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Review

Supramolecular assemblies in functional siRNA delivery: Where do we stand?

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ABSTRACT

The discovery of RNA interference (RNAi) has excited the scientific field due to its potential for wide range of therapeutic applications. The pharmacological mediator of RNAi, short interfering RNA (siRNA), however, has faced significant obstacles in reaching its target site and effectively exerting its silencing activity. Effective pharmacological use of siRNA requires 'carriers' that can deliver the siRNA to its intended site of action. The carriers assemble the siRNA into supramolecular complexes that display functional properties during the delivery process. This review will summarize non-viral approaches to siRNA delivery, emphasizing the current obstacles to delivery and the mechanisms employed to overcome these obstacles. The carriers successfully pursued in pre-clinical (animal) models will be presented so as to provide a glimpse of possible candidates for clinical testing. Supramolecular assembly of nucleic acids with carriers will be probed from thermodynamics and computational perspectives to understand supramolecular structures and their dynamics. The delivery and trafficking requirements for siRNA are then dissected and engineering approaches to overcoming these barriers will be articulated. The latter has been attempted both at the cellular levels, focusing on intracellular barriers, as well as systemic level, emphasizing macroscopic challenges affecting siRNA delivery. Clinical experience with non-viral siRNA delivery is summarized, highlighting the nature delivery modes attempted in clinical settings. We conclude with a perspective on the future of siRNA therapeutics, specifically concentrating on the possible impact of non-viral carriers in the field.

1. Background on siRNA carriers

Despite the promise of RNA interference (RNAi) and reported success of direct delivery of "naked" siRNA to some tissues [1], administered siRNA has little chance of *in vivo* efficacy if it is not structurally modified or accompanied with an engineered delivery system. The naked siRNA has a poor pharmacokinetics profile. It is almost instantly degraded by RNase A type nucleases [2] that leads to short serum half-life ($t_{1/2}$) on the order of <30 min [3]. The rapid siRNA clearance by the kidneys also contributes to its short $t_{1/2}$ (the glomerular molecular weight cut-off of ~60 kDa is larger than the ~14 kDa siRNA) [4]. An additional obstacle for naked siRNA is the negligible cellular internalization; the anionic charge of backbone phosphates (~40/molecule [3]) makes it impossible for siRNA to interact with anionic phospholipid cell membranes. Therefore, many strategies have been evaluated to design siRNA carriers to protect siRNA from *in vivo* degradation, to limit its premature elimination, and to deliver siRNA into target cells for effective silencing. Some of these strategies relied on viruses since the natural abilities of viruses to insert their genome into host cells make them effective delivery agents. Viral delivery is based on DNA-based expression cassettes designed to express double-stranded short hairpin RNA (shRNA) or microRNA (miRNA) [5–8]. Non-viral carriers aim to mimic viral-like delivery by relying solely on biomolecules to package the nucleic acids. Although other physical strategies, such as electroporation, ultrasonic delivery, hydrostatic and 'gene gun', have been attempted for this purpose, they will not be reviewed in this manuscript.

Abbreviations: RES, Reticuloendothelial system; IV, Intravenous; siRNA, Small interfering RNA; RNAi, RNA interference; PEG, Polyethyleneglycol; MW, Molecular weight; PEI, Polyethylenimine; CPP, Cell penetrating peptide; MD, Molecular dynamics; PAMAM, Poly(amidoamine); $t_{1/2}$, Half-life.

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1.1. Viral vectors for RNAi

Genes encoding hairpin structures (shRNA or miRNA) have been inserted into viral vectors so as to express the RNA molecules endogenously, which are then processed into siRNA in the cytoplasm of host cells [7]. An advantage of this approach is that a long-term expression of the interfering RNAs could be achieved with a single administration, which is especially desirable in chronic diseases such as HIV infection [5]. Viral-mediated RNAi has been extensively explored in 'hard-to-transfect' cells, such as the nervous system for treatment of several neurodegenerative diseases, including Huntington's disease, Alzheimer's, Amyotropic Lateral Sclerosis, and prion disease [6].

Adenoviral vectors have been commonly used in RNAi delivery. These are medium-sized, non-enveloped viruses with nucleocapsid and a linear double-stranded DNA genome [7]. These viruses are commercially available for RNAi expression and many studies have reported their use *in vivo* [9,10]; however, the risk of a strong immune response, liver toxicity [11] and diminished genetic vector stability arising from small shRNA expression cassettes could limit these vectors [12]. Adeno-associated viruses (AAV) are smaller viruses with single-stranded DNA, but they are attractive being non-pathogenic to humans [13]. AAV vectors have been investigated for shRNA delivery in animal models of tumor therapy [14] and have been even tested in clinical studies [15]. Similar to adenoviral vectors, however, the genetic information delivered by the AAV is transiently expressed [7]. Retroviruses, on the other hand, are single-stranded RNA viruses that have been employed in clinical gene delivery early on due to their ability to integrate into the host genome for a long-lasting expression. However, safety concerns associated with these vectors have motivated alternative strategies [16]. A recent study has shown successful HIV-1 suppression in a T-cell line using shRNA delivery by retroviral delivery [17]. Lentiviruses, a subclass of retroviruses, possess two single-stranded RNA genomes in an enveloped capsid and are able to transduce both dividing and non-dividing cells. Ability to better target non-dividing cells, such as neurons [8], is an important advantage and many studies have shown successful regulation of specific targets in brain after local injection of shRNA-carrying lentiviral vectors [18]. Lentiviral delivery of an shRNA into hematopoietic stem cells is currently in clinical testing, where the *ex vivo*-transduced cells are re-infusion to HIV-positive hosts [19]. Finally, insect baculoviral systems have been employed for RNAi since they are unable to replicate in mammalian cells and provide a safer alternative [20]. shRNA expression with baculoviruses has been reported to target viral infections, such as Influenza A and B [21], and HCV [22].

1.2. Non-viral carriers for RNAi

Carriers that assemble with siRNA to form supramolecular complexes have been engineered for siRNA delivery. Despite significant variations in the design and characteristics of these carriers, the end goal is to overcome the shortcomings of the naked siRNA. Once at the target site, efficient intracellular trafficking and release from the carriers are paramount for effective silencing. In addition to chemical modification of the siRNA molecule [23,24], carriers developed for DNA packaging and delivery are being redesigned for siRNA delivery, while new nanotechnology-based strategies are adopted for siRNA delivery. Non-viral carriers offer a more acceptable immunogenicity and safety profiles [25], although clinical validation of this claim remains to be demonstrated. Promising non-viral carriers (Fig. 1) have been reviewed in the next section.

1.2.1. Liposomes

Highly ordered lipid aggregates at the nanoscale, liposomes are distinguished by an internal aqueous phase and a lipid bilayer envelope, which is reminiscent of naturally occurring phospholipid membrane in cells. Liposomes have been particularly successful for delivery of water-soluble drugs entrapped in the hydrophilic core. "Stealth" liposomes increase the circulation times (longer $t_{1/2}$) and systemic dose (i.e., area under plasma/blood concentration vs. time curve, AUC) of the encapsulated drug, which is a reflection of a decrease in the clearance (CL) and/or volume of distribution (Vd) [26]. Liposomes have been explored extensively for siRNA delivery due to their suitable size (~100 nm), biocompatibility of their components, and especially ease of preparation [27]. For example, neutral 1,2-oleoyl-sn-glycero-3-phosphocholine (DOPC) can encapsulate ~65% of siRNA by simply mixing the solutions of the two components [28]. Dioleoyl phosphatidylethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and phosphatidylcholine (PC) are other neutral lipids employed in preparation of liposomes [29]. Landen *et al.* reported EphA2 (a tyrosine kinase receptor associated with poor clinical outcome in ovarian cancer) down-regulation in a nude mice model using DOPC liposomes [28]. Liposomes formed with DOPC have been also employed for Protease-activated receptor (PAR-1) down-regulation to inhibit melanoma growth and metastasis by decreasing angiogenesis [30] and for adhesion kinase silencing to eradicate ovarian cancer cells [31]. DOPE liposomes have been reported in siRNA delivery for Ubc13 [32] silencing.

1.2.2. Lipoplexes

Cationic lipids complexed with nucleic acids form complexes known as lipoplexes [27]. The main advantage of cationic lipids is the spontaneous interaction with anionic siRNA as well as cell membranes, which lead to higher cell internalization [33]. However, higher toxicity compared to neutral liposomes, shorter serum $t_{1/2}$ (partly due to uptake by reticuloendothelial system, RES) and higher immunogenicity (due to uptake by macrophages) are among the risks associated with lipoplexes [34]. Use of cationic liposomes has been accordingly confined to *in vitro* systems. Polyethylene glycol (PEG) coating in lipoplexes helps to minimize these risks [34]. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [35] lipoplexes have been successfully used for siRNA delivery against Tumor Necrosis Factor (TNF) by intravenous (IV) injection [36], and against Vascular Epithelial Growth Factor (VEGF) by subretinal injection [37] in mouse models. Cardiolipin, a cationic analog of phospholipids found in the cardiac muscle, has been used for siRNA-mediated C-raf silencing in different animal models [38,39]. A more comprehensive review of lipoplexes in siRNA delivery could be found in [1,27,29].

1.2.3. Stable nucleic acid lipid particles (SNALP)

SNALPs are typically composed of multiple lipids, including neutral, cationic and PEGylated lipids [1] and present a more complicated siRNA formulation. This allows better functionalization of siRNA particles for a variety of purposes, but it may also bring additional complications in the development studies. SNAPL formulations of siRNA has been successfully employed for Apolipoprotein B (ApoB) silencing in cynomolgus monkeys [40] and for polo-like kinase 1 (PLK1) silencing in subcutaneous tumors in mice (~75% reduction in size) [41]. Recent developments in SNALP-mediated delivery of siRNA [42,43] indicated excellent potential for their systemic applications.

1.2.4. Cationic polymers

Supramolecular complexes of siRNA formed with cationic polymers (polyplexes) have evolved into a dominant strategy for

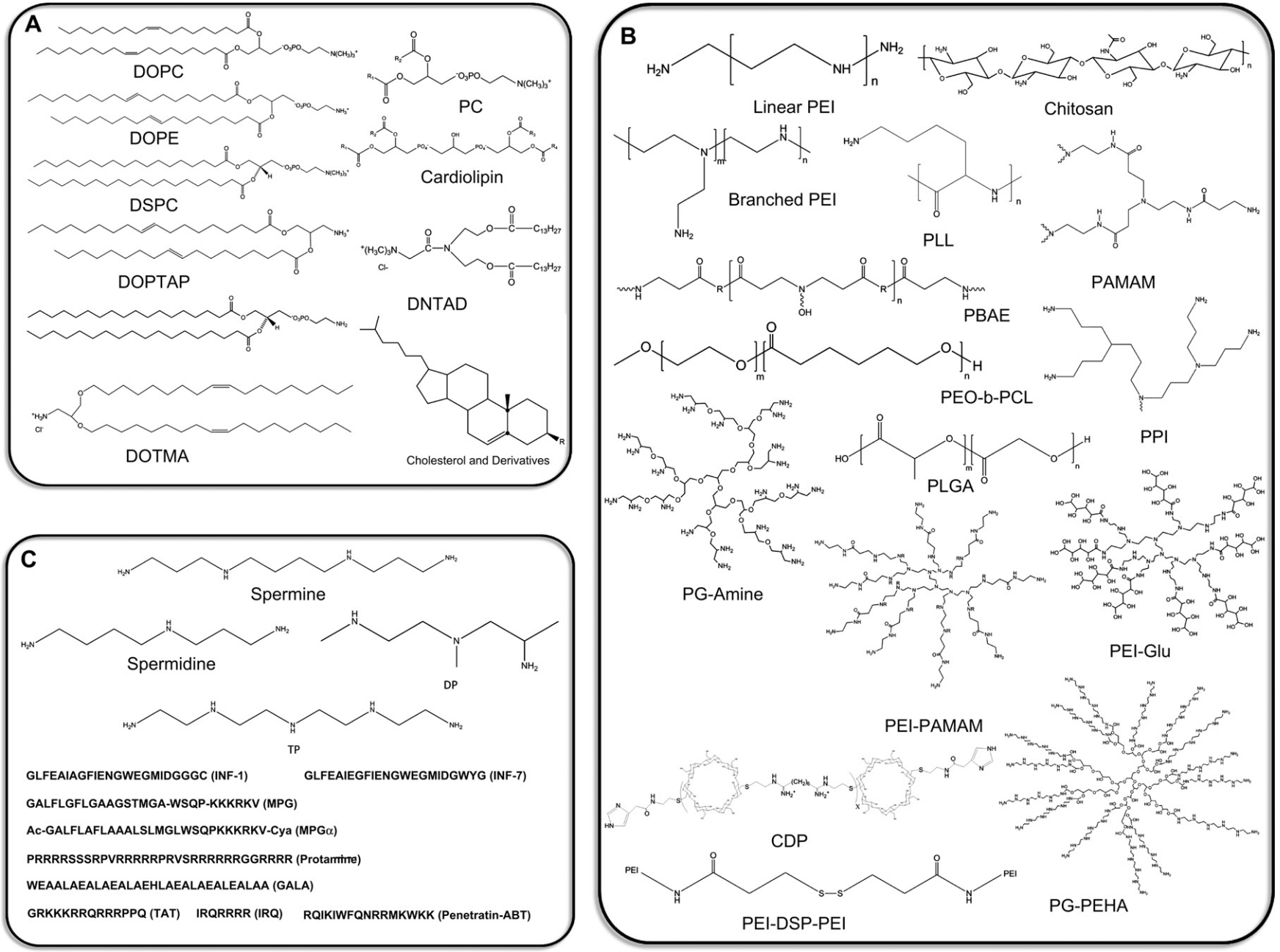

Fig. 1. Chemical structure of select carriers used for siRNA delivery. The carriers shown in this Figure were discussed in the manuscript and categorized into (A) cationic lipids, (B) polymers, and (C) peptides and polyamines. The functional carriers constitute a diverse group of molecules that range from cationic lipids to polymeric molecules with repeating cationic and neutral groups. CDP: Cyclodextrin-polycation; DOPC: 1,2-oleoyl-sn-glycero-3-phosphocholine; DOPE: Dioleoyl phosphatidylethanolamine; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; DP: N,N-dimethyldipropylenetriamine; DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine; DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; GALA: Glutamic acid, Alanine, Leucine, Alanine; PEI-Glu: PEI-gluconolactone; PAMAM: Poly(amidoamine); PBAE: Poly(beta-amino ester)s; PC: Phosphatidylcholine; PEI: Polyethylenimine; PEO-b-PCL: poly(ethylene oxide)-block-poly(ε-caprolactone); PG-Amine: Polyglycerolamine; PG-PEHA: polyglyceryl pentaethylene hexamine carbamate; PLGA: Poly(lactic-co-glycolic acid); PLL: Poly-L-L-lysine; PPI: Polypropylene imine; TP: Tetraethylenepentamine.

siRNA delivery. Self-assembly of complexes results from ionic interaction between the repetitive cationic moieties on polymers and anionic phosphates on siRNA. Depending on the extent of polymer:siRNA ratio, the charges are neutralized to a desirable extent and siRNA is physically protected in the complex against RNase degradation. The main advantage of polymers is their structural flexibility that allows convenient manipulation of the physicochemical characteristics of the delivery system; polymer properties such as molecular weight, charge density, solubility, and hydrophobicity could be engineered at will, as well as addition of desired chemical groups for further functionalization. Both natural and synthetic polymers have been explored for this purpose.

1.2.4.1. Chitosan. A naturally occurring polysaccharide containing repeating glucosamine and N-acetylglucosamine units, chitosan is derived from deacetylation of chitin [25]. PEGylation of chitosan, like other polymers, was effective in enhancing the stability of siRNA complexes and serum $t_{1/2}$ [44]. Chitosan is biodegradable (readily digested by lysozymes and chitinases *in vivo*; [45]) and is practically non-toxic in mammals (with LD_{50} of 16 g/kg in rats; [46]). Chitosan/siRNA complexes are characteristically $\leq$200 nm [47], a proper size for *in vivo* delivery. Despite the relative safety and biocompatibility of chitosan, there are only a few *in vivo* studies using chitosan/siRNA complexes, possibly due to limited efficiency of the polymer for delivering siRNA to its target. Effective siRNA delivery has been reported (against a model target, green fluorescent protein, GFP) in lung epithelial cells after intranasal administration in mice [47]. Intraperitoneal administration of anti-TNF-α siRNA with chitosan showed a ~44% silencing in mice, leading to inhibition of inflammatory response in a collagen-induced arthritis model [48]. Chitosan has been also used as a 'coating' to improve efficiency of other delivery systems. Chitosan-coated polyisohexylcyanoacrylate particles have been reported for *in vivo* delivery of anti-RhoA siRNA to breast cancer xenografts in nude mice, which inhibited tumor growth by >90% [49].

1.2.4.2. Other natural polymers. Cyclodextrin, a funnel (or toroid) shaped molecule usually investigated in pharmaceutical delivery formulations, has been used as a component of a cationic polymer to form complexes with siRNA via ionic interactions. Cyclodextrin was proposed not only to protect siRNA from degradation, but also to block immunogenicity of siRNA *in vivo*, even in presence of immune stimulatory sequences in siRNA [50]. Transferrin-targeted cyclodextrin/siRNA complexes were capable of silencing the oncogene EWS-FLI1 in transferrin receptor-expressing Ewing's sarcoma cells [51] and luciferase in Neuro2A-Luc cells [52]. This delivery system was well tolerated in non-human primates [50]. Atelocollagen (~300 kDa; purified from pepsin-treated Type I collagen; [53]) is another cationic carrier that has been used for siRNA silencing against different tumor targets in mice with considerable success [54].

1.2.4.3. Polyethylenimine (PEI). Considered by many to be the 'gold standard' in non-viral gene delivery, PEI is a potent carrier due to its exceptional cellular uptake and endosomolytic activity [55]. High MW (25 kDa) PEI has been extensively investigated for siRNA delivery [56]. High charge density of the polymer facilitates strong binding to siRNA and effective protection against enzymatic degradation. However, the toxicity and limited biodegradability of this polymer posed obstacles for its clinical use [57]. Low MW (<2 kDa) PEIs display acceptable toxicity profiles but they do not display efficacious siRNA delivery into cells. It has been hypothesized that PEI and, other cationic polymers, increase cellular uptake of genomic material via creation of transient nanoscale holes in cell membrane, which could enhance material exchange across the cell membrane [58]. The same destabilizing action on membranes has been proposed as the mechanism of cytotoxicity [59]. It is, therefore, not surprising that the polymers more efficient in delivering nucleic acids are also more cytotoxic. Another structural factor affecting the efficiency and toxicity of PEI is the degree of branching in the polymer structure [60]. The branched PEI contains primary, secondary and tertiary amines at an approximate ratio of 1:2:1, whereas the linear polymer is composed of all secondary amines except for the primary amines at terminals [35]. In general, branched PEI was found superior to linear structure in nucleic acid delivery [61]. Despite remarkable potential of this polymer, structural modifications might be required to optimize the efficiency and overcome the limitations that prevented its clinical use.

1.2.4.4. Dendrimers. Highly branched polymers developed in 1980s, dendrimeric molecules from poly(amidoamine) (PAMAM), polypropylenimine (PPI), poly(L-lysine) (PLL), and carbon-silanes [62] have been explored for siRNA delivery. An appropriate concentration of PAMAM was shown to provide the necessary charge density to form stable siRNA complexes [63]. PAMAM polymers are commercially available (Polyfect™ and Superfect™) for siRNA delivery [64]. A biodegradable arginine ester of PAMAM was effective for siRNA delivery to neurons *in vitro* and *in vivo* (intracranial injection to rabbits) with minimal toxicity [65]. A Luteinising Hormone Releasing Hormone (LHRH)-conjugated PAMAM formulation, capable of restricting its electrostatic charges inside a core, displayed reduced toxicity and effectiveness in tumor targeting [64]. A PEI-related polymer, PPI has been specifically designed for siRNA delivery and functionalized with a PEG and LHRH; growth of human lung A549 xenografts in mice was retarded, while minimizing the liver and kidney concentrations of the carrier/siRNA [66].

1.2.4.5. Other synthetic polymers. The linear PLL has a high density of cationic charge suitable for siRNA neutralization. Using PLL/siRNA complexes, a significant silencing of lipoprotein ApoB expression was observed in C57BL/6 mice, without hepatotoxicity and reduction in serum low-density lipoprotein in ApoE-deficient mice (a model of hypercholestromia, [67]). Our lab reported ineffective siRNA delivery with the native PLL, suggesting significant variations in the performance of this polymer depending on the context of silencing. P-glycoprotein (P-gp) down-regulation in a drug-resistant breast cancer xenogratfs (MDA435/LCC6 MDR1) was possible with a PLL-based delivery system, but only after lipid substitution on the polymer. This led to effective tumor growth retardation in NOD-SCID mice after systemic administration of the chemotherapeutic drug DOXIL™ [68]. Several lipids (ranging from C8 to C18) were capable of imparting siRNA delivery capability to the native PLL, although stearic acid substitution functioned better than the others [69].

Poly(beta-amino ester)s (PBAE) are degradable cationic polymers that are synthesized from the conjugate addition of amines to diacrylates [35]. PBAEs have been investigated on their own for DNA delivery, as polycationic coatings on gold nanoparticles or multilayer structures formed with oppositely charged polyelectrolytes. Gold-siRNA nanoparticles coated with PBAEs led to >95% gene silencing, whereas non-coated particles were unable to mediate silencing [70].

Micellar structures from poly(ethylene oxide)-block-poly(ε-caprolactone) (PEO-b-PCL) block copolymers have been explored for siRNA delivery after adding polyamine side chains on the PCL block, including spermine (PEO-b-P(CL-g-SP)), tetraethylenepentamine (PEO-b-P(CL-g-TP)), or N,N-dimethyldipropylenetriamine (PEO-b-P(CL-g-DP)). *In vitro* P-gp silencing in MDA435 breast cancer cells has been demonstrated with these micelles [71]. The

efficacy was improved after functionalizing the polymer with an integrin αvβ3 targeting peptide (RGD4C) and the cell penetrating peptide TAT [72]. Poly(lactic-co-glycolic acid) (PLGA) microparticles have been reported for antigen-coding DNA delivery in Balb/c mice [73]; siRNA delivery with these particles is in its initial stages [74].

1.2.5. Peptides

Short (<30) amino acid (a.a.) sequences were introduced in 1990s for therapeutic delivery. Peptides are versatile molecules due to considerable variety in the chemical characteristics of the building blocks and are efficient delivery systems that can enhance cellular uptake of siRNA. Basic a.a.s such as arginine and lysine are needed for complex formation with siRNA. Highly charged peptides, however, are impeded by RES, and incorporation of cysteine (and formation of disulphide bonds) in a lysine-rich peptide was reported to improve intracellular delivery due to lower opsonisation [75]. A special class of cationic peptides, known as cell penetrating peptides (CPP; 5-40 a.a. long), have been extensively explored for transferring their cargo across cell membranes. Several CPPs were derived from viral proteins known to be responsible for cell penetrating capability: for example, TAT from HIV-1 [76] and INF-1 and INF-7 from influenza virus [77]. Many mechanisms have been suggested for this efficiency, including signal-activated endocytosis, macropinocytosis, and direct translocation routes (including "inverted micelle" model) [78]. CPPs were used in two approaches for siRNA delivery, one based on covalent binding and one based on electrostatic complexation with the siRNA. The main strategy for covalent linkage between siRNA and CPP is through a disulphide linker (and thioether linkers to a lesser degree), which can degrade in cytosol. Even though this strategy offers a higher siRNA carrier association, a lower silencing activity may result if the linkage is too stable to prevent siRNA entry into RNA-induced silencing complex (RISC) [79]; however, effective silencing with peptide-conjugated siRNA has been reported [80]. Electrostatically interacting peptides was employed for siRNA delivery against GAPDH [81]. A CPP peptide known as MPG was also investigated for silencing cyclin B1 in athymic nude mice, with effective inhibition of tumor growth [82].

2. Supramolecular assembly of nucleic acids with non-viral carriers

While diverse carriers are pursued to deliver siRNA effectively, it is clear that their success will depend on the nature of supramolecular complexes formed with the nucleic acids. The dynamic physical and chemical properties of the complexes are expected to dictate the silencing behavior. Experimental investigation of the thermodynamics of the assembly process is indispensible to gain insight into the nature of complexes. Computational studies could provide further details on complexes that are not readily available via experimental techniques. Below, we summarize the current literature of these two aspects of siRNA complexes.

2.1. Thermodynamics of siRNA complexation with carriers

Binding and complexation of siRNA into supramolecular assemblies suitable for cell uptake are driven by thermodynamic forces between cationic carriers and the siRNA. Thermodynamic forces will dictate the stability of siRNA complexes, cellular delivery and possibly the siRNA efficacy in silencing. Investigation of interactions with probes (e.g., nucleic acid binding dyes) and microscopic techniques (e.g., atomic force microscopy) may provide indirect evidence for binding parameters, but measurement of fundamental thermodynamics parameters is paramount for better understanding of siRNA complexes. Differential scanning and isothermal titration calorimetry are the appropriate technique for analyzing interactions of biomolecules in solution, since they do not require a reporter probe and are not susceptible to solution turbidity. The heat exchange during the interactions could be measured to obtain the 'observed' enthalpy change (ΔH), along with the binding stoichiometry (n) and affinity (K_a), in order to calculate Gibb's free energy (ΔG): $\Delta G = -RTln K_a = \Delta H - T\Delta S$, where R, T and ΔS are the Gas Constant (8.314 J mol^{-1} K^{-1}), temperature (K) and entropy change, respectively This analysis assumes an equilibrium process for binding, which could be valid in the initial stages of binding, but will not be valid for aggregation events when siRNA is saturated with carrier binding and a defined stoichiometry is lost.

Despite its promise, relatively little work has been reported on the interactions of cationic carriers with nucleic acids, and most of this work has been in the context of DNA interactions [83–89]. Binding constants are elusive at times due to significant aggregation effects [83] and, when obtained, significant variations in the measured parameters impede accurate assessment of the parameters [84]. Unlike a single mode binding, dual-binding modes may be more appropriate to analyze the data [90], as was the case with PEI and salmon DNA [91]. In that case, one binding mode was attributed to carrier binding to DNA groove, and the other to the external phosphate backbone, which also incorporated DNA condensation. In the former case, a positive ΔH was observed, indicating the dominance of hydrophobic interactions (i.e., ΔS) driven by displacement of tightly bound water in the DNA groove [92]. The lipophilic carriers are expected to display increased endothermic ΔH due to burial of acyl chains in DNA groves [93]. In the case of carrier binding to phosphate backbone, little or no ΔH is expected, which is characteristic of ionic binding and DNA condensation [91]. The binding stoichiometry and K_a significantly vary between the two binding modes and carriers could get protonated or de-protonated during the binding processes.

While the protonation state of the carrier will be dictated by medium pH, the phosphodiester backbone should be constantly protonated given its pK_a of ~3. The nucleic acids will remain charged at all times under the physiologically relevant pH conditions and, as expected, siRNA was not observed to alter its configuration (assessed by CD spectra) during acidification to low pH values typically seen in endosomes [94]. The carriers such as branched PEI contains a mixture of protonated and un-protonated Ns, where the protonation state was observed to change from 47% to 19% (i.e., percentage of protonated Ns) when the pH was increased from 6.0 to 9.0 [91]. In a study aimed at probing the thermodynamics of siRNA complexation with rigid, dendritic PAMAM carriers, undetectable change in the shape of PAMAM was seen upon siRNA binding [95] (note that MD simulations suggested 1–2% change in molecular radius of G4-G7 PAMAMs, a value too small for experimental resolution [96]). An apparent (i.e., buffer-dependent) ΔH of -869 ± 90 kcal/mol was observed in the initial binding phase (excluding the effects of condensation and aggregation), which was consistent with computational results [95]. In contrast, similar PAMAM molecules displayed endothermic binding to much longer DNA [97], clearly highlighting the difficulty of translating results from one type of nucleic acid to another. The extent of deprotonation/protonation during nucleic acid interactions might be a key in reconciling the differences, but this has not been probed to-date with siRNA carriers. The rigidity of PAMAM may also pose a steric impediment for additional (e.g., hydrophobic) interactions with the siRNA molecule, since the rigid PAMAM core might not be accessible for siRNA interactions.

As siRNA complexes are mainly internalized through endocytic pathway (see next section), the carriers could experience a pH change from 7.2 to 7.4 on the cell surfaces to 5.5–6.0 in the

endosomes, to ~5.0 in acidic endosomes/lysosomes [98]. If the carriers undergo protonation during this process (typical of PEI and histidine moieties), carrier binding to siRNA will be progressively altered, significantly affecting thermodynamics parameters and especially binding stoichiometry [99]. The number of carriers bound per unit nucleic acid, for example, could decrease, resulting in endosomal release of carriers from the nucleic acid cargo. This may result in endosomal destabilization with lipid membrane-destabilizing carriers. Upon endosomal release, cationic carriers might additionally elevate the cytosolic pH by as much as 0.4 units [100] and manifest a lower affinity to its cargo, leading to more effective siRNA dissociation. The pH-dependent binding to siRNA was observed with a particular peptide carrier, Endo-porter™; whereas the peptide did not display any interaction with siRNA at neutral pH, a weak exothermic interaction was evident at lower pH (6.0) [94]. This was indicative of an electrostatic interactions and possibility of protonated form of the carrier binding to siRNA. The pH-dependent siRNA binding behavior of carriers might be a key reason for the efficiency of the carriers.

2.2. Computational simulations of siRNA complexation with carriers

With advances in computational hardware and methodology, computer simulations are playing an increasing role in nucleic acid delivery. Studies involving all-atom molecular dynamics (MD) simulation can illuminate the structures of biological and synthetic molecules at atomic resolution, as well as predict and interpret the intra- and inter-molecular interactions that are the basis of functional responses in biological systems. In designing siRNA carriers, MD simulations can be employed to understand interactions of siRNA with its carriers and dynamics of their complexes, so as to evaluate and screen the designed carriers before experimental evaluation. In one of the first studies of its kind, Yingling and Shapiro reported on the computational design of all-RNA nanoring and nanotube structures capable of siRNA delivery [101]. An RNA nanoring was constructed from six helical building blocks, could be extended to an RNA nanotube with the appropriate design of building blocks that are held together by non-covalent interactions. By incorporating bioactive silencing sequences (siRNA) into the proposed all-RNA structures, one can theoretically generate delivery systems without the need for additional carriers. This work mainly served as a demonstration of how the optimal design of nanoparticles for delivering siRNAs can be realized relatively fast and inexpensively via simulations.

MD simulations of more practical systems involving siRNA molecules complexed with polymeric carriers have been recently reported [95,102–109]. Ouyang *et al.* performed a series of simulations to investigate complexation of siRNA with carriers derived from PAMAM dendrimers, and dendritic and linear PLL [102] (all MW <2000). Two molecular species were studied for each molecule: one carrying 4+ charges and the other carrying 8+ charges. The carriers with 4+ charges were found to only bind to the major grooves of siRNA, while the carriers with 8+ charges showed less binding specificity, in that they bound to both the major and minor grooves. The energy analysis for siRNA carrier interactions revealed the electrostatic interaction to be the primary contributor [102,108], followed by van der Waals interactions [102]. This relative contribution was found to change with rigid molecules sterically hindered for electrostatic interactions; van der Waals interactions gained more prominence in that case [102]. Upon exposure of multiple carriers to a single siRNA [103], all cationic polymers could bind to the siRNA at low polycation:siRNA charge ratios of 0.6:1 and 1:1, while only a fraction of polycations could bind to the siRNA at the high charge ratio of 2:1. The fluctuations in the siRNA structures were found to be reduced when complexes with multiple polymers were formed in solution, suggesting more stable structures in this case. Since carrier charge is intimately linked to the medium pH, these studies are indicative of dynamic siRNA/carrier complexes depending on the environmental conditions to which the complexes are exposed. Release of bound water (or counter ions), resulting in entropic gains in the system, has been often attributed to be the driving force for complexation, and this was evident in this simulation work [102,103].

The MD simulations of higher generation PAMAMs were reported by Pavan *et al.* [104,105]. The flexibility of the dendrimer and its ability to reorganize its structure for interactions with siRNA were found to be important for binding affinity [104]. Specifically, fourth generation (G4) PAMAM displayed adaptability (i.e., underwent conformational change) for siRNA binding, while G6 PAMAM behaved like a rigid sphere with a lower binding affinity with siRNA. G5 PAMAM molecule showed an intermediate behavior that strongly depended on the pH: at pH ~7.4 it behaved like a G4 dendrimer with good flexibility, but at pH<5 its flexibility was dramatically reduced and it behaved similar to the G6 PAMAM. This effect was attributed to varying cationic nature of the G5 PAMAM as a function of pH, ultimately influencing the rigidity of the carrier. The siRNA were shown to penetrate into high generation PAMAM structures [107], although the terminal amines formed the primary contacts [108]. The authors demonstrated that (i) the calculated structural parameters of the PAMAMs were in good agreement with experimental measurements, and (ii) the diffusivity of siRNA/PAMAM complexes agreed well with experimental data [105]. Other studies also reported good agreement between the calculated dimensions of PAMAMs and experimental measurements (e.g., G7 PAMAM analyzed with SAXS) [95]. However, experimental binding of PAMAM to siRNA (with gel electrophoresis) was indifferent to the generation number [105], somehow contradicting to previously reported computational differences observed with different generations of PAMAMs.

The effect of carrier flexibility on siRNA affinity was explored by using MD simulations of triazine dendrimers [106]. By comparing rigid vs. flexible dendrimers, rigid structures were shown to display higher affinity to siRNA as compared to flexible dendrimers. As with PAMAM, terminal groups of the triazine dendrimers were primarily responsible for making contact with siRNA and rigid structures appeared to have more concentrated contacts, making use of their interacting groups more effectively [106]. Flexible linkers appeared to 'retract' the carrier onto itself, making it unavailable for siRNA interactions. While flexibility in PAMAM was expected to lead to better wrapping of the dendrimer around the siRNA molecules (simulations with G3 vs. G4) [108], the flexibility in triazine dendrimers seems to be not beneficial for this reason. In the presence of excess carriers (e.g., multiple PAMAMs per siRNA), the flexibility of the carrier might not be a significant factor in siRNA binding [108]; crowding of carriers might not allow significant conformational changes in individual molecules to wrap siRNA, but rather the terminal amines in crowded PAMAM structures might impede carrier wrapping around siRNA due to electrostatic repulsions [108]. Since practical systems often involve multiple carrier molecules per siRNA, simulating single siRNA carrier systems might be misleading. This was the case especially for G7 PAMAMs [95], where no changes in the structure were evident upon siRNA binding. The salt concentration in media was critical for the number of actual contacts formed between a carrier and siRNA [108], a parameter often ignored in MD simulations. For example, up to ~75% loss of contacts could be obtained as a result of increasing salt concentration to the physiological levels of 150 mM NaCl in MD simulations (also linked to the calculated ΔG in the presence/absence of NaCl). The implications of this result on cellular uptake

of siRNA are obvious, and it will be important to correlate these computational results to experimental uptake values.

The relevant thermodynamics parameters of siRNA carrier interactions, specifically ΔG, ΔH and ΔS, were computed for several carrier-siRNA systems [95,102,104–108]. Without experimental data that closely matches the simulated system (i.e., similarity in molecular size, buffer composition, siRNA sequence, etc.), it is difficult to judge the validity of the reported values; however, they could be used as a 'qualitative' guide to understand the influence of carrier properties (e.g., generation number) or environmental conditions (e.g., salt concentration) on the thermodynamics of siRNA interactions. One study [95] reported an experimental value of apparent molar enthalpy of binding (ΔH_{bind}) of -869 ± 90 kcal/mol (interaction in HEPES buffer) for G7 PAMAM, which was argued to be in line with ΔH_{bind} calculated in that study (-850 ± 15 kcal/mol; not taking into account the buffer effects) using the MM-PBSA method [109]. However, the calculated ΔH_{bind} is actually not the binding enthalpy, but the summation of the carrier-siRNA binding enthalpy in vacuum (ΔH_{gas}) and the solvation free energy (ΔG_{sol}) [110]. Hence, it is incorrect to make such a direct comparison. The same oversight was also committed in other studies [95,104–108] in interpreting the free energy results from the MM-PBSA calculations, leading to apparent "mismatch" with experimental values. For instance, in independent studies, calculated "ΔH_{bind}" values were significantly different and ranged from approximately −480 to −6880 kcal/mol for G3 and G4 PAMAMs (simulated in the absence and presence of NaCl) [108]. Even the most relevant theoretical "ΔH_{bind}" (G4 in 150 mM NaCl) was significantly different from the experimental value, so that computational values of the thermodynamics parameters should be viewed with caution.

One direct use of calculated ΔG could be to predict the stability of siRNA carrier complexes. The ΔG values could predict the cellular uptake of complexes since this event is closely tied to ability of carriers to form stable siRNA complexes for passage through lipid cell membrane. However, a more important feature of complexes might be their ability to dissociate. Liao *et al.* found experimentally that incorporating the anionic poly(γ-glutamic acid) (γ-PGA) into chitosan/siRNA complexes did not alter the complex formation ability between chitosan and siRNA, but enhanced the cellular uptake significantly [111]. The inclusion of γ-PGA greatly expedited the onset of gene knockdown, and enhanced the inhibition efficiency. MD simulations suggested that the chitosan/siRNA complex remained stable in the cytosol environment while the chitosan/siRNA/γ-PGA complex was disintegrated. The less stable chitason/siRNA/γ-PGA complex was proposed to facilitate intracellular release of siRNA and contribute to higher gene silencing efficiency. This avenue of research exploring complex stability is a fruitful avenue of research since dissociation seems to be critical for the activity of the delivered siRNA.

3. A mechanistic look at cellular delivery of siRNA complexes

Silencing a target mRNA can be achieved only after supramolecular siRNA complexes reach target cells, interact strongly with cell surfaces, proceed to be internalized and trafficked to appropriate cytoplasmic destination(s) for the siRNA to integrate into RISC complexes without hindrance of the carriers. The ability to navigate each sub-cellular stage contributes to the resulting silencing efficiency and it is critical to understand and optimize each step of this process. Although one is tempted to compare the efficiencies of various supramolecular complexes reported in the literature, it is practically impossible to undertake this task due to extensive variability in experimental parameters, such as the cell type employed, the intrinsic properties of siRNA and target mRNA (e.g., turn-over rate) and dose/duration of treatment. Nevertheless, we attempted to summarize two basic features of supramolecular complexes, namely size and ζ-potential, as well as the silencing potency (at both protein and mRNA levels) for a select set of studies with different carriers (Fig. 2). The size of complexes did not appear to drastically vary among carriers, where most complexes were typically ~200 nm or less (Fig. 2A). The ζ-potentials of complexes were usually positive (typically 0 to +40 mV; Fig. 2B), but some did exhibit negative ζ-potential. Most studies employed ≥100 nM siRNA in order to achieve effective silencing, a concentration range difficult to translate into pre-clinical and clinical settings (20–50 nM is preferred), but some carriers were effective at <100 nM siRNA (Fig. 2C). Not surprisingly, there is no correlation between the extent of silencing and effective siRNA concentration, owing to large numbers of uncontrolled variables among these studies. We recently conducted a similar analysis for silencing a specific target, P-gp, involved in multidrug resistance in cancer [112], and a large range of effective siRNA concentrations was also evident with various non-viral carriers for this specific case. It is not immediately clear as to why some carriers are functional at the desirable 20–50 nM range while others require >200 nM siRNA. Defining a minimal effective concentration for each delivery system will clearly identify promising carriers, but this has not been a common practice in the field. In some cases, effective siRNA concentrations were not clearly reported and, more importantly, scrambled siRNA/carrier complexes have been missing as treatment controls, a critical issue since any kind of cellular treatment is bound to give a response. Below, we investigate various steps involved in intracellular transfection pathway.

3.1. Cell surface binding

Rather than the interactions with individual components, cell surface interactions of the supramolecular complex as a whole are critical for effective entry. Sufficient binding strength is necessary to prevent dissociation of complexes at the cell surface interference from higher concentrations of competing polyelectrolytes [113], keeping in mind that the complex has to dissociate once in the cytoplasm. Charged carriers, such as cationic liposomes, polymers and CPPs, can interact with extracellular matrix components as well as proteoglycans and/or phospholipids at the cell surface (summarized for CPPs in [114]). Rather than the charge of cationic carriers, ζ-potential of the assembled siRNA/carrier complexes dictates the membrane interactions. The nature of charged moieties in a carrier and carrier:siRNA ratio used for supramolecular assembly are obvious determinants of the ζ-potential; however, other factors that promote or hinder the supramolecular assembly can affect the ζ-potential. We have seen this when lipid-modified polymers were employed for siRNA delivery (Fig. 3). The siRNA complexes with 2 kDa PEI gave little siRNA delivery across cell membranes and gave an overall charge close to neutrality; however, upon lipid modification of PEI, ζ-potential of complexes became positive and siRNA delivery efficiency was significant [115]. Lipid moieties presumably ensured a robust affinity among the assembled components under aqueous conditions.

Anionic species are not the obvious siRNA carriers, but they have been occasionally employed for siRNA delivery (Fig. 2). When polyglycerol-based dendrimers including a cationic dendrimer (+12.4 mV; polyglycerolamine) and anionic dendrimers (−2.2 to −0.614 mV; poly(glyceryl pentaethylenehexamine carbamate), PEI-PAMAM and PEI-gluconolactone) were utilized, the cationic dendrimer was more effective, demonstrating 50% silencing at carrier concentration over 4-fold less than the lowest anionic dendrimer with mid-range cytotoxicity [116] (note that a thorough optimization of complex charges and siRNA:carrier ratios was missing in that study). A targeting ligand (LHRH peptide) was also

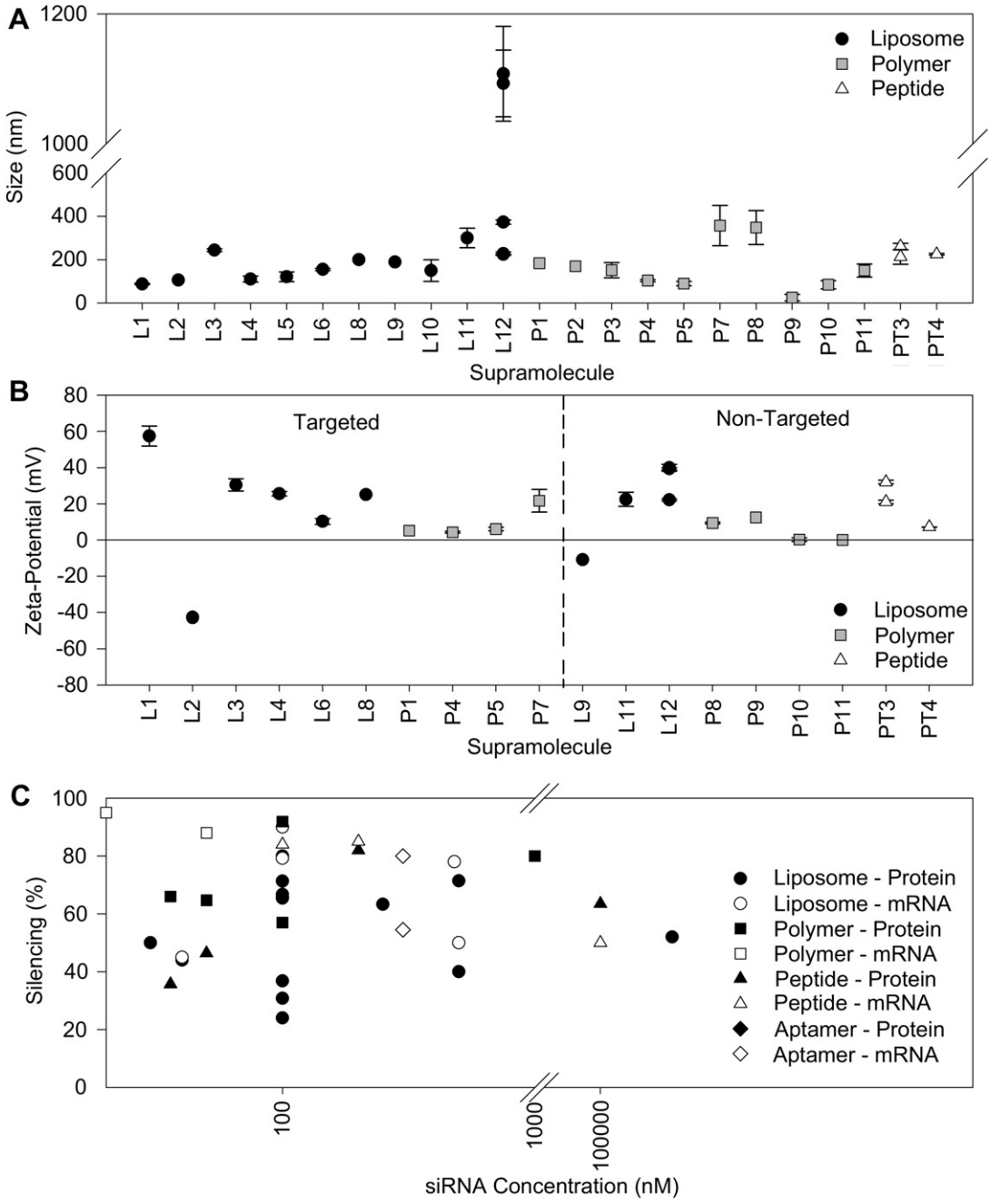

Fig. 2. A summary of select studies reporting on the size (A; error bars represent standard deviation or reported range), ζ-potential (B; error bars indicate standard deviation, if reported) and silencing efficiency as a function of siRNA concentration (C). The data were chosen from articles reviewed for this manuscript, where siRNA-mediated silencing was reported. Where appropriate, most potent carrier was selected among several carriers reported and some values were estimated from the provided graphs and/or calculated from others units and described methods. References: Liposomes (L): L1 [113], L2 [118], L3 [138], L4 [167], L5 [250], L6 [253], L7 [245], L8 [252], L9 [119], L10 [122], L11 [136], L12 [137]; Polymers (P): P1 [120], P2 [131], P3 [133], P4 [166], P5 [237], P6 [242], P7 [164], P8 [115], P9 [116], P10 [121], P11 [149]; Peptides (PT): PT1 [130], PT2 [239], PT3 [171], PT4 [155]; Aptamer (A): A1 [247].

required for neutral (+0.11 mV; internally cationic but surface neutral) PAMAM (85% quarternized-PAMAM-OH) dendrimer for silencing, but a high siRNA concentration (1000 nM) was needed even in this case [117]. Whereas cationic complexes do not necessarily require targeting ligands (although they were shown to be beneficial as articulated below), anionic ones usually do. Such effect is seen with a liposome-fusion phage protein (DMPGTVLP) system targeting PRDM14, where the liposomal system did not demonstrate silencing unless it was combined with the phage protein (40–50% silencing at both mRNA and protein levels) [118]. In case of liposomes formulated with a shortened GALA-peptide (for endosomal release), the anionic assembly (−11 mV) was supportive of silencing, but again at exceedingly high siRNA concentrations *in vitro* (480 nM) and at exuberant doses *in vivo* (4 × 4 mg siRNA per kg mouse weight) [119]. A targeting ligand could have been beneficial in this case and made the system effective at more practical siRNA concentrations. The ubiquitous interactions of cationic complexes, however, with soluble anionic species and non-target cells (and resultant uptake) are undesirable. A weak positive charge (<+5 mV) has been suggested as ideal to balance the

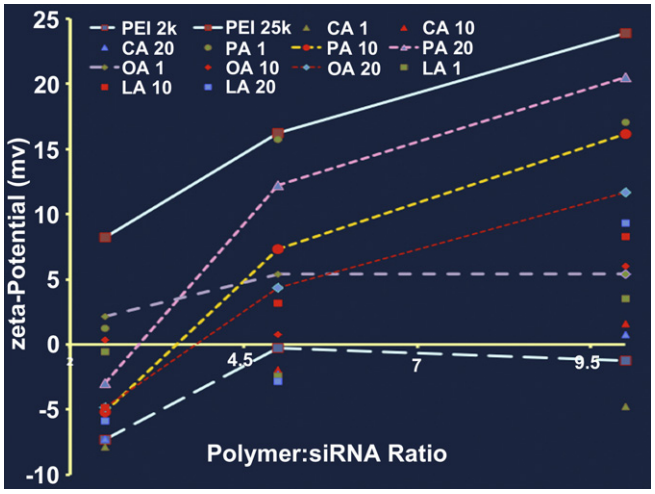

Fig. 3. ζ-potential of polymer/siRNA complexes for native PEI (2 and 25 kDa) and lipid-substituted 2 kDa PEIs. The complexes were formed at polymer:siRNA weight ratios of 2.5:1, 5:1 and 10:1 for ζ-potential measurements. Substituting the PEI2 with lipids increased the ζ-potential of complexes and brought it closer to the ζ-potential of the PEI25 complexes. The substituents on PEI are CA (caprylic acid), PA (palmitic acid), OA (oleic acid) and LA (linoleic acid), each substituted at three different ratios (indicated as 1, 10 and 20).

needed cell surface interactions while minimizing non-specific target carrier binding [120,121], as long as the propensity for complex aggregation at near neutral charge is addressed.

Hydrophobic moieties in supramolecular complexes should enhance cell membrane affinity non-specifically. Cholesterol has been incorporated into siRNA delivery systems by a variety of means for enhancing interactions with cell membranes. Cholesterol plays a role in many cellular membrane-related events such as membrane fusion, macropinocytosis, caveolin-mediated and lipid raft-mediated endocytosis [122], and cholesterol-containing carriers are expected to improve DNA/RNA transfection through an enhanced interaction with cell membrane [123]. Cholesterol conjugated to siRNA was reported to decrease serum degradation [124], improve siRNA pharmacokinetics and biodistribution, and enhance cellular uptake due to cholesterol interaction with lipoproteins. Cholesterol has been also shown to stabilize the liposomal structure [29] and act as a targeting moiety for liver cells [125]. Aliphatic lipids have been also used to functionalize otherwise non-efficient polymeric carriers (e.g., low MW PEIs) for nucleic acid delivery [126]. We initially speculated that the substituted lipids could enhance the interaction supramolecular complexes with lipid membranes, but subsequently realized the increased ζ-potential could be also responsible for increased siRNA delivery (Fig. 3). Our experiments have shown the functional silencing with select lipid-modified polymers against P-gp [115], Breast Cancer Resistance Protein [127], and survivin [128], three molecular targets whose expressions are changed in an undesirable manner in tumorigenic cells.

Incorporating cell surface binding ligands into supramolecular complexes is the preferred approach for generating effective and cell-specific binding. Ligands targeting endocytosed receptors, especially in the case of cancers where particular receptors are overexpressed, are preferred for improved internalization (as discussed in [129,130]). Ligand-mediated binding provides better internalization especially for shielded (e.g., PEGylated) complexes; amphiphilic surfactant and siRNA complexes demonstrated significant reduction in silencing due to substantial decrease in siRNA delivery when PEGylated, but the use of a targeting peptide (bombesin) enabled delivery and silencing at the pre-PEGylation levels [131]. However, targeting ligands could be prone to immunogenicity. Their targets could be low in abundance and display variability from patient to patient [132]. Typical ligands include endogenous molecules (e.g., carbohydrates), synthetic (e.g., phage-displayed derived) and natural proteins/peptides, and antibodies (Table 1). Positive bias is naturally expected in the disseminated studies with ligand-targeted complexes, where negative outcomes are likely under-reported. Increased cellular delivery by receptor-mediated binding is evident even for cationic supramolecular assemblies after incorporation of a ligand, as is the case of cationic DOTMA/DOPE liposomes modified with the K_{16}GACYGLPHKFCG peptide [113] and a CPP system (CPP-conjugated PLGA with spermidine/siRNA complexes) modified with folate [133]. Multimodal interactions that involve both receptor-mediated and non-specific binding to cell surfaces (e.g., by cationic species and CPPs) can enhance the overall cell association in this way [113,133]. It is important to note that the beneficial effect of ligands may not be always observed in certain contexts; (i) an RGD/PEG modified branched peptide was found effective at silencing *in vivo* unlike the *in vitro* studies [134], and (ii) a PAMAM-RGD carrier, where improvement in *in vitro* siRNA delivery and silencing was not observed with RGD functionalization, gave enhanced delivery when applied to an *in vitro* spheroid tumor model [135]. One has to be aware of this issue since promising systems could be dismissed under selective testing conditions and their true performance could only be manifested after testing in pre-clinical (*in vivo*) models [134].

3.2. Cellular internalization

Intracellular entry of supramolecular complexes may occur by direct transfer through cellular membranes or by energy-dependent membrane buddings known as endocytosis. In the latter case, the specific pathways include clathrin-mediated and clathrin-independent pathways such as caveolae-mediated, clathrin-independent, caveolae-independent, and macropinocytosis. All of these pathways were functional for siRNA internalization

Table 1
A selection of targeting ligands used with carriers for creating functionalized supramolecular assemblies. The compiled list was not meant to be exhaustive, but rather representative of the range of ligands used for facilitating cell surface interactions in siRNA delivery. The nature of specific carriers used for functionalization was also provided.

Category	Ligand	Nature of Carrier
Natural proteins and peptides	RGD	Peptide [134], polymer [135,166,237]
	TAT	Peptide [169], polymer [166]
	Bombesin	Polymer [131]
	LHRH	Polymer [117,238]
	Transferrin	Polymer [161]
	Rabies Virus Glycoprotein	Peptide [239]
	Hexapeptide (antagonist G)	Liposome [139]
Synthetic proteins and peptides		Liposome [113,118,138], polymer [240–242], fusion protein/peptide [130,243], aptamer [244]
Endogenous molecules	Folate/folic acid	Liposome [245], polymer [120,133,246], aptamer [247]
	Prostaglandin E2	Polymer [248]
	Anisamide	Liposome [167,249,250]
	Mannose	Polymer [164]
	Galactose	Liposome [251]
	Hyaluronic acid	Polymer [140]
Antibodies		Liposome [252,253], peptide [254]

depending on the specific siRNA carrier [122,136–140] and, although not completely understood, each pathway has distinct features and varied intracellular trafficking that then can affect the fate of complexes. Clathrin-mediated pathway follows the traditionally assumed pathway, where the complexes are trafficked from endosomes to lysosomes with a gradual drop in pH and ultimately exposure to degradative conditions. Complexes in caveolae-mediated pathway are directed to caveosomes with a less defined fate, but may escape the drop in pH and degradative conditions that are destructive to siRNAs. Macropinocytosis, also a regulated process, takes up a large amount of liquid by plasma membrane ruffles for intracellular trafficking at a slower speed as compared to other methods [141]. Determining the native and/or optimal endocytosis pathway followed by a supramolecular assembly is a challenging task. A clear consensus on the reliability of endocytosis inhibitors used in mechanistic studies is absent and one needs to optimize the inhibitors for each cell line studied (i.e., to ensure that the effects are not due to non-specific cytotoxicity on the cells) and to further validate the outcomes with additional inhibitors and/or independent methods [142]. Cells might utilize multiple pathways for internalization of same complexes, displaying rapid adaptation (or compensation) to experimental interventions [143,144]. Such an adaptation might be displayed as a function of siRNA dose, where low concentrations of complexes undergo clathrin-/caveolae-mediated endocytosis or macropinocytosis, and internalization becomes non-endosomal at high concentrations as commonly observed for CPPs [114]. A large number of physical characteristics of assembled complexes can affect the internalization method, including size, charge, presence of a ligand and polydispersity [144–147]. As the endocytosis characteristics can change depending on the payload (drugs, DNA or siRNA), we will focus our analysis solely on siRNA studies, which are few in number, but are beginning to provide insight for effective siRNA delivery.

Effective silencing may not result from the major endocytosis pathway employed, but from secondary pathways that may be more conducive for siRNA release into cytoplasm. This could be one reason why intracellular delivery percentages may not correlate with silencing efficiencies. In one study, siRNA formulated with cationic lipoplexes (DharmaFECT1) entered the cells mostly by endocytosis, but silencing was attributed to siRNA fraction that directly fused with the cell membrane [122]. Liposomal fusion in the case of DNA delivery was found to be undesirable, unlike the siRNA delivery in this case, obviating the efforts previously taken to optimize delivery with plasmid DNAs. Simple alterations in preparative procedures may greatly affect endocytosis pathways and resulting silencing efficacy. When siRNA was formulated with the cationic liposome LipoTrust^{TM}-ST (a mixture of O,O′-ditetradecanoyl-N-(α-trimethyl ammonioacetyl) diethanolamine chloride, DOPE and cholesterol) by vortexing, rather than by spontaneous assembly, decreased size of complexes (possibly due to less aggregation) gave better siRNA accumulation in cytoplasm due to a change in internalization from membrane fusion to clathrin-mediated endocytosis, along with increased silencing [136]. A contradiction is evident from the latter 2 studies on the optimal pathway for siRNA delivery. In another study, several cholesterol derivatives (amido- and carbamate-linked hydroxyethylated cationic cholesterol) were used for siRNA delivery to human PC-3 prostate tumor cells. Amido-linked complexes prepared by different methods led to different internalization pathways; the internalization of complexes prepared in water involved faster silencing kinetics via clathrin-mediated uptake and membrane fusion, whereas complexes prepared in 50 mM NaCl (resulting in larger complexes) gave slower and more effective silencing, and employed clathrin and caveolae-mediated endocytosis. The carbamate-linked complexes, on the other hand, displayed similar high silencing efficiencies under both conditions [137]. These studies highlight the importance of the physical nature (size, shape or elasticity) of the complexes rather than the chemical nature of supramolecular assembly. Aside from the usual variability in the experimental settings (cell type, mRNA target and size/charge of supramolecular assemblies), a 'universally' effective pathway for siRNA entry might be elusive. It is likely that nature of the supramolecular complex (especially the nature of carrier) might dictate the appropriate pathway [148]. However, the fact that one can alter or optimize the uptake pathway by adjusting simple preparation variables is encouraging in order to quickly identify the most efficacious pathway for silencing.

The nature of the ligand is expected to affect the endocytosis pathway. In one study, a novel IRQ-peptide grafted cholesterol/phosphatidylcholine liposome was compared to an $(arginine)_8$-grafted liposome (known to undergo macropinocytosis at high concentrations) for siRNA delivery in NIH3T3 fibroblasts [138]. The IRQ-peptide changed the internalization to caveolae- and clathrin-mediated endocytosis where a portion of the peptide was suggested to interact with caveolae and clathrin receptors. In another study, hexapeptide antagonist G-grafted cationic liposomes were used for siRNA delivery to small cell lung carcinoma (SCLC H69) cells [139]. The hexapeptide directed internalization by clathrin- and caveolae-independent mechanisms with possible small contributions from clathrin-mediated and macropinocytosis; however, no silencing was achieved with either the ligand-modified or unmodified liposomes and lack of caveolae-mediated pathway in the chosen cell line was suggested as a possible reason for this observation. With hyaluronic acid grafted onto hydrophobic amines and spermine (polymer micelle formulation), caveolae-mediated pathway was the major mode of internalization [140]. Although silencing was obtained by this delivery approach, the lack of a control siRNA in silencing studies does not allow a clear assessment of its efficacy. This literature indicates that directing endocytosis along the caveolae-mediated pathway is preferable to avoid late endosome/lysosome degradation. One can envision designing carriers whose supramolecular complexes with siRNA employ this desirable pathway.

3.3. Crossing lipid membranes for cytoplasmic release

The supramolecular complexes have to cross lipid membranes to gain access to cytoplasm for siRNA release. This can be achieved by non-contact mechanisms (such as inducing endosomal swelling) or by direct endosome membrane interactions leading to disruption or fusion. Carriers that exhibit non-physical contact often utilize H^+ buffering, a unique mechanism for endosomal escape. Also termed as 'proton-sponge effect', this mechanism has been initially recognized in the context of PEI [55]; protonation of PEI amines prevents the endosome from reaching the acidic pH needed for lysosomal nucleases and causes swelling of PEI/siRNA complexes. The influx of Cl^- to balance the H^+ influx causes osmotic swelling, eventually bursting the endosome to release the cargo [55]. Such a mechanism might occur with other carriers with similar buffering capacities, for example, with PAMAM-PEG-PLL carrier where PAMAM was intended to increase the buffer capacity for endosomal release, leading to significantly improved silencing [149].

Direct interactions causing membrane disruption, destabilization or fusion are the more straight-forward approach to penetrate cellular membranes. Membrane interaction with the lipid components of supramolecular complexes are paramount for this purpose and this can occur via a mechanism termed mesomorphic phase behavior: the cationic lipids form charge-neutral pairs with anionic lipids of cellular membranes, causing a localized change from the

usual lamellar structure to a hexagonal phase. The alteration in membrane structure along with carriers' cationic lipid components can allow for siRNA to pass through the membrane. Although details of this mechanism have not been completely elucidated, carriers were designed to promote this phase transition [150,151]. The cationic lipid carrier and anionic cell membrane interaction is dependent on the strength of the cationic charge of the carrier. Thus, the ionization constant (Ka) of the lipid headgroups can be optimized to promote the interaction. For endosomal escape, an amino lipid pKa within the range of 6–8 should allow for increased protonation at endosomal pH, thereby increasing membrane interaction and resulting crossing while minimizing interactions at physiological pH, that may lead to increased cytotoxicity or serum protein interactions [152]. Along the same lines, hydrophobicity of complexes has been found to increase silencing through lytic disruption of the cellular membrane. A diblock copolymer made up of butyl methacylate and propylacrylic acid (which gets protonated at endosomal pH and significantly elevates hydrophobicity of the carrier) [121] and hydrophobically-modified oligoethylenimine (with hexyl acrylate) [153] demonstrated increased hemolytic activity with increasing hydrophobicity content, which correlated with the siRNA activity. Peptides, such a CPPs and fusogenic peptides, can also mediate transfer across cellular membranes. Various membrane disruption mechanisms was attributed as the mechanism for peptide-mediated delivery, such as pore formation or rearrangement of the lipid bilayer [154]. Hydrophobic peptides, such as arginine [155], have been suggested to promote escape by fusion with endosomal membranes. Peptides are often used in conjunction with other carriers. Such designs include a liposomal siRNA delivery system utilizing the fusogenic peptide (GALA). The fusogenic peptide was introduced into the supramolecular complex because the PEG, intended for 'stealth' properties, also interfered with endosomal escape, thereby almost completely inhibiting silencing activity. The GALA undergoes a conformational change from a random coil structure due to the repulsion of negative charged-glutamic acid at physiological pH to an α-helix at low endosomal pH as the glutamic acid is neutralized, inducing membrane fusion, thereby increasing endosomal escape for subsequent silencing ability (summarized in [119]). How CPPs are incorporated into complexes can influence the functionalities of the CPPs and in some case diminish their effectiveness [156].

3.4. Transport within the cytoplasm

After achieving cytoplasmic entry, the siRNA must be available (dissociated from carrier) in sufficient quantities in order to silence the target mRNA. Competitive binding with the components of supramolecular complex can lead to desirable disassembly of electrostatically-held complexes. Anionic molecules such as cytoplasmic RNA (mRNA, tRNA, etc.) and glycosaminoglycans are thought to aid siRNA release [113,157], especially after the intra-complex interactions are weakened during endosomal escape due to interactions with lipid membranes [151]. A decrease in electrostatic binding among carriers and siRNA molecules can also occur during supramolecule swelling in endosome and changes in overall charge [113]. With a lipid-modified 2 kDa PEI library, the highly bound complexes, although they show efficient uptake, displayed decreased silencing compared to weakly bound complexes [115]. CPPs covalently bound to siRNA are not intended to dissociate, instead the linkage must be located appropriately as to not impede the RNAi mechanism; linkage at the 3′ end of the sense strand (passenger strand) of siRNA has been found to be optimal [156]. Rather than relying on of supramolecular disassembly with endogenous molecules, it is possible to design biodegradable carriers so that the complexes are disassembled by taking advantage of cleaving agents in the cytoplasm. Disulfide linkages are one such type of labile linkage that are susceptible to reducing environments for siRNA release. Cross-linking low MW PEI using agents such as dithiobissuccinimidylpropionate (DSP) and dimethyl-3,3′-dithiobispropionimidate (DTBP) [158], or 1,3- butanediol (or 1,6-hexanediol) diacrylates [159] has been reported as a strategy to create an efficient carrier with extensive disulphide (-S-S-) and amide (-C(=O)-N(H)-) linkages for degradation. The smaller building blocks will presumably clear in the body on their own without an adverse effect.

Once the siRNA is delivered to cytoplasm, comparing the amount of siRNA within the cell for target mRNA suppression (of similar targets) can provide us with a sense of carrier efficiency. Only a few studies with supramolecular complexes have provided clues on this aspect; MPGα-mediated (a CPP) siRNA required ~10,000 copies, the cationic liposomal Lipofectamine™ 2000 required ~300 copies and physical methods such as electroporation and microinjection required, respectively, ~400 and <300 copies of siRNA for 50% silencing; large variations in assessed silencing efficiency was evident in these studies (reviewed in [160]). This was indicative that the vast majority of siRNA copies in supramolecular complexes not being available for silencing. What happen to excess siRNA (and associated carrier) is an important issue, as well as elucidating the reasons for sub-optimal release. Determining the number of siRNA copies delivered per complex provides another perspective. A transferrin-targeted cyclodextrin system was suggested to contain ~2000 siRNA copies in a 70 nm nanoparticle [161]. Based on the estimated siRNA copies needed per cell, ~15% release of supramolecular assembled siRNA (300/2000) will be needed for 50% silencing. Timing from cell exposure to cytoplasmic detection is expected to depend on carrier features, among other variables, but delivery typically occurs fairly fast. Delivery within 0.5–6 h is typical for a range of carriers including a liposomal-targeting peptide system, cationic liposome (Lipofectamine™ 2000), dendrimer (polygycerolamine), linear PEI, micellar systems (PEO-b-polyester with RGD and/or TAT) and a peptide (arginine) carrier [113,116,162–166]. Significant silencing at the mRNA/protein levels occurs in the next 24–96 h, although the duration of silencing is not always reported. Duration of one week is an optimistic estimate, for example ~5 days for a targeted liposome system [167] and 6–7 days for the lipid substituted 2 kDa PEI [127].

Once the siRNA is available in the cytoplasm, RISC (including argonaute 2 and GW182) association is needed to direct the mRNA cleavage. The exact details of this process remain to be elucidated. It is reasonable to assume that intra-cytoplasmic targeting could improve efficiency, as mRNA [168], and possibly RISC components are asymmetrically located within the cytoplasm, leading to greater silencing and/or less siRNA required. However, as it is not known how RISC forms (i.e., which components initiate assembly and how do they form) or how it localizes to proximity of the target mRNA, targeting possibilities in the cytoplasm include components of RISC, specific cytoplasmic organelles and structures, or the location of target mRNA itself. Targeting P-bodies is one possibility, as it was found that when siRNA was delivered by Lipofectamine™ 2000, the siRNA localized to P-bodies (whose role in RNAi still remains unclear) prior to binding to RISC [163]. Various carriers including liposomes, peptide-targeted liposomes, siRNA/peptide complexes and dendrimers were found to localize to perinuclear region. Additionally, perinuclear localization has been observed to correlate with RNAi activity, suggesting that RISC, at least when activated, is located in this region [113,155,169]. If this is in fact the case, targeting microtubules may improve efficiency since they participate in shuttling of cargo between nucleus and cell periphery. An arginine and TAT-peptide delivery systems as well as liposomes

(Lipofectamine™ 2000) were found to localize to perinuclear region both in the absence and presence of an mRNA target (e.g., with luciferase, GFP, and endogenous CDK9) [155,169], suggesting that supramolecular complex targeting to the nuclear periphery is independent of the presence of mRNA [169]. Active delivery to mRNA targets or their general location is another approach to improve silencing; although variability in sub-cellular distribution of mRNA is noted, the reasons for asymmetrically distribution of mRNA is not well understood [155,168]. It is not clear how targeting could be achieved apart from the complementary pairing of the siRNA and the target mRNA. However, charge and lipophilicity may play a factor in intracellular localization; in CPPs designed for mitochondrial-penetration, lipophilicity and overall charge affected their intracellular localization (mitochondria vs. cytoplasmic) and nuclear localization [170]. In rare cases, when the siRNA target is in the nucleus, nuclear targeting can be utilized. In one study, siRNA against an essential promoter region of EF1A gene were trafficked to the nucleus by incorporating the nuclear targeting NLS peptide into CPPs, which achieved highly significant silencing [171]. Finally, the state of cellular physiology has been found to contribute to silencing efficiencies. Loss of RNAi function can occur due to cell stress causing the human argonaute 2 protein being re-located to stress granules, as was seen with the cationic liposome Lipofectamine™ 2000 [172]. Delivery methods should therefore minimize cytotoxicity and stress related factors not only for off-target effects on other cells, but to ensure that the RNAi system targeted remains functional. Half-life of the target protein (i.e., its rate of synthesis) is another factor influencing silencing; efficient silencing will occur with proteins produced in low quantity with short half-lives – i.e., a siRNA residence time 3 fold higher than the half-life of the protein target is desirable [173].

4. In vivo siRNA delivery

Considering the obstacles for siRNA delivery, it is not surprising that effective delivery systems require significant structural design and multiple functional moieties to fulfill different tasks. This is especially true when siRNA delivery is attempted in animal models and clinical studies, where additional measures have to be considered to address organ-wide obstacles. The choice of the administration route is the foremost consideration. A close match between the pathophysiology of the disease and the pharmacokinetics of siRNA obtained from a given route needs to be considered. The stability of siRNA in body fluids is the next consideration and measures to protect siRNA by chemical modification as well as physical barriers have to be considered. A major concern for any systemically administered supramolecular complex is uptake and elimination by the RES, mainly macrophages in blood circulation and Kupffer cells in liver [174]. When stability of these complexes is limited and interactions among the complexes lead to aggregation, large particles will be quickly removed by the RES. A desirable feature of siRNA delivery is target specificity and active along with passive targeting have been relied on for siRNA delivery, similar to other nanoparticulate delivery systems. The intent is to concentrate the siRNA in target cells for increased potency. Active targeting takes advantage of a moiety that seeks, recognizes and attaches to cell-specific surface markers. Attaching the targeting molecule directly to the siRNA itself is one approach for this end, assuming that siRNA functionality is not jeopardized. Alternative strategies have been investigated for attaching targeting ligands onto siRNA carriers [27,62,175]. The final obstacle is the extracellular matrix, whose dense and charged structure offers a difficult path of reach cell surface. The following sections explore these considerations in more detail to analyze the means for optimum delivery of siRNA *in vivo*.

4.1. Administration route

The siRNA delivery has been attempted through topical (including intratumoral, intravitreal, or intrathecal injection, and inhalation route for lung delivery) and parenteral routes, as well as oral administration (Tables 2 and 3). A local route of administration, if clinically feasible, usually ensures maximum siRNA accumulation at the site of action and eliminates concerns related to systemic exposure and non-specific distribution of supramolecular complexes. Intratumoral injection is one local route that can be employed for accessible tissues (e.g., melanomas) and it has been widely used as “proof-of-concept” in pre-clinical studies exploring the feasibility of siRNA delivery, either as the sole route [176], or supplementary to IV injection (Table 2). Studies based on intratumoral injection of siRNA typically lack any pharmacokinetic or biodistribution data (due to limited presence of siRNA in blood circulation) and were excluded from Table 2. Intravitreal injection of siRNA has been employed for treatment different types of age-related macular degeneration (AMD). The efficacy of this strategy was first evaluated in animal models of ocular neovascularization and scarring by silencing VEGF [177] or VEGF receptor (VEGFR) [178]. This strategy entered the clinical trials (Table 4). Intrathecal injection of siRNA to silence pain-related cation-channel P2X3 for treatment of chronic neuropathic pain [179] and to silence delta opioid receptor (DOR) to treat nociception [180], intratesticular injection of FGF-4 siRNA to inhibit tumor growth [53], subcutaneous injection near tumor sites to silence bcl-2 [181], intranasal administration of siRNA against different targets for treatment of pulmonary conditions [1] and intravaginal administration of siRNA targeting HSV-2 to protect against herpes simplex virus (HSV) infection [182] have been reported with some success.

IV injection seems to be the prominent route of administration, and most practical for clinical treatments of systemic pathophysiology. Due to immediate exposure to all organs, however, this delivery route may pose significant toxicities. Several studies have reported higher siRNA accumulation in liver, kidney or lungs when associated with clinically feasible carriers (e.g., in treatment of Hepatitis B virus using a SNALP formulation; [183]). Intraperitoneal (IP) injection has been performed as an alternative to IV injection for systemic delivery. The siRNA uptake and efficacy were comparable to IV injection with a liposomal delivery system in this case [184]. The IP injection could lead to a ‘depot effect’ compared to the immediate availability associated with bolus IV injection [185]. This depot effect could minimize the risk of toxicity associated with some carriers. Although ease of administration, less volume restrictions, and better reproducibility are advantages of IP route in pre-clinical testing, the IP route is less practical in a clinical setting.

4.2. Stability in systemic circulation

The two strategies to improve stability *in vivo* are chemical modification of siRNA (reducing nuclease specificity) and physical protection provided by supramolecular complexes (preventing nuclease access). Attempts on siRNA modification aim to delay its enzymatic degradation without jeopardizing its silencing efficiency. It has been shown that nuclease stability can be achieved by introducing a phosphorothioate (PS) backbone linkage at the 3′-end for exonuclease resistance and a 2′ modifications (2′-O-methyl and 2′-fluoro) for endonuclease resistance [186], without significantly affecting the silencing capability [187]. On the other hand, modification at the 5′-end of the antisense strand seems to decrease the silencing activity more than modification at the 3′-end; the 2′-F residue is well tolerated on the antisense strand, whereas the 2′-O-methoxyethyl modification on either strand leads to loss of activity [175]. PEG grafting to siRNA has been also

Table 2
Select siRNA delivery studies in pre-clinical animal models. Studies that reported pharmacokinetics and organ targeting data were highlighted in this Table. The studies were categorized based on the route of administration and broken down to indicate the type of carrier used for siRNA delivery.

Route of administration	Delivery System	Protein Target	Animal model	Therapeutic Target	PK/Biodistribution data	Effect (side effect)	Ref.
IV Injection	Peptide (Atelocollagen)	EZH2 p110-α	Athymic nude mice	Bone metastasis	↑siRNA in tumor, liver, lung, liver, kidneys	↓ metastasis (None)	[255]
	Peptide (Derived from RVG)	JEV	NOD/SCID mice	Viral encephalitis	↑siRNA in brain	↑Survival Rate (NR)	[204]
	Cationic Liposome (DOPE, RPR209120)	TNF-α	DBA/1 mice	Arthritis	↑siRNA in liver and spleen	↓Collagen-induced arthritis	[36]
	PEGylated cationic Liposome and peptide	MDM2 VEGF	C57BL/6 mice	Lung cancer	↑Tumor delivery	↑Survival Rate (None)	[256]
	PEGylated cationic Liposome and peptide	Luciferase (model)	C57BL/6 mice	Lung cancer	↑Tumor accumulation (not spleen or kidney)	NA (None)	[167]
	Neutral Liposomes and protamine	Cyclin D1	C57BL/6 mice	Colitis	↑siRNA gut levels in presence of colitis	Reversal of colitis (None)	[257]
	Polymer (PEI-PEG-RGD)	VEGF R2	Nude mice	N2A Tumor	↑Tumor delivery (compared to PEI)	↓Tumor growth (NR)	[237]
	Various models	NA	Balb/c mice	NA	↑blood circulation and lung accumulation	NA	[124]
	Liposome (DOPC)	EphA2	Athymic nude mice	advanced ovarian tumor	↑siRNA in liver, kidney, and lungs	↓Tumor growth (None)	[28]
	SNALP (containing DSPC)	HBV	A/J mice	HBV infection	↑blood circulation and liver levels	↓serum HBV DNA (None)	[183]
	Galactosylated liposomes (DOPE)	Ubc13	ICR mice	NA	↑blood circulation and liver, kidney, and lungs	NA (IFN Induction)	[32]
	Cationic liposome (CCLA)	c-raf	BALB/c mice	Human breast cancer	↑siRNA in lungs	↓Tumor growth (Toxic with MD‡)	[38]
	Liposome (DSPE-PEG-Protamine)	EGFR	Athymic nude mice	Lung cancer xenograft tumor	↑t1/2, AUC, MRT, tumor siRNA ↓CL	↓Tumor growth (signs of toxicity)	[190]
IV & Intratumor Injection	jet-PEI Cationic Liposome	RecQL1	BALB/c nu+/+ mice	Hepatic cancer	↑in liver (no change in circulation time)	↓Tumor growth (None)	[258]
	Polymer (PEI-PEG)	VEGF	Nude mice	Prostate carcinoma tumor	↑Tumor delivery and circulation time	↓Tumor growth (NR)	[188]
	Peptide (MPG-Cholesterol)	Cyclin B1	Athymic nude mice	SCK-3-HEK2 Tumor	↑ blood circulation	↓Tumor growth (None)	[82]
IV & SC Injection	Cationic liposome (LIC-101)	Bcl-2	BALB/c nu+/+ mice	Liver metastasis and prostate cancer	NR	Strong antitumor activity	[181]
IP Injection	Liposome (Phospholipid DOPC)	FAK	Athymic nude mice	Ovarian cancer tumors	NR	↓Tumor weight (None)	[31]
	Liposome (DOPC)	IL-8	Athymic nude mice	Ovarian cancer tumors	NR	↓Tumor weight (None)	[259]
	Polymer (JetPEI)	HER-2	Athymic nude mice	Ovarian Carcinoma	↑siRNA in muscles, liver, kidney, tumor	↓Tumor growth (NR)	[185]
Oral	Polymer (PEI - β 1, 3-D-glucan)	TNF-α & Map4k4	C57BL6/J mice	Systemic inflammation	↑siRNA in MPs, spleen, liver, and lungs	↓in TNF-α levels (None)	[260]

AUC: Area under the serum concentration vs. time curve; CCLA: Cationic cardiolipin analog; CL: Clearance; EGFR: Epidermal growth factor receptor; EZH2: Zeste homolog 2; FAK: Focal adhesion kinase; HBV: Hepatitis B virus; HER-2: c-erbB2/neu; IP: Intraperitoneal; IV: Intravenous; IL-8: Interleukin-8; IP: Intraperitoneal; JEV: Japanese Encephalitis Virus; MD: Multiple dosing; MRT: Mean residence time; NA: Not Applicable; NR: Not Reported; p110-α: Phosphoinositide 3′-hydroxykinase p110-α-subunit; PEG: Polyethylene Glycol; PEI: Polyethylenimine; PLK-1: Polo-like kinase 1; RVG: Rabies Virus Glycoprotein; SC: Subcutaneous; $t_{1/2}$: half-life in serum; TNF: Tumor Necrosis Factor; VEGF: Vascular Endothelial Growth Factor; VEGF R2: Vascular Endothelial Growth Factor Receptor 2.

‡ 100 mg/kg of CCLA-based liposome for 3 consecutive days caused 33.3% mortality.

Table 3
Select siRNA delivery studies in pre-clinical animal models where active targeting was attempted. Representative studies that employed different targeting ligands were highlighted in this Table.

Targeting ligand		Delivery system	Route of administration	Targeted tissue	Protein for silencing	Ref.
Antibody	BsAb against EGFR	Minicells	IV Injection	Xenograft Tumor	PLK1 or KSP	[261]
	ScFv against CD7	$(arginine)_9$ peptide	IV Injection	T cells and HIV Virus	CD4 and CCR5	[262]
	TfRScFv	Liposome	IV Injection	Xenograft Tumor	HER2	[208]
	Ab against LFA-1	Protamine	IV Injection	Activated Leukocytes	NA (CY3-siRNA)	[254]
Peptide	Cyclic RGD	PAMAM	NA	Tumor cells	EGFP	[135]
	RGD	PEG-PEI	IV Injection	Xenograft Tumor	VEGF R2	[237]
	RGD	EHCO-PEG	IP Injection	Xenograft Tumor	HIF-1α	[263]
	Transferrin	Cyclodextrin	IV Injection	Xenograft Tumor	Luciferase	[52]
Aptamer	Oligonuleic acid (Binds to PSMA)	None-siRNA conjugate	Intratumoral Injection	Xenograft Tumor	PLK1 and BCL2	[264]
	Oligonuleic acid (Binds to PSMA)-PEG	None-siRNA conjugate	IP Injection	Xenograft Tumor	PLK1	[189]
	Oligonuleic acid (Binds to CD4)	None-siRNA conjugate	Vaginal	$CD4^+$ T cells	CCR5	[265]
Ligand	HA	PEI	Intratumoral Injection	Xenograft Tumor	VEGF	[266]
	Folate	PEG-DSPE-F	Intratumoral Injection	Xenograft Tumor	HER 2	[210]
	Galactose	DOPE-DOPC	IV Injection	Hepatocytes	Ubc13	[32]
	Anisamide	DSPE-PEG	IV Injection	Xenograft Tumor	EGFR	[190]

Ab: Antibody; BCL2: B-cell lymphoma 2; BsAb: Bispecific Antibody; CCR5: C-C chemokine receptor type 5; DSPE: distearoylphoshatidylethanolamine; EGFP: Enhanced Green Fluorescent Protein; EGFR: Epidermal growth factor receptor; EHCO: (1-aminoethyl) iminobis[N-(oleicylcysteinylhistinyl-1-aminoethyl)propionamide]; HA: Hyaluronic acid; HER2: Human Epidermal growth factor Receptor 2; HIF-1α: Hypoxia inducible factor-1α; IP: Intraperitoneal; IV: Intravenous; KSP: Kinesin spindle protein; LFA-1: Lymphocyte function-associated antigen-1 PAMAM: Poly(amidoamine); PLK1: Polo-like kinase 1; PSMA: Prostate-specific membrane antigen; TfRScFv: Anti-Transferrin receptor single-chain antibody fragment; Ubc13: Ubiquitin conjugating 13; VEGF R2: vascular endothelial growth factor receptor-2.

reported for enhanced stability [188]. PEGylation of a siRNA/aptamer conjugate increased the serum $t_{1/2}$ of siRNA from <35 min to ˃30 h after adding PEG at the 5′-end of non-coding siRNA strand [189]. Steric protection of the siRNA against nucleases as well as other molecules responsible for siRNA clearance from the circulation presumably led to this observation.

Although physical protection of siRNA in supramolecular assemblies with oppositely charged carriers is effective, the risk of dissociation after administration that will leave siRNA susceptible to enzymatic degradation is a concern. Several factors could affect the propensity of complex dissociation, such as the charge density and distribution in carriers, the molecular weight of carriers, and the possibility of ionic interaction with other charged moieties such as proteoglycans and other polyelectrolytes including cellular DNA and RNA molecules. Supramolecular packaging of siRNA into complexes is most beneficial in avoiding renal elimination and hepatic uptake. An increase in the mean residence time (MRT) or AUC and a decrease the clearance (CL) of siRNA are expected pharmacokinetics benefits [183]. Li *et al.* explored siRNA delivery against epidermal growth factor receptor (EGFR) using a liposomal system containing distearoyl phosphatidylethanolamine (DSPE), PEG, and protamine (as targeting ligand) via IV injection [190]. The study showed ˃15-fold increase in AUC, ˃100-fold increase in MRT, ˃7-fold increase in volume of distribution in steady state (VD_{SS}), and ˃15-fold decrease in CL for the targeted liposomes compared to free siRNA in mice. A higher tumor accumulation of siRNA was expected with the targeted liposomes, and the increase in VD_{SS} could be attributed to the additional distribution "sink" provided by tumor for the targeted delivery (same increase in VD_{SS} was not observed for the targeted delivery system in tumor-free animals). In fact, the tumor concentration of siRNA was reported as 70–80% of the dose/g of tissue for liposomal delivery compared to ~10% for the free delivery. The siRNA concentration in lungs was doubled for liposomal system (indicating retention in pulmonary vascular bed), while kidney, spleen and liver concentrations did not show a significant difference when compared to free siRNA.

4.3. Immune recognition

Immunogenicity has been a constant concern for both siRNA and some carriers. Certain motifs in siRNA are immunogenically sensitive; however, the risk of immune response by siRNA is significantly less than plasmid DNA, and could be even further decreased by incorporation of 2′-O-methyl modifications [191]. This off-target effect could be advantageous to exploit immune stimulation as an adjuvant to RNAi-mediated silencing [192] in cancer therapy. Even though siRNA complexation with carriers may conceal the reactive motifs, immunogenic reactions have been reported for monoclonal antibodies (used as targeting moiety in siRNA carriers, [29]), CPPs (affecting the innate immune system through interactions with the Toll-like receptor pathway; [193]), and anionic lipids integrated in liposomal structure (due to the negative charge [194]), and cationic lipoplexes [195]. While PEGylation can minimize the RES uptake of siRNA by shielding the surface charges of particles, and may lower immune reactions, PEG itself could be immunogenic after repeated administration and it can promote antibody, especially IgM, responses [196].

4.4. Biodistribution and target delivery

Supramolecular carriers of siRNA are intended to alter biodistribution of siRNA by limiting its deposition at non-target organs and enhancing siRNA concentration at the site of action. This is normally achieved through active and passive targeting strategies.

4.4.1. Passive targeting

Passive targeting takes advantage of the tendency of particulate carriers to accumulate in a specific organ due to characteristics of the particle or the unique physiology of the tissues. The particle size and particle surface features are keys for this behavior. An effective siRNA delivery system needs to be large enough to avoid extravasation from the continuous capillaries to minimize distribution throughout the body. At >10 nm, particles become large enough to avoid elimination by kidneys but particles >100 nm are easily uptaken by RES,

Table 4
A summary of clinical studies conducted with siRNA therapeutics (last updated in October/2011). The studies were categorized based on the type of delivery system used for clinical application. The target protein chosen was indicated along with the outcome of the clinical study. Published studies were referenced where appropriate but outcome from private web sites or clinical database was employed.

Carrier Category	Product name (Carrier)	Administration route	Target protein	Disease	Trial phase	Results	Ref.
Naked or Modified siRNA	Bevasiranib	Intravitreal Injection	VEGF	Exudative AMD; Diabetic macular edema; Wet AMD	III (Terminated)	Well tolerated,"Unlikely to achieve its primary endpoint"	[267]
	AGN211745 or siRNA-027	Intravitreal Injection	VEGF Receptor-1	Wet Age-Related Macular Degeneration	II (Terminated)	Well tolerated, Foveal thickness decreased within 2 weeks	[175]
	RTP801i-14 or PF-655	Intravitreal Injection	RTP801 (DDIT4)	Diabetic Macular Edema; Wet AMD	II	Well tolerated Higher doses needed	[24]
	QPI-1002 or I5NP	IV Injection	P53	Acute Kidney Injury Kidney transplant	II	Well tolerated	[218]
	ALN-RSV01	Intranasal or inhalation	RSV Nucleocapsid	Respiratory syncytial virus (RSV)	IIb	Well tolerated, Significant antiviral activity	[268]
	TD101	Intradermal Injection	Keratin 6a	Pachyonychia congenita	Ib	Well tolerated Regression of callus	[221]
Stable nucleic acid lipid particles (SNALPs)	Atu027 (Multiple Lipids)	IV Infusion	Protein kinase N3	Solid Tumors	I	Well tolerated Inhibition of metastasis	www.silence-therapeutics.com
	PRO-020401 (Multiple Lipids)	IV Infusion	ApoB	Hypercholesterolemia	I	Well tolerated Improvements required	[40]
	ALN-VSP (Multiple Lipids)	IV Injection	KSP, VEGF	Advanced solid tumors with liver involvement	I	Well tolerated Signs of VEGF silencing	[224]
	ALN-TTR01 (Multiple Lipids)	IV Injection	Transthyretin	Transthyretin-mediated amyloidosis (ATTR)	I	Silencing in pre-clinical studies	[226]
	TKM-080301 (Multiple Lipids)	IV Injection	PLK1	Solid Tumors	I	Significant efficiency in animal models	tekmirapharm.com
Liposome	BCR-ABL siRNA	IV Injection	bcr-abl	Chronic myeloid leukaemia	I	Well tolerated Apoptosis of CML cells	[228]
Viral	SV40/BCR-ABL	NA	bcr-abl	Chronic myeloid leukaemia	I	Data not reported	Clinicaltrials. gov
Poly-cationic Polymer	CALAA-01 (Cyclodextrin)	IV Infusion	RRM2	Solid Tumors	I	Side-effects, but no dose-limiting toxicities Significant silencing	[269]
EP[a]	Dendritic cells	Intradermal injection of dendritic cells	IP beta subunits LMP2, LMP7 and MECL1	Metastatic melanoma	I	Recruiting for the Phase I studies	[270]

AMD: Age-Related Macular Degeneration; DDIT: DNA-damage inducible transcript; EP: Electroporation; IP: Immunoproteasome; KSP: Kinesin Spindle Protein; NA: Not Available; RRM2: Ribonucleotide reductase M2
[a] Cationic polymers used to transfect the cells with tumor antigen RNA.

resulting in larger concentrations in spleen. On the other hand, particles in the 10–100 nm range (especially <70 nm) are expected to easily perfuse through fenestrated capillaries in liver and show higher accumulation in this organ [26]. Therefore, size of the particles could be manipulated to modify biodistribution of siRNA.

Surface charge (or ζ-potential) of the particles is another important factor for biodistribution. A neutral surface charge could facilitate aggregation of the particles (due to lack of repulsing electrostatic forces) and expedite rapid clearance from circulation. Although cationic siRNA/carrier complexes display significant repulsion before *in vivo* administration (and form stable suspensions), they could absorb serum albumin and other anionic proteins in circulation and form clot-like accumulations in the blood [197]. A variety of serum proteins, also known as opsonins, can adsorb to complexes, which triggers recognition by macrophages and Kupffer cells, rapid clearance and accumulation in spleen and liver [198]. PEGylation seems to be an efficient strategy to minimize particle aggregation and opsonisation due to stealth properties of hydrophilic PEG shells. It has been shown that PEGylation could increase blood residence time of the particles and minimize RES uptake [199], increase $t_{1/2}$ and enhance plasma AUC (i.e., decrease in clearance and/or the volume of distribution) [87].

Passive targeting in tumors is a natural consequence of prolonged circulation time and enhanced permeability and retention (EPR) effect. The goal in tumor targeting is to minimize extravasation of particles through continuous capillaries of normal tissues and fenestrated capillary of liver, and to survive RES uptake and renal filtration, so that a maximal siRNA is deposited at tumor sites. The EPR effect is caused by the increased permeability of tumor vasculature, and lack of lymphatic drainage in solid tumors that normally remove macromolecules from normal tissue. The permeability of the vasculature in the tissue, due to inflammation and cancerous growth, is abnormally high, since rapid and unorganized formation of new blood vessels will allow extravasation of particles that would not normally pass through established endothelial layers [200]. Angiogenesis is stimulated by hypoxia of tumor cells, among other factors, that activates many genes, including VEGF and its receptors [201]. Formation of fenestrated (with transendothelial circular openings of 40–80 nm) and discontinuous capillaries (with openings of 100–1000 nm) in tumor capillaries is the main reason for the enhanced permeation of tumor vasculature [202]. In fact, many *in vivo* studies on tumor treatment with siRNA have reported higher tumor concentrations based on passive targeting (Table 2).

4.4.2. Active targeting

This strategy is based on the presence of a moiety that specifically seeks and binds to particular cells or tissue components. The key to an efficient delivery is stable association of the targeting moiety with the carrier, and the presence of a specific and overexpressed target at the desired site. The targeting moiety on the carrier could be peptides, antibodies, other endogenous molecules and synthetic aptamers (nucleic acid or peptides that are selected from a random library to bind to a specific target molecule) [62] (Table 3). Targeting moiety could be conjugated to siRNA itself, as reported for: (1) a luciferase-specific siRNA conjugated to cyclic RGD [203], (2) antiviral FvE[J] siRNA conjugated to rabies virus glycoprotein [204], (3) IGF-1 conjugated siRNA for increased cellular uptake *in vitro* [205], and (4) siRNA conjugated to rabies virus glycoprotein (RVG), which specifically binds to the nicotinic acetylcholine receptor expressed by neuronal cells, resulted in specific gene silencing within the brain [204]. siRNA conjugates of a prostate-specific membrane antigen as the aptamer [206], TAT [169], and a monoclonal antibody against transferrin receptor [207] have been also reported.

Different types of antibodies and antibody fragments have been used as a targeting moiety with considerable success. Immunoliposomes have been reported to target siRNA to xenograft tumors in animal models [208]. CPPs have been both conjugated directly to siRNA, or integrated into the delivery system as targeting moieties [78]. The RGD peptide has been a popular choice, and has been conjugated to different polymers for siRNA delivery (Table 3). Aptamers have been conjugated to siRNA to target cellular receptors and PEG addition to aptamer/siRNA conjugates created stealth supramolecular assemblies and increased the siRNA AUC in blood [189]. The folic acid receptor has been recognized as a marker for ovarian carcinomas and frequently overexpressed in a wide range of tumors [209], making folate a useful ligand for tumor targeting [210]. Galactose has been evaluated for targeted delivery to hepatocytes, which overexpress galactose-binding asialoglycoprotein receptors [211]. Other ligands for active targeting of siRNA include hyaluronic acid and anisamide (Table 3).

Adding ligands to siRNA complexes may lead to enhanced potency due to increased deposition in tumors as well as increased cell uptake. Bartlett *et al.* reported an *in vivo* study with particles formed by cyclodextrin and luciferase siRNA in NOD/SCID mice bearing luciferase-expressing Neuro2A xenografts [52]. Although similar biodistribution and tumor accumulation was observed for non-targeted and transferrin-targeted particles, targeted siRNA reduced the luciferase activity in xenografts to ~50% of the activity obtained by the non-targeted formulation after one day of IV injection. The authors concluded that the transferrin was beneficial in cellular uptake and, therefore, in silencing, by improving the association of the siRNA-containing particles with the target cells.

4.5. Extracellular matrix (ECM)

After extravasation, siRNA complexes need to navigate through ECM before cellular contact. ECM is a non-cellular tissue component that form a scaffold around the cells, and with a composition that is slightly different for each organ [43]. Two main ingredients of ECM are proteoglycans (PGs) and fibrous proteins. PGs are composed of sulphated and non-sulphated glycosaminoglycan chains and a protein core (except hyaluronic acid) [212]. The main fibrous proteins are collagens, elastins, fibronectins and laminins [213]. Some of ECM ingredients such as sulphated glycosaminoglycans are negatively charged, and could interact and sequester cationic siRNA complexes, or compete with anionic siRNA for carrier binding, destabilizing the carrier/siRNA assembly. In both cases, ECM could decrease the concentration of siRNA available for cell uptake. This effect has been shown for PEI based carriers even after PEGylation [214]. Since entrapment of siRNA particles in the fibres of ECM is size dependant, this might be a more significant issue for larger particles. CPPs, however, have been shown to be able to penetrate the ECM before cellular internalization [215].

5. Clinical studies employing siRNA therapies

Despite intense RNAi efforts since its discovery, siRNA has only recently entered the clinical setting (Table 4). Delivering naked or modified siRNA without a carrier accounts for most common approach, while employing SNALPs is a close second. The reason for omitting carriers, even though they were indispensible in preclinical studies, is presumably the desire to minimize exogenous agents in the body. Among the studies aimed at delivering siRNA without carriers, three are focused on AMD, where two of them target VEGF. Angiogenesis has been implicated in the pathogenesis of AMD and VEGF seems to be a critical component in this process. In pre-clinical studies, siRNA delivery significantly lowered VEGF-A production in response to hypoxia [216]. Bevasiranib is a double-

stranded RNA formed by the hybridization of two partially complementary RNAs in which the 3′-end are capped with 2-deoxyribose (dT) units [216]. It was recently evaluated in a Phase II study and, although no serious adverse effects were observed, the mean visual acuity of patients receiving all doses of Bevasiranib declined and the average lesion size increased [216]. The Phase III clinical trial of Bevasiranib was terminated in 2009 based on the recommendation that it was unlikely to achieve its primary endpoint of reducing vision loss. Among the viable ways to improve the efficacy of Bevasiranib is enhancing delivery with effective siRNA carriers. Silencing the VEGFR is another approach to inhibit VEGF action and studies with murine models demonstrated an effective reduction of VEGFR-1 [178] by using AGN211745, a siRNA targeted specifically against VEGFR-1 and not VEGFR-2. Intravitreal injection of a single dose of AGN211745 from 100 to 1600 μg was well tolerated in AMD patients, with no dose-limiting toxicity. Adjusted mean foveal thickness decreased within 2 weeks after study treatment. The decrease was most pronounced in the 100 and 200 μg doses, and not the highest doses [217]. However, the sponsor (Allergan) terminated Phase II trial in 2009 and its development after the drug failed to meet a key efficacy endpoint.

The RTP801 was evaluated for its potential role in AMD progression, since its expression seems to be rapidly up-regulated in response to ischemia, hypoxia and/or oxidative stress [24]. A Phase II study, conducted by Quarkpharma Inc., involving siRNA delivery against RTP801 was terminated 12 months after the study onset, based upon interim analysis suggesting that higher doses would be necessary to produce a therapeutic effect sufficiently superior to current standard of care, given emerging new therapeutic modalities. The siRNA was delivered without a carrier in this study as well.

One of the siRNAs in clinical trials, QPI-1002 or I5NP, is modified by 2′-O-methylation on alternating positions on both strands of siRNA, and is intended to block ischemic acute kidney injury by targeting the pro-apoptotic p53. In pre-clinical studies, naked p53 siRNA injected IV 4 h after ischemic injury maximally protected proximal tubule cells (site of oligonucleotide reabsorption within the kidney) and maintained kidney function [218]. Phase I studies indicated acceptable safety profile in both post-cardiovascular surgery and deceased donor renal transplantation. Another Phase I trial with a modified siRNA (ALN-RSV01) was started in 2006 using minimally modified, un-encapsulated siRNAs to treat RSV (respiratory syncytial virus) infection using intranasal nebulisation [24]. Interim reports suggest that subjects receiving ALN-RSV01 that targets the RSV nucleocapsid experienced a 38% reduction in infection rate [219]. Finally, naked siRNA has been delivered in pachyonychia congenital disorder that primarily affects the skin, nails and mouth, causing blistering on the hands and feet, mouth sores and cysts of various types. It is caused by mutations in keratin (K) genes and a siRNA (TD101) that selectively inhibits a mutant allele of K6a was described [220]. The results of a single-patient 17-week dose escalation trial have shown regression of callus on the siRNA-treated foot [221].

Several clinical studies on siRNA delivery by using SNALPs have been attempted. The Atu027 siRNA delivery system is derived from a cationic, a neutral and a PEGylated lipid, where a siRNA against protein kinase N3 was incorporated onto the liposome shell by ionic interaction [222]. Protein kinase N3 is involved in tumor metastasis, interferes with the endothelial lining of tumor blood vessels, causes cell migration, and reduces the oxygen supply of the tumor. After IV injection, Atu027 was shown to efficiently inhibit the growth of PC-3 xenografts in an orthotopic mouse model and to decrease the number of lymph node metastases [223]. A Phase I study evaluated the safety, tolerability and pharmacokinetics of Atu027 in patients with colorectal cancer metastasizing to the liver [224]; the industrial sponsor (Silence Therapeutics) reported "*preliminary data show disease stabilization and other indications of potential efficacy in cancer patients with advanced solid tumors.*"

A similar delivery system has been used for silencing of Apolipoprotein B (ApoB), an essential protein for assembly and secretion of Very-low-density lipoprotein (VLDL) and Low-density lipoprotein (LDL) [225]. A SNALP formed by cationic, fusogenic, and PEGylated lipids was designed to target ApoB gene in hypercholesterolemia. Pre-clinical studies in cynomolgus monkeys demonstrated significant reductions in ApoB protein, serum cholesterol and LDL levels [40]. Based on the information from www.clinicaltrials.gov, Phase I studies have shown transient moderate ApoB down-regulation; however, the studies have been terminated due to "potential for immune stimulation to interfere with further dose escalation." Same SNALPs have been used by Alnylam Pharmaceuticals to deliver siRNA targeting VEGF-A and kinesin spindle protein (KSP) in solid tumors with liver involvement, named ALN-VSP. The preliminary results showed that IV infusion of ALN-VSP has been well tolerated, with limited liver toxicity and indications of VEGF silencing [224]. A Phase I study was recently initiated with similar carriers for a siRNA that targets transthyretin-mediated amyloidosis (ATTR), based on successful attempts in mice and Cynomolgus monkeys [226].

Silencing tyrosine kinase Bcr-Abl is a viable approach for CML therapy, which is caused by a reciprocal translocation t(9; 22) forming active tyrosine kinase Bcr-Abl [227]. A clinical study on a single CML patient showed that a significant inhibition of over-expressed Bcr-Abl resulted in increased apoptosis of CML cells by siRNA formulated with an anionic liposome containing soya bean oil, glycerol and egg phospholipids [228]. According to www.clinicaltirals.gov, the only viral system used for siRNA delivery in clinics is based on simian virus 40 (SV40), targets Bcr-Abl, but the results of this study are yet to be published.

The first targeted delivery system for siRNA approved by FDA was CALAA-01 (Calando Pharmaceuticals), which consists of four components: a double-stranded siRNA, a cyclodextrin-containing polycation, a PEG-based stabilizing agent (to reduce steric hindrance), and transferrin capable of binding to transferrin receptor of tumor cells [62]. CALAA-01 delivers a siRNA against ribonucleotide reductase subunit M2 (RRM2), which catalyzes the formation of deoxyribonucleotides from ribonucleotides and whose inhibition results in loss of cell proliferation [229]. In a Phase I trial, CALAA-01 was administered to adults with solid tumors, refractory to standard-of-care therapies, as a 30-min IV infusion [230]. It was shown that the supramolecular siRNA complexes reduced the target gene expression and provided transient inhibition of tumor growth [51]. Even though moderate increases in blood urea nitrogen, creatinine, alanine aminotransferase, aspartate transaminase, and IL-6 were observed at the dose of 27 mg siRNA/kg, the study concluded that no clinical signs of toxicity could be attributed to the treatment [50]. This delivery system was recently adopted to target hypoxia inducible factor-2a (HIF-2a), which is overexpressed in many solid tumors [231].

6. Future prospects of siRNA delivery

The design and engineering of siRNA carriers gained significant momentum in recent years, as a result of accumulation of predictable and therapeutically promising molecular targets. The biomaterials and pharmaceutical scientists have taken enormous strides to create a diverse array of functional carriers that can assemble siRNA in supramolecular complexes. However, most practitioners in the field are in desperate need of developing good comparisons among the available carriers. One needs to understand their relative performance in well-controlled experimental

systems, with the purpose of identifying carriers with the highest potency. The latter could be defined based on the dose of siRNA needed for effective silencing or amount of carrier to be employed for therapeutic efficacy. Dose-response studies clearly revealing the IC_{50} of the developed systems will clarify some of the confusion in the literature on the relative efficacy of promising delivery systems. This is needed not only in *in vitro* studies but also in pre-clinical studies (similar to any pharmacological agent to be developed for clinical testing). With siRNA, however, non-specific effects of carriers and/or siRNA exposure need to be further assessed; dose-response studies ought to be carried out with non-functional (scrambled) siRNA sequences along with functional siRNAs to better reveal the magnitude of the observed therapeutic effect. This is rarely done and it is impeding the progress of the field at this stage.

Development of functional carriers has been mostly driven by experimental studies but we believe that computational simulations to characterize the interactions among siRNA and carriers could make a significant impact. Although the current computational hardware/software still limits the atomistic simulations to relatively small size (<1,000,000 atoms) and short time scale (<1000 ns) compared with practical conditions, the length and time scales accessible through simulations are rapidly increasing. We can anticipate atomistic simulations of many practical aspects of siRNA delivery to be realized: (i) carrier mediated siRNA aggregation and condensation, (ii) dynamics of siRNA/carrier complexes, (iii) interaction between siRNA/carrier complexes and cell membranes, (iv) dynamics and interaction of siRNA/carrier complexes inside cells with endosomal membranes and cell proteins, and (v) the dynamics of siRNA release from internalized complexes. Attempts to simulate multimolecular interactions have been reported [103,108]; however, considering that the practical sizes of siRNA/carrier complexes are >100 nm [95] and most extensive simulations to-date attempted structures with few molecules (e.g., one siRNA and 20 carrier molecules), simulations on the larger scales will be urgently needed. Simulations with 4 dodecamer DNAs and 28 PEI carriers recently revealed a highly dynamic exchange between the complex-bound and free carriers [232], and extension of this approach to endogenous molecules will make a significant impact in understanding intracellular behavior of siRNA complexes (Fig. 4).

The initial paradigm of siRNA therapy inherently assumed a single target for silencing. However, pathophysiological changes in tissues often result from changes in multiple targets. For example, in tumors undergoing chemotherapy, up-regulation of the drug efflux transporter P-gp is usually accompanied with changes in anti-apoptotic mediators such as Bcl-xL [233], mcl1 [234], and XIAB [235]. The combined effect is multidrug resistance, resulting from the efflux activity of the elevated P-gp molecules, but also from de-sensitization to drug action from the increased activity of anti-apoptotic mediators. Inhibiting the single target, P-gp or the associated anti-apoptotic proteins, alone is not likely going to reverse the evolved cellular phenotypes. Delivering multiple siRNAs against an integrated set of targets should be considered in therapy, and optimizing supramolecular complexes with multiple siRNAs might be needed. Little work has been done in this aspect, even though recent studies pursued supramolecular complexes from tailored carriers, siRNA and conventional small molecular drugs such as doxorubicin [120,166,236]. Packaging multiple siRNAs in supramolecular complexes should not be a significant challenge given the similarity in the overall physico-chemical features of siRNA molecules, but controlling their relative release profiles, if needed, might be a significant challenge.

Independent studies have overwhelmingly demonstrated the feasibility of siRNA-mediated down-regulation using both non-viral and viral vectors, but complete knockdown is rare. What happens to sub-populations of cells where the molecular target is not silenced is an open issue in the literature. Will those cells display selective resistance to therapy and take over the patho-physiology, ultimately creating a phenotype resistant to the therapy? Studies focusing on reason(s) for lack of complete down-regulation will be needed to better understand this issue. If inherent reasons prevent siRNA action (e.g., overwhelming the RISC pathway), other silencing agents, such as miRNA or anti-sense oligonucleotides that employ different mechanisms of actions, could complement the siRNA action. If delivery issues are limiting effective silencing, we need to urgently focus on mechanistic studies revealing critical impediments to the delivery.

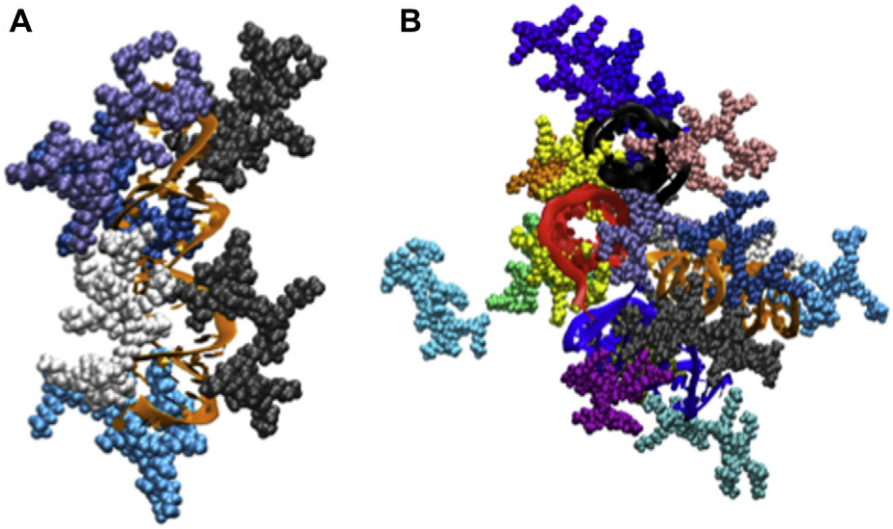

Fig. 4. Molecular dynamics simulations involving siRNA (~14 kDa) and 2 kDa branched PEI. (A) Simulations with a single siRNA and multiple (6) PEI molecules and (B) simulations with 4 siRNA and 18 PEI molecules. The helical siRNA molecule is shown saturated with 6 PEI molecules in A that forms a complex with excess cationic charge. The 4 siRNA molecules interacting with 18 PEI molecules in B forms an aggregate with inter-bridging PEI molecules linking multiple siRNA molecules as well as loosely bound PEI molecules that display interaction with complexes transiently.

Understudied areas on this front include: (i) role of ECM in supporting or impeding supramolecular access to cell surface, (ii) intracellular dissociation of supramolecular complexes, and (iii) long-term fate of dissociated carriers. Quantitative studies on the fate of intracellularly delivered siRNA will better reveal how effective the silencing attempts are. If one can probe whether all delivered siRNA molecules are used up in silencing (highly unlikely) and what fraction remains 'unfunctional' or sequestered, one can then assess the need for improved carriers that present siRNA to the biochemical machinery more effectively. Degradable and environmentally-sensitive carriers are likely to form the foundation of such carriers, but one has to assess the desired functional properties *in situ* and relying on *in vitro* characteristics (or functional responses) are likely to lead to misinterpretation of the perceived mechanisms of actions.

Despite the wide interest in using carriers to package siRNA in supramolecular complexes and considerable transfection efficiency obtained, only a few formulations from each approach were tested in clinics. While the immunogenicity, pathogenicity, and unsuitability of viral vectors for siRNA expression (compared to DNA) have limited the clinical progress of this strategy, the lack of significant testing with synthetic carriers is surprising. Pharmaceutical industry seems to prefer to employ siRNA on its own, rather than presumably risking an unknown/adverse effect to an additional entity, i.e., carrier. The toxicity of more effective polymers for siRNA delivery seems to be the major hurdle for wider clinical application. Enhancing the potency of carriers (hence lowering the administration dose) is most likely approach to minimizing chances of a clinical adverse reaction against the administered agents. Several approaches are also available to lower the toxicity of carriers without affecting efficacy and PEG conjugation is the prototypical approach. Carriers or complexes with minimal cell interactions are also attractive, but their efficacy needs to be somehow enhanced by functionalization. The latter includes employing cell-specific ligands or lipophilic moieties on non-cell interactive carriers. A systematic approach on carrier design and performance testing, as outlined in this review, is likely to increase the comfort level with clinical use of non-viral carriers, ultimately providing more opportunities for successful therapeutic use of the RNAi.

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