Chem Soc Rev

RSC Publishing

TUTORIAL REVIEW

View Article Online
View Journal | View Issue

Chemistry and formulations for siRNA therapeutics

Cite this: *Chem. Soc. Rev.*, 2013, **42**, 7983

Andrzej Gallas,[a] Cameron Alexander,[a] Martyn C. Davies,[a] Sanyogitta Puri[b] and Stephanie Allen*[a]

Received 19th December 2012

DOI: 10.1039/c3cs35520a

www.rsc.org/csr

Small interfering ribonucleic acids (siRNAs) form potentially the most important class of next generation therapeutics. However, achieving their efficient delivery in the correct dose, time and location in the body remains a significant challenge. Rapid developments in the chemistries of siRNA formulations are enabling new strategies to overcome the core obstacles to delivery which include poor ribonuclease (RNase) resistance, short biological half-life, lack of tissue targeting, inefficient cellular uptake and undesirable toxicity. In this review we describe these principal challenges and evaluate recent approaches proposed to overcome the chemical, biochemical and physiological barriers. The role of the specific chemical structure of siRNA is considered and an overview of selected literature-reported siRNA formulations is provided. These include chemically-modified siRNAs and analogues, aptamer–siRNA chimeras, self-assembled nanoparticles, lipid and polymer complexes, bioconjugates and fusion protein complexes. We conclude the review with an outlook for the clinical use of this highly promising, but pharmaceutically challenging biotherapeutic.

Key learning points

(1) Therapeutics based on siRNA are drug candidates which modulate protein production in the body. These offer remarkable potential advantages over currently available treatments.

(2) The main barriers that prevent siRNA therapeutics from reaching the market are poor resistances to enzymes such as RNase, short biological half-lives, a lack of cell/tissue/organ targeting, ineffective cellular uptake, toxicity and other formulation-related difficulties.

(3) Formulation strategies that improve RNase resistance and biological half-life are likely to be successful in enhancing siRNA delivery, as these processes are relatively well understood and the developed strategies to overcome them based on many years of research within the wider drug delivery field.

(4) Effective cellular uptake and toxicity are the most significant barriers for the targeted delivery of siRNAs. Progress is hindered by a poor understanding of the behaviour of siRNA *in vivo* and the natural phenomena underlying siRNA transport and intracellular processing.

(5) Although there are still many unresolved issues regarding the targeted delivery of siRNA, recent significant progress towards understanding the *in vivo* behaviour of siRNA suggests that siRNA therapeutics are nevertheless strong candidates that are likely to transform medicine in the near future.

Introduction

In little more than a decade, the science of small (short) interfering ribonucleic acids (siRNAs) has moved from the academic laboratory to become a Nobel Prize winning endeavour, with the generation of an entirely new class of potential medicines. It is not surprising that siRNA has become an extremely hot topic for the global pharmaceutical industry, with a small number of siRNA-based drug candidates already at the stage of clinical trials. However, although siRNAs can in theory be generated to knock down selectively the expression of almost any single protein in a biological system, the delivery of nucleic acids required to achieve this remains a considerable challenge.[1,2] The difficulties in delivery stem in part from the chemistry of ribonucleic acids, for example their polyanionic nature and their enhanced susceptibility to hydrolysis, but also from specific cellular and physiological barriers.[3] Indeed, in order to administer siRNA as a therapeutic it is necessary to transport a macromolecule across a range of exacting biological barriers, which have evolved to keep charged biopolymers such as exogenous RNA out.

Biochemistry of siRNA

siRNAs are polyanionic macromolecules, but are relatively small compared to many other biopolymers (size: <10 nm, molecular weight: ~ 13 kDa). The siRNA structure comprises

[a] *School of Pharmacy, Nottingham, NG7 2RD, UK. E-mail: stephanie.allen@nottingham.ac.uk; Tel: +44 (0)115 951 5050*

[b] *AstraZeneca, Pharmaceutical Development, Macclesfield, SK10 4TG, UK. E-mail: Sanyogitta.puri@astrazeneca.com; Tel: +44 (0)162 551 5847*

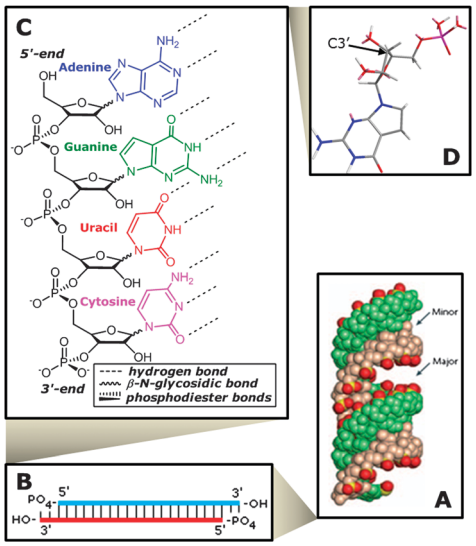


Fig. 1 The biochemical structure of siRNA. (A) A space-filling model of tightly-packed siRNA, with an A-form double-helix showing major and minor grooves; (B) schematic of the double stranded structure of siRNA highlighting the red guide and blue passenger strands and their ends (5′-phosphate and 3′-hydroxyl group-terminated, respectively); (C) the chemical structure of an RNA strand composed of phosphodiester bond-linked ribose moieties and the following bases: adenine (blue) guanine (green), uracil (red), cytosine (purple); (D) C3′-*endo* sugar pucker in ribose-GMP, where the nitrogen, oxygen and phosphorus atoms are shown in blue, red and yellow, respectively. (A) and (D) are adapted by permission from Macmillan Publishers Ltd: Nature ref. 4, © 2007.

two strands: the guide strand (or the antisense strand) and the passenger strand (or the sense strand) that are complexed with each other according to Watson–Crick base pairing (Fig. 1).

Usually, siRNAs are 19–21 base-pairs (bp) long, however longer sequences have been reported.[4] Both strands have their 3′- and 5′-ends oriented oppositely with free phosphate groups at the 5′-ends and 2-nucleotide overhangs at the 3′-ends. Chemically, each strand is composed of ribose residues that are linked with 3′α → 5′β-phosphodiester bonds building a strand backbone, with one of four possible bases (adenine (A), guanine (G), uracil (U), cytosine (C)) attached at every 1′β-position.

Hydrogen bonds formed between the bases of opposing strands of complementary sequence, are the important forces responsible for forming the double-stranded complex (duplex). Structurally, siRNA forms a right-handed (anticlockwise) and tightly packed A-form helix with deep and narrow major grooves, that are not easily accessible for protein interactions (diameter ≈ 23 Å, intrastrand phosphate–phosphate distance ≈ 5.9 Å, helical pitch ≈ 28 Å with 11–12 bp per helical turn). The reason why the structure is so compact, and thermodynamically stable is because the ribose residues have a C3′-*endo* configuration.[4]

siRNA mode of action

In 1998 Andrew Fire and Craig Mello discovered a regulatory mechanism of post-transcriptional protein expression, in the worm *Caenorhabditis elegans*, that they called RNA interference (RNAi).[5] Briefly, this phenomenon consists of triggering the selective destruction of messenger RNA (mRNA) after introducing a long (>23 bp) double stranded ribonucleic acid (dsRNA) into a cell. Initially, dsRNA is taken up by the cell and hydrolysed by the endoribonuclease, *Dicer*, into siRNA. Next, the siRNA binds to the holoenzymatic complex, RISC (RNA-induced silencing complex), that catalyzes binding and cleaving of a specific mRNA, which in-turn inhibits translation of the protein (Fig. 2). In 2001, Elbashir *et al.*[6] then demonstrated that it was also possible to silence gene expression by direct introduction of siRNA to mammalian cells, and hence avoid the Dicer-catalysed reaction. In retrospect it is now clear that Fire and Mello had identified a system that enables the selective knock down of gene expression, by transfecting cells with an RNA molecule. Soon after this discovery, other scientists started

Andrzej Gallas

Andrzej Gallas is a PhD student in the School of Pharmacy at the University of Nottingham. He is a part of an industrially sponsored Targeted Therapeutics Centre for Doctoral Training and his project combines the areas of biophysics, surface analysis and drug delivery. Andrzej is a pharmacist, graduating (with honours) from the Poznan University of Medical Sciences in Poland. Before his PhD, Andrzej was involved in several multidisciplinary research projects in the fields of molecular biology, polymer and organic synthesis, solid dosage formulation and tissue engineering in Poland, Germany and the UK.

Stephanie Allen

Stephanie Allen is Associate Professor and Reader of Molecular Biophysics in the School of Pharmacy at the University of Nottingham (UK). She received her PhD (1997) from the University of Nottingham, and following postdoctoral research, secured the 'Pfizer Lectureship in Pharmaceutical Sciences' post at Nottingham (in 1999). Based within the Laboratory of Biophysics and Surface Analysis (LBSA) (School of Pharmacy), she leads the experimental single molecule biophysics research. This is focussed on understanding biological processes at the molecular level and how this can be exploited for the development of new therapeutic approaches or new materials/devices.

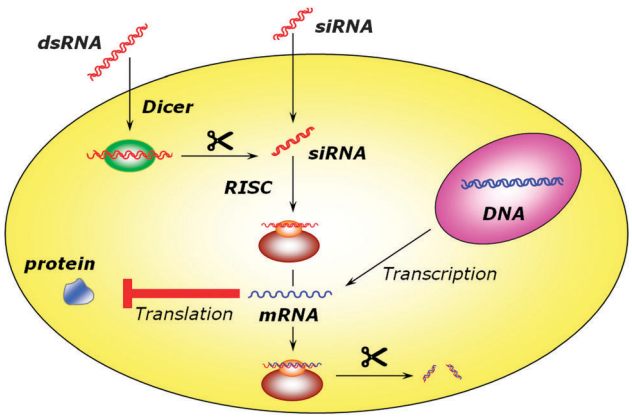


Fig. 2 The role of siRNA in gene silencing. After internalization into a cell, the siRNA duplex is recognized and dissociated by the RNA-induced silencing complex (RISC), which subsequently catalyses cleavage of a messenger RNA (mRNA) of a specific sequence and prevents it from being transcribed into protein. If longer (>23 bp) double stranded RNA (dsRNA) sequences reach the cytosol, initial processing by the endonuclease, *Dicer*, occurs.

using siRNA as a basic research tool for inhibiting the cellular biosynthesis of a desired protein. At the same time, the potential of siRNA as a promising next-generation therapeutic against various currently incurable diseases became apparent.

The potential of siRNA therapeutics

Over the past ten years the potential impact of siRNA therapeutics has been established. It was initially assumed that they held the potential to selectively silence the expression of any gene.[7] However, due to toxicity issues, more recent research suggests that the selective knockdown of all genes, in reality, remains a significant challenge. Nevertheless, siRNA-based drugs may still be an option for several currently untreatable diseases, such as age-related macular disease (AMD), Huntington's disease and cancer.[8] Since siRNA inhibits protein synthesis, its therapeutic effect should also last from days up to weeks.[7] Such a long-lasting therapy is attractive as it would not only reduce the expense of medical treatment, but also help with regards patient compliance. The efficacy of siRNA therapeutics is estimated at the picomolar level, which in addition to reducing the likelihood of adverse drug reactions (ADRs), may be of benefit for economic reasons. Finally, the large-scale manufacture of siRNA is potentially straightforward and efficient[7] as oligonucleotide synthesis is well-developed, allowing the requirements of good manufacturing practices (GMP) to be easily met.

Delivery of siRNA

According to John Rossi (Beckman Research Institute, California) the three biggest problems with siRNA-based medicines are "delivery, delivery and delivery".[7] Indeed, getting siRNA into a certain place at a certain time and at the required dose is not trivial due to the many obstacles that siRNA therapeutics face. The following section focuses on identifying the challenges for effective siRNA delivery, as well as outlining the possible ways to overcome them.

Barriers for siRNA therapeutics

The rational design of siRNA formulations (siRNA + delivery system) has to take into account the challenges that prevent them from being efficient therapeutic tools. We distinguish these as the following: poor ribonuclease (RNase) resistance, short biological half-life ($t_{\frac{1}{2}}$), lack of cell/tissue/organ targeting, ineffective cellular uptake, toxicity and other formulation-related difficulties.

To be used as a therapeutic, siRNA has to be stable in the environments to which it is exposed *e.g.* during manufacture, storage and delivery. Unfortunately, this nucleic acid is extremely susceptible to digestion by RNases, a class of commonly occurring enzymes (*e.g.* associated with dust, hands and the blood) which rapidly catalyse RNA hydrolysis. Inherently siRNAs therefore have poor RNase resistance, both *ex* and *in vivo*. The interaction between the siRNA backbone and the active site of the RNase lie at the basis of this instability (Fig. 3).[9]

Once administered, to evoke a desirable clinical effect the siRNA should not be eliminated from the body rapidly. Naked siRNA circulating in the blood stream is very unlikely to achieve this due to its very short biological half-life ($t_{\frac{1}{2}}$ = 2–6 min[10]). It is hydrolyzed by systemic RNases within seconds after injection and due to its relatively small size, is easily forced through the fenestrations during glomerular filtration in the kidneys and eliminated into urine. SiRNAs also lack the ability to strongly bind to plasma proteins and/or accumulate in a 'sink' compartment (a specific organ or tissue), which also manifests in a high renal clearance† (C_L = 17.6 ml min^{-1} (ref. 10)) (Fig. 4).

A sufficient dose of siRNA then has to reach a particular location in the body (organ, tissue, cell) and be retained there for a sufficient period. Since naked siRNAs do not have any cell-targeting properties they are not able to specifically accumulate

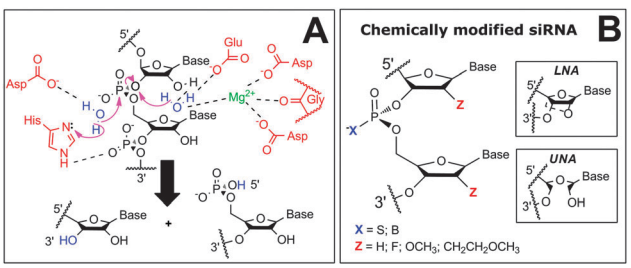


Fig. 3 (A) Proposed mechanism of RNase-catalysed hydrolysis of siRNA and (B) the types of chemical modifications used to prevent this. (A) The 2′-hydroxy groups and phosphodiester linkages of siRNA strands (black) form hydrogen bonds with the RNase active centre and water (red and blue, respectively), to facilitate hydrolysis. (B) Chemical modifications of red and blue groups preclude the enzymatic reaction. Alternatively, RNase resistance can be gained through changes in the conformation of ribose ring(s), *e.g.* locked or unlocked nucleic acid (LNA and UNA, respectively).

† Clearance (C_L) is a pharmacokinetic parameter expressing the ability of an organ (usually the kidneys) to eliminate drug from the plasma. By definition it is the amount of plasma cleared from the siRNA per unit of time [min ml^{-1}].

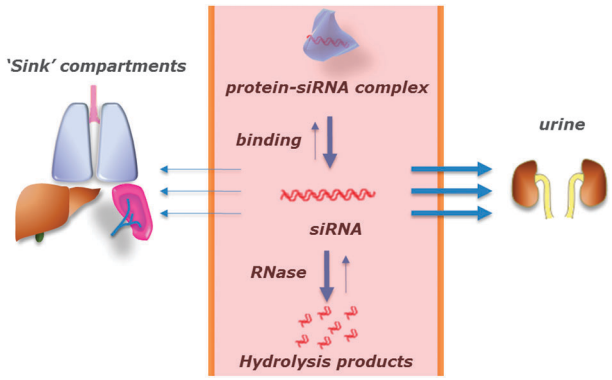


Fig. 4 Pharmacokinetics of naked siRNA. After systemic administration most siRNA is hydrolysed in the blood or eliminated *via* kidneys, instead of binding to plasma proteins and reaching the desired target site in the body.

in cells that express the protein(s) of interest. Being a generic issue faced within the broader drug-delivery field, two main strategies, classified broadly as passive and active targeting, can be employed to overcome this. Passive targeting takes advantage of various *in vivo* processes and occurs independently of specific changes in the formulation structure. Here several differences between organs can be exploited including in cell adherence, the sizes of membrane pores within tissues, blood filtration rates, tissue accessibilities for local drug application and organ/tissue/cell specific biological processes (*e.g.* circulation of gut-associated lymphatic tissue (GALT) macrophages[11]). Active targeting, on the other hand, strongly depends on the specific physico-chemical properties of the formulation and usually requires functionalization of the therapeutic with specific moieties (ligands) that can bind with high affinity to receptor-like structures close to the desired active site. Once a ligand-equipped siRNA formulation recognizes a specific receptor localized on specific cell, binding and cellular uptake of siRNA occur (Fig. 5B and C).

At the cellular level sufficient siRNA needs to cross the cytoplasmic membrane and reach the RISC, in order to induce the desired silencing effect. According to Lu *et al.* transport of about 95% of the siRNA entering the cell occurs *via* endocytosis.[14] Although various types of endocytosis have been described, the general mechanism consists of two key steps: (1) an interaction between the macromolecule/particle and cellular membrane resulting in the entrapment of the macromolecule/particle within a newly formed spherical vesicle, called the endosome, inside the cell (endocytosis triggering); (2) the release of the macromolecule/particle into the cytosol after lysis of endosome induced by pH changes within its interior (endosomal escape). Since siRNA is polyanionic and hydrophilic it is unlikely that interaction with the negatively charged, cytoplasmic membrane will occur. Even if the siRNA is incorporated into endosomes, its automatic release is unlikely, so that most of the nucleic acid will stay within the endosomal matrix and eventually undergo low pH-induced hydrolysis. In the event of successful endosomal escape, the siRNA will need to be recognised by an appropriate argonaute protein (AGO) (the catalytic component of RISC) within the complex

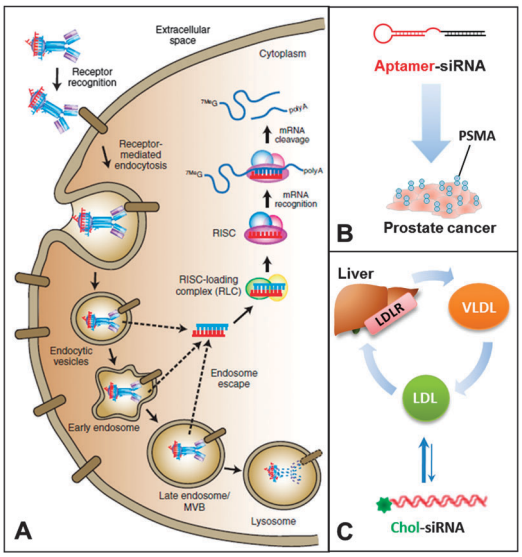


Fig. 5 (A) Endocytosis mediated cellular uptake of a bifunctional protein–siRNA complex. The targeting agent (antibody) recognises surface protein and triggers receptor-mediated endocytosis. After the fusion of endocytic vesicles into a late endosome/multivesicular body (MBV), the pH within its matrix decreases and the therapeutic is either transferred and hydrolysed in lysosomes or released into the cytosol and loaded onto RISC. Reproduced with permission from ref. 12. (B) The active targeting of aptamer–siRNA chimera that selectively recognize prostate-specific membrane antigen (PSMA) overexpressed on prostate cancer cells.[13] (C) The passive targeting of cholesterol–siRNA conjugates that are taken up by low-density lipoproteins (LDL), bind to LDL receptor (LDLR) and accumulate in the liver.[10]

environment of the cytoplasm. In summary, in order to take siRNA successfully to its molecular destination, delivery platforms‡ must be able to overcome three cellular barriers: the ability to trigger endocytosis, to escape from the endosome and to reach the RISC (Fig. 5A).

SiRNA therapeutics also cannot be toxic. Toxicity may originate from three sources: the siRNA itself, the formulation excipients or the complete siRNA formulation. Induction of an immune response (immunogenicity), and also the unselective silencing of additional genes (off-target effects) have been observed and reported in the literature as side-effects.[15] The reasons for siRNA-related toxicity are related to its sequence, which may contain motifs that are detected by pattern recognition receptors (PRRs) of the innate immune system or induce any other immunotoxic response, since chemically synthesized siRNA may be recognized as a foreign body *in vivo*. The sequence may also be suitably unspecific so that it pairs with more than one mRNA within the cell effecting expression of many genes at a time. For instance, duplex RNAs possessing a 5′-UGU-3′ motif have been demonstrated to bind to *Toll-like* receptors 7/8 (TLR7/8) and promote immunogenicity,[16] whereas the exposure of mammalian cells to siRNA of defined sequence non-specifically stimulated or repressed over 1000 different genes.[15]

‡ The term 'delivery platform' in this review stands for all the delivery excipients in one formulation that carry siRNAs. An exception are chemically modified siRNA formulations (*e.g.* aptamer–siRNA chimeras, sd-rxRNA) where the terms delivery platform and formulation are equal.

If the toxicity originates from the excipients or the formulation as a whole, the reasons can be various, as detailed in later sections of this review.

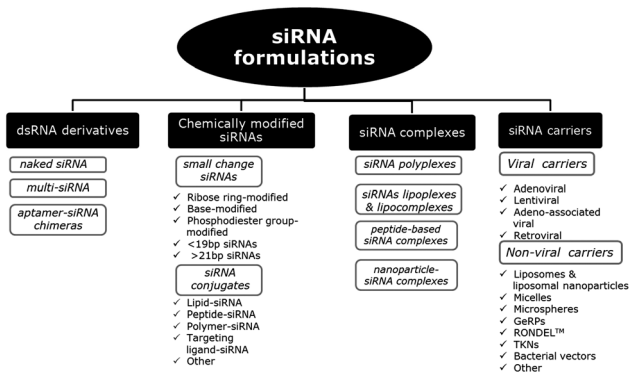
In summary, research performed over the last ten years has greatly facilitated our understanding of siRNA delivery science and, in particular, has identified the important factors that need to be carefully considered during formulation design. However, we have only recently begun to overcome the barriers which have prevented its realization as an efficacious therapeutic, as presented in the following section.

Formulation approaches for siRNA delivery

The various approaches that have been employed to create useful siRNA formulations can be organised according to their level of complexity (see Scheme 1). Here we review the potential approaches that are currently offered in the literature.

Increasing RNase resistance

Since poor siRNA stability is caused by ubiquitous RNases, approaches to deal with this issue have focussed on the prevention of enzyme access to the nucleic acid. Two main approaches have been suggested: (1) the use of serum-stable, siRNA-encapsulating or complexing vectors that build a mechanical barrier that prevents access of the RNases (Fig. 6); (2) small chemical modifications of the ribose 2′-OH and/or phosphate groups which are the key points of the siRNA–RNase interaction (Fig. 3B). In practice, the challenge of increasing siRNA stability is best managed when both of these approaches are employed.



Scheme 1 Types of siRNA formulations based on their relative complexity; from simple dsRNA derivatives to the relatively complex formulations based on viral vectors.

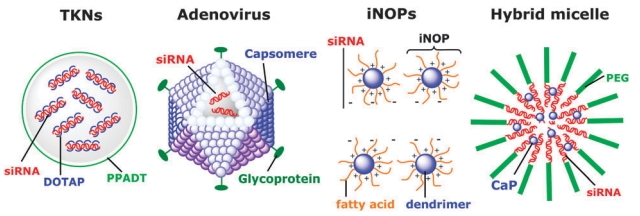


Fig. 6 Examples of vectors preventing RNase–siRNA interaction. SiRNA and the cationic lipid, DOTAP (1,2-dioleoyl-3-trimenthylammoniumpropane), form complexes encapsulated by a reactive oxygen species-sensitive polymer PPADT (poly(1,4-phenyleneacetone dimethylene thioketal)), to form thioketal nanoparticles (TKNs).[17] Similarly, adenoviral vectors incorporate siRNA molecules.[18] Interfering nanoparticles (iNOPs) composed of fatty acids covalently attached to fourth-generation lysine dendrimers, associate with siRNA into complexes.[19] Calcium phosphate (CaP) facilitates the formation of hybrid micelles by PEG–siRNA conjugates.[20]

Many examples in the literature utilise the first of these strategies. However, consideration of the RNA degradability at the stages of drug formulation and intracellular distribution seems to be lacking for such systems. For manufacture, encapsulation of a naked siRNA into a delivery system would require an absolute RNase-free environment, which is not attractive for the pharmaceutical industry for increased cost reasons. Furthermore, even if efficiently delivered and taken up into cells, within the cytosol an siRNA molecule will still have to encounter intracellular RNases that may cause its degradation prior to reaching the molecular target, RISC. Therefore, since a simple encapsulation of a naked macromolecule is unlikely to be a sufficient solution for all cases, alternative approaches have been developed.

Another method to enhance siRNA stability is through chemical modifications of the nucleotide strands. As the catalytic mechanism of RNases involves interaction with the ribose 2′-OH and phosphate groups of the RNA molecule, slight modifications of these groups are likely to increase the nuclease resistance. Indeed, 2′-O-methyl (2′OMe), 2′-deoxy (2′H), 2′-deoxy-2′-fluoro (2′F), 2′-methoxyethyl (2′-MOE), locked nucleic acids (LNAs) as well as phosphorothioate (PS) or boranophosphate (BP) derivatives have been demonstrated to increase the stability of siRNA therapeutics.[4,8] The reason why such a phenomenon takes place can be explained *via* Koshland's induced fit hypothesis for enzyme active sites.[21] According to this hypothesis, the additional groups in the structure of a substrate (*i.e.* the chemically modified siRNA) introduce a steric hindrance to the active site of an enzyme (*i.e.* the RNase), which prevents the required adjustment of their conformations. As a result, the interaction between the chemically modified siRNA and the RNase active site is so poor that the enzyme-catalysed reaction (*i.e.* hydrolysis) is inhibited. Obviously, the type, location and amount of chemical modification cannot be random. Careful consideration of this is needed before synthesis to reduce the risk of creating an siRNA with reduced silencing potency and/or increased toxicity.[4]

Increasing the biological half-life of siRNA

Overcoming a drug's short biological half-life is a common issue that needs to be addressed for many potential therapeutics, and is not restricted to siRNA. It is therefore not surprising that approaches to improve this for siRNA therapeutics have adopted strategies also used for non siRNA drugs. In addition to increasing RNase resistance, these include, binding to plasma proteins (lipoproteins and/or serum albumin), decelerating the kidney elimination process and/or pushing the drug into 'sink' biological compartments such as the liver, lungs or cancer tissue.

For example, Alnylam Pharmaceuticals Inc. demonstrated that conjugating the siRNA to a lipophilic moiety enhanced plasma protein binding after systemic administration to rats.

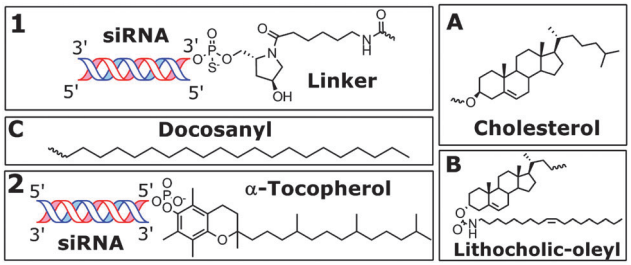


Fig. 7 Structures of lipophilic siRNA conjugates with prolonged biological half-lives. Cholesterol (1A), bile acid (1B) or fatty acid (1C) can be connected to 3′-end of siRNA guide strand (red) through a *trans*-4-hydroxyprolinol linker.[22] α-Tocopherol (2) has also been directly attached to the 5′-end of the siRNA guide strand. Such modifications have been made to increase RNase resistance.[23]

They managed to significantly improve the pharmacokinetic properties ($t_{\frac{1}{2}}$ = 95 min, C_L = 0.5 ml min^{-1}) by using Chol–siRNA, a stable siRNA which was conjugated to a cholesterol residue at the 3′-end of the sense strand (Fig. 7). Additionally, this formulation was reported to provide a broad tissue distribution (*i.e.* liver, heart, kidney, adipose, lung tissues) within 24 h after injection in mice.[7,22] Similarly, other lipophilic siRNA conjugates (*e.g.* Toc–siRNA,[23] bile- and fatty acid–siRNA conjugates[22]) were shown to bind more efficiently to plasma proteins and promote gene silencing (mainly in the mouse liver) (Fig. 7).

PEGylation is a common modification for any small-size therapeutic that tends to be rapidly cleared by the kidneys after administration, and for example has been successfully utilized to improve the plasma half-life of aptamer–siRNA chimeras.§ In such formulations, two major components are distinguished within the structure, *i.e.* a dsRNA portion and the aptamer, which are often connected through a covalent linker as illustrated in Fig. 8A.

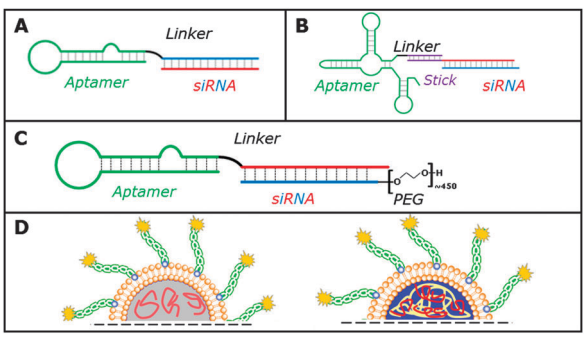


Fig. 8 (A–C) Structures of example aptamer–siRNA chimeras. A targeting aptamer (green) can be conjugated to a passenger siRNA strand (blue) *via* a short linker sequence[10] (black) (A) or to a guide strand (red) through a flexible sticky sequence[25] (purple on B). The passenger strand may be PEGylated on the opposite strand and end to the aptamer[10] (C). (D) The structure of LPD-NPs[26] (right), LPH-NPs[27,28] (right) and LCP-NPs[29] (left). The cores are composed of calcium phosphate composites (grey), siRNA (red), protamine (blue) and carrier DNA/hyaluronic acid (yellow). The liposomal coating (orange) postinserted with cationic lipid–PEG-targeting agent conjugates (blue, green, gold, respectively).

§ The term 'chimera' stands for nucleic acid construct composed of strands of different origin.

Aptamers are short oligo-ribonucleotide sequences (~25–40 bp) that can specifically and efficiently bind proteins on the cell surface with nano- or picomolar dissociation constants, and therefore serve as targeting agents and triggers of receptor-mediated endocytosis. The affinity and specificity of an aptamer to a target protein strongly depends on its sequence.[24]

The dsRNA component is around 29 bp long and is the source of the siRNA, which is released in the cytosol of a cell after Dicer-catalyzed hydrolysis. In order for the dsRNA sequence to be easily recognised by the Dicer/RISC enzymatic apparatus, certain structural modifications can be applied, *e.g.* 2-nucleotide overhangs at the 3′-end of the dsRNA guide strand, point mutations in the passenger strand and an attachment of the aptamer–linker conjugate to the 5′-end of the guide strand. The linker sequence can be designed in various ways. One simple way is for a small dinucleotide sequence connecting the 5′-end of the dsRNA passenger strand and 3′-end of aptamer sequence to be introduced to build a flexible link for the 'targeting' and 'silencing' parts of the formulation.[13] However, Zhou *et al.* reported the successful use of a '*sticky sequence*' linker design which not only assures the flexibility between the aptamer and dsRNA sequences, but also enables the delivery of multiple silencing siRNA sequences within the same platform[25] (Fig. 8B).

Importantly, the aptamer–siRNA chimeras are stable, relatively cheap and quick and easy to synthesize. This system can target cells with specificity comparable to antibody-targeted formulations, but with generally lower and more manageable immunogenicity, as well as a higher potential for cellular uptake due to the smaller molecular size. The disadvantages of this platform however, include the need for *i.v.* administration and a short plasma half-life ($t_{\frac{1}{2}}$ < 35 min in mice). In an attempt to overcome this latter drawback, it was recently demonstrated that the conjunction of a nuclease resistant aptamer–siRNA chimera to PEG could substantially increase its half-life (in mice), to greater than 30 h[10] (Fig. 8C).

The issue of short *in vivo* life times can also be solved by making the drug accumulate in a separate tissue (sink compartment), which prevents the siRNA from being eliminated in the urine. For instance, Huang's group reported a delivery platform employing self-assembled nanoparticles that preferentially accumulate in tumours. Their cationic non-viral vectors (size: ~120–150 nm; zeta potential: ~4.3 mV; encapsulation efficiency: >92%) consist of siRNA-containing liposome–protamine–DNA nanoparticles (LPD-NPs), post-functionalized with a shielding and targeting agent, DSPE–PEG–AA (distearoylphosphatidylethanolamine–polyethylene glycol–anisamide conjugate).[26] Each LPD-NP is thus a self-assembled spherical structure, composed of protamine, siRNA and a carrier DNA core complex coated with a DOTAP/cholesterol-containing cationic lipid bilayer. The main advantages of this system include an almost complete encapsulation of RNA, an easy preparation method, a high tumour delivery efficiency and significant gene silencing *in vivo*, with low levels of toxicity and immunogenicity. Furthermore, the formulation has been reported to both sensitise tumour cells to anticancer chemotherapy and facilitate their apoptosis (programmed cell death).

The high delivery efficiency was attributed to the tumour tissue acting as a sink compartment.[26] In an attempt to increase the therapeutic window and the chances for regulatory approval, Huang's group also replaced the carrier DNA with hyaluronic acid (HA), to form liposome–protamine–hyaluronic acid nanoparticles (LPH-NPs).[27] These were reported to efficiently knockdown protein expression in the lung metastasis, when additionally functionalized with tumour-targeting single chain antibody fragments (scFv).[28] Through the introduction of calcium phosphate composites in place of the carrier DNA, to form lipid coated calcium phosphate nanoparticles (LCP-NPs) the group were also able to reduce the potential immunogenicity of the LPD-NPs.[29]

Cell/tissue/organ targeting through formulation design

Both passive and active targeting strategies have been demonstrated to facilitate targeted siRNA delivery. The most straightforward way to achieve passive targeting is by direct administration into the location of interest. Such methods provide efficient delivery of siRNA, however only a small number of organ/tissues can be accessed in clinical practice, including the skin, lungs, muscle, rectum, vagina, eye and nervous system.[8,30] Passive targeting can also be achieved through systemic administration. For instance, the liver in mice can be passively targeted with siRNA by employing a quick high-volume and high-pressure *i.v.* administration (so called hydrodynamic tail vein (HTV) injection).[31] Although the exact mechanism is not fully understood, a high liver blood filtration rate, the plasticity of the liver microvasculature, as well as facilitated hepatocytic uptake of siRNA seem to contribute. For the LPD-NPs already discussed (sized from 120–200 nm) the passive particle-size dependent targeting mechanism appeared to be most important mechanism to allow their accumulation within tumours. This has been attributed to the organ packing and fenestrae sizes, which for cell-dense liver tissue is ~100 nm and for the more 'leaky' tumour structure, about 400 nm.[32]

β-1,3-D-Glucan-encapsuled siRNA particles (GeRPs) have been developed as an orally-administrated and passive-targeted platform for systemic siRNA delivery, at the University of Massachusetts. They are particles with a 2–4 μm outer glucan shell, and a core of tRNA/PEI/siRNA-endoporter complex/PEI arranged in a layer-by-layer format (Fig. 9A), which are able to selectively silence protein expression in mice. The mechanism of absorption following oral administration is reported to occur *via* gut-associated lymphatic tissue (GALT), in which macrophages take up the GeRPs with a high efficiency, undergo siRNA-derived gene silencing and accumulate in other tissues including the peritoneum, lungs, liver and spleen. The glucan shell provides a mechanical barrier to resist RNA nucleases, has a low level of non-specific binding to the gut, triggers macrophage particle uptake (*via* detectin-1- and other β-1,3-D-glucan-receptor-mediated pathways[11]) and promotes low pH-dependent siRNA phagosomal release due to its porous structure. The siRNA complexing agents PEI and endoporter appear to additionally protect the siRNA from hydrolysis and promote

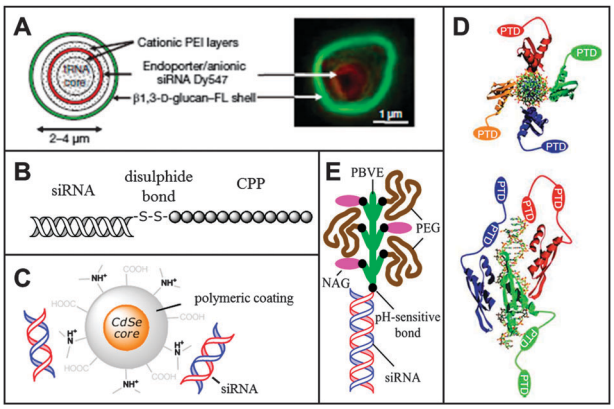


Fig. 9 Examples of siRNA formulations that employ different targeting strategies. (A) The structure (left) and confocal image (right) of fluorescent GeRPs.[11] The polyethyleneimine (PEI) (grey-spotted area) and Dy547-labelled-siRNA-endoporter complex (red) are adsorbed on the yeast tRNA core in a layer-by-layer format and encapsulated in fluorescein-labelled glucan shell (green). (B) A schematic of a CPP–siRNA conjugate. CPP (cell penetrating peptide (*e.g.* penetratin, transportan)) may be conjugated to the siRNA *via* disulphide bonds at the strand ends.[33] (C) The structure of QD–siRNA nanoparticles, where polymer-coated (grey) quantum dots (orange) are complexed with siRNA molecules (red/blue).[34] (D) A schematic of PTD–DRBD–siRNA complex. Each PTD–DRBD fusion protein is shown in a different colour.[35] A and D adapted by permission from Macmillan Publishers Ltd: Nature ref. 11 and 35; © 2009. (E) The structure of the siRNA dynamic polyconjugate.[36] The 5′-end of the siRNA guide strand (red) is conjugated to a polymer backbone, PABVE (green) functionalized with targeting moieties, (NAG (purple)), and shielding agents (PEG (brown)). All the junctions (black) are pH-sensitive.

phagosomal escape through proton-sponge and pore-forming mechanisms, respectively. GeRPs offer high silencing potency (20 μg kg^{-1} as an effective oral dose), possible formulation adaptability (efficient silencing for various siRNAs), great loading capacity and very low levels of toxicity.[11]

Active targeting can be achieved by functionalizing the siRNA therapeutic with a ligand that specifically recognises a defined receptor. Such an approach has been reported for many different siRNA formulations with success *in vivo* (Table 1). The RONDEL™ delivery system, developed by Calando Pharmaceuticals and Caltech, deserves particular attention (Fig. 10).

This formulation consists of four components: siRNA as a therapeutic molecule and three excipients: a cyclodextrin-containing polymer (CDP), adamantane–polyethylene glycol conjugate (AD–PEG) and AD–PEG–transferrin conjugate (AD–PEG–Tf). Prior to administration the components are stored in two vials, one containing the delivery excipients and the other, nucleic acid. After mixing the vials, the components self-assemble into nanoparticles (diameter: ~70 nm; zeta potential: −10 mV; an essentially complete encapsulation).

The CDP is composed of five main segments, each of which plays a separate role. The imidazole terminal groups correlate positively with delivery efficacy. The β-cyclodextrin (βCD) units ensure a satisfactory level of biocompatibility and strong binding with the formulation stabilizing and targeting agents (AD–PEG and AD–PEG–Tf, respectively). The positively charged amidine centres are responsible for forming the CDP–siRNA interaction. Ethylsulphide spacers linking βCDs to amidine

Table 1 Examples of siRNA formulations reaching targets *via* active targeting mechanisms

Ligand	Molecular target (tissue)	Formulation	Ref.
A10 aptamer	PSMA (prostate cancer)	Aptamer–siRNA chimera	13
Folate	FOLR (cancer cells)	siRNA conjugate	38
Retinol	RBP (hepatocyte)	siRNA lipocomplex	8
ApoA-I	SCARB1 (hepatocyte)	siRNA lipocomplex	39
Transferrin	TfR (neuronal cells)	siRNA lipoplex	39
Bombesin	BB1/3 (cancer cells)	siRNA polyplex	12
RVG	AchR (neuronal cell)	Peptide–siRNA complex	39
Lactose	ASGP-R (hepatocyte)	PIC micelle	40
Anti-integrin β_7 antibody	Integrin β_7 (leukocyte)	Liposomal nanoparticle	39

Abbreviations: PMSA – prostate-specific membrane antigen; siRNA – small interfering ribonucleic acid; FOLR – folate receptor; RBP – retinol binding protein; ApoA-I – apolipoprotein A-I; SCARB1 – scavenger receptor class B member 1; TfR – transferrin receptor; BB1/3 – bombesin receptors 1 and 3; RVG – rabies virus glycoprotein; AChR – acetylcholine receptor; ASGP-R – asialoglycoprotein receptor; PIC – polyion complex.

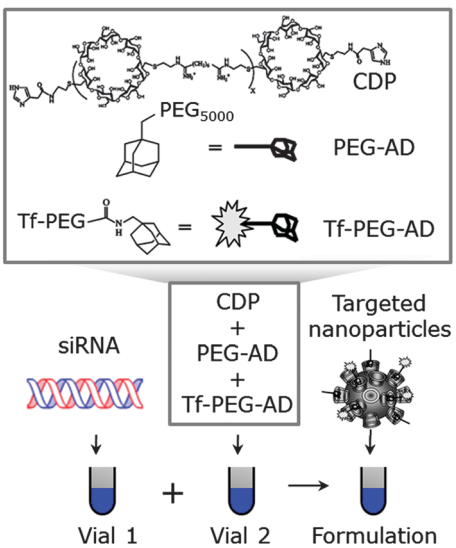


Fig. 10 The formation and delivery protocol for RONDEL™-based siRNA therapeutics. Vial 2 contains the delivery components (cyclodextrin-containing polymer (CPD), polyethylene glycol–adamantane conjugate (PEG–AD) and the transferrin-PEG–AD conjugate (Tf–PEG–AD)) which self-assemble to form nanoparticles when mixed with the siRNA/vial 1. Adapted with permission from ref. 37; © 2009 American Chemical Society.

centres provide the optimal distance between the segments, protecting the formulation from a toxicity increase and/or an efficiency decrease. 'Elastic' hexylene linkers probably facilitate the formation of particle-like formulation during self-assembly.[37]

Interestingly, RONDEL™ overcomes most siRNA delivery- and formulation-related obstacles. Both poor RNase resistance and short biological half-life are not an issue, due to the vesicle-like design, surface PEGylation and an appropriate surface charge. These also result in low toxicity and a lack of particle aggregation. Tumour tissue targeting is achieved primarily by keeping the particle size within the range of 10–100 nm, whereas the imidazole and transferrin functionalities support efficient uptake and siRNA release in cancer cells. The self-assembling nature of RONDEL™ also facilitates scale up and production within GMP environments, and enables formulation adaptability and efficient drug loading. However, the pharmacokinetic profiles of RONDEL™ based formulations are currently unclear, with *i.v.* injection the only associated administration route. Nevertheless, the RONDEL™-based anti-cancer therapeutic CALAA-01 remains the first systemic siRNA treatment that has been reported to induce gene silencing in human phase I clinical trials, involving patients with solid cancers.[41]

In summary, both active and passive targeting strategies remain currently of use within the field of siRNA delivery and both offer advantages. Passive targeting is often correlated with more specific accumulation in the cell/tissue/organ of interest, whereas active targetting mechanisms offer both a wider choice of tissues that could be targetted and an enhanced cellular uptake of the therapeutic that crosses cytoplasmatic membrane.

Effective cellular uptake

Since naked siRNA is a macromolecule, endocytosis is considered the preferred intracellular transport mechanism. Here, the triggering of endocytosis, endosomal escape and reaching the RISC within the cytosol are the major obstacles that need to be overcome within the cell expressing the protein(s) of interest.

Triggering of endocytosis. The lipophilic and negatively charged cellular membrane does not interact independently with hydrophilic and anionic nucleic acid molecules, thus efficient siRNA therapeutics must trigger endocytosis. In order to meet this requirement, drug delivery approaches have focused on masking the negative charge, increasing the lipophilicity and/or reducing the size of the siRNA. Alternatively, special modifications can be introduced to an siRNA formulation, so that it gets internalized *via* a particular type of endocytosis pathway.

A number of siRNA formulations are based on the formation of polymer complexes (*e.g.* with chitosan or PEI), which mask the siRNA negative charge and facilitate association with the cellular membrane. A complex is typically formed with siRNA in a self-assembly process due to the ionic interactions between the phosphate groups of the siRNA and cationic groups (such as ammonium residues) within the delivery platform, where the final zeta potential value of the delivery complex is the net charge of all of the ions involved. The ideal zeta potential is slightly above or close to zero mV, because the purpose of such

modification is to enhance binding to cell membranes. Overly positively charged siRNA complexes may inhibit complex disassembly, if the formulation is internalized into the cytoplasm, and also bind to various cells/cell components in a non-specific manner, which usually correlates with greater cytotoxicity. However, multimeric siRNAs (multi-siRNA) mixed with linear PEIs (LPEI) at 10 : 1 nitrogen : phosphorous molar ratio have been reported to knock down protein expression *in vivo*, suggesting that the surface charge may not be the only important factor for triggering endocytosis.[42]

Since the cellular membrane forms a hydrophobic barrier between the extracellular and intracellular matrix (ECM and ICM, respectively), making the siRNA more lipophilic appears to be a reasonable approach for more effective cellular uptake. Such a change would however, still need to provide the lowest possible cytotoxicity and maximal siRNA availability in the cytosol after internalization. Hence a 'lipophilic' siRNA should be (i) disruptable, so that, after crossing the membrane, free siRNA is released into the ICM, or (ii) amphiphilic enough, in order to provide functional siRNA both in the polar and non-polar media.

Increases of siRNA lipophilicity have been approached in several ways, including the introduction of chemical modifications to the siRNA molecule (*e.g.* replacement of phosphodiester groups with phosphorothioate linkers, conjunction with cholesterol,[10] fatty or bile acids[22]) or formation of siRNA-lipophilic excipient complexes (*e.g.* iNOPs,[19] siRNA lipoplexes[43]) and with siRNA encapsulation in hydrophobic vesicles (*e.g.* stable nucleic acid–lipid nanoparticles (SNALPs),[44] lipidoid nanoparticles[45]). All of these siRNA delivery systems have been demonstrated to knock down protein expression efficiently. However, for these systems, other mechanisms that improve cellular uptake are often also involved, *e.g.* masking the siRNA negative charge for iNOPs or surface modifications of SNALPs with ligands that trigger a specific type of endocytosis.

Cellular incorporation of siRNA therapeutics also depends on their size. In general, the smaller the formulation, the easier the incorporation occurs, hence small sized siRNA formulations permeate more effectively through the membrane and silence gene expression more effectively than large systems. For that reason, several pharmaceutical siRNA-oriented companies are keen on developing siRNA-based medicines that are either shorter than the native 19 bp siRNA (*e.g.* asymmetric interfering RNA (aiRNA) and self-delivering rxRNA (sd-rxRNA™)) or modified in the passenger strand, such as in mipomersen (Fig. 11).

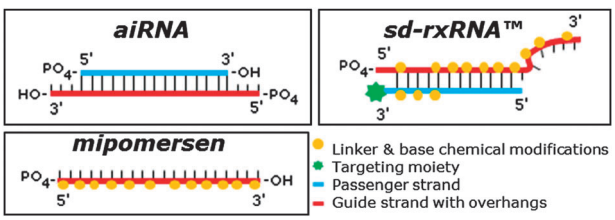


Fig. 11 The structures of aiRNA, sd-rxRNA™ and mipomersen.

Asymmetric interfering RNA (aiRNA) is a system developed by Boston Biomedical, Inc. and Harvard Medical School. Chemically, aiRNAs are <19 bp-long RNA duplexes with 3′- and 5′-end antisense overhangs. Interestingly, a 15 bp-long duplex was demonstrated to efficiently silence gene expression with picomolar efficiency in mammalian cells. The potential advantages of this system include a reduced number of off-target effects, a cheap, quick and easy production as well as improved cellular uptake.[46]

Self-delivering rxRNA is a therapeutic system patented by RXi Pharmaceuticals Corporation. The chemical structure of sd-rxRNA™ is based on asymmetric interfering RNA (aiRNA) but is chemically modified, with a lipophilic group (*e.g.* cholesterol) at the 3′-end of the passenger strand, phosphorothioate and other hydrophobic linkages, a phosphate group at the 5′-end of the guide strand, as well as 2′-O-Met, 2′-F and base modifications. A less rigid structure, with smaller negative charge and increased lipophilicity in comparison to naked siRNA are attributed to its increased cell membrane penetration. Good *in vitro* efficacy for this system is maintained due to the 19–23 nucleotide length of the guide strand, whereas ribose modifications and phosphorothioate linkers are of benefit for stability. The presence of phosphorothioate groups may also be the reason for the lower toxicity of sd-rxRNA™. This system should also be easy to scale-up and thus be suitable for production within GMP. Initial studies on mice suggest that sd-rxRNA™ is more suitable for local delivery applications such as injection into eye or skin or inhalation into lungs, assuming that the dose potency of this therapeutic is better than that of encapsulated drugs. Disappointingly, systemic delivery was reported to silence gene expression only in mouse liver after *intra-venous* injection of a high dose (50 mg kg^{-1}), with a relatively short half-life (~40 min).[47]

Mipomersen was introduced by Isis Pharmaceuticals which, in collaboration with Genzyme Corporation, developed this cholesterol-reducing drug, recently approved by the FDA for the treatment of homozygous familial cholesterolemia. Although it is targeted at reaching cytoplasmatic mRNA like other siRNAs, technically it is classified as an antisense oligonucleotide, because of its chemically-modified single-stranded structure. It contains ten 2′-MOE ribonucleotides at both ends (5 per each end) and 2′-deoxyribonucleotides in between. Additionally, all of the 20 nucleotides are linked with PS groups and every cytosine base is C5-methylated. Interestingly, such a design allows the molecule to achieve better stability and cell penetration, a longer circulation half-life and decreased non-specific binding to proteins that correlates with lower toxicity than double-stranded structures. The efficacy and manageable safety profile of mipomersen have been demonstrated throughout clinical trials.

SiRNA internalization into cells can be improved by functionalization of the delivery system with specific ligands that are known to induce a specific type of macromolecule cell internalization mechanism. It can be achieved by the introduction of active targeting moieties which bind selectively to surface receptors on the cytoplasmic membrane and trigger

receptor-mediated endocytosis. Alternatively, cell penetrating peptides (CPPs) conjugated or complexed with siRNA have also been demonstrated to promote cell permeation, probably *via* micropinocytosis.[18,35,39] Interestingly, the high positive charge of the arginine- and/or lysine-rich CPP oligopeptide sequences originating from viruses (*e.g. trans*-activator of transcription (TAT) peptide from HIV) seems to be the reason for the improved membrane trafficking of these drugs (Fig. 9B).

To summarize, since several successful solutions have been proposed for the triggering of endocytosis for siRNA delivery, the current understanding of this challenge should not be considered as poor. The introduction of the targeting moiety and size reduction of the siRNA formulations are the leading and most promising approaches.

Endosomal escape. One of the biggest remaining challenges for siRNA delivery is facilitating endosomal escape. Approaches to overcome this have mainly focused on promoting either fusion with or disruption of the endosomal membrane. Alternatively, photosensitizers (*e.g. meso*-tetraphenyl chlorine disulphonate ($TPPS_{2a}$)) or pore-forming proteins or peptides (*e.g.* viroporins, endoporter) have been demonstrated to mediate the escape *via* other mechanisms.[12]

The use of excipients such as positively charged fusogenic lipids or peptides facilitates the escape of siRNA by triggering the interaction with the anionic endosomal membrane. This approach has been employed for several lipid-based siRNA formulations, *e.g.* lipoplexes, SNALPs, lipidoid nanoparticles and other liposomal nanoparticles (LNs), which are enriched often with cationic lipids containing unsaturated fatty acids in their structure. Such lipids can assemble in an inverse hexagonal phase, forming a net of long, cylindrical structures which are thought to be disruptive to the endosomal membrane[48] (Fig. 12B).

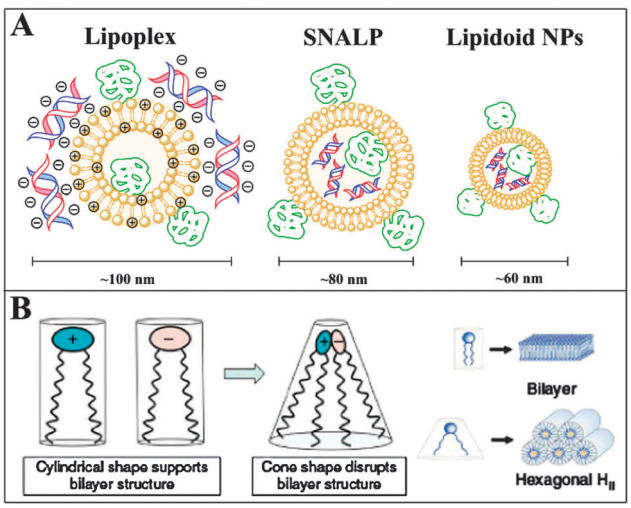


Fig. 12 (A) Schematics of lipoplex, SNALP and lipidoid nanoparticle structures and (B) the proposed mechanism of membrane disruption triggered by fusogenic lipids. The cationic lipids originating from lipid-based formulations associate with anionic lipids from the endosomal membrane into cone-shaped complexes, which do not support the formation of a bilayer structure and ultimately lead to the endosomal membrane disruption. Adapted by permission from Macmillan Publishers Ltd: Nature Biotechnology ref. 48; © 2010.

SiRNA lipoplexes were used as part of the complex-based siRNA delivery platform (AtuPLEX/AtuFECT) originally developed by Silence Therapeutics. Structurally, these are complexes of positively charged LNs composed of three types of lipids and 2′OMe-stabilized siRNA molecules. For example, each LN of Atu027 – a siRNA lipoplex formulation (size: ~102 nm; zeta potential: +38.9 mV) currently pending phase II clinical trials – contains a cationic lipid, a helper lipid and a PEGylated-lipid mixed at a defined molar ratio. Such a design provides RNase resistance and an improved half-life. This facilitates the cellular and organ uptake of siRNA and an efficient and potent silencing effect (siRNA dose: 1.88 mg kg^{-1} in mice), which does not seem to be associated with significant toxicities both *in vitro* and *in vivo* in mice, rats and non-human primates. In addition, the formulation is able to reach and cross epithelial cells as well as target organs including the lungs, liver and heart in animal studies.[43] According to data from phase I clinical trials, the lipoplex Atu027 was reported to be well tolerated up to a dose of 0.18 mg kg^{-1} in patients with advanced solid tumors. Dose escalation studies of this formulation in humans are currently ongoing.[49]

Stable nucleic acid–lipid particles (SNALPs) are a vector-like siRNA delivery system initially developed by Tekmira Pharmaceuticals, Roche and Alnylam Pharmaceuticals. They consist of 2′OMe and PS-modified siRNA, encapsulated in a liposomal nanoparticle (size: ~80 nm, encapsulation efficiency: ~95%) containing 4 types of lipids: cholesterol, a neutral phospholipid, an ionizable cationic and a PEG–lipid conjugate.[48] The PEGylated lipid component plays an important role in preventing particle aggregation, as well as extending the *in vivo* lifetime ($t_{\frac{1}{2}} > 6$ h). The ionisable cationic lipid is responsible for increased cellular uptake, which results in a high delivery efficiency. The neutral cholesterol and phospholipids participate in stabilizing the SNALP structure and shape. Interestingly, the delivery of Apo-B-targeted siRNA to cells using SNALPs has been reported as the most efficient and correlates with *in vivo* studies in non-human primates where the highest siRNA dose was 0.6 mg kg^{-1} and was not associated with significant toxicity.[44]

Lipidoid nanoparticles are another siRNA delivery platform developed by Alnylam Pharmaceuticals and collaborators. Structurally, they resemble SNALPs, encapsulating chemically modified siRNA (2′OMe, 2′F, PS), but the difference consists of using different components for the particle formulation, namely; cholesterol and/or other neutral lipids, lipidoids and polyethylene glycol–lipids. Lipidoids are novel, cationic, lipid-like substances composed of an amine-rich central part attached to long oxoalkyl chains.[45]

The introduction of lipidoids allows the formulation of stable cationic particles that self-assemble with siRNA (size: 50–80 nm, zeta potential: +2–34 mV, encapsulation efficiency: 90–95%). The formed particles possess an extended half-life, a potent silencing effect (siRNA dose: 0.01–6.25 mg kg^{-1}, with effects lasting even up to 40 days after *i.v.* administration) in several specific tissues (liver, lung, PEC macrophages) and minimal toxicity both *in vitro* and *in vivo* (mice, rats, non-human primates).

Also, many polymer-based delivery platforms have been developed to trigger the increase of osmotic pressure in the endosome, eventually leading to its rupture. Interestingly, such polymers due to their high proton-binding capacity (*s.c.* proton sponges) act as buffering agents preventing the pH decrease in late endosomes. Consequently, they promote the influx of protons and counter anions from the cytosol, which implicates high osmolarity in the endosomal matrix. Subsequently, diffusion of water into the hypertonic environment of endosomes results in endosomolysis. This mechanism has been employed by the Mirus Bio Corporation, which developed the siRNA dynamic polyconjugate (DPC) therapeutic system. It has also been employed by Yezhelyev *et al.*, who reported polymer-coated quantum dot-siRNA nanoparticles (QD-siRNA) (see Fig. 9 and 13). Chemically, the siRNA DPC consists of siRNA and amphipathic polymer backbone PBAVE (polyconjugate of butyl and amino vinyl ethers) units that are connected to an siRNA duplex through a reversible disulfide linkage at the 5′-end of the guide strand. Both strands are stabilized with 2′OMe groups and PS linkers, whereas the branched endosomolytic polymer is additionally functionalized with both hepatocyte targeting ligand NAG (*N*-acetylgalactosamine) and polymeric shielding agents *e.g.* PEG. Interestingly, NAG and PEG residues are attached to PBAVE through bifunctional and reversible maleamate linkages, which like disulfides, are cleaved under the low pH conditions of late endosomes, releasing the free PBAVE and siRNA molecules, promoting both endosomal escape and the siRNA–RISC interaction.

The presence of hepatocyte targeting ligands, shielding agents and the fact that the siRNA unit is covalently attached to the PBAVE, all contribute to the preferential accumulation of siRNA in the cytosol of hepatocytes instead of other cells and result in a good silencing efficiency *in vitro* and *in vivo*. After *i.v.* administration into mice, the silencing effect lasted up to ten days and only minimal toxicity was observed, with insignificant increases of liver enzymes and adverse inflammatory responses.[36] Unfortunately, it is unlikely that this system could be adapted for targeting anything other than liver-associated tissues.

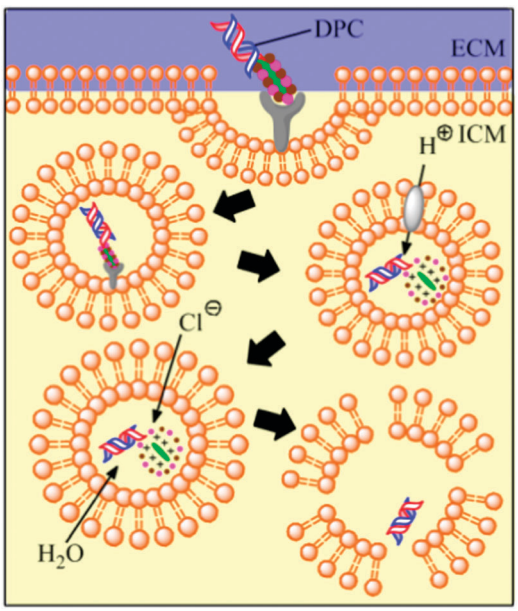


Fig. 13 The 'proton-sponge'-based mechanism of DPC endosomal escape. After DPC encapsulation in the endosome, the subsequent pH decrease within the endosomal matrix triggers conjugate hydrolysis and PABVE protonation. To counter the high osmotic pressure and positive charge, water and anions flow into the endosome eventually causing its rupture and the release of siRNA.

The approach of using quantum dots (QDs) as a delivery system for siRNA has been of interest to several scientific groups, with one of the most advanced examples reported in 2008 by Yezhelyev *et al.*[34] Their system is composed of a chemically modified siRNA complexed with positively-charged QD nanoparticles consisting of a cadmium selenide (CdSe) core and a proton-absorbing polymeric coating, with a balanced proportion of carboxylic acid groups as well as covalently-attached tertiary amines (Fig. 9). Cell-based studies demonstrated efficient gene silencing and little cytotoxicity. Although it seems unlikely that QD-based formulations will become therapeutic agents, currently they appear to be useful for imaging the intracellular trafficking of siRNAs.

Loading of RISC and reaching the target mRNA. After release from the endosome, siRNAs need to be loaded onto the RISC in order to be paired with the target mRNA and induce knock down of protein expression. The vast majority of siRNA therapeutics are designed to reach their molecular target straight after endosomal release. However, some designs (*e.g.* aptamer–siRNA chimeras) may initially need to be cut by Dicer into smaller chains, before the therapeutic sequence reaches the silencing complex that recognises RNA duplexes of ~21 bp only. Surprisingly, this additional step does not seem to be an efficacy limiting factor for siRNA-based chimeras, since their silencing effect has been reported to be more potent than for the corresponding siRNAs introduced to the cell as pure molecules.[50]

Should the siRNA be recognized by the RISC and the cleavage of an appropriate mRNA facilitated, the underlying siRNA structure has to fulfil certain requirements.[1] Firstly, it is crucial that the siRNA forms an A-form double helix, as its major groove appears to be necessary for mediating the siRNA pathway. Secondly, the siRNA should possess sticky ends or (unpaired) overhangs at the ends of one or both strands. The 5′-end of the guide strand must also possess a free hydroxyl of the phosphate group, while the other ends may and usually are functionalized with various moieties (polymers, peptides, lipids). siRNA DPC[36] and Toc–siRNA[23] demonstrate that such ends may be functionalized and the silencing effect maintained. Thirdly, chemical modifications of the 2′-OH-ribose groups around the 9th–11th bp from the 5′-end of antisense strand should be avoided, as this is the point of AGO-catalysed mRNA cleavage. In addition to guide strand modifications with bulky groups (*e.g.* 2′-MOE), small changes in siRNA structure are generally well tolerated, providing that the number of changes is not too high. Finally, chemical modifications of the purine and pyrimidine heterocyclic rings can affect the silencing activity differently; C5-halogenation of pyrimidines in

the guide strand does not affect the activity, whereas C3-methylation of uracil in both strands substantially does.[4]

It should be noted however, that as the mechanisms by which siRNA is recognized, loaded onto and unfolded by the human RISC, and also how the relevant mRNA is targetted and cleaved by the siRISC (siRNA guide strand-RISC active complex) are still not fully understood, the rules in the preceding paragraph are very likely to not yet be complete.

Toxicity

Since the toxicity of siRNA therapeutics can arise from three main sources *e.g.* the siRNA, the formulation excipients and the complete formulation, the current approaches oriented at minimizing this are focused on improving all of these aspects.

First, siRNA (and aptamer) sequences containing no known immunogenic motifs are of preference. The siRNA sequence must be chosen carefully, and the fact that siRNAs can potentially knock down the expression of other, non-target genes makes this a considerable challenge. A strategy for the complete encapsulation of siRNA can be successfully used,[37] so that it does not have the opportunity to interact with anything other than the targeted cells. However, even in such cases the siRNA sequence needs to be carefully preselected, since receptors such as TLR-3 are localized both extra- and intracellularly and may mediate an immune response after internalization at the target site.

Second, chemical modifications of one or two siRNA strands may help to decrease the toxicity originating from off-target silencing. Such modifications involve: the 2′-ribose ring of the guide strand (2′OMe, 2′F, 2′H, UNA[51]), purine and pyrimidine bases and alterations in the number of nucleotides for both siRNA strands. Interestingly, the introduction of 2′-OMe modifications to the guide siRNA strand has been found to reduce off-target effects, while the same modification to both strands decreased immunogenic potential.[51]

Third, strategies to avoid toxicity arising from formulation excipients, usually involve either development of novel, more biocompatible materials or modifications of existing ingredients. For example, the toxicity observed in early studies with viral gene delivery vectors has led to improvements in material science for alternative non-viral approaches.[52] Similarly, the motivation for the synthesis of the new class of lipidoid materials was to discover a lipid-like material(s) that possessed better properties than the lipids used in the formulation of SNALPs. Another example is the Peptide Transduction Domain-dsRNA Binding Domain (PTD-DRBD) fusion protein, which is an siRNA delivery system developed at the University of California in collaboration with Life Technologies (Fig. 9D).[35] The polyanionic siRNA forms a high-avidity complex with PTD-DRBD (at 4 : 1 protein : siRNA ratio) masking the negative charge of the nucleic acid. Within the structure of the fusion protein two units are distinguished: a small (~63-residue) DRBD unit responsible for binding ~12–16 bp of siRNA and an arginine-rich (Arg-rich) PTD unit which promotes the cellular penetration of siRNA, probably *via* micropinocytosis. The complex was efficient in gene silencing in both difficult-to-transfect primary cell types and transgenic mice (after local administration) and is non-immunogenic with low levels of off-target effects.[35] This is promising result since most protein-based delivery systems are considered immunogenic (toxic).

Alternatively, addition of PEGylated moieties to the formulation is thought to be beneficial in terms of reducing undesirable interactions *in vivo* (*i.e.* triggering an immune response, non-specific binding to cells or RNase activity). Such modifications make the formulation more biocompatible by increasing its hydrophilicity and forming a steric barrier, contributing to an increased biological half-life. A similar tendency can be noticed for excipients functionalized with targeting ligands that direct the siRNA molecules to the desired cell in the body and decrease the possibility of interaction with other tissues.

Finally, toxic effects may be evoked *in vivo* by the whole therapeutic, even if the exposure to pure excipients and/or siRNA does not cause toxicity. For example, delivery systems larger than 200 nm are often recognized by immune system as 'potential pathogens' and trigger an immune response.

Other challenges

In addition to the challenges associated with siRNA delivery, there are a number of practical issues related to formulation development and manufacture. The pharmaceutical industry for example, has a focus on developing non-invasive delivery systems, such as tablets, oral solutions, inhalers, patches or creams, as they are more convenient for patients. Currently, most macromolecular drug candidates (including siRNA-based) are administered locally or *via* injection, because their absorption from the gastro-intestinal tract, lungs or skin is not efficient or they are inactivated *en-route* to the site of action. Although some orally administrated siRNA delivery platforms, have been reported (*i.e.* GeRPs,[11] TKNs[17]), the possibility of translating them into a human model remains problematic. This is because orally-delivered systems potentially allow non-specific siRNA delivery to a variety of locations in the body, which significantly reduces their potential applicability.

Ideally, a therapeutic 'platform' for siRNA delivery is sought, with the potential to facilitate delivery of a range of different siRNAs, perhaps in conjunction with other therapeutics. Such multicomponent systems could be used for the treatment of many different conditions (*e.g.* by simply swapping the APIs) and would help in cases where combined therapy (with two or more drugs) are indicated. The drive to a universal formulation concept is exemplified *via* 'container' approaches (*e.g.* RONDEL™, LNPs), which are able to encapsulate a variety of different molecules. Similarly, the ability to generate aptamer–siRNA chimeras of many different binding selectivities and therapeutic targets allows a more biotechnology-focussed route to 'platform' siRNA systems.

However, it should be noted that if particle type delivery vectors are preferred for formulations, their low encapsulation efficiency and/or the potential for particle aggregation need also to be considered. Nanoparticle aggregation is usually caused by high surface free energy and can be avoided by

particle functionalization with polymeric molecules such as PEG, HA or polysaccharides. Such modifications are known to reduce non-specific cell membrane–nanoparticle interactions and increase the particle size, which usually results in lower toxicity, longer biological half-life and reduced cell transfection efficacy. The low encapsulation efficiency may be tackled by optimising the formulation composition. For instance, binding siRNA with a cationic excipient (*e.g.* protamine, PEI or other poly(amines)) may increase the total amount of siRNA incorporated into a vesicle-like system. Unfortunately, cationic additives may also result in a higher toxicity of the formulation and hinder the release of the siRNA. Alternatively, optimization of the fabrication procedure may improve encapsulation efficiency. For example, the concentration of poly(lactic-*co*-glycolic) acid (PLGA), as well as the oil-phase/water-phase volume ratio, have been shown to significantly influence the encapsulation efficiency for siRNA-loaded PLGA nanoparticles prepared by the double emulsion solvent evaporation method.[53] However, if the adjuvant solvent is not fully evaporated, the risk of formulation toxicity again increases.

The large scale production of siRNA therapeutics whilst maintaining GMP requirements is another important challenge for industry. Potential therapeutics that can be formulated in a single step by taking advantage of naturally occurring phenomena, such as self-assembly, are thus highly desirable. While the production of single component systems or simple mixtures, such as siRNA complexes or aptamer–siRNA chimeras, should not cause problems for large scale manufacture, more complex systems (*e.g.* nanoparticle or liposomal systems, peptide or protein-containing siRNA therapeutics or advanced siRNA conjugates) may not be suitable.

The current lack of screening methods for high throughput discovery and optimization of siRNA formulation performance also requires consideration. Most of the current methods for characterization of formulation performance involve comprehensive cell-based experiments which are time-consuming and often expensive. Attempts to improve this are focused on the development of methods that enable identification of the optimal composition of an siRNA therapeutic through high throughput screening of formulation (HTF) approaches. For example, Akinc *et al.* have demonstrated a number of approaches for the synthesis, characterization and identification of optimal siRNA formulations with the lipid derived molecules 'lipidoids'.[45] Future work in this area is likely to focus on either modifying already existing approaches (such as High Content Screening (HCS)) or developing novel methods which enable rapid, cheap and efficient discovery of optimal siRNA formulations.

SiRNA therapeutics therefore currently face not only delivery-related barriers, but also industrial production and formulation-related challenges which must be addressed before they reach the market. Thus, it is extremely important that issues relating to all aspects of the chemistry of siRNA and how this affects its application are considered at the early stages of siRNA formulation design.

Conclusions

Although significant progress in the field of siRNA formulation science has been made within the last ten years,[54–60] there are still no siRNA-based medicines on the market. Current research is moving towards practical siRNA therapy through enabling identification of the desired properties of the 'ideal' siRNA therapeutic. Importantly, our understanding in how we overcome challenges such as RNase resistance, short biological half-life time or tissue targeting is now maturing and the preparation of stable siRNA formulations able to accumulate in particular cell types is becoming possible. However, toxicity, effective cellular uptake as well as some formulation-related problems appear to be manageable only in some cases and usually these require special attention. Additionally, a growing clinical knowledge set regarding the behaviour of siRNA in humans suggests that a better understanding of *in vivo* exposure and stability of siRNA formulations is still needed to more efficiently address the preclinical and clinical issues surrounding these complex biotherapeutics.

Furthermore, given the large quantity and variety of RNAi formulation designs published, it is clear that this is a very competitive research area. The strong interest and financial support that has been provided by the large pharmaceutical companies in developing siRNA-based therapeutics is significant, but may not necessarily be sustainable. However, as innovative research takes time and resources and there are still uncertainties as to whether siRNA therapeutics will ever reach the market, the pharmaceutical industry requires an acceleration in the course of formulation development in order to reduce the associated expense. In this regard, since current methods for the discovery and optimization of siRNA formulation performance are inefficient, expensive and time-consuming, high throughput screening-based methodologies seem to be the solution of preference. Therefore, we believe the extension of the existing research methodologies for siRNA therapeutics with the approaches benefiting from high throughput materials chemistry techniques will significantly accelerate application of these fascinating and potent biomolecules.

References

1 M. E. Davis, J. E. Zuckerman, C. H. J. Choi, D. Seligson, A. Tolcher, C. A. Alabi, Y. Yen, J. D. Heidel and A. Ribas, *Nature*, 2010, **464**, 1067–1070.
2 K. A. Whitehead, R. Langer and D. G. Anderson, *Nat. Rev. Drug Discovery*, 2009, **8**, 129–138.
3 J. W. Gaynor, B. J. Campbell and R. Cosstick, *Chem. Soc. Rev.*, 2010, **39**, 4169–4184.
4 T. M. Rana, *Nat. Rev. Mol. Cell Biol.*, 2007, **8**, 23–36.
5 A. Fire, S. Q. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello, *Nature*, 1998, **391**, 806–811.
6 S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, *Nature*, 2001, **411**, 494–498.
7 J. M. Perkel, *Science*, 2009, **326**, 454–456.

8 K. A. Whitehead, R. Langer and D. G. Anderson, *Nat. Rev. Drug Discovery*, 2010, **9**, 412.
9 D. R. Yazbeck, K. L. Min and M. J. Damha, *Nucleic Acids Res.*, 2002, **30**, 3015–3025.
10 J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R. K. Pandey, T. Racie, K. G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan and H. P. Vornlocher, *Nature*, 2004, **432**, 173–178.
11 M. Aouadi, G. J. Tesz, S. M. Nicoloro, M. X. Wang, M. Chouinard, E. Soto, G. R. Ostroff and M. P. Czech, *Nature*, 2009, **458**, 1180–1184.
12 M. Dominska and D. M. Dykxhoorn, *J. Cell Sci.*, 2010, **123**, 1183–1189.
13 J. P. Dassie, X. Y. Liu, G. S. Thomas, R. M. Whitaker, K. W. Thiel, K. R. Stockdale, D. K. Meyerholz, A. P. McCaffrey, J. O. McNamara and P. H. Giangrande, *Nat. Biotechnol.*, 2009, **27**, U839–U895.
14 J. J. Lu, R. Langer and J. Z. Chen, *Mol. Pharmaceutics*, 2009, **6**, 763–771.
15 S. P. Persengiev, X. C. Zhu and M. R. Green, *RNA*, 2004, **10**, 12–18.
16 M. Robbins, A. Judge and I. MacLachlan, *Oligonucleotides*, 2009, **19**, 89–101.
17 D. S. Wilson, G. Dalmasso, L. X. Wang, S. V. Sitaraman, D. Merlin and N. Murthy, *Nat. Mater.*, 2010, **9**, 923–928.
18 D. Reischl and A. Zimmer, *Nanomedicine*, 2009, **5**, 8–20.
19 H. Baigude, J. McCarroll, C. S. Yang, P. M. Swain and T. M. Rana, *ACS Chem. Biol.*, 2007, **2**, 237–241.
20 M. Z. Zhang, A. Ishii, N. Nishiyama, S. Matsumoto, T. Ishii, Y. Yamasaki and K. Kataoka, *Adv. Mater.*, 2009, **21**, 3520–3525.
21 D. E. Koshland, *Proc. Natl. Acad. Sci. U. S. A.*, 1958, **44**, 98–104.
22 C. Wolfrum, S. Shi, K. N. Jayaprakash, M. Jayaraman, G. Wang, R. K. Pandey, K. G. Rajeev, T. Nakayama, K. Charrise, E. M. Ndungo, T. Zimmermann, V. Koteliansky, M. Manoharan and M. Stoffel, *Nat. Biotechnol.*, 2007, **25**, 1149–1157.
23 K. Nishina, T. Unno, Y. Uno, T. Kubodera, T. Kanouchi, H. Mizusawa and T. Yokota, *Mol. Ther.*, 2008, **16**, 734–740.
24 S. M. Nimjee, C. P. Rusconi and B. A. Sullenger, *Annu. Rev. Med.*, 2005, **56**, 555–583.
25 J. H. Zhou, P. Swiderski, H. T. Li, J. Zhang, C. P. Neff, R. Akkina and J. J. Rossi, *Nucleic Acids Res.*, 2009, **37**, 3094–3109.
26 S. D. Li, Y. C. Chen, M. J. Hackett and L. Huang, *Mol. Ther.*, 2008, **16**, 163–169.
27 S. Chono, S. D. Li, C. C. Conwell and L. Huang, *J. Controlled Release*, 2008, **131**, 64–69.
28 Y. C. Chen, X. D. Zhu, X. J. Zhang, B. Liu and L. Huang, *Mol. Ther.*, 2010, **18**, 1650–1656.
29 J. Li, Y.-C. Chen, Y.-C. Tseng, S. Mozumdar and L. Huang, *J. Controlled Release*, 2010, **142**, 416–421.
30 D. M. Dykxhoorn, D. Palliser and J. Lieberman, *Gene Ther.*, 2006, **13**, 541–552.
31 D. L. Lewis and J. A. Wolff, *Adv. Drug Delivery Rev.*, 2007, **59**, 115–123.
32 H. Maeda, *Adv. Enzyme Regul.*, 2001, **41**, 189–207.
33 J. H. Jeong, H. Mok, Y. K. Oh and T. G. Park, *Bioconjugate Chem.*, 2009, **20**, 5–14.
34 M. V. Yezhelyev, L. F. Qi, R. M. O'Regan, S. Nie and X. H. Gao, *J. Am. Chem. Soc.*, 2008, **130**, 9006–9012.
35 A. Eguchi, B. R. Meade, Y. C. Chang, C. T. Fredrickson, K. Willert, N. Puri and S. F. Dowdy, *Nat. Biotechnol.*, 2009, **27**, 567–571.
36 D. B. Rozema, D. L. Lewis, D. H. Wakefield, S. C. Wong, J. J. Klein, P. L. Roesch, S. L. Bertin, T. W. Reppen, Q. Chu, A. V. Blokhin, J. E. Hagstrom and J. A. Wolff, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 12982–12987.
37 M. E. Davis, *Mol. Pharmaceutics*, 2009, **6**, 659–668.
38 A. W. York, F. Q. Huang and C. L. McCormick, *Biomacromolecules*, 2010, **11**, 505–514.
39 N. Manjunath and D. M. Dykxhoorn, *Discovery Med.*, 2010, **9**, 418–430.
40 M. Oishi, Y. Nagasaki, K. Itaka, N. Nishiyama and K. Kataoka, *J. Am. Chem. Soc.*, 2005, **127**, 1624–1625.
41 M. E. Davis, J. E. Zuckerman, C. H. J. Choi, D. Seligson, A. Tolcher, C. A. Alabi, Y. Yen, J. D. Heidel and A. Ribas, *Nature*, 2010, **464**, U1067–U1140.
42 H. Mok, S. H. Lee, J. W. Park and T. G. Park, *Nat. Mater.*, 2010, **9**, 272–278.
43 A. Santel, M. Aleku, O. Keil, J. Endruschat, V. Esche, G. Fisch, S. Dames, K. Loffler, M. Fechtner, W. Arnold, K. Giese, A. Klippel and J. Kaufmann, *Gene Ther.*, 2006, **13**, 1222–1234.
44 T. S. Zimmermann, A. C. H. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M. N. Fedoruk, J. Harborth, J. A. Heyes, L. B. Jeffs, M. John, A. D. Judge, K. Lam, K. McClintock, L. V. Nechev, L. R. Palmer, T. Racie, I. Rohl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A. J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H. P. Vornlocher and I. MacLachlan, *Nature*, 2006, **441**, 111–114.
45 A. Akinc, A. Zumbuehl, M. Goldberg, E. S. Leshchiner, V. Busini, N. Hossain, S. A. Bacallado, D. N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. de Fougerolles, J. R. Dorkin, K. N. Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K. G. Rajeev, D. W. Y. Sah, J. Soutschek, I. Toudjarska, H. P. Vornlocher, T. S. Zimmermann, R. Langer and D. G. Anderson, *Nat. Biotechnol.*, 2008, **26**, 561–569.
46 X. G. Sun, H. A. Rogoff and C. J. Li, *Nat. Biotechnol.*, 2008, **26**, 1379–1382.
47 J. Cardia, W. Salomon, J. Kamens, J. Lam, A. Rodgers, W. Stanney, T. Woolf, L. Libertine, M. Byrne, J. Metterville, T. Drew, K. Flannery-Rossi, D. Samarsky, P. Pavco and A. Khvorova, ed. B. a. A. Keystone Symposia on RNA Silencing: Mechanism, Keystone, 2010.

48 S. C. Semple, A. Akinc, J. X. Chen, A. P. Sandhu, B. L. Mui, C. K. Cho, D. W. Y. Sah, D. Stebbing, E. J. Crosley, E. Yaworski, I. M. Hafez, J. R. Dorkin, J. Qin, K. Lam, K. G. Rajeev, K. F. Wong, L. B. Jeffs, L. Nechev, M. L. Eisenhardt, M. Jayaraman, M. Kazem, M. A. Maier, M. Srinivasulu, M. J. Weinstein, Q. M. Chen, R. Alvarez, S. A. Barros, S. De, S. K. Klimuk, T. Borland, V. Kosovrasti, W. L. Cantley, Y. K. Tam, M. Manoharan, M. A. Ciufolini, M. A. Tracy, A. de Fougerolles, I. MacLachlan, P. R. Cullis, T. D. Madden and M. J. Hope, *Nat. Biotechnol.*, 2010, **28**, 172–176.

49 D. Strumberg, B. Schultheis, U. Traugott, C. Vank, A. Santel, O. Keil, K. Giese, J. Kaufmann and J. Drevs, *Int. J. Clin. Pharmacol. Ther.*, 2012, **50**, 76–78.

50 D. H. Kim, M. A. Behlke, S. D. Rose, M. S. Chang, S. Choi and J. J. Rossi, *Nat. Biotechnol.*, 2005, **23**, 222–226.

51 D. M. Kenski, A. J. Cooper, J. J. Li, A. T. Willingham, H. J. Haringsma, T. A. Young, N. A. Kuklin, J. J. Jones, M. T. Cancilla, D. R. McMasters, M. Mathur, A. B. Sachs and W. M. Flanagan, *Nucleic Acids Res.*, 2010, **38**, 660–671.

52 B. Abdallah, L. Sachs and B. A. Demeneix, *Biol. Cell*, 1995, **85**, 1–7.

53 D. Cun, D. K. Jensen, M. J. Maltesen, M. Bunker, P. Whiteside, D. Scurr, C. Foged and H. M. Nielsen, *Eur. J. Pharm. Biopharm.*, 2011, **77**, 26–35.

54 C. Scholz and E. Wagner, *J. Controlled Release*, 2012, 554–565.

55 H. Lee, A. K. R. Lytton-Jean, Y. Chen, K. T. Love, A. I. Park, E. D. Karagiannis, A. Sehgal, W. Querbes, C. S. Zurenko, M. Jayaraman, C. G. Peng, K. Charisse, A. Borodovsky, M. Manoharan, J. S. Donahoe, J. Truelove, M. Nahrendorf, R. Langer and D. G. Anderson, *Nat. Nanotechnol.*, 2012, **7**, 389–393.

56 M. Naito, T. Ishii, A. Matsumoto, K. Miyata, Y. Miyahara and K. Kataoka, *Angew. Chem., Int. Ed.*, 2012, **51**, 10751–10755.

57 S. K. Hamilton, A. L. Sims, J. Donavan and E. Harth, *Polym. Chem.*, 2011, **2**, 441–446.

58 J. G. van den Boorn, M. Schlee, C. Coch and G. Hartmann, *Nat. Biotechnol.*, 2011, **29**, 325–326.

59 F. S. Du, Y. Wang, R. Zhang and Z. C. Li, *Soft Matter*, 2010, **6**, 835–848.

60 A. L. Jackson and P. S. Linsley, *Nat. Rev. Drug Discovery*, 2010, **9**, 57–67.