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## Engineering RNA for Targeted siRNA Delivery and Medical Application <sup>☆</sup>

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### ABSTRACT

RNA engineering for nanotechnology and medical applications is an exciting emerging research field. RNA has intrinsically defined features on the nanometre scale and is a particularly interesting candidate for such applications due to its amazing diversity, flexibility and versatility in structure and function. Specifically, the current use of siRNA to silence target genes involved in disease has generated much excitement in the scientific community. The intrinsic ability to sequence-specifically downregulate gene expression in a temporally- and spatially controlled fashion has led to heightened interest and rapid development of siRNA-based therapeutics. Although methods for gene silencing have been achieved with high efficacy and specificity *in vitro*, the effective delivery of nucleic acids to specific cells *in vivo* has been a hurdle for RNA therapeutics. This article covers different RNA-based approaches for diagnosis, prevention and treatment of human disease, with a focus on the latest developments of non-viral carriers of siRNA for delivery *in vivo*. The applications and challenges of siRNA therapy, as well as potential solutions to these problems, the approaches for using phi29 pRNA-based vectors as polyvalent vehicles for specific delivery of siRNA, ribozymes, drugs or other therapeutic agents to specific cells for therapy will also be addressed.

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One research area in the emergent popular field of nanotechnology involves modification, engineering and/or assembly of organised materials on the nanometre scale [1,2], thereby forming conjugative or alternated supramolecular structures [3–5]. The modified materials can then be used as building blocks in nanomedicine or nanotechnology.

Biological macromolecules, including DNA, RNA and proteins, intrinsically have defined features at the nanometre scale and can serve as unique and powerful building blocks for the bottom-up fabrication of nanostructures and nanodevices. RNA is a particularly interesting candidate for nanotechnology applications due to its amazing diversity, flexibility and versatility in structure and function [6–9]. RNA molecules are polymers made up of four nucleotides: A, U, G and C. Thus, a 30-nucleotide (nt) RNA polymer can generate as many as  $4^{30}$  (or  $10^{18}$ ) different RNA molecules. Three-dimensional RNA structures are of nanometre scale, and hence construction of RNA nanoparticles is feasible by a bottom-up approach. An example of one of the early applications of RNA bottom-up assembly is the construction of micrometre-scale RNA arrays derived from bacteriophage phi29 motor pRNA. For more details on pRNA-based nanostructures, please see Refs. [10] and [11].

RNA molecules can be designed and manipulated at a level of simplicity, characteristic of DNA [12,13], while possessing the flexibility in structure and function similar to that of proteins. For example, ribozymes are composed of RNA but have the enzymatic property of proteins. Likewise, RNA aptamers are similar to antibodies in that

they can bind small molecules as specific biosensing and targeting moieties.

In addition to pure nanotechnology applications of RNA, the idea to silence target genes involved in disease using nanosized therapeutic RNA has generated much excitement in the scientific community. In particular, the mechanism of RNA interference (RNAi) has prompted the development of several therapeutic strategies. RNAi is a sequence-specific gene-silencing mechanism, typically involving short double-stranded RNAs (dsRNAs) called small interfering RNA (siRNA). The intrinsic ability to sequence-specifically downregulate gene expression in a temporally- and spatially controlled fashion has led to heightened interest and rapid development of siRNA-based therapeutics. Although methods for gene silencing with high efficacy and specificity have been achieved *in vitro*, effective delivery of nucleic acids to specific cells *in vivo* has been a hurdle for RNA therapeutics.

Here, we review different RNA-based approaches for diagnosis, prevention and treatment of human disease. This article is an extension of the earlier publication [10], at the same time focussing on the latest developments of non-viral carriers of siRNA for delivery *in vivo*. The applications and challenges of siRNA therapy, as well as potential solutions to these problems, will also be discussed. Approaches for using phi29 pRNA-based vectors as polyvalent vehicles for specific delivery of siRNA, ribozymes, drugs or other therapeutic agents to specific cells for therapy will also be addressed.

## 1. RNA molecules with potential for diagnosis, prevention and treatment of human disease

Following the decoding of the human genome, a new era has opened for developing new gene therapy strategies employing nucleic acids. The rational design of RNA sequences that can specifically block the expression of selected genes responsible for various diseases, including cancer, viral infection and genetic disease, has received major attention in recent years. The research in this area has been fuelled by the idea that selective and specific inhibition of tumour growth with minimal side effects on normal cells can be achieved using RNA. The simplicity of RNA engineering combined with its versatility in structure and function has highlighted the use of nucleic acid- and RNA-based strategies for therapy. Next, we list several RNA-based therapeutic approaches.

## 2. Ribozymes

Ribozymes are catalytic RNA molecules with the capacity to bind and cleave other RNA molecules [14–19]. By intercepting and cleaving messenger RNA or viral genomic RNA, ribozymes are capable of regulating gene function for therapeutic purposes [20,21]. The structural variability of ribozymes translates into their functional versatility and diversity. Hammerhead ribozymes [22], hairpin ribozymes [23] and phi29 pRNA [24,25] are classified as small ribozymes, whereas larger ribozymes include introns and RNase P [26,27]. After being incorporated and escorted by the phi29 pRNA, the activity of ribozyme is enhanced due to the improvement in folding and stability [28,29].

## 3. Antisense RNA

Antisense RNA are single-stranded nucleic acid oligomers which can inhibit translation by Watson–Crick base pairing to target mRNA sequences to sterically block translation [30,31]. The oligonucleotide sequences were coined ‘antisense’ because most often they are transcribed in a direction opposite to that of the target RNA from the same DNA transcript. After the antisense strand binds to the target mRNA, the complementary or nearly complementary RNA strands form a stable and easily detectable complex which can be used to probe RNA/RNA interactions. The only oligonucleotide-based therapeutic currently on the market is fomivirsen (Vitravene; Isis Pharmaceuticals/Novartis) [32], an antisense therapeutic targeting cytomegalovirus (CMV) retinitis in AIDS patients [33].

## 4. Small interfering RNA (siRNA)

RNA interference (RNAi) is a post-transcriptional gene-silencing (PTGS) mechanism found in plants, some invertebrates and mammalian cells. RNAi exists in several forms, two of which siRNA [34] and miRNA (see section titled ‘Aptamers’) are discussed in this article. The use of RNAi technology for functional genomics has been highlighted by systematic RNAi-based genetic screening in *C. elegans* and *Drosophila* [34,35]. Baulcombe and Hamilton found that cells can control protein production by a novel mechanism through which small double-stranded RNA molecules bind to specific mRNA sequences and block the respective gene’s protein expression [34–36]. Furthermore, additional aspects of RNAi have been recognised over the past 7 years. In 2001, Elbashir *et al.* demonstrated that RNAi can be mediated by 21-nt siRNAs in cultured mammalian cells [37,38]. Then, Andrew Z Fire and Craig C Mello were awarded the 2006 Nobel prize for physiology or medicine for their fundamental work in RNAi and its therapeutic potential [39].

SiRNA is a key molecule in the RNAi pathway. SiRNAs are short (usually 21–25-nt-long) dsRNA strands with 2 nt overhangs at the 3′-ends [34,35]. In the cytoplasm, siRNAs are loaded into a protein complex called the RNA-induced silencing complex (RISC). The loaded RISC complex then scans all intracellular mRNA for a target mRNA with

a complementary sequence to the loaded siRNA. If a target mRNA is found by the loaded RISC, the target mRNA is cleaved and degraded, successfully inhibiting the translation of the target gene.

SiRNAs can be generated in several ways. In some cases, long dsRNA is introduced to a cell, either by a virus, endogenous RNA expression (i.e., microRNA), or exogenously delivered dsRNA. The enzyme Dicer cleaves the long duplex RNAs into siRNAs [35]. Another way to introduce siRNA into cells is to express short hairpin RNAs (shRNA) from plasmid vectors. ShRNA is transcribed under the control of RNA Pol-II or Pol-III promoters and folds into a structure resembling a siRNA duplex. ShRNAs are then processed by Dicer into siRNAs. Alternatively, chemically synthesised siRNA duplexes, mimicking the structure of Dicer-processed products, are commonly used in research for gene silencing. Chemically synthesised siRNAs simply bypass the Dicer cleavage step.

Gene silencing via siRNA offers a new paradigm for drug design and discovery compared to more traditional small molecules and monoclonal antibody inhibitors. In terms of drug discovery, conventional small-molecule drug discovery involves iterative screening and random modifications to lead to compounds. However, to design a therapeutic siRNA only requires knowledge of the target gene’s sequence. Moreover, the fact that siRNA-mediated RNAi mechanism takes place in the cytoplasm is a potential advantage over other gene-regulation mechanisms that require penetrating the nucleus [34]. Theoretically, RNAi can silence the expression of mRNA for any gene, including growth factors, viral genes, oncogenes, anti-apoptotic genes (e.g., survivin) and genes that were once deemed ‘non-druggable’ [40] by small molecule inhibitors. Consequently, not only is siRNA-mediated RNAi a useful biological tool for assessing gene function, but also equally important is siRNA’s therapeutic potential [41].

## 5. Aptamers

Aptamers are a family of RNA- or DNA-based oligonucleotides of 20–50 nt in length that can specifically bind to selected targets. They may be generated by *in vitro* screening via Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [42,43]. Utilising a library containing random RNA sequences with about  $10^{14-17}$  varieties, *in vitro* binding, elution and reverse PCR amplification techniques allows for the selection of RNA molecules that efficiently bind to a specific receptor or ligand with high affinity [42,44]. The approach is based on the folding ability of RNAs into complex structures, reflecting the diversity of RNA functions [43,45–47]. A natural adenosine triphosphate (ATP)-binding aptamer with structure similar to the ATP-binding aptamer by SELEX was also reported in the pRNA of bacterial virus phi29 DNA packaging motor [48]. Aptamers have been selected to bind proteins, organic compounds, small molecules and nucleic acids with  $K_D$ 's in the  $\mu\text{M}$  or nM range [42,49–53]. Aptamer-binding occurs with high specificity through the formation of strong binding pockets analogous to antigen–antibody interaction. SELEX allows screening for co-variation of several nucleotides and can be used to reveal non-canonical interaction, which is difficult to prove by classic genetic and biochemical approaches. For therapeutic purposes, aptamers can be used to bind and inhibit harmful molecules or serve as targeting ligands for nanomedical constructs [54]. RNA aptamers, transcribed or synthesised, simplify the need for chemical conjugation or mixing with other moieties. Aptamers have been used as ligands for specific delivery of siRNA to prostate cancer cells [55] and lymphocytes [54,56,57].

## 6. MicroRNAs

The microRNA (miRNA) pathway is another form of RNAi [58]. MiRNAs are an important class of short, non-protein-coding RNAs, originating from endogenous genome DNA sequences. MiRNAs are first transcribed in the nucleus as parts of long primary miRNA transcripts (referred to pri-miRNA) with 5′-caps and 3′-polyA tails. Pri-

miRNAs with hairpin structures are then processed into pre-miRNAs by the ribonuclease Droscha. The pre-miRNAs are subsequently transported out of the nucleus to cytoplasm by Exportin-5, and processed to mature miRNAs by Dicer. Similar to siRNA-mediated silencing pathway, miRNA is then loaded into RISC. However, its mode of action is dependent on the extent of sequence complementarity between the miRNA and the target mRNA. When an miRNA matches the sequence of the mRNA completely, the miRNA/RISC complex mediates the cleavage of the mRNA using the same mechanism as siRNA. For miRNAs that only partially match the mRNA's sequence, the miRNA/RISC complex induces translational inhibition and subsequent mRNA degradation. Endogenous miRNAs with partial sequence complementarity to their target mRNA commonly target the 3'- untranslated region of their target mRNAs.

The miRNA mechanism is not fully understood. MiRNAs silencing is arguably more complex than siRNA silencing, owing to the fact that miRNAs only require partial sequence complementarity to silence genes. In addition, it is hypothesised that a single miRNA may silence up to 100 different mRNA targets [59]. For this reason, therapeutics that use miRNA as the key therapeutic agent have not been reported.

Some diseases may be linked to aberrant miRNA expression and function. Thus, some research groups are targeting miRNAs for therapeutic purposes using siRNAs or 'antagomirs' to interfere with aberrant miRNAs [60]. For a review of this topic, readers may refer to Soifer et al. [61].

## 7. Challenges of siRNA in therapy

The use of siRNA as a research tool for functional genomics *in vitro* is now well established. RNAi has been applied quickly in various research fields, and numerous reports have emerged using siRNA as a powerful tool to perform gene knockdown. Currently, with the aid of commercially available siRNA and transfection reagents, siRNA-mediated gene silencing has become a common procedure to study gene function and identify disease gene targets. In addition to being a research tool, RNAi holds great promise in gene therapy to silence disease-causing genes.

The success of siRNA strategies for gene therapy *in vitro* has accelerated the efforts on the *in vivo* siRNA studies. Some researchers performing siRNA delivery *in vivo* in mice introduce naked siRNA by high-pressure tail injections [62]. High-pressure injections, however, would be fatal for humans and are not a realistic *in vivo* method of naked siRNA delivery [63]. Several siRNA therapeutics already are in clinical trials for targets that are superficial organs (e.g., the eye), where the therapeutic agent can be directly injected into the organ [64]. SiRNA therapeutic studies currently in clinical trials include age-related macular degeneration, respiratory infections, chronic myelogenous leukaemia (CML) and other types of cancer. However, this strategy is not feasible for deeper tissues, which demand vectors that can be delivered systemically (e.g., intravenous injection). The human body is well equipped with special functions to destroy foreign RNA present either in the bloodstream or in the tissues and prevent the dsRNA from entering the cells. The aim for *in vivo* delivery is to provide specific cells with therapeutic concentration of siRNAs for the length of time required for the inhibition of gene expression. Consequently, the ability to deliver therapeutic siRNA to specific ailing cells, and its stability in the extracellular and intracellular environments after systemic administration, are some of the most challenging aspects in the advancement of siRNA therapeutics.

## 8. Chemical stability

RNA's stability in physiological pH ranges, lack of detectable antibody responses by the adaptive immune system [65] and biocompatibility hint to the idea of using RNA constructs for gene therapy. *In vitro* transcription (or synthesis) and purification of siRNA are relatively

easy procedures, and simple gel electrophoresis can verify the correct sizes and stoichiometry of siRNA. However, naked siRNA oligomers are highly susceptible to nuclease degradation. In order for siRNA to survive long enough to maintain an acceptable level in the tissues, its degradation must be avoided or at least significantly delayed.

Chemical modifications of siRNA duplex have been used extensively to achieve enhanced resistance to nuclease-induced degradation and to reduce off-target effects for *in vivo* application. Chemical modifications can be introduced to the 5'- or 3'- terminus, backbone, sugar and nucleobase of siRNA. An ideal modification should enhance stability of siRNA duplexes yet retain their gene-silencing activity. Evaluation of chemically modified siRNA has been performed by cell-based assays as well as in animal studies. It has been found that, in general, the sense strand (non-guide strand) of siRNA can tolerate more modifications than the antisense strand (guide strand), and the terminal regions of siRNA can tolerate more modifications than the central region [66]. Several modifications that enhance stability have little or no effect on gene silencing. Some have even been shown to have enhanced efficacy and significantly reduced immunogenicity as described in the following sections.

## 9. Backbone modification

Phosphorothioate (P=S) backbone modifications have been used to modify antisense DNA oligonucleotides to enhance stability. However, siRNA carrying P=S modification on backbone [67] showed little enhancement of nuclease resistance [68], although the gene-silencing function was retained and siRNA uptake was enhanced [69]. Cytotoxicity was observed when the P=O was partially or completely replaced by P=S [70]. On the other hand, siRNAs with boranophosphonate (P=B) backbone modification have much higher nuclease resistance than non-modified ones. In addition, the gene-silencing activity of siRNA was not compromised when the boranophosphonate modification was introduced to the sense strand or the terminal regions of the duplex [68,71,72].

## 10. Sugar modification

Common modifications of siRNA on the sugar moiety are 2'-fluoro (2'-F) [73], 2'-O-methyl [74], 2'-halogen, 2'-amine [75] and a 2'-deoxy [76] and locked nucleic acid (LNA) [77], all of which have been shown to increase serum stability of siRNA significantly. LNA is a nucleotide derivative which links the 2'- and 4' positions of the sugar with an -O-CH<sub>2</sub>- bridge [77]. In addition, 2'-NH<sub>2</sub>, 2'-O-(2-methoxyethyl) and 4'-thio are also tested as alternative approaches. In contrast to most other 2'- modifications, which can be incorporated only through chemical synthesis, 2'-F can be incorporated through *in vivo* transcription. Long dsRNAs with all pyrimidines replaced with 2'-F-nucleotides retain the ability to be processed by recombinant human Dicer into short siRNA duplexes. In an *in vivo* study, 2'-F-pyrimidines-siRNA duplexes inhibited the target gene expression in mice and, as expected, exhibited a prolonged half-life in plasma as compared to non-modified siRNAs [62]. However, this prolonged half-life did not translate into increased potency in animals, presumably because of unfavourable pharmacokinetic features. Modification with 2'-O-methyl on the entire siRNA abolished its activity, while modification on only the sense strand did not. Currently, 2'-O-methyl modification at the terminal regions of the sense strand is commonly used for commercially available siRNA duplexes. In addition, 2'-O-methyl substitution at position 2'- in the guide strand reduced silencing of most off-target transcripts that contain complementary sequences to the seed region in the guide strand [78,79].

Mook et al. [80] tried end-modifying or heavily modifying siRNA strands with LNA. End-modified and heavily modified RNA enhanced serum stability to >40 and >90 h, respectively. Silencing ability,



however, decreased with the heavily modified strand, suggesting that the RNAi mechanism cannot tolerate radical LNA modifications to the siRNA chemical structure. One might assume that siRNA must be protected from nucleases both in serum and in the cytoplasm, but cytoplasmic stability is unclear. Chiu *et al.* showed that modified nucleotides last longer in the cytoplasm [81], while other literature reported that once siRNA is internalised, both modified and non-modified siRNA have similar stability [62,82].

### 11. Nucleobase modification

Nucleobase modifications to siRNA include the 2,4-difluorotoluidyl residue, 5-bromouridine residue, 5-iodouridine residue, 4-thiouridine residue, N-3-Me-uridine residue, 5-(3-aminoallyl)-uridine residue, inosine residue and 2,6-diaminopurine residue. Parrish and colleagues [83] found that siRNA with 4-thiouridine and 5-bromouridine remained active while the inclusion of 5-(3-aminoallyl)-uridine abolished the activity in *C. elegans*. For the other nucleobase modifications, they are more tolerated in the sense stand/terminal region than the antisense strand/central region on the siRNA duplexes. A recent study demonstrated that DNA nucleotides can be used in the seed region of siRNA, the 2–8 nt from the 5'-end of the guide strand, and still retain the silencing ability [84].

### 12. Terminal modification

End modification of siRNA allows conjugation of ligands, such as folate, cholesterol, biotin or fluorescent molecules, offering opportunities to enhance pharmacological characteristics or introduce special features to siRNA. Different studies reported that both 5'- and 3'-terminal modifications of the sense strand are well tolerated and retain siRNA functionality [40,85]. Conjugation of a cholesterol [86] or folate [87,88] group at the 3'- end of the sense strand conferred siRNA drug-like properties and enabled liver, jejunum or cancer cell-selective delivery.

For the antisense strand, it has been found that a 5'-phosphate group is essential for the RNAi activity of siRNA duplexes [71]. The 5'-phosphate group can be either introduced by chemical synthesis or phosphorylation through endogenous kinases. Blocking the 5'-hydroxyl terminus by functionalities on the antisense strand abolished the gene-silencing activity of siRNA [71]. However, modifications were still tolerated as long as the 5'- phosphodiester was present. The effect of 3'- terminal modification on antisense strand is less conclusive. For example, 3'-biotin is well tolerated, while 3'-2-hydroxyethyl-phosphate led to the loss of the silencing function [71].

Chemically synthesised siRNA duplexes often contain a two deoxy-nucleotide overhang at the 3'- ends, which resembles the 3'- overhang of siRNA duplexes produced by Dicer. Inclusion of this 3'- overhang increases the stability of the siRNA duplexes and facilitates the loading of the duplex into the RISC complex for subsequent gene silencing [71]. In addition, the presence of such 3'- overhang may help to reduce immune responses, as shown in a recent study where siRNA duplexes with various lengths activated the interferon system in the absence of 3'- end overhangs [89]. It has been reported that very potent induction of interferon alpha and beta by short single-stranded RNAs (ssRNAs) transcribe with T3, T7 and Sp6 RNA polymerases, and that the initiating 5' triphosphate triggers the interferon response [90].

### 13. Size and pharmacokinetics

Despite displaying a significant serum resistance and improved stability, chemical modifications to systemically delivered siRNA do not solve another challenging issue of delivering siRNAs *in vivo*: siRNA's size. The average size of a single siRNA molecule is well below 10 nm. In addition, the polyanionic nature of RNA makes it difficult for it to penetrate the cell membrane and non-formulated siRNAs have

been reported to be easily excreted by the body [91–93]. Therefore, these molecules, even if stabilised, are subject to renal filtration when administered into the bloodstream. SiRNAs would suffer from poor pharmacokinetics and be limited in the extent and duration of their effect. To increase the size, siRNA can be transcribed as a longer piece or chemically linked (i.e., by disulfide bonds, streptavidin/biotin, etc.) to other nucleic acids, proteins and surfaces to form larger particles [94]. Another approach, achieving both increased particle size and enhanced stability in the extracellular and intracellular environments, involves extranucleotide materials. Polymers and liposomes, which together with siRNA form nanoparticles, can increase the size and protect siRNA from nuclease degradation. These nanoparticles are formed by conjugating biologically inert polymers directly to siRNA or by preparing liposomes that envelop siRNA molecules. Both types of nanoparticles have proven successful in stabilising siRNA in serum.

Nanoparticle delivery of siRNA also has the potential to improve the pharmacokinetics, pharmacodynamics, biodistribution and toxicology of this newly emerging therapeutic modality. It is commonly accepted that the size of a nanoparticle is paramount for effective delivery to diseased tissues. Many authors suggest that particles ranging from 10–100 nm [95–97] or as large as 200 nm [98], are the optimal size for a non-viral vector because they are large enough to be retained by the body yet small enough to access the cell surface receptors and pass through the cell membrane by receptor-mediated endocytosis [96]. However, nanoparticles with a diameter greater than 100 nm are recognised by the reticuloendothelial system (RES). The RES is made up of mononuclear phagocytes that originate in the bone marrow and mature in the bloodstream. As the monocytes mature, they begin to localise outside the bloodstream in or near a number of tissues including lymph nodes, liver, lung, digestive track and spleen, transforming into fully mature macrophages. Macrophages recognise , internalise and degrade large particles through a process called opsonisation. Opsonisation is the process by which opsonins, originating from mature macrophages, coat foreign substances like bacteria or nanoparticles. The coating drives the binding and ingestion of the foreign substances by macrophages. This process is largely responsible for the short half-life of nanoparticles larger than 100 nm and must be taken into account when designing nanoparticles for siRNA delivery. While they avoid recognition by the RES, nanoparticles with a diameter less than approximately 5 nm are subjected to renal filtration. The kidneys, largely responsible for the whole body homeostasis, efficiently remove waste and other matter from the body. However, size limits of globular filtration preclude large molecules from being subject to this process. Specific challenges associated with the use of nanoparticles as systemic drug delivery vehicles continue to be stabilisation of the particles, increased circulation time and targeted delivery.

To produce RNA nanoparticles of appropriate size, the 20–40 nm chimerical RNA complex was constructed by fusing the siRNA with the pRNA of bacteriophage phi29 motor pRNA [29,54,99]. An increase in size was achieved by making the motor pRNA dimer and trimer formation. Such increase of the RNA particle size to 20–30 nm extended the half-life of the therapeutic RNA complex *in vivo* to avoid the short retention time of RNA molecules and avoid the problem of low delivery due to the larger size as described [10].

### 14. Biodistribution and uptake

Another challenge for delivering therapeutic siRNA *in vivo* originates from the non-specific distribution of siRNA throughout the body when administered systemically [100–102]. Systemic delivery decreases the local therapeutic concentrations in the ailing cells. Additionally, siRNA has to surmount the blood vessel endothelial wall and multiple tissue barriers before being able to reach the targeted cells. To exert its therapeutic effect on the diseased cells, intact double-stranded siRNA needs to be efficiently transferred into the cell.

siRNA cannot efficiently enter into the cell without assistance. One approach to enhance siRNA cellular uptake is by complexing siRNA molecules within lipid formulations similar to the widely used transfection reagents. In addition to enhanced optimising pharmacokinetics, siRNA nanoparticle delivery offers the additional advantage of being able to promote desired tissue distribution profiles.

Although it is likely that targeting strategies play some role in tissue selectivity, it is not clear that any targeting mechanism employed by a given nanoparticle singularly directs biodistribution. Depending on the disease pathology and the properties of the delivery system, passive targeting at the disease site can occur. The nanoparticle size and surface charge play a key role not only in the half-life of the nanoparticle but also in governing which tissues accumulate relatively high concentrations of nanoparticles. One of the driving forces behind this theory is that the barriers protecting tissues vary in porosity, both in pore size and number. In particular, some tumour tissues are surrounded by rapidly growing and/or disorganised vasculature that is not supported by efficient lymphatic drainage. Nanoparticles are able to extravasate and accumulate in the 'leaky' vasculature of these tumours. This phenomenon is called the 'enhanced permeability and retention' (EPR) effect [103–105]. Therefore, siRNA nanoparticles may be passively targeted to these tissues if they stay within the size range optimal for EPR.

While the EPR method can efficiently enhance uptake, it is highly non-specific and may not be sufficient to direct the tissue distribution of siRNA. To better direct the tissue distribution and simultaneously enhance uptake, a number of targeting moieties have been explored. Small molecules, single-chain monoclonal antibodies and receptor-binding aptamers have all been used to enhance uptake and, in some cases, direct tissue distribution (see section titled 'Ligand-targeted siRNA delivery'). Specific cellular uptake can occur via receptor-mediated endocytosis. In this case, nanocarriers functionalised with targeting ligands are internalised upon binding of the ligand-modified nanocarrier with the cell-surface receptor and then transported to the disease site through the endosome vesicles [54,99,106].

### 15. Potential therapeutic applications of siRNA for treatment of cancer

Cancer development is a gradual and complicated process, often accompanied by accumulation of genetic alternations, which lead to unregulated cancer-promoting oncogenes or disabled tumour suppressor genes. Oftentimes, the genetic abnormality dictates the malignant behaviour of tumours, allowing for unrestrictive cell proliferation, invasion of adjacent tissues, metastasis from the primary tumour site, formation of new blood vessels and resistance to chemotherapeutic drugs. Cancer gene therapy aims to correct these genetic abnormalities by suppressing pathological genes or re-expressing functional tumour suppressor genes. The potential of siRNA for cancer therapy was recognised early, as it can hypothetically regulate the expression of cancer cells while leaving normal genes unperturbed. In addition, the gene-silencing efficiency of an equivalent dose of siRNA has been proven higher than antisense oligonucleotide and ribozyme strategies, which were initially popular for their abilities for suppressing gene expression for the purpose of cancer gene therapy [107,108]. One important factor for successful RNAi-based cancer therapy is to choose the right target genes. Great efforts have been made to identify molecular targets in cancer therapy. The most common targets fall into the categories given in the following.

### 16. Target genes regulating apoptosis and cell cycle

The guardian of the genome, p53, is inactivated by point mutations in more than 50% of human cancers. siRNA has been used to suppress the expression of mutated p53 and restore the function of the wild-type gene. Programmed cell death, or apoptosis, is commonly deregulated in most cancers, especially in drug-resistant tumours. siRNA-based

therapeutics can induce apoptosis of cancer cells by targeting anti-apoptotic factors, such as Bcl-2, survivin and Akt1. Suppression of those anti-apoptotic genes by siRNAs was found to induce apoptosis in various cancers or sensitise them to chemotherapy drugs [109–115]. Specifically, survivin has attracted a considerable amount of interest since survivin is present only in foetal tissues or cancers, but absent in normal adult tissues [116]. Thus, silencing of survivin could be a tumour-specific therapy without damaging normal tissues [117–119].

### 17. Target genes involved in signalling transduction

Increasing knowledge of neoplastic cell-signalling pathways has resulted in several novel drugs for cancer therapy, including the inhibitor of protein tyrosine kinase Bcr-Abl (Gleevec®) and the monoclonal antibody against HER2/neu receptor (Herceptin®). Inhibition of critical signal transducers involved in proliferation or survival pathways, especially protein kinases, is considered to be a promising direction in developing siRNA-based cancer therapeutics. A study conducted by Wohlbold et al. [120] indicates that RNAi selectively inhibited Bcr-Abl-dependent cell growth in CML [120]. In addition, siRNA against Bcr-Abl significantly increased the sensitivity of CML cells to the treatment of anti-CML drugs. Several proof-of-concept studies using siRNA to silence HER2 expression have been performed in mouse models of pancreatic and ovarian cancers [121,122].

### 18. Target genes involved in angiogenesis

Vascular endothelial growth factor (VEGF) plays a critical role in the pathological angiogenesis during the development of cancer. Against VEGF, siRNA almost completely inhibited the secretion of the growth factor in a human prostate cancer cell line and dramatically suppressed tumour angiogenesis and tumour growth in a xenograft model in mice [98,123].

### 19. Target genes involved in drug resistance

P-glycoprotein and MDR1-mediated multiple drug resistance (MDR) is one of the major causes for unsuccessful chemotherapy in cancer patients. siRNA against MDR1 and P-glycoprotein genes resulted in a reduction of expression of these two genes by >90% *in vitro* or 75% in mice [124]. Inhibition of P-glycoprotein in cells by retroviral-mediated short hairpin (shRNA) conferred increased sensitivity of MDR1-transfected cells to cytotoxic drugs including vincristine, paclitaxel and doxorubicin.

### 20. Potential therapeutic applications of siRNA for treatment of viral infections

siRNA offers the opportunity to treat viral infection by suppressing viral transcripts in host cells. Viruses of different families have been inhibited by siRNA with high efficiency, including RNA, reverse-transcribing and DNA viruses. Many of these viruses pose major threats to human health, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), severe acute respiratory syndrome coronavirus, polio virus, influenza virus and herpes simplex virus. It has been suggested that RNAi can be applied to inhibit replication of every class of virus since the transcription of mRNA is a critical step needed for the replication of all viruses [125]. Both viral and host genes that are essential for viral replication have been used as targets of siRNA-based viral therapy to suppress the replication. Silencing of viral genes involved in the early events of the viral life cycle, such as binding to cellular receptors, entry and the transcription and translation of early genes, is considered to be more effective than silencing of late genes. An initial study performed by P Sharp's group [126] used siRNA to inhibit HIV replication *in vitro*. In a HeLa-derived cell line expressing CD4 and other co-receptors of HIV, siRNA against CD4 or

the viral gene *gag* resulted in inhibited viral production following HIV infection [126]. Since mutant strains of HIV emerge constantly, highly conserved sequences in essential genes such as *gag*, *pol*, *int* and *vpu* are often chosen to be targeted [127]. However, targeting those highly conserved sequences may not translate directly into effective gene-silencing, as only a few suppressed gene expression effectively when a panel of siRNAs were tested. In another study of influenza virus, siRNAs were designed against several conserved regions of the viral genome including NP, PA, PB1, PB2, M or NS genes. Two siRNA against nucleocapsid and RNA transcriptase were much more effective than the others [128]. Therefore, the actual inhibition efficiency of each rationally designed siRNA still needs to be verified by experiments. Another strategy to deal with potential viral genome mutation is the use of cocktail siRNA, which is a combination of siRNAs targeting one or several essential genes of the virus [129,130]. Cocktails with different siRNAs targeting the same mRNA target can also be used to combat viral genes that undergo frequent mutations. The downside of the cocktail approach is the potential difficulty in pinpointing any siRNAs that induce the interferon response or off-target effects. Targeting host genes by RNAi is also effective in the prevention and treatment of virus infection. Knocking down the expression of CD4, CCR5 or CXCR4 by siRNA effectively inhibited the entry and replication of HIV [126,131]. However, silencing host receptors with critical biological functions, such as CD4, may result in unacceptable side effects. Targeted delivery of siRNA to viral-infected cells then becomes an essential supplement to this approach. For example, siRNA conjugated to an anti-gp160 antibody was delivered specifically to a mouse melanoma cell line stably expressing HIV gp160, which mimics the antigen presented by HIV-infected cells [56,106,132].

## 21. Principles and approaches in engineering RNA for conjugation and bottom-up assembly

Transcription, chemical synthesis, fusion, mutagenesis and conjugation are the basic techniques in RNA technology. The self-assembly of nanoparticles from RNA or RNA/chemical conjugates is a prominent bottom-up approach to obtain nanometre-sized, nanostructured complexes in a variety of nanotechnology and nanomedicine applications. Combination of chemical and biological techniques can be successfully integrated into nanotechnology [11,54,99,133,134]. Such approaches for RNA nanotechnology rely upon the cooperative interaction of intra- or inter-RNA molecules that spontaneously fold or assemble to form larger two- or three-dimensional complexes with the appropriate structure and stoichiometry. Within the realm of self-assembly or folding, there are two main subcategories: non-templated assembly and template-directed assembly.

In non-templated assembly, methods are sought to ensure that the conjugation or modification did not affect the folding or functioning of the RNA molecules. Non-templated assembly involves the formation of a larger structure by individual components without any influence from external forces. Included in the non-templated category are ligation, chemical conjugation, covalent linkages, loop/loop interactions of RNAs and the formation of macromolecules by structural interaction in the formation of multimers [12,54,135–138]. The use of bacteriophage phi29 pRNA as a building block for the construction of micrometre-sized RNA arrays for potential tissue and wound repair has been reported [11].

Template-directed assembly involves the interaction of RNA molecules with one another under the influence of a specific external structure, force or spatial constraint. RNA transcription, hybridisation, replication, annealing of DNA/RNA and molding or replica production are part of this category.

## 22. Non-viral carriers for siRNA delivery

Delivery of novel therapeutics using nanoparticles is one of the most promising embodiments of nanotechnology. Without such improve-

ments, siRNA will not realise its full potential. The ideal systemic delivery vector should be ~10–50 nm in size, non-toxic, non-immunogenic, stable, capable of efficient intracellular delivery and possess specific targeting ability. That is one of the key reasons why antibody therapies currently have so many problems in the clinic, and scientists are looking for new solutions in nanotechnology. In order to rationally engineer an effective vector, key biological features and mechanisms should be identified and appropriately avoided or exploited for its design. These biological aspects include the choice of material, toxicity, systemic immune or cellular responses, dynamic pH or oxidative environments, RNA–protein or RNA–RNA interactions, covalent versus non-covalent bonding and passive versus active internalisation mechanisms. Combinations of poly(ethylene glycol), fusogenic and non-fusogenic lipids, dendrimers [139–141], liposomes, polycations, nucleic acid modifications, phi29 pRNA fusing systems [11,29,54,99] and targeting moieties have been used to improve the pharmacology of RNA-based therapeutics. Additionally, although the feasibility as a therapeutic approach has yet to be investigated, nanotubes have also been used as carriers of siRNA [142,143]. The following section will highlight current efforts using the nanoparticle technology to enable the systemic delivery of siRNA for therapeutic purposes.

## 23. Nucleic acid-based vectors

Several nucleic acid-based siRNA vectors have been reported. The rationale for using nucleic acid-based vectors stems from the fact that (1) construction of the vector may be simpler compared to other lipoplex or polyplex strategies; (2) without complexing to proteins, nucleic acids have not been reported to elicit an antibody response; (3) nucleic acids are within the nanometre regime and can construct larger structures via bottom-up assembly in a controlled manner; and (4) molecules can be conjugated directly to the ends of nucleic acid strands to add additional functionality. Reports have shown that, when designed correctly, nucleic acid-based constructs such as phi29 pRNA/siRNA chimera are still able to be processed by Dicer into functional siRNAs [29,54,99]. Reiterating two of the criteria for a successful non-viral siRNA vector, nucleic acid-based vectors must be of adequate size and, for RNA-based vectors, stable against RNase nucleases. Undoubtedly, if nucleic acid-based vectors are to be used, they must incorporate RNase-resistant nucleotide analogues if they are to be successful *in vivo*.

McNamara et al. [55] constructed an RNA-based siRNA vector through *in vitro* transcription. The construct featured both an siRNA domain as well as an RNA aptamer targeting prostate cancer cells [55]. Zhang et al. [88] constructed a hybrid DNA:RNA molecule using a 'tethering' approach; the sense strand of the siRNA contained a 15 nt overhang 'tether'. A single-stranded folate–DNA strand with a complementary 15 nt sequence was synthesised separately and annealed to the overhang [88]. The rationale was that the folate moiety would facilitate targeted delivery to folate-receptor positive tumour cells. Once internalised, RNaseH, which degrades DNA:RNA hybrid strands, would degrade the hybrid portion of the vector, liberating the siRNA strand to participate in silencing. Although the authors did not show data that the vector was being processed as hypothesised, the authors reported ~80% relative mRNA knockdown in HUVECs in a folate-targeted, sequence-specific manner. Their construct did not elicit a significant interferon response.

## 24. Liposomes

Liposomes are widely used in delivering cargo into target cells. Of the major classes of non-viral delivery vectors, liposomes were among the first to be studied and developed and therefore are the most characterised [96]. Neutral lipids exhibit low toxicity, low immunogenicity and easy production. Liposomes are also an attractive choice for gene delivery because they can be formulated as ~100 nm in size and their by-products are biocompatible. Lipid encapsulation of the payload (i.e., siRNA) simply involves mixing and incubation. Specific



delivery can be achieved by conjugating a ligand to the lipid molecule and then proceeding to form liposomes.

Common neutrally charged lipids include 1,2-Oleoyl-sn-Glycero-3-phosphocholine (DOPC) and 1,2-Dioleoyl-sn-Glycero-3-phosphoethanolamine (DOPE). Neutrally charged DOPC can encapsulate ~65% of siRNA in solution by simple mixing [144]. *In vivo* study revealed >50% reduction in xenographic tumour volume when compared to no treatment. The same group also demonstrated that DOPC could deliver siRNA against focal adhesion kinase to kill ovarian cancer cells implanted in mice [145].

Instead of using neutral lipid molecules, cationic lipids can be added to the neutral lipid formulation. The cationic charge can electrostatically complex with siRNA to achieve a more robust construct, while the neutral lipids facilitate fusion to the host cell's membrane. Cationic liposomes complexed with nucleic acids are termed 'lipoplexes'. 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-3-trimethylammonium propane (DOTAP) cationic liposomes conjugated to poly (arginine) improved the cell entry and protected siRNA against serum degradation [146]. A lipoplex analogue of phospholipids found in cardiac muscle tissue called 'cardiolipin' successfully delivered siRNA and exhibited less toxicity and increased delivery efficiency when compared to DOTAP. A cationic liposome has also been reported to deliver siRNA against tumour necrosis factor (TNF)-alpha in an experimental model of rheumatoid arthritis [122]. In the previous two cases, enhanced distribution of liposome at the targeted sites was believed to be mediated by EPR effects in inflammatory tissues and solid tumours, where the vasculature and endothelial cell junction become leaky [147].

The Chang lab [148] uses immunolipoplexes, which are DOTAP:DOPE mixtures attached to targeting ligands (i.e., a monoclonal antibody for the transferrin receptor) [148]. Although relatively large (200 nm diameter), these immunolipoplexes conjugated to repeating units of pH-sensitive histidine-lysine (HK) peptide, which buffers the acidity of endosomes to release the siRNA payload, and increased the silencing potency compared to lipoplexes without HK peptide *in vivo*. The authors found immunolipoplex:HK:siRNA treatment in conjunction with other traditional drugs had the best effect *in vivo* compared to immunoplex:siRNA or siRNA treatment alone [122].

Although lacking a targeting moiety, the development of stable nucleic-acid-lipid particles (SNALPs) represents an exciting and recent direction in lipid-based systemic RNAi. The ~100–150-nm particles are mixtures of neutral lipids, cationic lipids and poly(ethylene glycol) (PEG). The additional layer of PEG improves the retention time to as much as 10 h [96]. In a study of HBV replication in a mouse model, prolonged half-life of SNALP-siRNA in serum was observed. Three daily intravenous injections of a therapeutically viable dose (3 mg kg<sup>-1</sup>) of SNALP-siRNA reduced HBV levels significantly. The reduction of HBV lasted up to 6 weeks [149]. Another study showed that SNALP-siRNA rapidly silenced apoB gene in the liver of cynomolgus monkeys at a dose of 1–2.5 mg kg<sup>-1</sup>, resulting in a 65% reduction of serum cholesterol [150]. In addition, the silencing effect was found to last for 11 days after the RNA treatment. The size of SNALPs likely plays a significant role in their limited biodistribution. Morrissey et al. showed that SNALPs generally accumulate in the liver and spleen and did not deliver significant amounts of RNA to other tissues [149]. More so, SNALP-siRNA delivery is generally well tolerated; evaluation of its toxicity reveals only a transient increase in liver enzyme activity [149,150].

Lipoplex delivery of siRNA has been successful in several reports and mechanisms for the release of payload from lipoplexes have been suggested [151,152]. Continued research into the mechanism of release may lead to the development of more bioresponsive vectors that can achieve higher silencing for a given dose.

## 25. Cationic polymers (polyplexes)

Polymers are another class of materials that have been used to deliver nucleic acids and siRNA. They come in a variety of molecular

weights and functional groups. PEG is an uncharged polymer with high biocompatibility and is used in many biomedical applications. It is often used as a block element (i.e., in block co-polymers) or coating ('PEGylation') of polymers and biomaterials to prevent non-specific binding of proteins. PEG has also been used as the material basis for siRNA vectors. It was conjugated to other polymers and siRNA encapsulated in a PEG-polymer/siRNA-PEG pattern was used to produce nanosized block ionomers or spherical polyion complex (PIC) micelles. Exploiting the nature of the cytoplasm's endocytic environment, PEG was linked to the siRNA via a pH-sensitive bond [153]. The design rationale is that, upon internalisation into the endosome, the reductive and acidic environment breaks the bond, releasing the siRNA to participate in silencing.

In contrast to uncharged polymers, cationic polymers can electrostatically complex with the polyanionic backbone of nucleic acids to achieve a more robust non-covalent interaction. A variety of synthetic or biological polycations or polycation-containing block co-polymers have also been used to deliver siRNA. Examples of these materials are reviewed next.

## 26. Polyethyleneimine

Polyethyleneimine (PEI) is a synthetic polymer with a highly branched network and a high cationic charge density. Due to its ability to condense nucleic acids and mediate endocytosis, PEI of various molecular weights or degrees of branching have been used to deliver nucleic acids *in vitro* and *in vivo*. Low-molecular-weight PEI has been shown to effectively condense RNA and protect them from nucleases in a cell-free RNase digestion assay using 1% serum [154]. An additional benefit is that PEI exhibits a proton sponge effect and water swells the endosomes. As the amine groups on, the polymer buffers the lower pH of the endosome, subsequently rupturing the endosomal membrane and allowing more siRNA to escape the complex into the cytoplasm. Urban-Klein et al. found that PEI-complexed siRNA showed protection from degradation after 4 h *in vivo*, whereas uncomplexed siRNA was completely degraded within 15 min [154]. In another study, the PEI-siRNA complex was delivered through intravenous administration to prevent and treat influenza virus replication in mice [155]. It was found that PEI-siRNA was distributed to multiple organs but delivered to the lung preferentially after intravenous (IV) injection. However, whether the lung preference is due to the trapping of the polymer-siRNA complex or by lung macrophage remains to be investigated. The trapping of bronched nanoparticles by lung macrophages and liver Kuffer cells is one of the current challenges in siRNA delivery.

Some studies, however, show PEI exhibits high toxicity *in vitro* [156] and therefore raises concerns about *in vivo* use. In attempts to decrease the potential toxicity of PEI, a combination of PEI-PEG was able to stabilise and complex siRNA into a nanosized particle and also prevent non-specific binding to cells or proteins [157]. PEGylation reduced PEI's toxicity, but the particle size increased dramatically upon PEGylation.

## 27. Cyclodextrin-based polycations

Cyclodextrin-based polycations (CDPs) can deliver therapeutic molecules ranging from small molecule drugs, plasmids, and recently, siRNA. CDPs, 50–150 nm in diameter, are composed of cationic polymers with funnel-shaped cyclodextrin molecules integrated into the polymer strand. The cationic polymer complexes with the siRNA and the cyclodextrins serve as adapter molecules, where different functionalised adamantane molecules can be 'plugged' into the CDP. The functionalised adamantane molecules give CDPs additional functionality. As the individual components of CDPs were known to be relatively non-toxic, they were designed specifically as highly non-toxic and non-viral vectors. *In vivo* studies include CDPs delivered to non-human primates and CDPs containing siRNA against the cancerous

EWS–FLI1 fusion gene product. For the EWS–FLI1 study, both short- and long-term administration of CDP–siRNA decreased implanted tumour volume [158]. The authors claim that CDP protects RNA from serum, so modified nucleotides are not required. As an application of CDP functionalisation, they were functionalised with adamantane–transferin and adamantane–PEG conjugates. The resulting PEGylated and transferrin-targeted CDPs delivered siRNA in animals at dosages that are likely to be amenable to therapeutic use in humans. Perhaps the most astonishing characteristic of this approach is that the resulting siRNA complex, even when siRNA containing an immune stimulatory sequence is included, does not produce immune stimulation like that seen with other siRNA–lipid complexes. Generally, 2'-modified siRNA must be used to abrogate such a response.

## 28. Chitosan

Chitosan is a cationic polysaccharide polymer found in nature that has been well characterised and studied for biomedical applications ranging from nucleic acid delivery [159] to bandages. A major draw for chitosan is its biocompatibility. The optimal cationic charge of chitosan-based nanoparticles is controlled by the ratio of amines to phosphates (N:P). After determining the optimal N:P ratio to complex as much siRNA as possible, Howard et al. [160] used chitosan–siRNA nanoparticles in mice and specifically knocked down GFP expression in the lungs. The addition of siRNA dramatically affected the size of the particle, but particles (with or even without siRNA) were  $\geq 200$  nm. A recent study has shown that IV delivery of siRNA (anti-RhoA) loaded in chitosan-coated polyisohexylcyanoacrylate nanoparticles efficiently inhibited the growth of aggressive xenografted breast cancer in mice.

## 29. Dendrimers

Dendrimers are highly branched polymer molecules. They have proven useful in masking the charge of siRNA long enough for *in vivo* delivery. In this approach, polycationic dendrimers, conjugated with targeted lipid moieties, complex with 2'-modified siRNA. The resulting interfering nanoparticle (iNOP) has been used to deliver modified siRNA to the liver, knocking down the expression of apolipoprotein B (apoB) and lowering the serum cholesterol. Inhibition of apoB occurred at concentrations as low as  $1.25 \text{ mg kg}^{-1}$  in mice. This falls within the range generally agreed to be amenable to effective dosing in humans.

## 30. Dynamic polyconjugates

Dynamic polyconjugates are roughly 10 nm in size, much smaller than SNALPs and iNOP–siRNA complexes. They have also been reported to deliver siRNA *in vitro* and *in vivo*. A recent study has demonstrated their ability to target hepatocytes via an *N*-acetylgalactosamine (NAG)-driven targeting mechanism [161]. Dynamic polyconjugates were engineered so that all functional groups of the vector are stable at pH 7, but degrade once in the acidic environment of the endosome. Once internalised into endosomes, the endosome is disrupted via an endosomolytic agent and the siRNA is released into the cytoplasm. This could prove advantageous when developing siRNA complexes with unique biodistribution profiles. One drawback to the approach is that the chemistry necessary to synthesise the dynamic polyconjugates is more involved than other vector preparation.

## 31. Protein-based siRNA vectors

Proteins have been used as a nanosize platform for delivering siRNA. Positively charged proteins, like protamine, have been used to non-covalently bind siRNA, masking their negative charge until their intended target is reached. Such an approach has been used to prohibit HIV infection *in vitro* and slow tumour growth *in vivo* [162]. Recently, the use

of a protamine–antibody fusion protein to selectively target activated leucocytes was reported [163,164]. This demonstration proposes the possibility of interfering selectively with undesired pathogenic immune stimulation without inducing a global immunosuppressive response.

Minakuchi et al. [165] employed atelocollagen, a purified pepsin-treated form of type I collagen from cows [165], to deliver siRNA. This protein complexes with siRNA by simple mixing in a phosphate buffer. The atelocollagen–siRNA treatment method has been used in a localised injection method of xenograft tumours [165,166]. *In vivo* studies using atelocollagen–siRNA showed faster transfection and enhanced silencing of luciferase-expressing xenograft tumours compared to liposomes [40]. Atelocollagen-mediated siRNA delivery has also been used by Kinouchi et al. [167] who demonstrated efficient siRNA targeting against myostatin, a negative regulator of skeletal muscle growth.

## 32. SiRNA conjugates to metallic core nanoparticles

Stable metal core nanoparticles, with different coating layers, can serve as a foundation for building larger nanoparticles. Cores can be constructed from iron oxide, iron cobalt, iron gold or iron nickel. Coating materials include polymers, sugars or other compounds to generate a core–shell structure. SiRNA has been conjugated to metal core nanoparticles via thiols [168], dextran [169], biotin–streptavidin linkages or through metals coated with cationic polymers [170]. In addition, depending on the type of core material, the particles can be tracked via magnetic resonance imaging after systemic injection to evaluate targeting [169] or manipulated to target tissues using external magnets [169].

Superparamagnetic iron oxide has been coated with PEI to complex nucleic acids. These particles, complexed with siRNA against GFP, nearly completely silenced gene expression *in vitro* [171,172]. Another group used superparamagnetic cross-linked iron oxide coated with dextran and conjugated to polyarginine to facilitate cell entry and siRNA (GFP) to silence GFP expression in mice [169]. These metallic nanoparticle approaches deserve continued attention, but major hurdles for the *in vivo* delivery will be toxicity and a demonstrated advantage over other methods.

## 33. Ligand-targeted siRNA delivery

Targeting to the diseased cell, organ or tissue will increase the silencing potency of a given dose of siRNA nanoparticles [10,99,173,174]. Specific cell-targeting will also prevent side effects by avoiding non-diseased cells. At different stages of disease, abnormal cells such as neoplastic or viral-infected cells express a variety of unique markers such as specific antigens and receptors on their surfaces. Such signature molecules provide valuable recognition sites for developing targeted delivery of siRNA. Antibodies, aptamers, small peptides and other ligands that bind to the signature molecules with high specificity and affinity have been studied extensively for their ability to guide siRNA to the target tissues and cells. The attachment of targeting moieties to the siRNA vectors was mentioned briefly in some of the constructs discussed in the section titled 'Non-viral carriers for siRNA delivery'. Here, we categorise and elaborate on various targeting strategies. With the added benefits of stability enabled by various 2'-modifications, it is possible that direct conjugation to siRNA may serve as a simple yet effective way to target it selectively to diseased cells or tissues.

## 34. Small molecules (carbohydrates, folate and cholesterol) as targeting ligands

The asialoglycoprotein receptor (ASGP-R) binds a variety of carbohydrates and is predominantly and highly expressed in liver cells. The ASGP-R was the *in vivo* target for Kim et al. [175] delivering an siRNA-encoded DNA plasmid via a PEI vector. Galactose was conjugated

to PEI and PEG (GPP) via a peptide bond. After 30 min of systemically delivered GPP, gamma imaging of mice revealed high targeted delivery of GPPs to liver tumours compared to the heart, kidney and bladder. Oishi *et al.* also targeted ASGP-R with acid-cleavable lactosylated PEG: siRNA block polyionic complexes and showed competitive targeting and knockdown *in vitro* [153]. The carbohydrate NAG has been successfully used to target hepatocytes in a study using dynamic polyconjugates to deliver siRNA [161].

Folate receptors (FRs) are over-expressed on the cell surface in several types of cancers and diseased cells but are expressed in minimal quantity in all normal cells except kidneys [176,177]. Although only used *in vitro*, molecular folate was conjugated to the cationic polymers PEI and PLL and promisingly achieved high targeted siRNA delivery and target gene silencing in nasopharyngeal carcinoma cells. By incorporating the folate-AMP into the *in vitro* RNA transcription system, a single folate molecule can be placed at the 5'-end of the RNA molecule [87]. Using the dimer/trimer formation mechanism, a complex containing both siRNA and folate has been generated and specifically delivered to a variety of cancer cells that express the folate receptor [87,99].

Cholesterol and cholesterol analogues serve as targeting ligands [86,178,179]. Cholesterol is endocytosed by the cholesterol receptors on hepatocytes. siRNA conjugates were intrasystemically delivered to mice to knock down apolipoprotein B (apoB), an over-expressed lipoprotein involved in coronary artery disease. The cholesterol moiety confers siRNA with 'drug-like' properties such as good stability, cellular delivery and tissue bioavailability. Naked siRNA against apoB showed no localisation to any particular tissue, whereas siRNA-cholesterol molecules were delivered in considerable concentration in hepatocytes, followed by only small levels in the heart and kidney. Depending on which region of the apoB mRNA was targeted, the siRNA-cholesterol exhibited >57% reduction of apoB *in vivo*.

### 35. Peptides, proteins and antibodies as targeting ligands

Many peptides and proteins such as transferrin and antibodies have been used traditionally for the purpose of cell-targeting and gene delivery. Their specificity in target recognition is excellent. However, the large size of proteins and the induction of antibodies are part of the disadvantages in using proteins in gene delivery or nanotechnology.

### 36. Peptides

Peptide ligands are an obvious choice as a targeting moiety for nanoparticles due to their known specificity. One well-characterised targeting strategy exploits the phenomenon that tumours must produce blood vessels to provide nutrients for the fast-growing tumour cells in a process known as angiogenesis. RGD peptide represents another type of targeting peptide to mediate tumour-specific delivery. It binds to transmembrane integrins, which are over-expressed in many types of cancer, and mediates the entry of its cargo into cells. The RGD sequence has been coupled to the surface of several siRNA vectors aiming to target or kill tumour cells, while leaving normal cells relatively unharmed [180]. RGD was conjugated to PEI-PEG polymers, and fluorescent microscopy revealed FITC-siRNA complexed with RGD-PEI-PEG localised more to tumour cells than other tissues.

### 37. Transferrin

Some cancer cells upregulate certain cell surface receptors that correspond to a larger protein ligand, such as transferrin. Transferrin was conjugated to PEG molecules and then incubated with non-conjugated PEGs and CDPs. The three components self-assemble into a nanosize particle, with the CDP region forming the core and the PEG and PEG-transferrin molecules forming the surface [158]. The addition of the transferrin-targeting moiety improved delivery of a luciferase plasmid significantly [181]. The same lab also used transferrin-CDPs to

deliver siRNA to metastasised EWS-FLI1 tumours expressing luciferase. Bioluminescent images showed that non-targeted CDPs did not significantly decrease the size of metastasised tumours, but transferrin-targeted CDPs showed an 80% reduction in metastasised tumour growth compared to controls. A recent study in cynomolgus monkeys showed that multiple systemic doses of transferrin-conjugated CDPs containing siRNA can be safely administered to non-human primates [182].

### 38. Antibodies

Antibodies have been fused to various drug delivery platforms as a targeting agent. Monoclonal antibodies against oncogenes such as HER2 or VEGF have been well developed and widely used for cancer treatments in clinics. In attempts to create a trivalent particle, a recent study by Tan *et al.* [183] conjugated a HER2 antibody to a chitosan-siRNA-quantum dot nanoparticle. The results showed that the trivalent particle significantly silenced GFP. Instead of using the transferrin molecule directly, Pirollo *et al.* conjugated a single-chain monoclonal antibody fragment (scFv) targeted to the transferrin receptor to nanoimmunolipoplexes containing fluorescent siRNAs. These targeted nanoimmunolipoplexes showed a dramatic increase in targeting to primary and metastatic tumours compared to untargeted siRNA treatment. In addition, whereas systemically delivered nanoparticles commonly accumulate in the liver, the study showed that no considerable amount of siRNA was detected in the liver [184]. In a study performed by Lieberman's group [185], the heavy chain Fab fragment of an antibody against HIV-1 envelope protein was fused with nucleic acid-condensing protein protamine (Ab-protamine) to serve as a delivery vehicle. The antibody:protamine:siRNA construct targeted against the HIV *gag* gene effectively suppressed target gene expression and inhibited HIV replication in hard-to-transfect primary T cells infected with HIV. In addition, intravenous injection of the same antibody:protamine:siRNA complex in a mouse model led to substantial suppression of the growth of HIV envelope-expressing melanoma. In the same study, a fusion protein containing the single-chain antibody against HER2 receptor was also able to deliver siRNA specifically to HER2-positive breast cancer cells. These studies represent major landmarks in the successful design and *in vivo* delivery of antibody-targeted therapeutic siRNA particles. Following this approach, another study constructed an antibody-targeted cationic liposome to deliver siRNA against cyclin D to treat inflammatory bowel disease [164]. One drawback to these peptide-based targeting moieties is the induction of antibody response [185], which would abolish long-term dosing schedules unless a humanised antibody be utilised.

### 39. Aptamers as targeting ligands

Aptamers have also been explored as a targeting agent to deliver siRNA. Currently, the major challenge in ligand-based delivery is the short of useful specific cell receptors for targeting. In addition, many cell receptors will simply not internalise cargoes after the ligands interact with the receptors. Receptor-binding RNA aptamers may prove successful in this area. As mentioned in Section 2C, aptamers bind to small molecules (i.e., metabolites, proteins, cell surface receptor, etc.) with high affinity and specificity comparable to antigen-antibody interactions. Applications of aptamers in siRNA vectors include the use of CD4 aptamer targeted to lymphocytes [11] and PSMA aptamer targeted to prostate cancer membrane antigen, a signature surface molecule of prostate cancer (Guo S and Guo P, unpublished results) [55,186]. In the work by McNamara *et al.* [55], RNA aptamers were linked to siRNA to form an RNA chimera by *in vitro* transcription and annealing. Systemic administration of this construct specifically inhibited tumour growth in a xenograft model of prostate cancer in mice. In contrast to the system used by Guo *et al.* (see section titled 'Targeted delivery using phi29 pRNA nanocarriers'), McNamara *et al.* could not transcribe their



entire construct in one piece; the antisense strand of the siRNA was synthesised commercially and annealed to the sense strand separately. McNamara's approaches, however, allowed the incorporation of imaging nucleotides into the construct. It was shown that PSMA-siRNA was internalised by PSMA-positive cells and the treatment reduced xenograph tumour volume. In a similar study, prostate cancer-specific delivery of siRNA was observed when it was coupled to the PSMA aptamer via a streptavidin bridge.

Protein-free platforms using RNA aptamers as targeting ligands are attractive because RNA is not recognised by antibodies and thus suggests improved long-term dosing regimens. As more RNA and DNA aptamers are developed for clinically relevant targets and specific cancer or disease markers, the aptamer delivery approach will become more powerful.

#### 40. Targeted delivery using phi29 pRNA nanocarriers

##### 40.1. Unique features of phi29 pRNA-derived nanocarriers

An RNA-based siRNA vector was inspired by the molecular machinery within the phi29 bacteriophage DNA packaging motor. The motor comprises of a variety of packaging proteins, DNA and six RNA molecules, 117 nt in length, which form a hexameric ring by 'hand in hand' interactions. Extensive structural and functional studies of the motor revealed that the RNA hexamer plays an essential role in genomic DNA translocation and packaging. Thus, it has been termed 'packaging RNA', or pRNA since. Recently, the phi29 pRNA monomer has been designed to carry a variety of therapeutic agents, including siRNA [54,99], hammerhead ribozyme [29] and targeting ligands such as folate and receptor-binding RNA aptamers. Phi29 pRNA monomers with different cargo molecules can be subsequently used for the assembly of dimer, trimer or hexamer molecules, which enables the use of phi29-derived nanocarriers as polyvalent delivery vehicles. Dimeric, trimeric and hexameric pRNA structures can be easily formed via the interactions of rationally designed pRNA monomers with interlocking left and right loops. The nucleotide sequences in defined regions of the individual pRNA monomers can be easily manipulated to accommodate specific RNA sequences without altering the pRNA folded structure and its ability to form multimers via interlocking loops.

It is commonly accepted that RNAs can induce a lower antibody response compared to proteins. Hence, the use of RNA as a delivery vehicle has the potential to reduce the immune response and the rejection of protein vectors after repeated long-term drug administration for chronic diseases. pRNA-derived nanoparticles have a small size (20–40 nm) and narrow size distribution and are of the putative optimal size range for cell uptake (10–100 nm), making them particularly suited for *in vivo* systemic delivery. The ability to easily functionalise pRNA multimers for target-specific localisation and the feasibility of multiple therapeutic agents delivery using a single vehicle are other significant advantages of the pRNA approach. Each hexameric pRNA nanoparticle offers six positions available to carry molecules for cell recognition, therapy and/or detection. A variety of other molecules such as heavy metals, quantum dots, fluorescent beads or radio-isotopes can also be conjugated for detection via imaging of cancer signatures at different developmental stages. Therapeutic efficacy could be improved by conjugating pRNA to endosmolytic chemicals which can trigger the release of the internalised therapeutic reagents from the endosome. The pRNA nanocarriers could also be used for treatment of chronic viral infection by targeting specific virus-glycoproteins from the surface of virus-infected cells.

#### 41. Construction of pRNA monomers harbouring a therapeutic agent, a targeting ligand or a delivery marker

Studies of phi29 pRNA structure have shown that pRNA contains two distinct domains, which fold independently of each other. The first

domain is a double-stranded helical domain at the 5'/3' end and the second domain is an intermolecular binding domain. It has been previously reported that altering the primary sequence of any region of the helical domain does not affect the pRNA structure and folding provided that the two opposite strands remain complementary. The following section will focus on the design and construction of monomeric pRNAs with various cargo molecules as building blocks for dimeric, trimeric or hexameric pRNA nanoparticles.

#### 42. Stabilisation of pRNA

The stability of RNA oligos in the extracellular and intracellular environments is one of the major challenges for RNA-based therapies. Exonuclease degradation occurs within a short period of time and, consequently, the degraded RNA can no longer serve as a therapeutic agent at the diseased tissues. In addition to improved stability of siRNA by chemical modifications (see section 3A), the pRNA carrier can provide additional protection by connecting both ends of the foreign RNA with both ends of the pRNA. The expected exonuclease resistance of circularly permuted RNA likely makes it more stable than its linear counterpart [187,188].

#### 43. pRNA/siRNA

Taking also into account the double-stranded helical structure of siRNA, it is possible that phi29 pRNA can be a good candidate to carry a particular siRNA sequence encoded into its helical domain. To test this hypothesis, the wild-type nucleotide sequence of the helical domain was instead encoded with siRNA sequences targeting GFP, luciferase and survivin [99]. Upon transfection, specific inhibition of the targeted gene was demonstrated by mRNA and protein level. Targeted gene silencing was tested using two chimeric pRNA/siRNA constructs targeting either firefly luciferase or renilla luciferase. These constructs were found to silence the luciferase gene expression without showing non-specific inhibition to internal control luciferase gene expression. The knockdown efficacy of chimeric pRNA/siRNA was comparable or higher than that of chemically synthesised double-stranded siRNA or hairpin siRNA (GFP). Expression of survivin, which is necessary to downregulate genes involved in tumour development and progression, was suppressed by the chimeric pRNA/siRNA (survivin) and pRNA/ribozyme (survivin) and, subsequently, cell death by apoptosis was observed [99].

#### 44. pRNA/hammerhead ribozyme

The ability to alter the nucleotide sequence of pRNA, combined with the possibility to couple the pRNA 5'/3' end with variable sequences without affecting its folding and function, has two major advantages over other available methods. Using circularly permutation strategies, a pRNA-based vector was designed to harbour a hammerhead ribozyme at its 5'/3' ends [28]. The hammerhead ribozyme was targeting the HBV polyA signal. The chimeric ribozyme, flanked and processed by two *cis*-cleaving ribozymes was able to fold correctly while escorted by the pRNA and cleave the polyA signal of HBV mRNA *in vitro* almost completely. Inhibition of HBV replication by the pRNA-escorted ribozyme was more efficient than in the absence of pRNA as shown by Northern blot and Hepatitis B e-antigen assays in cell culture studies. pRNA could also carry another hammerhead ribozyme to inhibit a different gene thus making targeting of multiple genes possible.

#### 45. pRNA/receptor-binding aptamers

As stated in Section 9, pRNA molecules show remarkable folding consistency, even when several mutations are introduced. To achieve cell-specific targeting, a CD4-binding RNA aptamer was identified and used to construct chimeric pRNA/aptamer (CD4) via a mutual 5'/3' end



connection. Using circular permutation, the pRNA vector has been reorganised with the nascent 5'- and 3'- ends relocated to residues #71 and 75, respectively, of the original pRNA sequence. It is conceivable that other pRNA/RNA aptamer monomers can be constructed in the same way.

#### 46. pRNA conjugated to drugs, folate or other chemical moieties

Small molecules, such as folate or cholesterol, can be conjugated to pRNA to enable its targeted delivery to specific diseased cells. The folate molecule has been successfully incorporated into the pRNA 5'-end by *in vitro* transcription using folate-AMP. Alternatively, a variety of molecules such as drugs, chemicals for image detection or endosomal disruption may be desired to be delivered to target cells. RNA chemistry offers simple methods for the conjugation and delivery of such molecules using pRNA-based vectors. Nucleic acids can be modified with functional groups including NHS-, NH<sub>2</sub>-, COOH- and SH-. The NHS-reactive group is useful to conjugate RNA with molecules that have primary amine groups. The NH<sub>2</sub>-group can be used to label RNA with molecules containing COOH- and SH- groups via 1-ethyl-3-(3'-N,N'-dimethylaminopropyl) carbodiimide (EDC) and a variety of amine and sulphhydryl reactive heterobifunctional cross-linkers such as N-[13-Maleimidopropoxy]-succinimide ester (BMPS), respectively. The SH-groups can be further reacted with maleimide derivatives.

#### 47. Assembly of polyvalent dimeric, trimeric and hexameric pRNA complexes

Chimeric pRNA monomers carrying a different cargo such as molecules for specific cell recognition, image detection and therapeutic treatment were used as building blocks for the assembly of dimeric and trimeric polyvalent pRNA delivery vehicles. The assembly mechanism of dimeric or trimeric pRNA nanoparticles is based on interlocking loop interactions between pRNA subunits. The bases involved in these interactions were identified as 45–48 and 82–85 of wild-type pRNA in the left- and right-hand loops, respectively. The sequence in the left-hand loop is represented by a capital letter, while the sequence in the right-hand loop is represented by a lowercase letter and apostrophe. The left-hand loop of one monomer pairs with the right-hand loop in another monomer, and vice versa. In some cases, only two G-C pairs between the interacting loops were sufficient to allow the formation of pRNA dimers. However, a minimum of three base pairs were needed for pRNAs tertiary interactions. When four nucleotides were paired, at least one G-C pair was required. The maximum number of base pairs required to form pRNA multimers was five. Base-pairing can be used to control the multiplicity of the pRNA nanoparticle and the assembly of hetero- or homo-multimeric structures. Rational design of the interlocking base-pairs allows the formation of hetero- or homo- dimers and trimers. For example, a pRNA monomer with a loop sequence of Aa' can form a homo-dimer with itself. Meanwhile, Ab' cannot do that for a homo-dimer. However, an Ab' monomer incubated with a Ba' monomer can dimerise to form an Ab'/Ba' heterodimer. The capacity to control the number of subunits of a pRNA nanoparticle has the advantage of using pRNA-based vectors for the delivery of multiple therapeutic payloads with controlled stoichiometries.

Multivalent pRNA trimers were assembled from the individual chimeric pRNA monomers designed to have specific right or left loops to interact with other subunits [189]. The pRNA folded structure and competency in forming dimers or trimers with high efficiency were confirmed by gel electrophoresis, AFM imaging and sucrose gradient sedimentation. Dimeric or trimeric pRNA was formed simply by mixing individual chimeric pRNAs with their complementary partners with appropriate interlocking loops. Our group has designed a trimeric pRNA nanoparticle harbouring three individual functional subunits: (1) an RNA aptamer or other receptor-binding ligand, which spe-

cifically binds to a cell-surface receptor, (2) a therapeutic agent such as siRNA, ribozymes, antisense RNA or other drugs to be delivered and (3) a reporter molecule such as fluorescent tag. Thus, the pRNA-based vectors enable the simultaneous application of siRNA and aptamers against specific receptors, while tracking the delivery via the fluorescent marker. This allows the use of a single cargo carrier for the targeted delivery of therapeutic agents with confirmation of delivery. Our previous studies demonstrated that incubation of pRNA vectors containing a receptor-binding aptamer and a therapeutic siRNA resulted in binding and co-entry of the nanoparticles into cells, subsequently modulating the apoptosis of cancer cells in leukaemia model lymphocytes. Specific suppression of tumorigenicity of cancer cells has been also confirmed in *ex vivo* animal trials.

We note that due to high shear forces in the blood stream (20 dyn cm<sup>-2</sup>) [190], systemic *in vivo* application of pRNA nanoparticles must be carefully considered. In our previous pRNA multimer studies, intact pRNA dimers and trimers were successfully delivered to cancer cells. However, the cancer cells were only shortly incubated with pRNA trimers *ex vivo*. To support pRNA nanocarriers as systemic *in vivo* multivalent delivery systems, more quantitative results are needed to show that pRNA multimers are stable in high shear force environments.

#### 48. Delivering siRNA using phi29 pRNA dimers

pRNA-based vectors have a small particle size, narrow size distribution, can be modified to carry target-specific ligands and can be rationally and easily designed and controlled to carry various cargo molecules. These unique features make pRNA an ideal polyvalent delivery vector. Our results support the notion that pRNA dimers, trimers or hexamers can be used as polyvalent vehicles for the delivery of up to six therapeutic molecules to specific cells. Ongoing research aims to utilise the pRNA-based vectors *in vivo* for the treatment of lung cancer, brain and neck cancer, ovary cancer, breast cancer, prostate cancer and leukaemia.

To probe the specific cell binding of the folate-pRNA/pRNA-siRNA (survivin) dimer complex, folate-receptor positive human nasopharyngeal epidermal carcinoma KB cells were incubated with dimeric pRNA comprising of a folate labeled Ab' pRNA monomer and a radiolabelled Ba' pRNA monomer. Binding of the dimeric pRNA was confirmed via the radioactive emission and probed the successful delivery. Firefly luciferase gene knockdown was also achieved by simple cell culture incubation of dimeric pRNA nanoparticles containing both folate-labelling and chimeric pRNA/siRNA. To demonstrate specific dimeric pRNA harbouring both folate and siRNA against survivin, animal trials were also performed in athymic nude mice. KB cells pre-incubated with chimeric pRNA complexes with or without folate and siRNA (survivin) were axilla injected into the nude mice. Within 3 weeks after injection, the mice receiving KB cells without folate-pRNA and pRNA/siRNA (survivin) developed tumours while those who received cancer cells pre-treated with both folate-pRNA and pRNA/siRNA (survivin) did not. The specificity of tumour inhibition was probed by using pRNA-derived vectors without folate or containing mutations in the survivin siRNA sequence, which did not show any effect on the tumour development in other mice groups. The RNA chemistry used to conjugate folate to pRNA can be easily extended to other targeting or reporter molecules.

In an artificially CD4-expressing cell line, a dimer made from pRNA/siRNA (survivin) and pRNA/CD4 aptamer selectively and efficiently (>90%) delivered the dimer into cells. Subsequent siRNA knockdown killed more than 30% of the cells. Cancer cells, which did not express CD4, were not affected by the treatment, demonstrating that the activity was dependent on CD4. This work was done *in vitro* on an artificial and clinically non-relevant target receptor. However, it clearly illustrates the promise of pRNA-based vectors as a protein-free, nanosized and targeted siRNA delivery approach.

#### 49. Delivering imaging probes using pRNA multimers

To demonstrate the successful binding and entry of multivalent pRNA complexes into cells, pRNA trimers were constructed using Ab' pRNA/CD4-binding aptamer and Bc' and Ca' pRNA fluorescently labelled with fluorescein (FITC) and rhodamine (Rho), respectively. The binding and entry of the trimer was visualised using confocal microscopy and dual channel detection. The two emission wavelengths indicate that both, the Bc' pRNA and Ca' pRNA, have been conjugated to the Ab' pRNA/CD4 and have been co-delivered to the cell. Specific binding to the cells was demonstrated by the fluorescence emission originating from the cell surface which could not be detected on the cells which lacked CD4. The entry of the trimer into the cell via CD4-mediated endocytosis was visible through the fluorescent spots localised within the cell. These results strongly support the idea that pRNA trimers can serve as vehicles for the targeted delivery of multiple therapeutic components.

#### 50. RNA for nanotechnology and tissue engineering

DNA- and protein-based technologies have been extensively explored for nanotechnology and tissue engineering applications. However, RNA in general and pRNA in particular has only in the last decade been brought into attention as a good candidate for such purposes. RNA combines the ability to easily design and manipulate different constructs specific to DNA nanotechnology with the robust, versatile structure characteristic to proteins. Our work has demonstrated that pRNA can be engineered to assemble into a number of different structures and shapes, including dimers, twins, trimers, tetramers, rods, triangles and hexamers. Arrays tens of microns in size have been constructed that contain thousands of pRNA building blocks [11]. Arrays are collections of many subunits joined together in a repeating pattern that form a more large-scale structure. These arrays are stable under a wide range of temperatures, salt concentrations and pH levels. Protein components of the phi29 DNA-packaging motor have been also used for the construction of arrays, which can be engineered to serve as chips in the diagnosis of diseases or to function as ultra-high density data storage systems [191]. These ordered biological structural arrays can serve as templates for the further construction of superlattices. Another application of RNA arrays would be an antigenicity-free and biodegradable scaffold for wound and tissue repair in tissue engineering.

The novel ordered structures assembled from proteins or pRNA can also be converted into metal arrays by replica and metal spray coating technology. The potential applications of these arrays include molecular sieves for scaffolds of large-scale supramolecular structures, nanowires, nanochips for the detection of pathogens or ultra-high density data storage systems.

#### 51. Potential adverse side effects of siRNA therapy

Although siRNA holds great potential as a clinical drug to silence disease-causing genes, recent research has discovered several undesired side effects associated with siRNA. The mechanism for the side effects range from sequence-independent effects to choice of vector (i.e., viral or non-viral delivery). Some side effects are similar to those identified during the clinical development of therapeutic antisense oligonucleotides.

#### 52. siRNA-mediated induction of immune responses

In vertebrates, an immune response can be induced by dsRNA as a mechanism to defend viral infection. DsRNA can be sensed in cytoplasmic and endosomal compartments by RNA-dependent kinases (PKR) [192]. The activation of PKR induces interferon (IFN) responses, leading to global inhibition of protein synthesis by phos-

phorylation of eIF-2 $\alpha$ , and production of proinflammatory cytokines by activating NF-kappa-B. Besides PKR, recent studies showed that dsRNA within the cytoplasm can also be sensed by the helicase retinoid-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5), which may also contribute to the activation of NF-kappa-B and the production of IFN. RIG-I is an intracellular pathogen-recognition receptor. A variety of viruses are recognised by RIG-I which then triggers the innate antiviral response independent of the Toll-like receptor (TLR)-dependent pathways. MDA-5 gene is induced during differentiation, cancer reversion and cell apoptosis. In the past, only long dsRNAs (>30 bp) have been considered to induce interferon responses. However, more studies have found that both, chemically synthesised siRNAs and those generated from *in vitro* transcription with various lengths, can lead to the activation of PKR, and the longer the length of the dsRNA, the stronger is the effect on the host cells. A study performed by Rossi's group reported that a 5' triphosphate on T7 polymerase-transcribed RNA molecules is a potent inducer of IFN response [193].

In addition to being sensed within the cells, non-self RNA can be recognised on the cell surface by TLRs. A variety of TLRs are expressed on immune cells to identify non-self patterns from pathogens. Among them, TLR7 and TLR8 recognise viral or synthetic single-stranded RNAs (ssRNAs) containing GU-rich sequences. TLR3 is believed to recognise dsRNA and polyI-polyC sequences. Recognition of RNA by TLRs activates cellular-signalling pathways that lead to the activation of NF-kB and production of proinflammatory cytokines. In a very recent study it was shown that siRNA against VEGF in the eye can trigger TLR3 on the cell surface. VEGF is a pro-angiogenic factor that is involved in age-related macular degeneration. Although the intended effect (i.e., decreased blood vessel growth) was observed, the effect was seen regardless of the sequence; for example, siRNA-targeting luciferase also showed reduced vessel growth [194]. The silencing was attributed to sequence-independent triggering of TLR3 and subsequent cytotoxicity. Therefore, TLRs represent another important component of siRNA-mediated immune response. To avoid or reduce unfavourable immune responses, siRNAs need to be optimised to avoid GU-rich sequences or other patterns that may trigger TLR responses [195]. Optimising the length and/or reducing the dosing of siRNA may also help to alleviate IFN responses. Finally, experimental screening of multiple siRNAs against the same target is required to identify a candidate with minimal immune responses.

#### 53. Sequence-dependent off-target effects of siRNA

Off-target effects of siRNA refer to undesired silencing of genes other than the intended target. Off-target silencing has been the subject of intense study because it may lead to serious and unpredicted side effects. Typically, if siRNA induces immune responses in a sequence-independent manner (as mentioned in the section 'siRNA-mediated induction of immune responses'), it affects global RNA transcription and protein synthesis. On the other hand, sequence-dependent off-target effects come from the presence of a sequence in the siRNA that partially matches an mRNA sequence of non-target genes in the host. The existence of such homologous sequences will lead to the cleavage of the non-targeted mRNA or inhibition of its translation in a similar fashion to the action of miRNA. Using bioinformatics, careful comparison of the sequences of siRNA with the host genome will be necessary to exclude a possible match of sequences. A recent study has found that 2'-O-methyl modification at the position 2 in the guide-strand of siRNA can reduce off-target silencing of most transcripts that are complementary to the seed region in the siRNA guide strand [196]. However, it is unlikely to successfully avoid all sequence-dependent off-target effects by the current siRNA design algorithms. High-throughput mRNA and protein arrays have been used to determine the gene(s) affected by the siRNA, which helps to evaluate the off-target effects of each individual siRNA candidate.

## 54. Saturation of endogenous silencing pathways

A fundamental study has indicated that high levels of shRNA, a precursor molecule to siRNA, expressed by adeno-associated virus type 8 can cause severe liver toxicity and mortality in mice [193]. The hepatocyte toxicity has been associated with the saturation of endogenous miRNA pathways. The machinery to process and transport miRNA, such as nuclear karyopherin Exportin-5, can be saturated by excess exogenous shRNA that resembles miRNA. This study hinted at the potential danger of using viral vectors for siRNA delivery. In addition, the risk of disrupting endogenous small RNA pathways has been minimised by optimising the length of the shRNA and reducing the dose of virus. The optimisation of shRNA expression level can also be achieved by the use of different promoters of transcription.

## 55. Conclusion

Besides the challenge of specific delivery, other challenges of siRNA therapy are non-specific gene silencing, immune response and cytotoxicity. Currently, these areas are under extensive investigation and feasible solutions to these questions are emerging. The non-specific gene silencing and off-target effect can be reduced by alternating the siRNA size, targeting location and modifying the primary sequence. The sense strand is more tolerable to sequence modification without losing the gene-silencing potential. The immune response to siRNA includes PKR effect, toll-like immunity and dsRNA-induced interference or the induction of alpha/beta interferons. Readers are directed to other recent excellent reviews in this area [197–199].

The ideal systemic delivery vector should be ~10–50 nm in size, non-toxic, non-immunogenic, stable, capable of efficient intracellular delivery and possess specific targeting ability. That is one of the key reasons why antibody therapies currently have many problems in the clinic and scientists are looking for new solutions in nanotechnology.

RNA engineering and nanotechnology has emerged as novel and exciting areas for therapy and diagnosis of diseases. Among the therapeutic RNA categories, siRNA has arguably elicited the most excitement. To make the application of therapeutic siRNA a reality, future research must continue to focus on achieving efficient delivery to the desired cells, minimising off-target effects, increasing resistance to nuclease degradation, avoiding immune responses such as  $\alpha/\beta$  interferons, PKR effects and toll-like immunity and trapping of polymerised particles by Kuffer cell and lung macrophage.

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