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ATTRACTIVE APPROACHES IN siRNA DELIVERY USING POLYMER BIO-BASED CARRIER SYSTEMS

Şükrü TÜZMEN[1], Zülal YALINCA[2,*]

1 Molecular Biology and Genetics Program, Department of Biological Sciences, Faculty of Arts and Sciences, Eastern MediterraneanUniversity, Famagusta, Mersin 10, Turkey
2 Department of Chemistry, Faculty of Arts and Sciences, Eastern MediterraneanUniversity, Famagusta, Mersin 10, Turkey

ABSTRACT

Functional characterization of genes can be determined by disruption of gene expression. This provides powerful approach in designing novel treatment strategies. RNA interference (RNAi) is a natural phenomenon that can aid in the study of post-transcriptional regulation of genes. This mechanism can be stimulated via introduction of double stranded RNA (dsRNA) in a cell. Synthetic short/small interfering RNAs (siRNA) can be utilized to trigger down-regulation of desired genes via transfecting into mammalian cells. Recently, utilization of polymeric carriers has been an attractive approach in drug delivery for medical applications. This review article describes the advantages of exploiting polymeric carriers for siRNA delivery, as bio-therapeutics. Here, we report the current developments, safety and delivery of bio-based siRNAs via polymeric carriers. Additionally, cancer genetics and metabolic disorders including obesity and diabetes pertaining to the progress in clinical applications have been highlighted.

Keywords: siRNA, bio-therapeutics, Post-transcriptional regulation, Polymeric delivery systems

1. INTRODUCTION

RNA interference (RNAi) is a biological event in which double stranded RNA (dsRNA) molecules suppress gene expression, causing silencing of specific transcripts. RNAi mechanism is precise, efficient, stable and promising technology for bio-therapeutics [1-5]. Additionally, RNAi phenomenon is known to act as a defense mechanism by the hosts' system against parasitic [6], viral [7] and transposon infections [8]. RNAi machinery also has been illustrated to influence developmental stage of crops [9]. For whole manuscript, the Dot should be at the end of the reference square. This operation is triggered by dsRNA (<25 nucleotide) processed from long (200 nucleotides or longer) RNA duplexes. Following the introduction of dsRNAs into a cell, an enzyme called Dicer [10] cuts these long dsRNAs into approximately 21 nucleotide long small dsRNAs. These new entities are referred to as small/short interfering RNAs (siRNAs) [11, 12]. siRNAs are essential tools for RNAi technology, which enable gene silencing [9, 13-22]. To date this technology has been used in many areas of research including metabolic disorders and cancer genetics [23].

Major obstacles to RNAi mechanism involve poor pharmacokinetic property and biological handicaps such as interferon activation [5, 11, 20, 24-26] and off-target effects [27-29]. Long double stranded siRNAs (200 nucleotides or longer) and high concentration of siRNAs can trigger immune system activity in cells. Additionally, therapeutic applications facilitated by siRNAs may be affected by low transfection rates and poor delivery [3, 4, 19, 30, 31]. Nevertheless, additional obstacles still exist regarding the journey of siRNA involving clinical applications for therapeutic utilization [19]. Xu

*Corresponding Author: zulal.yalinca@emu.edu.tr

Cong-Fei. *et al.* [12] reported that siRNAs may be unstable under physiological conditions during the siRNA trafficking through the systemic delivery. siRNAs destroyed by enzymatic activity of the nucleases such as RNase in the serum can be excreted through kidneys. Furthermore, siRNAs can also be destroyed by phagocytes, and aggregation with serum proteins. To date, vast number of research experiments is carried out utilizing polymer-based carriers for effective siRNA delivery. In this review we will concentrate on siRNA loaded polymeric carriers.

2. RNAi: Mode of Action

RNAi mechanism depends on the encapsulation of the siRNA of interest, and its delivery to the target cell or tissue. Following the absorption by endocytosis, siRNAs need to be released into the target cell in order for gene suppression to take place. The major obstacles of siRNA functionalities include delivery[32, 33], siRNA degradation [3, 34, 35] and off-target effects [3, 27, 28, 36, 37]. As illustrated in Figure 1. RNAi mechanism can be synthetically triggered utilizingpolymeric carriers [15, 38].

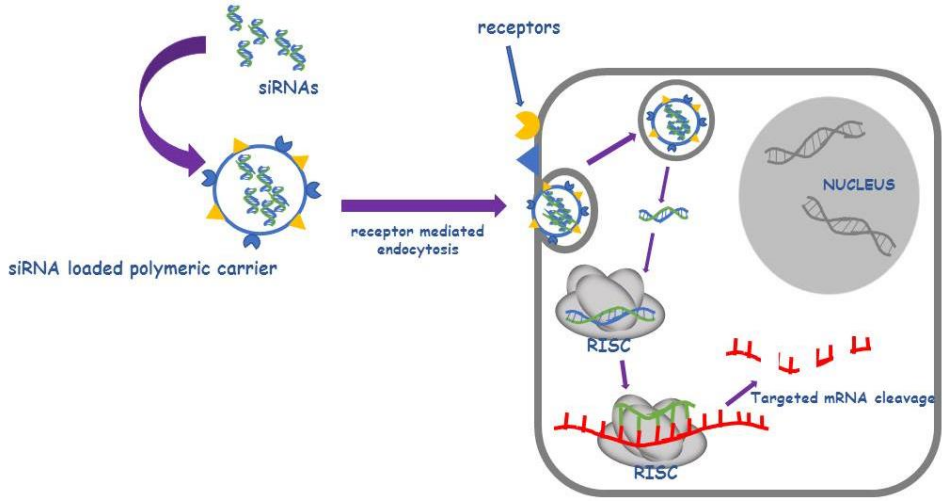

Figure 1. Mechanism of siRNA delivery via polymeric carriers (adapted from [1, 3, 16, 19, 30, 34, 38])

3. The Hallmarks of Effective siRNAs

3.1. siRNA Delivery

Gene delivery systems can be classified into viral and non-viral carriers. Currently, both viral and non-viral siRNA delivery systems are utilized and there exist advantegous and disadvntages for each approach. The efficacy of gene silencing approach depends on the efficiency of the carrier used for siRNA/shRNA delivery. Viral based systems usually utilize retroviruses, lentiviruses, adenoviruses or adeno/associated viruses as delivery vectors. The advantage of viral delivery system is to facilitate high transfection efficiency, but with a risk of off-target effect. In contrat to viral delivery systems, non-viral delivery systems are much less cytotoxic but with lower transfection efficiency. Additionally, it should be also noted that pharmaceutical grade purity needs to be considered as critical factor for marketability in formulation development of non viral carriers. Viral based delivery systems illustrated rather efficient transfection properties with high risks such as immunological reactions. Due to the drawbacks of viral based carriers, there has been increased attention in the utility of non-viral based delivery systems such as cationic lipid systems (liposomes, lipids, micelles, emulsions), polymeric delivery stsems (dendrimers, albümin, PEI) and antibodies for suitable siRNA delivery [21, 23, 39-41].

The site of action of siRNAs is the cytoplasm. Multiple hurdles for siRNA delivery may exist. In general, local delivery of siRNAs may be more effective than systemic delivery. There are many studies outlining physical and immunogenic barriers of siRNA delivery including delivery to the eye, skin, lung and brain [41]. Additionally, numerous studies pertaining to systemic delivery may involve intravenous injection, which can pose greater challenges including, increased stability [42-44]. It is worth noting that the challenges of endocytic uptake and stability of non-viral siRNA carriers are facilitated prior to initiating an experiment. It is also equally important that siRNA degradation be considered and designed accordingly to avoid siRNA destruction [39]. The ideal siRNA delivery system should possess the properties including high transfection efficiency, and controlled siRNA release [37, 39, 45]. siRNA transfection efficiency can be considerably increased by nano-carrier utilization. Additional information regarding intracellular delivery of nano-carriers can be found elsewhere [34].

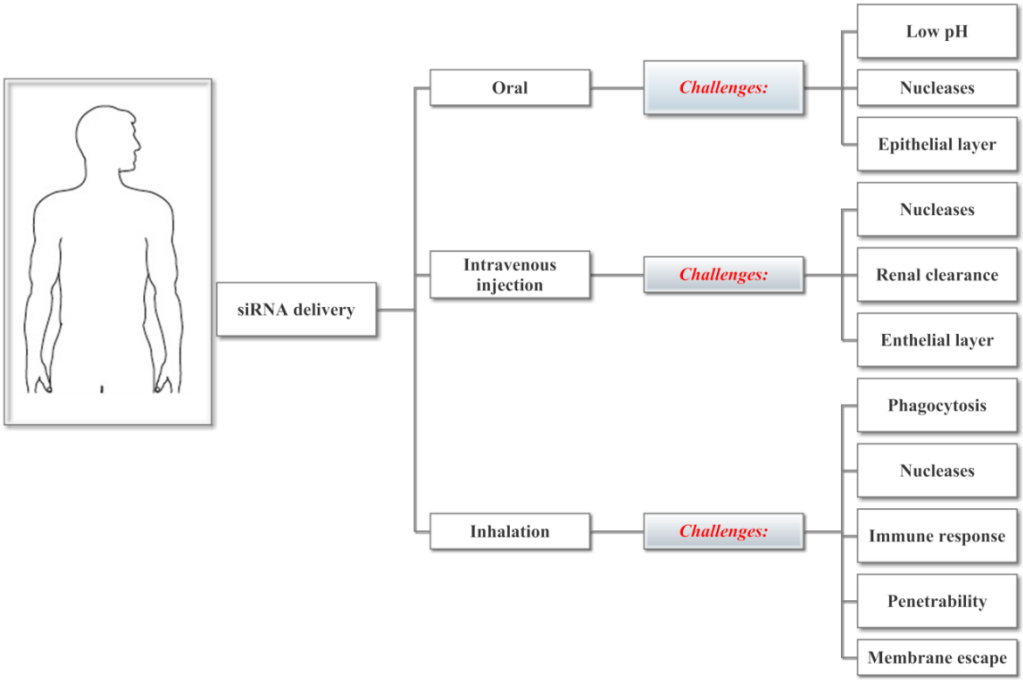

Figure 2. Routes and challenges of siRNA delivery. (adapted from[34, 37, 46, 47])

The choice of application for siRNA delivery is determined by the availability of the target tissue/organ within the body[37, 48]. The efficacy of delivering nanoparticles depends on the site of administration[34]. Nanoparticles possess the ability to escape endosomes. This escapability provides the efficacious gene silencing compared to the controls without ability to escape [34, 49].

3.2. siRNA Degradation

RNAi mechanism involves ~21nt long dsRNA (double stranded RNA) molecules that induce silencing of complementary mRNA target(s), facilitating post-transcriptional gene regulation. In the cytoplasm of cells, an enzyme known as Dicer initiates RNAi mechanism by binding and cleaving long dsRNAs (>200nt) to generate short interfering RNAs (siRNAs). The resulting siRNAs are incorporated into a RNA induced silencing complex (RISC) and siRNAs are unwound by the helicase action of this RISC holoenzyme complex yielding a sense and an anti-sense strands of siRNAs. Following this process, the RISC complex chaperones the anti-sense strand of the siRNA to the complementary RNA template for targeted degradation giving rise to gene silencing. Poor stability and short half-life can be listed amongst many obstacles of efficacious siRNA applications as therapeutic agents. Certain strategies have been investigated for improvement of siRNA stability [30].

There exist many hurdles in the use of siRNAs as therapeutic agents due to their size, surface charge and vulnerability towards enzymatic digestion [50]. Innovations in clinical utility of RNAi will be supported by non-cytotoxic and effective siRNA design [50].

3.3. Off-Target Effects

Generation of immune response and low transfection efficiency are the major hurdles that must be overcome urgently. Off-target effects can be caused by suppression of gene expression other than desired gene targeting resulting in repression of non-targeted genes causing undesired post-transcriptional regulatory outcomes [18, 29]. Partial binding of siRNAs with its target mRNA sequences or incorporation of sense strand into RNA Induced Silencing Complex (RISC) [17] can cause off-target effects. Cell death can be the consequence of off-target effect regardless of knocking down the targeted genes. Moreover, even scrambled sequences may sometimes cause off-target effects resulting in cell death based on siRNA concentration and cell types used [37]. Exogenous siRNAs may also trigger innate immune response. Strategies involving chemical modifications aid in avoiding immune response. However, the mechanism remains not fully understood [4, 51-54]. Currently, siRNA based therapeutics are considered to be at its infancy. This may involve the unresolved issues such as varying siRNA dosage, siRNA delivery, timing and duration, targeted gene of interest and disease choice.

4. siRNA Delivery Systems

It is feasible to utilize similar carrier molecules for siRNAs, which are already developed for DNA, since DNA and siRNAs share similar characteristics pertaining to the carriers administered. These carrier systems can be classified under two different categories:

a) Viral based carrier systems
b) Non-viral based carrier systems

The viral based delivery systems show rather good transfection properties, however large number of problems are associated with the use of these vectors. Given the problems with viral vectors, there has been an increasing interest in the use of non-viral lipidic and polymeric delivery systems and antibodies for suitable siRNA delivery [16, 31, 34, 55].

The handicap in siRNA delivery technology can be caused by extreme temperatures, which can inhibit the activities of these nucleic acid based drugs. Hence, inhibiting specific targeted effects. Encapsulation of siRNAs within nano vesicles will aid in protecting the siRNAs from being destructed by nucleases and immune response of the host. Therefore, enabling successful delivery of siRNAs. Moreover, delivery of siRNAs to their targets such as tumor cells can be enhanced by nano ligand bound vesicles [3, 19, 56].

The following properties of siRNAs and their carriers may hinder their clinical applications:[57-59]

a) Poor water solubility
b) Poor hydrophobicity of nano-carriers
c) Bioaccumulation of nano-carriers

In order to overcome these challenges, hydrophilic modifications may be required. Hence, improving the stability of nano-carriers and increased intestinal and oral absorption. Biodegradability and reduced size are significant factors for avoiding bioaccumulation [56-59]. It is advised to evaluate the type and the quantity of nano-carrier to be administered in order to avoid their immediate removal from the body [56].

Viral carriers in general have potential immunogenic effects, for this reason non-viral carriers are more popular alternatives.[60] Viral based carriers have been reviewed elsewhere [61-63]. siRNA and nano-carrier combination is a method of choice to avoid limitations of nucleic acid formulation [64-66].

Non-viral polymeric materials involved in siRNA delivery can be subdivided into two categories:[4, 37]

a) Natural polymeric carriers including chitosan [67-69], albumin [70-72], gelatin [64, 73].
b) Synthetic polymeric carriers including cyclodextrin [74-76] polyethylene glycol (PEG), polyethyleneimine (PEI) [12, 77], poly (d,l-lactideco- glycolic acid) (PLGA).

Enhanced stability, efficient delivery, and regulated release are amongst the advantages of biodegradable polymeric nanocarriers [19, 56, 59].:

The following steps are involved in non-viral siRNA carriers and siRNA complexing [41, 56].

a) Complex formation of siRNAs with cationic carrier
b) Conjugation of siRNAs with small molecules.
c) Encapsulation of siRNAs within nanoparticles.

Terrazass *et al*.[78] reported that RNA backbone modification enhanced the stability of siRNAs, with no disadvantage towards RNAi efficiency [78]. siRNA properties can justify the type of delivery system chosen. Positively charged polymeric materials that have high charge density introduces "proton sponge" properties, which can facilitate endosomal escape, protecting oligonucleotide degradation [79, 80] The desired properties of nano-carriers include siRNA protection from degradation, escape from immune system recognition especially in blood circulation [19, 40]. Surface charge of the carriers should be taken seriously as cationic siRNA complexes are likely to react with serum proteins [56].Amongst the positively charged polymers, PEI induce endosomal escape via proton sponge hypothesis. Boussif *et al*.[79] reported a study involving buffer capacity pertaining to cationic polymers with proton sponge characteristics. Cytotoxicity is a disadvantage of PEI and cytotoxic effect increases with an increase in the size of PEI. However, low molecular weight of PEI cannot sustain multi-molecular structure in physiological state. For this reason, low molecular weight (i.e. 800 Da) is usually conjugated with each other to increase the molecular weight to 10-20 kDa, through biodegradable linkage. PEI can also be utilized for systemic siRNA delivery via constructing ligand targeted systems [41, 81]. Osmotic swelling can be induced by PEIs leading to cytoplasmic release of siRNAs avoiding subsequent siRNA degradation [79, 82-84]. Biocompatible polymers such as PEG can be tagged to the ends of siRNAs in preclinical studies. Pegylating both ends of siRNAs provide protection of siRNAs more than 48 hours in serum. However, the extent of pegylation can prevent intracellular uptake of siRNAs owing to the steric effects of cellular membranes. In order to avoid steric effects, PEG-siRNA conjugate can be modified by addition of lactose and folate molecules. Furthermore, molecular size involving Nitrogen/Phosphorous (N/P) ratios indicated that efficacy of transfection significantly relies on characteristic composition of the non-viral carriers [85, 86]. Cytotoxicity may be induced via electrostatic interaction between polyplex and cell membrane stimulating undesired immune response [40]. Pegylated carriers can also be utilized for intravenous administration in order to be able to delivery siRNAs in a tumor-selective manner [81]. PEG facilitates the permeability and retention effect in delivery systems [86].

PLGA possesses characteristics such as biodegradability, biocompatibility, which enables its utility as a biopharmaceutical agent. However, as PLGA is not soluble in water on its own, it is not the agent of choice to be utilized alone with siRNAs, as a non-viral based system [40, 86]. Modification of PLGA has gained impetus to facilitate formation of positively charged carriers for increased cellular uptake. These efforts include coating PLGA with chitosan [41, 87, 88]. Combination of PEI into PLGA nano-carriers enhanced siRNA loading activity. This incorporation facilitated siRNA protection and

increased gene knockdowns in cultured cells [89, 90]. Dendrimers are also utilized as carriers of small molecule drugs and large biomolecules [91]. Structure, molecular weight and surface charge are amongst the properties of dendrimers that makes them biocompatible. The number of branching paths in dendrimers can be controlled during chemical synthesis. This provides an advantage in supplying increased surface groups, closer packing and high charge density. Thus, dendrimers are being used for nucleic acid delivery owing to their beneficial properties including structural homogeneity and multifunctional nature [40,92, 93]. Cyclodextrins and their derivatives are used in pharmaceutical formulations to enhance the solubility, stabilization, and absorption of small molecule drugs and proteins or peptides. Another frequently utilized natural polymer involves chitosan based systems. Chitosan is a copolymer of N-acetyl glucosamine and glucosamine with relatively low cytotoxicity and immunogenicity. However, there exist limitations of chitosan such as low solubility in physiological pH, reduced buffering properties and poor cytoplasmic dissociation kinetics. Numerous modifications of chitosan including pegylation, thiolation illustrated advantageous potential in cancer models [40, 41, 67-69].

5. siRNA Delivery in Cancer Treatment

Cancer is a genetic disease. Currently, small molecular therapeutics are being used to target defective genes causing cancer [18]. Scientists are working on finding optimal therapeutic approaches for cancer treatment. In spite of accumulated knowledge in the cancer field, cancer still remains to be the second leading cause of death in the world [94]. Owing to the new generation sequencing technologies, many genetic aberrations have been pinpointed in tumor samples. Hence, enabling siRNA targeting of mutant genes within many genetic disorders [23]. One of the options for cancer patients is to go through chemotherapeutic treatment, but the major obstacle for such treatments is the development of multi drug resistance. However, in situations where small molecular drugs have complex characteristics, RNAi therapeutics would be method of choice. In some instances, combination of siRNA treatment responds better towards the curable outcome. This can also be utilized as combination of small molecule therapies together with siRNAs. Utilization of RNAi therapeutics has revolutionized cancer treatment. Combination therapies can enhance drug therapeutic response and help outcome multi-drug resistance. Additionally, further combination approaches such as chemotherapy, immunotherapy, radiation therapy or photodynamic therapy together with siRNAs can drastically enhance the efficacy of cancer treatment. siRNAs have considerable advantage pertaining to the cancer treatment due to their potential to down-regulate genes not depending on the druggability of their protein products [18, 95-98].

Advantages of synergistic effects of siRNAs together with small molecule anticancer drugs [99, 100].

a) Prevention of multi drug resistance
    i. Reduction of drug concentration at the target site by small molecule drug
    ii. Increase in drug concentration at the drug target site by siRNA with small molecule drug
b) Facilitation of synergistic apoptotic effects
    i. Utilization of specific pathway through small molecule drug
    ii. Multiple pathway activation resulting in synergistic apoptotic effect by siRNA and small molecule drug
c) Reduction of toxicity
    i. Synergistic effect of siRNA and low dose of small molecule drug, which results in reduced side effects [99, 100].

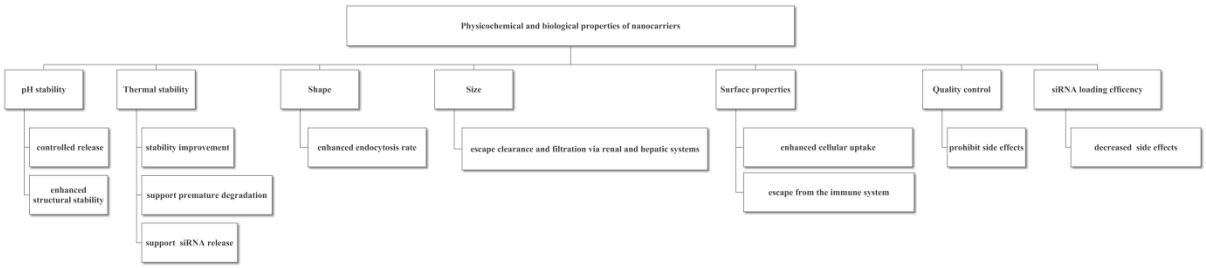

Figure 3. Physicochemical and biological properties of nano-carriers.(adapted from[56])

Liposomes and polymer mediated formulations for drug delivery in particular doxorubicin has been introduced into clinical cancer research in year 1995. However, more cancer specific approaches are required [42]. Thus far, gene knock-down methodologies for cancer treatment has been shown via numerous preclinical studies in order to investigate the efficacy of RNAi mechanism. Smart designs including multifunctional carriers integrated with certain responsive elements such as pH and magnetic sensitive modules may enhance the efficacy of siRNA targeting toward neoplastic sites. Nonetheless, chemical alterations of siRNAs will carry on to enhance their efficacy and reduce their off target effects and their undesirable side effects in order to expedite the translational applications of siRNAs from bench to bed-side [101].

6. siRNA Delivery in Metabolic Disorders

Metabolic disorders such as hypercholesterolemia, liver diseases, obesity and diabetes can involve specific post-transcriptional regulation in their treatment [102]. RNAi efficacy in vivo has been demonstrated in various infectious diseases including hepatitis HBV [23, 103], HCV [104], HIV [105, 106] and diabetes [16, 32, 34, 102, 107-109]. The current understanding of RNAi technology and its utilization in the pathogenesis of obesity and diabetes have been shown to target genetic signatures involved in insulin metabolism in liver [107].

There are several ways of applications pertaining to siRNA therapeutics including oral and topical administration, which exhibit a great potential for new siRNA therapeutics. Particularly, liver plays a critical role as a target for siRNA delivery. Liver is the site of vast number of viral infections, cancer and metabolic disorders [55]. Additionally, RNAi therapeutics provides promise for the development of new translational methodologies in order to treat diseases that are yet hard to cure and may facilitate therapeutic remedy of disorders such as depression and obesity [107, 110].

7. Challenges and Resolutions in Clinical Delivery of siRNAs

Individual genes correlated with a specific disease can be a potential target of siRNAs. A good example for this can be given in the case, where the vascular and endothelial growth factor (VEGF) mRNA transcripts are targeted, hence causing the RNAi protocols to be attractive therapeutic approach [43, 102]. Further investigation of the RNAi based mechanism of action for therapeutic intervention of genetic disease such as cancer is needed in order to facilitate therapeutically advantageous siRNA mediated agents to contribute to the future of medicine [102].

In different types of treatments of human diseases, siRNAs may be delivered either intravenously or intraperitoneally. Concerning the undruggable disease targets, siRNA therapeutics have the ability to knockdown almost any gene target [4, 96]. Even though, it is relatively quicker to identify and optimize siRNAs for a particular target and it is also easy to make modification in the synthesis of siRNAs, siRNA therapeutics for human disease treatment still needs to be further developed.

The main obstacle in clinical utilization of siRNAs remains within a selection of potential targets. Therapeutic efficacy in disease treatment utilizing siRNAs may depend on choice of target and route of delivery [4].

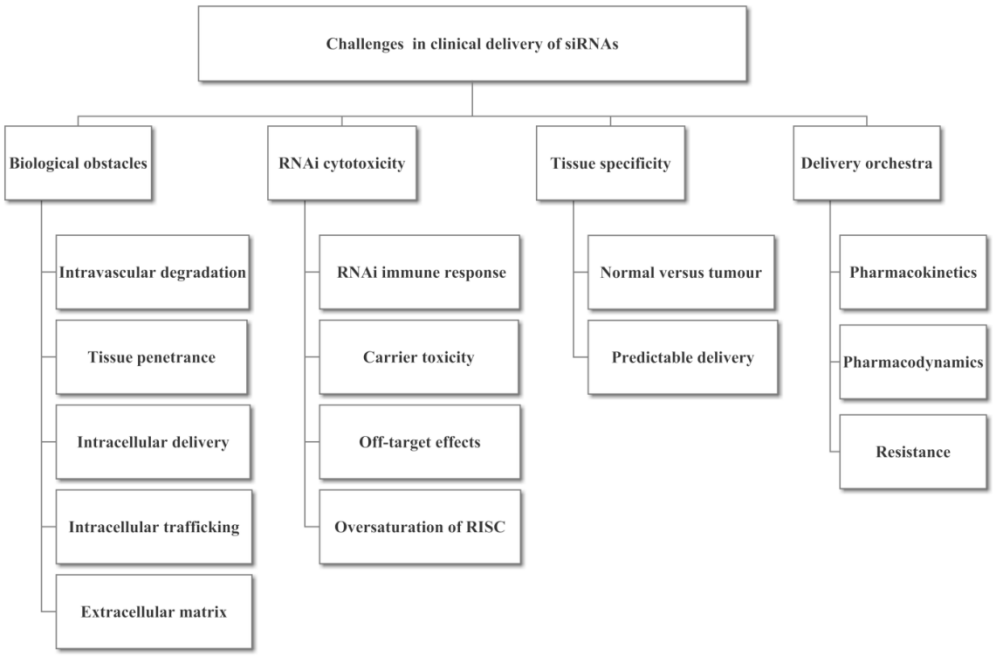

Figure 4. Challenges in clinical delivery of siRNAs.(adapted from [42])

There are several challenges that currently limit the use of RNAi mechanism in the clinic (Figure 4). Methods that overcome these are being developed and are discussed in [42] Figure 5.

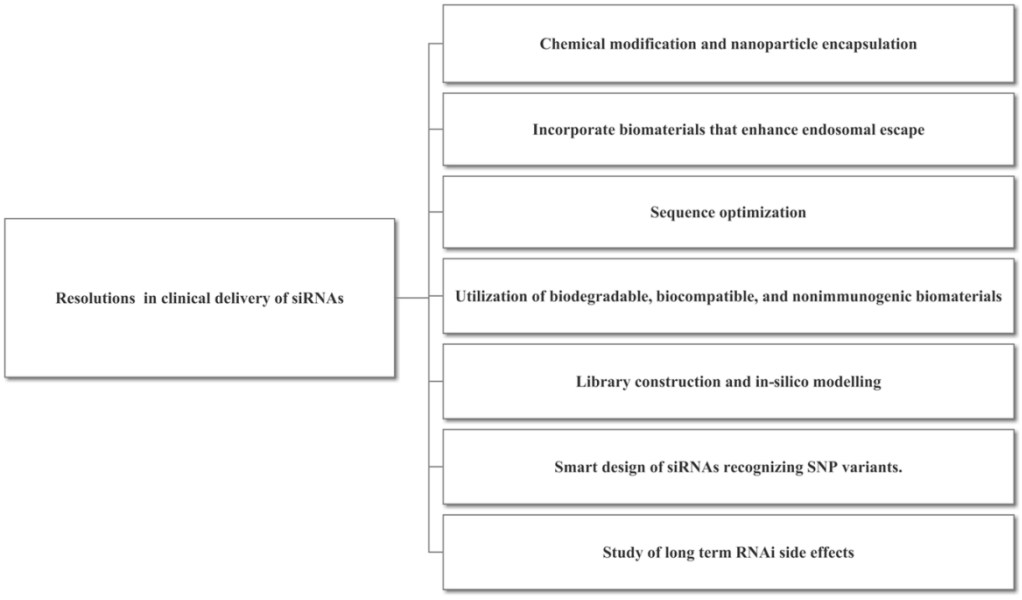

Figure 5. Resolutions in clinical delivery of siRNAs. (adapted from[42])

Although most advanced carrier systems are lipid-based particles for siRNA delivery, diverse delivery systems have been developed to expand applications to target diseases. One of the promising candidate systems is the siRNA-based conjugates. siRNA conjugates have major advantages,

including the simple fabrication process, superior biocompatibility, and targeted delivery. However, several challenges, e.g., endosomal escape and high dose requirements still remain. These challenges could be overcome by the design of potent siRNAs, multivalent conjugations and combinational formulations with additive polymers and lipids [42, 111].

The development of RNAi based drugs have been facilitated by the potential gene signature knock downs. Poor recognition of targeted cells is a disadvantage for lipid based carriers. This drawback can be overcome via utilization of antibodies, aptamers and peptides for cell type specific delivery of siRNAs [102].

8. Future Directions and Conclusions

To date, many attempts have been conducted in order to facilitate therapeutic strategies utilizing siRNAs owing to their capacity to establish post-transcriptional gene regulation. The major concerns regarding the siRNA targeted delivery includes unavailability of efficient and safe carriers. However, in the past few years new carriers have been designed that have succeeded in clinical applications. It is anticipated that siRNA nano-carriers will be utilized as potential therapeutics in the areas of oncology and metabolic disorders. siRNAs should be utilized in a manner to have the least side effects in normal tissues. Additionally, new therapeutic strategies and enhancing carrier systems for local and systemic delivery should be further developed for siRNA targeted therapies. Efficacious siRNA delivery and thorough target selection will facilitate the proper treatment of many disorders. Nanotechnology provides adaptability for targeted delivery stage for RNAi therapeutics. [16, 34]. Hence, it is important to engage in designing and constructing nano-carriers for nontoxic and efficacious siRNA delivery [34, 45]. The shape, size, surface to volume ratio, thermal stability, pH responsiveness and siRNA loading capacity are the important physicochemical properties of nano-vehicles [18, 56]. The inefficient uptake of naked siRNAs can be solved by designing materials for suitable delivery carriers. Hence, numerous studies were performed based on polymeric nano-vehicles for proper functional effects of siRNA delivery of specific genes. The targeted tumor tissues can receive the delivery carriers from their nearby blood vessels. These carriers can be modulated to have higher affinity towards the individual cancer cells. The delivery efficiency can be enhanced by designing smart delivery carriers in order to recognize the target cells [49]. The complex structure of the tumor micro environment can be altered to induce the efficient delivery of the siRNAs [49]. Low toxicity, biodegradability, reduction in immune stimulation and nucleic acid facile condensation are amongst the advantages of utilizing natural polymeric carriers in siRNA delivery. Bio-based polymeric carriers for siRNA delivery as bio-therapeutics have the great potential to provide the basis and model for new drug delivery systems.

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