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siRNA and Gene Formulation for Efficient Gene Therapy

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1. Introduction

Whilst small interfering RNA (siRNA, also known as short interfering RNA) has a somewhat chequered history with regard to its discovery and initial usage, the "mammalian" research community singularly neither reading nor citing the output from the "plant" research community, it is now recognised in terms of $bn being invested and spent that RNA interference (RNAi), sequence specific post-transcriptional gene silencing (PTGS) by siRNA, has many potential therapeutic applications [1] as well as being an important tool in the study of functional genomics. The site and mechanism of action of siRNA requires that these short double-stranded nucleic acids are delivered to the cytosol of target cells. Therefore, formulation is required in a strategy similar to that for gene therapy, although not requiring access to the nucleus. Efficient medicines design should come with an understanding of the problem at the molecular level. Our contributions are aimed at the use of non-viral gene therapy and this Chapter therefore has such a focus.

2. RNA interference

2.1. History and mechanism of RNA interference

siRNA is a double-stranded RNA (dsRNA) typically of 21-25 nucleotides per strand. siRNA operates as a part of the cellular mechanism called RNAi, which was first noticed in petunia flowers (*Petunia hybrida*) which showed reduced pigmentation on the introduction of exogenous genes that were meant to increase pigmentation [2, 3]. These experiments aimed at increasing the pigmentation of the petunia flowers by means of introducing additional gene constructs expressing either chalcone synthase [2, 3] or dihydroflavonol-4-reductase [2]. However, the resultant plants produced completely white flowers and/or flowers with white

or pale sectors on a pigmented background. The exact mechanism was not identified at the time and was simply termed co-suppression. The transcription level of the suppressed chalcone synthase genes in petunia flowers was found to be similar to that of the non-suppressed genes, and thus the co-suppression must have been at the post-transcriptional level [4]. Later in 1997, the suppression of chalcone synthase endogene in petunia flowers was suggested to be related to formation of RNA duplexes by intermolecular pairing of complementary sequences between the coding sequence and the 3'-UTR sequence of the transgene mRNA [5]. Indeed, the seminal contributions the plant RNAi community have made to this RNAi field are also reflected in the research of Hamilton and Sir David C. Baulcombe in the Sainsbury Laboratory, Norwich, UK, on PTGS as a nucleotide sequence-specific defence mechanism that can target both cellular and viral mRNAs with RNA molecules of a uniform length, ~25 nucleotides [6]. That RNA silencing involves the processing of dsRNA into 21-26 long siRNA to mediate gene suppression (correspondingly complementary to the dsRNA) was demonstrated in Arabidopsis, "RNA silencing pathways in plants that may also apply in animals" [7]. That Arabidopsis ARGONAUTE1 RNA-binding protein is an RNA slicer that selectively recruits microRNAs and siRNAs was shown to be by a key mechanism similar to but different from that found in animals [8]. In 1998, Fire, Mello and co-workers reported the reduction or inhibition (hence genetic "interference") of the expression of the *unc-22* gene *in Caenorhabditis elegans* by means of dsRNA that is homologous to 742 nucleotides in the targeted gene [9], a discovery that was awarded the Nobel Prize in medicine or physiology in 2006. The target gene expresses an abundant although nonessential myofilament protein. Decreasing *unc-22* activity resulted in an increasingly severe twitching phenotype, while complete inhibition resulted in impaired motility and muscle structural defects. The target gene inhibition was best achieved with dsRNA, while using the individual sense or anti-sense RNA strands resulted only in modest silencing. The authors also noticed that only few copies of the dsRNA are required per cell to initiate a potent and specific response, rejecting the hypothesis that the mechanism of interaction with target gene mRNA is stoichiometric in nature, and thus the role of the dsRNA in the interference machinery must be catalytic or amplifying.

Elbashir et al. reported in 2001 that sequence-specific gene silencing of endogenous and heterologous genes with 21 nucleotide siRNA occurs in mammalian cell cultures [10]. The reporter genes coding for sea pansy (*Renilla reniformis*) and firefly (*Photinus pyralis*) luciferases were silenced successfully in different cell lines including human embryonic kidney cells (293) and the cervix cancer cells (HeLa cell line, the first human cell line grown in vitro with success [11]), as well as the endogenous gene coding for the nuclear envelope proteins lamin A and lamin C in HeLa cells. The authors used dsRNA of length 21 or 22 nucleotides with 3'-symmetrical 2-nucleotide overhangs on each strand, as dsRNA with length >30 nucleotides initiates an immune response e.g. inducing interferon synthesis) that leads to non-specific mRNA degradation, which was evident from non-specific silencing of luciferase with 50 and 500 nucleotides dsRNA in HeLa S3 cells, COS-7 cells (kidney cells of the African green monkey), and NIH/3T3 cells (mouse fibroblasts) [10]. The RNAi mechanism of action continues to be investigated in detail and reviewed thoroughly [12-17]. The RNAi mechanism involves the incorporation of dsRNA segments (e.g. siRNA) that have a sequence complementary to the targeted mRNA in a protein com-

plex. This core complex which carries-out mRNA degradation is the RNA induced silencing complex (RISC) [18-20]. The degradation process requires the key argonaute family of proteins, which contain a domain with RNase H (endonuclease) type of activity that catalyse cleavage of the phosphodiester bonds of the targeted mRNA. RISC assembly and subsequently its function to mediate sequence specific mRNA degradation occur in the cytoplasm of the cell [16]. The source of the dsRNA segments incorporated in RISC can be endogenously processed microRNA (miRNA), short hairpin RNA (shRNA), or synthetic siRNA. miRNA is produced from endogenous DNA through the action of RNA polymerase II resulting in the formation of non-coding RNA called primary miRNA (pri-miRNA), which is processed in the nucleus by a protein complex containing an enzyme known as Drosha and a dsRNA binding protein cofactor called Pasha (DGCR8). Drosha cleaves pri-miRNA to produce (pre-miRNA), a dsRNA of 70-90 nucleotides and having a hairpin loop, which binds to Exportin 5 protein and is transferred from the nucleus into the cytoplasm. Pre-miRNA is processed by Dicer (RNase III enzyme) in the cytoplasm to give miRNA, typically of 22 nucleotides in length and having two nucleotide overhangs at the 3'-position [16, 21], shRNA is produced by transcription from an exogenous DNA that is delivered to the nucleus, and codes for a hairpin shaped RNA with segments of length 19-29 nucleotides and loop of 9 nucleotides [22, 23] which can then be processed by Dicer and incorporated in the RNAi machinery.

Once in the cytoplasm, the processed dsRNA (miRNA, processed shRNA, or siRNA) is then incorporated into a protein complex (RISC-loading complex, RLC). In *Drosophila* the RLC is composed of the dsRNA, heterodimer protein DCR2 (Dicer variant)/R2D2, possibly including the catalytic argonaute proteins as well in this complex. The active RISC is formed when one of the RNA strands in the complex is cleaved (the passenger strand) and the strand with the less thermodynamic stable 5'-end (guide/anti-sense strand) remains in the complex. The mRNA with complementary sequence to the guide strand binds to the active RISC and is cleaved by the endoribonuclease activity of the argonaute component of the complex (Figure 1).

2.2. RNA duplex structure

RNA is a polymer of ribonucleotides. Each RNA nucleotide is composed of one nucleobase, the monosaccharide pentose ribose, and one phosphate group. The nucleobases in RNA are adenine (purine base), guanine (purine base), uracil (pyrimidine base), and cytosine (pyrimidine base) (Figure 2). A nucleoside is formed when each base is connected via a glycosidic bond to the anomeric carbon 1' of ribose, thus when glycosylated, adenine, guanine, uracil, and cytosine nucleobases give adenosine, guanosine, uridine, and cytidine nucleosides. Each two nucleosides are connected via a phosphate diester bond between the 3' of one nucleoside and 5' of the next nucleoside to form the RNA polynucleotide strand. The main differences in the primary structure of RNA and DNA are that RNA pentose is ribose while DNA pentose is 2'-deoxyribose, and the RNA incorporates the nucleobase uracil instead of thymine.
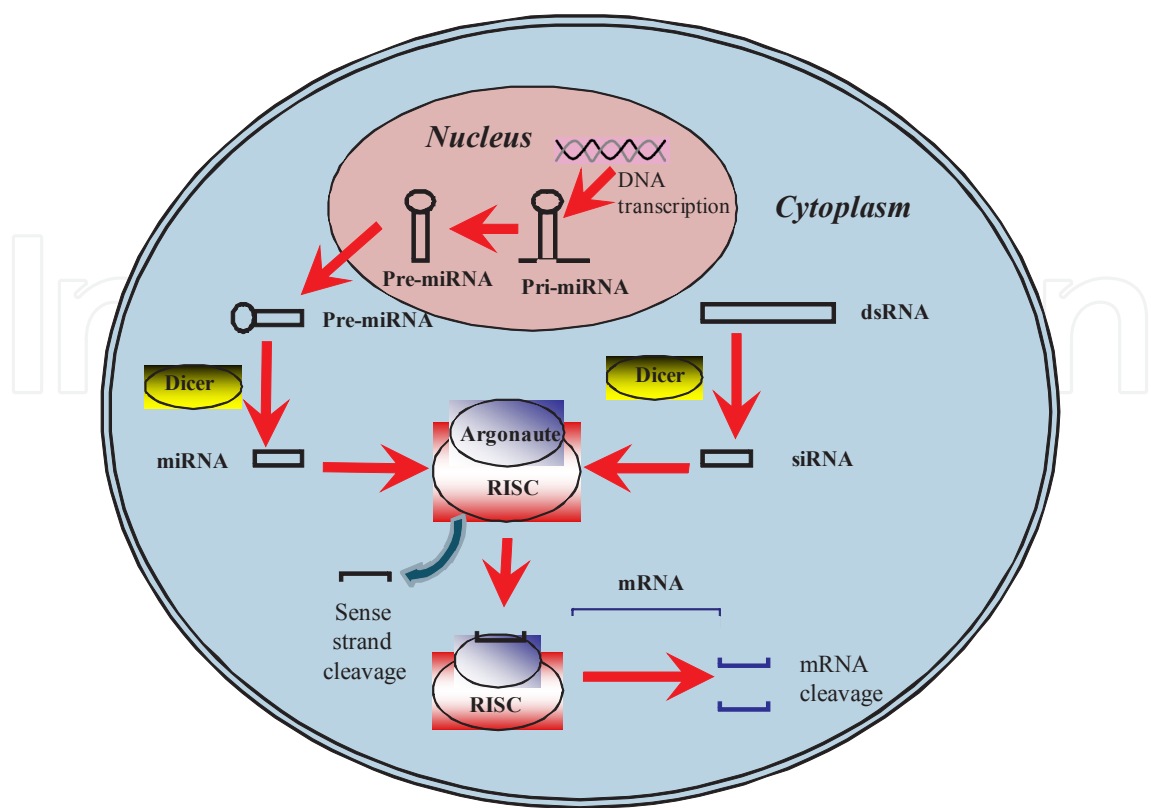
Figure 1. RNAi mechanism in a eukaryotic cell. The source of the antisense strand incorporated in RISC can be miRNA, processed exogenous long dsRNA, or synthetic siRNA delivered to the cell.
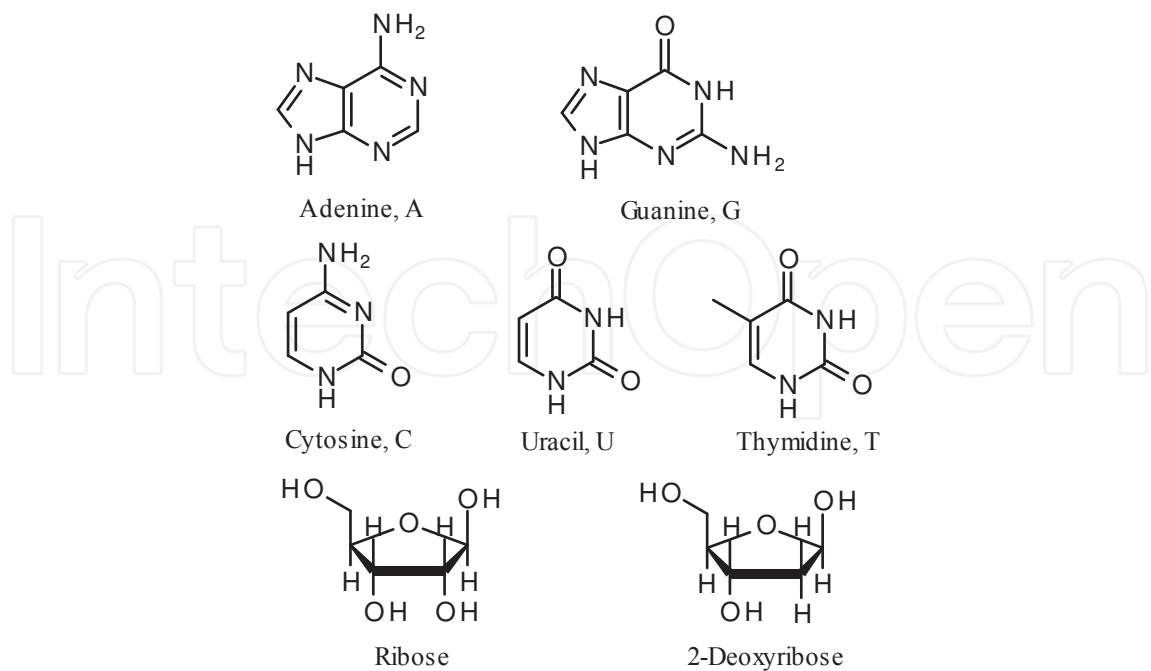

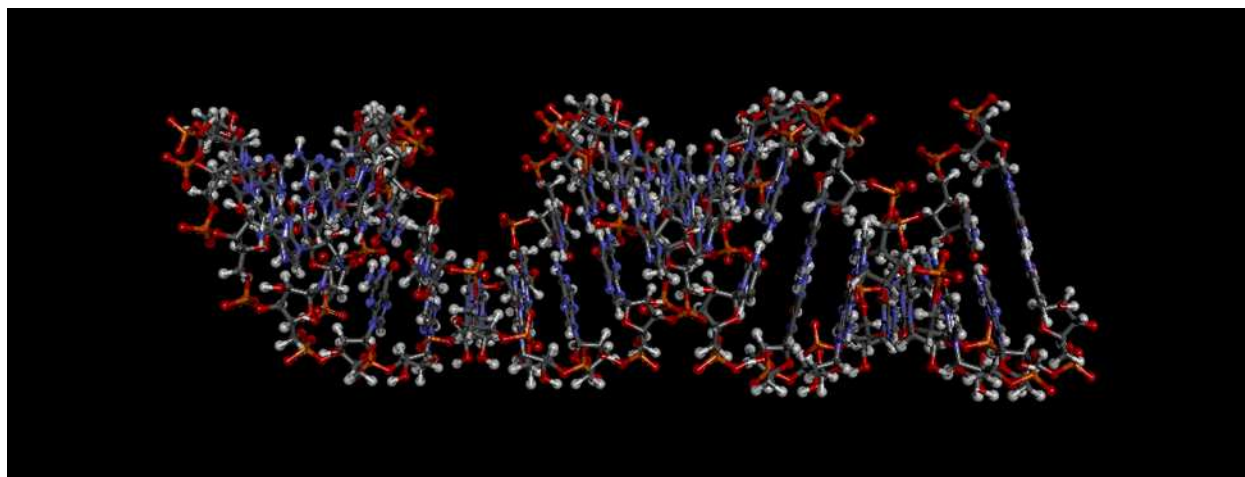**Figure 2.** Nucleobases and pentoses of RNA and DNA.

Figure 3. siRNA duplex 22-mer targeting the enhanced green fluorescent protein (EGFP) mRNA. The two deoxythymi-dine residues at the 3'-end of the sense strand are not shown. Sense strand: 5'-GCAAGCUGACCCUGAAGUUCAUTT-3' Anti-sense strand: 5'-AUGAACUUCAGGGUCAGCUUGCCG-3' Target DNA sequence: 5'-CGGCAAGCTGACCCTGAAGTT-CAT-3'

In order to form an RNA duplex (Figure 3), the strands with complementary nucleotide sequence bind together by hydrogen bonds. Adenine is bound to uracil with two hydrogen bonds while guanine is bound to cytosine with three hydrogen bonds, thus forming what is known as Watson-Crick base pairs. RNA duplexes under normal physiological conditions are in the form of A-helix. This type of duplex is a right-handed helix [24-26].

The presence of the 2'-hydroxyl group of the ribose and the lack of the methyl group on the nucleotide uridine (in contrast to the methylated thymidine) results in structural differences between RNA and DNA, with the 2'-hydroxyl group of RNA being the major cause of the differences. The sugar phosphate backbone of RNA duplexes is stabilized by the 2'-hydroxyl in the C3'-endo position, while DNA adopts the C2'-endo position (Figure 4). Thus, the RNA duplex takes the A-helix form while the DNA helix takes the B-form. The A-helix form is suggested to have a greater hydration shell, giving RNA duplexes more thermodynamic stability and more rigidity compared to DNA duplexes [24-26]. RNA A-helix completes one complete rotation in 11-12 base pair (bp) compared to 10 bp for DNA, with a rise of 2.7 Å per bp of RNA [27]. The A-helix geometry has been suggested to be the major factor explaining why dsRNA and not dsDNA is involved in the RNAi machinery [28], where the A-helix geometry between the guide strand and the complementary target mRNA is essential for the catalytic activity of the argonaute 2 protein in the RISC.

As a result of the presence of a hydroxyl group in the 2'-position of the ribose in the RNA backbone, the RNA phosphodiester backbone is more susceptible to hydrolysis by nucleases compared to the DNA which lacks the 2'-hydroxyl in its 2'-deoxyribose [29]. Incubation of
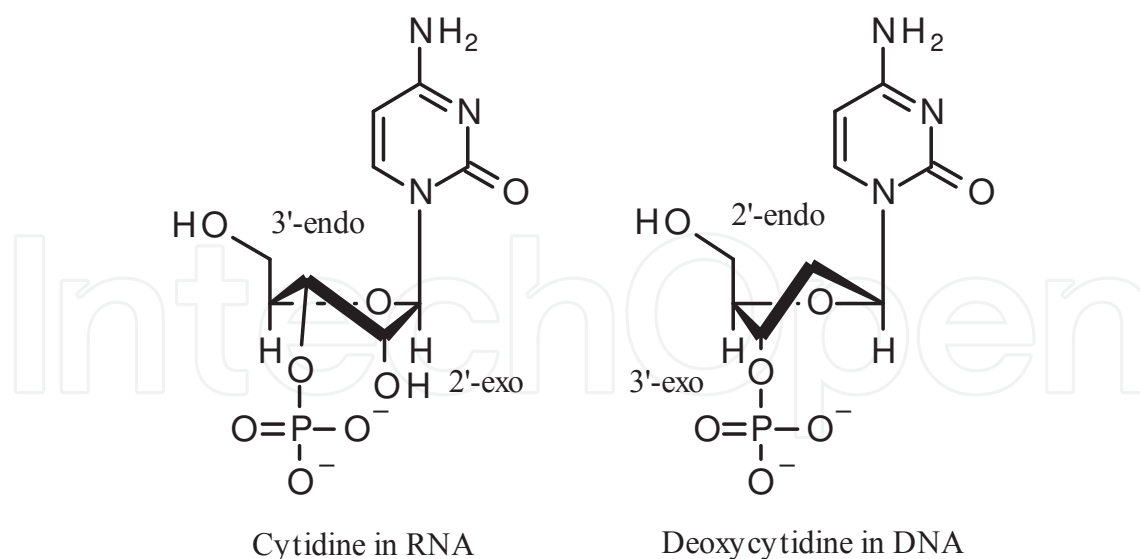
Figure 4. 3'-endo ribose configuration of RNA (left) vs 2'-endo (right) of 2'-deoxyribose in DNA. Shown is cytidine (RNA) and deoxycytidine (DNA) with the 3'-hydroxyl phosphorylated. The hydrogen atoms at C2' and C3' are not displayed for clarity.

siRNA in fetal bovine or human serum at 37 °C resulted in the degradation and partial or complete loss of activity [30]. When incubated in human plasma at 37 °C, more than 50% of the unmodified siRNA was degraded within one minute, and practically all siRNA was completely degraded within 4 hours [31]. Although Ribonuclease A (RNase A, an endoribonuclease) cleaves single stranded RNA, siRNA degradation in serum was reported to be mainly due to RNase-like activity[32], which is suggested to occur during transient breaking of the hydrogen bonds joining the two siRNA strands. In addition to the RNase A family of enzymes, blood serum contains phosphatases and exoribonucleases which can also affect degradation of siRNA at nuclease-sensitive sites on both strands [33].

2.3. Therapeutic potential of RNAi based therapies

RNAi based therapies emerged in the period following its discovery in 1998 in plants, and are promising therapeutic candidates to treat various types of diseases, ranging from age related macular oedema to respiratory tract infections to various types of cancer [34-36]. In addition to siRNA based therapies, shRNA [37, 38] and miRNA [39] are potential therapeutic tools. siRNA based therapeutics are already in phase I and phase II clinical trials; representative examples of clinical trials involving siRNA are shown in Table 1. The basic concept is the reduction or inhibition of the expression of a protein that is involved in the pathophysiological pathway of the target disease (silencing/knocking-down the target gene). This concept is evident from using Cand5 siRNA targeting the mRNA translating the vascular endothelial growth factor (VEGF), thus reducing/inhibiting angiogenesis and preventing progression of wet age related macular oedema (Table 1) [40]. Atu027 siRNA targets the biosynthesis of protein kinase N3 which plays a role in cancer metastasis [41].

siRNA	Disease	Vector/ *Route*	Phase	Sponsor
Cand5/ Bevasiranib	Diabetic macular oedema	None/ *Intravitreal*	Phase II	Opko Health (Miami, USA)
Cand5/ Bevasiranib	Age-related macular degeneration	None/ *Intravitreal*	Phase II (Phase III halted)	Opko Health (Miami, USA)
ALN-RSV01	Respiratory syncytial virus infection	None/ *Intranasal*	Phase II	Alnylam Pharmaceuticals (Cambridge, USA)
CALAA-01	Solid tumour/melanoma	Cyclodextrin nanoparticles/ *Intravenous*	Phase I	Calando (Pasadena, CA, USA)
Atu027	Colorectal cancer metastasizing to the liver	AtuPlex-Liposome/ *Intravenous*	Phase I	Silence Therapeutics (London, UK)
Two siRNA against TGFBI and COX-2 STP705	Wound healing	Nanoparticles/ *Intravenous*	Phase I	Sirnaomics (Gaithersburg, MD, USA)
I5NP	Protection from acute kidney injury after cardiac bypass surgery	None/ *Intravenous*	Phase I	Quark Pharmaceuticals (Fremont, USA)
TKM-080301	Against PLK1 gene product in patients with hepatic cancer	Lipid nanoparticles/ *Hepatic intra-arterial administration*	Phase I	NCI (Maryland, USA)

Table 1. Representative clinical trials using siRNA (http://clinicaltrials.gov/ct2/home, accessed on 5/8/2012).

The therapeutic application of siRNA requires overcoming several barriers (Figure 5) for its intracellular delivery and the subsequent functional gene silencing activity [42-44]. Those barriers are mainly due to siRNA specific characteristics, most important are having a highly negative charge due to their phosphate backbone (on average 40-50 negative charges per siRNA), being susceptible to degradation by nucleases, and having relatively large molecular weight (13-15 kDa) compared to conventional small drug molecules. First, local delivery (such as intravitreal) is different from intravenous delivery, where the latter will subject the siRNA to the serum ribonucleases, which results in degrading non-modified siRNA within time periods that vary from minutes to hours [31]. siRNA injected intravenously in rats was reported to be cleared rapidly from circulation and accumulates in kidneys within minutes of injection [45], making it useful only if the target organ is the kidney.

In order to gain access into the cytoplasm where siRNA can exert its biological activity, the polyribonucleotide must pass first through the interstitial space then through the target cell membrane. This will be a difficult task, since both the extracellular matrix in many tissue types

and the cell membrane incorporate negatively charged glycosaminoglycans (e.g. heparan sulfate) [46]. In addition, cell membranes contain negatively charged phospholipids (e.g. phosphatidyl serine) therefore the membrane is negatively charged [46, 47]. The net result is an unfavourable repulsive interaction with naked siRNA. As a result, different strategies are being developed to overcome the barriers to reproducible and functional siRNA delivery, and these approaches fall into two general categories. One category is modifying the siRNA, the other is deploying a vector to protect the siRNA and increase its efficiency of delivery.
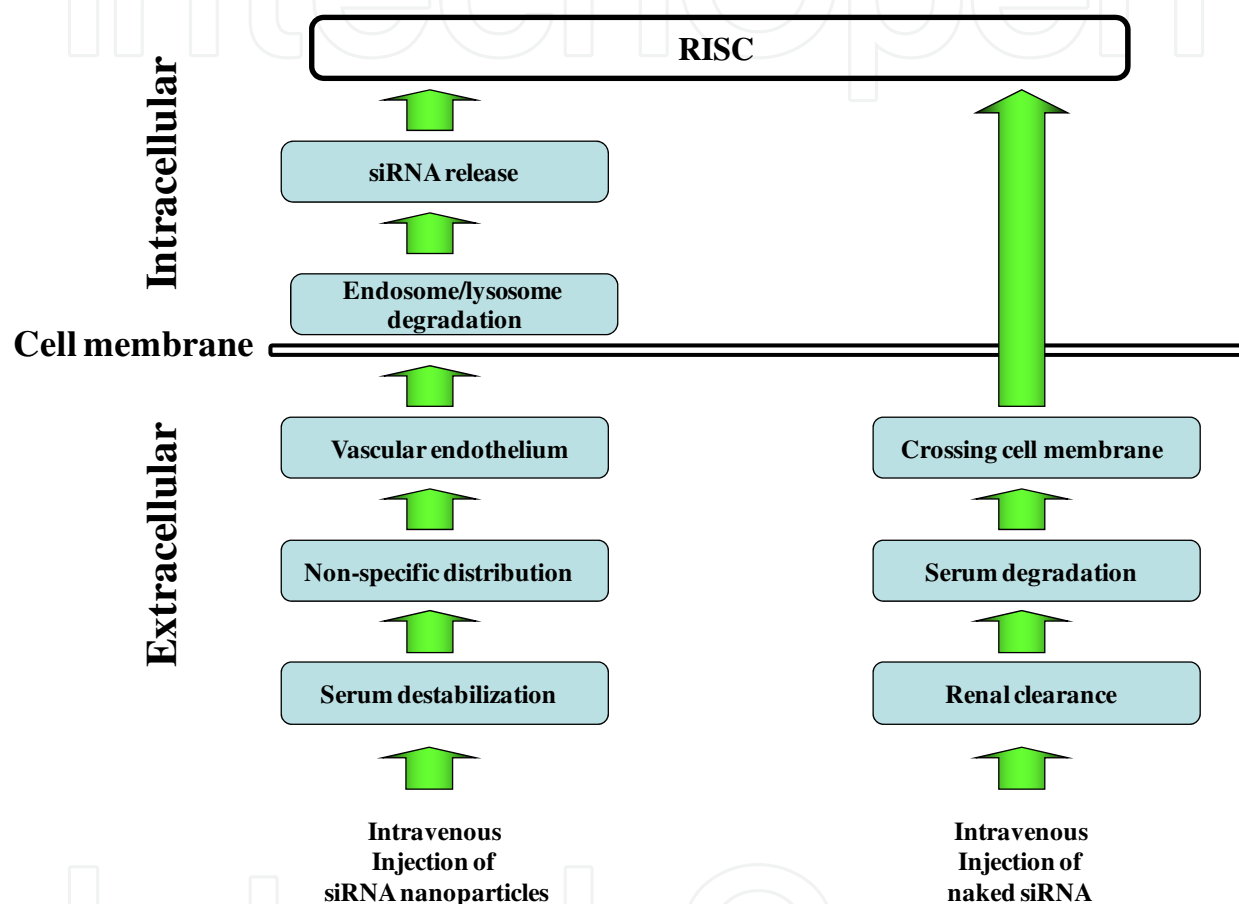

Figure 5. Summary of barriers to successful gene-silencing mediated by siRNA after intravenous injection, whether delivered naked or incorporated in nanoparticles.

3. Strategies to achieve efficient siRNA delivery and gene silencing

3.1. siRNA modifications

siRNA modifications include those carried out at the ribose residue, at the phosphate backbone, at the RNA nucleotides, the siRNA termini, and/or by conjugation of other molecules to the siRNA molecule. Modifications to the ribose at the 2'-position are common [48], and include 2'-*O*-alkylation (e.g. 2'-*O*-methyl and 2'-*O*-methylethoxy) modifications. 2'-Fluoro RNA is

another common modification. Locked nucleic acids (LNAs) have a methylene bridge connecting the 2'-*O* to the 4'-C of the ribose unit, locking the sugar in the 3'-endo conformation. These modifications led to increased ribonuclease resistance [48, 49]. Modifications at the phosphate backbone include phosphorthioate, boranophosphate, and methylphosphonate linkages [48, 49] and is reported to increase siRNA stability against various ribonucleases and phophodiesterases [50]. siRNA nucleotides can be substituted with DNA nucleotides to increase stability and/or decrease unwanted siRNA off-target effects [51]. Modifications of the 3'-overhangs (usually two nucleotides in length) include incorporating deoxyribonucleotides to reduce costs and increase stability towards 3-exoribonucleases. The 5'-terminus chemical phosphorylation of the antisense strand results in higher gene silencing efficiency, while blunt ended duplexes were reported to be more resistant to exonucleases. The advantages of each of the aforementioned techniques, other modification strategies, as well as the considerations related to the degree of modification and its effect on gene silencing efficiency and associated cytotoxic effects have been reviewed thoroughly [48, 52-54].

The conjugation of drug molecules, aptamers, lipids, polymers, and peptides/proteins to siRNA could enhance in vivo delivery [55]. The main aims of such conjugations are: to enhance siRNA stability, increase in vivo half-life, control biodistribution, increase efficiency of intracellular delivery, while maintaining the gene silencing activity.

One strategy is to increase the hydrophobicity of the siRNA. Cholesterol was conjugated to the 5'-terminus of siRNA, the cholesterol-siRNA conjugate (chol-siRNA) resulted in better intracellular delivery compared to unmodified siRNA and retained gene silencing activity in vitro in β-galactosidase expressing liver cells [56]. When cholesterol was conjugated to the 3'-terminus of the sense (passenger) strand of siRNA, the conjugate had improved in vivo pharmacokinetics as the intravenous administration of chol-siRNA in mice resulted in its distribution and detection in the fat tissues, heart, kidneys, liver, and lungs, even 24 h after intravenous injection [57]. No significant amounts of unmodified siRNA were detected in the tissues 24 h after the intravenous injection. Conjugation of siRNA to bile acids and long-chain fatty acids, in addition to cholesterol, mediates siRNA uptake into cells and gene silencing in vivo [58]. The medium chain fatty-acid conjugates, namely lauroyl (C12), myristoyl (C14) and palmitoyl (C16), did not silence the target apolipoprotein B mRNA levels in mouse livers after intravenous injection. However, siRNA fatty-acid conjugates having long saturated chains, stearoyl (C18) and docosanoyl (C22), significantly reduced apolipoprotein B mRNA levels.

Cell penetrating peptides (CPPs) are used to facilitate cellular membrane crossing of many molecules displaying various properties such as antisense oligonucleotides, peptides, and proteins and are already being tested in vivo [59]. siRNA was conjugated to penetratin and transportin, to silence luciferase and green fluorescent protein (GFP) in different types of mammalian cells [60]. However, in vivo lung delivery in mouse of siRNA conjugated to penetratin and TAT(48-60), targeting p38 MAP kinase mRNA showed that the reduction in gene expression was peptide induced and the penetratin conjugated siRNA resulted in innate immunity response [61].

siRNA functioning against the VEGF mRNA was conjugated to poly(ethylene glycol) (PEG, 25 kDa) via a disulfide bond at the 3'-terminus of the sense strand [62]. The siRNA-PEG

conjugate formed polyelectrolyte complex (PEC) micelles by electrostatic interaction with the cationic polymer polyethylenimine (PEI). The formed VEGF siRNA-PEG/PEI PEC micelles showed enhanced stability against nuclease degradation compared to the unmodified siRNA. These micelles efficiently silenced VEGF gene expression in prostate carcinoma cells (PC-3) and showed superior VEGF gene silencing compared to VEGF siRNA/PEI complexes in the presence of serum. PEG conjugation on its own enhanced the stability of the siRNA in serum containing medium. The prolonged stability of the PEC micelles was suggested to be due to the presence of PEG chains in the outer micellar shell layer, thus sterically hindering nuclease access into the siRNA in the micelle core [62]. Targeting molecules such as antibodies [63] and aptamers (peptides or single stranded DNA or RNA that have selective affinities toward target proteins) [64] have also been conjugated to siRNA, with the aim of increasing the efficiency of siRNA delivery to the target tissues.

Conjugating molecules to siRNA requires specific considerations. First, the site of conjugation (3'- and/or 5'-terminus, on sense and/or antisense strand) should be chosen such that it does not affect the activity of the siRNA and its ability to be incorporated in the RISC, or its ability to bind the target mRNA in the correct helix conformation. Second, the conjugated siRNA might have new properties that were not present in the unmodified parent siRNA. An example is the in vivo immune response resulting from the penetratin-siRNA conjugate [61]. Third, the conjugation process is multi-step, and the chemical reaction intermediates and products require efficient purification in order to meet the specifications of in vivo applications. These steps need to be repeated for each siRNA under investigation, which can be costly and time consuming. Thus, although there are clear advantages to synthesize siRNA conjugates, there are also disadvantages, and conjugation is therefore only one of two valuable approaches in the toolbox for preparing siRNA based therapies. The other valuable tool is complexation or incorporating the siRNA in a vector.

3.2. Viral vectors for shRNA delivery

Vectors for RNAi based therapies are either viral or non-viral vectors. Viral vectors (Table 2) are used to deliver genes encoding hairpin RNA structures such as shRNA and miRNA, which are then processed by the cellular RNAi machinery to the functional silencing dsRNA [65, 66].

Viral vectors offer two main advantages, the first is the very high efficiency compared to non-viral vectors [68], which can reach few orders of magnitude more than that achieved with non-viral vectors, and the second is the potential of long term expression of the delivered RNAi therapeutic, which is very useful in the treatment of chronic diseases such as HIV infection and viral hepatitis [69, 70]. Retroviruses are enveloped, single stranded RNA viruses and have a genome capacity of 7-10 kilobases (kb). They preferentially target dividing cells which limits their use to mitotic tissues (thus for example excluding brain and neurons). Retroviruses integrate their DNA in the host genome using an integrase enzyme, which provides the advantage of stable long term expression of the delivered transgene in the host cell and its descendants. However, integrating new DNA sequences into host genome carries the risk of

		Retrovirus/ Lentivirus	Adenovirus	Adeno- associated virus	Herpes virus
Viral vector properties	Genome	ssRNA	dsDNA	ssDNA	dsDNA
	Capsid	Icosahedral	Icosahedral	Icosahedral	Icosahedral
	Envelope	Enveloped	None	None	Enveloped
	Viral Polymerase	Positive	Negative	Negative	Negative
	Diameter (nm)	80-130	70-90	18-26	150-200
	Genome size (kb)	7-10	38	5	120-200
Gene therapy related	Infection tropism	Dividing*	Dividing/ Non-dividing	Dividing/ Non-dividing	Dividing/ Non-dividing
	Virus genome integration	Integrating	Non-integrating	Integrating	Non-integrating
	Transgene expression	Lasting	Transient	Lasting	Transient
	Packaging capacity (kb)	7-8	8	4.5	>30

* Lentiviral vectors can infect non-dividing cells as their pre-integration complex can traverse the nuclear membrane pores (NMP), in contrast to retrovirus pre-integration complex which does not traverse NMP, requiring the host-cell division to integrate the retroviral genome [67].

Table 2. Summary of properties of viral vectors that are commonly used in gene therapy (adapted from http://www.genetherapynet.com/viral-vectors.html, accessed on 5/8/2012).

insertional mutagenesis [68, 71]. shRNA expression cassette delivered by a retroviral vector was used in rats to silence a RAS oncogene in order to suppress tumour growth [72]. Herpes virus was used successfully to deliver shRNA targeting exogenous β-galactosidase or endogenous trpv1 gene mRNA in the peripheral neurons in mice by injecting once directly into the sciatic nerve of the animals [73].

Unlike other retroviruses, lentiviruses can infect dividing as well as differentiated and non-dividing cells. The lentiviral genome can accommodate 7.5 kb [66], and their genome is integrated in the host cell genome, lentiviral vectors are generally preferred for long-term expression of transgenes, and efficient delivery in vivo to the brain, eye, and liver to induce long-term transgene expression as reported [74]. A lentiviral vector was used to deliver shRNA targeting *Smad3* gene mRNA, and enhanced myogenesis of old and injured muscles [75].

Adenoviruses are non-enveloped viruses, with linear double stranded DNA. They preferably infect the upper respiratory tract and the ocular tissue. Their genome can accommodate up to 8 kb which can be extended to ≥25 kb in modified viruses that have their viral genes deleted [68]. These viruses can infect post mitotic cells and thus are good candidates for neurological diseases. Unless delivering genes that can exist as episomes in host cells, adenoviruses result only in transient expression of their cargo. However, although the host cells with the episome can express the delivered genes for the cell life time, these cells will eventually be removed by the host immune system [68]. shRNA targeting VEGF that was delivered by an adenoviral vector resulted in potent inhibition of angiogenesis and tumour growth in mice [76].

Adeno-associated virus (AAV) is a single stranded DNA non-pathogenic virus that can accommodate a 4.7 kb genome. They can infect dividing or non-dividing cells. The replication of AAV requires co-infection with adenovirus. The viral genome integrates into the host cell genome at a specific location on chromosome 19 [68]. Direct intracerebellar injection in a mouse model of spinocerebellar ataxia of an AAV vector delivering a cargo expressing shRNA targeting polyglutamine induced neurodegeneration significantly restored cerebellar morphology and improved motor coordination in mice [77].

Although highly efficient in delivering their cargo, viral vectors have their disadvantages. Adenoviral vectors have the disadvantage of triggering a strong immune (adaptive and innate) response by repeated administration, in addition to target organ immunotoxicity, specially hepatotoxicity [78-80], which resulted in 1999 in the death of one 18-year-old male who received high dose of adenovirus that was delivered directly in the hepatic artery in a clinical gene therapy safety study [81]. Clonal T-cell acute lymphoblastic leukemia caused by insertional mutagenesis in a gene therapy completed clinical trial involving patients suffering X-linked severe combined immunodeficiency (SCID-X1) was reported in one out of the 10 patients using a retroviral vector [82]. Integration of the vector genome material in the antisense orientation 35 kb upstream of the protooncogene (LMO2) caused over expression of the gene in the leukemic cells. In a similar study, 4 out of 9 patients developed leukemia within 3-6 years post-treatment mainly due to vector-mediated upregulation of host cellular oncogenes [83, 84]. In addition, immune responses (whether adaptive or innate) of varying degrees depending on the type of vector, dose, and target organs were reported for lentiviral, adenoviral, adeno-associated viral vectors [80].

Current research on viral vectors for gene therapy is focussed on approaches such as vector engineering e.g. modifying the viral capsid or pseudotyping the envelope, different delivery strategies, and administration to immune-privileged sites that can tolerate the delivered viral vectors without responding with an inflammatory response [80, 85]. Other research focusses on the essential scaling-up process of vector production and increasing the packaging efficiency of the vectors [85], the processes without which, the wide spread and successful therapeutic use of the viral vectors will be very difficult to achieve.

3.3. Non-viral vectors

Non-viral vectors for gene and siRNA delivery are an alternative to viral vectors, as they do not suffer many of the disadvantages of the viral vectors, especially immunogenicity and tumourigenicity. The non-viral vectors can be classified generally as peptides, polymeric based vectors, carbohydrate based, and lipid based [86]. CPPs, also known as peptide transduction domains (PTDs), have shown the ability to cross the cellular membrane despite their relatively high molecular weight and size (Table 3).

PTDs generally are short amphipathic and/or cationic peptides that can transport many hydrophilic molecules across the cell membrane. A wide range of molecules including liposomes [87, 88], peptides, proteins [89], peptide nucleic acids [90] and polynucleotides [91] are delivered intracellularly using PTDs and they have also been applied in vivo [59, 92, 93].

CPP	Sequence of CPP	Type of association with siRNA	Target mRNA
CADY	GLWRALWRLLRSLWRLLWRA	Non-covalent	GAPDH, p53 [94]
EB1	LIRLWSHLIHIWFQNRRLKWKKK	Non-covalent	Luc [95]
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Non-covalent	Luc, GAPDH [96] Oct-3/4 [97]
Poly-arginine	RRRRRRRRR	Non-covalent	VEGF [98]
Penetratin	RQIKIWFQNRRMKWKK	Covalent	Luciferase (Luc), EGFP [60]
		Covalent	SOD1, caspase-3 [99]
		Covalent	Luc, p38 MAP kinase [61, 100]
Transportan	LIKKALAALAKLNIKLLYGASNLTWG	Covalent	Luc, EGFP [100]
TAT	GRKKRRQRRRPPQ	Covalent	EGFP, CDK9 [101]

Table 3. Selected CPPs used for siRNA delivery [59].

It was reported by Frankel and Pabo in 1988 that the HIV-1 derived TAT protein could be taken up by cells growing in tissue culture [102], and that a small basic region of TAT (48-60) was essential for uptake by the cells [103]. PTDs include antennapedia homeodomain protein (Antp, penetratin), mitogen-activated protein (MAP), poly-arginine, transportan, VP22 [59, 92]. Two major pathways are involved in the uptake of PTDs and PTD-cargos: direct translocation at 4 °C and 37 °C and endocytosis-translocation at 37 °C. These mechanisms depend on many factors: cargo size, cell line, PTD concentration, and the type of PTD [59, 104, 105]. siRNA can be conjugated covalently to the CPP or can be complexed with the cationic groups of basic amino acids that are present in the backbone of the CPP. As a representative example of non-covalent complexation, CADY [94], which is basic due to its five arginine residues can complex with the negatively charged siRNA. Another example of non-covalent complexation is the poly-arginine CPP [98].

PEI (Figure 6) is an efficient, but toxic, plasmid DNA delivery vector. However, as a siRNA delivery vector PEI is reported to be much less efficient [106, 107]. This decreased efficiency is due to the dissociation of the siRNA/PEI complex upon interaction with the negatively charged cell membrane, which is suggested to be because of the short length of siRNA and the associated weak electrostatic interaction with PEI [108, 109]. Another drawback of PEI is its relatively high toxicity [110]. Thus, in addition to linear PEI, PEI polymers with a wide range of molecular weights were developed to increase PEI efficiency and/or decrease toxicity, although not all PEI are suitable for siRNA delivery [111]. The main advantage of PEI is the ability of its variety of amino groups to be protonated at lower pH (inside endosomes) leading to what is known as the "proton-sponge effect" [112], and efficient escape of the nucleic acid cargo from endosomes.

One approach to enhance siRNA delivery with PEI is increasing the hydrophobicity of PEI by covalently conjugating alkyl chains [113], where increasing the hydrophobic alkyl chain length generally improved the stability of the PEI/siRNA complex. In a similar strategy, cholesterol was conjugated to PEI with decreased toxicity of the conjugates [114]. Low molecular weight PEI (MW < 5 kDa) is less toxic than the higher molecular weight PEI (≈25 kDa), but less efficient
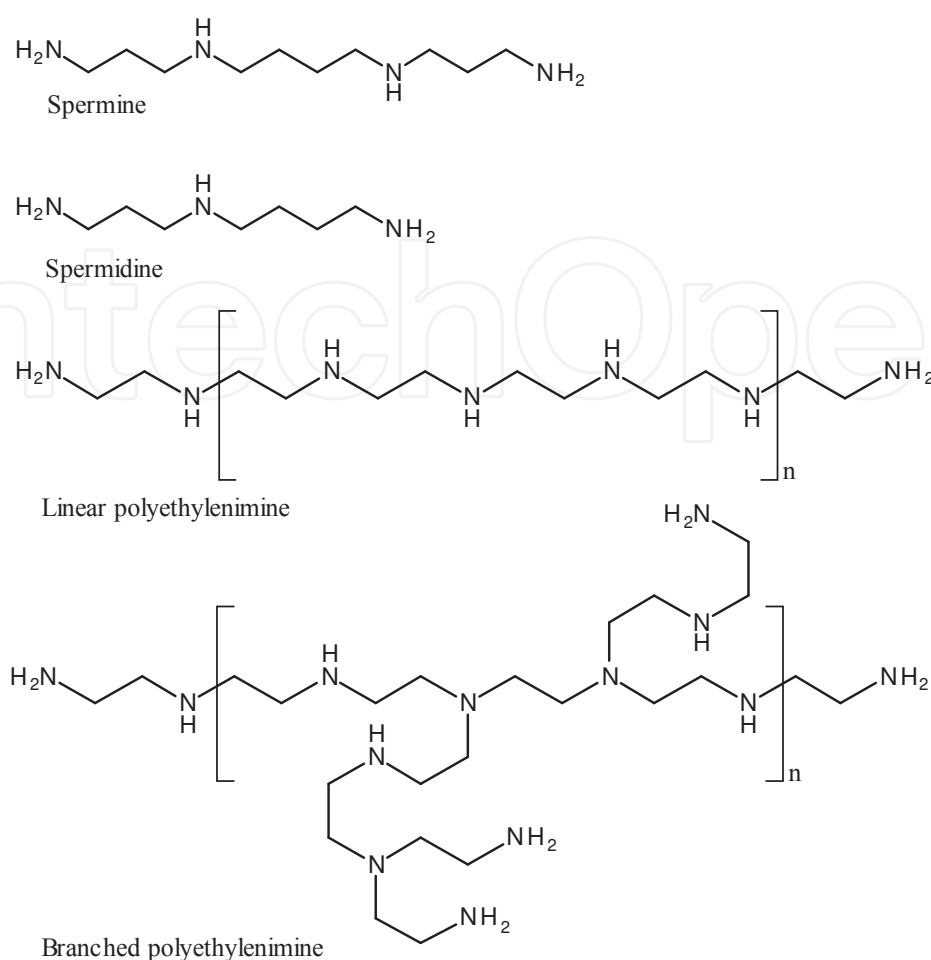
Figure 6. Representative examples of polyamines used in siRNA delivery either as in lipid conjugates of polyamine alkaloids e.g. spermine and spermidine, or as in polyethylenimine (PEI), a cationic polymer.

in polynucleotide delivery, thus, cross-linking of the low molecular weight PEI with disulfide bonds which are cleaved in the reducing environment of the cytoplasm increased the efficiency of siRNA delivery through the enhanced release of siRNA in the cytoplasm [115].

Chitosan is a biocompatible and biodegradable polysaccharide that is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine. Chitosan has weakly basic properties due to the presence of the D-glucosamine residue with a p*K*a value 6.2-7.0. The molecular weight of chitosan affects the complex stability, size, zeta-potential and in vitro gene knock-down of siRNA/chitosan nanoparticles [116]. High molecular weight (64.8-170 kDa) chitosan formed stable complexes with siRNA and resulted in high gene knock-down efficiency in human lung carcinoma (H1299) cells, while low molecular weight (10 kDa) chitosan could not complex the siRNA into stable nanoparticles and showed almost no knock-down [117]. The method of association affects gene silencing efficiency, where chitosan-TPP/siRNA nanoparticles (siRNA entrapped inside the nanoparticles, and TPP is sodium tripolyphosphate and used as a polyanion to cross-link with the cationic chitosan groups by electrostatic interactions) showed high siRNA binding and better gene silencing in vitro compared to siRNA/chitosan particles prepared by

simple complexation and adsorption of siRNA onto chitosan [118]. Although chitosan has good potential as a non-viral gene delivery vector, widespread use is largely limited by its poor solubility (because of their pKa, chitosan amino groups are only partially protonated at the physiological pH 7.4), poor stability of its siRNA complexes at the physiological pH, and low transfection efficiency. Various strategies have been adopted to overcome these drawbacks, such as covalently conjugating PEG to chitosan and binding targeting ligands to enhance cell specificity [116].

Cyclodextrins (CD) are cyclic oligosaccharides composed of 6, 7, or 8 D-(+)-glucose units, known as α-CD, β-CD, γ-CD respectively, bound through α-1,4-linkages. Polymers conjugated to β-CD lack immunogenicity and hence are attractive vectors for polynucleotide delivery. β-CD have a hydrophilic outer surface and a hydrophobic inner cavity which enable them to form inclusion complexes. Efficient cellular transfection of siRNA labelled with a fluorescent tag into human embryonic lung fibroblasts (MRC-5 cells) was observed by siRNA complexes with the β-CD guanidine derivatized bis-(guanidinium)-tetrakis-(β-cyclodextrin) tetrapod (having four β-CD units) [119]. The ability of β-CD to form inclusion complexes was used to develop a siRNA delivery vector. β-CD was covalently bound to a polycationic segment (to electrostatically bind siRNA), while adamantane-PEG-transferrin (adamantane can fit in the β-CD cavity) formed an inclusion complex which can enhance the stability of siRNA nanoparticles in vivo [120]. This system was used to deliver siRNA silencing the *EWS-FLI1* gene thus inhibiting tumour growth in a murine model of metastatic Ewing's sarcoma. The first experimental siRNA therapeutic to provide targeted delivery in humans was reported by Davis and co-workers [121]. siRNA was formulated into a nanoparticle (CALAA-01), which consisted of a cyclodextrin-containing polymer that contains amidine and primary amine functional groups, a PEG for steric stabilization in the in vivo environment (via inclusion complexes of β-CD with adamantine-PEG conjugate), and human transferrin (Tf) as the targeting ligand to binds to the transferrin receptors that are over-expressed on cancer cells. The siRNA/nanoparticles components self-assembled in the pharmacy. CALAA-01 was administered intravenously to the first patient with a solid cancer in a phase I clinical trial (safety study) in May 2008 [121]. Tumour biopsies from patients' melanoma after treatment (phase I clinical trial) showed the presence of intracellular nanoparticles. Reductions in the levels of both the specific mRNA (M2 subunit of ribonucleotide reductase, RRM2) and the protein (RRM2) were found when compared to levels in pre-dosing tissues. These results demonstrated that siRNA nanoparticles administered systemically to a human patient can produce a specific gene knock-down via an RNAi mechanism of action [122]. A recent and novel approach to the synthesis of cationic or neutral PEGylated amphiphilic β-CD used copper-catalysed "click" chemistry to modify selectively the secondary 2-hydroxyl group of the β-CD. The 6-position of these β-CD conjugates was conjugated to a dodecane alkyl chain. Complexation of cationic β-CD alone with siRNA resulted in good silencing of the luciferase reporter gene in Caco2 cells in culture. Co-formulation of cationic β-CD with a PEGylated β-CD and siRNA resulted in lower surface charges and reduced aggregation. The transfection efficiency of the cationic β-CD vector was lowered by co-formulation with the PEGylated β-CD, although the siRNA binding was not affected and the surface charge of the complexes did not reach complete neutrality [123].

Dendrimers have a central core to which are connected several branched arms in a manner that can be symmetrical or asymmetrical. During the synthesis of dendrimers, arms (branches) are added to the core structure. Each addition is called a generation and increases the previous generation number by one. Due to their unique structure, dendrimers can have a planar, elliptical, or spherical shape depending on generation number. Among the most widely used dendrimers are polyamidoamine (PAMAM) and polypropylenimine (PPI) dendrimers [124]. Dendrimers which have positively charged cationic groups on their outer surface are commonly used for polynucleotide delivery. The transfection efficiency of dendrimers increases with increasing the charge density or generation number [125]. However, dendrimers with high generation number are generally more cytotoxic compared to dendrimers with low generation number [126]. Usually the inner space near the core is larger compared to outer space near the surface due to the lower density of molecules (less number of arms) near the core, which allow small molecules to be incorporated in the inner space. Owing to the relatively large molecular weight of polynucleotides, they are usually bound to the surface of cationic dendrimers and not in the inner space of the dendrimer. Generally, the toxicity of dendrimers is lower than that of PEI or poly-L-lysine (PLL) [127]. One advantage of dendrimers is that they have pH buffering capacity (proton-sponge effect), an important feature for endosomal escape and enhancing the release of polynucleotides [125, 128].

PPI dendrimers with high generation numbers (4 and 5) were more efficient in forming discrete nanoparticles with siRNA and in gene silencing in human lung cancer (A549) cells than lower generation dendrimers (2 and 3). Generation 5 PPI dendrimers were more toxic, probably due to the increased positive charge density per dendrimer, than generation 4 dendrimers [129]. Complex formation between PAMAM dendrimers with an ethylenediamine core and siRNA as a function of three variables has been reported [130]. The ionic strength of the medium (without or with 150 mM NaCl), the generation number (4, 5, 6 and 7) and the N/P ratio (ratio of positively charged amine groups per negative phosphate) were varied. The size of the complexes depended on the ionic strength of the media, with the strong electrostatic interactions in medium without NaCl making siRNA/dendrimer complexes smaller than those obtained in 150 mM NaCl. Both the intracellular delivery and the silencing of EGFP expression in cell culture was dependent on complex size, with smaller complexes efficiently delivered, and resulting in the highest silencing of EGFP expression. siRNA complexed with generation 7 dendrimers resulted in the highest silencing of EGFP expression both in human brain tumour cell line T98G-EGFP (35%) and mouse macrophage cell line J-774-EGFP (45%) cells, in spite of having lower protection of siRNA against degradation with RNase A, showing the importance of formulation procedures on the efficiency of transfection [130].

4. Cationic lipids as non-viral vectors for siRNA and DNA delivery

4.1. Gene delivery by cationic lipids

Gene delivery (DNA transfection) with cationic lipids (Figure 7) dates back to 1987 when it was reported by Felgner et al. [131], and the term "lipofection" was coined. Small unilamellar

liposomes containing the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride (DOTMA) was reported to spontaneously complex DNA completely entrapping the DNA, and enhanced fusion with the cell membrane in vitro in cell cultures, resulting in efficient delivery and expression of the delivered DNA. The lipofection was 5-100-fold more effective than the commonly used transfection techniques at the time by either calcium phosphate or DEAE-dextran (diethylaminoethyl-dextran), depending on the cell line used [131]. Cationic lipids have polar and non-polar domains and thus are amphiphilic in nature, with three general structural domains: (a) a cationic hydrophilic head-group (positively charged). The head-group can carry a permanent positive charge as in quaternary ammonium groups, or can be protonated at the physiological pH 7.4, such as primary and secondary amine groups. There can be one cationic group per lipid molecule (monovalent cationic lipids) or more than one cationic group per lipid molecule (multivalent cationic lipids); (b) a hydrophobic domain covalently attached by a linker to the cationic head-group. This domain can be in the form of either alkyl chains (commonly 2 chains) of various chain lengths (with various oxidation states) or can be a steroid such as cholesterol; (c) the linker between the head-group and the hydrophobic domain [132, 133]. This linker controls the biodegradation of the cationic lipid and its stability under different conditions according to the type of chemical bonds (e.g. ester, ether, or amide). Each domain can be controlled to change a specific character of the cationic lipid, e.g. using a disulfide functional group as a bioresponsive linker [134] which is reduced in the intracellular environment by glutathione/glutathione reductase and enhance biodegradation characters of the lipid and decrease its cytotoxicity.

4.2. The cationic head-group

The cationic head-group's main function is to bind electrostatically the negatively charged phosphates of the polynucleotides. The complexes of cationic lipids with polynucleotides such as DNA and siRNA are called lipoplexes. This requires the presence of a positive charge on the head-group at the physiological pH 7.4, i.e. the pKa of the head-group is ideally at least one unit higher than the physiological pH. The most commonly used head-groups contain nitrogen (e.g. amines or guanidines). However other head-groups, e.g. arsonium and phosphonium have been reported [135]. Arsonium is less toxic than arsenic (III), and in vitro cytotoxicity evaluation showed that arsonium and phosphonium are surprisingly less toxic than the ammonium group [135, 136].

One property that can be changed by controlling the type of the head-group is the head-group cross-sectional area. The greater the difference between the cross-sectional area of the polar head-group and that of the hydrophobic domain, when the former is designed to be smaller than the latter, the greater is the ability of the cationic lipid to fuse with the cell membrane and endosomal membrane and the greater is the release of polynucleotides from their complex with the cationic lipid due to the decreased structural stability of the lipid assembly [133, 137]. The hydration of the head-group affects its cross-sectional area, thus, the conjugation of groups which decrease the hydration state (such as hydroxyalkyl groups that form intermolecular H-bonds) decreases the head-group cross-sectional area.

Thus, gene delivery by DOTMA and DOTAP (1,2-dioleoyloxy-3-(trimethylammonio)-propane) was enhanced by incorporation of a hydroxyethyl group to yield the lipids DORIE

(1,2-dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide) and DORI (*N*-[1-(2,3-dioleoyloxy)propyl-*N*-[1-(2-hydroxy)ethyl]-*N,N*-dimethyl ammonium iodide) respectively [138, 139]. The head-group cross-sectional area can be also controlled by subtle changes to the head-group structure. The DC-Chol (3β(*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl)cholesterol) with dimethylamino head-group resulted in more efficient transfection compared to DC-Chol with diethylamino or diisopropylamino head-groups, probably due to increased steric repulsion of the head-groups.

The in vitro gene transfer with six non-cholesterol-based cationic lipids (each having two alkyl chains) with a single guanidinium head-group in Chinese hamster ovary (CHO), COS-1, MCF-7, A549, and HepG2 cells has been reported [140]. These lipids were able to form lipoplexes with size-range 200-600 nm and ζ-potential +3.4 to -34 mV. The efficiencies of the lipids which had an extra quaternized cationic centre were 2-4-fold more than that of the commercially available reagent Lipofectamine in transfecting COS-1, CHO, A-549, and MCF-7 cells. MTT viability assay in CHO cells showed high (>75%) cell viabilities at the lipid/DNA charge ratios used. DNase I protection assays showed that the lipids having the extra quaternized centre protected DNA better against enzyme catalysed hydrolysis. These results shed light on the importance of choosing the type of head-group and number of cationic centres in designing cationic lipids [140].

A series of cationic cholesterol derivatives were synthesized by covalently attaching the heterocycles imidazole, piperazine, pyridine, and morpholine groups (the head-groups) to the parent cholesterol via a biodegradable carbamoyl linker [141]. These lipids were compared with the parent DC-Chol with the linear amine head-group, and they generally showed better or comparable transfection efficiency of pCMV-luciferase into human HepG2 cells (a human liver cancer cell line) in the presence or absence of FCS. The most efficient two of these lipids were with morpholine and piperazine head-groups, and they gave higher levels of gene expression in HepG2 and human melanoma cell line (KZ2) which are generally very hard to transfect with the commonly used reagents e.g. DC-Chol, Lipofectamine, and PEI. In vivo studies with lipids having morpholine and piperazine head-groups resulted in successful delivery of the reporter gene to the target cells through intrasplenic injection [141]. Cationic lipids which have more than one cationic head-group (multivalent cationic lipids) have more surface charge density than their monovalent (with one head-group) analogues, and they are generally expected to better bind and complex polynucleotides. Many multivalent cationic lipids contain a natural occurring polyamine such as spermidine and spermine, which are believed to interact with the minor groove of B-DNA [142].

The triamine spermidine and the tetramine spermine (Figure 6), and their diamine precursor putrescine, are organic polycations that are widely but unevenly distributed in both mammalian and non-mammalian cells and tissues. They have an essential role in controlling DNA, RNA and protein synthesis during normal and neoplastic growth, in cell differentiation, and tissue regeneration [143]. These polyamines exhibit many metabolic and neurophysiological effects in the nervous system, and are important for the developing and mature nervous system and affect modulation of ionic channels and calcium-dependent transmitter release [143-149].
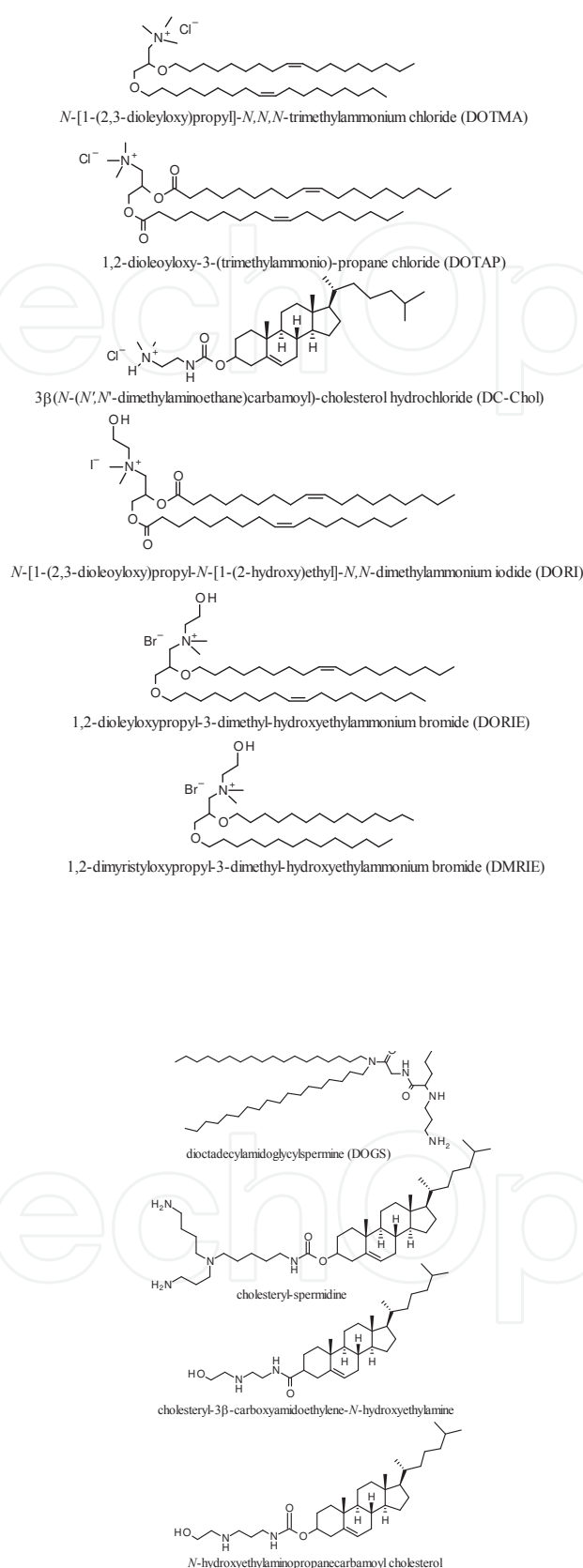
N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)

1,2-dioleoyloxy-3-(trimethylammonio)-propane chloride (DOTAP)

3β(N-(N',N'-dimethylaminoethane)carbamoyl)-cholesterol hydrochloride (DC-Chol)

N-[1-(2,3-dioleoyloxy)propyl-N-[1-(2-hydroxy)ethyl]-N,N-dimethylammonium iodide (DORI)

1,2-dioleyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (DORIE)

1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (DMRIE)

dioctadecylamidoglycylspermine (DOGS)

cholesteryl-spermidine

cholesteryl-3β-carboxyamidoethylene-N-hydroxyethylamine

N-hydroxyethylaminopropanecarbamoyl cholesterol

Figure 7. Representative examples of cationic lipids used in DNA and siRNA delivery.

Spermine is incorporated in the cationic polymer polyspermine imidazole-4,5-imine (PSI) and in dioctadecylamidoglycyl-spermine (DOGS) [150] (Figure 7); spermidine is bound in cholesteryl-spermidine [151]. The free amine groups of spermine in cholesteryl-spermine conjugates have different pKa values and provide a buffering effect in the endosomes facilitating the escape of lipoplex from the endosomes [152]. The length of the linear polyamine attached to the hydrophobic domain and the charge distribution on it affects the transfection efficiency of the cationic lipid [153]. Addition of amine groups separated by methylene groups to the linear polyamine attached to a cholesterol residue did not automatically increase transfection efficiency regardless of the increased charge density. Molecular modelling simulations suggested that increasing chain length led to an increased number of folded conformations due to greater flexibility of the conjugates, which is unfavourable for interaction with DNA [132, 153].

The central tetramethylene motif of spermine was reported to be essential in conferring high transfection efficiency in a series of cholesterol-polyamine conjugates [152]. It was suggested that the tetramethylene segment of spermine can bridge between the DNA complementary strands, while the polyamine with a central trimethylene segment would only bind with the adjacent phosphates on the same DNA strand. These results point to the importance of the structure of the polyamine head-group and the relation between its amine groups, and also point to the fact that increasing efficiency of transfection is not only a matter of increasing the number of positive charges per head-group.

4.3. The hydrophobic domain

The length, saturation state and type of the hydrophobic chains conjugated to cationic lipids affect their transfection efficiency [154-156]. Although these factors were studied extensively for the effect on transfection, and although the majority of studies accepted that the type of alkyl chains influence the outcome of transfection, it is difficult to set a definitive set of rules to describe the best type of alkyl chains to be conjugated to the polar head-groups. The contribution of the alkyl chains (and the hydrophobic domain) to the cationic lipid properties as a whole is what determines the transfection efficiency of the lipid.

Results obtained with DMRIE (1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl-ammonium bromide) [157], glycine betaine conjugates [138] with two alkyl chains, alkyl acyl carnitine esters having chains of length C12 to C18 [158], lactic acid conjugates of *N,N*-dialkyl amine group [159], lipids related to DOTAP with two alkyl chains (C12-C18) linked to the head-group through ether bonds [160], and cationic lipids with different hydroxyethyl or dihydroxypropyl ammonium backbones and esterified hydrocarbon chains and hydroxyl substituents [161] showed that a comparison of the cationic lipids based only on the lengths of the two saturated aliphatic chains led to the observation of the superior transfection efficiency of C14 chains over the longer C16 and C18 chains [132, 133]. It was proposed that a shorter chain length facilitates mixing with cellular membranes [138] which is important for endosomal escape [162].

In another set of experiments, we showed the longer chain C18 oleoyl (with one *cis*-double bond) to be more efficient than cationic lipids with shorter chain lengths. Varying the chain length in N^4,N^9-diacyl spermines from C10 to C18, for plasmid DNA delivery, resulted in us establishing that the conjugate with C18 oleoyl chains is both more efficient and less toxic than

the shorter chain conjugates [163]. A series of multivalent Gemini-surfactants with the hydrophobic chains systematically varied resulted in the conjugates with C18 oleoyl chains to be better in transfection than the C16 and C14 alkyl chains [164]. Chain saturation was also shown to affect the efficiency of transfection. The results of studies on a set of cationic triester phosphatidyl choline derivatives (each having two alkyl chains) show a strong dependence of their transfection efficiency on the lipid hydrocarbon chain characteristics, where transfection activity increases with increasing chain unsaturation from fully saturated to having two double bonds. Transfection efficiency decreased with increasing chain length (increasing the total number of carbons per lipid molecule ~30-50). Maximum transfection was with monounsaturated myristoleoyl 14:1 chains [156]. The data obtained from transfection experiments with 20 cationic phosphatidylcholine (PC) derivatives show that hydrocarbon chain variations results in transfection efficiencies that varies by more than 2 orders of magnitude. The most important variables were chain saturation state and total number of carbon atoms in the lipid chains. Transfection increased with decreasing chain length and increasing chain unsaturation. Best transfection efficiency was found for cationic lipids with monounsaturated (myristoleoyl) 14:1 chains [154]. Higher levels of transfection were also reported with lipids having oleoyl chains in comparison with stearoyl chains [157, 158]. Unsaturated chains promote lipid fusion between the lipoplexes and the various cellular membranes, which is essential for delivery and endosomal escape [133, 154, 165].

Cholesterol derivatives with various cationic head-groups were synthesized to investigate their efficiency as siRNA delivery vectors. The transfection efficiencies of siRNA lipoplexes prepared with the cationic cholesterol derivatives DC-Chol, cholesteryl-3β-carboxyamido-ethylene-N-hydroxyethylamine (OH-Chol), and N-hydroxyethylaminopropane carbamoyl cholesterol (HAPC) was investigated in human prostate tumour cells that stably express the luciferase gene (PC-3-Luc). When lipoplexes were prepared in water, HAPC was more effective in knocking-down luciferase activity than OH-Chol and DC-Chol [166]. The presence of NaCl while preparing the lipoplexes increased the gene silencing efficiency of luciferase, while it did not affect efficiency of HAPC. The commercially available transfection reagent, Lipofectamine 2000 (a cationic lipid liposomal preparation) resulted in strong gene silencing by siRNA, but exhibited increased toxicity (~40% cell viability), in contrast to OH-Chol, DC-Chol, and HAPC lipoplexes (~80–100% cell viability). These results indicated that siRNA lipoplexes prepared with OH-Chol, and HAPC can efficiently suppress gene expression without increased cytotoxicity [166].

4.4. The linker

The linker is dependent upon the type (hence properties) of the functional group and its length (number of carbon atoms). The linker has two main functions: (a) to conjugate covalently the polar head-group to the hydrophobic domain; (b) to control the biodegradability of the cationic lipid and/or introduce a new property to the cationic lipid, e.g. responding to the intracellular reducing environment [133, 167]. The most commonly used linker functional groups are: amide, carbamate, ester, ether, ortho ester, and disulfide.

Both amides and ester bonds are biodegradable and hence are hypothesized to be less toxic than other non-biodegradable bonds (e.g. ethers) [168]. Lipids with a pyridinium head-group (with palmitoyl 16:0 hydrophobic domains and with ester and amide linkers) were used to prepare liposomes with either DOPE or cholesterol at the cationic lipid/helper-lipid molar ratio of 1:1. Following transfection of CHO cells with lipoplexes delivering plasmids expressing EGFP, the cationic lipids having amide linkers significantly increased transfection efficiency in all liposomal formulations compared to their counterparts having the ester linker [169]. The high transfection efficiency of lipids with amide linker was suggested to be due to their lower phase-transition temperature which makes the liposome's bilayer structure more stable in aqueous media during the transfection process as well as liposome storage. The phase-transition temperature of a lipid is the temperature at which there is a change in the lipid's physical state from the ordered gel phase (where the hydrocarbon chains are closely packed and fully extended) to the disordered liquid crystalline phase (where the hydrocarbon chains are fluid and randomly orientated) [169].

Depending on the structure of the cationic lipid, the linker influence on transfection efficiency can be more than on cytotoxicity. Cholesterol-based cationic lipids that have different nitrogenous heterocyclic head-groups (*N*-methylimidazole, *N*-methylmorpholine, and pyridine) and acid-labile linkers (carbamate, ester, and *N,O*-acetal ether) were used to transfect human embryonic kidney 293 (HEK 293) cells with EGFP plasmid [170]. Choosing those linkers was based on the concept that incorporation of acid-labile bonds in the cationic lipid structure enhances the release of polynucleotides from the endosomes, therefore increasing the transfection efficiency [171]. *N,O*-Acetals are known to undergo hydrolysis in acidic environment [170, 171]. The results showed that the structure of these lipids only slightly affected their cytotoxicity but largely changes the efficiency of intracellular accumulation of the polynucleotides. The lipids having the cationic head-groups pyridine and/or methylimidazole head-groups with either an ester or a carbamate linker resulted in better transfection efficiency as compared with the cationic lipids with either the *N*-methylmorpholine head-groups and/or an ether linker. The lipid that has a pyridine head-group and a carbamate linker to deliver EGFP plasmid resulted in comparable transfection efficiency with that achieved with commercially available Lipofectamine 2000.

Two cleavable cationic lipids having a linear or a cyclic ortho-ester linker between the cationic head-group and the unsaturated hydrophobic domain (two oleoyl chains) were previously reported [172]. It is hypothesized that the acidic pH in the endosomes catalyzes the hydrolysis of the linker group to result in fragmentation products that destabilize the endosomal membranes. At pH 7.4, the lipids (with DOPE) formed stable lipoplexes with plasmid DNA. Decreasing the pH enhanced the hydrolysis of the ortho ester linkers which removed the cationic head-groups and caused lipoplex aggregation. At pH 5.5, the cationic lipid *N*-[2-methyl-2-(1',2'-dioleylglyceroxy)dioxolan-4-yl]methyl-*N,N,N*-trimethylammonium iodide that have a cyclic ortho-ester linker showed increased pH-sensitivity and caused the permeation of its lipoplexes to model biomembranes within the time span of endosomal processing before the lysosomal degradation. This lipid markedly increased gene transfection (~3-50-fold) of the luciferase reporter protein in monkey kidney fibroblast (CV-1) and human breast cancer (HTB-129) cells in culture compared to the pH-insensitive control lipid DOTAP lipoplexes [172].

Transfection with DNA lipoplexes of three thiocholesterol-derived gemini cationic lipids possessing disulfide linkages incorporated between the cationic head-group and the thiocholesterol backbone in order to render the lipids biodegradable has been reported [173]. Comparing transfection in a prostate cancer line (PC3AR) and a human keratinocyte cell line (HaCat) with two commercially available reagents showed comparable or better expression of GFP in the transfected cells. Cytotoxicity studies showed the nontoxic property of these lipid-DNA complexes at different N/P ratios used for transfection studies. The rationale behind this design was to ensure the destabilization of the lipid-polynucleotide lipoplexes in the cytoplasm after reduction of the disulfide linker by the intracellular glutathione (GSH), which is the most abundant low molecular weight thiol present in cells and is involved in controlling cellular redox environment. GSH is found at very high intracellular concentrations and at comparatively low extracellular concentrations e.g. blood plasma concentrations (2 μM) are 1000-fold less than concentration in erythrocytes (2 mM). This large difference between intra- and extracellular environments provides a potential mechanism for release of polynucleotides from lipoplexes of lipids that have a disulfide functional group linker and is now a well-trodden research path [115, 134, 173].

5. Conclusions and future avenues

In our research, symmetrical and asymmetrical acyl polyamine derivatives (fatty acid amides of spermine) [152] have been synthesized, characterized, and evaluated as non-viral vectors for siRNA [163, 174-177]. The intracellular delivery of siRNA and the subsequent sequence specific gene silencing has been quantified by flow cytometry techniques (FACS analysis) [163]. The ability of the spermine conjugates to bind siRNA and form nanoparticles has been investigated and the effect of the complexes of siRNA lipoplexes on the cell viability 48 h post-transfection has been quantified. Our SAR studies allow the identification of the most efficient fatty acids in terms of high gene-silencing efficiency and high cell viability [174-178].

Whilst we were completing this Chapter, four interesting papers, each one on a different aspect of this topic, were published. Langer, Anderson and co-workers at MIT reported on the delivery of immunostimulatory RNA (isRNA) to Toll-like receptor (TLR)-expressing cells to drive innate and adaptive immune responses. The specific activation of TLRs has potential for a variety of therapeutic indications including antiviral immunotherapy and as vaccine adjuvants. Effective lipidoid-isRNA nanoparticles, when tested in mice, stimulated strong IFN-α responses following subcutaneous injection, had robust antiviral activity that suppressed influenza virus replication, and enhanced antiovalbumin humoral and cell-mediated responses when used as a vaccine adjuvant. Their lipidoid formulations, designed specifically for the delivery of isRNA to TLRs, were superior to the commonly used N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate-RNA delivery system and may provide new tools for the manipulation of TLR responses in vitro and in vivo [179]. This paper follows after their other recent major contribution on delivering naked siRNA as part of a self-assembled (due to DNA complementarity) tetrahedral nanoparticle construct considering the presentation of folate as a cancer targeting ligand [180]. These monodisperse nanoparticles of

essentially naked DNA, carrying siRNA as the cargo, have a defined size of only a few nm. They show that at least three folate molecules per nanoparticle are required for optimal delivery of the siRNA into cells and that gene silencing only occurs when the ligands are appropriately orientated. In vivo, these naked DNA nanoparticles showed a longer blood circulation time than the parent siRNA [180]. In another exciting development, Geall and co-workers at Novartis have also advanced the field of nucleic acid vaccines by taking advantage of the recent innovations in non-viral systemic delivery of siRNA using lipid nanoparticles (LNPs) to develop a self-amplifying RNA vaccine. This technology elicited broad, potent, and protective immune responses, comparable with those achieved by a viral delivery system, but without the inherent limitations of viral vectors [181]. Even today, a biologically responsive cationic polymer system based on spermine has been reported for the intracellular delivery of siRNA [182]. This polyspermine imidazole-4,5-imine (PSI) (Figure 7) carrier is designed to be hydrolysed at the mildly acidic pH found in the endosome.

It is clear that both ssRNA to activate the immune system and RNAi brought about by siRNA delivery have high therapeutic potential. The major remaining barrier, that of efficient and potentially selective delivery to target cells in now being addressed. The non-viral delivery of siRNA is a major tool in modern functional genomics. Medicines design, the formulation of drugs, in this case siRNA and plasmid DNA, is an essential requirement for efficient gene therapy.

Acknowledgements

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