

Chapter

Perspective Chapter: Mastering RNA Interference (RNAi) Delivery – Strategies for Effective Targeting and Gene Silencing

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Abstract

RNA interference (RNAi), a mechanism for post-transcriptional gene silencing using small interfering RNA (siRNA) or microRNA (miRNA), has emerged as a promising approach for managing numerous genetic disorders by selectively targeting and degrading the mRNA of implicated genes. However, the clinical application of these therapeutics is hindered by significant challenges that limit their delivery to target sites. RNAi therapeutics face multiple extracellular and intracellular barriers post-administration, including rapid glomerular excretion, recognition, and opsonization by the reticuloendothelial system (RES), and catalytic degradation by nucleases, leading to poor cellular and tissue penetration. To address these challenges, various delivery strategies have been explored to efficiently transport these RNAi therapeutics to their intended tissues. These strategies encompass chemical modification, bioconjugation with specific ligands, and carrier-mediated approaches. Nanotechnology-based delivery systems have demonstrated remarkable capabilities in encapsulating and delivering these molecules to their specific cells. Therefore, there is an urgent need to develop innovative delivery systems that can effectively encapsulate and target RNAi therapeutics. By targeting key genes, RNA interference holds the potential to address numerous genetic, viral, and cancer diseases at an early stage. This book chapter explores several studies detailing diverse design strategies aimed at overcoming the hurdles encountered in RNAi delivery.

Keywords: active drug-targeting, nanotechnology, RNA interference, gene silencing, liposomes

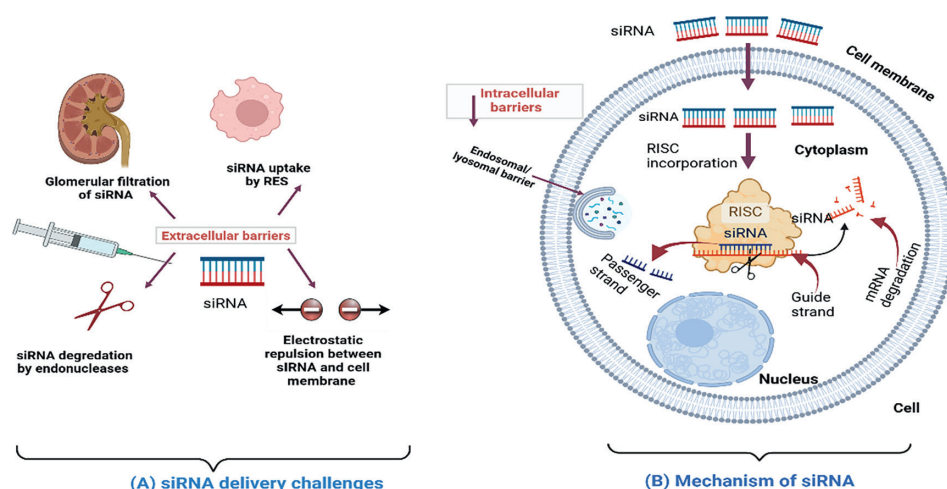
1. Introduction

Andrew Fire and Craig Mello received the Nobel Prize for their groundbreaking work on RNA interference (RNAi), a technique that holds immense promise for gene therapy in addressing various genetic disorders [1]. The identification of RNAi achieved a critical advancement in gene therapy, enabling scientists to elucidate

the gene silencing mechanism and harness this new technology for gene function studies. Additionally, the progress in high-throughput screening and bioinformatics has enabled the targeting of several disease-causing genes, facilitating therapeutic applications and designing more effective personalized treatments. RNAi utilizes small siRNAs and miRNAs for post-transcriptional gene silencing by degrading the messenger RNA (mRNA) of targeted genes. Gene silencing through RNA interference (RNAi) takes place in the cytoplasm. Initially, a double-stranded RNA (dsRNA) is processed into smaller dsRNA fragments, called siRNAs and miRNAs by a Dicer enzyme. These RNAi molecules then bind to the Argonaute-2 (AGO2) protein, forming part of the RNA-Induced Silencing Complex (RISC). Within this complex, the RNAi duplex separates into two single strands: the guide and passenger strand. The passenger strand is degraded, leaving the guide strand to bind to complementary mRNA molecules. This binding leads to the destruction of the mRNA, preventing the translation of the target protein [2–4]. While siRNAs target specific mRNAs, miRNAs can affect multiple targets [5]. Few siRNA therapies have gained FDA approval for rare genetic disorders, such as hereditary variant transthyretin amyloidosis, acute hepatic porphyria, primary hyperoxaluria type 1, and hypercholesterolemia [6]. Despite RNAi's promising therapeutic efficacy, its clinical application faces hurdles, mainly due to challenges in delivering RNAi molecules to their intended sites. The delivery of siRNA therapies faces significant challenges due to barriers both inside and outside cells. These barriers include rapid clearance from circulation, susceptibility to degradation, limited cellular uptake, and rapid excretion through glomerular filtration. Researchers have explored various strategies to overcome these obstacles [3, 7, 8]. Chemical modifications enhance the stability and uptake of RNAi molecules, while bioconjugation with specific ligands enables targeted delivery to tissues of interest, reducing off-target effects. Nanotechnology offers promising solutions, with several proposed nanocarriers, facilitating the delivery of RNAi therapies to target cells, thereby improving their circulation time and reducing the need for frequent administration [4, 9]. These advancements aim to enhance the pharmacokinetics and efficacy of RNAi therapies. The current book chapter explores several RNAi delivery strategies aimed at improving the delivery of RNAi therapeutics for various gene-related disorders, demonstrating the potential of these approaches in advancing precision medicine.

2. Mechanism of action of RNAi therapeutics

Briefly, the process of gene silencing begins with the cleavage of long double-stranded RNA (dsRNA) by an endonuclease Dicer protein, resulting in the formation of small dsRNA fragments known as siRNAs or miRNAs. These small molecules then interact with the Argonaute-2 (AGO2) protein, a crucial component located at the center of a complex protein assembly called the RNA-induced silencing Complex (RISC). RISC identifies a group of diverse molecular complexes that can be directed to silence any gene. Typically, RISC activation occurs upon the detection of dsRNA within the cytoplasm of a eukaryotic cell [10]. This interaction leads to the separation of these duplexes into two strands: the passenger strand and the guiding strand. The complex formed by the guide strand and the RISC then recognizes and binds the complementary mRNA sequence of the target gene. This binding triggers the degradation of the targeted mRNA to small non-functional fragments, effectively silencing the translation process of the target protein as shown in **Figure 1**.



Abbreviations: siRNA (small interfering RNA); RISC (RNA-Induced Silencing Complex); RES (Reticulo-Endothelial System)

Figure 1.

Illustrative diagram showing the mechanism of action of RNAi therapies along with the challenges of their delivery to target sites. This figure was adapted from Abosalha et al. with permission [4].

3. Delivery barriers and limitations

RNAi is a regulatory mechanism found in most eukaryotic cells, serving as an efficient tool for post-transcriptional gene silencing. Their high specificity, selectivity, and potency give rise to promising targeted gene-specific inhibition. Its biochemical mechanism of action has rendered its clinical relevance across a wide spectrum of diseases such as infections, cancer, cardiovascular, and neurodegenerative disorders [11]. Yet, many intracellular and extracellular barriers disrupt the *in vivo* pharmacodynamics and pharmacokinetic profiles of naked unmodified RNAi molecules attributed to their poor absorption, unsatisfactory stability and distribution, rapid systemic clearance, and increased off-target effects [12]. Firstly, their anionic nature which is attributed to their phosphate backbone as well as their hydrophilic nature challenge their ability to be absorbed and to diffuse through the negative lipid bilayer membranes. Moreover, as soon as RNAi therapeutics are administered, they are susceptible to degradation by endonucleases, which cleave the phosphodiester bonds destabilizing these nucleic acids. Moreover, they can induce the secretion of inflammatory cytokines type I interferons (IFNs) since specific sequence motifs are recognized by pattern recognition receptors (PRRs) like toll-like receptors (TLRs) [7]. Interfering RNA are also subjected to phagocytosis through the reticuloendothelial system (RES) and to renal clearance due to their anionic surface charge and their small size and molecular weight. This poses an obstacle for this therapeutic RNAi to reach its target cells as its biodistribution and circulation time have been restricted. If not excreted by the kidneys or even the liver, the phagocytosed RNAi up taken by non-targeted cells can lead to off-target effects and toxicity. Furthermore, following a successful uptake of RNAi into the targeted cell, it can also encounter intracellular challenges. While enclosed in endosomes, they face entrapment or experience a change of pH affecting the stability and half-life of these RNAi, reducing their effectiveness in gene silencing. Finally, among many intracellular components, RNAi must interact with the RNA-induced

silencing complex (RISC) [13]. Some limitations may occur in this process depending on RNAi's delivery/loading pathway onto RISC, its structure's thermodynamic properties, and competition with endogenous RNA. These delivery barriers may interfere with the successful application of RNAi therapeutics and should be addressed efficiently to optimize the pharmacological efficiency of these therapies. For example, although 2'-OH modifications do not directly impact the RNAi machinery and potency, 2'-F modifications enhance the stability and half-life of siRNA. Chiu et al. demonstrated that the degradation of unmodified single-stranded antisense siRNAs is almost instantaneously, and the majority of these silencing RNA was undetectable within 30 mins. While duplex-siRNAs face a more sustained degradation, only 7% of siRNA remained after an hour. Conversely, 2'-fluorinated double-stranded siRNA exhibited higher stability with around 68%–81% undegraded siRNA by the end of the experiment extending the persistence of RNA interference effects [14]. Another study showed that aptamer-conjugated siRNA enabled tumor-targeted siRNA delivery. STAT3 siRNA was specifically delivered to glioblastoma cells with the specific binding of the antagonist Gint4.T aptamer to the oncogenic receptor tyrosine kinase PDGFR β . Enhanced specificity leads to increased cellular uptake of the bioconjugated siRNAs reaching 73% internalization in a glioblastoma cell line after an hour as reported by Esposito et al. [15]. Long et al. pursued another approach to combat breast cancer by loading siRNA-vascular endothelial growth factor (siVEGF) onto PAMAM grafted halloysite nanotubes (PAMAM-g-HNTs). In vivo imaging showed the rapid excretion of freely intratumorally administered cys5-siVEGF by the mice. This obstacle was surmounted by the encapsulation of the cys5-siVEGF into the PAMAM-g-HNTs delivery system, where the majority of accumulation was seen in tumor sites. The observed enhanced permeability and retention (EPR) effect enables controlled gene release in the targeted tumor [16]. The above-mentioned examples highlight the integral role of the adopted strategies in effectively enhancing the biological activity of RNAi therapeutics. The reported delivery barriers are presented in **Figure 1**.

4. Strategies to overcome delivery barriers of RNAi therapies

The abovementioned barriers reveal that the design of a suitable delivery mechanism is essential for the effective clinical application of RNAi therapies. These delivery strategies should ensure the precise delivery of RNAi molecules to their intended target. Additionally, there is an essential demand for ongoing improvement of these strategies aimed at enhancing their targeting accuracy. Generally, three delivery strategies have been adopted to optimize the delivery of these therapeutics. The adopted techniques are chemical modification of naked RNAi molecules, bioconjugation with specific targeting ligands, and nanotechnology-based drug delivery systems (**Figure 2**).

4.1 Chemical modifications

The delivery of freely administered candidate RNAi is made feasible after undergoing chemical modifications. This strategy aims to mitigate the challenges arising from the original RNAi physicochemical properties. Mainly, the polyanionic phosphodiester skeleton of these molecules impinges the cell permeability and its cellular uptake as well as activates the RES leading to their opsonization followed by their rapid clearance [17]. Therefore, the masking of the negative charges along with the alterations of other characteristics such as hydrophilicity, its short structure, and sensitivity to ribonucleases

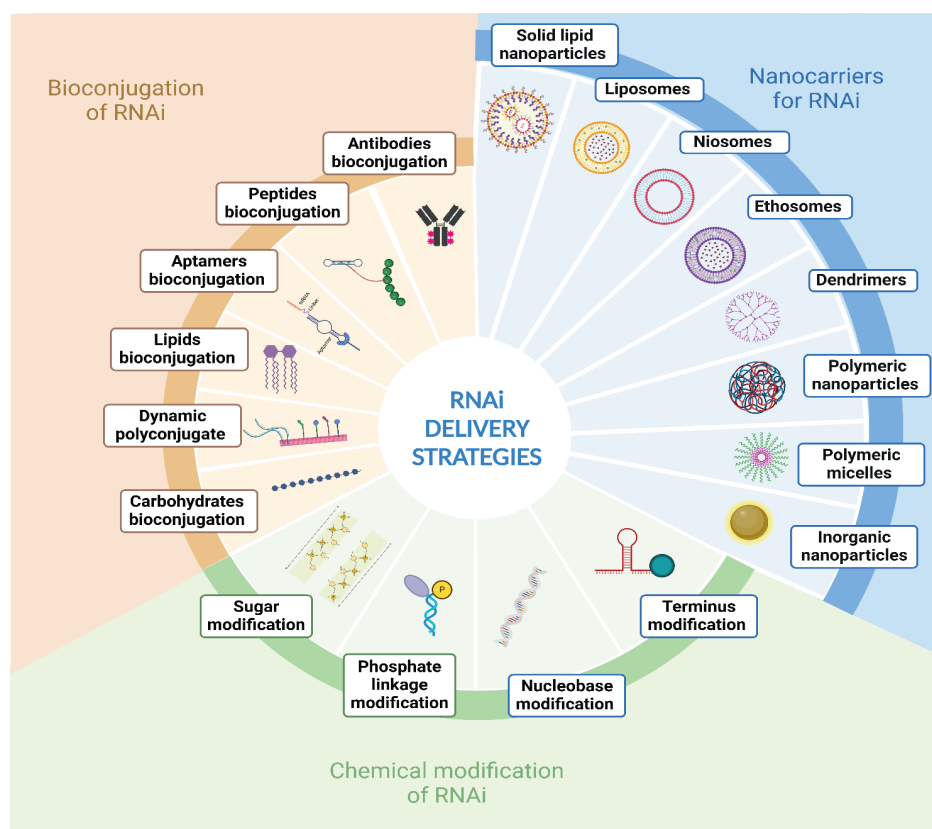


Figure 2.
 Schematic diagram illustrating the adopted strategies to overcome the delivery barriers of RNAi therapeutics.

through chemical modifications may enable the oligonucleotide therapy to reach its target with an improved serum stability and biological half-life. Moreover, pattern recognition receptors (PRRs) recognize unmodified and unprotected RNAi molecules as foreign RNA, initiating the release of inflammatory cytokines and type I interferon (IFNs), which induces off-target interactions and toxicity [18]. Thus, for siRNA or miRNA to effectively exhibit their silencing effects, non-specific gene targeting must be reduced along with their immune-stimulatory activities, which are also further triggered by the RNAi nuclease-degradation byproducts. The main types of chemical modifications optimizing the chemical architecture of RNAi for clinical productivity are ribose modifications, phosphate modifications, and nucleobase modifications.

4.1.1 Ribose modification

A common and established approach of chemical modification is the addition of ribose moiety. Particularly, ribose modifications at the 2' position have been widely explored as the 2'-OH group contributes, through hydrolysis, to the mechanism of endoribonucleases, hence RNAi cleavage [12]. Additionally, the 2'-OH group is not essential in RNAi silencing activity as it is not involved in the RISC, making this site a candidate target to be substituted with 2'-fluorine or 2'-o-methyl for enhancing properties of the oligonucleotide [11, 19]. Although 2'-OMe modification might

decrease the activity of the sense strand by sterically blocking interactions, the conjugation of a fluorine group to the ribose moiety may compensate for this adverse effect by mimicking the 2'-OH group in size and charge [20, 21]. Overall, the alternating 2'F/2'OMe-RNA pattern offers increased thermodynamic stability, resembling that of a highly functional RNAi, as well as a higher binding affinity and half-life with a reduced immunogenicity [22, 23]. Alternatively, bicyclic and acyclic ribonucleoside substitutions may be used known as locked nucleic acids (LNA) and unlocked nucleic acids (UNA), respectively [12]. They introduce a chemical asymmetry into the RNAi duplex causing a conformation change promoting RISC loading of the guide strand, thus an increase of the RNAi activity and affinity [24].

4.1.2 Phosphate modification

Phosphate modifications are also used to enhance RNAi efficacy. Modifications with several phosphonate analogs at the 5'- end of the guide strand improve siRNA activity as it is essential for RISC recognition. Specifically, since following a 5'- ribose modification of the guide strand, mentioned above, the intracellular phosphorylation may be impacted, the chemical addition of a 5'-phosphate can rectify the activity of the RNA strand [25]. Furthermore, Lima et al. developed a phosphate analog more resistant to dephosphorylation in cells named 5'-(E)-vinylphosphonate (5'-E-VP), compelling for in vivo efficacy of RNAi on account of its suitable conformation and stereoelectronic properties [26]. Other phosphate modifications used in oligonucleotides include 5'-C-methyl analog, phosphorothioate (PS), and peptide nucleic acids (PNA) [23]. Particularly, phosphorothioate (PS) linkage primarily provides hydrophobicity reinforcing plasma protein binding tendencies, granting stability and an improved biodistribution, cellular uptake, and half-life circulation. However, it is important to note that high PS amounts may provoke chemical-related toxicities [12]. Hence, adequate numbers, positioning, and PS isomer, where the desired chirality for each strand ends is dictated by either the Sp or Rp configurations, are crucial for enhanced RNAi performance [27].

4.1.3 Base modification

Base modification has also been reported using nucleic acid analogs. While their use is limited, they contribute to a better understanding of gene silencing mechanism as well as prevent off-target effects [11, 23]. For example, according to Zhang et al. the activity of the passenger strand can be reduced with a 5'-nitroindole modification of the siRNA at position 15 of the passenger strand as a strategy to mitigate off-target effects without affecting the guide strand [28]. To enhance therapeutic effects, 5'-fluoro-2'-deoxyuridine substitution not only silences the targeted gene but also triggers cell death making it a candidate application for cancer therapy [29]. On the other hand, siRNA can be conjugated with fluorescent nucleobases such as 6'-phenyl-pyrrolocytosine to be used as in vivo monitors allowing for the visualization of siRNA internalization and other mechanisms [30].

4.2 Bioconjugation

The bioconjugation of RNAi therapies acts as an effective and promising approach for mitigating the shortcomings associated with delivering these molecules. Some of the commonly reported challenges such as vulnerability to endonucleases, limited cellular permeability, and inconsistent pharmacokinetic parameters may be addressed

through their bioconjugation to specific ligands [31]. Literature reported a notable improvement in RNAi therapy's bioavailability, ability to target specific sites, stability in circulation, gene-silencing capability, and biological effectiveness because of their bioconjugation [3]. It is known that careful consideration of the conjugation site is crucial for determining the *in vivo* performance of the RNAi conjugates. Generally, the 5' end of the guide strand, responsible for initiating RNA interference, and the 3' end of the same strand are unsuitable for conjugation. Hence, the 3' and 5' ends of the passenger strand are preferred sites for bioconjugation. Moreover, bioconjugation to other sites like the 2'-OH group of ribose or nucleobases has also been explored [31, 32]. Several ligands have been investigated for their potential to overcome the delivery challenges of RNAi therapeutics as presented below:

4.2.1 Aptamers-RNAi bioconjugates

Artificial nucleic acid ligands, commonly referred to as aptamers, have risen as potent ligands for targeting numerous RNAi therapeutics. An aptamer stands as an artificially engineered, single-stranded RNA or DNA oligonucleotide, emerging as one of the most promising ligands for delivering RNAi therapeutics. These small three-dimensional oligonucleotide molecules exhibit remarkable specificity and high affinity toward a wide range of targets while remaining stable and adaptable to various chemical modifications. Aptamers pose additional advantages over other frequently used ligands for bioconjugation due to their low immunogenicity, high safety profile, cost-effectiveness, ease of synthesis, and extended shelf life. Based on these characteristics, aptamers serve as ideal delivery vehicles, imparting specificity, and selectivity to their conjugated molecules. Moreover, aptamers can efficiently enter cells via cell-mediated endocytosis, thereby overcoming poor cellular internalization of the delivered RNAi therapies and enhancing their cellular accumulation [33, 34].

4.2.2 Antibodies-RNAi bioconjugates

Generally, the antibody-drug conjugate approach holds considerable promise for targeted delivery of several drugs, peptides, nucleic acids, and genes. This technique of active drug targeting has got approval from regulatory authorities such as the FDA and EU for various cancer treatments [35, 36]. A variety of membrane receptors have emerged as crucial targets for therapeutic intervention in medical and pharmaceutical research. For example, antibodies designed to target overexpressed receptors on cancer cells, aim to disrupt the interaction between tumor and stromal cells, thereby impeding tumor progression and metastasis. Monoclonal antibodies targeting PD-1, PD-L1, and EGFR receptors have received approval for the treatment of various cancer types. Due to the high affinity and specificity of these ligands to their antigens, they are presently considered the conventional choice for bioconjugation for active targeting of RNAi therapies. Mostly, this conjugation method relies on the straightforward ionic attraction between positively charged antibodies and the anionic phosphate groups of RNAi molecules [3, 37].

4.2.3 Peptides-RNAi bioconjugates

Among various ligands of bioconjugation, cationic peptides have emerged as a promising strategy for the delivery of RNAi molecules owing to their facile synthesis, controllable dimensions, and adaptable structure for customizing physicochemical properties

and specific targetability of conjugated therapeutics. Additionally, peptides can promote the cellular internalization of RNAi molecules due to shielding of their negative charge. Additionally, peptides can uphold a high degree of specificity and binding affinity to targeted receptors the same as antibodies. Moreover, their capacity to selectively bind specific proteins on cell surfaces, owing to their distinct tertiary structures, may be employed for the targeted delivery of siRNAs and miRNAs. The cyclic RGD (cRGD) peptide stands out as one of the most utilized peptides for this purpose [38, 39].

4.2.4 Lipids-RNAi bioconjugates

The incorporation of lipids into drug delivery systems has introduced significant advancements in delivery and targeting strategies. Unmodified RNAi molecules often face challenges due to their negative charge and hydrophilicity, hindering their optimal delivery to the desired tissue. Moreover, the hydrophilicity of RNAi therapeutics reduces their tendency to bind plasma proteins, making them suitable candidates for rapid glomerular excretion, resulting in a short half-life in circulation. Cationic lipids offer a potential solution by neutralizing the surface charge of these molecules, thereby enhancing their cellular permeability. The chemical composition of lipids significantly influences the effectiveness of RNAi delivery *in vivo*. For example, siRNAs conjugated with lipids such as cholesterol preferentially bind with LDL and predominantly accumulate in the liver. On the contrary, bioconjugation with lipids such as lithocholic acid exhibits a greater affinity for HDL and albumin, leading to deposition in various tissues including the liver, kidney, ovary, intestine, and others [40, 41].

4.2.5 Miscellaneous RNAi bioconjugates

Several studies reported the bioconjugation of siRNAs or miRNAs with other carbohydrate ligands such as glucose, galactose, lactose, and their derivatives. This bioconjugation showed an enhancement in the targeting, bioavailability, half-life, distribution, and pharmacological efficacy of encapsulated RNAi molecules [42, 43]. Additionally, small molecules can serve as highly specific and potent ligands for siRNA or miRNA delivery. The bioconjugation of RNAi to ligands such as folic acid and 2-[3-(1,3-dicarboxypropyl) ureido] pentanedioic acid (DUPA) could effectively target it to malignant cells overexpressing the respective folic acid receptor and Prostate-specific membrane antigen (PSMA) receptor, respectively. **Table 1** shows examples of RNAi therapies-bioconjugates for the management of different gene-related disorders.

4.3 Nanotechnologies

Nanocarrier systems have revolutionized drug delivery by offering innovative strategies aiming to harness the principles of nanotechnology to encapsulate RNAi. This allows these molecules to reach a wide range of applications with enhanced drug efficiency and minimal side effects. These nanoplatforms exhibit diverse characteristics based on their type, size, shape, and physical/chemical properties. Advanced nanocarriers like lipid nanoparticles and their derivatives including liposomes, lipid nanoemulsions, solid lipid nanoparticles, nanostructured lipid carriers, and lipid-polymer hybrid nanoparticles exhibit high biocompatibility when delivering encapsulated nucleic acids into the body. Other organic nanocarriers can be polymer-based such as PLGA and chitosan nanoparticles offering tunable sustained release of RNAi [53].

RNAi therapy	Conjugating ligand	Silenced gene	Targeted disease	Reference
3'-biotinyl-siRNA	The human insulin receptor-monoclonal antibody (HIR-MAb)	Luciferase	Neurological disorders	[44]
STAT3 siRNA	Humanized monoclonal antibody (Hu3S193)	Signal Transducer And Activator of Transcription 3 (STAT3)	Cancer therapy	[45]
siRNA	Anti-CD22 monoclonal antibody (Anti-CD22 Mab)	Glyceraldehyde-3-dehydrogenase (GAPD)	Lymphoma	[46]
Luc-siRNA	Cyclic- (arginine-glycine-aspartic) (cRGD)	luciferase gene	Melanoma	[47]
IRS1 siRNA	Insulin-like growth factor 1 (IGF1)	Insulin receptor substrate 1 (IRS-1)	Breast cancer	[48]
IGF-1R siRNA	Mucin-1 (MUC1-apt)	Insulin-like growth factor receptor 1 (IGF-1R)	Metastatic breast cancer	[49]
Delta-5-desaturase siRNA	Epithelial cell adhesion molecule aptamer (EpCAM)	Delta-5-Desaturase (D5D)	Colon cancer	[50]
p-gp siRNA	Aptamer A6	P-glycoprotein transporter	Breast cancer	[51]
let-7 g miRNA	GL21.T Aptamer	let-7 g target genes	Lung cancer	[52]

Table 1.
Examples of RNAi therapies bioconjugation.

4.3.1 Solid lipid nanoparticles (SLNs)

Concurrent advancements in drug delivery involve lipid nanoparticles. They are the most commonly used carrier systems for RNAi encapsulation and delivery. SLNs are characterized by their amphiphilic properties encompassing hydrophilic head groups and hydrophobic tails [54]. These polar interactions enable the formation of a lipid monolayer or bilayer surrounding an aqueous core serving as a protecting environment for encapsulated payloads. Additionally, SLNs may be surface-modified with targeting ligands or PEGylation to enhance target-specific delivery and circulation time with the inhibition of RES uptake as well as inhibit SLNs aggregation [55]. Particularly, cationic SLNs, composed of ionizable cationic lipids or PEGylated lipids, are commonly used for nucleic acid delivery. 1,2-dioleoyl-3-trimethylammonium propane, also known as DOTAP, is one of the two most common cationic lipids [54]. When combined with DOPE in a 1:1 ratio, a reduction in toxicity is reported [56]. These nanoparticles are synthesized through high-frequency sonication between the lipid phase and the aqueous phase containing PEGylated surfactant [54]. The entrapment of the therapeutic RNAi is based on electrostatic interactions, which are also responsible for the controlled release kinetics of RNAi via endocytosis triggered by the contact of the positively charged cationic lipid and the negatively charged cell membrane [57]. Yang et al. have formulated a cationic lipid–polymer hybrid nanoparticle delivering siRNA toward breast tumor tissue for cancer therapy [58]. The cationic lipid monolayer, in which the siRNA was efficiently loaded via electrostatic forces, facilitated the carriage and escape of loaded siPlk1 into BT474 cells, inducing downregulation of the Plk1 oncogene and thereby cancer cell apoptosis. This hybrid nanoparticle shield with the cationic lipid is an attractive siRNA-delivering platform with significant potential in suppressing tumor growth and cancer therapies [58].

4.3.2 Liposomes

Liposomes are self-assembled amphiphilic lipid bilayers surrounding an aqueous core that may contain hydrophilic cargoes, including RNAi molecules. Its application range is further broadened with its ability to also encapsulate lipophilic and amphiphilic biologically active therapeutics. These lipid-based vesicles can be found as unilamellar, multilamellar, or multivesicular vesicles [59]. A common method of preparation involves oil-in-water emulsion where the lipids dissolved in organic solvent constitute the dispersion phase [59]. Their bilayer structure grants these carriers high biocompatibility and non-immunogenicity as well as stability and controlled release. Additionally, their ease of fabrication and customizable physicochemical properties promote cellular uptake and pharmacokinetics. Furthermore, optimized versions of traditional liposomes are being developed such as elastic liposomes (ELs) [60]. With the addition of surfactants, namely edge activators, ELs offer increased deformability and stress-dependent adaptability, enabling enhanced transdermal penetration for topical delivery targeting the efficient delivery of siRNA for the treatment of psoriasis and cervical carcinoma [61, 62].

4.3.3 Transfersomes

Transfersomes are a derivative of liposomes that are composed of edge activators. These vesicles are designed to particularly enhance the delivery of the payloads, including nucleic acid-based agents, across biological barriers such as skin or cell

membranes [63]. These nanocarriers belong to the group of ultra-deformable hydrophilic vesicles characterized by their high malleability giving them the ability to deform and penetrate through narrow pores. In addition, the edge activators, which contain both lipophilic and hydrophilic moieties, enhance the diffusion of transferosomes through the skin via osmotic gradient activating via water evaporation [64]. Similar to liposomes, transferosomes are composed of lipid biomimetic bilayer, which supports their biocompatible and biodegradable properties. Moreover, anionic transferosomes could be employed as a strategy for mitigating foreign body reactions *in vivo* through the prevention of protein adsorption and immune system activation. Dorrani et al. reported that DOTAP/NaChol (6:1) transferosomes can penetrate the skin layer and effectively deposit BRAF-siRNA for the management of melanoma [65].

4.3.4 Ethosomes

Ethosomes are also ultra-deformable hydrophilic vesicles. They are composed of phospholipids, water, and a high content of ethanol ranging between 20 and 45%. Particularly, ethanol contributes to the formation of a highly elastic nanovesicle with improved deformability and fluidity enhancing skin absorption. Ethanol also increases the solubility of encapsulated drugs and imparts higher stability and smaller-sized non-aggregated vesicles attributed to the negative charge on the surface of ethosomes conferred by ethanol [66]. According to Chen et al. penetration enhancer such as SPACE-peptide can decorate the surface of cationic ethosomes optimizing the internalization of the DOTAP-SPACE-ethosomal system into epidermal keratinocytes [67]. The two conventional methods of preparation of ethosomes are the cold method and the hot method. The latter consists of heating the organic phase containing the phospholipids and the water phase to 40°C [66]. The final vesicles are obtained by either sonification or extrusion for both methods. Furthermore, additional alcohols such as propylene glycol (PG) and isopropyl alcohol can be included in ethanol-based vesicles forming binary ethosomes. These enhancers provide higher penetration and drug retention while penetrating the dermis layers. Also, as a humectant, PG aids with the hydration of the stratum corneum making it less susceptible to causing skin irritation or inflammatory [66].

4.3.5 Niosomes

Niosomes are non-ionic surfactant self-assembled vesicles. The amphiphilic surfactant consists of a polar and a non-polar group which allow for a specific orientation of the hydrophilic head toward the aqueous phase and hydrophobic tail shielded from water molecules. This configuration reduces the free energy at the interface, which effectively lowers the surface tension and facilitates the self-assembly of the niosomes [68]. Also, as an alternative to phospholipid-based carriers, niosomes present an enhanced *in vivo* stability against oxidation and degradation than phospholipids [69]. Additional advantages including high biocompatibility, permeability, encapsulation efficiency, and low production cost make niosomes potential candidates for commercialization [70]. The surface of these nanocarriers may also be decorated with hydrophilic polymer (e.g., PEG), cationic lipids (e.g., DOTAP), or conjugated with active targeting ligands/antibodies, among other modifications. For instance, Yang et al. developed a theranostic cationic niosome using a nonionic surfactant sorbitan monooleate (Span 80). This system efficiently mediated the intracellular delivery of siRNAs and miRNAs to human mesenchymal stem cells (hMSCs). A successful specific inhibition of miR-138 promoted osteogenic differentiation of hMSCs. In addition to RNAi delivery, this platform allows

for cell labeling capabilities with the encapsulation of an amphiphilic dye, indocyanine green [68]. Alternatively, following the encapsulation of RNAi inside the hydrophilic core of niosomes, inorganic nanoparticles, such as superparamagnetic iron-oxide nanoparticles, can be entrapped within the niosomal barrier. Thereby, a targeted delivery via an external magnetic manipulation is achievable leading to an improved cellular uptake, as demonstrated by Maurer et al. on BT-474 cells for a breast cancer therapy [70].

4.3.6 Polymersomes

Polymersomes are vesicular carriers formed by the self-assembly of amphiphilic copolymer blocks. The formed polymeric bilayer offers this nanoparticle distinctive properties for the delivery of encapsulated cargoes. The covalent crosslinking of the copolymer blocks leads to greater stability of the vesicle with the ability to modulate the membrane properties such as permeability, flexibility, and surface charge [71]. Also, the modification of the membrane surface is feasible for the conjugation of targeting ligands that present high binding affinity to proteins or receptors expressed on the surface of targeted cells. This active targeting enables selective drug delivery improving cellular uptake and minimizing off-target effects. Zheng et al. constructed a polymersome delivery system for the co-delivery of temozolomide (TMZ) and siRNA for the synergistic therapy of glioblastoma (GBM) [72]. The brain-targeted polymersome is decorated with angiopep-2, a peptide that binds to the lipoprotein receptor-related protein-1 (LRP-1) receptor. As the latter is overexpressed on the surface of endothelial cells of blood-brain-barrier and GBM cells, this actively targeted nanocarrier showed an increased circulation time of siRNA/TMZ in the blood as well as an improved crossing of the blood-brain-barrier [72]. Alternatively, the polymersome membrane can be modified with stimuli-responsive moieties enabling the drug release rate to be programmable by external stimuli such as temperature, pH, and light [71].

4.3.7 Dendrimers

The term “dendrimer” originates from the Greek word “dendron,” signifying tree or branch. Dendrimers represent specially designed structured polymers characterized by their three-dimensional, extensively branched architecture, and uniformity in size and composition. Comprising a central core, branching arms, and functional groups at their terminals, dendrimers offer an ideal platform for drug delivery. Additionally, their internal structure provides a reservoir for drug encapsulation, while their surface can be decorated with diverse chemical groups, enhancing their targeting for specific clinical applications. Notably, dendrimers exhibit superior properties compared to conventional polymer-based delivery systems, including enhanced water solubility, excellent biocompatibility, and precise control over molecular weight. The utilization of dendrimers as a drug delivery system began to gain attraction in the late 1990s. They are typically synthesized via two methods: divergent and convergent synthesis. Divergent synthesis involves outward growth from the core, while convergent synthesis starts from the outer surface toward the core. Dendrimers offer advantages such as precise control over size and properties during synthesis, as well as the ability to attach various functional groups for specific targeting. They also exhibit prolonged circulation in the body, making them promising for delivering therapeutic molecules like RNAi therapeutics [73–75]. For instance, one study utilized a PAMAM dendrimer with a triethanolamine core to deliver Hsp27 siRNA to prostate cancer cells, demonstrating significant gene-silencing activity [76].

4.3.8 Polymeric nanoparticles

Polymeric nanoparticles have gained a special concern among all drug delivery techniques. These polymer-based nanocarriers showed interesting features as drug delivery vehicles. The ability to control the physicochemical characteristics of the formulated nanoparticles by controlling the selection and characteristics of utilized polymers has changed the landscape of drug delivery. Polymeric nanoparticles represent a class of polymer-based nanoscale structures that exhibit a uniform size distribution pattern. These nanoparticles show excellent biocompatibility and biodegradability, employing materials such as Poly-lactide co-glycolide (PLGA), Poly-lactic acid (PLA), Polycaprolactone (PCL), and chitosan. Their morphology, either nanocapsules or nanospheres exhibits heightened stability [77, 78]. Some of the commonly applied polymeric nanoparticles in drug delivery are discussed below.

4.3.8.1 PLGA nanoparticles

PLGA acts as a highly successful polymer in the encapsulation of several pharmacologically active molecules for medical applications. Its approval by the FDA for human administration underscores its exceptional biocompatibility and biodegradability. PLGA nanoparticles have gained significant interest in recent years owing to their versatility in transporting various types of drugs. Additionally, the capability to tailor their surface characteristics enhances their targeting and cellular internalization. PLGA nanoparticles were investigated across diverse therapeutic conditions, including vaccinations, chemotherapies, neurological disorders, inflammation, and other disorders. PLGA represents a meticulously studied biodegradable copolymer that undergoes degradation into non-toxic byproducts (H_2O and CO_2). PLGA nanoparticles degrade *in vivo* via hydrolysis of ester bonds to form monomeric anions (lactate and glycolate). PLGA is well-known for its ability to sustain the encapsulated drugs through a prolonged period. This sustained release profile extends the duration of action and decreases the frequency of administration of loaded drugs. Several methods were adopted to fabricate PLGA nanoparticles, including emulsion-solvent evaporation, double emulsion solvent evaporation, nanoprecipitation, salting-out, and supercritical fluid [79–81]. According to Cun et al. the encapsulation of small interfering RNA into PLGA nanoparticles, prepared by the double emulsion solvent evaporation method, may be optimized with the adjustment of the concentration of PLGA, the w/o phase ratio, the sonication time, quantity of loaded siRNA, and the acetylated bovine serum albumin (Ac-BSA) content in the inner water phase [82]. This strategy allows the obtention of high encapsulation efficiency of siRNA while preserving the negative particle zeta potential and small size. With a mean size of 220 nm and zeta potential of -7.3 mV, a hyaluronic acid-PLGA nanoparticle developed by Byeon et al. encapsulated paclitaxel (PTX) and focal adhesion kinase (FAK) siRNA with a 77–85% entrapment efficiency. The co-delivery of siRNAs permitted for the targeted delivery against chemoresistant ovarian cancer causing their apoptosis [83].

4.3.8.2 Glycogen nanoparticles

Glycogen is a natural hyperbranched polymer. It belongs to the polysaccharide class and is composed of glucose-repeating units connected by linear α -d-(1–4) glycosidic bonds and α -d-(1–6) branched chains [84, 85]. Glycogen-based nanoparticles are emerging as promising biocompatible and biodegradable delivery platforms. With

a hydrodynamic diameter of 50 nm, these nanocarriers successfully accumulated in solid tumors via an enhanced permeability and retention (EPR) effect [85]. This cost-effective polymer is highly available since it is a renewable resource isolated from animals or plants. Similarly to other nanoparticles, glycogen's surface may be modified to confer anionic and hydrophobic properties using octenyl succinate (OS) groups for the delivery of antigens to dendritic cells [86]. Wojnilowicz et al. synthesized lactosylated glycogen-siRNA nanoparticles that demonstrated efficient targeting and penetration into 3D multicellular human prostate cancer spheroids which overexpress lectins [84]. A further optimization includes the engineering of the construct with pH-sensitive moieties using ethylenediamine (EDA). As the escape of RNAi from endosomes post-endocytosis is a limiting step in nucleic acid delivery, EDA enhances the proton sponge effect, promoting a more efficient endosomal escape of encapsulated RNAi and improving gene silencing effects [84].

4.3.8.3 Chitosan nanoparticles

Chitosan has gained attention as a promising natural biopolymer-based carrier in drug delivery owing to its biocompatibility, ability to adhere to biological surfaces, and capacity to enhance drug absorption. Nanoparticles formulated from chitosan and its derivatives typically exhibit a positively charged surface charge and possess mucoadhesive characteristics, enabling adhesion to mucous membranes and facilitating the sustained release of the encapsulated payload. Chitosan nanoparticles are characterized by their biodegradability, biocompatibility, low toxicity, and ease of preparation, introducing them as highly effective and promising in clinical applications. Moreover, chitosan nanoparticles can be readily functionalized to achieve targeted delivery and have received Generally Recognized as Safe (GRAS) status from the FDA. Unlike PLGA, The preparation of chitosan nanoparticles is conducted under mild conditions facilitated by chitosan's solubility in acidic aqueous solutions, eliminating the need for toxic organic solvents. These nanoparticles offer advantageous features in drug delivery for oral, ophthalmic, nasal, pulmonary, and transdermal delivery. Multiple methods are adopted

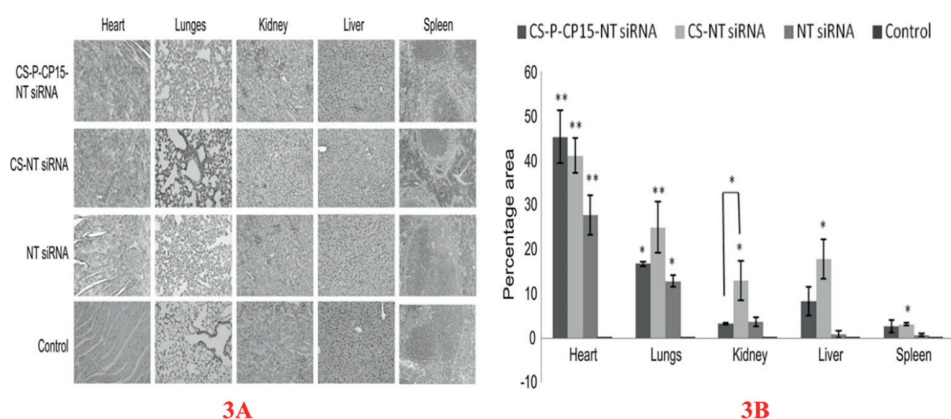


Figure 3. The analysis of biodistribution of siRNA-loaded chitosan nanoparticles in various tissues 4 hrs after the administration of various formulations. **Figure 3A** shows histopathological staining of the heart, lungs, kidney, liver, and spleen, administered at a dose of 0.5 mg/kg) CS-P-CP15-NT siRNA: chitosan-PEG-CP15, CS-NT siRNA: unmodified chitosan nanoparticles, NT-siRNA: non-targeting biotin-siRNA alone, and control as untreated. **Figure 3B** illustrates an image analysis of the mean percent area stained in the tumor tissues. This figure was adapted from Prakash et al. (US patent US 11,766,486 B2) with permission [90].

for the fabrication of chitosan nanoparticles same as mentioned in PLGA nanoparticles preparation [87–89]. Prakash et al. prepared siRNA-loaded chitosan nanoparticles for the management of several types of cancer. The surface of the prepared nanoparticles was functionalized with TAT protein which enhances internalization and cellular uptake of the formulated siRNA nanoparticles. The results revealed the potential of the prepared siRNA-chitosan-TAT nanoparticles in targeting the encapsulated siRNA with a high concentration in different tissues [90] as shown in **Figure 3**.

5. Conclusion

The discovery of small interfering RNAi as therapeutic agents for gene silencing has generated considerable interest in the scientific community. RNAi shows significant promise for treating a wide range of diseases, including cancer, hepatic disorders, viral infections, and neurological conditions. Its efficacy in downregulating disease-associated genes and proteins has been demonstrated in various global health challenges. Few RNAi therapies have already been approved for clinical use, yielding remarkable outcomes. For instance, lumasiran has shown effectiveness in managing primary hyperoxaluria type 1 (PH1). However, delivering RNAi therapeutics to target tissues remains a significant challenge, limiting its effective application. Various strategies have been developed to address this issue, including chemical modifications to enhance RNAi molecules stability and biodistribution, bioconjugation with various ligands such as peptides and antibodies to improve cellular uptake and minimize adverse effects, and the use of nanotechnology-based carrier systems like liposomes, niosomes, dendrimers, solid lipid nanoparticles, and polymer-based nanoparticles to encapsulate RNAi and facilitate targeted delivery. The continuous optimization of appropriate strategies for RNAi therapeutics targeting is imperative to fully realize the therapeutic potential of this exciting therapeutic modality across various diseases.

6. Future of RNAi therapy

RNAi therapeutics may offer a novel approach to target numerous progressive genetic diseases by silencing their defective genes. They can also inhibit growth-promoting proteins in cancers, such as glioblastoma multiforme. Additionally, they may slow neurodegenerative diseases by targeting degenerative mediators, and treat viral infections by targeting virus mRNA. The COVID-19 pandemic has highlighted the potential of siRNA, particularly against the SARS-CoV-2 virus. Despite vaccine development, the emergence of viral variants always poses challenges. RNAi therapeutics can be potentially forwarded to silence viral gene expression and replication in various viral infections. Thus, advancing the delivery techniques of RNAi therapeutics is crucial for future therapeutic and vaccine development.

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Conflict of interest

The authors declare no conflict of interest.

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