgunes [100] demonstrated that oligonucleotides against HIV-1, encapsulated in sterically stabilized pH-sensitive liposomes, have prolonged circulation in vivo and efficiently inhibited virus replication. Oligonucleotides targeted against the Friend retrovirus encapsulated in pH-sensitive liposomes were more active against the virus that those encapsulated in non-pH-sensitive liposomes [101].

2.3. Targeted liposome delivery

Cationic lipids for targeted delivery can be engineered by the covalent attachment of targeting ligands. Novel PEG-PE lipids allow proteins or antibodies to be conjugated at the distal end of the PEG spacer, thus providing both long circulation times and effective target binding in vivo [102–104]. Liposomes can also be targeted by attached antibodies directed against antigens expressed on the target cells, resulting in the antigen-specific binding and cell uptake of the complexes [105–107]. Anti-CD32 or anti-CD2 immunoliposomes improved the delivery of antisense oligonucleotides to leukemic cells carrying the appropriate receptor [108]. The uptake of the oligonucleotides was twice that of the liposome-encapsulated or non-specific immunoliposome-encapsulated oligomers. Meyer [82] demonstrated that complexes containing conjugated anti-HER2 F(ab′) fragments at the distal termini of PEG chains efficiently delivered antisense oligonucleotides into the cytoplasm and nuclei of HER2-overexpressing SK-BR-3 and MCF-7 cancer cells, and greatly enhanced the biological activity of the conjugated antisense oligonucleotides.

Ligands of cellular receptors, such as folate, which may provide targeted delivery to tumor cells [109–111], have been used to target cells via specific cell-surface receptors. Antisense oligodeoxyribonucleotides targeted to the EGF receptor were encapsulated into liposomes linked to folate via a PEG spacer, and were efficiently delivered into cultured KB cells via folate receptor-mediated endocytosis [112].

Positive, neutral and negatively charged liposomes, containing galactocerebroside or complexed with lactosylated polyethylenimine (PEI), provide an efficient targeted delivery of the oligonucleotides to hepatocytes via the asialoglycoprotein receptor. The negative liposomes and 25-kDa lactosylated PEI provided the most intense nuclear fluorescence with the fluorescein-labeled oligonucleotides in rat hepatocytes [113].

For efficient targeting of Leishmania-infected macrophages with antisense oligonucleotides, Chaudhuri [114] used liposomes coated with maleylated bovine serum albumin, an artificial ligand for macrophage scavenger receptors. De Lima [115] employed transferrin in association with cationic lipids to promote internalization by receptor-mediated endocytosis. They also used a synthetic fusogenic peptide (influenza hemagglutinin N-terminal peptide HA-2) in a complex with 1,2-dioleoyl-3-trimethylammonium propase (DOTAP) and DOPE to promote endosomal destabilization and release of genetic material into the cytoplasm.

Heme (ferric protoporphyrin IX) was conjugated to the aminolipid, DOPE, and used to form cationic lipid particles with DOTAP to promote the receptor-mediated delivery of antisense oligonucleotides into hepatocytes [116]. These lipid particles protect oligonucleotides from degradation in human serum and increase their uptake into 2.2.15 human hepatoma cells. Lipid particles with a net negative charge delivered oligonucleotides into both the 2.2.15 cell cytoplasm and nucleus.

A new class of lipid-like delivery agents, cationic amphiphiles or ‘umbrellas’, has been described recently [117,118]. These cationic amphiphiles contain cholic acid moieties that interact with the lipid bilayer and alkylamino side chains for binding of nucleic acids. Umbrellas efficiently deliver oligonucleotides, in contrast to plasmid delivery.

In an attempt to develop a controlled delivery system for the ocular administration of antisense oligonucleotides, Bochot et al [119] dispersed liposomes with oligonucleotides in poloxamer 407 gel. This system allows a slow release of oligonucleotides from the liposomes, and could be used for the systemic delivery of antisense oligonucleotides.

3. Protein and peptide delivery vehicles

As mentioned above, several novel techniques are currently available to enhance the cytoplasmic and nuclear delivery of antisense oligonucleotides and nucleic acids. Stable phosphorothioate oligonucleotides enter the cells through the endosomal pathway but cannot permeate the endosomal membranes. Commonly used permiabilization techniques include microinjection, electroporation, transfection with cationic lipids, and the use of chemically-modified oligonucleotides. However, all of these techniques have limitations [120]. For example, cationic lipids are toxic and exhibit a markedly decreased activity in the presence of serum and antibiotics.

During the past few years, a novel class of delivery agents have been developed, based on the use of several types of proteins and peptides which have the ability to penetrate cell

membranes. One of the common methods of the formation of transport complexes is to conjugate DNA with poly-L-lysine (PLL) and a cell-specific carrier molecule that is the ligand for a surface receptor. These carriers include glycoproteins [121–124], transferrin [125,126], insulin [127], antibodies [128], EGF [129], lectins [130], polymeric immunoglobulins [131], mannose [132] and folic acid [133]. The proteins are frequently molecules which are cationic at a physiologic pH, such as PLL, histones and protamine [121–123,126,127,130,131,134,135]. Other types of protein vectors include pH-sensitive fusogenic peptides [136–139], Antennapedia-type peptides [140,141], peptides containing hydrophobic motifs [120,142], the HIV Tat protein [143,144], a short sequence from the signal peptide segment of acidic Kaposi fibroblast growth factor (KFGF) [145,146] and PNAs [147–149].

The use of an asialoglycoprotein conjugate of PLL (ASGP) for the transfection of a luciferase-containing plasmid has been demonstrated by Fisher and Wilson [123]. They introduced the transmembrane domain of diphtheria toxin into a DNA–PLL conjugate complex. This domain was expressed in *E. coli* as a maltose-binding fusion protein. For conjugation with PLL through the heterobifunctional cross-linker sulfosuccinimidyl 6-(3′-(2-pyridyldithio)-propionamido)hexanoate, one Trp-residue was substituted with Cys. Incorporation of this conjugate into the complex formed by a luciferase-containing plasmid with an asialo-orosomucoid–polycation conjugate significantly increased transfection efficiency.

A highly efficient receptor-mediated delivery system for DNA and oligonucleotides to avian liver cells has been established, using complexes of non-modified human adenovirus particles and a protein conjugate consisting of *N*-acetyl-glucosamine-modified bovine serum albumine, streptavidin and PLL. Using this delivery system, an antisense oligonucleotide targeted to the encapsidation of hepatitis B virus (HBV) pregenome causes strong inhibition of HBV replication in vitro [150].

Rajur [151] eliminated the polylysine and covalently attached multiple (six) oligonucleotides to each ASGP molecule by a stable disulfide linkage. These conjugates were used to deliver antisense oligonucleotides complementary to the mRNA encoding gp129, an IL6 signal transduction protein. These conjugates inhibited the cytokine-stimulated upregulation of the acute phase protein, haptoglobin, in HepG2 cells.

Transferrin covalently linked to PLL was employed to introduce c-myb antisense oligonucleotides to leukemic HL60 cells [152–154] through the transferrin receptor. An alternative approach was based on the use of oligonucleotides conjugated with human monoclonal antibodies targeted to the transferrin binding site [128]. The use of complexes of another peptide, folic acid–polylysine, conjugated to a c-myc 15-mer phosphorothioate, caused a significant decrease in the intracellular c-myc protein level, resulting in a reduction of the growth rate and colony-forming capacity of the human melanoma M14 cell line [133].

Liu showed that the *N*,*N*-dipalmitylglycyl–apolipoprotein E peptide (dpGapoE) was highly effective for the delivery of both oligonucleotides and plasmids via LDL receptors. In these experiments, antisense oligonucleotides selectively inhibited cholesteryl ester transfer protein (CETP) expression in human CETP-stably transfected CHO cells [155].

Another strategy for oligonucleotide and gene delivery is based on short peptide vectors [120,142,156]. Morris [120] developed a 27-residue peptide, called MPG, with the sequence GALFLGFLGAAGSTMGAWSQPKSKRKV, composed of a hydrophobic N-terminal domain derived from the fusion sequence of HIV gp41, which is known to be essential for membrane fusion activity; it also contains a hydrophilic C-terminal domain derived from the nuclear localization signal of the SV40 large T-antigen. This domain is required for improving the nuclear localization of the peptide. The formation of complexes of peptide vectors and oligonucleotides occurs through electrostatic interactions between the five basic residues of the peptide and the charges on the phosphate groups of oligonucleotides. It has been demonstrated that the complexes of MPG with fluorescein-labeled single or double-stranded oligonucleotides in a 1:20 ratio (oligomer/MPG) were delivered into mammalian cells (HS68 and NIH-3T3 fibroblasts) in less than 1 h with relatively high efficiency (90%), and that delivery was independent of the endosomal pathway. The intracellular localization of the oligonucleotides was nuclear. It has also been shown that this peptide strongly protects the oligonucleotide from degradation in cell culture media. This nuclease-protective effect is believed to be due to peptide–peptide interactions leading to the formation of a peptide cage around the DNA molecule.

MPG was used for the delivery of not only short oligonucleotides, but also for a plasmid expression vector [142]. MPG promoted the delivery and expression of the pRL-SV40 plasmid in several different cell lines (HS68 and NIH-3T3 fibroblasts and COS-7 and C2C12 myoblasts). The degree of transfection was two- to seven-fold times higher than that obtained with Lipofectamine®. It has also been shown [142] that MPG efficiently delivered a full-length antisense cDNA encoding human cdc25C, which consequently reduced protein expression levels and promoted cell cycle arrest at the G2/M transition. Vidal [156] developed the MPG peptide vector for RNA delivery into HS68 human fibroblasts. They demonstrated that in the presence of this vector, fluorescently-labeled mRNA was delivered into the cytoplasm in less than 1 h with relatively high efficiency (80%).

Another example of the use of peptides containing a hydrophobic motif is the work relating the internalization of the complex of an oligonucleotide with a synthetic import peptide derived from Kaposi's growth factor, which had been covalently conjugated with polylysine [146].

There are two known types of pH-sensitive peptides for plasmid and oligonucleotide delivery. They include fusogenic peptides based on sequences derived from viral

proteins, such as the influenza virus hemagglutinin [136,137], and amphipathic helix peptides with the prototype being the 'GALA' peptide [138,139]. Both of these change the conformation at acidic pHs, causing destabilization and the fusion of bilayer membranes in a pH-sensitive manner.

Pichon [137] used an amphiphilic, anionic peptide to introduce oligonucleotides into the cytosol and the nucleus of different cells. The delivery vehicle was an E5 peptide analog derived from the N-terminal segment of the HA2 subunit of the influenza virus hemagglutinin protein. In the presence of this peptide, fluoresceinated single- and double-stranded oligonucleotides were taken up by adherent (HepG 2, HeLa, COS, Rb-1), as well as non-adherent cells (CEM-T4 and U937), and diffused into the nucleus. Bongartz [157] described the use of a similar peptide in order to improve the antiviral potency of antisense oligonucleotides (anti-TAT) and oligonucleotide phosphorothioates (SdC28) in de novo HIV-infected CEM-SS lymphocytes. Deshpande [129] described the ability of the fusogenic peptides, polymyxin B and the influenza HA2 peptide, to promote the internalization of oligonucleotides via the EGF receptor pathway. When A549 cells were incubated with complexes of fluorescently-labeled oligonucleotides and the EGF–PLL conjugate of either of these peptides, a significant dose-dependent increase (four- to six-fold) in intracellular fluorescence was observed.

To improve intracellular delivery, pAntennapedia can be coupled to a novel type of DNA analog, PNAs, as designed by Nielsen [147]. PNAs are DNA mimics, in which the phosphodiester backbone is replaced by a homomorphous backbone consisting of (*N*-2-aminoethyl) glycine units bearing the nucleobases attached through methylenecarbonyl linkers. They are resistant to proteases and nucleases, much more stable in cells than regular DNA, and form stable and tight complexes with complementary RNA or DNA [158]. Interestingly, PNAs exert their antisense effect not by an RNase H-based mechanism; they probably inactivate target mRNAs by steric blockade of translation [159,160]. Pooga [161] showed that a 21-mer PNA, complementary to the human galanin receptor mRNA, coupled to the pAntennapedia peptide, was efficiently taken up into Bowes melanoma cells, where they suppressed the expression of galanin receptors.

Recently, Derossi [140,141] discovered that a class of peptides called penetratins, of which pAntennapedia is a member, has potentially valuable delivery capacities. This class of cationic peptides binds DNA through specific sequences of 60 amino acids (the homeodomain), which is composed of three α-helices, with one β-turn between helices 2 and 3. The penetration mechanism of the homeodomain of the Antennapedia peptide is not related to the classical adsorptive endocytosis mechanism. According to one model of internalization [162], the third α-helix associates directly with the cell membrane. This association is presumably due to electrostatic interactions between the positive charges of the basic amino acids and the negative charges of the membrane lipids or sugar moieties. The stability of this association is sufficient to allow peptide accumulation at the water–membrane interface, and to alter the membrane organization from a lipid bilayer to an inverted micelle. Internalization of antisense oligonucleotides covalently linked to the homeodomain of pAntennapedia resulted in a marked transient decrease in amyloid precursor protein neosynthesis [163].

Hughes [164] found that the use of the GALA peptide conjugate with an antisense oligonucleotide resulted in an approximate 38% reduction in the expression of chloramphenicolacetyl–transferase in CHO cells. Chaloin [165,166] designed a series of amphipathic vector peptides containing both a hydrophobic and a hydrophilic sequence separated by various peptidic linkers. The presence of a linker appeared to play a role in cellular localization. The most efficient peptide was covalently linked to an antisense oligonucleotide complementary to an mRNA encoding the β-subunit of the L-type calcium channel. This conjugate improved internalization into cardiac H9C2 cells [167].

Cationic peptides, such as those contained in the HIV-1 Tat protein or some of its fragments [143,144], have also been used for oligonucleotide delivery. In contrast to the Antennapedia homeodomain peptide, the internalization of Tat is receptor-mediated [168]. Hughes [169] showed that conjugation of a Tat derived peptide containing the basic sequence RKKRRQRRR with a 2′-*O*-methyl-substituted 21-mer oligoribonucleotide increased its uptake into HeLa cells. Modification of the Tat peptide with biotin increased its uptake activity by six-fold [170].

Pichon [171] noted the inhibitory activity of oligonucleopeptides containing a KDEL motif. Antisense phosphodiester oligonucleotides, complementary to the AUG initiation site of the HIV-1 *gag* gene, were covalently linked to a peptide terminating with the Lys–Asp–Glu–Leu (KDEL) motif at the carboxyl-terminal position (oligodeoxynucleotide-*p*-KDEL). Lys–Asp–Glu–Ala (oligodeoxynucleotide-*p*-KDEA) was the control, inactive peptide. They found that although the uptake of oligodeoxynucleotide-*p*-KDEL was four-fold less than the corresponding peptide-free oligonucleotide, it was five-fold more effective in the inhibition of gene expression in HepG2 cells. The internalization of oligodeoxynucleotide-*p*-KDEA was similar to that of oligodeoxynucleotide-*p*-KDEL, but in this case, the antisense activity was lower, and similar to that of the free oligonucleotide. The IC_{50} for oligodeoxynucleotide-*p*-KDEL was 0.2 ± 0.05, compared with 1.0 ± 0.2 for the peptide-free oligonucleotide and 0.7 ± 0.1 μM for oligodeoxynucleotide-*p*-KDEA. The authors hypothesized that the linking of the oligonucleotide to a KDEL motif changed the intracellular trafficking pattern of the oligonucleotide.

Bachmann [172] described an interesting approach to facilitate the delivery of oligonucleotides into mammalian cells, presumably through receptor-mediated endocytosis. Their approach was based on the use of relatively small

synthetic peptides which contained two functionally important sequences: (a), a short sequence specific for a cell-surface receptor; and (b), a sequence responsible for complex formation with the oligonucleotide, which leads to the protection of the oligonucleotide against nucleases and neutralization of its overall negative charge. The peptides used in this study contained two motifs: an RGD-containing sequence specific for several integrin receptors; and the nucleocapsid protein (NCp7) of HIV-1, or shorter fragments thereof. Treatment of HL-60 cells with a complex of an 18-mer antisense oligonucleotide, complementary to the c-myb mRNA and the peptide vector, caused inhibition of cell proliferation. The effective dose of oligonucleotide was reduced by more than 50-fold after its ionic complexation to the peptide. It has also been shown that the RGD sequence was responsible for the efficient oligonucleotide delivery. The authors demonstrated that the deletion of this sequence, replacement with lactosyl residues, or saturation of the RGD receptors on the cell surface with integrin peptide (FMDV), did not lead to an inhibition of cell proliferation.

Meunier [173] described the capacity of C5-propynylated antisense oligonucleotides conjugated with a short peptide sequence, termed the nuclear export signal (NES), to enhance the inhibition of protein expression from a gene transcribed in the cytosol. HeLa cells were co-transfected with two plasmids: one containing the T7 RNA polymerase gene under the control of SV40 large T-antigen promoter, T7 (RNA polymerase lacks a nuclear localization signal, and does not enter the nucleus); the other containing the luciferase gene controlled by the T7 promoter. The oligonucleotide–NES peptide conjugates were introduced into the cells and exported from the nucleus to the cytosol. These conjugates efficiently inhibited (75%) the cytosolic expression of luciferase, whereas at the same concentration, the oligonucleotides lacking peptides were inactive.

4. Other oligonucleotide delivery strategies

As noted previously, internalized naked oligonucleotides accumulate in endosomal vesicles within cells [57,174], severely limiting their access to targeted mRNA [175]. To improve the access of the oligonucleotides to their targets, several additional methods have been proposed. A recent study has demonstrated the use of pressure-mediated transfection for the effective delivery of oligonucleotides to human venous endothelium ex vivo and to non-vascular cells of the rat myocardium [176]. The optimal delivery of oligonucleotides was achieved at a pressure of 300 mmHg, established by a standard angioplasty insufflator while the tissues were secured to a cannula. Nuclear localizations of 90 and 50% of the oligonucleotide was reported in vein and myocardium cells, respectively. It has been suggested that the application of pressure changed the pattern of intracellular trafficking, which in turn affected the intracellular distribution of the oligonucleotides. The oligonucleotides were no longer internalized via receptor-mediated endocytosis and avoided lysosomal degradation. However, although about a 50% downregulation of targeted protein vs. control has been observed, this downregulation did not necessarily correspond to a higher nuclear accumulation. In fact, a problem arose because the efficiency of delivery of the ^{32}P-labeled oligonucleotides was determined by scintillation counting. This method might be misleading, since the oligonucleotide could be degraded and lose the [^{32}P] label after being exposed to intact cells. The method is new and promising, but seems to have its limitations, being applicable only to non-luminal solid organs. Further investigation seems appropriate.

Antisense oligonucleotides need to go through a series of biological barriers before reaching their target site. These barriers, viewed simply, represent a series of membranes with various pore sizes, and distinct electrostatic and hydrophobic properties. The ideal antisense oligonucleotide delivery vehicle should be small enough ($<$30 kDa) to be able to pass through the capillary walls to exit the bloodstream, and should promote oligonucleotide penetration into the target cell. Most importantly, it must stably bind oligonucleotides until it reaches the target. Recently, several promising delivery agents have emerged.

The association of block polymers in aqueous medium has potential for pharmaceutical application. A pair of oppositely charged polymers, a negatively charged oligonucleotide and a positively charged block copolymer, with poly(ethyleneglycol) (PEG) segments, spontaneously form a polyionic complex micelle with a spherical shape and narrow size distribution. The micelles formed in aqueous media have a small size of only about 40 nm, a hydrophobic core derived from the neutralized polycation and a hydrophilic corona derived from the PEG chains [177,178]. One recent work demonstrated the use of these complex micelles bound to an avidin/biotin construct. The effective diameter of these particles was approximately 99 nm. A conjugated complex of an antisense oligonucleotide and a cationic copolymer displaying transferrin on its surface was reported to promote an 80-fold increase in oligonucleotide uptake in multiple drug-resistant human epidermoid carcinoma cells, KBv, compared with oligonucleotides alone [179]. The authors suggested that these conjugates promoted oligonucleotide uptake and transfection as a result of the interaction of transferrin with its cell-surface receptor. In fact, fluorescent microphotographs of the treated KBv cells confirmed the accumulation of the complexes in cells. However, the ultimate goal of the experiment was to improve the antisense downregulation of gene expression. Antisense phosphorothioate oligonucleotides that were complexed with the micelles were used to target the human *mdr* 1 mRNA. Ideally, this should have resulted in the downregulation of P-glycoprotein expression. However, although a decrease of *mdr* 1 mRNA levels of 42% was observed, a Western blot failed to demonstrate downregulation of protein expression.

However, *mdr* 1 is a very long-lived protein, and if the experimental time did not provide sufficient time for protein turnover, antisense effects might not have been detectable. It is also possible that the observed mRNA downregulation might not be a specific antisense effect, or alternatively, that the extent of the mRNA was simply not great enough to produce an antisense effect at the protein level.

A variety of delivery agents have been used to enhance the cytoplasmic and nuclear accumulation of antisense oligonucleotides. Encouraging new technologies to enhance DNA delivery employ polymer particles made from polyvinylalcohol (PVA), polyvinylpyrolidone (PVP) [180], or biodegradable nanoparticles as carriers of antisense molecules. Nanoparticles are solid colloidal drug carriers ranging from 10 to 990 nm. They consist of macromolecular materials, in which a biologically active substance is entrapped, encapsulated, or adsorbed onto the surface. The polymers most often used for nanoparticles are polyalkylcyanoacrylate (PACA) [181], polybutylcyanoacrylate (PBCA), polyisohexylcyanoacrylate (PIHCA) [182,183] and polyhexylcyanoacrylate (PHCA) [184]. Hydrophobic cations, such as the quarternary ammonium salts, tetraphenylphosphonium chloride or hexadecyltrimethylammonium bromide (CTAB), or diethylaminoethyl (DEAE)-dextran were usually used in combination with the nanoparticles to promote oligonucleotide binding [184–187]. Oligonucleotides are attached to the surface of nanoparticles via ionic interactions with adsorbed quaternary ammonium surfactant molecules.

PIHCA nanoparticles, complexed with antisense oligonucleotides and targeted against Ha-*ras*, were reported to successfully inhibit the growth of the HBL99 human mammary cells, which were transformed by a plasmid carrying the EJ/T24 bladder carcinoma Ha-*ras* oncogene [185]. In the presence of nanoparticles, growth inhibition was achieved at 99-fold lower oligonucleotide concentrations than during treatment with free oligonucleotides in vitro. However, the effectiveness of the antisense treatment was extrapolated from a change in cell proliferation rate, and was not based on a Western blot. In addition, significant inhibition of tumor growth was also observed in vivo after cells were treated with free oligonucleotides.

In another work which also targeted the Ha-*ras* gene in the T24 human bladder carcinoma cell line, the PIHCA nanoparticles were employed, but the antisense oligonucleotide was coupled to a cholesterol moiety to increase oligonucleotide lipophilicity [186]. While cell growth was not affected by the treatment with cholesterol-bound oligonucleotides, 30% inhibition of cell proliferation was reported after 96 h of treatment with the nanoparticle associated cholesterol antisense oligonucleotide. However, the effect might have been non-sequence-specific, at least in part. The oligonucleotide concentration at which an effect was observed was 200 nM, and an increase in oligonucleotide concentration of up to 1 μM did not produce any change in proliferation inhibition.

In addition, several recent reports suggested that nanoparticles can enhance oligonucleotide effects in biological systems by improving cellular uptake [187,188]. Oligonucleotide delivery associated with nanoparticulates was improved by about 99%, compared with free oligonucleotide uptake, in DU144 human prostate cancer cells after 24 h treatment [189]. However, as encouraging as these data are, direct measurements of targeted protein and mRNA levels are necessary to confirm the effectiveness of the nanoparticles on antisense efficacy.

Recently, a new kind of nanoparticle for oligonucleotide delivery was proposed [190]. Oligonucleotides were entrapped into the nanoparticle's core, which was formed from alginate nanosystems cross-linked by PLL. These nanosponges were reported to be stable in serum, and were able to sufficiently protect oligonucleotides from cleavage by serum nucleases. However, the study did not address antisense efficacy, and further investigation is necessary to optimize this promising carrier system.

Other delivery techniques have included the use of dendrimers [191–193] and surfactants [173]. Dendrimers have attracted interest as delivery agents for both genes and oligonucleotides. Starburst polyamidoamine (PAMAM) dendrimers are spherical, highly ordered, arborizing (i.e. dendrimeric) polymers. They contain positively charged primary amino groups on their surfaces at a physiological pH [194,195]. It has been demonstrated that dendrimers form stable, non-particulate complexes with plasmid DNA or oligonucleotides, have limited cellular toxicity, and are stable in the presence of serum proteins [196,197]. Experiments assessing efficacy were performed in HeLa cells using an indirect method, consisting of a luciferase reporter assay that requires an antisense oligonucleotide to correct the splicing of a mutated intron [198]. The assay is based on the fact that only oligonucleotides that reach the cell nucleus permit the correct splicing which results in luciferase activity. An approximately eight-fold increase of luciferase activity was reported at a 1:70 charge ratio of oligonucleotide to dendrimer. The effect was independent of the serum concentration in the media. However, since oligonucleotide delivery itself was not evaluated in the study, it is possible that the dendrimers simply increase the interaction of the antisense oligonucleotide with the mRNA; although for several reasons this seems unlikely. Other researchers, who also utilized dendrimers, reported a 50-fold increase in the delivery of oligonucleotide into the cell, but did not assess antisense activity [192]. Overall, the use of dendrimers appears promising, especially considering that the molecular size of the complex is relatively small, in fact, comparable to that of peptide–oligonucleotide conjugates.

5. Oligonucleotide delivery in vivo

5.1. Delivery of 'naked' oligonucleotides in vivo

In vitro, delivery vehicles are necessary for antisense oligonucleotides to be effective. However, as noted previously,

oligonucleotide uptake in vitro may differ from oligonucleotide uptake in vivo. It has been claimed that in vivo, naked oligonucleotides exhibit activity [4,199]. The systemic mice treatment with murine-specific antisense phosphorothioate oligonucleotide reduced a ICAM-1 expression and the amount of infiltrating leukocytes in a mouse model of colitis [200]. Efficient antisense oligonucleotide downregulation of c-Haras [201] and c-myc [202] oncogenes has been shown in mouse models. For the treatment of leukemia, c-myb [203] antisense phosphorothioate oligonucleotides have been tested in the severe combined immunodeficient (SCID) mouse model. Animals receiving c-myb antisense oligonucleotides survived at least 3.5 times longer than the various control animals. Bcl-2 antisense oligonucleotide treatment improved the chemosensitivity of human melanoma cells grown in SCID mice [39]. Antisense phosphorothioate oligonucleotides effectively inhibited the expression of hepatitis virus B gene in duck [204] and mouse [205] models for hepatocellular carcinoma. The uptake of oligonucleotides by scavenger receptors on endothelial liver cells has been claimed for in vivo delivery [206,207].

Antisense inhibition of gene expression in utero was reported for the first time [208]. A single injection of an antisense oligonucleotide targeting VEGF into pregnant mice resulted in a lack of primary angiogenesis. It is noteworthy, that only 2′-*O*-methyl-modified phosphorothioate oligonucleotides, which possess an increased stability in vivo, are suitable for embryo delivery of oligonucleotides.

Recently, the ability of analogs of DNA, e.g. PNAs, to enter neuronal cells in vivo has been demonstrated [209]. An unmodified antisense PNA complementary to mRNA of the rat neurotensin receptor was demonstrated to penetrate the blood–brain barrier and cause a reduction in binding sites for neurotensin both in the brain and small intestine [210].

Poly-2′-*O*-(2,4-dinitrophenyl) modification of antisense oligoribonucleotide targeted against the polymerase gene of duck hepatitis B virus is an additional interesting example of nuclease protection for 'naked' antisense oligonucleotides in vivo. These modified oligonucleotides were used to treat infected ducks in vivo, and completely inhibited duck viremia in a dose of 1 mg/kg (i.v.) per day for 25 days [211].

In general, naked oligonucleotides supply some advantages for successful delivery, because any delivery moiety will add to the size and complexity of the antisense oligomer, thus possibly limiting its access to certain sites. However, delivery agents also provide advantages in that they protect antisense oligonucleotides against degradation, increase cell uptake, and may target the drug to specific cells or tissues [175]. Although naked oligonucleotides can reach their biological targets in vivo, they are eliminated relatively rapidly (within minutes) from the bloodstream after injection due to extensive liver and kidney uptake [212,213]. Thus, a delivery system allowing the controlled and sustained release of oligonucleotides in vivo should increase the in vivo efficacy of antisense technology.

5.2. Liposome delivery of oligonucleotides in vivo

Encapsulation of antisense oligonucleotides in conventional liposomes can enhance their biological activity in vivo [214–216]. One of the advantages of using liposomes as delivery agents is their preferential accumulation at sites of infection, inflammation [217] and tumors [218–220]. Liposome-encapsulated oligonucleotides targeted against ICAM-1 mRNA demonstrated a significant anti-inflammatory effect in a model of acute inflammation in mice [221]. Liposomes have been efficiently used as delivery agents for antisense oligonucleotides against angiotensin in the rat model of spontaneous hypertension [222], and against transforming growth factor beta2 (TGF-β2) in the malignant mesothelioma mouse model [223]. Phosphorothioate oligonucleotides against MAP 1A mRNA were efficiently delivered into rat brains using liposomes and a miniosmotic pump, and efficacy in the growth suppression of rat C6 glioma cells was demonstrated [224]. Liposome-encapsulated antisense raf oligonucleotides inhibited Raf-1 protein expression in vivo, and sensitized radioresistant tumors to ionizing radiation [225]. Electroporation of a bcl-2 antisense oligonucleotide encapsulated in liposomes could inhibit rat hepatocarcinogenesis [226]. Liposomes were efficiently used to increase the hepatic delivery and antiviral efficacy of phosphorothioate antisense oligonucleotides employed in the in vivo treatment of hepatitis B virus infection in the duck model [227].

Circulating oligonucleotides carried in vivo by liposomes were intact for at least 24 h, compared with 5 min for free oligonucleotides [225,228,229]. Liposomes stabilized with PEG had an increased mean half-life time of plasma clearance (up to 57.8 h) [230]. Both protection of the oligonucleotide in the circulation and its targeted delivery in vivo have been achieved using HJV-conjugated liposomes [231–233]. The transfer of FITC-labeled oligonucleotides enclosed in HJV-liposome complexes resulted in the sustained nuclear localization of fluorescence in the vessel wall of rat carotid arteries for at least 1 week [95,231] and in murine retina for up to 3 days [234]. The inhibitory effect of antisense oligonucleotides against cdc2 kinase and proliferating-cell nuclear antigens delivered with HJV liposomes into the rat carotid artery persisted for up to 8 weeks after a single transfection [235].

Liposomes may also be used in vivo for the delivery of oligonucleotides in the therapy of intracellular infections (e.g. HIV infections, especially when targeting the pool of virus harbored in macrophages) [236].

The major disadvantages of liposomes (e.g. toxicity, serum instability) may affect their use in in vivo delivery [67,79]. Nevertheless, novel liposomal compositions possess tolerable toxicity [237], are sufficiently stable in the presence of plasma and serum [80]. For example, the

phosphorothioate oligonucleotides targeted against the influenza A virus and encapsulated into serum-resistant Tfx-10 liposomes inhibited viral growth in lung tissues, and increased the overall survival rates of mice infected with influenza A [238].

Another limitation on the use of liposome technology for systemic drug delivery is their preferable accumulation in the liver and spleen [228,229]. This problem can be partially ameliorated, though not entirely prevented, by the use of long-circulating liposomes and/or antibody targeted liposomes [104].

5.3. Macromolecular carrier systems

Macromolecular carrier systems can act as alternatives for liposome delivery agents in vivo. The criteria for a carrier suitable for targeting nucleic acids to organs in vivo are the same as for liposomes; a carrier should be sufficiently stable in the bloodstream, non-covalent binding with oligonucleotide is preferred, and a complex of the carrier with an oligonucleotide should be water-soluble [134]. Phosphorothioate antisense oligonucleotides targeted to fibronectin transcripts were coupled to water-soluble, non-toxic copolymer carriers consisting of polyoxyethylene and polyspermine chains. These were used to specifically reduce fibronectin expression in retinal vascular cells in rats [239]. Fluorescently-labeled oligonucleotides were detected in retinal vascular cells until day 6, and the fibronectin protein level on day 6 was significantly reduced to $61.4 \pm 16\%$ of the control. A linear PEI derivative was used to deliver fluorescent oligonucleotides to the liver after intravenous injection into ducks [240]. PEI was also used to deliver fluorescent oligonucleotides into embryonic mice neurons with no apparent toxic effects [241].

Nanoparticle-adsorbed antisense oligonucleotides directed to a point mutation (G → U) in codon 12 of the Ha-ras mRNA markedly inhibited Ha-ras-dependent tumor growth in nude mice after subcutaneous injection [185,242].

For site-specific delivery of antisense oligonucleotides and genes, positively charged macromolecules, such as PLL, histone, protamine, etc., have been linked to a tissue-specific ligand, and then allowed to bind to nucleic acids via an electrostatic interaction. The resulting complexes retained their ability to interact specifically with cognate receptors on target cells (e.g. glycoside-modified PLL and the asialoglycoprotein receptor uniquely expressed by parenchymal liver cells), leading to receptor-mediated internalization of the complexes. At the same time, the protein conjugate may coat and condense nucleic acids, protecting them from the nuclease attack [243]. A [^{32}P]-labeled 67-mer antisense oligonucleotide complexed with native and asialo-human α-1 acid glycoprotein–PLL conjugates was successfully targeted to hepatocytes after intravenous injection of the complex into rats [244]. Asialoglycoprotein–PLL oligonucleotide complexes were used to suppress CETP in cholesterol-fed rabbits [245]. Glycosylated PLL conjugates with antisense oligonucleotides were successfully employed to target oligonucleotides to murine [246] and rat [247] liver cells in vivo.

Another approach employs conjugates of nuclease-resistant antisense oligonucleotide with a modified glycotripeptide, composed of, beginning at the N-terminus, a single tyrosine (Y) residue and two glutamate (E) residues. This tripeptide YEE (aminohexyl *N*-acetylgalactosamine)$_3$, known to bind to galactose/*N*-acetylgalactosamine receptor sites on hepatocytes [248]. These conjugates have low toxicity (vs. PLL, which is toxic at certain concentrations), even at high concentrations and are structurally homogeneous. Such neoglycoconjugates had high selectivity in targeting liver cells ($69.9 \pm 9.9\%$ of the injected dose) and were extensively metabolized in mice [249].

Kang, et al. [250] linked biotinylated phosphodiester ODNs to a conjugate formed between a neutral avidin and a monoclonal antibody directed to the rat transferrin receptor (OX-26). This conjugate and a similar conjugate with streptavidin could penetrate the blood–brain barrier and deliver oligonucleotides [251] and PNAs [252] into the brain after intravenous injections in rats. However, because of the strong binding of phosphorothioate oligonucleotides to plasma proteins, oligonucleotide conjugates were poorly transported into the brain through the blood–brain barrier by this delivery vector following intravenous administration [252]. Thus, because of decreased plasma protein binding, PNA–antibody–streptavidin conjugates may represent more optimal antisense molecules for drug delivery to the brain [253]. Replacement of the chemical conjugate for a novel antibody–avidin-fusion protein provides both antibody- and avidin-related activities. In the rat model, the fusion protein conjugate had a much longer serum half-life, and demonstrated superior [^{3}H]biotin uptake into brain parenchyma than the chemical conjugate between the OX-26 antibody and avidin [254].

Cellular transporter peptides could be used as delivery vectors for antisense compounds, such as PNA. A 21-mer PNA, complementary to the human galanin receptor type 1 mRNA, coupled either to the peptide transportan or pAntennapedia (43-58), was efficiently taken up in the rat spinal cord and resulted in a decrease in galanin binding in the dorsal horn, which modified the pain response [161].

There has been rapid progress in the development of antisense drugs and delivery systems for in vivo applications. However, site-specificity, cellular membrane permeability and distribution in cellular compartments must be improved for better therapeutic effects. Most of the existing nucleic acids/carrier complexes are too large to reach the targeted tissues. Strategies that can reduce the size of these complexes and improve their intracellular trafficking are required to enhance their in vivo efficacy.

In summary, the number of potential cellular delivery systems for antisense oligonucleotides has expanded tremendously over the past several years. However, it is not clear which of the many currently available technologies

provides optimal delivery, defined as maximal antisense activity for the least unit cost. Undoubtedly, these are some of the many challenging problems in this area that will occupy numerous scientists over the next decade.

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