Antisense Technology: Oligonucleotides and its Delivery Strategies

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ABSTRACT

Aim: The present study highlights antisense technology where oligonucleotides have predominant role in its delivery for various diseases. Objective: Antisense compounds are biological molecules consisting of small ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) segments (Oligonucleotide) which have enormous potential for the treatment of number of diseases. Methods: All the methods analyzed were obtained through high impact articles. Results: Several impediments were made to Oligonucleotide for its widespread usage as them as drugs to overcome their lack of stability in physiological fluids and their poor penetration into cells. Experiments like, association with or encapsulation within nano and micro sized drug delivery systems, lipids and polymers could help to increase the efficiency of oligonucleotide delivery. This review discusses the use of cationic lipids, Cell penetrating peptides, nanoparticles, microparticles and several novel methods that have recently been explored as delivery vehicles. Conclusion: The use of naked Oligonucleotide given a clear cut way to new targets for potential therapeutic compounds. Apart this, antisense therapy represents a promising and evolving approach to the management of numerous diseases in which a specific abnormality has been identified as a primary or major contributing etiologic factor. In near future, authors strongly believe that antisense therapy will give a positive way for number of In vitro experiments and break through for many life threatening diseases.

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Introduction

Basic medical and genetic research in recent years has shown many incurable diseases are caused by defective regulation of certain genes. This manifests as excessive or faulty synthesis of proteins that can trigger diseases and promote their progression. The use of antisense molecules to inhibit the synthesis of such pathogenic proteins represents an innovative therapeutic approach. Normally gene expression is the result of molecular changes of the protein. For this the cell must undergo two major complex processes like transcription and translations. During this process a copy of RNA (Ribonucleic acid) is made from DNA (Deoxyribonucleic acid) with the involvement of many enzymes like polymerase, helicase, exonuclease, ligase. These enzymes unwind the double helix base pairs of DNA and direct this to match with RNA base pairs with help of single stranded binding protein, results heterogeneous nuclear RNA (hnRNA). This particular hnRNA spliced, Protected from cellular environment and shifted from nuclear membrane to cytoplasm, immediately triggers the protein production by hooking up with ribosomes. Nucleic acid is termed an "anti-sense" because of its base sequence is complementary to the gene's messenger RNA (mRNA), which is called the "sense" sequence (so that a sense segment of mRNA $5'$-AAGGUC-3' $ would be blocked by the anti-sense mRNA segment $3'$-UUCCAG-5' $). The use of antisense oligodeoxynucleotides (ODNs) both in research and therapy have emerged as powerful alternative or complement to small molecule inhibitors employing traditional drug design strategies. ODNs are short pieces of synthetic and chemically modified DNA designed to hybridize to specific mRNA sequences. They inhibit gene expression mainly through RNase-H activation or hybrid arrest, steric blockage of translation. They are being explored as potential therapeutic tools against viral infections, cardiovascular, inflammatory and hematological diseases and cancer. Occasionally, a bad mRNA molecule is synthesized so that the resulting protein cannot function properly. Abnormalities of proteins cause many diseases that afflict humans. Therefore, it seems logical to conclude that if the expression of these malfunctional proteins (Figure 1) could be stopped, the sources of disease would be obliterated and the disease will be treated, if not cured.

The first report of usage of this particular antisense oligodeoxynucleotides to inhibit Rous sarcoma virus gene expression, there has been tremendous progress in the understanding and application of antisense oligodeoxynucleotides. However in this modern world antisense technology has become an essential laboratory tool to study and understand the functions of any newly discovered genes. This antisense approach should allow the design of drugs that specifically intervene with the expression of any gene whose sequence is known by that it will be more convenient for the treatment for genetic disorders or infections. When the genetic sequence of a particular gene is known to be causative of a particular disease. It is possible to synthesize a strand of nucleic acid (DNA, RNA or a chemical analogue) that will bind to the messenger RNA (mRNA) produced by that gene and inactivate it, effectively turning that gene "off". This is because mRNA has to be single stranded for it to be translated. Alternatively, the strand might be targeted to bind a splicing site on pre-mRNA and modify the exon content of mRNA. Antisense drugs are being researched to treat a variety of diseases such as cancers including lung cancer, colorectal carcinoma, pancreatic carcinoma, malign-antglioma and malignant melanoma,
diabetes, amyotrophic lateral sclerosis (ALS), Duchenne muscular dystrophy and some other diseases such as asthma, arthritis and pouchitis etc. Most potential therapies have not yet produced significant clinical results, though two antisense drugs have been approved by the U.S. Food and Drug in istration (FDA), fomiviren (marketed as Vitavene) as a treatment for cytomegalovirus retinitis and mipome rsen (marketed as Kynamro) for homozygous familial hypercholesterolemia. Most potential therapies have not yet produced significant clinical results, though two antisense drugs have been approved by the U.S. Food and Drug in istration (FDA), fomiviren (marketed as Vitavene) as a treatment for cytomegalovirus retinitis and mipome rsen (marketed as Kynamro) for homozygous familial hypercholesterolemia. Most potential therapies have not yet produced significant clinical results, though two antisense drugs have been approved by the U.S. Food and Drug in istration (FDA), fomiviren (marketed as Vitavene) as a treatment for cytomegalovirus retinitis and mipome rsen (marketed as Kynamro) for homozygous familial hypercholesterolemia. Most potential therapies have not yet produced significant clinical results, though two antisense drugs have been approved by the U.S. Food and Drug in istration (FDA), fomiviren (marketed as Vitavene) as a treatment for cytomegalovirus retinitis and mipome rsen (marketed as Kynamro) for homozygous familial hypercholesterolemia.

Antisense oligonucleotides- chemistry

In general, to target the total pool with in the cells, short and hybridize unique sequence which containing short oligonucleotides (13-15 nucleotides) were considered. These short nucleotides are out product (as unmodified or chemically modified) of single stranded DNA molecules. This synthesis was direct towards 3'5' direction. Product such as phosphodiester oligonucleotides which may not be used in antisense experiments due to its unidirectional functions on key proteins which results in cell growth inhibitions. By many chemical modification methylphosphonates oligonucleotides (First Chemically synthesized) were synthesized by the replacement of the methyl group in the place of non bridging oxygen atom which has an excellent stability in biological system. Even though, due to many limitations, application of phosphodiester oligonucleotides was minimized or better to say as restricted in antisense technology. The phosphorothioates are the most widely studied oligonucleotides, because of their nuclease stability, ease of synthesis. In this particular oligonucleotide non-bridging oxygen atom was replaced by sulphur at each phosphorus group. By that it attains oligonucleotide chain with high solubility and excellent antisense activity. During last two decades, it was clearly evidenced by publishing many data’s which stands as a key setup to generate antisense effects in tissues cultures, both in In vivo and In vitro, driven latter in to clinical therapeutic trials, naming few among this are G3139, an 18-mer which were targeted as initiation codons of the bcl-2 mRNA (now being evaluated clinically in melanoma, chronic lymphocytic leukemia, and other tumors) and ISIS 3521(lung carcinoma). The main mechanism is known to induce sequence-independent effects attributable to its length dependent high affinity for various cellular proteins, especially heparin-binding growth factors, such as acidic fibroblast growth factor, basic fibroblast growth factor, platelet-derived growth factor, vascular endothelial growth factor, and a host of other heparin-binding molecules, such as laminin, fibronectin, and Mac-1. Further these phosphorothioates will continue to be extensively and exclusively used in the clinical trial settings. Peptide nucleic acid (PNA, s) are the another excellent outcome product of basic oligonucleotide modifications. This contains nucleic acid analogous in uncharged, flexible, polyamide back bone which is comprised of repeating N-(2-aminoethyl) glycine units. To this glycine, via methylene carbonyl linker’s nucleobases are attached. These oligomers will form very stable duplexes or triplexes with nucleic acids, i.e with double or single-strand DNA or RNA. Morpholino oligomer is another compound which has several positive factors to claim as antisense compound. In this deoxyribose moiety is replaced by a morpholine ring and was linked by the uncharged phosphorodiamidate linkage instead of charged phosphor di-ester inter subunit linkages. By this these compounds attains maximum stability over biological system and exhibit efficient antisense activity in cell free translation systems. This system was strengthened by using scrap loading
technique and permeation with streptolysin-O, thus facilitating the oligonucleotide penetration with increases antisense effect. Another example of a “second-generation” oligonucleotide is the N3’-P5’ phosphoramidate (PN), which result from the replacement of the oxygen at the 3’ position on ribose by an amine group. These oligonucleotides can, relative to form very stable complexes with RNA and single- or double-stranded DNA and can exhibit highly selective and specific antisense activity in vitro and in vivo. It clearly demonstrated by the experiment in mice treated with 900µg/day of oligonucleotide were survived for the weeks of 30 were as the control mice treated with mismatched oligonucleotide has survived only for 7 weeks.

Mechanism of action of Antisense compounds

The concept behind antisense technology is quite straightforward: the use of a sequence, complementary by virtue of Watson-Crick base pairs hybridization. These Oligonucleotide are modulated to a specific mRNA which can inhibit its expression and then induce a blockade in the transfer of genetic information from DNA to protein, but mechanism of induction of its biological effect is subtle and complex. On basis of mechanism of action oligonucleotides can be discerned in to two types: (a) degradation of mRNA by RNase H-dependent oligonucleotide; (b) the steric-blocker oligonucleotides, which physically prevent or inhibit the progression of splicing or the translational machinery.

Delivery of Oligonucleotides

In order for an antisense oligonucleotide to gene expression, it reaches targeted cells effectively. As such to date, the precise mechanisms involved in oligonucleotide penetration is not clear. Uptake is influenced by many factors like active transport, which in turn depends on temperature, the structure and the concentration of the oligonucleotide, and the cell line used. At the present time, it is strongly believed that high concentration of oligonucleotide having influence on its internalization which depends upon the major mechanism of adsorptive endocytosis and fluid phase pinocytosis. In case of low concentration internalization is initiated via interaction with a membrane-bound receptor and to be observed as poor strategies over the effector site.

Need of Developing Delivery systems:

The major limiting step in antisense compounds application is the inefficient delivery of them to cells and poor bioavailability to intracellular targets due to rapid degradation by nucleases, cellular uptake and subsequent intracellular trafficking. The poly-anionic nature and the large size of antisense compounds render them practically impermeable to cell membranes and consequently their biological activity is significantly compromised. So, it creates an demand to develop novel antisense delivery system towards exits one. Although viral vectors have been widely used to transfer genetic material into cells they bear an inherent risk for the patient to encounter severe immunological responses or even develop other deceases. As a result of these problems, much attention has been paid in recent years to develop non-viral delivery systems. Among different non-viral systems- cationic polymers, cationic liposomes, polymeric nanoparticles and cell-penetrating peptides (CPPs) represent an attractive concept to bypass the problem of poor membrane permeability of charged macromolecules.

Delivery strategies of Oligonucleotides

The aims for optimal delivery of antisense compounds are therefore to
enhance cellular uptake and improved to exit from sub-cellular compartments as correct targeting (spatial and temporal) to a particular site of action. On based on this following are important non viral drug delivery forces and described in brief.

**Poly Cations**

Polycations and polyanions are poly electrolytes. These groups will dissociate in aqueous solutions (water) and make the polymers charged. Charged molecular chains, commonly present in soft matter systems plays a fundamental role in determining structure, stability and interactions of various molecular assemblies. Poly cationic water-soluble block copolymers consisting of polyoxyethylene (PEO) and polyspermine (PS) chains have been developed for the delivery of antisense oligonucleotides (Figure 2) into the target cells. Cationic polymers constitute one of the most promising approaches to the use of viral vectors for gene therapy.

**Liposomes**

Liposome is vesicular structures consisting of hydrated bilayers. This structures used for pharmaceutical purposes with phospholipids as back bone. Liposomes could also be effective delivery systems for DNA and for nucleic acid-based therapeutics such as antisense oligonucleotides (Figure 3) and siRNA. There are several new methods of liposome preparations are available which are based on lipid drug interaction and liposome disposition. Mechanism involved is inhibition of rapid clearance of liposome by controlling particle size, charge and surface hydration. The liposomes are characterized with respect to physical, chemical and biological parameters. This mode of drug delivery lends more safety and efficacy to administration of several classes of drugs like antiviral, antifungal, antimicrobial, vaccines, antitubercular drugs and gene therapeutics. Present applications of the liposome deliveries are in immunology, dermatology, vaccine adjuvant, eye disorders, brain targeting, infective disease and in tumor therapy. The new development in this field is specific binding properties of a drug-carrying liposome to a target cell such as a tumor cell and specific molecules in the body (antibodies, proteins, peptides etc.). A number of general principles have emerged from the large and rapidly growing literature in the field of nucleic acid delivery. Liposome’s are considered very promising delivery systems for antisense therapeutic approach, offering drug protection and facilitating oligonucleotide cell internalization. Liposomes are the most widespread non-viral carriers for nucleic acid delivery. Although anionic and neutral liposomes have been studied for ODN delivery, their poor nucleic acid entrapment efficiency has limited their uses.

**Cationic Lipids**

Writhing Delivery of oligonucleotides and genes to their intracellular targets is a prerequisite for their successful use in medical therapy. Recently a novel group of cationic amphiphiles has afforded protection to the nucleic acids has been elucidated. Cationic liposome’s are another most commonly used and promising delivery systems for oligonucleotides and genes. These entrap nucleic acids efficiently through formation of complexes which are called lipoplexes. Lipid fusion plays an important role in the cationic liposome-mediated delivery of these compounds. Fusion is involved in complex formation between the nucleotides and lipids lays in between extracellular materials with the complexes, as well as in the intracellular trafficking of the delivery system and its load. Since lipid fusion is such a crucial factor in polynucleotide delivery, its controlled use is
important for the success in oligonucleotide and DNA delivery\textsuperscript{55-58}.

Nanoparticles

In recent years, there has been a rapid increase in nanotechnology in the fields of medicine and more specifically in targeted drug delivery\textsuperscript{59}. Nanoparticles are also considered to have potential as novel intravascular or cellular probes for both diagnostic and therapeutic purposes (drug/gene delivery), which is expected to generate innovations and play a critical role in medicine. Target-specific drug/gene delivery and early diagnosis in cancer treatment is one of the priority research areas in which nanomedicine will play a vital role. Major classes of nanoparticles include, drug conjugates and complexes, dendrimers, vesicles, micelles, core shell particles, microbubbles, and carbon nanotubes. Most of these formulations have been described as carriers of either drugs or contrast agents (Figure 4)\textsuperscript{60}. Nanoparticles can be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. Nanoparticles have been prepared most frequently by three methods: 1. dispersion of preformed polymers, 2. polymerization of monomers and 3. ionic gelation or coacervation of hydrophilic polymers. Other methods include supercritical fluid technology\textsuperscript{61} and particle replication in non-wetting templates (PRINT)\textsuperscript{62,63}.

Dendrimers:

Dendrimers can act as vectors, in gene therapy; these belong to a family of nanosized, three-dimensional polymers characterized by a unique tree-like branching architecture and compact spherical geometry in solution. Dendrimers are synthesized by a repetitive step-growth polymerization process. Intrinsic viscosity is another important characteristic that distinguishes dendrimers from more conventional polymers\textsuperscript{64}. The internal cavity of an appropriately designed dendritic structure could be used for the entrapment of drugs with the possibility of successive controlled release. Studies by several research groups have shown that the interior of a dendrimer is capable of encapsulating guest molecules. The first strategy for the entrapment of guest molecules in dendrimers is physical encapsulation. The second strategy for the encapsulation of guest molecules in dendrimers is based on multiple noncovalent chemical interactions, such as hydrogen bonding, between guest molecules and the dendritic arrangement. Monodispersity, polyvalency, biodegradability and non immunogenicity are the various significant properties for dendrimers which makes them more effective and useful in drug encapsulation\textsuperscript{65}.

Cell penetrating peptides:

The difficulty of delivering large molecules like proteins, peptides, and nucleic acids into cells through the cell membrane (cellular uptake) has proven a significant impediment to medicinal chemists and the pharmaceutical industry as a whole. The plasma membrane prevents direct translocation of hydrophilic macromolecules by acting as a barrier to efficient and controlled intracellular delivery. Thus, novel efficient carrier delivery methods have to be developed to impart good bioavailability of drug molecules (Figure 5). Peptides which are able to penetrate the cell membrane are known as cell-penetrating peptides (CPPs). They are generally 10 to 30 amino acid residues in length, and either arginine-rich, amphipathic and lysine-rich or hydrophobic\textsuperscript{66}. CPPs can be broadly classified as protein derived, chimeric (derived from two or more genes which are coded for separate proteins), or synthetic. CPPs share common features such as positively charged amino acids,
hydrophobicity, and amphipathicity. The discovery of CPPs’ ability to traverse the cell membrane opens up a new avenue for drug delivery. Attaching therapeutically significant biomolecules to CPPs provides a means to transport them across the cell membrane. A major breakthrough in the field was the delivery of peptide-nucleic acids (PNAs) using the chimeric peptide transportation.

Conclusion

Over the past two decades, the antisense oligonucleotide technology has emerged as a valid approach to selectively modulate gene expression in all the ways and means. By adhering to a strict set of specific rules, ongoing in vitro studies using antisense oligonucleotides have given a clear cut way to new targets for potential therapeutic compounds. Apart this, antisense therapy represents a promising and evolving approach to the management of numerous diseases in which a specific abnormality has been identified as a primary or major contributing etiologic factor. The number of in vitro experiments has increased continuously, and this has led to numerous therapeutic trials, a few of which now appear preliminarily to be positive. This review highlights the large diversity of particulate systems that have been designed for the delivery of antisense compounds. There are still many difficulties to be overcome before any of them can proceed into clinical trials. The development of ‘smart’ nanotechnologies able to control the interactions with biological fluids and to be recognized by target cells should be pursued. The research on alternative routes of administration other than parenteral and transdermal systems should also be extended. However, the optimal usage of antisense oligonucleotides requires the attention towards the effective design, in enhancing its efficacy towards its biological activity and pinpoint delivery. Authors strongly believe that current research towards the antisense therapy will be continued to shed light on ways to increase specificity, increase therapeutic and increase therapeutic efficacy.

Authors Contribution

All authors are equally contributed towards this manuscript in Conception, Design, Manuscript writing, Financial support, Administrative support, Collection of study materials, Data analysis and Interpretation.

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**Fig. 1** Basis concepts in antisense technology in protein modification.

**Fig. 2** Mechanism involved in cross-linked polycation and PEG network with oligonucleotides binding.
Fig. 3 Encapsulated Oligonucleotide with liposomes.

Fig. 4 - Chemically modified oligonucleotides and the development of Nanoparticles-linked oligonucleotide probes.
Fig. 5 - Oligonucleotide penetration mechanism through cell membrane and its release mechanism in cytoplasm.