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REVIEW

A comprehensive update of siRNA delivery design strategies for targeted and effective gene silencing in gene therapy and other applications

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ABSTRACT

Introduction: RNA interference (RNAi) using small interfering RNA (siRNA) is a promising strategy to control many genetic disorders by targeting the mRNA of underlying genes and degrade it. However, the delivery of siRNA to targeted organs is highly restricted by several intracellular and extracellular barriers.

Areas covered: This review discusses various design strategies developed to overcome siRNA delivery obstacles. The applied techniques involve chemical modification, bioconjugation to specific ligands, and carrier-mediated strategies. Nanotechnology-based systems like liposomes, niosomes, solid lipid nanoparticles (SLNs), dendrimers, and polymeric nanoparticles (PNs) are also discussed.

Expert opinion: Although the mechanism of siRNA as a gene silencer is well-established, only a few products are available as therapeutics. There is a great need to develop and establish siRNA delivery systems that protects siRNAs and delivers them efficiently to the desired sitesare efficient and capable of targeted delivery. Several diseases are reported to be controlled by siRNA at their early stages. However, their targeted delivery is a daunting challenge.

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1. Introduction

Andrew Fire and Craig Mello were awarded the Nobel Prize in 2006 for their discovery of 'RNA interference-gene silencing by a double-stranded RNA.' This new theory represented an advanced technique of gene therapies to control numerous gene-associated diseases. The process of gene silencing describes the employment of a 21–25-nucleotide, double-stranded RNA molecule known as 'siRNA' to control the post-transcriptional regulation of the mRNA of the targeted gene. The siRNA duplex is composed of two strands: a sense strand (i.e. passenger) and an antisense strand (i.e. guide). The guide strand is complementary to the mRNA of the targeted gene so that this strand can easily recognize it [1,2]. Four siRNA therapies are present in the market after the approval of the FDA and regulatory agencies. Patisiran was approved in 2018 for the management of a rare genetic disorder termed 'Hereditary Variant Transthyretin Amyloidosis' [3]. Givosiran was authorized in 2019 for the treatment of acute hepatic porphyria [4]. In 2020, Lumasiran was the third accepted siRNA for controlling primary hyperoxaluria type 1 [5]. Recently, Inclisiran was approved for the treatment of hypercholesterolemia [6]. The delivery of these therapies was achieved by either encapsulating the siRNA into lipid nanocarriers such as liposomes (e.g. Patisiran) or through conjugation using N-acetylgalactosamine (e.g. Givosiran, Lumasiran, and Inclisiran) [7]. Currently, there are several other siRNA therapies at different phases of clinical trials including Vutrisiran, Nedosiran, Fitusiran, Cosdosiran, Tivanisiran, and Teprasiran.

The pharmacokinetic profile of siRNA therapies is highly restricted by several intracellular and extracellular barriers that interfere with the delivery of siRNA successfully to the target site. siRNA is rapidly cleared from the blood with a very short circulation half-life (a few minutes) as a result of many combined factors. Firstly, siRNA is highly retained by the reticulo-endothelial system (RES), endosomes, and lysosomes. Secondly, it is highly vulnerable to degradation by endoribonucleases and exoribonucleases in the plasma and tissues [8,9]. Additionally, siRNAs are mainly excreted by glomerular filtration due to their small size. Moreover, the cellular internalization of siRNA is limited by the electrostatic repulsion between its negatively charged phosphate backbone and the anionic lipid bilayers of cell membranes. Also, the size of siRNA (7–8 nm in length) is larger than the thickness of the cell membrane (5 nm) [10–14]. The above-mentioned barriers provide an explanation for the limited number of approved siRNA therapies currently on the market. It also reveals the urgent need to develop new strategies that can deliver siRNA efficiently to targeted organs with minimum off-target effects.

There are three major techniques to overcome the obstacles of siRNA delivery: chemical modification, bioconjugation of siRNAs to specific ligands, and nanocarriers. The chemical modification of the naked siRNA molecule results in an

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Article highlights

- siRNA therapeutics are designed to degrade targeted mRNAs, blocking the expression.
- The therapeutic efficacy of siRNA is challenged by numerous intracellular and extracellular barriers, resulting in rapid clearance of siRNA from systemic circulation.
- Modifications of naked siRNA molecules have the potential to increase their resistance to degradation by endonucleases, prolong their circulation time, and penetrate barriers.
- The conjugation of siRNA therapies to specific ligands has emerged as a leading strategy to direct siRNA to specific sites of action.
- Nano-based drug delivery systems such as liposomes, niosomes, SLNs, PNs, and dendrimers may optimize the delivery of naked siRNA therapeutics for effective targeting and gene silencing in gene therapy and other applications.

This box summarizes key points discussed in the article.

increased cellular uptake, better resistance against nucleases, low toxicity, and a sustained circulation half-life of the modified siRNA therapies as compared to the unmodified counterparts [15,16]. The conjugation of siRNA molecules with different ligands such as antibodies, peptides, aptamers, lipids, and polymers represented an effective strategy for the active targeting of siRNA therapies to the targeted tissues with the highest possible concentration while mitigating off-target effects [14]. Finally, numerous nanocarriers such as liposomes, niosomes, dendrimers, PNs, and SLNs appeared as promising drug delivery systems that can encapsulate siRNA effectively and transfer it to targeted cells with a prolonged circulation time which may be advantageous in decreasing the frequency of administration of naked siRNA therapies [17]. This review outlines recent advances in siRNA delivery to improve their pharmacokinetic and therapeutic efficacy.

2. The mechanism of action

The process of gene silencing via RNAi occurs at the cytoplasm. First, a double-strand RNA (dsRNA) is cleaved into small dsRNA fragments (siRNA) with the aid of an endonuclease Dicer protein. Subsequently, siRNA binds to Argonaute-2 (AGO2) protein which is considered the heart of a multiprotein complex known as RNA-Induced Silencing Complex 'RISC' with consequent separation of siRNA into two single strands: the guide and passenger strands. The passenger strand is then degraded while the guide strand-RISC complex links to the complementary targeted mRNA and destroys it into nonfunctional moieties, thereby blocking the translation of the target protein [18–20] as demonstrated in Figure 1.

3. Challenges that restrict the direct delivery of naked siRNA

The siRNA encounters several extracellular and intracellular barriers following its administration. The relatively small size of siRNA makes it an excellent substrate for renal excretion through glomerular filtration. The RES involves noncellular and cellular components differentiated from monocytes and plays a significant role in the phagocytosis of foreign particles. These cells are mainly concentrated in the liver, spleen, and lymph nodes. The siRNAs are easily detected and opsonized by RES. These factors result in the rapid clearance of siRNA from the systemic circulation, minimizing its half-life circulation (i.e. less than 10 minutes). Additionally, siRNA undergoes extensive catalytic degradation by nucleases which limits its delivery to the target tissue. Regarding intracellular barriers, siRNA is negatively charged due to the phosphate backbone which creates an ionic repulsion between siRNA and lipid

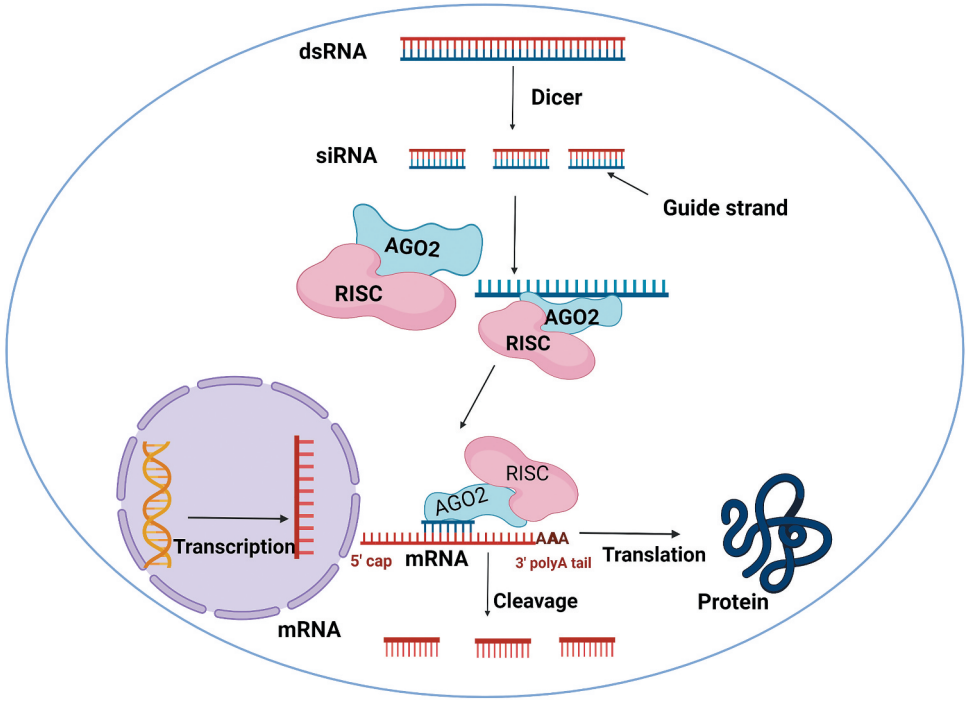

Figure 1. The mechanism of action of siRNA. Created with BioRender.com/.

bilayers of cell membranes. Consequently, poor cellular, blood-brain barrier, and tumor penetration ensue. Even after the cellular internalization, the acidic environment of endo/lysosomal compartments may lead to further degradation of siRNA. Therefore, there is an urgent need to develop and optimize effective carriers for secure siRNA delivery to the target tissue. Failing to do so may cause a gradual decline in the promising medical value of siRNA for gene silencing [13,21–26].

4. Strategies to overcome obstacles of siRNA delivery

Numerous biologically active moieties have experienced a loss in therapeutic value due to the improper establishment of an optimized delivery system that delivers it to the target organ. Hence, previous research and literature have focused efforts on developing suitable techniques for targeting active pharmacological substances in order to maximize their desired effect while minimizing off-target effects. Many studies reported promising strategies to counteract barriers that impede the pathway of siRNA to target cells. These technologies can be classified into three main strategies: chemical modification, bioconjugation, and carrier-mediated vesicles. These technologies have been employed to optimize the safety, efficacy, and stability of siRNA therapies [27,28] (Figure 2).

4.1. Chemical modification of siRNA

The negatively charged phosphate groups of siRNAs represent the most significant problem toward the cell permeability of siRNA [15,29–32]. An early approach to overcome this barrier includes masking the negative charge of siRNA through chemical modification. Other chemical modifications were also adopted for increasing the stability of circulating siRNA. An integral factor that should be taken into consideration during chemical modification is the balance between the stability and biological activity of the modified siRNA. The target site for modification must be non-essential for its gene silencing effect. Four possible sites for modification have been previously examined: the sugar moiety, phosphate linkage bond, nucleobase, and strand terminus, which can be performed individually or in combination [15]. Modifications of ribose sugars of siRNA are very common for improving the potency and stability of siRNA. The 2′-hydroxyl group is highly involved in chemical modification for many reasons. First, it has a prominent impact on the hydrolysis and stability of siRNA, making it highly susceptible to hydrolytic degradation. Additionally, it appears to not affect biological activity [16]. The 2′-methoxy and 2′-fluro are common substitutions that increase siRNA stability significantly. Other modifications have been performed including bicyclic derivatives such as locked nucleic acids (LNA) and acyclic derivatives like unlocked nucleic acids (UNA) [29,30]. Cell penetration may also be enhanced by covering the negative charge of phosphate groups of inter-nucleotide linkage. Overall, siRNA compromises four modifiable phosphate groups throughout its two strands (i.e. passenger and guiding). The 5′, 3′ terminus of the passenger strand and the 3′ terminal of the guiding strand are commonly suggested sites for modification. However, modification of the 5′ terminal (i.e. the seed region) of the guiding strand is not recommended due to its vital role in gene silencing activity [14,16,33–35]. The chemical modification of nucleobases is less familiar, but it also has a considerable effect on the heat stability and immunogenicity of siRNA [31]. One particular study by Nawrot et al. revealed the effect of chemical modification on siRNA activity. The authors showed that the activity of siRNA can be increased by including 2-thiouracil or pseudouracil bases at the 3′-terminal of the guide strand in addition to a dihydrouracil base at the 3′-

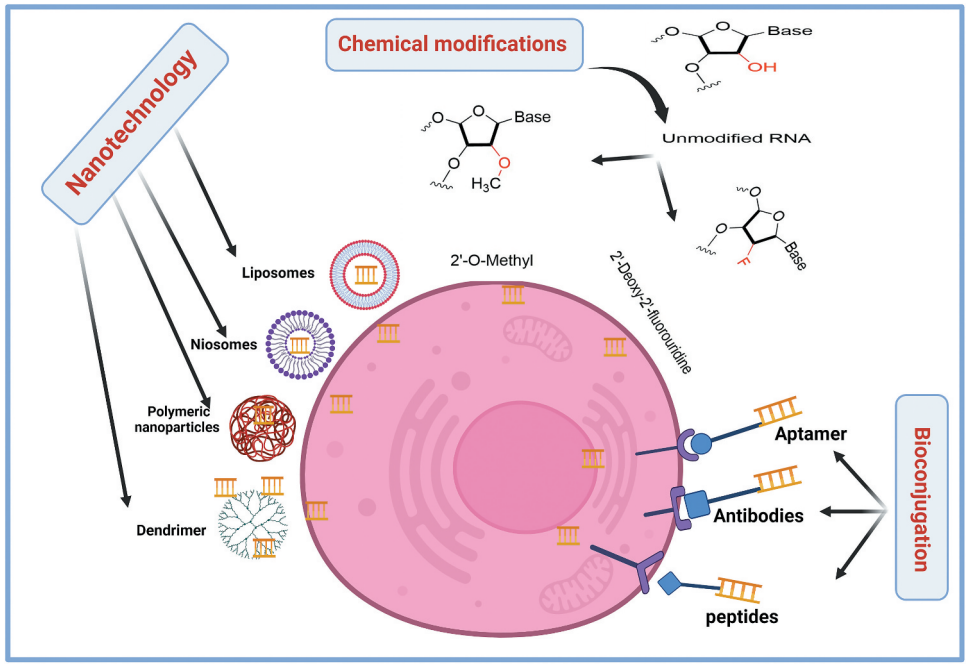

Figure 2. Various siRNA delivery techniques. Created with BioRender.com.

terminal of the sense strand. However, incorporating 2-thiouracil or pseudouracil to the 3′-terminal of the sense strand (i.e. opposite to the seed region) resulted in a significant decline in siRNA activity. Such findings were attributed to the improved thermodynamic asymmetry of the duplex terminus, supporting the 'opening' of the duplex at the 5′ -terminus of the guide strand [36].

4.2. SiRNA bioconjugation

The bioconjugation of siRNA represents an influential strategy to overcome the drawbacks of naked siRNA delivery. Numerous complications such as short systemic circulation time, susceptibility to endonucleases, poor cellular permeability, fluctuated pharmacokinetic profile, and off-target side effects can be optimized through bioconjugation. The siRNA bioconjugate exhibits significantly better bioavailability, targetability, prolonged half-life, and enhanced biological activity while maintaining the desired gene silencing property [37]. The site of conjugation is critical for the in vivo behavior of conjugated-siRNA and should be selected carefully. Previously, phosphate groups have gained the interest of several researchers in search of optimizing siRNA properties. The two strands of siRNA, each compromise two phosphate groups. It is well-established that the 5′ end of the guiding strand operates the RNAi machinery, acting as an unsuitable site for conjugation. Similarly, the 3′ end of the antisense is also not recommended for conjugation. Thus, the 3′ and 5′ ends of the passenger strand are the best available options for siRNA bioconjugation [16,35,38]. Additionally, bioconjugation to other sites such as the 2′-OH group of ribose or nucleobases has been reported [39]. The siRNA bioconjugate should pose a cleavable linkage that enables it to dissociate readily for proper incorporation into RISC. Acid labile linkers and disulfide linkage are commonly designed cleavable linkages. The acidic environment of endosomal/lysosomal vesicles will encourage the dissociation of siRNA from its acid-labile linker. Whereas the reductive characteristics of the cytosol environment will drive the cleavage of disulfide bond linkage [40]. In some cases, the bioconjugate itself contains a dicer recognition site through which the cleavage occurs by the dicer enzyme [41,42].

4.2.1. Antibody-conjugated siRNA (ACS)

Based on its high affinity and specificity toward its antigens, antibodies currently represent the standard bioconjugate for active siRNA targeting. This form of conjugation depends on the simple ionic attraction between a positively charged antibody and anionic phosphate groups of siRNA molecules via covalent linkage. Despite being an easy and reproducible method for siRNA-complexing, this nonspecific electrostatic interaction may result in an unfavored aggregation that can affect the bioavailability, distribution, and efficacy of the complexed siRNA [43–46]. The ACS has been used to deliver siRNA to various tissues such as cancer, skeletal muscles, cardiac, and liver cells [47–49]. In 2016, Tsukasa Sugo et al. reported the efficacy of delivering siRNA to skeletal and cardiac muscles; in vitro and in vivo through ACS theory using bioconjugation between siRNA and anti-CD71 antibody. Simply, CD71 is the receptor of transferrin, an iron-binding protein that is responsible for transferring iron to various cells. The CD71 is highly expressed in numerous types of cells including skeletal and cardiac muscle cells. Intravenous delivery of siRNA-anti CD71 complex showed a significantly strong robust and sustained myostatin silencing effect [49]. Recently, Zhili Yu et al. discussed the bioconjugation of siRNA to antibodies using a multifunctional peptide, involving a cell-penetrating peptide (CPP) and a substrate peptide (SP) as a linker, which is overexpressed in several types of solid tumors. The prepared SP-CPP-siRNA complex carried siRNA to tumor cells by the definite binding affinity of the antibody with an improvement of siRNA gene silencing efficiency both in vitro and in vivo [50].

4.2.2. Peptide-conjugated siRNA (PCS)

Peptides can be used as an alternative approach to ACS targeting for several reasons. Primarily, peptides are relatively smaller in size with low molecular weight, affordable, and easy to synthesis, especially after the extensive application of the phase display technique [51]. Furthermore, peptides can maintain a high degree of specificity and binding affinity to specific receptors also associated with antibodies [52]. As a result, peptides have evolved as one of the primarily selected ligands for active targeting and drug delivery [53,54]. Gandioso et al. studied the probability of targeting siRNA to integrins, somatostatin, and human epidermal growth factor receptor 2 (HER2) via bioconjugation to RGD-containing cyclopentapeptide, octreotide, and anti-HER 2 peptides, respectively. Their findings proved the efficacy of these conjugates to deliver siRNA effectively to numerous tumor cells [55]. Wagner et al. functionalized a naked siRNA with atherosclerotic plaque-specific peptide-1 (AP1) that binds efficiently to the interleukin-4 (IL4) receptor which is highly expressed in various solid tumors. Additionally, this siRNA-AP1 complex was furtherly conjugated to other apoptotic peptides (i.e. KLK, BAK, and BAD) for improved antitumor and gene silencing efficacy [56]. In 2021, Eyford et al. conducted a unique study describing the bioconjugation of siRNA to a 12 amino acid peptide known as MTfp that crosses the blood–brain barrier and delivers siRNA to the brain to block the expression of NOX4, a gene responsible for oxidative stress in ischemic stroke. This study represents a new approach toward treating oxidative stress-mediated neuroinflammation in several neuropathologies [57]. Cell-penetrating peptides (CPPs) are a diverse class of tailored peptides with well-developed cell permeability characteristics that can deliver siRNA to the target site significantly [58]. Generally, there are two categories of CPPs: cationic CPPs and activatable CPPs [14]. Cationic CPPs improve the cellular uptake of siRNA by posing a positive charge that shields the negative charge of siRNA and transports it safely to the cytosol. Transportan, Penetratin, and Tat are common examples of cationic CPPs. Moschos et al. investigated the effect of siRNA conjugation with two CPPs (i.e. TAT and Penetratin) on the knockdown of p38 MAP kinase. They determined that the decoration of siRNA with Penetratin or TAT (48–60) resulted in a significant reduction in p38 MAP kinase expression while the administration of penetratin-siRNA

complex alone triggered innate immunity [59]. Activatable CPPs are considered CPPs prodrugs wherein the positive charges of the cationic CPP are neutralized by a counterion. After activation, the counterion becomes dissociated and the peptide acts as a typical CPP [60,61]. Hairpin-structured peptide containing a polycationic CPP moiety and polyanionic counterion, as well as an enzymatically cleavable linkage, is an example of this type of CPP peptide [62].

4.2.3. Aptamer-conjugated siRNA (APCS)

The term 'aptamer' originates from the Latin name 'Aptus,' which is translated to 'to fit,' and the Greek term 'meros,' meaning 'part' [63]. An aptamer is an artificial, single-stranded RNA or DNA oligonucleotide, that is a promising ligand for siRNA targeting [14]. Notably, its characteristics combine the features of both antibody- and peptide-bioconjugates. Like antibodies, aptamers offer a high degree of specificity and binding affinity to certain proteins and receptors based on their unique three-dimensional structure. Moreover, aptamers are of value given their low immunogenicity, high safety, cost-effectiveness, ease of synthesis, and prolonged shelf life [64]. McNamara et al. were the first to report the conjugation of siRNA to a highly specific prostate-specific membrane antigen (PSMA) aptamer which enabled the targeted delivery of siRNA to prostate cancer cells due to the overexpression of PSMA receptors on its surface. This complex resulted in a significant silencing of survival genes and cell death [40]. Additionally, Jiehua Zhou and John Rossi synthesized a unique siRNA-aptamer complex for the treatment of HIV-1 viral infection. The aptamer was selected to bind specifically to HIV-1 envelope glycoprotein (gp120) with the goal of targeted delivery of siRNA to infected cells for mRNA knockdown and the inhibition of virus entry by blocking the interaction between HIV-1 gp120 and its CD4 receptor [63]. Interestingly, Khanali et al. designed an APCS that delivered siRNA directly to SARS-COV-2 infected cells as an attempt to control COVID-19 infections. Spike protein of SARS-COV-2 virion is responsible for its cell entry through interaction with the angiotensin-converting-enzyme-2 (ACE2) receptor. The designed anti-spike protein aptamer can efficiently bind to the ACE2 receptor and mediate the cellular entry of siRNA, subsequently blocking mRNA and protein translation [65]. Examples of other studies reporting bioconjugation to peptides, antibodies, and aptamers are further described in Table 1.

4.2.4. Lipid-conjugated siRNA (LCS)

The utilization of lipids as drug delivery systems has added important features to drug delivery and targeting. Pure lipid-based platforms such as liposomes and solid-lipid nanoparticles have been already employed to deliver several drugs and biologically active agents. The negative charge and hydrophilicity of native siRNAs are two primary barriers to achieving optimal targeting and gene silencing efficiency. Cationic lipids may overcome this problem by masking the siRNA-surface charge and converting it to a neutral molecule that can penetrate the cell membrane directly [75]. Additionally, the hydrophilicity of siRNA minimizes its plasma protein-binding tendency, making it an ideal candidate for glomerular excretion with a very short half-life circulation [76]. The LCS shows a higher affinity to circulating plasma proteins and lipoproteins like albumin, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) with a prolonged circulation time and better biodistribution profile as compared to the non-linked siRNA [14]. Thus, the conjugation of siRNA to lipid molecules may resolve two of the main challenges. Among many available lipids for bioconjugation, cholesterol has been extensively explored. Chernikov et al. linked cholesterol to siRNA through a methylene unit to examine its cellular penetration using three different cell lines (K2562, MOLT-3, and HL-60) and blood mononuclear cells. The data demonstrated higher cellular accumulation ratios in all types of cells, suggesting a promising strategy for the management of leukemia [77]. Osborn et al. examined the effect of hydrophobicity of lipids on the pharmacokinetics of siRNA after linkage of siRNA to four different lipids (i.e. cholesterol, docosanoic acid, lithocholic acid, and cosahexaenoic acid). The authors concluded that the lipid bioconjugation may result in a pronounced increase in the plasma half-life of siRNA throughout the binding affinity of the conjugate to lipoproteins, LDL, and HDL depending on the degree of hydrophobicity of the lipids [78].

4.2.5. Carbohydrate-conjugated siRNA (CCS)

Glucose, galactose, lactose, and their derivatives represent a promising approach to optimizing the targeting, bioavailability, half-life, distribution, and pharmacological activity of siRNA with simple and cost-effective ingredients [79–81]. Zhu et al. conjugated siRNA with two carbohydrate derivatives,

Table 1. Examples of reported literature for siRNA bioconjugation.

Type of ligand	Name of ligand	Targeted receptor	Gene of interest	Ref.
Antibodies	The human insulin receptor-monoclonal antibody (HIR-MAb)	The human insulin receptor (HIR)	Luciferase	[66]
	Humanized monoclonal antibody (Hu3S193)	Lewis-Y (Le^y) receptor	Signal Transducer And Activator of Transcription 3 (STAT3)	[67]
	Anti-CD22 monoclonal antibody (Anti-CD22 Mab)	CD22	Gyceraldehyde-3-dehydrogenase (GAPD)	[68]
Peptides	Cyclic- (arginine-glycine-aspartic) (cRGD)	αvβ3 integrin	luciferase gene	[69]
	12 amino acid peptide (YSA)	Erythropoietin-producing hepatocellular (Eph) A2	Epidermal growth factor receptor (EGFR)	[70]
	Insulin-like growth factor 1 (IGF1)	Insulin-like growth factor 1 (IGF1) receptor	Insulin receptor substrate 1(IRS-1)	[71]
Aptamers	Mucin-1 (MUC1-apt)	Mucin-1(MUC1) recptor	Insulin-like growth factor receptor 1 (IGF-1 R)	[72]
	Epithelial cell adhesion molecule aptamer (EpCAM)	Epithelial cell adhesion molecule (EpCAM)	Delta-5-Desaturase (D5D)	[73]
	Aptamer A6	Human Epidermal growth factor 2 (Her-2) receptor	P-glycoprotein transporter	[74]

namely, galactosylated poly (ethylene glycol) and mannose 6-phosphate poly (ethylene glycol), for enhanced silencing of luciferase and transforming growth factor-beta 1 (TGF-β1) genes in hepatocytes. These sugar-conjugated siRNAs achieved a significant block of the aforementioned genes efficiently with no reported adverse effects [80]. Nair et al. designed a highly specific siRNA-N-acetylgalactosamine (GalNAc) complex that targeted the asialoglycoprotein receptor in the liver. The subcutaneous administration of this complex revealed a five-fold enhancement of gene silencing as compared to the native siRNA with no adverse effects [47].

4.2.6. Dynamic polyconjugate of siRNA

The complex journey of siRNA begins with its administration and is followed by its arrival to the cytoplasm to block the expression of a specific gene. With several barriers along its path, designing an effective platform to carry siRNA to the target site of action has been challenging. Prior experimental strategies to overcome such obstacles have been oriented toward overcoming one barrier. Interestingly, the theory of dynamic polyconjugate has opened the door to the synthesis of a multi-conjugated tool that can overcome more than one barrier. Briefly, a dynamic polyconjugate is a multi-conjugate drug delivery system that offers numerous conjugation sites exposed from a polymeric backbone [14]. One or more ligands can be coupled to this backbone with added desired features. Many substances can be linked to this polymer skeleton such as a lipid component for better cell membrane permeability, a polymer for prolongation of the systemic circulation, and a specific targeting ligand for binding to surface receptors [75]. Rozema et al. were the first to design a dynamic polyconjugate composed of poly butyl amino vinyl ethers (PBAVE) as a backbone and possess many amine groups as conjugation sites. Two distinctive ligands were attached to the polymer skeleton: PEG for prolonged siRNA circulation and N-acetylgalactosamine for proper binding affinity with hepatocytes. Moreover, the two ligands were attached to the polymer by the acidic sensitive linker (CDM) which covered the negative charge of PBAVE for the desired endosomal escape effect. Effective silencing of the apoB gene in mouse hepatocytes was achieved following the administration of the dynamic polyconjugate [82].

4.3. Nanotechnology strategies

Since its introduction in 1959 by the American scientist Richard Feynman, nanotechnology has intrigued the mind of researchers worldwide. Simply, nanotechnology describes the employment of materials at a nano-scale size for different practical applications [83,84]. Nearly all fields of research have incorporated the concept of nanotechnology with different purposes. Concerning medical and pharmaceutical literature, nanotechnology has been employed to achieve treatment, prophylaxis, and diagnostic goals [85]. Many obstacles that hinder the effective delivery of therapeutics to specific sites of action may be mitigated through the use of proper nano-based drug delivery techniques [86–88]. Low membrane permeability [89,90], poor aqueous solubility [91], short circulation half-life [92], hepatic first-pass metabolism [93], rapid renal clearance [94], immediate drug release [95], and off-target side effects [96] are some examples of drug delivery limitations that could be resolved by nanotechnology-based strategies. Below, we discuss recent nano-based techniques for the improvement of siRNA delivery.

4.3.1. Dendrimers

Dendrimers are unique artificial macromolecules in the nano-scale size range with symmetric and monodisperse characteristics [97]. They display a three-dimensional, branched, and flexible structure with a highly exposed surface area [98]. Dendrimers are specialized polymeric materials composed mainly of three parts: a central core, radiating branches, and peripheral functional groups [99]. The structure of dendrimers is designed with tree-like arms that branch off the central core. Dendrimers exhibit several advantages as drug delivery platforms for many drugs, peptides, genes, and various biologically active moieties. Generally, dendrimers are synthetized by two distinctive techniques: a divergent and convergent synthesis. Divergent synthesis includes the outward addition of polymeric monomers from the central core through a series of polymerization reactions till dendrimers are formed. On the other hand, convergent strategy describes the inward branching starting from the outer surface of dendrimers to the core. In the convergent synthesis, dendrons are formed firstly by stepwise coupling reactions between branching units with a subsequent attachment to the central core [100]. In contrast to other polymeric platforms, they have very symmetric structures with uniform size, shape, and physicochemical characteristics that may be easily controlled during synthesis [101]. Additionally, a wide range of dendrimers with tailored molecular weight, density, physical, and chemical characteristics can be designed depending on the selected monomeric units [102]. Furthermore, the large number of peripheral functional groups enables dendrimers to bind to several molecules that can target desired organs [103]. Finally, dendrimers are characterized by their long circulation time with a prolonged duration of action [104]. Overall, the combination of these features results in dendrimers as a promising tool for siRNA delivery. Polyamidoamine dendrimers (PAMAMs) were the first designed class of dendrimers [105]. The radiating branches were constructed of aliphatic aminoamides, while the core was synthesized from ethylenediamine, triethanolamine, or ethylene diamine [106–108]. The peripheral functional groups can be coupled to a wide variety of substances such as PEG for sustained circulation time, fatty acids for optimized membrane penetration, or specific peptides for targeted delivery [109]. Amino groups are frequently used as traditional terminal groups as they are able to bind siRNA proficiently [110]. Liu et al. synthesized a PAMAM dendrimer, composed of triethanolamine (TEA) core to evaluate the gene silencing activity of Hsp27 siRNA using prostate cancer (PC-3) cells for the management of prostate cancer. Findings showed the delivery system to be highly efficient in protecting siRNA from enzymatic degradation and enhancing its cellular uptake with improved gene silencing activity [111]. Another multifunctional PAMAM dendrimer was prepared by Dong et al. for targeting siRNA to cancer cells. The dendrimer was decorated with RGDK peptide to bind efficiently to integrin and

neuropilin-1 receptors which are overexpressed on tumor cells. This dual-targeting dendrimer was able to deliver siRNA with a high affinity to cancer cells, higher cellular uptake, and powerful gene knockdown behavior [112].

4.3.2. Liposomes

Liposomes are known to be the most common and well-established nanocarriers utilized for drug delivery purposes. Liposomes are comprised of phospholipid vesicles arranged together in the form of unique lipid bilayers and a central hydrophilic core. This unique structure enables the encapsulation of a wide variety of hydrophilic and lipophilic materials. Aqueous components can be entrapped in the inner hydrophilic core while the hydrophobic moieties remain at the outer lipid bilayers [113,114]. Generally, liposomes are classified into two main categories: unilamellar vesicles and multilamellar vesicles. Unilamellar liposomes are composed of a single phospholipid bilayer surrounding an aqueous core. Whereas multilamellar vesicles show a multilayered structure of repeated unilamellar vesicles with concentric phospholipid bilayers separated by aqueous layers [115]. Adopted techniques for the preparation of liposomes are numerous with similarities. The general scheme for the preparation of these vesicles starts with dissolving the lipid in an organic solvent, followed by drying of these lipids. After that the dried lipids are dispersed in an aqueous media, permitting the spontaneous assembly of liposomal vesicles. These vesicles are further purified and characterized [116]. Depending on its fatty nature, it is assumed that liposomes exhibit excellent cellular and tissue uptake properties. Moreover, liposomes offer a high degree of stability to the encapsulated ingredients with optimized biodistribution and bioavailability. Additionally, these lipid vesicles show extraordinary features such as good compatibility, ease of self-assembly and formulation, high drug loading efficiency, and several advanced physicochemical characteristics that may be easily controlled [117,118]. Liposomes emerged as a promising carrier to entrap siRNA and bypass the several reported barriers including poor cellular uptake, vulnerability to degradation enzymes, and uncontrolled distribution within the body [118]. Man et al. formulated peptide-based liposomal vesicles to deliver Myocardin-related transcription factor (MRTF-B) siRNA for the management of conjunctival fibrosis. The liposomes-siRNA were linked to targeting peptides (Y and ME27) and the gene-silencing efficacy was examined using human Tenon's fibroblasts cells. The MRTF-B gene expression was significantly blocked by 76% and 72% with liposomal-Y-peptide-siRNA and liposomal-ME27-peptide-siRNA, respectively [119]. Another study that adopted liposomes for siRNA encapsulation was conducted by Mokhtarieh et al. who aimed to reach the highest possible entrapment efficiency of siRNA inside of the vesicles. The vesicles were constructed from cationic lipids in the inner compartment that could bind the negative siRNA while the neutral lipids were located at the external layer. The surface of liposomes was functionalized using a poly-arginine peptide (R12) or anti-human epidermal growth factor receptor antibody body (anti-EGFR). Their findings showed a improved siRNA cellular uptake and gene silencing in NCI-H322 cells [120]. Recently, Khabazian et al. synthesized cRGD peptide-liposomes that could successfully carry anti STAT3-siRNA to melanoma cells, overexpressing the integrin receptors. Liposomal vesicles transporting siRNA demonstrated higher stability and improved cellular uptake with respect to the control group [121].

4.3.3. Niosomes

Above, we discussed the benefits of liposomal vesicles that make them attractive nanocarriers for drug delivery in pharmaceutical research. However, an important parameter to discuss is the physical and chemical instability of liposomes due to their lipid composition [122]. Consequently, a new type of vesicles called 'niosomes,' which resemble liposomes in shape and assembly but differ in composition, has emerged as a possible alternative carrier. Niosomes are composed of non-ionic surface-active agents that show an amphiphilic structure, combining hydrophilic and lipophilic functional groups in the same compound [123]. The high interfacial tension between the fatty tails and hydrophilic heads facilitates the self-assembly and association of individual molecules. Additionally, the repulsion between similar head groups forces head groups outwards with direct contact with the aqueous environment. This assembly mostly requires a source of energy like heat or stirring [124]. Niosomes and liposomes share the same categories of types (e.g. unilamellar and multilamellar). Many methods of preparation were reported for the fabrication of niosomal vesicles such as thin-film hydration, reverse phase evaporation, microfluidization, supercritical carbon dioxide fluid, and ether injection methods. Niosomal vesicles are assembled in a form that is very similar to liposomes core and bilayers, allowing them to offer the same advantages as liposomes with regard to the encapsulation ability of both hydrophilic and hydrophobic drugs [125]. In contrast to liposomes, niosomes display excellent physical and chemical stability due to the absence of unstable fatty ingredients. Moreover, they have demonstrated higher encapsulation efficiency for entrapped substances when compared to liposomes. Furthermore, niosomes can easily be PEGylated to prolong blood circulation. Considering their characteristics, niosomes may act as an efficient and affordable substitute for liposomes [126]. Recently, Hemati et al. prepared positively charged PEGylated niosomes encapsulating siRNA, doxorubicin, and quercetin using Tween 60 as a surfactant. The designed niosomes showed optimized characteristics regarding, vesicle size, zeta potential (electrokinetic value relating to an accurate magnitude of surface charge), entrapment efficiency (the amount of drug encapsulated within the nanocarrier), and drug-release profile. The niosomes were reported to have powerful anticancer activity on prostate, breast, and gastric cancers cell lines [127]. Other synthesized siRNA-niosomes nanocarriers were achieved by Maurer et al. who investigated the downregulation of the Lifeguard (LFG) gene in BT-474 cells. The LFG-silencing siRNA was enclosed in the aqueous core of niosomes, while magnetic iron-oxide nanoparticles were incorporated into niosomal bilayers for successful siRNA-targeting under the influence of an external magnetic field. This novel delivery system achieved a significant knockdown of the gene of interest [128].

4.3.4. Solid lipid nanoparticles (SLNs)

SLNs are colloidal lipid-based nanocarriers composed of high melting point glycerides or waxes with a surfactant-coated surface [129]. SLNs may be considered the recent generation of traditional lipid nanocarrier as liposomes and microemulsion where the liquid oils are replaced by solid lipids in an attempt to optimize the stability of these hydrophobic platforms. SLNs exhibit numerous advantages as drug delivery vehicles based on their small size, high surface area, high drug loading efficiency, and stability [130]. The preparation of SLNs mostly requires a high source of energy either by using high-pressure homogenizers or ultrasonication [131]. Compared to liposomes or niosomes, SLNs demonstrate superior physical and chemical stability while maintaining the ability to entrap both hydrophilic and hydrophobic compounds and are affordable allowing ease of formulation and scale-up [132,133]. Furthermore, surface modification with PEG can be easily conducted, thus inhibiting RES uptake and sustaining the overall circulation time [134]. Moreover, SLNs demonstrate a well-developed permeability through strictly-tight biological membrane barriers such as the blood–brain barrier [135] and stratum corneum [136]. Given this, SLNs appear to be a promising technique for siRNA transport. SLNs could effectively sustain the release profile of siRNA over 10–13 days with reserved gene silencing after intradermal injection. This controlled-release behavior decreases the necessity for frequent administration of siRNA and its related side effects. Additionally, there is no threat of residual SLNs within the body during its release period due to the excellent biocompatibility and safety properties of the lipids [137]. Oner et al. developed new siRNA loaded cationic SLNs to block the expression of EphA2 receptor tyrosine kinase (siEphA2) which is highly expressed in numerous solid tumors including prostate cancer. The authors reported high cellular accumulation and gene downregulation effects [138].

4.3.5. Polymeric nanoparticles (PNs)

Polymeric nanoparticles are colloidal systems fabricated from natural or synthetic polymers in the nanoscale range [139,140]. PNs furnish numerous advantages over other nanocarriers such as liposomes, niosomes, SLNs, and polymeric micelles in terms of synthesis, bioavailability, stability, biocompatibility, and release of entrapped components. Such polymer-based nanoparticulate systems exhibit extraordinary stability and can be stored for a long period after lyophilization [141–143]. One of the most interesting properties of PNs is their ability to control the release of active agents depending on the utilized polymer characteristics ranging from immediate release to prolonged sustained release [144,145]. Additionally, the excellent biodegradability and biocompatibility of the used polymers decrease the risk of toxicity and immunogenicity [146]. Also, surface modification can be easily conducted, thereby optimizing the targeting of PNs to the desired tissue [147]. Finally, polymeric nanoparticles alone have demonstrated the ability to perform targeted delivery to cancer cells due to the enhanced permeability retention (EPR) effect of these tissues [148]. Several polymers have been utilized to formulate PNs like poly (lactic acid) (PLA), poly (glycolide) (PLG), co-polymer poly(lactide-co-glycolide) (PLGA), polyalkylcyanoacrylates, poly (caprolactone), Eudragit, and chitosan. Below, some of the most utilized polymers to deliver siRNA are discussed.

4.3.5.1. PLGA nanoparticles. PLGA is the most frequently used polymer to synthesize PNs due to its outstanding characteristics. It is a negatively charged copolymer that has been approved by the FDA and European medicines agency for human use following the demonstration of its excellent biodegradability and biocompatibility [149]. PLGA nanoparticles exhibit a sustained release profile which prolongs drug circulation time [150]. The synthesis of PLGA nanoparticles depends on the incorporation of a high source of energy to encapsulate the active ingredient within the matrix or the core of the polymer. Several methods were adopted to formulate PLGA nanoparticles such as nanoprecipitation, emulsion solvent evaporation, and double emulsification. The general preparation strategy involves the mixing between two phases: the organic phase and the aqueous phase, where the drug or polymer is dissolved completely according to its nature. This mixing is conducted via homogenization or ultrasonication to form an emulsion. Then, the solidification of these nanoparticles occurs spontaneously as a result of either the evaporation or the diffusion of the organic phase [144,151]. Many studies focused on the ability of PLGA nanoparticles to encapsulate siRNA reporting good stability, bioavailability, and therapeutic efficacy. Pantasiz et al. formulated PLGA-(RNA-binding motif protein 3) siRNA polymeric nanoparticles for the optimization of siRNA targeting release and gene silencing efficacy using a double emulsion solvent-evaporation technique. To improve the entrapment and release of siRNA, a small amount of a cationic polymer polyethyleneimine was added during nanoparticle processing. Results showed the developed delivery and gene expression downregulation of the PLGA-siRNA nanosystem [152]. Additional findings of the importance of PLGA nanoparticles to transport siRNA have been discussed by Bilecen et al. for the management of osteoporosis through inhibition of the receptor activator of nuclear factor kappa B(RANK) gene expression. The authors prepared RANK-loaded PLGA nanoparticles and investigated RANK gene silencing potential. These nanoparticles could significantly block RANK-mRNA levels by 47%. A recent study (2021) targeted the silencing of the inhibitor of NF-κB kinase subunit beta (IKBKB) as a potential strategy to control neuropathic pain. The results displayed the role of siRNA-PLGA nanoparticles in decreasing the secretion of proinflammatory mediators in rats [153].

4.3.5.2. Chitosan nanoparticles. Chitosan is a natural mucopolysaccharide polymer that has been approved by the FDA. Notably, its biodegradability and biocompatibility have led to its status as one of the commonly used polymers for the preparation of PNs in drug delivery research [154,155]. Unlike PLGA, chitosan is freely soluble in an acidic aqueous solution thus minimizing the need to use organic solvents with toxic and irritant properties [156]. Additionally, its cationic nature may facilitate the encapsulation of anionic siRNA molecules.

Ragelle et al. prepared PEGylated siRNA-loaded chitosan PNs and investigated their effects on gene expression knockdown in B16 melanoma cells overexpressing luciferase. They achieved an excellent siRNA entrapment efficiency (99.8%) and demonstrated the high stability, extended circulation time, and improved silencing activity of the presented nanoparticulate drug delivery system [157]. Furthermore, Tianyu Li et al. recently demonstrated the efficacy of chitosan nanoparticles to deliver lncRNA NEAT1 gene silencer to colon cancer cells with subsequently pronounced inhibition of tumor growth [158].

4.3.6. Inorganic nanoparticles

Recently, inorganic nanoparticles have gained more interest as potential carriers for siRNA therapies due to their prominent characteristics [159,160]. Inorganic nanoparticles posse unique physical, chemical, and optical properties that enable them to deliver siRNA efficiently to the desired cells. Besides their small size, inorganic nanoparticles experience a high surface area, allowing the encapsulation of a high amount of siRNA. Also, the ease of surface modification of these nanoparticles mitigate several challenges of siRNA delivery [161]. Common examples of this type of nanocarriers are gold NPs, magnetic NPs, and mesoporous silica NPs. Gold NPs are the most common and investigated type of inorganic nanoparticles for siRNA delivery. The inert, safe, and biocompatible core of gold NPs makes it a prominent platform for siRNA encapsulation. In addition to its ideal surface chemistry which can be easily conjugated with other ligands for targeted delivery [162]. Rahme et al. utilized gold NPs platforms to deliver the siRNA efficiently to prostate cancer cells. These gold nanoparticles were subjected to further surface modifications through the conjugation to folic acid to target the folate receptors in cancer cells. This system was very efficient for the targeted delivery of siRNA in vitro to prostate cancer cell lines with a significant gene silencing activity [163].

5. Conclusion and future prospective

The discovery of siRNA as gene-silencer therapeutics is promising in a variety of diseases like cancer, hepatic, viral, and neurological complications. siRNAs showed a prominent efficacy in the downregulation of several genes and proteins to treat the above-mentioned diseases. Currently approved siRNAs therapies are now present in the market with highly pronounced outcomes. For example, lumasiran is used for the management of a rare, genetic, and lethal disease termed 'primary hyperoxaluria type 1(PH1).' This metabolic disorder results in an increased plasma level of toxic oxalate with consequent kidney dysfunction due to the excretion of high amounts of this toxic mediator through the kidney. The subcutaneous injection of lumasiran achieved normal or pre-normal levels of urinary oxalate within 6 months in patients with (PH1) [164]. The delivery of siRNA to targeted tissues represents the most challenging problem which limits its effective utilization. Several techniques have been adopted to optimize the targeted delivery of siRNA therapies. Chemical modifications to original siRNA molecules may help to protect the siRNA from catalytic degradation by serum and tissue endonucleases, sustaining its blood circulation and improving its biodistribution to the selected organs. The siRNA may also be linked to various ligands such as peptides, antibodies, aptamers, lipids, and carbohydrates through bioconjugation. The bioconjugates of siRNA optimize its delivery and significantly increases its cellular uptake by the targeted cells while minimizing side effects. Nano-mediated carrier systems represent an advanced strategy for siRNA targeting and delivery. Numerous nanocarriers such as liposomes, niosomes, dendrimers, SLNs, and polymer-based nanoparticles demonstrate a safe, effective, and promising encapsulation for siRNA, with a powerful gene silencing activity. Moreover, siRNA therapies are promising in targeting different types of complicated diseases such as solid tumors, neurological disorders, skeletal muscle diseases, cardiomyopathy, leukemia, HIV infection, osteoporosis, and SARS-COV-2 viral infection and may be extended in the future toward other life-threatening diseases. Currently, four siRNAs therapies have been approved by the FDA and other regulatory agencies for human use. The advances in siRNA delivery and targeting have the potential to treat various diseases and assist in vaccine development.

6. Expert opinion

The role of siRNA as therapeutics is promising as it can be useful for a large number of diseases. RNAi has evolved as an advanced strategy of gene therapy that involves the utilization of short nucleotides known as 'siRNAs' to degrade the mRNA of defective genes and thus downregulate them. The knockdown of causative gene expression slows the progress of many lethal diseases and allows the better management of these complications at an early stage. The use of siRNA has shown potential in a host of diseases including cancer, hepatic, viral, and neurological complications, and others. siRNA shows a prominent efficacy in the downregulation of several genes and proteins in diseases that are impacting humans globally. The siRNA therapies may represent an advanced alternative strategy for targeting cancer tissues with high therapeutic efficacy and minimum off-target side effects [165]. Moreover, viral infections which are mainly progressed by the replication of viruses and the translation of various viral essential proteins may be targeted by siRNA therapies [166]. The downregulation of SARS-COV-2 expression and replication using siRNA therapies was suggested and some studies deliberated the probability of utilizing siRNA for the management of the COVID-19 [167]. Unfortunately, there is a current lack of optimum carriers to transfer these candidates to lung tissue and the passive administration of these molecules may result in several side effects. The design of a suitable delivery system to deliver siRNA to the target site is a prerequisite for the successful employment of these therapies. This delivery system should transfer siRNA efficiently to intended sites of action and provide a high degree of resistance against serum nucleases. Additionally, the selected delivery system may be functionalized by a ligand that will bind specifically with cell surface receptors for active targeting of siRNA. Overall, nanocarriers represent effective platforms for siRNA encapsulation. The small size and high surface area of

nanoparticles or nanovesicles may optimize the biodistribution of siRNA therapies. The combination between the above-mentioned strategies can act as an alternative approach for effective siRNA delivery. There is an urgent need for the continuous optimization of appropriate strategies for siRNA targeting.

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