

MINIREVIEW

Innovations in Oligonucleotide Drug Delivery

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ABSTRACT: Oligonucleotides (ONs) are a new class of therapeutic compounds under investigation for the treatment of a variety of disease states, such as cancer and HIV, and for FDA approval of an anti-CMV retinitis antisense molecule (VitraveneTM, Isis Pharmaceuticals). However, these molecules are limited not only by poor cellular uptake, but also by a general lack of understanding regarding the mechanism(s) of ON cellular uptake. As a result, various delivery vehicles have been developed that circumvent the proposed mechanism of uptake, endocytosis, while improving target specific delivery and/or drug stability. This review describes various traditional and novel delivery mechanisms that have been employed to improve ON cellular delivery, cost effectiveness, and therapeutic efficacy. © 2003 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 92:1559–1573, 2003

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INTRODUCTION

The approval of an antisense oligonucleotide (ON) for the treatment of HIV-associated CMV retinitis (VitraveneTM, Isis Pharmaceuticals) has signaled the birth of a new class of therapeutically useful compounds. Since their conceptual origins,^{1,2} ONs have been investigated as novel therapeutic agents, and they are currently under investigation for the treatment of a wide variety of diseases, such as cancer and viral diseases [human immunodeficiency virus (HIV), hepatitis, cytomegalovirus (CMV)].

ONs are short sequences of single stranded nucleic acid or nucleic acid analogs designed to bind intracellularly to target nucleic acids (RNA or

DNA) or proteins in a sequence or conformation-specific manner.^{3–5} Additionally, ONs containing a specific CpG motif can elicit an immune response.^{6–9} Although the clinical relevance of ONs has been demonstrated, poor stability and inefficient cellular uptake, both *in vitro* and *in vivo*, limit the efficacy of ONs and have been a barrier to therapeutic development. Cellular uptake can be <2% of the dose,^{10,11} depending on ON chemistry and cell type, whereas further intracellular barriers hinder transport of the internalized ON to the active site. As a result, ON concentration at the active site, especially in terms of *in vivo* drug delivery, may be too low for an effective and sustained outcome. At the very least, the efficiency of ON delivery is rather poor in terms of cost-effective drug development. Indeed, a majority of the current and past research focuses on the improvement of ON stability and delivery through modification of the ON structure itself^{5,12–14} or via the use of delivery vehicles, which can both protect

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the drug from degradation as well as improve ON delivery into cells. The purpose of this communication is to review the mechanism of ON cellular uptake as well as both some of the common and more novel ON cellular delivery methods.

OLIGONUCLEOTIDE CELLULAR UPTAKE

Mechanisms of Cellular Uptake

Advances in ON drug delivery by a variety of mechanisms have developed from a need to circumvent poor cellular uptake of “naked” ONs. However, a basic understanding of the proposed and potential actual mechanism(s) of ON uptake would be most useful for not only delivery of naked ONs but also development of optimal delivery systems. The most widely accepted mechanism of ON uptake is adsorptive endocytosis, whereby adsorption of the ON to the cell membrane results in transport of ON into the cell via endocytosis.^{15,16} However, intracellular localization of ON varies depending on cell type or experimental method. In some uptake studies, a significant proportion of internalized ON remains solely sequestered within the endosomal compartment, as evidenced by a punctate distribution of fluorescently labeled ON in the cell.^{15,17} If the ON is unable to escape the endosome, lysosomal degradation is likely. However, other research suggests that the ON escapes the endosome and is either rapidly transported to the nucleus or accumulates in perinuclear organelles.^{11,18,19} Currently, the mechanisms by which ONs escape lysosomal degradation and are transported into the cytosol and nucleus remain unclear. Other mechanisms of ON cellular uptake have been proposed such as pinocytosis²⁰ and caveolar potocytosis.¹⁸

The multitude of proposed mechanisms indicates that ON uptake is not easily defined. Indeed, the mechanism can vary depending on ON chemical structure. A number of modifications to the native phosphodiester (PO) DNA backbone have been synthesized to improve the target affinity, intracellular delivery, or stability of ONs in the presence of abundant exo- and endonucleases. Currently, the most commonly investigated ONs are the phosphorothioate (PS), methylphosphonate (MP), and chimeric backbones containing mixed backbone chemistries, and peptide nucleic acids (PNA; Figure 1). Although the PS modification retains the aqueous solubility of the native PO

backbone while increasing ON stability in the presence of nucleases, the anionic charge hinders uptake through the lipophilic membrane, resulting in poor cellular uptake (Table 1). However, ONs with an anionic backbone, like the PO and PS modifications, bind to cell surface proteins resulting in adsorptive endocytosis. In contrast to anionic ONs, neutral molecules like the MP and PNA have surprisingly low cellular uptake, resulting from low cell surface protein binding, and therefore exhibit decreased adsorptive endocytosis.

Cellular uptake is also highly variable depending on cell type and cell cycle. For example, cellular uptake of a *c-myc* ON into Rauscher erythroleukemia cells occurs via a nonspecific protein and energy-dependent pathway immune to endocytic inhibition, which is inconsistent with endocytosis alone.^{21,22} Cellular uptake involves significant surface binding as well as both cation and cell cycle dependency,²³ thus, multiple mechanisms of ON uptake in the Rauscher cell line may be occurring, some of which have yet to be completely characterized. Current evidence may also suggest a porin-like transporter or pore may be involved in cellular uptake.^{21,24} Finally, some researchers have demonstrated nuclear or perinuclear fluorescence without a punctate fluorescence pattern, suggesting either a nonendocytic uptake mechanism or rapid endosomal escape.^{11,25–27} Therefore, alternate nonendocytic or multiple mechanisms of ON cellular uptake are plausible.

Barriers to Efficient Cellular Uptake

Despite the unknown mechanism of uptake for ONs or other hydrophilic macromolecules, intracellular transport and activity of “naked” ONs are observed, although uptake is quite inefficient. Indeed, several barriers to cellular uptake exist, which must be overcome to improve ON cellular delivery. The first barrier is the lipophilic cell membrane, through which these large, anionic molecules must pass to reach the site of action. Additionally, many ONs appear to enter the cell by endocytosis, as already mentioned, resulting in sequestration of the molecules inside endosomes. Again, the ON must pass through the endosomal membrane or risk lysosomal degradation. Finally, although evidence exists that once inside the cytosol ONs are rapidly transported to the nucleus,^{28,29} nucleases within the intracellular milieu can degrade the ON, rendering it inactive. Ideally, optimal delivery of ONs to the

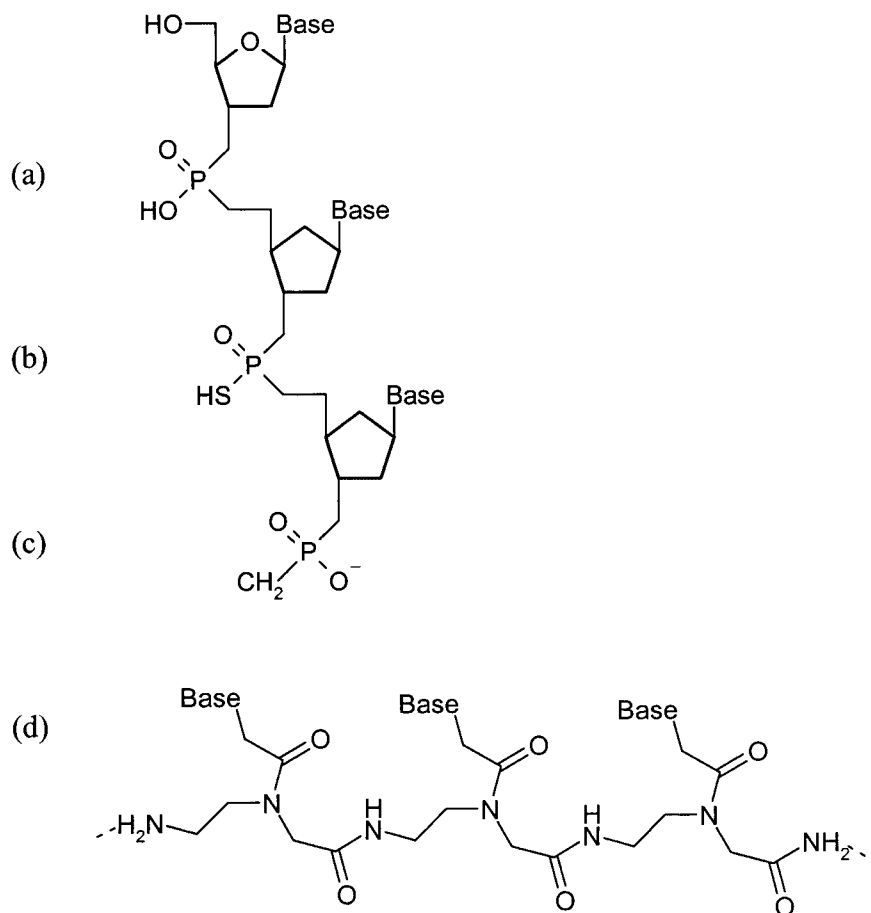


Figure 1. Backbone structures for commonly used oligonucleotides: (a) phosphodiester (PO); (b) phosphorothioate (PS); (c) methylphosphonate (MP); (d) peptide nucleic acid (PNA).

site of action would be based on a thorough understanding of the mechanism of ON uptake. However, because the mechanism of ON transport into the cell remains to be fully elucidated, many researchers have focused on the empirical development of various novel delivery vehicles that circumvent the barriers to uptake to improve the efficacy and ultimately the activity of ONs.

Table 1. Oligonucleotide Stability and Cellular Uptake^a

Backbone Chemistry	Nuclease Stability	Cellular Uptake	Charge
PO	↓	↓	—
PS	↑	↓	—
MP	↑	↓↓	∅
PNA	↑	↓↓	∅

^aKey: (↓) low; (↓↓) very low; (↑) high; (—) negative; (∅) neutral.

GENERAL DELIVERY METHODS

Liposomes

The most widely used method for improving ON stability and intracellular delivery utilizes ON-lipid conjugates or liposome formulations. Liposomes are composed from artificial cationic lipids such as *N*-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and *N*-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethylammonium methyl sulfate (DOTAP), where the cationic head group associates with negatively charged ONs, neutralizing the anionic charge and facilitating delivery of the large anionic molecules through the lipophilic cell membrane.^{30–33,35} Uptake occurs via endocytosis, in which the cationic liposomes induce membrane “flip-flop”, releasing the ON from the liposome and into the cytosol.³⁴ Additionally, the addition of dioleyl

phosphatidylethanolamine (DOPE) to liposome formulations aids in destabilization of endosomal membranes, facilitating the release of ON to the site of action.^{35,36} Encapsulation of ONs within liposomes also affords protection against abundant nuclease degradation in the intracellular milieu.^{37,38}

However, cationic liposomes are cytotoxic, especially at high concentrations, which limits their usefulness both *in vitro* and *in vivo*.^{38,39} To combat some of the drawbacks associated with cationic liposomes, modifications to the basic liposome formulation, such as changing the lipid, composition of lipids, or lipid concentration, have been extensively investigated. For example, a novel nontoxic liposome formulation has been used to deliver a phosphorothioate ON antisense to neuropeptide corticotropin-releasing factor receptor (CRF-R) mRNA in CHO cells using the amphiphilic pyridinium-based lipid SAINT-2.⁴⁰ ON–lipid complexes were formed using 20 μ M SAINT-2/DOPE (1:1) and 100 nM PS or PO anti-CRF-R ON. A 100–250-fold increase in cell-associated ON was observed following a 24-h incubation of complex in CHO cells compared with free ON. In addition, *in vitro* antisense activity resulted in a 50% decrease in CRF-R mRNA expression using complexes, whereas no effect was observed for free ON or lipid controls.

Alternatively, pH-sensitive fusogenic liposomes are used to bypass endosomal sequestration. pH-sensitive liposomes are composed of amphipathic lipids, such as cholesteryl hemisuccinate (CHEMS) and DOPE. At physiologic pH, CHEMS stabilizes DOPE into lamellar phase liposomes. However, at acidic pH, such as that in the early endosome, CHEMS undergoes protonation, resulting in formation of a hexagonal H_{II} phase and subsequent fusion or destabilization of the endosomal membrane and release of ON to the cytosol.^{41–45} By altering their lipid composition, pH-sensitive liposomes can have increased stability at physiologic pH, which would improve circulation time *in vivo*. Additionally, pH-sensitive liposomes also have fusogenic properties at low pH, resulting in release of the ON from the endosome to the cytosol.⁴⁴ pH-sensitive liposomes were used to deliver antisense ON against the Na⁺/*myo*-inositol co-transporter mRNA in astrocytes.⁴³ Cellular uptake of ON was more efficient when pH-sensitive liposomes were used, compared with free ON or non-pH-sensitive liposomes (lecithin), as determined using fluorescence microscopy. In addition, pH-sensitive liposome delivery

resulted in a 50-fold decrease of target mRNA expression compared with free ON, suggesting that liposome encapsulation can have a significant impact of ON delivery to the site of action. However, evidence suggests that small (<200 nm) pH-sensitive liposomes composed of DOPE/OA are stabilized in the presence of human plasma via insertion of hydrophobic plasma molecules into the liposome.⁴⁶ Consequently, the liposomes lose their pH sensitivity and therefore may have decreased drug delivery capability *in vivo*. Therefore, the size, lipid composition, and interactions with plasma of pH-sensitive liposomes need to be carefully characterized and adjusted to achieve optimal drug delivery.

Liposomes have been modified for targeted delivery by coupling receptor ligands^{47–49} or antibodies (immunoliposomes)^{50–56} to the liposome. For example, maleylated-ON–BSA conjugates effectively delivered ON into *Leishmania*-infected macrophages via the scavenger receptor system, resulting in nearly a 5-fold decrease in parasite-infected macrophages compared with the results with free ON.⁴⁸ Pagnan et al.⁵⁰ encapsulated *c-myc* antisense ON into cationic liposomes coated with neutral lipids and covalently coupled to anti-ganglioside GD₂ monoclonal antibody (GD₂-CCL).⁵⁰ Cellular uptake of ON into GD₂ positive neuroblastoma cells was significantly higher for the immunoliposome-encapsulated ON compared with free ON, resulting in a significant target-specific decrease in *c-myc* expression. Additionally, immunoliposome-directed uptake was specific for GD₂ receptors because no increase in uptake compared with free ON was observed in HeLa cell, a GD₂ negative cell line, or when a nonspecific immunoliposome was used. The results suggest that non-target GD₂ negative cells may be largely unaffected if the GD₂-CCL delivery system is used *in vivo* in the antisense treatment of neuroblastoma.

Carrier Molecules

Receptor-Mediated Endocytosis (RME)

RME-directed uptake utilizes import mechanisms already present in the cell membrane for the uptake of biomolecules necessary for cell function. ONs can be linked directly to a carrier protein via a covalent bond or noncovalently via poly-L-lysine (PLL)–carrier conjugates. The choice of carrier is dependent on its known ability to bind to specific cell membrane receptors and accumulate in the

cell via endocytosis. Not only can internalization of ON potentially be improved, but cell-specific delivery can also be achieved by targeting receptors solely expressed or over-expressed on certain target cells.

For example, although folate receptors are found on all dividing cells, they are overexpressed in epithelial cancer cells and therefore have been investigated for cell-specific targeting of drugs. Ginobbi and co-workers⁵⁷ used folic acid-PLL-ON conjugates to target antisense phosphorothioate *c-myc* ON to M-14 human melanoma cells. An increase in intracellular delivery of ON was observed, resulting in a significant decrease in both *c-myc* protein levels and cell growth. As a control, the investigators used a transferrin-PLL-ON conjugate, for which no RME should be observed due to the lack of transferrin receptors on M-14 cells. Indeed, no improvement of ON uptake and activity were observed, suggesting that targeting specific receptors is a viable means to limit drug delivery to specific cell populations. However, effective drug-targeting by RME depends not only on the affinity of the ligand for the receptor, but also on limitation of the selected receptor to target cells, especially *in vivo*. Additionally, the ligand itself must either be inactive or augment the therapeutic outcome to avoid potential toxicity associated with the delivery vehicle. Therefore, thorough characterization of the selected receptor and its *in vivo* ubiquity, or lack thereof, is a prerequisite to developing RME ON delivery.

Hepatocyte-specific ON drug delivery for the treatment of hepatic diseases has been investigated by targeting asialoglycoprotein receptors.⁵⁸⁻⁶³ Biessen and co-workers⁵⁹ delivered a random sequence ON to parenchymal liver cells by derivatizing ON with L₃G₄, a lysine-based galactoside synthetic ligand with high affinity for asialoglycoprotein receptors. Although conjugation of ON to L₃G₄ slightly decreased the affinity of L₃G₄ for ASOR receptors ($K_d = 23$ versus 6.5 nM), binding of ON to the target sequence in an *in vitro* assay was not significantly affected by the presence of L₃G₄, suggesting that the *in vitro* activity of the ON would not be affected by the ligand. The authors also suggested that uptake occurred via active transport because intracellular ON concentrations were 40 nM, which is 4-fold higher than the extracellular concentration, whereas cellular uptake decreased by at least 70% on the addition of various uptake inhibitors. In addition, uptake of ON-L₃G₄ conjugate was significantly decreased by almost 35-fold in the presence of the competitive

ASOR receptor ligands GalNAc and ASOR. However, confocal analysis of ON-ligand uptake using rhodamine-labeled ON showed mainly a punctate fluorescence pattern with minor amounts of fluorescence in the cytosol, suggesting ON is sequestered within endosomal compartments.

In vivo intravenous administration of ³²P-ON-L₃G₄ conjugates to mice demonstrated a significant alteration of ON distribution. Plasma clearance for ON was rapid; that is, at 2 min after injection, only 14.3 and 11.5% of free and conjugated ON, respectively, remained in plasma. However, whereas only 19.1% of free ON was found in liver, L₃G₄ conjugation increased delivery to the liver 4-fold to 77.6%. Not only did conjugation increase selective ON delivery to the liver, but also conjugates successfully bypassed reticuloendothelial clearance and scavenger-receptor uptake within the liver. A 25-fold increase of ON conjugate delivery to parenchymal liver rather than Kupffer or endothelial liver cells was observed compared with free ON hepatic distribution. Therefore, ON conjugates may potentially be employed to selectively deliver ONs using RME both *in vitro* and *in vivo*. However, further studies regarding the *in vitro* or *in vivo* activity of ON conjugates are necessary to determine the overall efficacy of ASOR RME delivery of ONs to the liver.

Many other cell-specific receptors may prove useful for targeted ON delivery. Enhancement of ON delivery has been investigated using mannose delivery to alveolar macrophages⁶⁴ and human tumor targeting using ErbB-2⁶⁵ and transferrin^{66,67} or anti-transferrin antibody.⁶⁸ Anti-transferrin antibody-ON conjugates have also been used to target ON delivery into the brain,⁶⁹ CD14-ON conjugates have been used to target monocytes,⁷⁰ and epidermal growth factor (EGF) receptor has been targeted for delivery of EGF-ON conjugates to epithelial cancer cells.⁷¹ However, RME generally results in sequestration of ON in endosomal compartments, thereby limiting the utility of this method for delivering therapeutic levels of drug to intracellular targets. Additionally, ON-protein conjugates must be carefully designed such that the ON does not hinder receptor-ligand binding by blocking receptor-binding sites on the carrier protein.

Fusogenic Peptides

Fusogenic peptides have been used to promote peptide fusion of ON-peptide conjugates with

either cell or lysosomal membranes.^{72–74} For example, Morris et al. developed a peptide vector containing hydrophobic HIV gp41 fusion peptide and hydrophilic SV40 T-antigen to create ON-peptide conjugates via electrostatic interaction between ON and the hydrophilic peptide.⁷⁴ Using confocal microscopy, cellular uptake of the conjugates was observed to occur via a non-endocytic pathway because ONs were localized to the nucleus within 1 h in 90% of cells, whereas very little punctate fluorescence was observed. In contrast, following endocytosis of ON conjugates to influenza virus hemagglutinin (HA) envelope fusion peptide, acidification of the endosomal compartment resulted in a conformational change in HA, destabilization of the lysosomal membrane, and release of ON conjugate to the cytosol.^{75–77} Freulon and co-workers investigated the uptake of the anti-HIV-1 antisense ON GEM 91 in A549 and HeLa cells by coinubating unconjugated ON and either monomeric or dimeric E5 C-terminal subunit of HA2 with A549 and HeLa cells in slightly acidic media.⁷⁷ Under acidic conditions, HA2 peptides destabilized endosomal membranes, allowing for ON delivery into the cytosol. Complete uptake of 0.1 μM fluorescently labeled ON occurred using 40 μM E5 monomer at pH 5.8 following a 30-min incubation at 37°C. Uptake was similar using different fluorophores, cell lines, and ON backbones but was dependent on pH, supporting the role of endosome acidification in HA fusion peptide-mediated delivery. Confocal analysis indicated that ON and E5 peptide were co-localized in endosomal compartments prior to acidification. However, following acidification and endosomal membrane destabilization, ONs were localized to the nucleus, whereas E5 remained in the cytosol. Although *in vitro* activity studies were not done, the potential for increased activity at lower ON concentrations was suggested because ON remained free (uncomplexed to the peptide vector) and delivery to the nucleus was improved compared with ON alone.

Signal Peptides

Selective import of nuclear proteins from the cytosol into the nucleus is mediated by short peptide sequences called nuclear localization signals (NLS). NLS peptide derivatives of SV-40,^{78–80} transcription nuclear factor κB ,⁸¹ influenza viral nucleoprotein,⁸² and yeast $\alpha 2$ protein⁸² have been used to facilitate ON transport to the

nucleus. In addition to NLS, signal import peptides (IP), which promote cellular uptake of IP or DNA, have been derived from Kaposi's fibroblast growth factor (K-FGF)^{81,83} and DNA uptake stimulating factor (DUSF) of *Neurospora crassa*,⁸⁴ respectively.

The synthetic IP from K-FGF promoted transient pore formation in cell membranes of P59, A549, SV40, and RAW264.7 cells.⁸³ Uptake of ON-PLL-IP conjugates was dose dependent at 50–500 μM IP-PL and resulted in strong intracellular cytoplasmic and nuclear fluorescence via a nonendocytic pathway, as demonstrated by confocal microscopy and endocytosis inhibition studies. Competition studies also indicated that the uptake mechanism was not receptor mediated. However, similar studies using ON-K-FGF disulfide conjugates in monkey kidney fibroblast CV1-P, CHO, and human retinal pigmented epithelial D407 cells resulted in punctate distribution of ON, with faint nuclear and cytosolic fluorescence, suggesting that the mechanism of K-FGF-mediated ON delivery varied among cell lines or that the method of ON-peptide conjugation strongly affected the delivery mechanism.

Finally, multiple import mechanisms may be combined for optimal ON delivery. Peptides derived from HIV tat or *Drosophila* antennapedia proteins serve as both cell membrane translocation signals as well as nuclear transport signals.⁸⁵ Fusion peptide-NLS conjugates are often used to deliver ONs and include delivery assemblages like K-FGF-NLS,⁸¹ gp41-NLS,^{74,80} and protamine-NLS.⁷⁹

NOVEL DELIVERY METHODS

Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides that consist of a hydrophobic central cavity and multiple hydroxyl groups on their outer surface (Figure 2). Because of their unique ability to associate with other molecules, their water solubility, and the potential for derivatization of the hydroxyl groups in the central cavity, CDs have been investigated for the delivery of a variety of drug molecules.^{86,87} CD delivery systems have also been used to minimize drug toxicity⁸⁸ or enhance bioavailability via multiple delivery routes.^{88,89} β -CDs were used to deliver a 25-mer phosphorothioate ON into a variety of human T-cell leukemia cells lines.⁹⁰ The authors reported

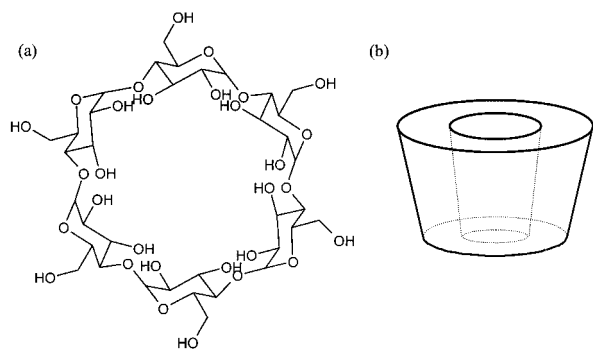


Figure 2. Chemical structure (a) and schematic diagram (b) of β -cyclodextrin.

that not only did cellular uptake of ON improve in the presence of CD (2- to 3-fold over 24 h), but that uptake was dependent on both the concentration of CD and ON. Importantly, ONs remained active when CD delivery was used.⁹¹ The antiviral activity of an antisense ON targeted to coronavirus in adenocarcinoma cells increased almost 8-fold when 7.5 μ M ON was delivered using a CD derivative, 6-deoxy-6-S- β -D-galactopyranosyl-6-thio-cyclomaltoheptaose, compared with free ON. No viral inhibition or cell toxicity was associated with the CD itself.

CDs have also been used to deliver ONs *in vivo*.^{92,93} Zhao and co-workers used CDs to deliver ONs containing immunogenic CpG sequences to mice.⁹³ At a dose of 50 mg/kg ON in varying concentrations of CD, the authors found that CD-mediated delivery resulted in a decrease in the mitogenicity of the ON, a lack of splenomegaly, and increased IgM production compared with free ON. However, when coadministered with ON, the protective effects of the CD against ON-associated toxicity decreased over time because the clearance of CD was much more rapid than that of the ON. Although the mechanism by which CDs inhibit immune stimulation by ONs was unclear, the authors suggested the possibility that inclusion of ON within the CD cavity blocks potential ON-protein binding with proteins involved in lymphocyte activation. Therefore, CD-ON formulations must be optimized to retain the improved delivery and stability of ONs while lengthening the time frame in which CD are also protective. Similarly, Barrett et al.⁹² found that cellular uptake of β -CD-adamantyl-ON conjugates in PC12 cells was enhanced compared with free ON. Additionally, following a 2-week systemic administration of the conjugate to mice, uptake to dorsal root ganglia, liver, and kidneys, but not brain, increased

compared with free ON, suggesting CD may be used to target specific disease sites *in vivo*.

Dendrimers

Dendrimers are highly branched macromolecules synthesized by multiplication of a series of repetitive units, typically polyamides (Figure 3). The molecules have surface functional groups, such as primary amino groups, which interact with other molecules via electrostatic interaction. As a result, rapid and highly reproducible complex formation occurs, yielding complexes with weak cytotoxicity. An increase in research investigating the potential of dendrimers as ON and/or DNA delivery vehicles has recently occurred.⁹⁴⁻⁹⁷

Bielinska et al. investigated the cellular uptake and *in vitro* activity of a 27-mer anti-luciferase ON, either as free ON or complexed with generation 3 starburst PAMAM (polyamidoamine) dendrimers, using luciferase expressing U937, Jurkat, D5, and Rat 2 cells.⁹⁸ Dendrimer-ON complexes at various charge ratios resulted in a 4- to 5-fold increase in cellular uptake and 30-60% decreases in luciferase expression compared with free ON in all cell lines, suggesting increased ON cytosolic delivery. In addition, ONs delivered using dendrimers had significantly higher retention in cells, up to 4 days, compared with <24 h with free ON. Similarly, Yoo and Juliano investigated the mechanism of dendrimer-mediated ON cellular uptake in HeLa cells using TAMRA-labeled ON-Oregon Green

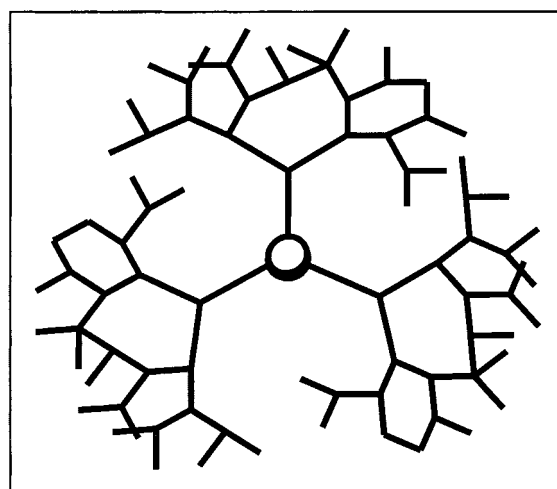


Figure 3. Schematic diagram of a generation 4 dendrimer.

488-labeled generation 5 PAMAM dendrimers.⁹⁴ Dendrimer–ON complexes remained intact intracellularly and, although presumably bound to the plasma membrane and in endosomal compartments, also co-localized in the cytoplasm and nucleus to a much higher extent than free ON. Similar to the results of Bielinska et al., the data suggested that dendrimers enhanced the extra-endosomal delivery of ONs. Additionally, delivery of ON in the presence of 30% FCS was possible using dendrimers, whereas liposome formulations, such as lipofectamine, are typically inactive in the presence of serum. Therefore, the lack of cytotoxicity and enhanced delivery of ONs, even in the presence of serum, has possibly given dendrimers an advantage over traditional delivery vehicles like liposomes.

Nanoparticles and Microparticles

Cyanoacrylate nanoparticles have been studied as drug delivery tools since the late 1970s^{99,100} and as ON delivery tools since the early 1990s.^{101–103} Drug–nanoparticle association is achieved through hydrophobic van der Waals interactions. The anionic charge of the ON necessitates formation of ion pairs using various hydrophobic cations prior to loading nanoparticles. Typically, alkylcyanoacrylates, such as polyisohexyl- (PIHCA), polyisobutyl- (PIBCA), or polyhexacyanoacrylate (PHCA), are used to form the nanoparticles, although a variety of lipophilic cation–ON ion pairs have been tested.^{103–105} Adsorption of ON to nanoparticulate systems is dependent on not only the type of cyanoacrylate used and its respective hydrophobicity, but also ON length and the type of hydrophobic cation used. In addition, ON adsorption to nanoparticles is inversely related to ionic strength during formulation.¹⁰³

Using ON–CTAB (hexadecylmethylammonium)–PIHCA nanoparticles, Helene and co-workers investigated the cellular uptake and stability of ONs in U937 cells.¹⁰¹ In cell-free systems, nanoparticle-encapsulated ON was stable for >20 h in the presence of phosphodiesterase at 37°C, whereas free ON was degraded within 10 min. Similarly, in serum-containing cell culture medium, nanoparticles were able to protect ONs from nuclease degradation for up to 3 h, whereas they were rapidly degraded when unencapsulated. Finally, uptake of ON nanoparticles was ~6-fold higher than unencapsulated ONs following a 24-h incubation with U937 cells, with no observed cytotoxicity.

Little *in vivo* work was done using nanoparticle ON delivery systems.^{102,106,107} For example, tumor growth in nude mice was inhibited by intratumoral injection of anti-Ha-ras ON adsorbed to PIHCA nanoparticles and required significantly less ON than injection of free ON alone.¹⁰² Similarly, Lambert et al. found that Ewing sarcoma-related tumor size in nude mice was ~3-fold smaller following intratumoral injection of anti-EWS Fli-1 ON adsorbed to PICA nanoparticles compared with free ON following ~15–20 days of treatment.¹⁰⁶ Finally, studies using ³³P-d(T)₁₆-CTAB-PIBCA nanoparticles to deliver ON to mice indicated that ON stability was improved compared with free ONs in both plasma and liver.¹⁰⁷ Additionally, while plasma half-life (*t*_{1/2}) was not significantly altered, distribution of ONs, when encapsulated by nanoparticles, was shifted to the liver and out of other organs.

Nanoparticle encapsulation can increase *in vitro* and *in vivo* cellular uptake of ONs with little cytotoxicity, while increasing *in vitro* ON nuclease stability. Although cell uptake and presumably activity are limited by endosomal sequestration, chemical modifications to nanoparticles to improve cytosolic and nuclear delivery of ONs *in vivo* may be possible. In addition, nanoparticles may be an advantageous delivery system for targeted delivery of ONs to the liver.

Recently, the use of microparticulate delivery systems has been investigated for sustained-release delivery of ONs.^{108–110} Microparticles or microspheres are micron-sized delivery systems typically composed of the biodegradable polymer poly(D,L-lactide-co-glycolide) [P(LA-GA)]. The advantage of microsphere formulation is slow release of encapsulated drug. For example, De Rosa et al. investigated the cellular uptake of poly dT_(4–16) ONs encapsulated in 30–60 μM P(LA-GA) microspheres in HeLa cells.¹⁰⁸ Release of ON from microspheres was triphasic, indicating an initial burst phase, a slow-release phase occurring over ~15 days, and eventually bulk erosion of the microsphere. Additionally, both encapsulation efficiency and release rate of ON could be controlled by the addition of polyethylenimine (PEI). Interestingly, confocal microscopy of HeLa cells incubated with ON, ON/PEI, or microsphere-encapsulated ON/PEI demonstrated increased cytosolic and nuclear delivery of rhodamine-labeled ON. The authors proposed that the extracellular controlled release of ON from microspheres prevented saturation of pinocytotic uptake, resulting in increased cytoplasmic accumulation and

nuclear delivery of ONs. Similar results have been demonstrated following encapsulation of *c-myc* or *tat* PO-ONs in 10–20 μm P(LA-GA) microspheres.¹¹⁰ The authors found that ON release was triphasic, with an initial burst phase during the first 48 h and microsphere degradation after 25 days when microspheres were incubated in physiologic buffer. In addition, the stability of the ONs was significantly improved in the presence of serum, yet ON-RNA hybridization was not hindered by microsphere encapsulation. Therefore, the development of microsphere delivery systems for ONs may not only improve cellular delivery of ONs, but will minimize the need for multiple dosing of ONs.

Physical Methods

In addition to molecular- and chemical-based strategies for delivering ONs into cells, physical methods of delivery have also been reported. Such delivery methods utilize the physical forces of electricity, pressure, and sound.

Electroporation and Iontophoresis

Electroporation is a delivery technique that utilizes high intensity electric fields to destabilize lipid bilayers, thereby permeabilizing cell membranes and allowing for increased transport of drug or ionized drugs (iontophoresis) into target cells. Electroporation has been successfully used to enhance the transdermal delivery of ONs^{111–116} and deliver ON in *ex vivo* and *in vivo* studies.^{114,117} Zewert and co-workers successfully increased transdermal delivery of *c-myb* and *c-myc* antisense-ONs through heat-stripped stratum corneum using electroporation.¹¹⁵ Increases of 6- to 12-fold in *c-myb*- and *c-myc*-ON transport, respectively, were observed when a 1.1-ms pulse of 80 V was applied every 5 s for 1 h compared with pre-pulsed ON transport.¹¹⁵ Transport occurred as a result of electrophoresis through the skin, rather than through preexisting pores or hair follicles.

Electroporation has been applied successfully to the *in vivo* delivery of *bcl-2* antisense-ONs in the rat.¹¹⁴ Application of 0.5 A through a 0.3–0.6-cm section of rat liver *in vivo* resulted in selective transfer of liposome-encapsulated ON to the liver within the target zone, yielding an increased apoptosis of precancerous cells. Therefore, delivery of anticancer ONs in conjunction with surgical removal and/or exposure of cancerous tissue may

prove to be an effective combination treatment. In addition to *in vivo* experiments, *ex vivo* treatment of bone marrow with *c-myc* antisense-ON and electroporation has been investigated for the purging of bone marrow contaminated with cancer cells for autologous transplantation.¹¹⁷ Studies in mice have shown an immediate decrease in *myc* protein in ON-treated samples, rapid and selective cell death of cancerous cells, and little observed effect on hematopoietic reconstitution. The combined results from the literature suggest that electroporation will potentially be an effective method for delivery of ONs via the transdermal route and may facilitate selective ON delivery either *in vivo* or *ex vivo*.

Pressure-Mediated Delivery

Application of pressure to “naked” ON delivery systems has been investigated both *in vitro* and *ex vivo* to inhibit bypass graft and cardiac allograft rejection.^{118,119} Studies in rat carotid arteries have demonstrated a pressure-dependent uptake of FITC-ON.¹¹⁹ Using an infusion pressure of 760 mmHg, ~84, 64, and 92% neointimal, medial, and adventitial cell nuclei, respectively, were positive for FITC-ON after 4 days. In an *ex vivo* experiment, excised rat hearts were submersed in an ON solution followed by injection of anti-IL-6- or ICAM-1-ON solution into the coronary circulation under 0.5–2 atm of pressure above ambient pressure.¹¹⁸ The hearts were then transplanted and harvested 90 days postoperatively, and levels of IL-6 or ICAM-1 were measured. A maximal uptake efficiency of 53% was observed when ON was applied both to the myocardial surface as well as into the circulation for 30 min at 2 atm, as already described, whereas only 3.5% uptake occurred when the ON was applied under ambient pressure. No neutrophil infiltration, edema, or histological changes were observed, but microscopy of myocardial cells suggested that uptake bypasses endosomal sequestration, resulting in direct transport of ON to the nucleus. Although ON delivery into cells and/or tissues was improved using pressure-mediated delivery, the need for a closed system under pressure may limit the technique to *ex vivo* procedures.

Shockwaves

The use of shockwaves, acoustic high-energy pressure pulses, as a novel method of cytoplasmic ON delivery has recently been introduced.¹²⁰

Shock waves are delivered through a fluid medium, focused on a targeted area of delivery. Sequential positive and negative pressure waves cause air bubbles and secondary shock wave emission resulting in transient pore formation in cell membranes (cavitation). A lithotripter (80 nF, 1 Hz, 25 kV) was used to deliver shockwaves to PBMC and HeLa cells in the presence of 50 μ M anti-TNF α -ON. Following the shockwave, cells were washed and placed under 3 bar of pressure. FITC-ON uptake was measured by flow cytometry and fluorescence microscopy, and TNF α production was measured by ELISA. Cell viability remained >95%. Cell uptake was dependent on the number of shockwaves and ON concentration. ON distribution in the cell was initially homogenous, followed by accumulation in the nucleus. At 50 μ M ON and using 250 shockwaves, up to 62% inhibition of TNF α was observed compared with cells treated with ON in the absence of shockwaves. Comparatively, electroporation resulted in spotty fluorescence on the cell membrane, with little nuclear accumulation and lower *in vitro* ON activity. Shockwaves therefore may be an advantageous delivery mechanism because non-endosomal delivery, the level of which can be adjusted by altering the concentration of ON, is achieved. Additionally, delivery is reproducible and can be applied to various cell types. Although the high volume of ON solution required for the technique may somewhat limit its widespread applicability, improved *in vivo* gene transfer into mouse melanoma cells has been demonstrated by administration of 800 shockwaves following injection of air (10% tumor volume) to improve acoustic cavitation. Therefore, shockwave-mediated delivery may potentially be a successful method for ON delivery.¹²¹

Ultrasound

Ultrasound is an acoustic technique similar to shockwaves but one that uses higher frequencies (MHz versus Hz) and shorter overall application times (seconds to minutes). Recently, the use of ultrasound as an enhancer of DNA transfection in gene therapy has been investigated.^{122–124} In *in vitro* experiments, ultrasound waves were transmitted through cell culture flask walls, permeabilizing cell membranes and allowing DNA to enter the cell. At 1-MHz continuous wave frequency, primary rat fibroblasts or chondrocytes were transiently transfected with lacZ or neo gene-containing plasmids at an efficiency of

up to 2.4%.¹²² Additionally, if the duration of 1 MHz ultrasound treatment was <60 s, little decrease in cell viability was observed.¹²³ Huber et al. compared shockwave and ultrasound in the transfection of β -gal and luciferase expressing plasmids into HeLa cells.¹²⁴ Luciferase expression was maximal following 100 Hz ultrasound for a duration of 2 min under 1.5 mPa pressure. This treatment resulted in 3% transfection of viable cells, which is about a 5-fold increase over transfection in the absence of ultrasound. In comparison, although shock-wave-mediated transfection increased as the number of waves delivered increased, only a 0.08% transfection rate was observed, partially due to a reduction in cell viability. Therefore, ultrasound may prove to be a more efficient and less toxic means than shockwaves of delivering DNA into cells. Future studies may prove that ultrasound can also be used to effectively deliver shorter DNA strands, like ONs, into cells or tissues whether *in vitro* or *in vivo*.

CONCLUSIONS

In vitro uptake of naked ONs into cells is highly inefficient and necessitates novel delivery methods. In addition, ON endosomal sequestration is commonly observed, limiting ON concentration at the target site in the cytosol and/or nucleus. This review has described various standard and novel techniques currently in use for the enhancement of ON delivery into cell culture. Several of the techniques (electroporation, liposomes, nanoparticles, pressure, and dendrimers) have also been investigated *in vivo* as potential delivery systems for therapeutic application of ONs. In addition, delivery methods that bypass or destabilize the endosome may prove to be most useful because endosomal escape is a major limiting factor for drug accumulation at the target site.^{28,74,83} Significant progress has been made in the area of ON drug delivery and, although questions remain regarding toxicity, stability, and optimization of the various delivery systems, the therapeutic use of such systems may prove to be necessary for the effective use of ONs in clinical settings.

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