Introducing Antisense Oligonucleotides into Cells

Contents

1. Introduction

The choice of sequence and chemistry of the antisense oligonucleotide is crucial to the success of any given experiment [1]. In addition to choosing the optimal chemistry, the researcher must decide upon a target site within the gene of interest. This entails looking at the possible cross-reactivity with non-target genes, as well as considering the conformational and thermodynamic characteristics of both the site and the probe. In addition, the investigator must carefully check the antisense oligonucleotide to make certain it doesn't contain sequences that might evoke biological responses unrelated to the down regulation of the targeted gene.

A successful antisense experiment depends on the following characteristics [2]:

- Unique DNA sequence
- Efficient cellular uptake
- Minimal nonspecific binding
- Target specific hybridization
- Non-toxic antisense construct
- Nuclease resistant chemistry to protect the antisense oligonucleotide (ASO)
- Minimal inflammatory or immune response
- Demonstration of reduction in target mRNA
- Appropriate controls

Once the antisense sequence is specified, the investigator must then design the controls. It is important to assess any unforeseen effects of the antisense oligonucleotide. The researcher must also consider the best way to deliver the probe to the internal workings of the cell where the probe can down regulate the gene of interest.

2. Controls

Experienced researchers have learned that antisense oligonucleotides can incite unexpected biological and pharmacological effects. These effects highlight the need for good controls. Researchers wishing to use antisense as a tool are not always aware of the pitfalls and, thus, many studies are still poorly controlled. The behavior of an oligonucleotide in a cell or other biological environment may be influenced by its base composition and by global properties such as conformation. No perfect control exists; therefore multiple controls should always be used [3]. Controls are divided into four types:

- Oligonucleotide sequences that maintain structural features, such as hairpins, but have different base compositions from the antisense.
- Scrambled oligonucleotide sequences that have the same base compositions as the antisense but not the same structural features, such as hairpins.
- Antisense oligonucleotide sequences with one or more mismatches to show whether the target is selectively hybridized.
- Use of cell lines in which the target gene is mutated or deleted or rescue of phenotype with transfection of a codon-varied version of the target gene that the ASO cannot target.

Using a variety of controls strengthens confidence in interpreting antisense experiments. Moreover, results will have the greatest believability when the levels of the target RNA and its expressed protein are directly measured.

3. Oligonucleotide Uptake

Oligonucleotides may be introduced to cells by a variety of methods:
- Receptor-mediated endocytosis
- Microinjection
- Cationic Lipids/Liposomes
- Electroporation
- Other methods of facilitated entry
- *In vivo* delivery systems

3.1 Receptor-mediated endocytosis

Receptor-mediated endocytosis occurs when the plasma membrane of a cell buds inward to form a vesicle which contains receptor sites. Molecules from outside the cell specifically bind to these receptor sites and are internalized into the cell. Oligonucleotide entry into living cells has been shown to occur through receptor-mediated endocytosis with small oligonucleotides taken up more rapidly than long oligonucleotides. This uptake can be competitively inhibited by other small oligonucleotides, especially if they contain a 5' phosphate. Uptake is temperature-dependent, occurring more rapidly at 37°C than at 4°C. Modifying the oligonucleotide can change its uptake efficiency. Tissue culture cells bind and internalize phosphorothioate-modified oligonucleotides better than they do phosphodiester oligonucleotides or methylphosphonates [4]. Oligonucleotide uptake by this mechanism is inefficient and seems to deposit the oligonucleotides into nonnuclear intracellular compartments that are largely inaccessible to RNA [5]. As a general rule, it appears more effective to use methods to directly facilitate oligonucleotide entry into the cell. Some investigators have suggested that any effects shown by endocytosed oligonucleotides may have been induced through sequence-independent mechanisms [6].

3.2 Microinjection

Microinjection is the process whereby a micropipette is used to deliver materials into single living cells. Microinjecting oligonucleotides into cells results in rapid accumulation of the oligonucleotide in the nucleus [7, 8] . It is important to note that when delivering an oligonucleotide directly into cells, the researcher can limit toxicity by controlling the purity of the preparation. One method used to ensure purity for microinjection is to use a series of HPLC and gel filtration steps to further purify the oligonucleotide [9]. Unfortunately, microinjection cannot be used in many *in vivo* studies and can only treat a limited number of cells.

3.3 Cationic Lipids

Cationic lipids are positively charged lipids which interact with the negatively charged DNA and cell membranes. Through these interactions, they are able to internalize the negatively charged DNA into the cells. Cationic lipids are commonly used to facilitate the entry of a variety of compounds, including antisense oligonucleotides, into living cells [10]. In one series of experiments, the cationic lipid reagent Lipofectin (Invitrogen Life Technologies) was shown to enhance the entry of an 18-mer phosphorothioate oligonucleotide into HUVEC cells by 20-fold and to effect a 1000-fold increase in biological antisense activity. These results were obtained with an 8 μM concentration of Lipofectin; the reagent became toxic when increased to 16 μM.

Cationic lipids do not appear to directly transport DNA into the cellular cytoplasm. Rather, a complex multistage process occurs. First, a DNA:cationic lipid complex is internalized in an endosome. Anionic lipids on the cytoplasmic face of the endosome fuse with the cationic transfection agent, and the oligonucleotide is displaced into the cytoplasm [11]. Oligonucleotides that have been introduced through lipofectin-induced uptake are diffusely distributed in the cytoplasm and the nucleus [12]. This distribution presumably results in greater oligonucleotide bioavailability and a subsequent enhancement in the antisense effect [13-15].

Cationic lipids must be used with caution and with appropriate controls as cell growth is inhibited by DOTMA, the cationic detergent in Lipofectin, in a linear dose-dependent fashion in some cell lines. [16]. Cationic detergents may not be useful in promoting oligonucleotide uptake in all cell types. In one study, keratinocytes were found to take up phosphorothioate oligonucleotides equally well both with and without Lipofectin [17]. Lipid-based agents other than Lipofectin have been used to enhance oligonucleotide uptake into cells. Different cell lines and different forms of DNA (single-stranded oligonucleotides, circular plasmids, etc.) may each have a different optimal lipid agent. Further, different chemically modified forms of DNA may require re-optimization of the transfection procedure [18]. The precise agent used, the ratio of DNA to lipid, and the liposome complexation volumes may each require optimization [19]. In addition, not all lipid-based agents can be used in the presence of serum [20].

By encapsulating concentrated oligonucleotides in lipid vesicles, the oligos can be protected from attack by nucleases in serum. In this way, the lipid vesicles can support delivery of intact oligos into the cells. In one described method, cardiolipin, phosphatidylcholine, and cholesterol were mixed at a 0.5:10:7 molar ratio, and combined with an antisense oligonucleotide. This formulation resulted in a final product that contained 60–70 μg oligonucleotide/mg lipid. Natural phosphodiester oligonucleotides are stable in serum for days when encapsulated in such liposomes and liposomal delivery increased oligonucleotide uptake about 20-fold into MOLT3 cells and resulted in diffuse cytoplasmic and nuclear localization [21].

Even with the use of lipid agents to promote transfection, oligonucleotide entry into individual cells can vary dramatically. Vaughn et al. used a fluorescence-activated cell sorter to assess cell-to-cell variability [22]. Cells were incubated with fluorescein-labeled phosphorothioate antisense oligonucleotides targeting the ERBB2 message. Cells were later incubated with a cascade blue–labeled anti-ERBB2 antibody. Evaluation of dual-fluorescence spectra indicated that cells having higher levels of antisense oligonucleotide had much lower levels of ERBB2 protein. Potent antisense effect was observed with approximately 10^7 antisense molecules per cell.

3.3.1 Modified Liposomes
Liposomes can be made for this application by a number of slightly different techniques [23]. Moreover, liposomes can be modified in a number of ways that enhance their ability to deliver nucleic acids into living cells. The addition of certain viral coat proteins can increase the efficiency of fusion and uptake several fold. Polyoma virus pseudocapsids have been shown to be efficient carriers of nucleic acids into mammalian cells. These transfection vehicles contain pure viral VP1 protein produced *in vitro* using the baculovirus insect cell system and are free of other viral proteins and genetic material. Their use can enhance delivery of transfected DNA many fold over unmodified liposomes [24].

It is also possible to take advantage of natural import pathways to introduce foreign material into living cells. Vitamins such as folic acid are actively imported in this way [25]. Folate-binding protein (FBP) resides in the cell membrane and is responsible for the active uptake of folate via

receptor-mediated endocytosis. This transport system is very efficient and has a K_d in the nanomolar range. If liposomes are tagged with folate using a long 250-Å polyethylene glycol (PEG) spacer, steric hindrance is minimized and the liposomes are imported [26]. PEG addition also seems to prolong liposome survival *in vivo* by interfering with their clearance by the reticuloendothelial system. Oligonucleotides packaged into liposomes and delivered to cells are protected from serum nucleases. Unmodified phosphodiester oligonucleotides encapsulated in folate–PEG liposomes have been shown to invoke antisense activity equivalent to that of similarly encapsulated phosphorothioate-modified oligonucleotides.

3.3.2 Available at IDT: TriFECTin™ Transfection Reagent

TriFECTin is a proprietary cationic lipid formulation that has been optimized for delivery of IDT's Dicer-Substrate siRNAs into a wide variety of cell types with minimal toxicity. For more information and to order TriFECTin, please visit the IDT website.

3.4 Electroporation

Electroporation is a process used to transform or transfect a wide variety of cell types through the use of an externally applied electrical field. The charge affects the permeability of the cell plasma membrane which allows for the entrance of the intended insert. Electroporation is efficient at delivering a large amount of nucleic acid to a cell. It is a useful procedure for delivery to cells which are difficult to transfect. Electroporation requires a large amount of delivered material (typically 500 nM – 2 ⌷M) in order to work efficiently. The process does introduce an increased risk of toxicity to the cells which must be treated very carefully following the applied electrical field in order to survive.

3.5 Other Methods of Facilitated Entry

- Pretreatment of cells with streptolysin *O* led to a 100-fold increase in oligonucleotide permeation with minimal cellular toxicity [27].
- Cell Penetrating Peptides (CPPs): The attennapedia homeodomain protein is translocated through cell membranes and targeted to nuclear localization. A 16 amino acid peptide fragment from the third helix has been shown to confer this property to the protein [28]. Investigators have used this peptide coupled to an antisense oligonucleotide to facilitate direct entry of the oligonucleotide into the nucleus, giving high efficiency of penetration with low dosing. Use of this agent was compatible with serum-containing tissue culture medium and seemed to protect the oligonucleotide from nucleolytic degradation which enabled the use of phosphodiester DNA as an antisense agent [29]. This peptide is commercially available with a sulfhydryl-modifier enabling its attachment to a SH-modified oligonucleotide (Penetratin by Qbiogene).
- Small molecule tags used to modify oligonucleotides improve their uptake efficiency. These types of tags include cholesterol-modification, membrane-permeant peptides, folate, antibiotics, VITE, and VITA [30].
- Cationic polymers can bind to large nucleic acids and condense them into stable nanoparticles and can, thus, serve as efficient transfection agents.

3.6 *In vivo* delivery systems

- Proteins derived from the coat of Sendai viruses are known to promote fusion of lipid bilayers. In one series of experiments, oligonucleotides were packaged in liposomes which were complexed with coat proteins derived from the hemagglutinating virus of Japan (HVJ, a Sendai family virus) and were infused into rat carotid arteries where they fused with the vascular endothelium and neointima. The oligonucleotides were able to curtail intimal hyperplasia following vascular injury [31, 32].
- It is possible to use fluorescein-conjugated antisense oligonucleotides to track their fate *in vivo*. Oligonucleotides delivered via liposome encapsulation can be detected in the nucleus for up to two weeks after administration [31].
- Antisense uptake into tissues in living organisms using intravenous, subcutaneous, or intraperitoneal injections has been higher than expected based on previous experience with cell culture [33].
- Certain tissues are accessible to topical or localized administration of siRNA including the eye, mucus membranes, and local tumors [30].
- Phosphorylated DNA antisense oligonucleotides have appreciable uptake *in vivo*.

4. Conclusion

Antisense technology represents a powerful technology for functional genomics and rational therapeutics. A number of parameters need to be considered when designing Antisense experiments including oligonucleotide sequence, structure and purity, secondary structure of the target RNA, mechanism of action, and the process chosen to introduce the Antisense oligonucleotide into the cell. Cellular uptake of phosphorothioate oligonucleotides is highly variable depending on a number of conditions including cell type, kinetics of uptake, tissue culture conditions and oligonucleotide chemical class [34, 35]. Conclusions about *in vitro* uptake must be made carefully and generalizations are impossible [34].

5. References

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