REVIEWS

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SYSTEMIC DELIVERY OF SMALL INTERFERING RNA THERAPEUTICS: OBSTACLES AND ADVANCES

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ABSTRACT

RNA interference (RNAi) has emerged as a critical tool for genetic therapeutics. Within the last two decades, there have been extensive investigations in the systemic delivery of these drugs but the obstacles in *in vivo* delivery have possessed a great difficulty in their administration. These developments led to the advances in systemic administration with designing and incorporation of various drug carriers, including bioconjugate systems, polymeric complexes, organic and inorganic nanoparticles. However, successful translation of these drug delivery systems for the clinical application has still been a great setback for these class of pharmaceuticals. In order to improve their competency and applicability, systemic evaluation of currently available carrier systems is required. In this review, we focus on the obstacles in the systemic administration of small interfering RNAs (siRNAs) and the advances with various carrier systems to overcome the limitations of these obstacles. General obstacles in siRNA delivery have been discussed with major emphasis on the barriers at different stages of systemic administration. Then, advances in their application for targeted delivery with rationally designed carrier systems such as bioconjugates, polymeric complexes, and nanoparticles have been introduced. Lastly, we discuss the progress and state of clinical studies of siRNA therapeutics with perspectives of clinical potential.

Keywords: Bioconjugate, Clinical study, Nanoparticles, RNAi, siRNA, Systemic administration

1. Introduction

RNA interference (RNAi) is a conserved self-defense mechanism found in most eukaryotes, which was initially discovered in *Caenorhabditis elegans* (Fire *et al.*, 1998). In this phenomenon, small double-stranded RNA (dsRNA) triggers a series of biochemical events and results in sequence-specific gene suppression (Fire, 1999; Hammond *et al.*, 2001; Sharp, 2001; Tuschl, 2001). The capability of this tool to potentially silence any gene, including the intracellular (undruggable) targets, has inspired the scientific community to understand and exploit it for efficient application as a new class of pharmaceuticals (Wu *et al.*, 2014; Finan *et al.*, 2017; Tushir-Singh, 2017). Particularly, RNA duplexes 21-23 nucleotides in length, also known as small interfering RNAs (siRNAs), have emerged as the leading candidates with the evidence of RNAi in mammalian cells (Elbashir *et al.*, 2001a). However, the translation of siRNA drugs to the clinical application has remained slow, with a number of challenges posing a barrier for the efficient

intracellular delivery of these therapeutics.

The very first clinical trial involving siRNA was conducted in 2004 with patients suffering from age-related macular degeneration (AMD) (Garba and Mousa, 2010). In 2006, Andrew Z. Fire and Craig C. Mello were awarded the Nobel Prize in Physiology or Medicine for discovering RNAi. And now, in 2018, the latest development has been the approval of the first siRNA drug ONPATRO, for the treatment of the polyneuropathy of hereditary transthyretin-mediated (TTR) amyloidosis in adults (Adams *et al.*, 2018). It has taken nearly two decades and ample efforts for the approval of the first drug, which can be attributed to the problems associated with siRNA such as hydrolysis by nucleases, large size, and hydrophilic nature (Sarett *et al.*, 2015). Chemical modifications of siRNAs have provided them considerable stability in most of the cases, whereas shielding the siRNA with carriers have further regulated the targeted systemic delivery (Shukla *et al.*, 2010; Xu *et al.*, 2015; Ku *et al.*, 2016; Lee *et al.*, 2016). Between the local

and systemic administration, the former has advantages of reduced off-target side effects and enables higher gene silencing but failed in initial clinical trials, disappointing the scientific community (Haussecker, 2014; Xue *et al.*, 2014).

Design and development of advanced delivery systems have notably increased the siRNA therapeutics in clinical trials and most of these are targeted via systemic delivery (Ozcan *et al.*, 2015; Chakraborty *et al.*, 2017). Many groups have reported systemic delivery of siRNA with different carrier systems such as bioconjugates (Ming and Laing, 2015; Lee *et al.*, 2016; Sivakumar *et al.*, 2018; Srimanee *et al.*, 2018), complexes (Biswas and Torchilin,

2013; Nussbaumer *et al.*, 2016; Takemoto and Nishiyama, 2017) and nanoparticles (Patil and Panyam, 2009; Kozielski *et al.*, 2013; Lin *et al.*, 2014; Tezgel *et al.*, 2018). Recent developments have been the combinatorial formulation of carriers to form complexes on the nanoscale for the systemic delivery of siRNA therapeutics (Garg *et al.*, 2016; Lee and Ahn, 2018; Zheng *et al.*, 2018).

Since RNA therapeutics are still at an early stage, many researchers have been actively working in this domain but only a few studies provide such comparative study of carrier systems and their developments at different phases. Therefore, in this review, we present recent remarkable contributions to systemic

Fig.1 A. The structure of siRNA, sense and antisense strands, and target mRNA complementary seed region. B. Schematic of siRNA-mediated gene silencing.

delivery of siRNA to fight various barriers. First, extracellular and intracellular obstacles in the systemic administration of siRNA are described. Second, we introduced advances with each carrier system by utilizing their respective mode of action, their pros, and cons for effective siRNA delivery. Lastly, the current state of siRNA therapeutics in clinical studies and application have been discussed.

2. siRNA-Mediated Gene Silencing Mechanism

siRNAs are short, double-stranded regulatory RNAs that contain two signature characteristics: a two nucleotide 3'-end overhang and a phosphorylated 5'-end (Elbashir *et al.*, 2001a, 2001b). Cutting long dsRNAs using the dicer endoribonuclease leads to the formation of these siRNAs. The functional strand, complementary to the target mRNA, is called the guide strand, whereas the non-functional strand is called passenger strand (Fig. 1A). The Argonaute (AGO) protein recognizes and guides the siRNA for target mRNA degradation by detecting the seed region complementary to the target mRNA (Frank *et al.*, 2010; Schirle and MacRae, 2012). The siRNA-mediated gene silencing follows recognition by Ago2, sense strand cleavage, RNA-induced silencing complex (RISC) activation, and target mRNA degradation in a sequential manner (Fig. 1B).

3. Delivery of siRNA Therapeutics and Obstacles

3.1. Local and systemic delivery

The delivery method of siRNA can be broadly classified on the basis of route of administration i.e. local administration and systemic administration. The local delivery is targeted for topical and oral application for the treatment of diseases related to eye, skin, vagina and nasal, where the target sites are easily accessible (Martirosyan *et al.*, 2014; Pan *et al.*, 2015). Whereas, the systemic administration is required for the treatment of metastatic or deep-seated tumors like the liver diseases (Gujrati and Lu, 2013; Hattori *et al.*, 2015; Zuckerman and Davis, 2015). The inhibition of respiratory viruses by nasally administered locoregional delivery of siRNA demonstrates efficient drug activity upon regional delivery (Bitko and Barik, 2008). Systemic delivery of siRNA to the liver and lung helps in eradicating metastatic tumors (Yang *et al.*, 2012; Huang *et al.*, 2015). Therefore, systemic administration is a preferred method for siRNA delivery over the local delivery. Also, since most of the cancers are metastatic in nature, their treatment requires systemic siRNA delivery to the target tissue for highly efficient activity.

3.2. General obstacles in siRNA delivery

Translation of siRNA therapeutics to clinical application is strenuous and still remain an enormous challenge. While ample efforts have been applied for the development of these synthetic drugs but the major limitation is their delivery to the site of action

as they face a number of extracellular as well as intracellular barriers, as shown in Fig. 2 (Borna *et al.*, 2015; Roberts *et al.*, 2016). The extracellular barriers comprise of the degradation by nucleases, renal clearance, and uptake by the reticuloendothelial systems. Many chemical modifications have evidently enhanced the stability of siRNAs and protected against hydrolysis by the nucleases (Ueno *et al.*, 2008, 2009; Chernikov *et al.*, 2017; Gvozdeva *et al.*, 2018). Small size and molecular weight lower than 40 kDa render the siRNA prone to the kidney filtration process. Therefore, carrier systems with a hydrodynamic size of over 6 nm and increased the molecular weight are required (Zuckerman *et al.*, 2012; Bienk *et al.*, 2016). Immunostimulation is another barrier in the delivery of siRNA as it leads to degradation of siRNA after recognition as an alien moiety by the macrophages and kupffer cells (Whitehead *et al.*, 2011; Khairuddin *et al.*, 2012). In order to tackle this issue, 2'-position of the ribose sugar had been modified with 2'-O-methyl and 2'-fluoro groups to suppress the immune response and improve serum provide stability (Valenzuela *et al.*, 2014; Haraszti *et al.*, 2018).

Intracellular barriers inhibit the siRNA to permeate the lipid bilayers due to charge repulsion. Hence, cationic delivery systems are utilized for the transport of siRNA inside the cell via endocytosis. The endosomal pH is lower than that of the cytosol and the release can be mediated through a proton pump, inability to do so leads to degradation by the lysosome limiting the bioavailability of siRNA to interact with RISC machinery in the cytosol (Ma, 2014; Tuttolomondo *et al.*, 2017).

4. Obstacles in the Systemic Administration of siRNA

Systemic siRNA delivery is more advanced and complicated in comparison to the local administration. In the last decade, 50% percent of the clinical trials have been executed with systemic delivery as they efficiently distribute siRNA to metastatic cancer. Obstacles in systemic delivery viz. the nuclease degradation in the blood vessels, uptake by reticuloendothelial cells, biodistribution to the target site, extravasation from blood vessels to target tissues, and permeation to the target site (Fig. 3). Lastly, endocytosis by the cell and endosomal escape constitute the complete list of obstacles in the systemic administration. Although the requirement of a carrier system for different routes of delivery has a considerable overlap, it is necessary for a carrier system to deliver the drug systematically rather than topically or locally.

4.1. Blood Circulation: Degradation by Nucleases and Renal Filtration

Systemic delivery is initiated with intravenous injections siRNA to traverse through blood vessels and reach the target tissue/organ. But the blood plasma contains several nucleases

Fig.2 General obstacles in the delivery of siRNA-based therapeutics, (A) extracellular and (B) intracellular.

which hydrolytically degrade the siRNA. Chemical modifications and substitution of phosphate backbone with phosphorothioate linkage yields serum stable siRNAs (Ueno *et al.*, 2008; Sierant *et al.*, 2013; Chernikov *et al.*, 2017; Ogata *et al.*, 2017). Thereby, chemical modifications have become vital tools in enhancing the nuclease resistance of siRNAs. Also, bioconjugates such as cholesterol have provided longer half-life due to sustained stability against serum as these lipoproteins are transported in the bloodstreams (Ding *et al.*, 2012; Khan *et al.*, 2016).

Rapid renal clearance is subjected for small molecules with molecular weights of less than 40 kDa to pass through glomerular of the kidney. Hence, siRNAs are liable enough to flush out of the system through kidney filtration unit, resulting in loss-of-function

of gene regulation. Therefore, conjugation or encapsulation siRNAs to carrier systems help in improving its size and mass to avoid renal filtration (Zuckerman *et al.*, 2012).

4.2. Biodistribution: Off-target Delivery and Accumulation in the Reticuloendothelial System

During the systemic delivery of siRNA, many off-target sites are recognized due to seed region complementarity and accumulation takes place (Gresham, 2003; Lin *et al.*, 2005). Some structurally modified siRNAs and conjugated systems have shown on-target delivery (Suter *et al.*, 2016; Janas *et al.*, 2018). Therefore, siRNA modification and a suitable delivery agent can help in reducing off-target activity. Secondly, the

Fig.3 Schematic of Systemic administration of siRNA with various barriers at each stage.

reticuloendothelial system (RES), also known as the macrophage system, an immune system in the blood, connective tissues, liver, spleen, lungs, and bone marrow, activates and captures the siRNA as an immune response (Whitehead *et al.*, 2009; Wang *et al.*, 2010). Nanoparticles, which are relatively smaller than polymers, have been shown to escape RES uptake (Huang and Guo, 2011). Circulation time and on-target delivery have been reported to increase upon siRNA conjugation with polyethylene glycol (Bao *et al.*, 2013; Miteva *et al.*, 2015).

4.3. Cellular Uptake: Extravasation and Cell Internalization

Localization of siRNAs at the target organ happens by transcytosis, hence it is important to have a small particle size for easy movement between the cells and reach the target site. Cell internalization is limited due to the anionic nature of the membrane surface causing repulsion of likely charged siRNA (Whitehead *et al.*, 2009). Cellular uptake of siRNA is usually done via endocytosis, where membrane-bound endosomes trap the siRNA and later release it into the cytosol. Naked siRNA cannot permeate cell membrane, hence cationic molecules or nanoplexes are used to mediate the delivery through the cell membrane. The anionic proteoglycans on the cell surface interact with the cationic

complexes and enable the integration of complex into the cell endosome (Juliano *et al.*, 2012; Lee *et al.*, 2012). The delivery carriers are modified for improved cell internalization through modified surfaces (Nourbakhsh *et al.*, 2015; Ly *et al.*, 2017).

4.4. Endosomal Escape: Release into the Cytosol

Following the endocytosis and internalization of the siRNA-carrier complex into the cell, siRNA endosomal release is essential for degrading target mRNA in the cytoplasm. Often, this endosomal escape has been attributed to the proton sponge effect, which is caused by cationic molecules and results in endosome swelling and disruption (Dominska and Dykxhoorn, 2010). Recently, the release of siRNA from endosome has been recognized as an acute problem because, even upon successful systemic delivery into the cell, no efficient RNAi activity could be observed in some cases. Therefore, researchers are now focusing on how to quantitatively release siRNA into the cytosol through endosomal escape. Different classes of delivery agents have been devised to this end, such as cationic peptides, lipid nanoparticles, and polymers (Shrestha *et al.*, 2012; Gilleron *et al.*, 2013; Lechanteur *et al.*, 2018).

5. Advances with Carriers to Escape the Systemic Delivery Barriers

The basis for the design and selection of siRNA carriers solely depends on its physiochemical properties. The similar characteristics of siRNA to DNA provided the basis for conducting the initial trials using previously developed carriers for DNA. These carriers are broadly divided into two categories: viral and non-viral. Viral carriers possess high toxicity and are detrimental to the cell, hence, non-viral carriers have been extensively studied to replace them (Beilvert *et al.*, 2012). Previous reviews have imparted basic information about such delivery carriers for siRNA therapeutics (Kanasty *et al.*, 2013; Lee *et al.*, 2016). Here, we will discuss the basis of their mechanisms and the advances in siRNA delivery by evading the obstacles.

5.1. siRNA Bioconjugates

Upon conjugation to siRNA, certain molecules have been shown to enhance siRNA stability, cellular uptake, and on-target delivery. These can be collectively categorized as bioconjugates, each with differing features (Fig. 4). Examples of bioconjugates that have been extensively utilized recently are cholesterol, *N*-acetyl-galactosamine (GalNAc), aptamers, and antibodies.

5.1.1. Cholesterol Bioconjugates

The basis of introducing cholesterol for siRNA delivery relates to its importance as a structural component of the animal cell membrane. The presence of cholesterol promotes intracellular transport and has been found to provide nuclease resistance during siRNA delivery. Two cationic cholesterol derivatives, cholesteryloxypropan-1-amine (COPA) and cholesteryl-2-

aminoethylcarbamate (CAEC) have been reported for liposome-based, stability-enhanced siRNA delivery (Han *et al.*, 2008). Other reports have also shown cationic cholesterol-modified nanoparticles as the carriers for delivery of siRNA into tumor cells (Kadengodlu *et al.*, 2013; Hattori *et al.*, 2015). The interaction between cholesterol and serum apolipoproteins and the subsequent recognition by hepatocyte receptors has been utilized in the development of a biomimetic nanovector for the targeted delivery of cholesterol-conjugated siRNA (Ding *et al.*, 2012). Chol-siRNA-loaded rHDL nanoparticles were found to have efficient cellular uptake and targeted cytoplasmic delivery in human hepatocellular carcinoma cell lines. Modification of the passenger and guide siRNA strands with a cholesteryl moiety to exploit the interaction with recombinant human serum albumin (rHSA) revealed a 4- and a 6-fold increase in plasma life with single and double cholesteryl modifications *in vivo*, respectively (Bienk *et al.*, 2016). Cholesterol-conjugated siRNAs have also been examined for carrier-free gene silencing of MDR1 (multi-drug resistance) genes (Chernikov *et al.*, 2017). The ability of cholesterol to circulate through blood vessels helps in guided systemic delivery of siRNA to the liver and conjugation with this moiety have shown a successful application in numerous case (Ming and Laing, 2015; Sebestyén *et al.*, 2015; Khan *et al.*, 2016).

5.1.2. Aptamer Bioconjugates

siRNA-chimeras represent an extensive RNA-mediated approach for targeted delivery. Protein-binding RNAs are initially identified with the systematic evolution of ligands by exponential enrichment (SELEX) and conjugated to siRNA. The delivery is facilitated by the interaction of these aptamers with membrane

Fig.4 Bio-conjugate carrier systems for siRNA delivery.

proteins. Prior reports have stated cell-specific targeted delivery using such aptamer-siRNA chimeras for prostate cancer cells and tumor vascular endothelial cells overexpressing PSMA, a cell surface receptor (McNamara *et al.*, 2006). Evidence for the dual activity of such carriers, such as the inhibition of HIV replication by the aptamer and the target mRNA degradation by the anti-HIV siRNA. (Zhou *et al.*, 2008, 2011). HER2 aptamer-Bcl-2 siRNA conjugates selectively internalized into HER2+ cells and silenced Bcl-2 gene expression (Thiel *et al.*, 2012; Li *et al.*, 2013). Aptamer AS1411 has shown specific binding to cell surface receptors and complexation with a cationic liposome improved intracellular trafficking to target the BRAF gene in melanomas (Li *et al.*, 2014). This gene mutation is found in nearly 60 % of malignant melanomas, which are difficult to treat. A recent review concerning aptamer-siRNA chimeras can be referred to for recent developments (Kruspe and Giangrande, 2017). Hence, this method of delivery improves intracellular delivery with enhanced interaction with membrane-bound proteins.

5.1.3. Cell Penetrating Peptides

Cell-penetrating peptides (CPPs), cationic, amphipathic peptides less than 30 amino acids in length, has emerged as a vital carrier for the transport of siRNA and other drugs across the cell membrane. CPP-siRNA complexes are non-covalently bonded, arising from electrostatic interactions that neutralize the anionic nature of siRNA to yield an overall cationic complex (Meade and Dowdy, 2007). These complexes provide an overall positive charge which helps in permeating through the cell membrane and have also been found helpful in the endosomal escape. Synthetic polypeptide, PVBLG(n)-8, was reported to have a helical structure assisting in amplified cellular uptake by destabilizing the cell membrane (Gabrielson *et al.*, 2012). Amphipathic peptides consisting of lysine and arginine have been evaluated for efficient delivery of siRNA, as well as histidine-rich peptides have also shown enhanced intracellular trafficking with these cell-penetrating peptides (Crombez *et al.*, 2009; Langlet-Bertin *et al.*, 2010; Bartz *et al.*, 2011; Kichler *et al.*, 2013). Octaarginine (R8)-modified lipid nanoparticles have been reported for increased gene silencing, blood circulation, and liver-specific distribution and accumulation *in vivo* (Hayashi *et al.*, 2011; Chu *et al.*, 2015).

The CPP TAT has been extensively utilized for the cell-specific delivery of siRNA. The anti-cancerous drug camptothecin was attached to TAT to effectively kill cancer cells through membrane disruption (Fang *et al.*, 2013; Song *et al.*, 2015). Methoxypolyethylene glycol-polycaprolactone (MPEG-PCL)-conjugated CPP (CH₂R₄H₂C) (C, cysteine [Cys]; H, histamine [His]; R, arginine [Arg]), when complexed with siRNA, resulted in augmented uptake, early endosomal escape, and facile cytosol

release from the complex (Tanaka *et al.*, 2013). Myristic acid conjugated to a CPP with a transferrin binding receptor peptide has shown targeted delivery of siRNA to brain cells through encapsulation (Youn *et al.*, 2014). Cationic liposomes encapsulated the siRNA and the surface is modified with photolabile-caged CPP and asparagine-glycine-arginine peptide (NGR). NGR guided these inactive particles to CD13+ tumor cells where near-infrared (NIR) light illumination activated them by uncaging the lysine from the photosensitive groups (Yang *et al.*, 2015). Cyclic RGD (cRGD) has provided extensive systemic siRNA delivery both *in vitro* and *in vivo* (Khatri *et al.*, 2014; Liu *et al.*, 2014; Huang *et al.*, 2015; He *et al.*, 2017; Zhou *et al.*, 2017).

5.1.4. Antibody Bioconjugates

Antibody-conjugated siRNAs have been an ideal candidate to eliminate off-target effects by mediating cell-specific delivery with an affinity towards the respective antigen. HIV suppression was achieved in humanized mice through conjugation of a CD7 specific single-chain antibody to an oligo-9-arginine peptide for T-cell specific delivery and further, conjugated to chitosan and siRNA nanoparticles, to show enhanced association with the CD4 T-cell line as well as improved gene silencing (Kumar *et al.*, 2008; Lee *et al.*, 2012). Conjugation of anti-STAT siRNA through non-covalent interaction with a monoclonal antibody against Lewis-Y, an antigen expressed in cancer cells, yielded better silencing activity with targeted delivery than seen with a covalently linked vehicle (Ma *et al.*, 2011).

Tumor-specific antibody addition to liposome-polycation-DNA (LPD)-based carriers created TLPD nanocarriers for breast cancer therapy with enhanced binding affinity and gene silencing both *in vitro* and *in vivo* (Gao *et al.*, 2011). Augmented HER2 mRNA and protein silencing was also reported using antibody-polymer conjugates (APCs), with aminoxy-derivatized cationic block polymer conjugated to an anti-HER2 Fab (Lu *et al.*, 2013). Endosome-disruptive biotinylated polymers complexed with streptavidin-conjugated HER2 antibody were found to silence protein expression in breast and ovarian cancer cells (Palanca-Wessels *et al.*, 2016). Antibody-conjugated carriers present great possibilities for effective siRNA delivery (Bäumer *et al.*, 2017).

5.1.5. GalNAc Bioconjugates

GalNAc binds to asialoglycoprotein receptors that are highly expressed on hepatocytes and guides siRNA for targeted delivery to the liver. Nair and colleagues reported that siRNA conjugated with GalNAc showed 5-fold higher gene silencing in the liver. Later reports stated use of a non-nucleoside linker with monovalent units to develop trivalent GalNAc-conjugated oligonucleotides, which mimicked the activity of the parent trivalent GalNAc conjugates and elicited robust gene silencing *in vivo* (Nair *et al.*, 2014; Rajeev

et al., 2015). Alnylam Pharmaceuticals conducted Phase 1 trials of Resuviran, an RNAi-based drug for ATTR amyloidosis treatment with GalNAc-mediated delivery. The target protein, transthyretin, displayed reduced levels up to 90 % with multiple subcutaneous doses (Zimmermann *et al.*, 2017). Their high receptor-specific delivery provides unaltered silencing activity even with a 50 % reduction in receptor expression (Willoughby *et al.*, 2018). Although these classes of delivery agents have been efficient for targeted delivery, rat hepatotoxicity renders them not able to advance into clinical development. This has been attributed to off-target effects and can be corrected with chemical modifications in the siRNA seed region with more thermally destabilizing nucleosides (Janas *et al.*, 2018).

5.2. Nanoparticle-Based Carriers

The nanoparticle carrier systems can be broadly divided on the basis of their compositional unit into two groups i.e. organic and inorganic nanoparticles. Liposomes, micelles and lipid nanoparticles comprise the organic group whereas gold nanoparticles and quantum dots belong to the inorganic class (Fig. 5).

5.2.1 Organic Nanoparticles

Liposomes and lipid-based carrier systems have already been employed in the delivery of oligonucleotides due to similar architecture like a cell membrane. Many clinical trials have been done using lipid-based carrier systems. siRNAs are complexed with these carrier systems either encapsulated or surface immobilized and easily delivered inside the cell. Lipid-coated calcium phosphate nanoparticles (LCP NP) emerged as the successor of liposome-polycation-DNA (LPD) owing to the

biodegradable core and highly negligible immunotoxicity. LCP NP has been found to efficiently inhibit lung metastasis in B16F10 melanoma cells with targeted delivery. The treatment increased the mean survival time of the animals by around 30 % in comparison to the control group (Li *et al.*, 2010; Yang *et al.*, 2012).

A multifunctional envelope-type nano device (MEND) was utilized for PEG-peptide-dioleoyl phosphatidylethanolamine (DOPE) ternary conjugate (PPD) delivery with enhanced cellular uptake and endosomal escape. Further, higher silencing was observed with PPD-MEND co-modified PEG-distearoylphosphatidylethanolamine (DSPE) with no evidence of hepatotoxicity or an innate immune response (Hatakeyama *et al.*, 2011). Stable nucleic acid lipid particles (SNALPs) complex provided efficient transport across the cell membrane when compared to the widely used polycationic carrier linear polyethyleneimine (Kong *et al.*, 2012). A small lipid nanoparticle (SLNP) and mono arginine-cholesterol (MA-Chol) complex assembled by direct attachment via a single arginine on the cholesterol revealed a high level of gene knockdown *in vitro*, whereas *in vivo* experiments displayed elevated gene silencing and enhanced tumor localization of siRNA (Lee *et al.*, 2016). Temperature responsive polymer-modified liposomes change from hydrophilic to hydrophobic above the lower critical solution temperature (LCST). Temperatures higher than the LCST enhanced their delivery into the cell and higher gene silencing with no cytotoxicity was reported (Wang *et al.*, 2017). Low molecular weight chitosan-lipid nanoparticles (CS-LNPs) exhibited ~40 % gene knockdown and no significant cytotoxicity up to 500 $\mu\text{g/mL}$ (Tezgel *et al.*, 2018). Biocompatible gelatin based nanoparticles have also been reported to deliver polymerized siRNA with targeted administration (Lee *et al.*, 2013).

Fig.5 Nanoparticle based siRNA carriers. A. Stable Nucleic Acid Lipid Nanoparticle (Organic); B. Gold Nanoparticle complexed siRNA carrier (Inorganic).

5.2.2. Inorganic Nanoparticles

Nanoparticles architecture with gold, magnetic ores, and other inorganic components have also gained importance with a different mechanism of cellular uptake. They either conjugate the siRNA covalently or encapsulate for intracellular delivery (Jiang *et al.*, 2016). The gold nanoparticles have been surface immobilized with peptides and lipids for efficient delivery of these siRNA therapeutics. Whereas, the magnetic nanoparticles utilize their property to move under magnetic fields and get internalized into the cells through magnetofection. Polymer modified mesoporous silica nanoparticle have revealed targeted delivery in HER2+ breast cancers (Ngamcherdtrakul *et al.*, 2015).

5.3. Complexes as siRNA Delivery Carriers

Cationic polymers have served as a great candidate for transfecting nucleic acids, such as siRNA, across the cell membrane (Fig. 6). The facile complexation of anionic nucleic acids with cationic polymers through electrostatic interactions helps charge neutralization, shield from degradation and improve cell uptake. These complexes have been found to be nuclease resistant (Wagner, 2012; Amjad *et al.*, 2017). Poly-L-lysine, polyethyleneimine (PEI), and poly-D,L-lactide-co-glycolide (PLGA) have gained importance owing to their upregulated transport through the cell membrane (Nimesh, 2012; Ragelle *et al.*, 2013). Encapsulation of PEI/siRNA polyplexes with (DexS/pARG)₅ created degradable capsular structures with better gene silencing in HeLa cells (Ganas *et al.*, 2014). Linear PEI nanoparticles (LPN) showed improved silencing *in vitro* and *in vivo* (Goyal *et al.*, 2012). Co-delivery of siRNA and hypericin to hypoxic human nasopharyngeal carcinoma cells by hyaluronic acid-modified PLGA-PEI nanoparticles showed improved accumulation in the tumor due to increased permeability and retention. Bioreducible polymer based targeted delivery of

siRNA have also been revealed for human cancer gene therapy (Xia and Lin, 2012; Kozielski *et al.*, 2014). Recent reports have shown co-delivery of siRNA and plasmid DNA using novel PEI/poly- γ -glutamic acid nanoparticles (Li *et al.*, 2016; Peng *et al.*, 2017). PLGA-PLL biodegradable nanoparticles exhibited controlled release, efficient gene silencing, endosomal escape, siRNA potency, and resistance against nuclease degradation (Zhou *et al.*, 2012). Targeted systemic delivery has also been achieved with other polymeric micelles (Xiong *et al.*, 2010; Liu *et al.*, 2013; Jhaveri and Torchilin, 2014). HER2+ breast cancer has also been targeted with siRNA using cationic polymer-modified silica nanoparticles (Ngamcherdtrakul *et al.*, 2015; Barrett and Guidry, 2016).

Dendrimers have been a vital addition to the category of siRNA carriers. They are small, compact and cationic in nature, providing a strong binding affinity of siRNA for transfer into the cell. They impart non-toxic delivery of siRNA with a significant increase in silencing activity (Biswas and Torchilin, 2013; Wu *et al.*, 2013; Liu and Peng, 2016). Cationic poly (amidoamine) (PAMAM) and poly(propyleneimine) (PPI) are two common dendrimer systems employed in the targeted delivery of siRNA (Taratula *et al.*, 2009; Kesharwani *et al.*, 2015). PEG-modified PAMAM dendrimers displayed improved resistance against nucleases and gave high transfection efficiency, *in vitro* gene knockdown similar to Lipofectamine 2000-mediated transfection (Tang *et al.*, 2012). Similarly, triethanolamine-modified PAMAM dendrimers showed gene silencing in prostate cancer cells and arginine-modified dendrimers showed prominent drug delivery *in vitro* and *in vivo* (Liu *et al.*, 2012, 2014). PAMAM-PEG-peptide dendriplex with gene-specific peptides were incorporated using a PEG linker and the resulting gene silencing was significantly enhanced. In addition, these dendriplexes were non-toxic, with a cell viability of approximately 75 % (Urbiola *et al.*, 2018).

Table 1. Examples of human diseases targeted with siRNA-mediated RNAi.

Disease	Target	Reference
Age-related Macular Degeneration	Vascular endothelial growth factor	(Garba and Mousa, 2010)
Acute Kidney Injury	P53	(Molitoris <i>et al.</i> , 2009)
Colorectal Cancer with Hepatic Metastases	Macrophage migration inhibitory factor	(He, 2017)
Glaucoma	β 2 adrenergic receptor	(Martínez <i>et al.</i> , 2014)
Dry Eye Disease	Transient Receptor Potential Vanilloid 1	(Moreno-Montañés <i>et al.</i> , 2018)
Chronic Myeloid Leukemia	BCR-ABL gene	(Valencia-Serna <i>et al.</i> , 2014)
Hepatocellular Carcinoma	Kinetochore-associated protein 2	(Makita <i>et al.</i> , 2017)
Hypercholesterolemia	Proprotein convertase subtilisin-kexin type 9	(Kosmas <i>et al.</i> , 2018)
Pachyonychia Congenita	Keratin 6a	(Leachman <i>et al.</i> , 2008)
Primary Hyperoxaluria Type1 (PH1)	Alanine-glyoxylate aminotransferase	(Liebow <i>et al.</i> , 2017)

Table 2. Major siRNA therapeutics in Clinical trials for humans.

Drug	Target	Vehicle	Condition	Participants	Phase/Status	Investigator (Reference)
TKM-080301	Polo-kinase-1	SNALP	Cancer Neuroendocrine Tumors Adrenocortical Carcinoma	68	I, II/Completed	Arbutus Biopharma Corporation, United States (Demeure <i>et al.</i> , 2016)
AGN211745	VEGFR1	NC	Choroid Neovascularization AMD	138	II/Terminated	Allergan, Ireland (Barakat and Kaiser, 2009)
ALN-PCS02	PCSK9	SNALP	Elevated LDL- Cholesterol	32	I/Completed	Alnylam Pharmaceuticals, United States (Fitzgerald <i>et al.</i> , 2014)
ALN-RSV01	RSV-N Gene	-	Respiratory Syncytial Virus Infections	87	II/Completed	Alnylam Pharmaceuticals, United States (J. <i>et al.</i> , 2016)
ALN-TTR01	TTR	LNP	Transthyretin mediated Amyloidosis	32	II/Completed	Alnylam Pharmaceuticals, United States (Coelho <i>et al.</i> , 2011)
ALN-TTRSC	TTR	siRNA-GalNAc	Acute Kidney Injury	341	II/Completed	Alnylam Pharmaceuticals, United States (T. <i>et al.</i> , 2013)

Drug	Target	Vehicle	Condition	Participants	Phase/Status	Investigator (Reference)
Atu027	PKN3	Cationic lipoplex	Advanced Solid Tumors	34	I/Completed	Silence Therapeutics GmbH, Germany (Strumberg <i>et al.</i> , 2011)
Atu027-I-02	PKN3	Cationic lipoplex	Metastatic Pancreatic Cancer	29	II/Completed	Silence Therapeutics GmbH, Germany (Schultheis <i>et al.</i> , 2016)
Bevasiranib	VEGF	NC	Age-related macular degeneration (AMD)	338	III/Terminated	Opko Health Inc., United States (Garba and Mousa, 2010)
CALAA-01	RRM2	CyD NP, transferrin, PEG	Cancer Solid Tumors	24	I/Terminated	Calando Pharmaceuticals, United States (Zuckerman <i>et al.</i> , 2014)
PF-04523655	RTP801	NC	Choroidal Neovascularization Diabetic Retinopathy Diabetic Macular Edema	258	II/Completed	Quark Pharmaceuticals, Israel (Nguyen <i>et al.</i> , 2012)
QPI-1002	P53	NC	Acute Kidney Injury	1088	III/Recruiting	Quark Pharmaceuticals, Israel (Demirjian <i>et al.</i> , 2017)

CyD: cyclodextrin, GalNAc, *N*-acetylgalactosamine, KSP: kinesin spindle protein, LNP: lipid nanoparticle, RRM2: M2 subunit of ribonucleotide reductase, NC: no carrier, PEG: polyethylene glycol, PCSK9: proprotein convertase subtilisin/kexin type 9, PKN3: protein kinase N3, RSV-N gene: respiratory syncytial virus nucleocapsid gene, SNALP: stable nucleic-acid lipid particles, TTR: transthyretin, VEGF: vascular endothelial growth factor

Complexation of siRNA with PAMAM, via a tadpole dendrimer structure conjugated to a peptide through PEG linker, significantly indicated specific gene knockdown over 3 days of administration in mice (Liu *et al.*, 2013). Maltose-modified poly (propyleneimine) dendrimers have been employed with specific conjugated antibodies for cell-specific delivery, coupled with efficient delivery using polyplexes. (Tietze *et al.*, 2017).

6. Current Clinical Trials

With advanced research and extensive efforts in formulating nucleic acid-based therapeutics, siRNA treatment has been studied for various diseases (Table 1). Bevasiranib was the first siRNA-based drug to enter the clinical trials for the treatment of age-related macular degeneration (AMD) (Garba and Mousa, 2010). Drugs targeting the RTP801 gene are also for the treatment of AMD (Nguyen *et al.*, 2012). Treatment for respiratory syncytial nasal virus infection was administered therapeutically in single and multiple dose regimens using the ALN-RSV01 conjugate (Alvarez *et al.*, 2009). Acute renal failure and kidney injury have

been evaluated as critical ailments after cardiac surgery. QPI-1002, a siRNA-based drug, has been analyzed for curing these problems (Demirjian *et al.*, 2017). PCSK9 silencing using a siRNA drug significantly reduced the level of blood LDL cholesterol in healthy patients and provided evidence for their safe administration against hypercholesterolaemia (Fitzgerald *et al.*, 2014). Cancer cells have been reported to overexpress VEGF; hence, siRNA drug targeting inhibition of this gene has notably demonstrated anticancerous activity with sufficient tolerance to the drug (Fitzgerald *et al.*, 2017). Mutation in the transthyretin (TTR) gene results in elevated levels of amyloid and accumulation of these fibrils in various tissues results in heart failure, central nervous system breakdown, and postural hypotension (Rizk and Tüzmen, 2017). This drug already passed the clinical evaluation and approved by FDA as already mentioned in this review.

These are certain classified examples of siRNA-based drugs in clinical trials (Table 2). However, there are other drugs which are awaiting trial recruitment for investigating their action in disease treatment. Therefore, this field provides enormous potential

to extend new domains into genetic therapeutics for numerous undruggable targets.

7. Conclusions

The current developments with therapeutic siRNAs provide many researchers with the scope to utilize this technological tool for effective gene suppression in critical diseases with desired modifications and very soon these drugs will significantly translate into clinical application. Although, lipid based systems have been the initial strategy, now numerous systems play an important role in targeted delivery of siRNAs. At present, it is the combinatorial formulations with different carrier systems which provides the effective systemic delivery of siRNAs. Unique mode of action of each delivery system helps in overcoming the various barriers. They possess all the characteristics at the nanoscale, required for the systemic administration of nucleic acid drugs. The current development with the approval of Onpatro drug by the FDA has provided researchers with the belief of efficiently translating siRNA drugs for clinical application with improved carrier systems. Additionally, RNAi tool have also gained importance as a promising tool for gene regulation in plants also. Advanced breeding technologies are utilizing this tool for creating disease resistant plants or insect resistant plants (Younis *et al.*, 2014; Mamta *et al.*, 2016). Hence, in addition to the mammalian targets, this technological development will also bring reform in the agriculture sector.

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