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REVIEW ON NON-VIRAL DELIVERY SYSTEMS FOR siRNA

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ABSTRACT: The emergence of RNAi, has found new horizons in siRNA as drug molecule. This technology offers a great promise in the achieving efficient treatment. There is a demand for designing efficient siRNA delivery systems/ vehicles that can achieve targeted delivery and replicate the invitro success. The siRNA delivery system selection is based on target cell type. The siRNA delivery systems are categorized as viral and non-viral delivery systems. Non-viral delivery system includes liposomes; polymers; peptides etc. are the widely studied delivery systems for siRNA. Non-viral delivery systems have advantages of control over functionality and industrial scalability. Liposomes were one of the first studied siRNA non-viral delivery systems which with time have evolved on account of modifications on certain attributes like surface charge, surface tagging, composition etc. Peptides and Polymers are the recent addition to the non-viral siRNA delivery vehicles on account of biocompatibility factor. This review clubs the non-viral siRNA delivery systems which have attracted research and have shown efficiency in the clinical trials.

INTRODUCTION: RNAi (RNA interference) is one of the approaches for the treatment of diseases which occur due protein over expression. The RNAi mechanism acts at the molecular level and hence degrade the mRNA that in future will be translated to protein. The RNAi technology utilizes siRNA (short interfering RNA) and miRNA (micro RNA) as its action molecules. Studies in the past have demonstrated siRNA as a successful drug candidate in inhibiting various target classes like transcription factors, growth factors, factor receptors, ion channels and neurotransmitters [1].

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siRNA is double stranded RNA (dsRNA) which is about 21-25 nucleotides in length and is highly specific for complimentary mRNA sequence [1]. The invitro and/or invivo success of siRNA has not been translated to the clinical trials, one of the reasons being delivery challenges and hence there is a demand for safe and effective siRNA delivery systems [2, 3].

The first therapeutic application siRNA in mammals was demonstrated by down regulation of Fas in a mice model with Fas mediated liver fibrosis. It was observed that 82% of mice treated with siRNA against Fas were able to survive for 10 days as compared to 3 days for the control group [4]. One of the first examples for cell type specific delivery was shown when siRNA conjugated with protamine/antibody specifically acted on cells expressing the HIV-1 envelope protein [5].

This paper presents the non-viral siRNA delivery systems that have shown promise and have travelled to clinical trials. The non-viral vector designing and selection needs a careful understanding of the target cell type to have an efficient response. As shown in **Fig. 1**, the siRNA delivery system/vector should also be non-toxic, biocompatible, non-immunogenic with high efficiency to ensure commercial acceptance.

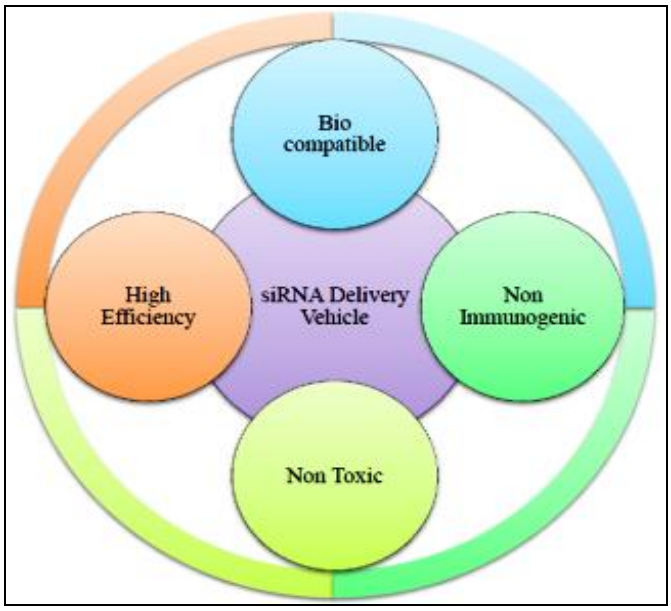

FIGURE 1: DELIVERY SYSTEM/VECTORS FEATURES FOR IDEAL siRNA FORMULATION

Delivery Systems: The delivery system for siRNA has been grouped broadly into two major categories as: viral vectors and non-viral vectors. Non-viral delivery systems offer advantages of ease of synthesis and industrial scalability. As shown in **fig. 2**, liposomes, peptides and polymers are the extensively studied non-viral delivery vehicles [6]. Different types of reported non-viral delivery systems have been discussed in this section. There are constant efforts to achieve siRNA mediated RNAi by playing with the delivery systems and route of administration to treat diseases like cancer, CNS disorders and CVS disorders [7–14].

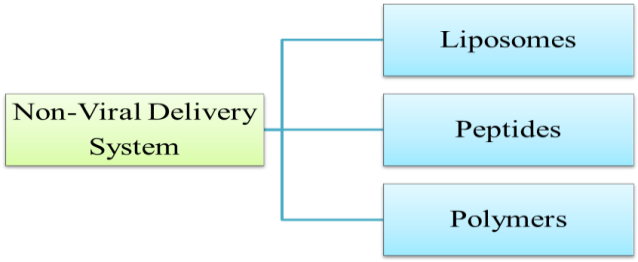

FIGURE 2: NON-VIRAL DELIVERY SYSTEMS FOR siRNA

A. **Liposomes:** Interaction of the lipids with the nucleic acid leads to the formation of either coated vesicles having nucleic acid in the core or the aggregates, both of which are studied as lipoplexes [15].

Alnylam Inc., (Cambridge, Massachusetts) and Massachusetts Institute of Technology (MIT) collaborated on a project to synthesize a library of more than thousand different lipid-like molecules and screened them for their efficiency to deliver siRNA. They tested these delivery systems in mice to treat the respiratory ailment and found that some of their molecules are ten times more efficacious in delivering siRNA in comparison to the existing non-encapsulated siRNA delivery [16].

Lipofectamine- siRNA formulation achieved 50% knock down of the transgenic mutant huntingtin expression, when it was given by i.v. injection to Huntington's disease mouse model, R6/2. This formulation decreased the number and size of intra nuclear inclusions in striatal neurons by the end of eighth week [17]. Successful delivery of siRNA was observed against Japanese encephalitis and West nile viruses utilizing iFECT and JetSI/DOPE [18].

1. **pH sensitive Lipoplex:** The purpose of designing the pH sensitive liposomes is to ensure an enhanced siRNA release from the endosomal compartment. The lipoplexes can be made pH sensitive in one of the two ways; first, by fusing them with the pH sensitive membrane active polyanions which are termed as ternary complex; secondly, conjugation of pH sensitive liposomes to the polyplexes, which show enhancement of transfection efficiency [15].

   Ternary complex prepared with either poly(propyl acrylic acid) or copolymers of MAA/ethyl acrylate (EA)/butyl methacrylate (BMA) have shown enhanced efficiency in comparison to parent, polymer free complex [19–21]. These pH sensitive ternary complexes have been reported stable in the presence of serum components [22].

2. **Cationic lipid based Liposomes:** siRNA formulation with a new cationic, amino acid derived lipid, N′,N″-dioleylglutamide (DG), incorporating the fusogenic lipid, 1,2-dioleyl-sn-glycero-3-phosphoethanolamine (DOPE) and cholesterol had high transfection efficiency and low cytotoxicity in comparison with the conventional cationic lipid based liposomes, Lipofectamine® 2000. This formulation achieved a higher viability and delivery of the fluorescent siRNA in the A549, HeLa, and WM266.4 human cancer cell lines in comparison with Lipofectamine® 2000 and cholesterol (DC-Chol) - based liposomes and was found effective in lowering surviving mRNA levels [23].

3. **Multifunctional Envelope-Type Nano Device (MEND)**: MEND assembles multiple devices in a single delivery system [24]. The lipid envelope of MEND is prepared by condensation with polycation. Modifications of MEND are also reported like; Stearylated octaarginine (STR-R8) modification on the lipid envelope of MEND resulted in enhanced siRNA cellular uptake by macro pinocytosis. MEND modification by addition of dioleoylphosphatidyl ethanolamine (DOPE) and phosphatidic acid (PA) led to an increase in siRNA release. MEND modification by conjugation of pH sensitive fusogenic peptide GALA (WEAALAEALAEALAEHLAEALAEAL EALAA) along with cholesterol favored endosomal release of siRNA. STR-R8-MEND modification with a cleavable PEG–peptide–DOPE conjugate (PPD) showed efficiency in *in-vivo* tumor targeting [25–27].

Nanoparticle in Microparticle Oral System (NiMOS), is a delivery technology for shorter nucleotides, and has been successfully applied to murine IL-10 gene expressing plasmid DNA and later to anti-TNF Alpha siRNA [28].

4. **Stable Nucleic Acid Lipid Particle (SNALP):** Tekmira Pharmaceuticals, in partnership with Alnylam Pharmaceuticals, developed a specialized liposome nanoparticle, Stable Nucleic Acid Lipid Particle (SNALP) which is one of the major achievements in systemic siRNA delivery[29]. SNALPs are different from the conventional liposomes as their lipid bilayer consists of cationic lipids, neutral lipids and PEG-lipid fusion regulators. SNALP formulations are reported to have a longer half-life in plasma and liver [30]. SNALP also protects the siRNA from RNase degradation [31]. Anti- hepatitis B virus (HBV) siRNA formulated in SNALP successfully inhibited the HBV replication [32].

   Studies on cynomolgus monkeys showed more than 90% silencing of the Apolipoprotein B messenger RNA (ApoBmRNA) in liver 48 hrs after i.v. administration of SNALP formulated siRNA against apolipoprotein B (siApoB). This formulation was shown to have an immediate, potent and lasting biological effect of siRNA treatment [33]. SNALP has attracted much of the research as evident from the 6 clinical candidates (ALN-VSP02, TKMApoB, ALN-TTR01, TKM-PLK1, ALN-PCS02, TKM-EBOLA) [34]. The phase 1 clinical trials of SNALP-formulated siRNA drugs of Tekmira (TKM-ApoB) targeting ApoB and Alnylam (ALN-PCS) targeting proprotein convertase subtilisn/kexin type 9 (PCSK9) both of which are for hypercholesterolemia were found to be safe and well tolerated without any serious adverse events [35, 36].

5. **AtuPLEX:** Recent modification to liposomal carrier includes 'AtuPLEX', a mixture of cationic and fusogenic lipids developed by Silence Therapeutics plc (formerly Atugen AG) for enhanced transfection efficiency. They reported successful silencing of CD31/Platelet endothelial cell adhesion molecule 1 by i.v. injection of anti-CD31 siRNA-AtuPLEXes. This silencing inhibited tumor growth and suppressed metastases in a PC-3 prostate xenograft model. Repeated systemic administration of the anti-CD31 siRNA-AtuPLEXes did not induced any toxicity [37, 38].

6. **Lipidoids:** Lipidoids are the liposome analogues with different chemical modifications of lipids to meet challenges of degradation, solubility and siRNA release. The siRNA-lipidoid formulation "98N12-5", which comprises five 12-carbon alkyl-acrylamide chains attached to an amine core, successfully knocked down the blood clotting factor, Factor VII in hepatocytes, mice and rats [16].

   Efficient silencing of Hmox1, a liver gene encoding the protein heme oxygenase-1 in mice was achieved using siRNA-lipidoid formulation. The group believes this silencing might also find application in malaria [39]. The lipidoid molecule C12-200 is reported as the most efficacious lipidoid to date [40].

B. **Polymers - Chitosan and Cyclodextrin:** Chitosan is a cationic polysaccharide having muco adhesive properties and which is reported to have low cytotoxicity. Chitosan is reported to have low transfection efficiency due to endosomal release challenges [41]. It is reported that fully de-N-acetylated chitosan are superior siRNA carriers in comparison to conventional partially N-acetylated chitosan [42].

   Chitosan modification by polyethylenimine (PEI) conjugation, Chitosan-graft-polyethylenimine (CHI-g-PEI) made the acid soluble chitosan easily soluble in water at the physiological pH and showed enhancement of transfection efficiency both invitro as well as invivo upon aerosol delivery. The complex CHI-g-PEI and siGFP/ scrsiRNA (scrambled siRNA) showed efficient siRNA condensing and efficiency in silencing of the oncoprotein Akt1.

   The group also reported a higher silencing of EGFP expression in A549 using anti-EGFP siRNA [41]. siRNA-chitosan formulation was effective in decreasing the population of EGFP expressing epithelial cells in the bronchioles by 43%, upon intranasal administration to EGFP expressing transgenic mice [43].

   Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene silencing was observed using siRNA- chitosan formulation in HEK293, HeLa and H1299 cell lines. This formulation also showed its efficacy in to primary cells (HUVEC) and MCF-7 cells which were difficult to transfect [42].

   Besides chitosan cyclodextrin is another polymer that has also been studied as siRNA delivery system. Cyclodextrin–siRNA nanoparticle formulation of Calando Pharmaceuticals (CALAA-01), against the M2 subunit of ribonucleotide reductase, a critical enzyme in cancer cell proliferation, has entered Phase 1b clinical trial, which is the first-in human study involving systemic siRNA administration to patients (NCT00689065) [44,45].

C. **Peptides:** Ease of synthesis, control over functionalization and stability of the peptide oligonucleotide complex make the low molecular weight peptides as the favorable candidates over lipoplexes as siRNA delivery vehicle. Peptides are viewed as alternative to the cationic polymers for the siRNA delivery due to four major reasons a) Their efficient packaging b) Cell specific delivery c) Efficient membrane transport and d) pH based membrane disruption [46].

   The cell penetrating peptides (CPPs) are the widely studied peptides as siRNA delivery system. **Table 1** shows some of the extensively studied CPPs. CPPs are less than thirty amino acids and have a positive charge at the physiological pH [6,47]. The CPPs are studied under three classes: a) naturally derived peptides (e.g. HIV-1 transactivating protein TAT and penetratin); b) chimeric peptides (e.g. transportan, a chimeric peptide composed of galanin and mastoparan) and c) synthetic ones (e.g. oligoarginine) [48–53].

   Suppression of VEGF production in CT-26 cells and in mouse model was observed upon local administration of Chol-R9/siVEGF complexes to subcutaneous tumor [57]. Enhanced silencing of p38 MAP kinase mRNA in mouse lung was reported using delivery systems containing cholesterol and cell penetrating peptides (CPPs) TAT and penetratin [58].

TABLE 1: CPPs FOR siRNA DELIVERY

Peptides	Sequence
HIV TAT	CGRKKRRQRRRPPQC [46]
JTS	GLFEALLELLESLWELLLEA [46]
Transportan	GWTLNSAGYLLGKINKALAALAKKIL [52]
TAT 48-60	GRKKRRQRRRPPQ [54]
Penetratin (Antp 43-58)	RQIKIWFQNRRMKWKK [55]
MPG	GALFLAFLAAALSLMGLWSQPKKKRKV [56]

Complexing of siRNA with CADY (Ac-GLWRALWRLLRSLWRLLWRA-cysteamide) (1:20 molar ratio) protects siRNA from serum nucleases. siRNA-CADY formulation successfully reduced GAPDH mRNA and protein levels in various cell lines including THP1 and primary HUVEC and 3T3C which are reported as difficult to transfect [59].

The silencing of galanin receptor in human bowes cells was one of the first demonstrations of antisense activity utilizing a 21-mer PNA disulfide having conjugation to the CPPs penetratin or transportan (hybrid of a section of the neuropeptide galanin and the wasp venom peptide mastoparan). This formulation was reported nontoxic at the working concentration and reflected pain modifying activity upon intrathecal injection to rats [60].

The Membrane perturbing peptides (MPPs) are also studied as peptide delivery systems. The MPPs are studied in two categories depending upon the DNA release behavior:

1) Fusogenic peptides

2) Endoosmolytic peptides. The fusogenic peptides act by mediating the DNA release at the endosomal pH.

The endoosmolytic peptides act by endosomal lysis followed by DNA release. Examples of fusogenic peptides include HA-2, N terminal end of influenza virus hemagglutinin and its derivatives like GALA, KALA and synthetic peptides like JTS [61].

D. **Hybrid Non-viral Delivery Systems:** Combination of two or more types of delivery vehicles have led to generation of this category. These combinations may be categorized as Liposome-Peptide, Liposome-Polymer, Peptide -Polymer and any other combination thereof [62, 63]. Studies have demonstrated invitro effectiveness of Liposome-siRNA-peptide complexes (LSPCs) in delivering PrP siRNA specifically to AchR-expressing cells, leading to suppressed PrP^{C} expression and eliminated PrP^{RES} formation. This formulation was reported to be stable in serum upon i.v. injection to mice [9].

Liposomal-siRNA- peptide formulation with siRNA against endogenous luciferase and GAPDH successfully silenced these genes in 3 cell lines (1HAEo- human airway epithelial, B104 rat neuroblastoma, Neuro2A-Luc mouse neuroblastoma) with 80% efficiency, similar in efficiency to Lipofectamine® 2000. This formulation had an additional advantage of siRNA protection from RNAse A [64].

Kodama *et al* (2013) reported binary and ternary complexes of liposomes and polymers for safe and effective siRNA delivery. They utilized cationic polymers and cationic liposomes like polyethylenimine (PEI), polyamidoamine (PAMAM) dendrimer, poly-L-arginine (PLA), trimethyl[2,3-(dioleoxy)-propyl]ammonium chloride (DOTMA), and cholesteryl 3β-N-(dimetylaminnoethyl) carbamate hydrochloride (DC-Chol) for the binary complexation and added γ-polyglutamic acid (γ-PGA) to form the ternary complex.

The ternary complexes showed significant suppression of luciferase activity upon direct injection into the tumors of mice bearing Colon26/Luc cells [65].

PATENTS: In a short span of its existence, RNAi via siRNA have resulted in generation of significant intellectual property. **Table 2** shows some of the patents on siRNA delivery vehicles.

TABLE 2: PATENTS ON NON-VIRAL SIRNA DELIVERY SYSTEMS

S. No.	Patent No.	Title	Publication Date
1	WO/2004/045582A1	High-Efficiency Fusogenic Vesicles, Methods of Producing them, and Pharmaceutical Compositions Containing them [66]	06/03/2004
2	US20050008617A1	Compositions and methods for delivery of short interfering RNA and short hairpin RNA [67]	01/13/2005
3	WO/2006/016097A2	Vector Comprising Polymer Modified siRNA Liposomes [68]	02/16/2006
4	WO/2009/006905A1	Dehydrated Chitosan Nanoparticles [69]	01/15/2009
5	WO/2010/113172A1	Amphoteric Liposomal Compositions for Cellular Delivery of Small RNA Molecules for use in RNA Interference [70]	10/07/2010
6	US20130072424A1	Compounds and Methods for Peptide Ribonucleic Acid Condensate Particles for RNA Therapeutics [71]	03/21/2013
7	WO/2013/075244A1	Peptide Sequence Design and Use thereof for Peptide-Mediated siRNA Delivery [72]	05/30/2013
8	EP2626427A2	siRNA conjugate and preparation method thereof [73].	08/14/2013

CONCLUSION: siRNA delivery has been attempted by both viral vector and the non-viral vectors. Limitations with the viral vector are a high probability of immune response and random binding of the nucleotide to the non-target sequences, both of which are not desired. The non-viral vectors on the other hand have a lower efficiency in comparison to the viral counterparts but they show very less immune response besides targeted binding. It is these attributes that have made them as the favorable siRNA delivery vehicle.

It is the liposomal delivery systems which appeared in most of the clinical trial formulations of siRNA. The role of proteins and polymers is also under research for its efficient targeting. In the near future it will be the protein/ peptide delivery systems that will challenge the liposomal delivery systems for the efficient silencing. As these proteins/ peptides upon degradation form amino acids which can be taken up by the cell itself. Role of polymers is equally valued although it is more stressed on the polymers from the natural sources like bacteria etc.

One of the sections the paper discusses the hybrid vehicles which are actually the intra non-viral hybrid vehicles. These hybrid siRNA delivery systems will be the future attraction of the siRNA delivery research. These hybrid siRNA delivery systems play with the surface properties of the liposomes which have proved its efficiency. Hybrid non-viral vectors like, liposome –siRNA- peptide are also reported to be efficient and have advantage of serum stability which is very vital for successful application of RNAi.

The selection of the appropriate delivery system is very critical decision. This selection has to be made on account of certain attributes like the surface properties, nucleotide loading capacity, and stability of the delivery systems besides the invivo behavior. In order to achieve targeted delivery various modifications in the non-viral siRNA delivery vehicles have been attempted like, surface charge, peptide tagging or covalent attachment of target cell receptor's activators for efficient entry into the cell.

The non-viral vectors have advantages of industrial scale up which favor commercialization possibility. It is the industrial scale up property of the non-viral siRNA delivery vehicles which have made them travel from the lab to the clinical trials. It is evident from the literature that the RNAi technology is growing but there is still a missing link that needs to be filled to convert the clinical failure into success and siRNA delivery system designing is a critical feature of that link.

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