Available online on 15.06.2020 at <http://jddtonline.info>

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

© 2011-18, publisher and licensee JDDT, This is an Open Access article which permits unrestricted non-commercial use, provided the original work is properly cited

Open Access

Review Article

Strategies and Approaches for siRNA Delivery

Soni Dhruvkumar M.*

Bombay College of Pharmacy, Santacruz (East), Mumbai, India: 400098

ABSTRACT

The present review discusses about RNA interference (RNAi) and its significance in gene therapy. The review mainly focuses on small interference RNA (siRNA) as a mediator of RNAi, its therapeutic benefits and various formulation strategies employed to overcome siRNA delivery hurdles. RNAi is a regulatory process which occurs endogenously within the cell wherein short double-stranded RNA (siRNA) effects sequence-specific posttranscriptional gene silencing. Even though siRNA assists researchers with its powerful therapeutic benefits, there are significant hurdles in developing efficient delivery systems for its systemic administration. These are extracellular and intracellular barriers for siRNA delivery. The present review addresses about pros and cons of gene therapy and superior advantages provided by siRNA over plasmid DNA in gene therapy. It also discloses about the discovery, mechanism of action, significance and applications of siRNA based gene therapies, challenges in its delivery and strategies for overcoming delivery hurdles. Furthermore, emphasis is provided on viral and non-viral vector based siRNA delivery and the significance of lipid based siRNA delivery, the lipoplexes over polymer based siRNA delivery - the polyplexes, followed by recent advances in siRNA based technologies directed against variety of diseases.

Keywords: Endosomal escape, gene therapy, lipoplexes, polyplexes, siRNA, vectors.

Article Info: Received 27 March 2020; Review Completed 19 May 2020; Accepted 28 May 2020; Available online 15 June 2020

Cite this article as:

Soni DM, Strategies and Approaches for siRNA Delivery, Journal of Drug Delivery and Therapeutics. 2020; 10(3-s):239-250. <http://dx.doi.org/10.22270/jddt.v10i3-s.4086>

*Address for Correspondence:

Dhruvkumar M. Soni, M. Pharmacy (Pharmaceutics), Bombay College of Pharmacy, Kalina, Sunder Nagar, Santacruz (East), Mumbai, India 400098

1. Gene therapy¹

Gene therapy is vital approach employed to treat diseases characterized by abnormal genetic function. Gene therapy cures dysfunctional cellular processes by either modifying the expression of a gene or by correcting an abnormal gene in the genome of a cell. Unlike traditional pharmaceuticals, gene therapy has the potential to treat almost any disease. Gendicine, a recombinant Adenoviruses-p53 gene therapy for head and neck squamous cell carcinoma was first approved as a commercial gene therapy medicine in 2003.^{2,3}

1.1 Need for gene therapy¹

There are varied types of diseases characterized by intracellular events that are controlled by specific gene transcription and mRNA translation. The alteration in these processes lead to the production of proteins which play vital role in inducing pathophysiological disorders. Best example that requires attention is production of Amyloid protein entanglements responsible for memory lost in Alzheimer's disease.⁴⁻⁶ Similarly, production of various intracellular messenger proteins inducing cell division play key role in neoplastic progression. Pathological proteins responsible for age induced macular degeneration⁷ and neoplastic

angiogenesis (vasoactive endothelial growth factors)⁷⁻¹³ are considered as macromolecules which are synthesized intracellularly during mRNA translation process. Hence, any change in the normal routine process whether in the transcription and/or translation produces drastic changes intracellularly which then lead to fetal abnormalities. Gene therapy is aimed at treating such pathophysiologies both at transcription and translation level with higher probability of success compared to conventional therapies.

1.2 Hurdles in gene therapy^{1,14}

There are many delivery hurdles that have prevented gene therapy from its widespread use for the effective treatment of various diseases. Some of these hurdles are listed as under:

1.2.1 Limited membrane permeability of nucleic acids:

Since DNA, siRNA, and other nucleic acids are hydrophilic and negatively charged macromolecules, they cannot efficiently cross cell plasma membrane which is essentially composed of hydrophobic lipid bilayer and typically have a net negative charge. While naked DNA can be introduced into cells through physical methods such as Electroporation,^{15,16}

a gene gun,¹⁷ ultrasound,^{18, 19} or direct injection into target tissue,²⁰ the clinical relevance of these methods is limited.

1.2.2 Short-lived nature of gene therapy:

Before gene therapy can become a permanent cure for a disease, the therapeutic RNA introduced into target cells must remain functional and the cells containing the therapeutic nucleic acid must be long lived and stable. Problems with integrating therapeutic nucleic acid into the genome and the rapidly dividing nature of many cells prevent gene therapy from achieving long-term benefits.

1.2.3 Immune response:

When any xenobiotic (foreign material viz. siRNA) is introduced into human tissue, the immune system attacks the invader, often reducing gene therapy effectiveness. Furthermore, the immune system's enhanced response to previous treatments makes it difficult for the repeat gene therapy in same group of patients.

1.2.4 Multigene disorders:

Conditions or disorders that arise from mutations in a single gene are the best candidates for gene therapy. Unfortunately, some of the most commonly occurring disorders such as heart disease, arthritis, diabetes, vasculature, and alzheimer's diseases are caused by the combined effects of variations in many genes. Multigene or multifactorial disorders such as these would be difficult to treat effectively today using gene therapy.

2. Birth of siRNA

2.1 Historical development of RNA interference:

The phenomenon of small interference RNA was first observed in plants in 1989.²¹ Post-transcriptional gene silencing by RNA interference, popularly known as RNAi, is a phenomenon that is exploited by cells to conduct gene regulation. The uniqueness and impact of this discovery can be judged from its mechanism²² and the fact that the initial discovery was done in the 1990s. In 1998, "breakthrough of the year" declared by the Science magazine and the Nobel

Prize for Medicine or Physiology was jointly awarded to its discoverers, Andrew Fire and Craig Mello in 2006.

2.2 Definition

RNA interference is the phenomenon by which post translational potency of messenger RNA is lost by silencing action of complementary single stranded RNA. Mediators of RNA interference are small interfering RNA (siRNA),²³ microRNA (miRNA),²⁴⁻²⁸ Short hairpin (shRNA). However, shRNA produces more stable transfection as compared to siRNA.

3. Molecular mechanism of small interference RNA in gene silencing⁷

In mammalian cell, siRNA is generated by the cleavage of larger double-stranded RNA (dsRNA) precursors by the RNase III endonuclease Dicer.²⁹ Dicer is complexed with the TAR-RNA binding protein (TRBP) and hands off siRNA to the RNA-induced silencing complex (RISC). RISC contains the protein that carries out the silencing activity by cleaving the target mRNA molecule between bases 10 and 11 relative to the 5' end of the antisense siRNA strand. In the core of RISC lies the Argonaute (Ago)³⁰ family members and in humans, Ago-2 (previously known as eIF2C2) carries out the catalytic cleavage activity. It has been observed that although the siRNA transferred to RISC are double stranded, Ago-2 cleaves and releases the "passenger" strand, leading to an activated form of RISC that contains a single-stranded "guide" RNA molecule. It is this guide RNA molecule that confers the specificity to the RISC and helps it in target recognition by intermolecular base pairing. Ago-2 is composed of three domains: PAZ, MID, and PIWI31 domains. The function of PAZ and MID domains are docking and anchoring the RNA. PIWI domain is the one with slicer activity. Messenger RNA molecule that displays perfect or near-perfect complementarity to the guide RNA are recognized and cleaved by Ago-2. Partial complementarity between a siRNA and target mRNA may in some cases repress translation or destabilize the transcripts if the binding mimics microRNA (miRNA) interactions with target sites (Figure 1).

Figure 1 Molecular Mechanism of Gene Silencing By siRNA³²

4. Therapeutic benefits of siRNA compared to conventional gene therapy (plasmid DNA)^{7, 33}

As the process of RNAi interferes with translation and not with DNA transcription, siRNA may not interact with chromosomal DNA. This lack of DNA interaction greatly reduces concerns about possible adverse gene alteration that might result from DNA-based gene therapy. The interaction of siRNA with mRNA and not with protein molecules also makes it possible to reduce the production of harmful proteins. siRNA is considered to be more potent as only a few siRNA molecules per cell are required to produce effective gene silencing. siRNA activation takes place in the cytoplasm and hence siRNA only needs to cross single barrier of cell membrane unlike viral vectors which have to cross multiple barriers such as nuclear membranes to reach cell genome. Moreover, conventional targets of traditional chemical drugs have been limited to certain classes of receptors, ion channels, and enzymes. Plasmid DNA can transfect during cell division phase only. However, siRNA can transfect cells both during cell resting and dividing phases, thus making it more effective.

5. Applications of siRNA therapy

5.1 Genetic diseases:

siRNA provides great promise for successful treatment of a large number of genetic diseases. Preliminary studies revealed single nucleotide polymorphism^{4, 34} in mutant allele transcripts can be used as effective targets for RNAi. One of the challenging targets for siRNA therapeutics is the disease causing polyglutamine proteins encoded by the CAG repeat containing transcripts. Such repeats are found in several neurological diseases. Recently, siRNA has been systematically tested for their ability to discriminate wild type from mutant alleles for disorders related to superoxide dismutase (SOD1) and huntingtin (HTT).

5.2 Viral diseases:

Initial application of the RNAi technology had been directed in the treatment of infections caused by hepatitis B (HBV) and hepatitis C (HCV) viruses. A significant knockdown (99%) of HBV core antigens was achieved in liver hepatocytes by using shRNA directed against them. Similarly, anti-HCV siRNA has shown to be effective in curing Huh-7.5 cells bearing persistently replicating HCV replicons. Moreover, inhibition of HIV replication has been achieved in numerous human cell lines and primary cells including T lymphocytes and hematopoietic stem cell derived macrophages.

5.3 Cancer:

The use of RNAi as a part of cancer therapy has great potential in revolutionizing cancer treatment. However, inherent challenges for cancer are not different compared to other diseases.

RNAi based cancer therapies include:

- i. Cessation of angiogenesis via inhibiting VEGF-signaling,⁷⁻¹³
- ii. Inducing apoptosis via inhibiting WNT and NF- κ B pathways;
- iii. Inhibiting EGFR, HER-2/NEU, telomerase, MDM2 and p53 or Bcl-2 molecules.

6. Delivery of siRNA^{4, 7, 35}

6.1 Challenges in siRNA delivery:

The delivery target location of siRNA is the cytoplasm of the target cells. Although this is one barrier less than that required for delivering plasmid DNA (which requires crossing the nuclear membrane for entry into the cellular genome), delivering siRNA still remains a great challenge. The first concern is the stability of siRNA itself. Naked siRNA is more prone to degradation and has plasma half-life in minutes. Their relatively small size also leads to rapid clearance by kidney filtration after systemic injection. Together with siRNA's negative charge that prevents its association with cell membrane, it's unlikely to simply use siRNA as a drug formulation. When formulated into a delivery vehicle, the stability of the delivery formulation is another issue. The siRNA loaded formulation has to escape from nonspecific uptake by the reticuloendothelial system (RES), especially the Kupffer cells in the liver and the macrophages in the spleen. Even if the siRNA stays in the blood circulation long enough, the siRNA formulation has to extravasate and gain access to the target tissue if the target is not blood cells or blood vessel cells (vascular endothelium). All the above is considered as the "kinetic barriers" which prevent the siRNA formulation from getting "access to the target cells". The other category of barrier represents the "physical barrier". Physical barriers are the hurdles that impede "the access of siRNA to the cytoplasm". Initially, siRNA needs to cross the cell membrane. Most commonly used delivery strategy for macromolecules including siRNA is to take advantage of the receptor-mediated endocytosis. After endocytosis, siRNA will need to escape the endosome to reach the cellular cytoplasm where the RISC locates. Another challenge facing siRNA therapy is 'immune stimulation'; this is recognition of siRNA duplex by the innate immune system. Introduction of too many siRNA molecules is found to result in nonspecific events.

6.2 Strategies in overcoming hurdles involved in siRNA delivery

6.2.1 siRNA delivery using vectors:

6.2.1.1 Viral vectors³⁵

6.2.1.2 Non-viral vectors.

- A. Lipid based vesicular systems (lipoplexes)
- B. Polymer based vesicular systems (polyplexes)

6.2.2 Physical delivery of siRNA,

6.2.3 Chemical modifications of siRNA.

6.2.1 siRNA delivery using vectors:

6.2.1.1 Viral vectors:^{34, 35}

Viral vectors possess certain advantages and disadvantages which are as follows:

Advantages:

1. They have higher transfection efficiency,
2. They show stable transfection, the phenomenon is useful for treating chronic conditions.

Disadvantages of viral vectors:

1. Susceptibility to immunostimulation,
2. Limited carrying capacity of genetic material in terms of number of base pair units/ kilodalton,
3. Chances of recombination,

4. High cost in formulation and processing,
5. low scalability,
6. Not useful in instances where stable transfection is not needed i.e. expression of genes during periodic remodeling following pathogenesis. Periodic remodeling during acute illness may be transient and may be regulated in a short period of time needing more temporary intervention. Hence, though high transfection efficiency and stable transfection, disadvantages of viral vectors led to increased strategic work for non-viral vectors such as lipoplexes and polyplexes.

6.2.1.2 Non-viral vectors:

Non-viral vectors are aimed to overcome following barriers during siRNA delivery:

Kinetic barriers include:

1. In vivo degradation of siRNA by plasma nucleases;
2. Off target gene silencing (non-specific gene silencing).

Physical barriers include:

1. Repulsion by cellular plasma membrane (which is embedded by anionic components viz. heparin, peptidoglycans etc.) due to anionic charge on siRNA.
2. Endosomal escape and cytoplasmic release.

A. Lipid mediated siRNA Delivery³⁵

All kinetic and physical barriers to siRNA delivery are likely to be overcome by formulations containing cationic lipid, especially cationic lipoplexes. These formulations help in efficient siRNA delivery by following mechanisms:

- i. Overcoming kinetic barriers (barrier to the transfer of siRNA in circulation to the target site):

In vivo degradation of siRNA by plasma nucleases and mobile scavengers (macrophages) could be overcome by complexing siRNA with functional cationic lipid having cationic lipid to siRNA charge ratio higher i.e. p/n ratio (phosphorous to nitrogen ratio) greater than one so that net excess positive charge on the lipid surface (net cationic) could help in retaining siRNA molecules since these molecules are anionic. Off target gene silencing can be avoided by active and passive targeting. Active targeting includes tagging lipoplexes with moieties such as transferrin, asialoglycoprotein, integrin, folate for tumor targeting. PEGylating the surface of cationic lipoplexes increases steric hindrance on the surface and prevents engulfment and scavenging by reticuloendothelial system.

- ii. Overcoming physical barriers (barrier to the entry into the target cell):

Cationic lipoplexes accumulates to the target cell membrane and gets endocytosed into the cytoplasm due to the anionic nature of plasma membrane. Endosomal escape (**Figure 2**) and release of siRNA into the cytoplasm following endocytosis remain the critical parameter for siRNA delivery into the cytoplasm. Cationic lipids together with its fusogenic property and pH buffering tendency assist in endosomal escape and siRNA release in the cytoplasm. Various mechanisms of endosomal escape are as follows:

a. Ion pair formation

After endocytosis, cationic lipids form ion pairs with the anionic lipids in the endosome membrane and thus, destabilize the endosomal membrane by excluding the surface bound water. Electrostatic interaction between cationic lipid and anionic lipid could further promote the formation of the inverted hexagonal (HII) phase which then assist in endosomal escape^{37, 38}. Lipids with small and/or less hydrophilic head group and bulky acyl or alkyl chains favor H II phase formation. Cationic lipid with a C18:2 alkyl chains showed higher delivery efficiency than the one with C18:1 chain. DOTAP containing two C18:1 acyl chains, is commonly used for transfection. However, DSTAP, a close analog of DOTAP but with two C18:0 chains, is not commonly used. HII phase is an intermediate structure when two lipid bilayers fuse with each other. In the process of fusion, both bilayers are destabilized. Thus, the ion-pair formation between the cationic lipids in the lipoplex and the anionic lipids in the endosome membrane not only destabilizes the endosome membrane, but also promote de-assembly of the lipoplex. Ion- pair formation requires close contact of the lipids with opposite charges. PEGylation of liposomes or LPD may significantly reduce the interactions between the cationic lipids and the endosomal lipids due to steric hindrance. PEGylation of the vector is often required to overcome the kinetic barriers. Cleavable PEG-lipid linker or acid labile PEG molecule design could be the answers to this problem. Many protein transduction domains contain arginine, and not lysine. It was showed that the presence of multiple guanidinium cations is important to their ion-pair formation in endosome that leads to efficient endosome escape. It was proposed that guanidinium cation containing delocalized positive charge can interact with an anionic group with delocalized negative charge. It is interesting to note that naturally occurring anionic groups in biological molecules, including phosphate, carboxyl and sulfate, all contain the delocalized negative charge which demonstrates the ion-pair formation between the delocalized positive charge of arginine and the delocalized negative charge of the phosphate group. The natural DNA condensing molecule in the sperm, i.e., protamine, is a polypeptide containing many arginine residues (**Figure 3**).

Figure 2 Mechanisms of endosomal escape

Figure 3 Ion pair effect in endosomal release³⁸

b. Proton sponge effect

Unlike lipoplex that relies on the fusogenic property of the lipid bilayer to mediate endosomal escape, polymeric carriers such as polyethyleneimine (PEI) are supposed to use the proposed "proton sponge" effect to enhance endosomal release of the endocytosed polyplex.³⁹

c. Deassembly

The siRNA complex must de-assemble sufficiently in the endosome or in the cytoplasm so that the released siRNA could access the RISC complex for gene silencing.

Different types of lipid used in the formulation of lipoplexes are as follows:^{1, 35}

i. pH sensitive lipids Trigger sensitive lipids:³⁵

The mechanisms of pH-triggered endosomal destabilization include:

1) Neutralization of negatively charged lipids in the bilayers via protonation leading to a lamellar to hexagonal phase transition. For eg. phosphatidylcholine and N-palmitoyl homocysteine (PHC). Upon exposure to lower pH, PHC undergoes a transition from a charged open form to an uncharged thiolactone ring form which destabilizes the bilayer and releases the encapsulated drug cargo.⁴⁰

2) Acid-catalyzed hydrolysis of bilayer-stabilizing lipids into destabilizing detergents or conical lipids. To circumvent potential problems due to the negatively charged surface of pH-sensitive liposome, cleavable liposomes with functional groups whose hydrolysis is catalyzed by acidic conditions have been designed. Different head groups, lipid chains, linker groups, and linkage configuration can be introduced. For example: acid sensitive vinyl ether linkage between head group and hydrocarbon chains^{41,42} (**Figure 4**).

Key:

- Acid sensitive lipids with vinyl ether linkage: (diplasmenylcholine and BCAT)
- Non acid sensitive divinyl ether linkage lipid (DCAT).

Figure 4 Acid and non - acid sensitive lipids

ii. Temperature sensitive lipids:

Lipids which are temperature sensitive and get destabilized at the body temperature are under clinical trials.

iii. Charge reversal/charge switching lipids:⁴³

The charge-reversal lipid has a cationic ammonium head group to bind DNA, lipophilic acyl chains to form a bilayer, and benzyl esters at the terminus of the acyl chains for enzymatic hydrolysis (**Figure 5**). When cationic in charge, charge-reversal lipid binds to nucleic acid molecules and then releases nucleic acid when it is anionic.

Figure 5 Charge reversible lipids

iv. Reducible lipid:⁴⁴

Cytoplasm has relatively high concentrations of reductive species such as 10 mM glutathione and the enzymes such as thioredoxin and glutaredoxin. Once internalized into the cell,

the lipoplex (liposomes loaded with siRNA) possessing a redox sensitive disulfide bond within its structure undergo S-S cleavage. Hence, reduction of the disulfide bond affords degradation of the lipid followed by destabilization of the liposomal membrane and release of DNA (**Figure 6**).

Figure 6 Reducible lipid

v. Mixed Lipids

Lipids used for formulating lipoplexes are as follows:

i. N(1-(2,3dioleyloxy) propyl) N, N, N-trimethylammoniumchloride (DOTMP) used as Cationic lipid for siRNA transfection. ii. Helper lipids such as cholesterol

and DOGS enhance lipoplex protection in the serum and increase transfection efficiency. iii. Fusogenic lipids such as dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) assist in endosomal escape mechanism (Figure 7).

Figure 7.1 Dioleoylphosphatidylethanolamine

Figure 7.2 Dioleoylphosphatidylcholine

Importance of fusogenic lipids (viz. DOPE):

Di-unsaturation in the fusogenic lipid increases the fluidity and fusogenic tendency irrespective of the position of unsaturation in the molecule. Fusogenic lipids maintain the lamellar phase of the lipoplexes in the plasma which help in decreasing lipoplex interaction with plasma proteins. However, fusogenic lipids also undergo phase transition from lamellar phase to inverted hexagonal phase inside the endosome which then assist in enhancing its interaction with the endosomal membrane followed by siRNA release.

vi.) Charged lipids⁴⁵

Cationic lipids are typically composed of three structural domains:

- Cationic head group,
- Hydrophobic part and
- Linker between the two domains.

Some examples of commercially available lipid reagents include

N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethyl-ammonium chloride (DOTMA),

2,3-dioleyloxy-N-(2(sperminecarboxamido)ethyl)-N,N-dimethyl-1-propanaminiumtrifluoroacetate(DOSPA), 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and dioctadecylamido-glycylspermine (DOGS) (Figure 8).

Figure 8 Dioctadecyamidoglycylspermine (DOGS)

I. Different methods employed in the manufacturing lipoplexes are categorized as follows:³⁴

1. Dehydration rehydration,
2. Ether injection,
3. Reverse phase evaporation;
4. Detergent dialysis technique.

General procedure for Lipoplex preparation using Film Rehydration Technique is as follows:

1. Initially, small unilamellar cationic liposomes (SUV) were prepared from a 1:1 (mol. ratio) mixture of 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and cholesterol (Chol), by extrusion of multilamellar liposomes (MLV).
2. Lipids were dissolved in chloroform (CHCl_3) and then dried under vacuum in a rotatory evaporator.
3. The dried lipid films were hydrated with high purity water and then the resulting MLV thus formed were sonicated followed by its extrusion through two stacked polycarbonate filters of 50 nm pore diameter.
4. The resulting liposomes (SUV) were then diluted with deionized water and filter-sterilized utilizing 0.22 μm pore diameter filters.

5. Lipoplexes were prepared by sequentially mixing liposomes with siRNA and followed by vortexing.

II. Characterization of cationic lipoplexes^{34, 46}

Cationic lipoplexes are characterized for following properties:

1. Lipoplexes morphology:^{34,35}

Two fundamentally different types of models have been employed in order to interpret the cationic lipoplex structures which include an “external” model in which DNA is adsorbed onto the surface of cationic liposomes and an “internal” model in which the siRNA is surrounded or “coated” by a lipid envelope.

Phase Behavior:

Lamellar structure is confirmed to be present during the condensation and transport of the nucleic acid molecules whereas a more aggressive inverted hexagonal structure is formed upon its contact with the cell membrane. The appearance of the lipoplexes is often a highly ordered tubular structure when they are endocytosed by cells and assume perinuclear localization in these endosomes. Cryo-electron images demonstrating the presence of such structures are shown in **Figure 9**.

Figure 9 Phase morphology

2. Particle Size:

Essentially particle size should be within the safe range which can be injectable in vivo. However, larger sized lipoplexes usually show high transfection efficiency due to larger surface available to contact with plasma membrane for endocytosis and again larger surface of the endosomal membrane remaining in contact with the liposomal membrane. Particle size increases with increase in lipid to siRNA ratio too.

3. Zeta potential measurements:

Zeta potential measurement is one of the vital characterization parameters of cationic lipoplexes. Positive zeta potential measurement implies net positive charge of cationic liposome to siRNA ratio and siRNA embedded inside the core of lipoplexes whereas negative zeta potential value indicates presence of siRNA on the surface of liposomes.

4. Surface topological profile:

Scanning electron microscopy using negative staining using phosphotungstic acid is generally performed. Cryo-fracture electron microscopy technique is also been performed for surface morphological measurement.

5. Target cell transfection efficiency:

Transfection efficiency of cationic lipoplexes is generally characterized by confocal laser scanning microscopy. The expression of green fluorescence peptide is quantitatively measured by measuring the intensity of fluorescence.

6. Cell viability studies:

Cell viability studies are generally performed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. MTT gets reduced in the mitochondria of viable cells to a blue formazan product which absorbs maximally at 570 nm. Greater the color intensity of formazan crystals under U.V., higher is the viability of the cells.

7. ¹H-NMR studies:

¹H-NMR studies help in understanding the presence of physical bonds formed between siRNA and cationic lipoplexes.

8. Gel permeation studies:

GEL permeation studies help in quantitating the amount of siRNA remaining unbound within the lipoplexes.

9. Gel retardation assay (GRA):

GRA also assist in quantitating the amount of free siRNA. Certain polypeptides have tendency to bind to naked siRNA. The resultant increase in molecular size of siRNA polypeptide complex retards the moment of the complex within the gel system.

III. Optimization of lipoplexes formulation:^{34, 36, 37}

Optimization of lipoplex formulation is a prerequisite exercise for enhancing lipoplex properties which include:

1. Transfection efficiency,
2. siRNA formulation stability,
3. Degradation of siRNA in circulation (plasma),
4. Off-target toxicity,
5. Delivery efficiency and endosomal escape,
6. Cytotoxicity of cationic lipoplexes;
7. Charge density on the lipoplex surface.

Following factors needed to be optimized for lipoplex mediated siRNA delivery include:

1. lipoplex morphology:³⁵

It is proposed that the absence of a propensity for transition to the HC II phase may result in a lower transfection potential compared to the lipoplexes that exhibit a higher-order inverted hexagonal structures. The difference of transfection efficiency between LC α phase and HC II phase comes from the fact that HCII lipoplexes fuse and release nucleic acid when in contact with anionic vesicles which are cell-free models of cellular membranes, in particular, anionic endosomal vesicles, but LC α lipoplexes remain stable when they come in contact with vesicle membranes confirmed by optical microscopy. On the other hand, HC II phase facilitates the interaction with the cell membrane and/or enables it to escape from the endosome.

2. Lipoplex size, plasma stability, transfection efficiency and endosomal escape.

Another parameter of morphology affecting transfection efficiency is lipoplex size.

Formulation stability:

Stability of siRNA depends on the charge ratio of lipid to DNA.

Transfection efficiency and particle size inhibition by serum.

Lipofection inhibition by serum is largely due to the tendency of serum in preventing lipoplex size growth which may be overcome by using large, stable lipoplexes. Lipoplexes size of less than 250 nm measured by dynamic light scattering showed efficient transfection efficiency in cells in absence of serum. Conversely, lipoplexes of over 700 nm mean diameter induce efficient transfection in the presence or absence of serum.

Endosomal escape:

Large lipoplexes size is more efficient to transfer gene because large particles are easily taken up by cells leading to the formation of large intracellular vesicles which are more easily disrupted, thus releasing siRNA into the cytoplasm. In addition, the advantageous effect of large particles upon lipofection has been attributed to maximum contact with cells, increased phagocytic activity accompanied by endosomal escape and faster sedimentation and better cellular trafficking.

Overcoming cell cytotoxicity by cationic lipoplexes:

Cationic lipoplexes induced a significant cytotoxicity towards HeLa cells, B16BL6 cells and RGC-6 cells compared to cationic lipid alone and the cytotoxicity increases as the cationic lipid content in the lipoplex increased. Hence, anionic polymers are incorporated for decreasing toxicity and enhancing in vivo delivery of siRNA complexed with cationic liposome. Addition of anionic polymers into siRNA lipoplexes lead to efficient lipoplexes mediated delivery. Divalent calcium ions are used to complex siRNA and anionic polymer within the cationic lipoplexes.^{36, 38}

B. Polymer mediated siRNA delivery (polyplexes)^{34, 39}

Polymer-based delivery systems have been extensively used for plasmid DNA and more recently for siRNA. As with lipid-based delivery systems, polymeric delivery of siRNA usually involves a cationic moiety as a core component of polyplex.

Distinct features of polymers used for siRNA delivery include:³⁴

i. Cationic polymeric delivery system provides higher simplicity for the formulation of siRNA and polymeric complexes (polyplexes),

ii. Unlike cationic lipoplexes, which require several processing steps in formulation, cationic polymers usually form complex with negatively charged siRNA upon simple mixing,

iii. Polyplexes are soluble in water;

iv. However, cationic polyplexes cannot destabilize endosomal membrane by surfactant like activity due to lack of hydrophobic domain.

Cationic polymers are generally classified into synthetic and natural polymers.³⁹

✓ Synthetic polymers include:

Polyethyleneimine (PEI), poly-L-lysine (PLL) and cyclodextrin-based polycations.

Natural polymers include:

Chitosan, atelocollagen and cationic polypeptides.

III. Biodegradable polymers³⁹ include:

Poly(lactide-co-glycolide).

i.) Synthetic polymers

Polyethyleneimine (PEI)⁴⁰

1. PEI is extensively used cationic polymer, has a wide range of molecular weights and multiple protonable amino groups leading to a higher cationic charge at physiological pH.

2. Complexation of nucleic acids with PEI is achieved by electrostatic interactions between the positively charged amino groups of PEI and the negatively charged phosphate groups of siRNA.

3. The advantage of using PEI over other polymers is the high transfection efficiency due to proton sponge effect". PEIs have buffering capability in the low pH of the endosome, thereby releasing nucleic acids into the cytoplasm. The magnitude of this effect depends on charge density in the lipoplexes surfaces.

4. To reduce the cytotoxicity induced by high molecular weight PEI and to increase serum nuclease resistance, various approaches are employed which include:

In one of the studies, wide range of PEG moieties was introduced to PEI for production of block copolymers. Increasing the hydrophilicity of PEG is expected to reduce the toxicity of the copolymer, improve the poor solubility of the PEI and siRNA complexes and help introduce degradable bonds by reaction with primary amines in the PEI. Moreover, the PEG moieties can form a shielding layer that protects the polyplex structure against undesirable interactions with degradation enzymes or proteins in the bodily environment. With the PEG derivatives of PEI, the chain length and graft density of PEG were found to strongly influence siRNA condensation and stability. PLL-graft-PEG copolymers have been used to increase the circulation time of siRNA in vivo.

Disadvantages of PEI:

A problem with the use of PEI is appreciable cytotoxicity. PEI has been shown to induce cell death in a variety of cell lines through cellular mechanisms such as necrosis and apoptosis. The cytotoxicity of PEI tends to increase at high molecular weights and with increasing branching units. The removal of uncomplexed PEI after complexation reaction with siRNA was shown to be effective in reducing the toxicity of PEI.

Purified complexes without free PEI had to be given at increased concentrations to achieve high transfection levels, but it also ensures improved toxicity profiles.

ii.) Natural polymers³⁹

Chitosan⁴¹

Chitosan is a naturally occurring biocompatible, biodegradable polycation with low toxicity, thus could serve as a promising carrier for delivering siRNA. Owing to the interaction of anionic siRNA and cation chitosan, siRNA can be complexed and loaded into chitosan nanoparticles. Chitosan has been used successfully to form complex with siRNA against green fluorescence protein (GFP). The spherical and stable chitosan siRNA nanoparticles with 83 - 94% complex efficiency can be formulated under mild electrostatic interaction. Nearly 80% gene silencing efficiency of chitosan-siRNA nanoparticles could be achieved.

iii.) Biodegradable polymers⁴²

Poly(lactide-co-glycolide):

Poly(DL-lactide-co-glycolide) (PLGA) is an FDA-approved biodegradable polymer. PLGA nanoparticle has been used as gene vector for siRNA.

6.2.2 Physical methods of siRNA delivery³⁹

Physical method is another strategy for introducing siRNA into cells and tissues.

Advantages:

1) Physical methods may avoid the possible nonspecific immune stimulations which might arise in some of chemical delivery systems.

2) Moreover, owing to their non-invasiveness and relative convenience for clinical application, there are increasing interests in physical methods for delivery of siRNA. Various physical methods include hydrodynamic injection, particle bombardment and electroporation, ultra sound and direct injection into the cell. Electroporation has been most frequently studied to stimulate the cellular and localized in vivo delivery of siRNA through electric pulses.

6.2.3 Chemical Modifications²⁶

To enhance the stability of siRNA for prolonged circulation in vivo, chemical modification of siRNA has been attempted. Chemical modification increases gene expression in liver by preventing siRNA phagocytosis. Chemical modifications at particular position reduce off target gene silencing and increase the efficiency of gene silencing. Various positions within the siRNA duplex have been chemically replaced or modified to provide nuclease resistance. One of the common approaches is replacement of the phosphodiester (PO₄) group with phosphothioate (PS) at the 3'-end. Moreover, the introduction of an O-methyl group (2'-O-M), a fluoro (2'-F) group, or a 2-methoxyethyl (2'-O-MOE) group resulted in prolonged half-lives and RNAi activities in cultured cells and plasma.

7. Evidence of improved siRNA mediated therapeutic efficacy:

Vast literature reports demonstrating superior advantages of siRNA are documented. Some of these reports are described here briefly. For instance, siRNA loaded cationic liposomes were developed with new polyarginine-conjugated PEG-lipid.⁴³ The cationic liposome formulation using a new PEG-lipid (PLR-PEG) showed not only enhanced intracellular

delivery of siRNA but also decreased cytotoxicity on H4II-E and HepG2 cell lines. The siRNA delivered by new cationic liposomes using PLR-PEG was effective in reducing the protein expression levels of the gene. The results suggested that the cationic liposomes could be used for efficient delivery of siRNA therapeutics. In another study, anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery was established.⁴⁴ In this study, various batches of polyethylene glycol (PEG)-coated cationic lipoplexes were prepared and subsequently screened for the avidity of these siRNA-lipoplexes upon angiogenic tumor vessels. Lipoplex having a lipid composition of DC-6-14/POPC/CHOL/DOPE/mPEG2000-DSPE=20/30/30/20/5 (molar ratio) and a charge ratio of cationic liposome and siRNA = 3.81 (+/-), showed a higher binding index to newly formed blood vessels. However, in another study, ultra-deformable cationic liposomes were developed for delivery of small interfering RNA into human primary melanocytes.⁴⁵ Blockade of the expression of a specific Myosin Va exon F containing isoform that is physiologically involved in melanosome transport in human melanocytes was intended. Ultra-deformable cationic liposomes (UCL) to deliver siRNA in hard to transfect human primary melanocytes were prepared and investigated in this study.

Additionally, a novel cholesterol derivatized cationic liposomes were developed for enhanced delivery of siRNA has been developed.⁴⁶ Two cholesterol derivatives, cholesteryloxypropane-1-amine (COPA) and cholesteryl-2-aminoethylcarbamate (CAEC) were evaluated. Uptake visualization using fluorescent labeled COPA and CAEC lipoplexes was studied. The presence of serum demonstrated varied effects on the cellular delivery of siRNA when siRNA was complexed to different cationic liposomes. CAEC based liposomes showed significantly reduced cellular delivery of siRNA in serum containing media as compared to serum free media. However, COPA-based liposome (COPA-L) showed serum enhanced delivery of siRNA in Hepal-6, A549 and Hela cell lines. In a separate study, anionic polymers were used for decreased toxicity and enhanced in vivo delivery of siRNA complexed with cationic liposomes.³⁸ In this study a new vector of cytokine targeted siRNA based on cationic lipid was developed for intravenous injection. Weekly injections of siRNA targeted to IL-1, IL-6 or IL-18 delivered in combination and formulated with cationic liposomes significantly reduced all pathological rheumatoid arthritis features. siRNA vector was formed by mixing lipopolyamine-containing cationic liposome with premixed solution of siRNA and plasmid DNA (p-DNA) acting as an anionic "cargo". Addition of pDNA cargo to siRNA prior to forming the complex with cationic liposome, led to enhanced gene silencing efficiency with reduced siRNA concentration. However, addition of DNA molecule in siRNA lipoplexes will not be acceptable in a clinical application since pDNA is a molecule that contains coding sequences. In this report, feasibility study was performed to replace pDNA by an anionic polymer that would be clinically approvable. Thus, siRNA lipoplexes with anionic polymers were prepared and their gene silencing efficiency as well as their physicochemical characteristics were evaluated. When added to siRNA lipoplexes, these anionic polymers increased their gene silencing efficiency. Upon i.v. injection in mice, siRNA lipoplexes prepared with polyglutamate led to significantly increased recovery of siRNA in liver and lung compared with lipoplexes without polymer.

8. Conclusion

Gene silencing by RNAi mechanism represents one of the most successful means of achieving gene therapy against genetic disorders. Silencing of pathological genes by small

interference RNA (siRNA) is observed as an efficient means of treating diseases which have been originated due to genetic malfunctions. Even though siRNA empowers researchers with its efficient gene silencing capabilities, its delivery to the target site has always been an issue. Viral and non – viral vectors are developed to overcome gene delivery problems. However, non – viral vectors are highly explored because of its advantages of safety and efficacy over viral vectors. Non – viral vectors include polymeric and lipidic carriers. However, lipidic carriers have emerged as most superior and compatible delivery carriers for site specific siRNA delivery. Lipoplexes have been widely explored as pioneer lipidic vesicles for siRNA delivery and have proved to be efficient not only in overcoming delivery barriers for siRNA but also have showed to be beneficial in achieving higher loading both for siRNA and other molecules which could be beneficial when used in combination. Lipoplexes can also be surface functionalized via pegylation and active targeted ligands for achieving optimum pharmacokinetic and tissue specific siRNA delivery. Hence, siRNA mediated gene therapy embarks prominent means of achieving efficient treatment modality for variety of genetic disorders.

References:

1. Zhang XX, McIntosh TJ, Grinstaff MW. Functional lipids and lipoplexes for improved gene delivery. *Biochimie*. 2012; 94(1):42-58.
2. Pearson S, Jia H, Kandachi K. China approves first gene therapy. *Nat Biotechnol*. 2004; 22(1):3-4.
3. Wilson JM. Gendicine: the first commercial gene therapy product. *Hum Gene Ther*. 2005; 16(9):1014-5.
4. Tseng YC, Mozumdar S, Huang L. Lipid-based systemic delivery of siRNA. *Adv Drug Deliv Rev*. 2009; 61(9):721-31.
5. Davidson BL, Paulson HL. Molecular medicine for the brain: silencing of disease genes with RNA interference. *Lancet Neurol*. 2004; 3(3):145-9.
6. Pardridge WM. shRNA and siRNA delivery to the brain. *Adv Drug Deliv Rev*. 2007; 59(2-3):141-52.
7. Oh YK, Park TG. siRNA delivery systems for cancer treatment. *Adv Drug Deliv Rev*. 2009; 61(10):850-62.
8. Patil S, Lalani R, Bhatt P, Vhora I, Patel V, Patel H, et al. Hydroxyethyl substituted linear polyethylenimine for safe and efficient delivery of siRNA therapeutics. *RSC Advances*. 2018; 8:35461-73.
9. Xie FY, Woodle MC, Lu PY. Harnessing in vivo siRNA delivery for drug discovery and therapeutic development. *Drug Discov Today*. 2006; 11(1-2):67-73.
10. Das B, Yeager H, Tsuchida R, Torkin R, Gee MF, Thorner PS, et al. A hypoxia-driven vascular endothelial growth factor/Flt1 autocrine loop interacts with hypoxia-inducible factor-1alpha through mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 pathway in neuroblastoma. *Cancer Res*. 2005; 65(16):7267-75.
11. Patil S, Bhatt P, Lalani R, Amrutiya J, Vhora I, Kolte A, et al. Low molecular weight chitosan-protamine conjugate for siRNA delivery with enhanced stability and transfection efficiency. *RSC Advances*. 2016; 6(112):110951-63.
12. Kou R, SenBanerjee S, Jain MK, Michel T. Differential regulation of vascular endothelial growth factor receptors (VEGFR) revealed by RNA interference: interactions of VEGFR-1 and VEGFR-2 in endothelial cell signaling. *Biochemistry*. 2005; 44(45):15064-73.
13. Takei Y, Kadomatsu K, Yuzawa Y, Matsuo S, Muramatsu T. A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. *Cancer Res*. 2004; 64(10):3365-70.
14. Potter H. Electroporation in biology: methods, applications, and instrumentation. *Anal Biochem*. 1988; 174(2):361-73.
15. Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud JM, et al. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc Natl Acad Sci U S A*. 1999; 96(8):4262-7.
16. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. DNA vaccines: protective immunizations by

- parenteral, mucosal, and gene-gun inoculations. *Proc Natl Acad Sci U S A*. 1993; 90(24):11478-82.
17. Wells DJ. Electroporation and ultrasound enhanced non-viral gene delivery in vitro and in vivo. *Cell Biol Toxicol*. 2010;26(1):21-8.
 18. Yoon CS, Park JH. Ultrasound-mediated gene delivery. *Expert Opin Drug Deliv*. 2010; 7(3):321-30.
 19. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer into mouse muscle in vivo. *Science*. 1990; 247(4949 Pt 1):1465-8.
 20. Napoli C, Lemieux C, Jorgensen R. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell*. 1990; 2(4):279-89.
 21. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998; 391(6669):806-11.
 22. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet*. 2002;3(10):737-47.
 23. Guo P, Coban O, Snead NM, Trebley J, Hoeprich S, Guo S, et al. Engineering RNA for targeted siRNA delivery and medical application. *Adv Drug Deliv Rev*. 2010; 62(6):650-66.
 24. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 2004; 5(7):522-31.
 25. Lim LP, Lau NC, Garrett-Engle P, Grimson A, Schelter JM, Castle J, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*. 2005; 433(7027):769-73.
 26. McBride JL, Boudreau RL, Harper SQ, Staber PD, Monteys AM, Martins I, et al. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc Natl Acad Sci U S A*. 2008; 105(15):5868-73.
 27. Soifer HS, Rossi JJ, Saetrom P. MicroRNAs in disease and potential therapeutic applications. *Mol Ther*. 2007; 15(12):2070-9.
 28. Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. Single processing center models for human Dicer and bacterial RNase III. *Cell*. 2004; 118(1):57-68.
 29. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell*. 2004; 15(2):185-97.
 30. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*. 2009; 10(2):126-39.
 31. Monaghan M, Pandit A. RNA interference therapy via functionalized scaffolds. *Adv Drug Deliv Rev*. 2011; 63(4-5):197-208.
 32. Yuan X, Naguib S, Wu Z. Recent advances of siRNA delivery by nanoparticles. *Expert Opinion on Drug Delivery*. 2011; 8(4):521-36.
 33. Miller VM, Gouvion CM, Davidson BL, Paulson HL. Targeting Alzheimer's disease genes with RNA interference: an efficient strategy for silencing mutant alleles. *Nucleic Acids Res*. 2004; 32(2):661-8.
 34. Tros de Ilarduya C, Sun Y, Duzgunes N. Gene delivery by lipoplexes and polyplexes. *Eur J Pharm Sci*. 2010; 40(3):159-70.
 35. Ma B, Zhang S, Jiang H, Zhao B, Lv H. Lipoplex morphologies and their influences on transfection efficiency in gene delivery. *J Control Release*. 2007; 123(3):184-94.
 36. Srinivasan C, Burgess DJ. Optimization and characterization of anionic lipoplexes for gene delivery. *J Control Release*. 2009; 136(1):62-70.
 37. ur Rehman Z, Hoekstra D, Zuhorn IS. Mechanism of polyplex- and lipoplex-mediated delivery of nucleic acids: real-time visualization of transient membrane destabilization without endosomal lysis. *ACS Nano*. 2013; 7(5):3767-77.
 38. Schlegel A, Largeau C, Bigey P, Bessodes M, Lebozec K, Scherman D, et al. Anionic polymers for decreased toxicity and enhanced in vivo delivery of siRNA complexed with cationic liposomes. *J Control Release*. 2011; 152(3):393-401.
 39. Yuan X, Naguib S, Wu Z. Recent advances of siRNA delivery by nanoparticles. *Expert Opin Drug Deliv*. 2011; 8(4):521-36.
 40. Katas H, Cevher E, Alpar HO. Preparation of polyethyleneimine incorporated poly(D,L-lactide-co-glycolide) nanoparticles by spontaneous emulsion diffusion method for small interfering RNA delivery. *Int J Pharm*. 2009; 369(1-2):144-54.
 41. Liu X. [Chitosan-siRNA complex nanoparticles for gene silencing]. *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi*. 2010; 27(1):97-101.
 42. Yuan X, Li L, Rathinavelu A, Hao J, Narasimhan M, He M, et al. SiRNA drug delivery by biodegradable polymeric nanoparticles. *J Nanosci Nanotechnol*. 2006; 6(9-10):2821-8.
 43. Kim HK, Davaa E, Myung CS, Park JS. Enhanced siRNA delivery using cationic liposomes with new polyarginine-conjugated PEG-lipid. *Int J Pharm*. 2010; 392(1-2):141-7.
 44. Tagami T, Suzuki T, Matsunaga M, Nakamura K, Moriyoshi N, Ishida T, et al. Anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery. *Int J Pharm*. 2012; 422(1-2):280-9.
 45. Geusens B, Lambert J, De Smedt SC, Buyens K, Sanders NN, Van Gele M. Ultradeformable cationic liposomes for delivery of small interfering RNA (siRNA) into human primary melanocytes. *J Control Release*. 2009; 133(3):214-20.
 46. Han SE, Kang H, Shim GY, Suh MS, Kim SJ, Kim JS, et al. Novel cationic cholesterol derivative-based liposomes for serum-enhanced delivery of siRNA. *Int J Pharm*. 2008; 353(1-2):260-9.