Rekindling RNAi Therapy: Materials Design Requirements for In Vivo siRNA Delivery

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With the recent FDA approval of the first siRNA-derived therapeutic, RNA interference (RNAi)-mediated gene therapy is undergoing a transition from research to the clinical space. The primary obstacle to realization of RNAi therapy has been the delivery of oligonucleotide payloads. Therefore, the main aims is to identify and describe key design features needed for nanoscale vehicles to achieve effective delivery of siRNA-mediated gene silencing agents in vivo. The problem is broken into three elements: 1) protection of siRNA from degradation and clearance; 2) selective homing to target cell types; and 3) cytoplasmic release of the siRNA payload by escaping or bypassing endocytic uptake. The in vitro and in vivo gene silencing efficiency values that have been reported in publications over the past decade are quantitatively summarized by material type (lipid, polymer, metal, mesoporous silica, and porous silicon), and the overall trends in research publication and in clinical translation are discussed to reflect on the direction of the RNAi therapeutics field.

1. Introduction

1.1. Brief History of Gene Editing Tools

The Human Genome Project, which successfully mapped the complete human DNA sequence in 2003,[1] was motivated in large part by the hope that diseases might be effectively treated by appropriate manipulation or modification of genes. This concept of disease treatment by genetic modification emerged in the 1960s, when the first in vitro observations that viral DNA could induce cellular modulation during an infection were made.[2] An overview of gene modification tools that are currently available is summarized in Table 1. The 1970s saw initial attempts at gene modification using recombinant DNAs (rDNAs; a combination of more than one DNA sequence from one or more species)[3] with viral infection or calcium phosphate as the primary transfection methods. This discovery led to developments of cell lines,[4] genetically modified mice,[5] and means to produce human proteins (e.g., insulin) in bacteria (e.g., Escherichia coli).[6] By 1978, liposomes had been harnessed to transfec messenger RNA (mRNA) in vitro, resulting in expression of rabbit globin in mouse lymphocytes.[7] Antisense oligonucleotides (ASOs) were also developed to bind to target mRNA sequences in the cell cytoplasm for inhibition of protein translation or to induce exon skipping.[8] During this period, the rapidly developing field catalyzed important discussions in the ethics, politics, and economics of genetic engineering, resulting in regulations limiting the different hierarchies of gene modification research (i.e., modifications made to bacteria, plants, mammalian systems, and humans) and guidelines for patentable inventions and systems subject to approval by the U.S. Food and Drug Administration (FDA).[2,9]

The late 1980s and early 1990s saw emergence of new classes of gene editing tools. The zinc finger motif in transcription factor IIIA (TFIIIA) was discovered in 1982,[10] which led to the 1994 discovery and development of the zinc finger nuclease (ZFN) that is able to selectively cleave a target DNA domain by tuning the DNA-binding motif of the system.[11] Nearly 20 years later, similar restriction enzyme-based systems—TALEN[12] and CRISPR/Cas9[13]—were discovered and developed into potential tools for gene therapy (in 2010 and 2011, respectively).

In the meantime, transient gene silencing via sequence-specific mRNA degradation using double-stranded RNA (dsRNA) was discovered in 1997 by Mello,[14] then further developed by Fire[15] in 1998; the phenomenon was termed “RNA interference (RNAi).” The mechanism for RNAi was elucidated soon after, with the identification of short interfering RNAs (siRNAs) and the RNA-induced silencing complex (RISC).[16,17] The discovery was ground-breaking in that RNA-based gene modification offered a simpler, reversible, and more transient effect than the DNA- and restriction enzyme-based approaches, and thus presented a more favorable option when relatively short-term effects were desired (i.e., in acute disease treatment). This review focuses on the use of RNAi for gene therapy.
1.2. RNA Interference

RNA interference is a transient gene silencing mechanism found endogenously in eukaryotic cells. Figure 1 details the mechanism by which siRNAs silences gene expression. The pathway is initiated by the presence of siRNAs (either artificially synthesized and delivered into the cell, or endogenously produced by dicer-mediated cleavage of dsRNAs into shorter 21–25 base-pair sequences) in the perinuclear region of the cell cytoplasm. Here, the siRNA binds to the RISC, and is unwound into a single-stranded RNA by Argonaut 2 (AGO2; a component of the RISC)-mediated cleavage of the unnecessary strand\(^{[18]}\). Next, the RISC guides the now single-stranded siRNA to its complementary sequence on the cell's endogenous mRNA for base-pair binding, at which point the RISC cleaves the mRNA for degradation\(^{[16,18–20]}\). After cleavage of the mRNA, the RISC and single-stranded siRNA are free to bind to other mRNAs, effectively preventing translation of the complementary mRNA sequence into proteins. Thus, siRNA-mediated RNAi silences the ability of the gene to express proteins.

Another nucleic acid sequence that is able to induce RNAi is microRNA (miRNA), as shown in Figure 2. There are three major differences between siRNA and miRNA. First is that miRNA may be synthesized artificially or it may be endogenously generated from the cell's nucleus as a hairpin structure that loops on itself to form a double-stranded sequence (pre-miRNA). When exported out of the nucleus to the cytoplasm, endogenous miRNAs are cleaved by the dicer to remove the hairpin loop. Second, miRNA is not able to completely bind to the complement mRNA sequence due to its non-linear structure; instead, only 2–7 of the nucleotide sequences at the 5’ end (called the “seed sequence”) of the miRNA bind to the mRNA\(^{[18]}\). This short binding region results in weaker attachment to the mRNA, which leads to lower specificity, but to a consequently wider range of mRNA targets that can be silenced. Thus, whereas siRNAs silence a specific target sequence, miRNAs may regulate a family of gene expressions. Lastly, there are two classes of miRNA; one that undergoes the same RNAi pathway as siRNAs, and another that undergoes an alternative pathway that simply binds to the complementary mRNA sequence to block its translation into protein\(^{[21]}\).

Overall, RNAi is an innate cellular machine that allows one to silence the expression of essentially any target gene simply by switching the sequence; this gives it the potential to attack almost any disease. However, for therapeutic applications RNAi is only feasible with a supportive technology to deliver the oligonucleotides to the site of action: the cell cytoplasm.

2. Limitations and Material Design Requirements in RNAi Therapy

Whereas there is a wide range of gene modification tools available (Table 1), there is an unmet need for platforms that can effectively and selectively deliver them to targeted cells in vivo. A successful system must overcome substantial extracellular and intracellular barriers to RNAi delivery. The design requirements can be broken into three components: 1) protection of the siRNA payload; 2) targeting this payload to a specific subset of cells; and 3) effective release of siRNA into the cytoplasm.
Table 1. Summary of gene modification tools that can be considered as potential payloads for delivery vehicles. The tool is defined, the year it was discovered or first applied for gene editing is listed, and the mechanism by which it edits gene expression is described.

| Payload | Definition | Year | Mechanism of action |
|---------|------------|------|---------------------|
| DNA/RNA |            |      |                     |
| cDNA    | Complementary DNA; single-stranded DNA that is reverse-transcribed from mRNA (found naturally in retroviruses). | 1970 | cDNA is inserted into cyclic vectors that can self-replicate and potentially recombine into host DNA genome. Vectors with promoters drive transcription of the cDNA into mRNA for gene expression. |
| rDNA    | Recombinant DNA; combination of more than one DNA sequence from plasmids of one or more species. | 1972 | When transfected in a plasmid, it can recombine into host DNA genome. Plasmids with promoters drive transcription of the cDNA into mRNA. |
| mRNA    | Messenger RNA; single-stranded sequence transcribed DNA for protein translation. | 1978 | In the cytoplasm, the mRNA is translated into proteins for expression. |
| siRNA   | Short interfering RNA; 21–25 bp sequence of double-stranded RNA (either artificially synthesized, or endogenous products dsRNA cleavage) that undergo RNAi. | 2000 | siRNA is bound by RISC and unwound into single-strands that bind to the complementary mRNA sequence. The pairing leads to cleavage at target sequence, and the cleaved mRNA is degraded and prevented from translation. |
| miRNA   | MicroRNA; endogenous ≈22 bp-long hairpin structure of non-coding sequence that regulates gene expression through RNAi. | 1993 | miRNA is cleaved by the dicer into a dsRNA (undergoing the same pathway thereof as siRNA). Alternatively, miRNAs bind to the mRNA and inhibit protein translation. Overall, miRNAs target wider range of mRNA targets than siRNA. |
| ASO     | Anti-sense oligonucleotide; short single-stranded RNA complement to target mRNA. | 1967 | Binds to mRNA to inhibit protein translation, or to induce exon skipping. |

Restriction enzymes

| ZFN     | Zinc Finger Nuclease; artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. | 1994 | DNA-binding domain is modified to a selective locus on the DNA sequence, and the DNA-cleavage domain cuts DNA to induce either: (1) gene knockout by NHEJ; or (2) gene insertion by HR when co-delivered with a DNA fragment. |
| TALEN   | Transcription activator-like effector nuclease; restriction enzyme generated by fusing a TAL effector DNA-binding domain to a DNA-cleavage domain. | 2010 | DNA-binding domain is modified to a selective locus on the DNA sequence, and the DNA-cleavage domain cuts DNA to induce either: (1) gene knockout by NHEJ; or (2) gene insertion by HR when co-delivered with a DNA fragment. |
| CRISPR/Cas9 | CRISPR is repeating DNA sequences in prokaryotic genome for recognizing DNA fragments; Cas9 is an enzyme that cleaves DNA that complement the CRISPR sequence. | 2011 | Guide RNA (gRNA) is modified to a selective locus on the DNA sequence, and the Cas9 may cut the DNA to induce either: (1) gene knockout by NHEJ; or (2) gene insertion by HR when co-delivered with a DNA fragment. |

Figure 1. RNA interference mediated by siRNA. 1) siRNA is either delivered exogenously or generated endogenously by the dicer (blue) of the RISC loading complex; 2) siRNA is unwound to a single-stranded form by the Argonaut 2 (AGO2; orange); 3) single-stranded siRNA activates the RISC into forming base-pairs between the siRNA and its complementary mRNA sequence; 4) the RISC-siRNA complex dissociates from the mRNA after cleavage of the complementary mRNA; 5) the cleaved mRNA sequence is fragmented for degradation. With degradation of the target mRNA sequence, the cell is unable to translate the sequence into protein synthesis.
2.1. Extracellular Barriers

The RNAi therapeutic must traffic from the site of administration to the target cell it needs to modify, and this is a particular challenge when local administration is not available. On its way to the targeted cell, an siRNA therapeutic must overcome many obstacles; primary among these are: a) degradation by nucleolytic enzymes; b) uptake by cells of the immune system; and c) inefficient tissue penetration (Figure 3). As a consequence of its strong anionic charge, its impermeability to cells, and its (often) sequence-dependent toxicity, free siRNA is typically degraded and cleared from circulation with a plasma half-life of a mere 10 min.[18,19,22–27]

Serum endonucleases are largely responsible for the degradation of siRNA post-administration (Figure 3a). In particular,
ribonucleases bind specifically to RNAs in blood circulation and generate fragments for degradation. The enzymes attack the unstable ends of the siRNA to begin separation of the strands and degradation of the RNA fragments; many of the early RNAi studies implemented various chemical strategies to modify the ends of siRNA (i.e., by hairpin formation, sequence modification, or locked nucleic acids (LNAs)) as a protection strategy. There are also numerous chemically modified and stabilized siRNAs—for example, the N-acetylgalactosamine (GalNAc)-conjugated siRNAs that have been used for targeting the asialoglycoprotein receptor (ASGPR) in liver hepatocytes, as a potential alternative treatment for diseases requiring liver transplantation. Because even chemically modified siRNA has the potential for degradation or other complications, researchers have resorted to the use of nanocarriers to temporarily encapsulate the oligonucleotide therapeutics, thereby limiting access of enzymes and other potential inhibitors.

As shown in Figure 3b, the mononuclear phagocytic system (MPS; also known as reticuloendothelial system (RES)) plays a strong role in clearance of siRNAs from blood circulation, especially when a nanocarrier is used to protect the oligonucleotide. While siRNAs and other small molecules (with sizes <8 nm) are removed via renal clearance, larger nano-scale objects are readily identified by the body's immune system to be phagocytosed by macrophages in the major RES organs, such as the liver, spleen, and lungs. The primary mechanism by which the immune cells recognize and clear these objects is by opsonization, where molecules that recognize foreign body (opsonins; e.g., antibodies) bind to the surface of the target to label them for phagocytic clearance by the complement immune response. The effect may be diminished by adjusting the particle properties, such as size and shape, surface chemistry (e.g., PEGylation), and surface charge (decreased clearance is observed in positive compared to neutral and negative surface charges). While this phenomenon can be used for passive homing when the target disease is localized in the RES organs, it remains a major barrier not only in siRNA delivery, but also in other nanodelivery systems.

Lastly, tissue penetration to transport the siRNA from the lumen of the blood vessel into the target tissue is required if the target cell for gene silencing is in tissues far removed from the vascular walls (Figure 3c). The strongly anionic nature of siRNA repels it from the cellular membrane, which is also negatively charged. Thus, oligonucleotides have difficulty undergoing extravasation, and they avoid clearance by diffusing either through (transcellular diffusion) or between (paracellular diffusion) endothelial cells; these diffusion pathways are of critical importance when targeting the brain through the blood–brain barrier (BBB). This obstacle may be overcome when a nanocarrier is used to deliver siRNA, but as the pores in the vascular capillaries are in the 60–80 nm diameter range, larger carriers can require additional features in order to penetrate tissues.

2.2. Intracellular Barriers

Once the siRNA reaches its target cell, it must get into the cell to perform its gene silencing task. As aforementioned, the siRNA must localize in the cytoplasm, particularly in the perinuclear region. However, due to the anionic charge, siRNA is generally cell impermeable in its native state. While a number of proteins have been reported as oligonucleotide-specific receptors or transporters in cells, none have been fully validated as of yet.

Nano-carriers that are employed to overcome this obstacle must be able to induce cytosolic release of their siRNA payload. A common means of entering the cell is through endocytosis. The endocytic pathways are mediated by different proteins and are often differentiated as either dynamin-dependent (e.g., caveolin, clathrin) or dynamin-independent (e.g., macrophage, CLIC) routes. However, cellular uptake by way of endocytosis entraps the siRNA-loaded carriers within endosomal vesicles that steadily progress from early (pH 6–6.5) to late (pH 5–5.5) endosomes, and finally to lysosomes (pH 4.5–5).

As a primary nano-carrier used for siRNA delivery, lipid nanoparticles (to be discussed in the following section) are one of the only systems to have been rigorously studied for quantification and mechanism of cytosolic siRNA delivery. Studies investigating the primary uptake pathway of lipid nanoparticles and the intracellular fate of their siRNA payloads demonstrated that approximately 70% of the siRNA molecules that initially enter the cell are excreted within the first 24 h (Figure 4a), and that only 1–2% of the total siRNA is able to escape from the early endosomes into the cytoplasm to undergo RNAi (Figure 4c). These findings suggest that a great majority of the delivered siRNA undergoes exocytosis and lysosomal degradation (Figure 4b) if it is unable to localize in the cytoplasm by the early endosome stage. While there are numerous nanosystems in addition to lipid nanoparticles that have been used to enhance siRNA delivery across a wide range of cell types, and many report significant RNAi-induced gene silencing, there is very little information available on the efficiency of cytosolic delivery of siRNA. So it is unclear how limiting the endocytosis pathway is for siRNA delivery in general. Nevertheless, overcoming endocytosis by way of escaping the endosome (e.g., proton sponge), or bypassing endocytosis (e.g., via fusion, membrane penetration) is a critical consideration in the particle design is expected to greatly improve RNAi efficiency.

3. Protective Carriers for siRNA Delivery

As opposed to restriction enzyme-based tools that require the delivery of multiple payloads (e.g., enzyme, guide nucleotide sequence, and DNA fragments for gene insertion), RNAi therapeutics present a simpler and more feasible payload for genetic modification, as they are singular anionic oligonucleotides. There has been a robust effort to develop delivery vehicles for RNA-based therapeutics to diminish their vulnerability to degradation and clearance. Delivery vehicles can offer a dual function, both in protecting the oligonucleotide from the extracellular barriers leading to clearance, and in overcoming the intracellular barriers that limit cytosolic delivery of siRNAs. While viruses serve as one of the more common transfectants (albeit more so for DNA vectors), they carry concerns of immunogenicity and cytotoxicity. Focusing on non-viral nanoparticle-based carrier systems for siRNA delivery, Figure 5 shows a generalized schematic of each carrier type to be discussed, with representative electron micrograph images.
Table 2 and Figure 6 compile materials used in the last decade for in vitro and in vivo siRNA delivery, and their knockdown (KD) efficiency in selected in vitro and in vivo models. Of note, we have listed only those publications that reported the in vivo KD efficiency via quantitative measurements (e.g., qRT-PCR), rather than qualitative or indirect observation (e.g., Western blot, therapeutic outcome). The average gene silencing effect seen across the literature seems to be relatively similar regardless of material type; with in vitro rates ranging from approximately 60–80%, and in vivo rates ranging from approximately 55–80%. As might be expected, the in vitro effects are greater than in vivo effects overall, and lipid-based systems performed consistently strongly both in vitro and in vivo.

### 3.1. Lipid-Based Nanoparticles

Nanoparticles made of lipids represent the most widely used material for gene delivery.[22,29,34,46] Within the umbrella of lipid nano-carriers, there are micelles (small unilamellar vesicles with a hydrophobic core and a hydrophilic exterior), solid lipid nanoparticles (SLNs; micellar vesicles with a hydrophobic solid lipid core that prevents lipid permeation and degradation[100]), and liposomes (large vesicles with a lipid bilayer that forms a hydrophilic core and exterior[46]) (Figure 5a). The primary advantage of these lipid constructs is the simplicity in synthesis and versatility of particle function that can be achieved by varying the lipid composition. However, they suffer from relatively low loading capacity for siRNA and premature leakage of payloads, and they require additional cationic materials to condense the highly anionic payloads into the liposomal core. Commonly used condensers include protamine, peptides, and polyethylenimine (PEI).[70,103–105]

As can be seen in Table 2 and Figure 6, lipid-based systems tend to report relatively strong in vitro and in vivo gene silencing effects, with an average knockdown efficiency of 80.4 ± 13.8% in vitro and 79.3 ± 15.2% in vivo. These numbers are representative of a wide range of lipid particles, including liposomes, SLNs, and peptidomimetic lipoplexes, which have been documented in numerous reviews over the years.[18,22,24,27,29,45,46,55,102,106,107]

Among those considered here, a promising system is the stable nucleic acid lipid particle (SNALP; now simply called the lipid nanoparticle (LNP)), which is a formulation that was optimized specifically for siRNA delivery by a thorough library screening of natural and artificial lipidoid materials.[73,108,109] The lipid shell is composed of a mix of natural and artificially synthesized materials—DLinDMA, DSPC (1,2-distearoyl-sn-glycero-3-phosphocoline), cholesterol, and PEG-c-DMA (3-N-[ω-methoxypoly(ethylene glycol)]aminoCarboxymethyl)dimethylamino-propylamine). The ionizable DLinDMA lipid comprises 40% of the molar ratio, and provides an essential cationic surface charge that helps attract the particle toward the cell membrane to better facilitate uptake. The DSPC is a minor component at 10% molar ratio of incorporation, but it plays a key role in stabilizing the structure during synthesis...
and in circulation. Cholesterol (typically 40% of the molar ratio), plays a stabilizing function in many liposomal formulations; by incorporating itself into the lipid bilayer to decrease membrane fluidity and permeability, cholesterol helps reduce premature payload leakage from the liposomes.[110] Last, the PEG-c-DMA lipid displays an interesting property that is not commonly observed in other PEGylated systems. While PEG is known to provide particle stability, hydrophilicity, and stealth properties, the PEG-c-DMA has an activating component, such that the molecule dissociates from the complete liposome soon after it is introduced to the body via intravenous injection. Once the PEG-c-DMA leaves, the particle returns to its transfection-optimal cationic charge and is able to transfet cells efficiently.[72,108] LNP formulations have been reported to load as much as 5 wt% siRNA,[72] and to silence genes in a dose-dependent manner in animals ranging from mice to non-human primates.[72,73,108,109] Indeed, LNP formulations have completed multiple clinical trials for delivery of siRNA, such as those against PLK-1 (TKM-080301) for the treatment of neuroendocrine tumors (NET), adrenocortical carcinoma (ACC), and primary/secondary liver cancer.[111] With the substantial achievements in siRNA loading, lipid design, and lipid composition, lipid-based nanoparticles have enjoyed the greatest success in translation from benchtop development to clinical trials, which led to the first FDA-approved siRNA-based LNP therapeutic, Patisiran.[72,112–114]

3.2. Polymeric Nanoparticles

After lipids, polymers represent the second most widely used material type for siRNA delivery. Based on published data accumulated over the past decade, average gene silencing
Table 2. Summary of technologies used for RNAi. Representative publications are listed by the material type (Lipid, Polymer, Metal, Mesoporous Silica, and Porous Silicon), cell targeting moiety (Local: local administration; RES: uptake into clearance organs), strategy to overcome endocytosis, name of the gene silenced, the in vivo model used, and knockdown (KD) values (% relative mRNA expression compared to appropriate controls) as quantified and reported in the indicated references. NP stands for nanoparticle, NR stands for nanorod.

| Delivery vehicle | Cell targeting | Endosome strategy | Target gene | Disease model | In vitro KD | In vivo KD | Ref. |
|------------------|----------------|-------------------|-------------|---------------|-------------|-----------|------|
| **Lipid**        |                |                   |             |               |             |           |      |
| Liposome         | Local          | —                 | Luc2p       | Healthy       | —           | 77%       | [66] |
| Liposome         | RES            | —                 | Ssb         | Healthy       | —           | 87%       | [67] |
| Liposome         | Hyaluronic acid| —                 | MMP2        | Healthy       | 73.4%       | 54.8%     | [68] |
| Lipoplex         | RES            | Endosome escape (DoGo3) | LxRalph | Healthy       | 75%         | 90%       | [69] |
| Lipoplex         | RES            | —                 | GAPDH       | Healthy       | 90%         | 90%       | [70] |
| SNALP/LNP        | —              | —                 | Antitransthyretin | Transthyretin amyloidosis | —           | 86.8%     | [71] |
| SNALP/LNP        | —              | —                 | TTR         | Healthy       | —           | 80%       | [72] |
| SNALP/LNP        | —              | —                 | PLK-1       | Hepatic Neuro2a xenograft | —           | 58%       | [73] |
| **Polymer**      |                |                   |             |               |             |           |      |
| PEG NPs          | —              | —                 | Bcl2        | SKOV3 xenograft | 43%         | 62%       | [74] |
| PEG-PPTMA-P(CMA-S-DMA) | — | Endosome escape (Proton sponge) | ApoB | Healthy       | 95%         | 63-80%    | [75] |
| Poly(amine-co-ester) NPs | RES | — | Nogo-B | Healthy | 80%         | 60%       | [76] |
| Chitosan NP      | —              | —                 | GAPDH       | Healthy       | 75%         | 50%       | [77] |
| Chitosan NP      | RGD peptide    | —                 | POSTN       | A2780 orthotopic | —           | 80%       | [78] |
| Cyclodextrin NP  | hTrigand       | —                 | RRM2        | Human tumor   | —           | 32-77%    | [79] |
| Alkyl-PEI:IO NP  | Local          | Endosome escape (Proton sponge) | Luc | 4T1-flu xenograft | 80%         | 30%       | [80] |
| PEI-PEG lipid NP | —              | Endosome escape (Proton sponge) | ICAM-2 | Emphysema, lung primary, metastasis | 85%         | 50-92%    | [81] |
| **Mesoporous Silica (MSN)** | | | | | | | |
| PEI-MSN          | —              | —                 | Pgp         | MDR          | —           | 75%       | [82] |
| Magnetic PEI-MSN | —              | —                 | VEGF        | SKOV3 orthotopic | 80%         | 60%       | [83] |
| PEI-MSN-KALA     | —              | —                 | VEGF        | A549 xenograft | 80%         | 48%       | [84] |
| PEI-MSN          | —              | —                 | TWIST1      | MDA-MB-231 (Luc) | 86%         | 77%       | [85] |
| PEI-MSN-PEG      | Trastuzumab    | Endosome escape (Proton sponge) | PLK-1 | LM2-/H2N metastasis | 87%         | 84%       | [86] |
| PEI-MSN-PEG      | Trastuzumab    | Endosome escape (Proton sponge) | HSP47; NOX4 | HER2-positive breast cancer | 95%         | 55%       | [87] |
| PEI-MSN-PEG      | Trastuzumab or Rituximab | Endosome escape (Proton sponge) | HER2 | HCC1954 orthotopic | 87%         | 58.6%     | [88] |
| **Porous Silicon (pSi)** | | | | | | | |
| Fusogenic lipid-coated pSiNP | CRP peptide | Membrane fusion | IRF3 | S. aureus pneumonia | 96%         | 83%       | [98] |
efficiencies of 64.6 ± 24.7% in vitro and 61.5 ± 19.6% in vivo have been achieved (Figure 6 and Table 2). The three most frequently employed types of polymer nanoparticles for siRNA delivery have been solid polymeric nanoparticles, dendrimers, and hydrogels,[22,24,36,112] but across the wide range of structural designs in polymeric delivery systems, there are a number of proven components that are regularly used: poly(lactic-co-glycolic acid) (PLGA), poly-L-lysine (PLL), chitosan, and polyethyleneimine (PEI).[23,41,74,77,85,115–118] PLGA, PLL, and chitosan generally play a structural role in forming the primary skeleton for siRNA loading. PLGA nanoparticles have high stability and biocompatibility, and the PLGA polymer is an FDA-approved material. However, PLGA nanoparticles require additional cationic condensers for effective siRNA loading, such as PEI.[106,115,119] On the other hand, PLL is a polycation, and so it can form complexes with siRNA without the need for an additional condenser. PLL’s primary advantage is in its relatively high biocompatibility compared to the fairly toxic PEI, but PLL systems suffer from diminished transfection efficiency in the presence of high serum content; in an environment that is clinically relevant and reflective of our vasculature (which has high serum content), the PLL systems have difficulty forming stable structures due to competition with serum proteins (which are generally anionic) for binding to the siRNA payloads.[116,120] Thus, recent efforts have focused on developing PLL derivatives and co-polymers to reinforce their vulnerability to high serum content environments.[85,116,120–122] Lastly, chitosan is a cationic polysaccharide, which offers high biocompatibility and many amine and hydroxyl groups available for chemical modification. However its low in vivo solubility is a critical drawback; PEGylation or hyaluronan-conjugation of the particles have been reported to increase solubility, as well as to offer a specific and controllable site for further surface modifications (e.g., attachment of targeting moieties), but much work remains to develop a platform with reliable and consistent performance.[78,116,123]

As a condenser, PEI is the most widely used polymer. It is used in numerous all-polymer systems as well as in hybrid systems (containing lipids, metals, silica/silicon, etc.) that require a cationic component for stable siRNA loading. A key design factor in PEI-based nanoparticles for siRNA delivery is the N/P ratio, which is the molar ratio between the nitrogen in the PEI to the phosphorus in the siRNA. An N/P ratio greater than 10 is recommended, as it accommodates stronger siRNA integration that leads to decreased payload leakage and degradation. However, while stronger and greater siRNA binding to the polymer mitigates its excessively cationic charge, the increased PEI content inevitably leads to rapid RES-mediated clearance and high cytotoxicity.[37,124] On the other hand, an N/P ratio less than 10 results in more anionic particles that have difficulty loading siRNAs and being taken up by target cells.[124] Thus, there is growing trend in developing novel polymeric nanoparticles that use non-PEI condensers to enable biosafe and effective siRNA loading and delivery.[74,75,82]

3.3. Metallic Nanoparticles

Metallic vehicles (primarily gold or iron oxide nanoparticles) for siRNA tend to underperform in vivo based on measurements of gene silencing efficiency, with average efficiencies of 72.3 ± 16.3% in vitro and 54.7 ± 19.6% in vivo (Table 2 and Figure 6). This trend may be attributed to the solid structure of the constructs, which leads to formulations in which the siRNA payloads are covalently bound or chemically adhered to the surface of the particles (e.g., thiol-gold chemistry).[128,43,81,85,87,125] This surface-loading mechanism leaves the siRNA molecules exposed to the degrading conditions of the in vivo environment during circulation, which underscores the necessity for an siRNA-protective design; thus, metallic systems generally employ cationic polymer/lipid

Table 2. Continued.

| Delivery vehicle | Cell targeting | Endosome strategy | Target gene | Disease model | In vitro KD | In vivo KD | Ref. |
|------------------|----------------|-------------------|-------------|---------------|-------------|-----------|------|
| Fusogenic lipid-coated pSiNP | IRGCD peptide | Membrane fusion | REV3L | CAOV-3 IP xenograft | 95% | 76% | [99] |
| Fusogenic lipid-coated pSiNP | LyP-1 peptide | Membrane fusion | P13Kg | CAOV-3 IP xenograft | 85% | 81% | [99] |
| PEI-pSiNP | — | — | MRPI | U87 xenograft | 30% | 82% | [100] |
| Lipid-pSiNP | — | — | EphA2 | HeyA8 IP orthotopic | — | 80% | [101] |

Figure 6. Comparison of reported in vitro and in vivo knockdown (KD) efficiencies across the literature, sorted by nanomaterial type. The data are representative of results presented in 111 publications from 2008–2019 that quantitatively reported the gene silencing effect by their siRNA-loaded nano-carrier in the selected cell line and in vivo model. Bars represent standard deviation. It should be noted that the relative siRNA dose, potential cytotoxic effects, targeting efficiency, and therapeutic outcome are not represented nor normalized in the figure. The table details the average values and standard deviation.
coatings to facilitate loading and protection of the siRNA payload.\textsuperscript{[38,80,81,85,87,90,113]}

Nonetheless, metallic particles have advantages over other organic material-based systems in their longer shelf life, their facile surface chemistry, their higher physical stability (to temperature or pH excursions), their precisely tunable physical properties, and their potential for multi-modality (e.g., photothermal therapy, MRI imaging).\textsuperscript{[126,127]} The surface chemistry of most metallic systems is amenable to attachment of a wide variety of targeting moieties or components that can facilitate endosomal escape (Table 2).\textsuperscript{[81–87,128,129]} In particular, gold-based systems have demonstrated the ability to induce controlled release of siRNAs via light-activation,\textsuperscript{[110] as well as providing a photothermal therapeutic effect as a secondary function.\textsuperscript{[129,131]} Iron oxide-based systems have also demonstrated unique characteristics, such as magnetofection,\textsuperscript{[132]} magnet-guided particle accumulation and release,\textsuperscript{[133,134]} and MRI-based particle tracking and tumor imaging.\textsuperscript{[80,128,135,136]}

While metallic nanoparticles show an average in vivo gene silencing effect that is relatively low compared to other materials, they offer innate multi-modality that other systems lack. In particular, the ability to directly image and track the siRNA carriers can provide important pharmacokinetic and pharmacodynamic information (e.g., bioavailability and biodistribution of the nanoparticle) in RNAi therapeutics;\textsuperscript{[89,127,128,134,135]} many studies use indirect fluorescent dye-tracking methods, or simply analyze mRNA or protein level changes in the target site post-administration. In order to set itself apart from the organic materials that excel in effective siRNA delivery and transfection of genes, the metallic delivery systems could perhaps benefit most by focusing on their unique material properties for multimodal theranostic applications.

3.4. Mesoporous Silica Nanoparticles

Unlike the above systems, which are comprised of hollow spherical, core-shell, or solid or semi-solid structures, mesoporous silica nanoparticles (MSNs) consist of a network of pores within a silicon oxide framework. These materials display relatively large and tunable pores that are able to sequester payloads akin to a rigid sponge. The porous structure affords a much greater surface area for binding compared to other systems, and it is thus able to load large quantities of siRNA.\textsuperscript{[127]} However, the native silicon oxide surface is anionic, necessitating cationic condensers. With the aid of cationic lipids and polymers to form hybrid structures, MSNs have reported average gene silencing efficiencies of 75.7 ± 19.4\% in vitro and 63.2 ± 17.0\% in vivo (Figure 6 and Table 2)—quite promising considering that these particles have not been studied for siRNA delivery nearly as thoroughly as lipid- and polymer-based vehicles.

A major drawback to MSN delivery systems is their questionable biosafety, where MSNs have shown dose-, size-, shape-, and administration-dependent toxicity in various drug delivery applications.\textsuperscript{[137]} This concern is particularly important for siRNA delivery, as the majority of MSN studies use the cytotoxic PEI polymer as a condenser.\textsuperscript{[93,97,138]} Moreover, most studies of PEI-MSN hybrid systems fail to demonstrate successful endosomal escape using the theorized proton sponge effect induced by the PEI, and most do not report rigorous dose-dependent toxicity studies; the general publication trend leans toward demonstration of a positive gene silencing effect and a lack of toxicity at the selected dose. In order to validate MSN-mediated RNAi therapeutics for clinical translation, more fundamental studies on the individual components of the hybrid system (the MSN, the condenser, and the siRNA) are needed. The oxide surface on MSNs provides entry into a wide pallette of surface chemistries that may offer a better alternative to PEI and other potentially toxic condensers for siRNA loading. While there is much room for further investigation, MSNs have so far demonstrated excellent in vivo gene silencing effects.

One of the more innovative MSN-based transfection agents that has been developed is the Protocell from the Brinker group.\textsuperscript{[139–142]} Protocells were first introduced in 2009 as MSNs that were encapsulated within cationic liposomes to enhance delivery of anionic and cell-impermeable fluorescent dye (calcine) as a model oligonucleotide.\textsuperscript{[140]} Then in 2011, the group demonstrated the ability of a Protocell to conjugate targeting peptide (SP94 peptide) and a endosomolytic peptide (H5WYG) to deliver a wide range of payloads (small molecule anticancer drugs, quantum dots, siRNAs, etc.) to target human hepatocellular carcinoma (HCC) cells.\textsuperscript{[139]} The particles demonstrated up to 90\% gene silencing efficiency in vitro.\textsuperscript{[142]} This hybrid organic/inorganic system provides a prime example of how hybrid nanoparticles can combine the advantages of organic (e.g., liposome’s biosafety and facile modification of properties by adjustment of lipid composition) and inorganic (e.g., ten-fold higher loading capacity and fivefold diminished payload leakage by loading siRNA within MSNs) components. The promising in vitro performance\textsuperscript{[141]} has yet to be tested in vivo.

3.5. Porous Silicon Nanoparticles

Porous silicon (pSi) is a relative newcomer in the field of RNAi. However, the few reports on in vivo siRNA delivery are extremely promising. With tunable pore sizes ranging from micropores (d < 2 nm) to meso- (d = 2–50 nm) and macropores (d > 50 nm), facile surface chemistry, and biosafe and biocompatible degradation, porous silicon boasts a relatively high loading capacity and compatibility with a diverse set of payloads. Moreover, when synthesized as a hybrid nanoparticle with lipids or polymers, pSi-based nanoparticles have demonstrated average gene silencing efficiencies of 75.6 ± 21.3\% in vitro and 80.4 ± 2.7\% in vivo (Figure 6 and Table 2) that rival the best liposomal systems.

While structurally similar to MSNs, porous silicon retains a crystalline silicon core in the porous skeleton. With dimensions on the order of 5 nm, the crystalline silicon domains in the skeleton function as quantum dots, producing an intrinsic photoluminescence.\textsuperscript{[143]} This unique property allows pSi materials to take advantage of both MSN’s large surface area and porous structure to attain high loading capacities, as well as the metallic system’s potential for multi-modal imaging.

Within the limited number of studies on pSi-based materials for siRNA delivery, there have been a variety of effective structures. The Ferrari group has primarily focused on the development of a multi-stage delivery system that consists of a larger
pSi microparticle loaded with liposomal siRNA. This yielded an extended gene silencing effect, showing in vivo knockdown efficiency as high as 80% for a period of 20 days after systemic delivery.\cite{101,144} The group of Stevens developed a related pSi system, but in the form of nanoneedles for transdermal delivery of siRNA. That work demonstrated >90% transfection efficiency in vitro, as well as a demonstrable phenotypic outcome (e.g., neovascularization) in vivo.\cite{145} At the smaller scale, the Voelcker group developed PEI-functionalized pSi nanoparticles for systemic administration, attaining 30–63% gene silencing effect in vitro and up to 82% in vivo.\cite{100,146} While the use of PEI raises the abovementioned concerns regarding dose-dependent cytotoxicity, silicon is amenable to other condenser chemistries. The common silica functionalization reagent (3-aminopropyl)-dimethylethoxysilane places a positively charged amine group on the surface of the inner pore walls, and this combined with a graphene oxide shell allowed entrapment of siRNA in pSi nanoparticles that induced 65% gene silencing effect in vitro. By targeting the compromised blood-brain barrier (BBB) in a mouse brain injury model and through the agency of the RVG (rabies virus glycoprotein) peptide, silicon is amenable to other condenser chemistries. The common silica functionalization reagent (3-aminopropyl)-dimethylethoxysilane places a positively charged amine group on the surface of the inner pore walls, and this combined with a graphene oxide shell allowed entrapment of siRNA in pSi nanoparticles that induced 65% gene silencing effect in vitro. By targeting the compromised blood-brain barrier (BBB) in a mouse brain injury model and through the agency of the RVG (rabies virus glycoprotein) peptide, this system was able to achieve enhanced siRNA-delivery efficiency in vivo.\cite{145} A similar structure, lacking the graphene oxide shell and conjugated with the CAQK brain injury targeting peptide attained 70% gene silencing effect in vitro.\cite{148} An alternative condenser involving calcium chloride-mediated formation of a calcium silicate binder with the pSi nanoparticles has also been developed. This method successfully trapped siRNA and was able to attain a modest 52.8% gene silencing efficiency in vitro.\cite{25}

A most recent development for pSi-based siRNA therapeutics is fusogenic lipid-coated pSi nanoparticles (FNPs; Figure 7).\cite{98,99,149} Using the above calcium silicate condenser chemistry,\cite{25} the FNPs were able to obtain loading capacity up to 25 wt% of siRNA, a significantly higher loading capacity compared with other materials of similar size and structure.\cite{25,98} Like Brinker’s MSN-based Protocell, the inorganic pSi core prevents premature leakage of siRNA payloads from the liposomal shell, and inversely, the liposomal shell is able to protect the pSi core from dissolution.\cite{99} Currently, the FNP system represents the most thoroughly investigated pSi-based siRNA delivery technology, as its material properties, biological mechanism of action, and its in vitro and in vivo behavior and therapeutic effects have been delineated.\cite{98,99,149} Overall, the FNPs are able to undergo a plasma membrane fusion to directly deposit the siRNA-loaded pSi core particles in the cytoplasm, where the pSi quickly degrades to release the siRNA for RNAi. Moreover, the FNPs have demonstrated strong selective homing when decorated with targeting peptides, while retaining their fusogenic uptake characteristic. These factors led to 85–96% in vitro gene silencing efficiencies across different cell lines (macrophages and tumor cells), as well as 76–83% in vivo gene silencing efficiencies post-intravenous administration. Moreover, FNPs represent a modular therapeutic platform; by exchanging the siRNA sequence and the homing peptide, the FNPs have been configured for three different disease targets (anti-inflammatory, chemo-sensitizing, and tumor-associated macrophage reprogramming). This system has demonstrated remarkable therapeutic effects in bacterial infection models (Gram positive \textit{Staphylococcus aureus}, Gram negative \textit{Pseudomonas aeruginosa}, and Methicillin-resistant \textit{S. aureus} (MRSA)), as well as in ovarian peritoneal carcinomatosis.\cite{98,99}

Figure 7. Fusogenic porous silicon nanoparticles (FNPs). a) Outline of the uptake mechanism for FNPs; b) transmission electron microscope (TEM) image of FNPs; scale bar represents 100 nm; c) in vivo gene silencing effect of FNPs delivering siRNA against Irf5 gene to activated macrophages using the macrophage-targeting CRV peptide (F-siIRF5-CRV), in a mouse model of \textit{S. aureus} pneumonia. Reproduced under the terms of the CC BY 4.0 license.\cite{98}
4. Selective Tissue Targeting

Realization of RNAi therapy requires the delivery vehicle to overcome the aforementioned extracellular barriers by either local administration or controlled homing to the target site. For tumor models, nanoparticles have traditionally relied on the enhanced permeation and retention (EPR) effect, where the porous tumor vasculature allows greater uptake and retention compared with normal tissues.\[150\] A recent review of the literature by Chan, which focused on the quantity of nanoparticles accumulated in solid tumors via the EPR effect, highlighted the very poor efficiency of this process; on average, a mere 0.7% of the injected dose of particles actually reaches the tumor.\[151\] Active targeting, via the agency of a pendant molecular species that has some affinity for the tumor, showed a minor improvement over EPR-mediated particle accumulation. However, in other (non-tumor) animal disease models, the use of selective homing agents can generate more substantial accumulation of siRNA.\[79,82,106,112,125,139,151–154\] For applications beyond local administration and passive MPS-mediated homing to clearance organs (e.g., liver, lungs, spleen), the literature contains a wide variety of homing moieties that can be decorated on the surface of carrier particles for in vivo targeting, including aptamers and receptor-specific ligands.\[155,158\] But currently peptides and antibodies have been the most widely used targeting agents.

4.1. Antibodies

With several FDA-approved entities already in clinical use, antibodies offer a reliable means of targeting cells.\[155,159,160\] Homing antibodies fall into two categories: 1) monoclonal antibodies (mAb); and 2) antibody fragments (or antigen-binding fragments, Fab). Monoclonal antibodies are those generated from a single cell lineage that bind to a single epitope on their target (as opposed to polyclonal antibodies that are generated by a range of B cells that bind to multiple epitopes on their target). The most commonly used mAb for targeted delivery of siRNA is Trastuzumab, an IgG1 mAb against human epidermal growth factor 2 (HER2) for selective targeting of breast cancer.\[95,97,161\] Another common antibody, useful for targeting the wider epidermal growth factor receptor (EGFR) family is Cetuximab.\[134,162,163\] A major concern in using mAbs with nanoparticles is their large size (>150 kDa), which limits the number of mAbs that can be placed on a single nanoparticle. The rationale here is that when multiple targeting groups are placed on a nanoparticle, the targeting efficiency is enhanced by a factor larger than the scalar number of targeting groups—so-called multivalency. Another issue with antibodies is that they contain the Fc domain, which can trigger cellular responses that may or may not be desirable—such as an antigenic reaction. The problem is mitigated somewhat when the Fab domain alone is used rather than the full antibody (which is composed of two Fab domains for binding to target receptors and one Fc domain for activity).\[160,163,164\]

Fragment antibodies make use of only the Fab binding motifs, making the molecule much smaller (approximately 50 kDa) and more economical to produce.\[155,164\] Moreover, the lack of the Fc domain reduces the chance that the targeting moiety will inadvertently cause adverse downstream activities (e.g., immunogenicity, cytotoxicity).\[24,155,160,164\] The argument has been made that the Fc domain is necessary in order to better stabilize the antibody against RES clearance and that, with their smaller size, Fabs are too quickly cleared from the body.\[164\] However, it appears that conjugation to nanoparticles can mitigate these effects for siRNA delivery. For example, studies have demonstrated effective siRNA-mediated gene silencing in vivo using Fab-conjugated nanoparticles.\[165\] Moreover, although Fab technology is relatively new compared to whole antibodies, three Fabs have already been approved for use by the FDA (Abciximab, Ranibizumab, Certolizumab pegol).\[166\]

4.2. Peptides

Targeting peptides are a sequence of linear or cyclic amino acids that are able to take advantage of specific binding sites on transmembrane receptors to selectively guide their carrier to the target cells. Specific peptide sequences may be identified through phage display either in vitro or in vivo, where populations of bacteriophages displaying different peptide sequences (that altogether form a library of peptides) undergo multiple rounds of binding assays to select for the sequence with the highest binding affinity.\[167–170\] While an in vitro phage screen can identify peptides that bind to a specifically chosen binding receptor, an in vivo phage screen isolates peptides that bind to the disease tissue of interest—even though the receptors or the cell types may be as yet unknown. A further advantage is that the peptides that survive an in vivo screen are more likely to be able to perform the same in vivo tissue targeting task when they are bound to nanoparticles other than a phage.\[148,167–172\] With an in vitro screen, the major question of whether or not the targeting peptide identified will survive all the varied clearance mechanisms of the in vivo environment remains unanswered.

Table 3 shows a representative list of peptides that have been used for targeting disease-specific cells or tissues, with emphasis on diseases that are currently of high interest (e.g., cancer, bacterial infection), or regions of the body known to be difficult to access (e.g., brain), and which might benefit from more effective RNAi therapeutic delivery. While most peptides bind to specific receptors on the cellular membrane and are known or expected to initiate receptor-mediated endocytosis, there are only few peptides where the true target and uptake mechanisms have been clearly demonstrated. Of particular significance are peptides classified as “CendR,” which stands for C-end-Rule, discovered in 2009 by the Ruoslahti group.\[168,173\] In general, these peptides display an R/KXXR/K sequence (e.g., iRGD and LyP-1 peptides; Table 3), with an essential C-terminal arginine (or occasionally lysine) motif that is exposed for activation by a primary receptor, which initiates a proteolytic cleavage of the peptide. The cleaved inner peptide sequence is then activated for a secondary binding to the neuropilin (NRP1/2) receptor to undergo cellular endocytosis. While many peptides carry the CendR motif (e.g., TAT cell penetrating peptide), the peptide homing must be primary receptor-specific, and cellular uptake must be activated by the initial docking; as opposed to cell-penetrating sequences that are active independent of binding and receptor recognition events.\[170,173\] The net result is a very selective peptide.
Table 3. Summary of effective targeting peptides used for selective homing to different cell targets. Name and amino acid sequence of the peptides are listed, and ordered by potential applications (cancer, bacterial infections, brain targeting, cell penetrating). Target cell types, the primary receptor, and mechanism of uptake (if known) are given.

| Peptide   | Target tissue          | Receptor | Mechanism                                                                                     | Refs.       |
|-----------|------------------------|----------|-----------------------------------------------------------------------------------------------|-------------|
| iRGD      | Tumor cells            | αvβ3     | CendR: RGD binds to αvβ3, cleavage at K, then RGD binds to NRP1, then receptor-mediated endocytosis/ transcytosis | [99,152,167,168,170,174] |
| CRGDKGPD  |                        |          |                                                                                               |             |
| CGKRK     | Tumor neovasculature   | p32      | Binds to p32, may then bind to NRP1, inducing cell penetrating properties that allow binding to mitochondria. | [153,169,175] |
| LyP-1     | Tumor/lymphatics;      | p32      | CendR: binds to p32, then KRT binds to NRP1 (and NRP2 in lymphatics), then receptor-mediated endocytosis/transcytosis | [99,168,176] |
| CGKRTGRC  | Tumor cells; TAMs      |          |                                                                                               |             |
| CRV       | Activated macrophages, | RXRB     | Unknown (not CendR, not receptor-mediated endocytosis)                                        | [98]        |
| CRVLRGSC  | TAMs                   |          |                                                                                               |             |
| CARG      | S. aureus, MRSA (+intracellular) | Unknown | Unknown                                                                                       | [171]       |
| EKR       | P. aeruginosa          | Unknown  | Unknown                                                                                       | [171]       |
| (KLAKKLAK)₂ | Bacteria, mitochondria | Membrane (receptor unknown) | Pro-apoptotic (usually need another targeting peptide); permeabilizes mitochondrial membrane, and activates caspases to induce cell death by apoptosis | [177]       |
| CAQK      | Brain injury           | Versican-(Hapln4)-tenascin-R complex | Perineuronal nets (PNN) complex in brain ECM is upregulated in brain injury. CAQK binds to PNNs. Exact uptake mechanism unknown | [148]       |
| CAQK      | Brain injury           |          |                                                                                               |             |
| RVG       | Neuronal cells         | Acetylcholine receptor | α7 subtype of nAChR-mediated transcytosis. GABA receptors, which are commonly found in neurons, glial cells, and brain capillary endothelial cells are also known to bind to RVG | [25,147,178] |
| YTIWPENP  | Neuronal cells         |          |                                                                                               |             |
| PRPGTPCD  | Neuronal cells         |          |                                                                                               |             |
| IFITNSRGK | Neuronal cells         |          |                                                                                               |             |
| KRASNG    | Neuronal cells         |          |                                                                                               |             |
| TAT       | Plasma membrane        | Heparan sulfate | Varying; different types of endocytic pathways are involved in the uptake mechanism of TAT alone and when conjugated with the cargo molecules | [179,180] |
| GRKKRRQRR  | Plasma membrane        | Proteo-glycans |                                                                                               |             |
| PQQ       |                        |          |                                                                                               |             |

With regard to in vivo siRNA delivery, the fusogenic porous silicon nanoparticles (FNPs) demonstrated effective use of targeting peptides to induce siRNA-mediated gene silencing in multiple cell targets within a single mouse model. [99] Figure 8 shows a significant example of the versatility of the approach, where three different targeting peptides were used to bring FNPs to their three respective targets with high selectivity. First, in a FNP platform decorated with the CRV peptide, the particles were selectively taken up by the activated macrophages in an infection site (Figure 8a). Second, FNPs decorated with the LyP-1 peptide selectively homed to tumor-associated macrophages (TAMs) in an ovarian peritoneal carcinomatosis model (Figure 8b) to induce knockdown only in the TAMs. Third, FNPs with iRGD peptide homed to ovarian cancer cells in the same tumor model (Figure 8c) and induced a significant gene silencing in those cells. All three formulations resulted in a significant therapeutic outcome in diseased mice. [98,99]

5. Strategies against Endocytosis

Whereas most drug delivery systems could benefit from payload protection and selective tissue targeting, intracellular drug release may not be necessary. However, cytosolic delivery is critical for RNAi therapeutics. Thus, approaches that either avoid endocytosis or escape from endocytic vesicles are essential to activating the RNAi machinery for gene silencing. Natural viral or bacterial transduction mechanisms have built-in endosomal escape pathways (e.g., membrane fusion, pore formation), which have inspired development of artificial systems that can avoid or escape the endosome. [181,182] Table 4 lists some techniques that have been used to escape or bypass endocytosis in nanoparticle-mediated siRNA delivery.

5.1. Endosomal Escape

One method to induce cytosolic localization of siRNA is to escape the endosome before degradation or excretion (Figure 4c). The most utilized method to instigate endosomal escape is via a mechanism called the “proton sponge” hypothesis. [73,188,189] As the early endosomes fuse with late endosomes and lysosomes, the intravesicular pH decreases to initiate degradation of the contents. But if an endosmolytic molecule (e.g., histidine-rich molecule, poly(amido amine) polymers) [65,181] is trapped within, it will buffer the pH of the vesicles and allow a continuous influx of H⁺, Cl⁻, and water molecules that increase the osmotic pressure. Eventually, the vesicle swells and...
ruptures to release its entrapped payload.\textsuperscript{[45,48,118,181]} For inorganic and polymeric nanoparticles, polyethyleneimine (PEI) is the most commonly used molecule, with its abundance of nitrogen groups available to buffer the pH and sponge up protons.\textsuperscript{[80,83,93,115,190]} While the proton sponge mechanism of PEI is still under debate,\textsuperscript{[189,190]} its endosomal escape phenomenon has been replicated numerous times, and has been extensively used to demonstrate effective in vivo gene silencing effect in numerous nanosystems, as shown in Table 2. More recently, alternative models to explain polyplex-mediated endosomal membrane disruption have been proposed and are under investigation.\textsuperscript{[191]}

Endosomal escape may also occur as a result of membrane fusion and pore formation. Herpes simplex virus innately carries glycoprotein H, which induces fusion in a pH-sensitive manner to help its virus escape lysosomal degradation. One notable study has used the peptide analogue of the fusogenic domain of glycoprotein H for in vitro transfection and

Table 4. Summary of techniques used for endosomal escape or bypass in siRNA delivery. Technique indicates the possible routes of endosomal escape or bypass by siRNA-delivery systems that have been presented in the literature. Example indicates molecules or structures that have been used to induce each technique. The hypothesized mechanism for each route of endosomal escape or bypass is given.

| Technique               | Example              | Mechanism                                                                 | Refs.                  |
|-------------------------|----------------------|---------------------------------------------------------------------------|------------------------|
| Proton sponge           | PEI, Poly(L-histidine), Chloroquine, PAsp(DET-CDM/DBCO) | Endosmology of vesicle; buffering molecule prevents pH drop in late endosome/lysosomes, and induces continuous influx of ions and water that eventually swells the vesicle for rupture. | [115,116,121,183] |
| Pore formation          | KALA peptide, GALA peptide | Peptides engineered from innate bacterial pore-forming mechanisms or derived from fusion domains of viral proteins; peptides can embed into the cell membrane to create gaps in a pH-sensitive manner by causing membrane re-orientation to a more energetically favorable pore-form | [92,93,181,184,185] |
| Membrane fusion         | DOPE:DOTMA, DMPC:DOTAP:DSPE-PEG | Fusogenic liposomes are composition-dependent, and undergo plasma membrane fusion to directly deposit its payload into the cytoplasm. | [98,99,149,186] |
| Cell penetration         | TAT peptide, Penetratin peptide, Pep14 peptide, P1 peptide | Theorized to diffuse through the plasma membrane either with or without receptor binding. Different uptakes are shown by cell type and carrier type. | [46,179–181,187] |
demonstrated membrane fusion and enhanced transfection efficiency.[192] Two other fusion peptides were derived from the haemagglutinin subunit HA-2 of the influenza virus, called KALA and GALA.[92,93,181,184,185] Both KALA and GALA peptides behave in a pH-sensitive manner, the peptides undergo a conformational change after embedding into the membrane of acidic endosomal or lysosomal vesicles, and form a helical channel through which the payloads may escape.[181,185] Table 2 shows two mesoporous silica-based systems that employed KALA by the Gu group to attain gene silencing efficiencies of 80% in vitro and approximately 50–60% in vivo.[92,93] While not as widely used as the PEI and other polycations, these peptides offer a promising and potentially safer method to induce endosomal escape.

5.2. Endosome Bypass

There are several reported methods to bypass endocytosis, such as receptor-mediated membrane penetration (e.g., scavenger receptors)[193], direct membrane penetration, and membrane fusogenic uptake.

Cell penetrating peptides (CPPs) are employed for diffusive uptake into the cell through the plasma membrane and directly into the cytoplasm. Although the exact uptake mechanism is highly debated, TAT, penetratin, and other CPPs have demonstrated successful cytosolic delivery of their carriers.[22,46,47,188,194] While some penetrating peptides are observed to undergo endocytosis with translocation afterward to transit to the cytoplasm, penetratin has been observed to cytoplasmically localize in cells even at 4 °C, by electrostatically penetrating through anionic phospholipids without an external driving force (e.g., pH-sensitivity, osmotic gradient).[187,195] On the other hand, the efficient penetrative activity of CPPs has led to reports of cell line-, cargo-, peptide size-, and dose-dependent cytotoxicity.[198] At biosafe doses, these peptides may provide a synergistic effect when combined with tissue-targeting peptides to help guide the nano-carriers to selective cells and allow them to efficiently activate RNAi in the cell cytoplasm. Conde, et al. demonstrated this idea on gold nanoparticles that were surface modified to display penetratin have been observed to cytoplasmically localize in cells even at 4 °C, by electrostatically penetrating through anionic phospholipids without an external driving force (e.g., pH-sensitivity, osmotic gradient).[187,195] On the other hand, the efficient penetrative activity of CPPs has led to reports of cell line-, cargo-, peptide size-, and dose-dependent cytotoxicity.[198] At biosafe doses, these peptides may provide a synergistic effect when combined with tissue-targeting peptides to help guide the nano-carriers to selective cells and allow them to efficiently activate RNAi in the cell cytoplasm. Conde, et al. demonstrated this idea on gold nanoparticles that were surface modified to display PEG, siRNAs (against c-myc), RGD tumor-targeting peptide, and the TAT cell penetrating peptide; the particle was able to demonstrate gene silencing efficiencies of 70% in vitro and 65.2% in vivo.[86] Additionally Mahajana, et al. decorated superparamagnetic iron oxide nanoparticles (SPION) with dextran, myristoylated polyarginine peptides (MPAPs), EPPT1 (tumor antigen-targeting peptide), and siRNAs (against PLK-1), which attained a modest 35% gene silencing efficiency in vivo.[200] The process of fusogenic uptake into cells (Figure 4d) may be mediated by specific compositions of liposomes.[182,197] Various fusogenic liposome compositions have been developed, although they were initially intended for biological studies on membrane fusion, rather than for siRNA delivery.[198] The most widely used compositions contain 1,2-dioleoyl-3-glycero-phosphatidyethanolamine (DOPE), and a cationic lipid such as N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP).[46,105,153,157] While the exact mechanism and role of these lipids are not clear, it is theorized that a mix of aromatic molecule, cationic surface charge, and conical lipid structure are involved in membrane fusion.[200] Another effective fusogenic composition is that of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000), and DOTAP.[98,99,149,186] Lee, et al. first demonstrated cell line-dependent fusogenicity of this composition (Figure 9a) that was independent of the major routes of endocytosis (e.g., macro-pinocytosis, caveolin-, clathrin-mediated uptake), and were able to retain fusogenic uptake in the absence of, or with downregulation of other major markers of vesicle traffic (e.g., Rab11, Rab5A). However, knockdown of intracellular vesicle fusion pathways prevented fusogenic uptake, and the fusogenic liposomes were found within the lysosomal compartments, indicating endocytosis (Figure 9b).[200] Although the specific cellular mechanisms are as yet unknown, it is clear that there is pathway-dependence of liposomal fusion to the plasma membrane.

6. Future Prospective

6.1. Trends in siRNA Therapeutics

Using the PubMed search engine of the MEDLINE database, we attempted to track research trends based on generic query terms, such as “siRNA,” “delivery,” and “nanoparticle,” relevant to publications on siRNA therapeutics. Out of over 3000 papers published on nanoparticle-mediated delivery of siRNAs for therapeutic applications since 2009, over 1000 publications report in vivo gene silencing effects either directly (e.g., qRT-PCR, Western blot) or indirectly (e.g., therapeutic or downstream screening effect). Among them, over 300 studies met at least two of the three design requirements described in this review: 1) siRNA protection; 2) selective targeting; and 3) cytoplasmic localization of siRNA.

Figure 10 shows the number of publications on siRNA delivery systems by material type in the last decade. Ten years after the initial discovery of siRNA, research articles relating to nanoparticle-mediated delivery of siRNA reached nearly 200 publications in 2010, and continued to produce increasing numbers of publications with a peak at over 400 publications in 2016 (grey bars, Figure 10). By material type, the lipid-based (which included liposomes, micelles, and solid lipid nanoparticles; yellow bars, Figure 10) and polymeric nanoparticles (green bars, Figure 10) were far more studied for siRNA delivery, compared to metallic (which included gold and iron oxide; blue bars, Figure 10) and silica/silicon-based systems (red and orange bars, Figure 10).

With the discovery of CRISPR systems in 2011, and its subsequent exponential increase in research publications (black dotted line, Figure 10), the overall number of publications on siRNA delivery began decreasing from 2017. The decline also coincides with a controversial shutdown of a large RNAi program in the pharmaceutical industry; Alnylam
Pharmaceuticals, one of the industry giants in RNAi therapy, had found potential toxicity in their lead siRNA formulation for the treatment of ATTR-amyloidosis.[201]

However, Alnylam made a breakthrough only two years later with another formulation for the same disease;[114] Patisiran demonstrated significant therapeutic outcome in patients and became the first FDA-approved RNAi/siRNA therapeutic in 2018. This pioneering achievement signals that we may be only at the beginning of the RNAi therapy story.

6.2. Clinical Translation

Table 5 lists all siRNA-based therapeutics that have been, or are currently under clinical trials, or have gained FDA-approval. The first time an siRNA therapeutic underwent Phase 3 clinical trials in the United States was in 2007, when Bevasiranib (Cand5) was formulated as a free-siRNA against VEGF-A in age-related macular degeneration (AMD). Since then, there have been over 30 clinical trials with siRNA-based therapeutics that were used to silence over 25 genes in over 40 different diseases (approximately 40% of which were cancer models). From the initiation of the first clinical trials of siRNA-based therapeutics in 2004, it took 14 years for the first siRNA-based therapeutic to be approved. This trajectory aligns closely with

![Figure 9. Fusogenic uptake bypasses endocytosis. a) Cell line-dependence of liposomal fusion to the plasma membrane by fusogenic liposome (MFL) versus endocytosis by non-fusogenic liposome (NFL) in HeLa, B16F10, and CT26 cell lines (scale bar represents 5 µm); Reprinted with permission.[186] Copyright 2015, American Chemical Society; b) schematic of endocytic uptake of Dil-loaded non-fusogenic liposome-coated pSiNPs (NNPs) with confocal microscopic image of CAOV-3 cells (scale bars represent 10 µm); c) schematic of fusogenic uptake of Dil-loaded fusogenic liposome-coated pSiNPs (FNPs) with confocal microscopic image of CAOV-3 cells (scale bars represent 10 µm); Adapted with permission.[149] Copyright 2019, JoVE.](image)

![Figure 10. Publications on siRNA delivery systems from 2009–2019, identified via the PubMed search engine of the MEDLINE database. Results for "Nanoparticle" were obtained using query terms "siRNA + nanoparticle"; results for "Lipid" were obtained using query terms "siRNA + liposome", "siRNA + lipid nanoparticle", and "siRNA + micelle"; results for "Polymer" were obtained using query terms "siRNA + polymer nanoparticle"; results for "Metal" were obtained using query terms "siRNA + metal nanoparticle," "siRNA + gold nanoparticle," and "siRNA + iron oxide nanoparticle"; results for "MSN" were obtained using query terms "siRNA + mesoporous silica nanoparticle"; results for "pSi" were obtained using query terms "siRNA + porous silicon"; and results for "CRISPR" were obtained using query terms "CRISPR + delivery."](image)
Table 5. siRNA formulations that have been, or are currently in clinical trials in the United States. Trials are organized by the carrier material (SNALP/LNP, GalNAc-conjugates, Others, and No-carriers), then by the year. “Formulation” indicates the generic name of the tested drug, “Gene” indicates the siRNA target to be silenced, “Disease” indicates the recruited patient conditions, “Status” indicates the current activity of the trials. Phases are indicated (P1: Phase 1; P2: Phase 2; and P3: Phase 3). ID number indicates the NCT number designation from clinicaltrials.gov. Trials with active or recruiting status are bolded.

| Formulation | Gene | Disease | Phase | Status | Duration | ID number |
|-------------|------|---------|-------|--------|----------|-----------|
| **SNALP/LNP** | | | | | | |
| Patisiran (ALN-TTR02) | TTR | TTR-mediated amyloidosis | Approved | NCT02939820 |
| CALAA-01 | M2 | Cancer; Solid tumor | Terminated | NCT00689065 |
| PRO-040201 | ApoB | Hyper-cholesterolemia | Terminated | NCT00927459 |
| ALN-VSP02 | KSP/VEGF | Solid Tumors | Completed | NCT00882180 |
| TKM 080301 | PLK-1 | Cancer with hepatic metastases | Completed | NCT0158079 |
| **CALAA-01** | | | | | | |
| CALAA-01 | M2 | Cancer; Solid tumor | Terminated | NCT00689065 |
| PRO-040201 | ApoB | Hyper-cholesterolemia | Terminated | NCT00927459 |
| ALN-VSP02 | KSP/VEGF | Solid Tumors | Completed | NCT00882180 |
| TKM 080301 | PLK-1 | Cancer with hepatic metastases | Completed | NCT0158079 |
| **ALN-VSP02** | | | | | | |
| ALN-VSP02 | KSP/VEGF | Solid Tumors | Completed | NCT00882180 |
| TKM 080301 | PLK-1 | Cancer with hepatic metastases | Completed | NCT0158079 |
| **TKM 080301** | | | | | | |
| TKM 080301 | PLK-1 | Cancer with hepatic metastases | Completed | NCT0158079 |
| **ND-02-s0201** | | | | | | |
| ND-02-s0201 | HSP47 | Fibrosis | Completed | NCT01858935 |
| DCR-MYC | Myc | Solid tumors; Non-Hodgkins lymphoma, etc. | Completed | NCT01437007 |
| siRNA-EphA2-DOPC | EphA2 | Advanced cancers | Completed | NCT01262235 |
| **Fitusiran (ALN-AT3SC)** | | | | | | |
| Fitusiran (ALN-AT3SC) | Antithrombin | Hemophilia A; Hemophilia B | Recruiting | NCT01858935 |
| **GalNAc-Conjugated siRNA** | | | | | | |
| Revisiran (ALN-TTRSC) | TTR | TTR-mediated amyloidosis | Completed | NCT01437007 |
| Inclisiran (ALN-PCSSC) | PCSK9 | Hetero/homozygous familial hyper-cholesterolemia, etc. | Completed | NCT01262235 |
| Givosiran (ALN-AS1) | ALAS1 | Acute intermittent porphyria | Completed | NCT02452372 |
| Lumasiran (ALN-GO1) | Glycolate oxidase (GO) | Primary hyperoxaluria type 1 | Completed | NCT02706886 |
| DCR-PHXC-101 | LDHA | Primary hyperoxaluria | Completed | NCT02706886 |
| **Other Carriers** | | | | | | |
| siG12D LODER (Polymer) | KRASG12D | Pancreatic cancer | Completed | NCT01188785 |
| STP705 (Polymer) | TGF-β1 COX-2 | Hypertrophic scar | Completed | NCT01676259 |
| iExosomes (Exosome) | KrasG12D | Pancreatic cancer | Completed | NCT01676259 |
| **No Carriers** | | | | | | |
| Bevasiranib (Cand5) | VEGF-A | AMD | Terminated | NCT00658086 |
| Sirna-027 (AGN211745) | VEGFR-1 | AMD | Terminated | NCT00499590 |
| ALN-RSV01 | RSV-N | Respiratory syncytial virus infections | Completed | NCT01065935 |
the history of antibody-based therapeutics, which started in 1975 and received their first approval (for a monoclonal antibody) in 1986.[79,114,202]

Based on Table 5, siRNA formulations without protective carriers have all been terminated or discontinued with the exception of the QPI-1007 formulation for local administration into the eye. Also, while GalNAc-siRNA conjugate systems (developed and pursued primarily by Alnylam Pharmaceuticals) are showing consistently strong performance with continued passages from Phases 1 to 3 (with the exception of Revusiran, which showed toxicity in Phase 3, and has been discontinued,[71,203]), liver-targeted applications are somewhat limited.[32] On the other hand, increasing numbers of carrier-based siRNA therapeutics have entered clinical trials since the late 2000s, and there are currently six trials that are active (four of which are SNALP/LNP formulations) for a wide range of applications (e.g., fibrosis, cancer, hemophilia, etc.).

7. Conclusions

While many challenges remain in the development of clinically translatable RNAi therapeutics (e.g., biosafe siRNA sequence selection,[203] mass production[204]), it appears we are quickly approaching a point when multiple therapeutic options will be approved for human use. In particular, the clinical trials are moving increasingly toward nanoformulations for siRNA protection and delivery, despite the declining number of publications on siRNA delivery systems from the academic community in recent years. This is clearly an opportunity for those of us working on advanced materials (and for the authors and readers of Advanced Materials). Moreover, while this review focused on siRNA, the materials design criteria presented in this review should be generally applicable to the problems encountered with the delivery of a wide range of gene modification systems currently under development (e.g., ASOs, CRISPR/Cas9, etc.). We hope this review has illustrated how rational materials design can generate new classes of nanoformulations that are able to protect, target, and deploy gene modifying therapeutics to the desired cell types for safe and efficacious in vivo outcomes.

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Conflict of Interest

M.J.S. holds an appointment as a “High-Level Talent” at the Key Laboratory of Organosilicon Chemistry and Material Technology of Hangzhou Normal University, China, he is a Guest Professor at Zhejiang University, China, and he is a scientific founder of Spinnaker Biosciences, Inc., a member of the Board of Directors, and has an equity interest in the company. Although one or more of the above grants have been identified for conflict of interest management based on the overall scope of the project and its potential benefit to Spinnaker Biosciences, Inc., the research findings included in this particular publication may not necessarily relate to the interests of Spinnaker Biosciences, Inc. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies.

Keywords

drug delivery, gene therapy, nanoparticles, RNA interference, siRNA

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References

[1] a) F. S. Collins, M. Morgan, A. Patrinos, Science 2003, 300, 286; b) F. S. Collins, A. Patrinos, E. Jordan, A. Chakravarti, R. Gesteland, L. Walters, Science 1998, 282, 682; c) M. V. Olson, Proc. Natl. Acad. Sci. U. S. A. 1993, 90, 4338; d) B. J. Wilson, S. G. Nicholls, Risk Management Healthcare Policy 2015, 8, 9.
[2] T. Friedmann, Nat. Genet. 1992, 2, 93.
[3] D. A. Jackson, R. H. Symons, P. Berg, Proc. Natl. Acad. Sci. USA 1972, 69, 2904.
[4] T. Lai, Y. Yang, S. K. Ng, Pharmaceuticals 2013, 6, 579.
[191] a) T. Bus, A. Traeger, U. S. Schubert, J. Mater. Chem. B 2018, 6, 6904; b) S. Vaidyanathan, B. G. Orr, M. M. Banaszak Holl, Acc. Chem. Res. 2016, 49, 1486.
[192] Y. Tu, J.-s. Kim, J. Gene Med. 2008, 10, 646.
[193] M. Yang, H. Jin, J. Chen, L. Ding, K. K. Ng, Q. Lin, J. F. Lovell, Z. Zhang, G. Zheng, Small 2011, 7, 568.
[194] J. C. LeCher, S. J. Nowak, J. L. McMurry, Biomol. Concepts 2017, 8, 131.
[195] C. Y. Jiao, D. Delaroche, F. Burlina, I. D. Alves, G. Chassaing, S. Sagan, J. Biol. Chem. 2009, 284, 33957.
[196] K. Saar, M. Lindgren, M. Hansen, E. Eiriksdóttir, Y. Jiang, K. Rosenthal-Aizman, M. Sassian, Ü. Langel, Anal. Biochem. 2005, 345, 55.
[197] C. Kleusch, N. Hersch, B. Hoffmann, R. Merkel, A. Csiszár, Molecules 2012, 17, 1055.
[198] a) V. Sokolov Iu, V. K. Lishko, Ukr Biokhim Zh 1980, 52, 700; b) N. Duzgunes, J. Wilschut, R. Fraley, D. Papahadjopoulos, Biochim. Biophys. Acta, Biomembr. 1981, 642, 182; c) R. Blumenthal, M. J. Clague, S. R. Durell, R. M. Epand, Chem. Rev. 2003, 103, 53; d) T. M. Allen, K. Hong, D. Papahadjopoulos, Biochemistry 1990, 29, 2976; e) S. Martens, H. T. McMahon, Nat. Rev. Mol. Cell Biol. 2008, 9, 543.
[199] A. Koshkaryev, A. Piroyan, V. P. Torchilin, Cancer Letters 2013, 334, 293.
[200] R. Kolasinac, C. Kleusch, T. Braun, R. Merkel, A. Csiszar, Int. J. Mol. Sci. 2018, 19, 346.
[201] K. Garber, Nat. Biotechnol. 2016, 34, 1213.
[202] J. K. Liu, Annals of Medicine and Surgery 2014, 3, 113.
[203] a) O. Snove Jr., T. Holen, Biochem. Biophys. Res. Commun. 2004, 319, 256; b) D. Haussecker, Molecular Therapy - Nucleic Acids 2012, 1, e8.
[204] a) P. Zhang, J. Xia, S. Luo, Materials (Basel) 2018, 11, 623; b) J. Jeevanandam, A. Barhoum, Y. S. Chan, A. Dufresne, M. K. Danquah, Beilstein J. Nanotechnol. 2018, 9, 1050.