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Therapeutic Targeting in the Silent Era: Advances in non-viral siRNA Delivery

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Abstract

Gene silencing using RNA-interference, first described in mammalian systems almost a decade ago, is revolutionizing therapeutic target validation efforts both *in vitro* and *in vivo*. Moreover, the potential for using short interfering RNA (siRNA) as a therapy in its own right is also progressing at a significant pace. However, the widespread use of such approaches is contingent on having appropriate delivery systems to achieve clinically appropriate, safe, and efficient delivery of siRNA. There are many physicochemical and biological barriers to such delivery, and a growing emphasis on the design and characterisation of non-viral technologies that will overcome these barriers and expedite targeted delivery. This review discusses the considerations and challenges associated with use of siRNA-based therapeutics, including stability and off-target effects. Speculation is made on the properties of an ideal delivery system and the non-viral delivery approaches used to date, both in vitro and in vivo, are classified and discussed. Moreover, the ability of cyclodextrin-based delivery vectors to fulfil many of the criteria of an ideal delivery construct is also elaborated.

Introduction:

"Silence is the perfectest herald of joy"

William Shakespeare (1564 - 1616), "Much Ado about Nothing", Act 2 scene 1

The promise of gene therapy-based medicine is slowly being realised and heralding much joy to patients suffering from a host of hitherto incurable disorders. The goal of gene therapy, is to either silence aberrant disease causing genes, amplify the functions of other genes or interfere with the expression of infectious agents or malignant cells[1]. Among those most widely used have been the introduction of plasmid DNA and the use of antisense technology. The discovery of gene silencing by RNA interference (RNAi) has revolutionarised this area and presents new opportunities for the successful achievement of gene-based therapies. Moreover, modern biomedical research heavily depends on genomic and proteomic strategies that help uncover disease mechanisms and detect potential target genes relevant to the disease pathology[2]. Therapeutic targets identified from the use of such high-throughput technologies warrant comprehensive *in vivo* validation of their aetiological role in the disease[3]. Gene silencing by RNAi thus offers itself as very suitable ways to identify how a system functions with the given gene abrogated. However, the future success of RNAi-based approaches is dependent on the design of efficient and effective delivery technologies. Design of delivery systems has been greatly informed by earlier experiences with delivery of other nucleic acids such as plasmid DNA (pDNA), oligonucleotids, ribozymes and DNAyzmes. Although there are some intrinsic differences between delivering each of these candidates, they also share many common characteristics. This review highlights the synthetic material-based delivery approaches in the rapidly developing area of short interfering (siRNA) therapy and details the barriers to, and the methods currently used to deliver siRNA both *in vitro* and *in vivo*.

The RNAi pathway and siRNA

RNAi is a process in which double-stranded RNA (dsRNA) inhibits gene expression in a sequence dependant manner through degradation of the corresponding mRNA. Fire *et al.* 1998[4] described the use and potency of dsRNA, first seen and investigated in plants[5] , to elicit changes in gene expression in the nematode *Caenorhabditis elegans*. This work led to Andrew Fire and Craig Mello being awarded the Nobel Prize in 2006. The first demonstration in 2001 that siRNA duplexes could be used in mammalian cells to suppress gene expression[6], provided a new tool for studying gene function in mammalian cells and that may eventually be developed into gene-specific therapeutics. Since these initial reports of RNAi

have emerged, it is hoped that this system can be exploited to develop a new class of drugs and numerous review articles have been published focusing on the evolution of RNAi as a therapeutic strategy[7-9].

RNAi is a mechanism that has been evolutionarily conserved among species (protozoa, fungi, plants and animals) and plays an innate immune security role in defending harmful effects of invasive nucleic acids from viruses, transposable elements and other pathogens, i.e. bacteria[10, 11]. RNAi pathways are maintained by small RNAs that mainly include microRNA (miRNA) and siRNA (see Figure 1 a pictorial representation of the major compants of the RNAi pathway). miRNA induces translational suppression and transcript deprivation for imperfectly complementary messenger RNA (mRNA), whereas siRNA mediates gene knockdown via sequence-specific cleavage of perfectly matching targets [7].

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Insert Figure 1 about here

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It is possible to utilize the RNAi mechanism for siRNA in three ways, including: A) the introduction of a plasmid or viral-based vectors to the nucleus in order to express shRNA; B) the introduction of shRNA into the cytoplasm to allow dicer processing and subsequently get loaded into the RISC; C) the introduction of synthetic siRNA into the cytoplasm to directly allow RISC loading (see Figure 1).

Viral-based vector approaches have largely dominated clinical trials in gene therapy to date due to their relatively high transfection efficiency[12]. However, such gene carriers have many potential side effects, such as immunotoxicity and mutagenesis[12]. The recent case, in which a retroviral vector's site of insertion into the genome appears to have activated a cancer-causing gene, has come further endorsed the search for efficient non-viral delivery technologies[12].

While the molecular understanding of the RNAi pathway is progressing, and new therapeutic targets for the treatment of a wide range of diseases are continuously identified, none of these therapies can be put into practice until efficient delivery of siRNA is achieved.

Challenges and considerations associated with the use of siRNA

Naturally occurring small RNAs exist in a relatively perfect equilibrium with their precursors and targets, undertaking evolutionary conserved cellular functions[13]. A variety of harmful/unexpected consequences may result from the non-optimized siRNA candidates, as this may disturb the existing

balance in the endogenous small RNA processed pathways within the cells[13, 14]. Therefore, there are a number of considerations that must be taken into account in overcoming the challenges for siRNA knockdown including minimising off-target effects including immunogenicity, and increasing the stability of synthetic siRNAs in the physiological milieu.

Avoiding Off-target Effects

The goal of siRNA-based gene therapy strategies is that it silences only the gene of interest. However, a number of important factors need to be taken into account when selecting siRNAs to ensure that specificity is achieved. There are two major classes of off-target effects described for siRNA including[13, 14], these being, i) non-specific silencing resulting from binding of siRNA to sequences other than the specific target sequence, and ii) initiation or enhancement of the innate immune response by some motifs or patterns in the siRNA. Efforts to overcome such non-specifity are driven at many levels. Based on the secondary structures of siRNAs/targets and the nucleotide sequences of siRNAs/targets, many computational methods have been developed to seek siRNA sequences that are able to silence the expression of their complementary genes[15-18]. BLAST (Basic Local Alignment Search Tool) is one of the most commonly used bioinformatics programmes and provides identifications of target sequences. Genome-wide expression analysis, i.e., by microarray technology, can afterwards be used to confirm, validate and further refine the algorithms[19]. However, it has been reported that siRNA with as few as 11 nucleotides homology can lead to off-target knockdown[20]. Birmingham et al.[21] reported that off-target effects mostly occur due to 3'-UTR seed matches, but not overall homology between siRNA and its target. Moreover, it has been reported that siRNA-induced toxicity can be promoted by a preponderance of specific motifs such as –UGGC- and other –AU- rich pentamers including -AUUUG, GUUUU, AUUUU, CUUUU, UUUUU, GUUUG-[22] thus use of such motifs should be minimized if possible.

One way to reduce off-target effects is by means of nullifying the formation of siRNA-sense-RISC and enhancing the incorporation of siRNA-antisense-RISC[23, 24]. As the 5'-phosphate group is essential for the siRNA strand to act as a guide strand, modification of the 5'-phosphate group of the sense strand to a 5'-*O*-methyl, e.g., can effectively avoid sense-RISC incorporation[23]. Moreover, position-specific and sequence-independent chemical modifications such as 2'-*O*-methyl ribosyl substitution in the seed region (2-8 nucleotides within the 5' end of the guide strand) of antisense strands of the siRNA can also reduce off-target effects[24].

The innate immune response can be triggered as siRNA is also exposed to endosomal and cytoplasmic RNA-sensing receptors of the immune system while being transfected into cells. An initial study demonstrated that dsRNA (double stranded RNA) with longer than 30 bases can induce recognition of

serine/threonine protein kinase receptor (PKR-a cytosolic RNA-binding receptor)[25]. Activation of PKR is considered to be responsible for virus- and dsRNA-induced production of type I interferons (IFNs) found in most mammalian cell types. In some instances, siRNAs can also trigger this pathway[26], potentially leading to non-specific effects and toxicity from the ensuing inflammatory response. Moreover, as siRNA can possess the same molecular signatures as viral RNA, i.e., unmethylated deoxycytidyl-phosphate-deoxyguanosine (CpG) dinucleotides, which are prone to induce an immune response via toll-like receptor (TLR) activation. TLRs are a family of receptors which act on the cell surface to detect pathogen-specific molecules [9, 13, 14]. siRNA[27, 28] can activate TLR 3 and TLR 7/8 [29, 30] and produce pro-inflammatory cytokines upon stimulation. TLR 7/8 activation, in some cases, is sequence-dependent as immuno-stimulatory siRNA sequence motifs, such as 5'-UGUGU-3'[31] or a 9-nt bases 5'-GUCCUUCAA-3' have been identified which activate this receptor[30]. In addition, the immune response can be also induced by many other sequences, which do not contain any GU bases and dsRNAs with high uridine motifs[32].

Certain approaches have been suggested to alleviate the undesired off-target effects and decrease the immune responses. These include[13, 14]:

a) avoiding the use of immuno-stimulatory sequence motifs and high uridine content
b) nullification of the immune response by introducing modifications (like 2'-deoxy, 2'-*O*-methyl, 2'-fluoro and etc.) in the nucleotides and/or their backbone while maintaining silencing activity[32, 33]
c) ensuring that siRNA has a 2-nt 3' overhang, as siRNAs without such overhangs activate the interferon system [34].

Moreover, synthetic siRNAs have recently reported to saturate some components of the cellular RNAi pathway, resulting in a limited capacity to assemble the RISC complex on exogenous siRNAs[14, 35]. It is suggested that considering of the dose of siRNA is employed, which should be as low as possible to preclude saturation of some factors in the RNAi pathway, like Exportin-5, leading to dysregulation of critical mechanisms involved in the endogenous RNAi pathway[14, 35].

Increasing stability of siRNA via chemical modifications

One of the main issues that must be addressed before the therapeutic promise of siRNA therapy can be completely fulfilled is the stability of the siRNA in the appropriate bodily fluid, with plasma/serum being the most widely studied given the propensity for degradation of the siRNA by serum nucleases[9]. Increasing efforts have been focused on enhancing siRNA stability by a variety of chemical modifications whilst not compromising silencing ability *per se*. Certain chemical modifications can be

introduced almost all bases of both RNA strands, whereas other specific modifications must be placed only at certain positions within the siRNA strands[36]. For example, stability against nuclease degradation can be achieved via the introduction of a phosphorothioate (P=S) backbone linkage at the 3' end for exonuclease resistance and a 2' modifications (2'-*O*-methyl and 2'-fluoro) for endonuclease resistance[37-40]. In relation to maintaining RNAi silencing activity, exonuclease-stabilizing modifications are well tolerated. Many studies have indicated that the introduction of P=S linkages into siRNA does not alter the silencing activity of siRNAs[37, 41, 42]. Although substitution of P=S oligonucleotides is the most common stabilizing modification for both antisense and siRNA, P=S oligonucleotides tend to bind nonspecifically to proteins, thus increasing the risk of toxicity[43]. However, most current siRNA designs now only include a few P=S linkages thus minimising the risk of protein binding. Moreover, it has been demonstrated that boron phosphorus (BO) linkages could similarly enhance gene silencing activity at lower concentrations and improve resistance to degradation by nucleases compared with unmodified siRNA[44].

Other modifications that have received a lot of attention are the 2'-*O*-methyl (Me), 2'-*O*-methoxyethyl (MOE) and 2'-fluoro (F) modifications which demonstrate high specificity in addition to protecting against nuclease degradation[23, 37]. Introduction of internal sugar modifications that are resistant to endonucleases is also generally well tolerated but is dependent on the position of the modification within the duplex: the sense strand being more apt to modification compared with the antisense one. The incorporation of 2'-*O*-Me and 2'-*O*-MOE in the sense strand of siRNA does not appear to have a significant effect on activity[45]. The siRNAs with modified residues at the 5' end of the antisense strand seem to be less active than those modified at the 3' end. The 2'-F residue is commonly well tolerated on the antisense strand, whereas the 2'-*O*-MOE modification leads to loss of activity irrespective of the location in the construct.

Other highly enhanced oligonucleotide chemistries have also been developed, such as 2'-OH modifications, peptide nucleic acids (PNAs), morpholino compounds, locked nucleic acids (LNAs) and hexitol nucleic acids (HNAs)[46, 47]. Of these LNAs, a family of conformationally locked nucleotide analogues, have by far the most promise as they are bestowed with very high affinity nuclease resistance to DNA and RNA oligonucleotides[166]. Studies in the antisense field over the past decade have shown that LNAs combine substantially increased potency *in vitro* and *in vivo* with minimal toxicity over other chemistries[167]. PNAs on the other hand are neutrally charged molecules, solubility and cellular uptake are serious problems that have to be addressed for the widespread application of PNAs as antisense agents[46]. Moreover, the target affinity of morpholino compounds is generally lower than the strength of RNA binding achieved with many of the other modifications[46]. With recent progresses in nucleotide

chemistry, these modifications described in this section may stabilise the siRNA duplex against nuclease degradation and last longer than naked siRNAs when they are formulated into various carrier systems for *in vivo* delivery[48].

Challenges and considerations associated with the delivery of siRNA

Once siRNA has been refined and synthesised to have a high specificity of action and be non-immunogenic, the next major and perhaps the most important obstacle to its use as a therapeutic agent, or in therapeutic target validation research efforts, is how to achieve efficient and specific delivery of the siRNA. While much research has gone into the development of viral vectors for use as delivery carriers for siRNA therapies, as alluded to earlier there are a number of safety issues associated with their use in humans[12]. The immune response to viral-based vectors limits their application in repeated administration schedules. Moreover, the potential for mutagenesis may also cause unexpected consequences to the host[12]. Clinical trials involving non-viral-vector based gene therapies have dramatically increased from 2004 to 2007, while the number of viral vector trials is steadily dropping over the same period[49, 50]. Thus, we will focus our attention for the remaining part of this review on the significant advances and improvements in non-viral delivery technologies and how they can be applied to siRNA-based therapeutics.

When designing a drug delivery system for siRNA one of the first considerations to be taken is whether to develop a delivery formulation for systemic or local administration. Local delivery offers several advantages over systemic, including lower doses, ease of access, less potential side-effects and facilitation of site-specific delivery. Alternatively, systemic delivery via intravenous (i.v.) or oral administration is more applicable where the disease site is not easily accessible. Before the delivery can successfully occur however the barriers encountered by siRNA in the extracellular and intracellular environments must be addressed.

Extracellular barriers to siRNA delivery

Gene packaging. The negative charge (nearly 40 anionic charges) and size (two turns of a nucleic acid double helix) of siRNA suggests that it is unlikely to bind or cross the cell membrane unaided[51]. Much of our knowledge regarding gene packaging to facilitate siRNA delivery has come from decades of research focused on studying DNA delivery aided by cationic non-viral vectors. Cationic materials can also bind to and condense siRNA into small, compact structures through electrostatic interactions between positive charges exposed on the vector material and the negative phosphates on the RNA backbone, thus spontaneously forming polyplexes[48]. The resulting particles are capable of protecting

siRNA from nucleolytic enzymes by steric blocking, thereby prolonging the half-life of siRNA substantially. In addition, complete siRNA encapsulation is also capable of avoiding the recognition of TLR 3[27] which as stated earlier is an important facilitator of off-target immune activation.

Serum stability. Research on cationic polyplexes has illuminated the potential drawbacks that hinder their successful *in vivo* application[48]. The major stability problems encountered with siRNA complexes are aggregation and decomposition. The serum stability of the siRNA-vector constructs depends on the polymeric structure, as well as on the overall zeta potential (surface charge of polyplexes in colloidal systems). As unmodified neutral polyplexes quickly form large aggregates in physiological salt environments, they are generally ineffective gene-delivery agents and can even be toxic *in vivo* due to embolization of the aggregations in the lung[48, 52]. It is of important to note that as positively charged polyplexes can adsorb serum albumin and other anionic proteins, aggregation of clot-like accumulations in the blood can occur[53]. These large masses subsequently are either entrapped in the lung endothelial capillary bed or taken up by reticuloendothelial system (RES)[54].

One of the best characterised methods to overcome uptake by RES is the addition of polyethylene-glycol (PEG). The process of PEGylation was first described in the 1970s[55, 56]. This polymer is highly soluble in aqueous solution and is approved for use by the Food and Drug Administration (FDA) as a result of its non-toxic, non-immunogenic and non-antigenic properties[57]. It has since been used extensively, to improve the pharmacokinetic and pharmacodynamic properties of drugs including biologicals, and as a component in gene delivery constructs[57-60]. PEGylation is generally used to increase the water solubility of molecules, prolong their systemic circulation, limit toxicity in addition to avoiding uptake by the RES[57-60]. siRNA-based carrier systems can escape from RES when they have a diameter less than 100nm and a neutral surface charge or if the particle surface is protected by polymers PEG[54]. When grafted onto the polymer as a ‘brush’, PEG can shield positively charged surfaces, thus stabilizing polyplexes against salt-, protein- and complement-induced inactivation[48, 54]. The charge shielding and steric effects provided by PEGylation result in reduced particle-particle and particle-protein interactions. There are, however, a few limitations in the use of PEG including polydispersity inside the body and excretion from the body[57]. Interestingly it has been shown that whilst PEGylation conferred salt stability to particles it also produced a reduction in gene expression[61], possibly resulting from significant affects of PEGylation on cellular uptake and intracellular trafficking of non-viral gene delivery particles. Moreover, it should be noted that there is accumulating evidence suggesting that the PEGylated component of liposomes (vesicles with a water compartment entrapped in a phospholipid bilayer), maybe immunogenic in its own right and promote antibody, especially IgM, responses against a second dose of such liposomes. This suggests that any PEGylated liposomal formulation may display

unexpected pharmacokinetic behavior upon repeated injection and, as a consequence, may show less therapeutic efficacy or even cause undesirable side effects[62].

Cell-specific targeting. Polypolexes normally do not have the capacity for cell-specific targeting, especially after PEGylation, due to the shielding of the cationic surfaces. However, PEG allows for flexible chemistry to enable the attachment of targeting moieties that allow both cell specificity and increased cell uptake[48, 58]. The detection of receptors overexpressed in certain tissues or disease states lends itself to the design of a targeted delivery system. Conjugating targeting proteins such as antibodies, which have high specificity for their corresponding antigens, to the delivery vector has been shown to be an effective method to increase cellular uptake of siRNA both *in vitro* and *in vivo*[63-65]. Oh and colleagues[66] have shown that the aminopeptidase P antibody specifically targeted nanoparticles to the caveolae of rat lung endothelium, thus providing a novel targeting delivery system for siRNA. On the other hand, Kumar *et al.*[67] reported the T cell specific delivery of anti-HIV siRNA in a mouse model. Treatment with siRNA conjugated to a peptide and an anti-CD7 antibody ensured selected delivery of the siRNA and led to suppression of the virus and prevention of the loss of CD4 T cells[67].

Integrins, adhesion molecules that bind to components of the extracellular matrix and act as sensor/signalling molecules, can also be used as targeting moieties[68-70]. In particular, the integrin lymphocyte function-associated antigen-1 (LFA-1) expressed on all leukocytes, which mediates heterotypic adhesion to intercellular adhesion molecules on other immune cells, undergoes remarkable conformational changes in response to stimulation[68]. Peer *et al.*[68] utilised this approach to target siRNAs to primary leukocytes, more specifically those expressing the high affinity adhesive form of LFA-1 as opposed to the low affinity non-adhesive LFA-1 found on naive cells and thus interrupting unwanted pathogenic immune stimulation. Intravenously injected siRNA complexes have been shown to selectively target K562 cells expressing the high affinity LFA-1 engrafted in the lungs of mice[68].

On the other hand, antibodies targeting the β7 integrin, found on the surface of inflammation associated helper T cells, were covalently attached to lipid-based nanoparticles and used to selectively deliver siRNA specific for the cyclin D (CyD1) (a cell cycle regulatory molecule) gene to the target cells to knock down the protein[69]. Following intravenous administration this approach resulted in a reduction of intestinal inflammation in a mouse model[69]. In a separate approach the efficacy of self-assembling nanoparticles containing siRNA and constructed from polyethylenimine (PEI), PEG and a peptide ligand to target integrins found in tumour neovasculature were investigated[70]. These were used to successfully target vascular endothelial growth factor-receptor 2 (VEGF-R2) and thus inhibit tumour angiogenesis and growth rate[70].

Another targeting ligand of particular interest is the transferrin receptor (TfR), which is overexpressed in many cancer tissues[71]. A number of different polymers have been modified to target transferrin including cyclodextrins[72] and liposomes[63, 64, 73]. Martlett and Davis[72] have shown activity of transferrin (Tf)-targeted siRNA-cyclodextrin particles against ribonucleotide reductase subunit 2 (RRM2) in a murine tumour model. Alternatively, PEGylated immunoliposomes modified with two monoclonal antibodies against the mouse TfR and the human insulin receptor were able to cross the blood brain barrier, via the TfR and once inside the compartment were able to enter brain tumour cells via the insulin receptor and resulted in a prolongation of survival[73].

Since its discovery, the folic acid receptor (FR) and its expression has been extensively characterised. There are five isoforms of the receptor, α, β, γ, γ' and δ. The α form of the receptor is upregulated in many forms of cancer including ovarian, lung, breast, kidney, brain and colon[74] while expression in normal tissues is minimal. The non-epithelial isoform FRβ is expressed on activated macrophages found in arthritic joints and a host of other inflammatory diseases such as, psoriasis, systemic lupus erythematosus and ulcerative colitis[74]. Zhang *et al.*[75] demonstrated the cell specific uptake of siRNAs which were noncovalently attached to folate-conjugated oligodeoxynucleotides (ONDs) *in vitro* to cells overexpressing FRα. Furthermore, FR-dependent silencing of αV integrin in human umbilical vein endothelial cells (HUVEC) and of survivin (an apoptosis inhibitor gene) in human nasopharyngeal carcinoma (KB) cells has been demonstrated[75]. An advantage to the use of folic acid is the relative ease with which it can be conjugated to both therapeutic and diagnostic agents [76].

The sigma receptor, a membrane bound protein showing high affinity for antipsychotic drugs, although expressed on some normal tissues such as the brain, is overexpressed on a range of tumours including, melanoma, prostate and non-small cell lung tumours[77-80]. Banerief *et al.*[77] first demonstrated the use of the selective sigma ligand namely anisamide to mediate efficient targeting of liposomal drugs to sigma receptor-expressing prostate cancer cells *in vitro* and *in vivo*. Since then there have been several articles reporting the application of anisamide targeting nanoparticles for the cell specific delivery of therapeutic siRNA[78-80].

The asialoglycoprotein receptor is expressed on hepatocytes and is able to recognise the galactose units on the oligosaccharide chains of glycoproteins or on chemically synthesised galactosylated carriers[81, 82]. Thus siRNAs complexed with galactosylated cationic liposomes can be used as a specific targeting tool. For example, systemic administration of such complexes was directed specifically towards liver parenchymal cells and produced a knockdown of the endogenous gene Ubc13[81]. In the treatment of hepatitis C (HCV) it is essential that any treatment is specifically targeted to the liver. Lactosylated cationic liposomes intravenously administered to mice accumulated primarily in the liver throughout the

hepatic parenchymal cells. The inclusion of anti-HCV siRNA in the targeted liposomes led to a dose-dependent suppression of intrahepatic HCV expression[82].

Intracellular barriers to siRNA delivery

Endolysosomal escape. Having generated siRNA-delivery vectors which successfully overcome the abovementioned challenges, the complexes are ready for internalization by the cells of interest in which even further barriers await. Untargeted cationic polyplexes bind to the surface of cells via electrostatic interaction and are generally internalized through adsorptive pinocytosis (a process of taking in fluid together with its contents into the cell)[83]. Alternatively, polyplexes modified with targeting ligands bind to the specific cell-surface receptors and enter into cells by receptor-mediated endocytosis[84]. In either pathway, the polyplexes become entrapped inside the endososomes, which represent a hostile environment[84]. Once inside the early endosomes, the internalized material can be transported back to the membrane and out of the cell by exocytosis. More commonly however, polyplexes are considered to be transported to late endosomes in which the pH quickly drops to ~5-6. Polyplexes can subsequently be delivered into lysosomes in which the pH further decreases to ~4.5 and there are a variety of degradative enzymes awaiting[84]. Unprotected nucleic acids entrapped in these vesicles are eventually degraded. Only siRNA that escapes intact from the endosomes/lysosomes can arrive safely at the cytoplasm to enter into the RNAi pathway.

Several approaches have been employed to overcome the endo-lysosomal barrier. Chloroquine is a buffering agent known to disrupt the endosomal membrane by raising the pH of the endosome environment. Chloroquine when simultaneously added to cells at the time of transfection results in enhanced gene transfection with some polymers[85]. Although this strategy has been commonly used *in vitro,* it is impractical for *in vivo* gene delivery due to its toxicity[86]. Additionally, inactivated adenovirus particles tethered to polylysine achieved up to a 2000-fold gene transfer[87] possibly due to virus-induced endosomal escape. This method is also impractical *in vivo* due to the increased difficulty of preparing the vector and safety issues, especially immunogenicity and mutagenesis. Alternatively, fusogenic viral[88] or synthetic[89] peptides can be attached to various polymers to assist endosomal escape. These peptides are normally pH-triggered amphiphiles that undergo a structural change in the acidic environment and then burst the vesicle membrane. Finally, certain materials, such as PEI and polyamidoamine (PAM), can escape from endosomes by what has been referred to as the ‘proton-sponge hypothesis’ by which the H^+ buffering capacity of polyamines results in endosomal Cl^- accumulation during acidification resulting in osmotic endosome swelling and enhanced polyplex escape[90].

Gene-unpacking. Once released from the endosomal compartments, poleplexes must subsequently dissociate from the siRNA at some point in the cytoplasm for entering into the RNAi pathway. Much of our understanding of this process comes from investigations with plasmid DNA. Several studies have found that gene expression is improved by reducing the number of positive charges[91], conjugation of PEG chains[92], or decreasing the polymer molecular mass[93]. Therefore, synthetic vectors must clearly be designed to incorporate a mechanism for nonspecific or environmentally responsive release of siRNA.

Physiochemical characterization and formulation of delivery vehicles

Several physical factors, including the size, charge and ability to complex or encapsulate the siRNA, have been shown to influence the efficacy of non-viral delivery systems[48, 54]. The size of particles for use as delivery vectors can be modified during the formulation process. Small particles (with diameter less than 500nm, usually <150nm) have an enhanced permeation and retention (EPR) in inflammation sites and solid tumours due to the leaky vasculature and endothelial junctions in those tissues[54]. An additional vital characteristic of the delivery vector is the surface charge. As described earlier, a positive charge will promote serum instability and non-specific interactions with both target and non-target cells. As outlined previously, PEG is often used to shield cationic surface and additionally act to prevent aggregation of the delivery vehicles and improve *in vivo* circulation. Encapsulation efficiency of nanoparticles can be increased using detergent or ethanol dialysis[54]. Formation of nanoparticles can be controlled by adjusting the concentration, charge ratio, ionic strength of solutions and rate of mixing[54]. The ratio of lipid/siRNA used is also an important factor found to affect transfection by influencing the particle size of the constructs[54]. The aqueous medium in which the lipid/siRNA complex forms influences the degree of transfection[94]. By using OptiMEM medium rather than water for the dilution of liposomes and siRNA and their mixing, significantly improved gene knockdown was generated[94].

Taking all of the abovementioned considerations, both biological and physicochemical, into account one can speculate on the ideal properties of a non-viral delivery system. These are outlined in Figure 2 and include biological stability, nanosized particles and cell-specific targeting. The achievement of these properties is dictated by the selection of materials, the formulation method used and the eventual therapeutic application.

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Insert Figure 2 about here

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In the light of the wide permutations of synthetic non-viral vector systems that have emerged over the last decade, Kostarelos and Miller[95] introduced an interesting and rational classification system to aid in the selection and comparison of the different technologies available. This **ABCD** concept is thus an appropriate structural paradigm for modified polymer-based carrier systems employed for *in vitro*, *ex vivo* and/or *in vivo* application. In **ABCD** nanoparticles [Figure 3], nucleic acids (termed **A**) are generally condensed within functional concentric layers of polymeric components that help cellular entry/intracellular trafficking (termed **B**); this is subsequently modified by addition of a biological stability group (termed **C**), and a distal biological targeting ligand (termed **D**). A variety of siRNA delivery systems will now be reviewed based on this **ABCD** concept.

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Insert Figure 3 about here

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"A"system - based delivery

Several studies have shown that delivery to certain tissues, such as the eye[96, 97], lung[98], liver[99], limb[99] and central nervous system (CNS) [100, 101] of 'naked' siRNA is effective. The term 'naked' siRNA refers to the delivery of siRNA (unmodified and modified) in saline or in simple excipients such as a solution of 5% dextrose in water (D5W). In a laser-induced choroidal neovascularisation in a non-human primate model of age–related macular degeneration (AMD) intravitral injection of saline-formulated vascular endothelial growth factor (VEGF) siRNA was well tolerated and efficient[96]. Intravitreal injection of saline-formulated siRNA targeting VEGF receptor-1 was also effective in reducing the area of ocular neovascularisation in two different mouse models[97]. siRNA formulated in D5W administered intranasally in a non-human primate model of severe acute respiratory syndrome (SARS) corona virus infection significantly reduced interstitial infiltrates and pathological changes, and inhibited viral replication in the lung[98]. The first report showing siRNA transfer into selected grafts (liver and limb) was achieved by a catheter-based intravenous rapid-injection method[99]. Hydrodynamic delivery involves injection of a large volume of nucleic acid solution and this rapid and large volume injection of solution exceeds the cardiac output and causes a transient overflow in the inferior vena cava, which induces nucleic acid solution to flow into the liver. This is followed by a quick increment of intra-hepatic pressure, liver expansion and reversible disruption of the liver fenestrae. This approach may be applicable to humans by using balloon catheter-based and occlusion-assisted injection to a specific vein

of an expandable organ[102]. The modification of balloon catheters and the development of a minimally invasive technique to allow selective isolation of liver segments for hydrodynamic gene therapy in the animals and humans are promising for siRNA specific-delivery[102]. Direct administration of siRNA in saline has also been utilized in the CNS to confirm disease targets *in vivo*. Direct instillation of saline-formulated siRNA by intracerebroventricular, intrathecal or intraparenchymal infusion led to silencing of specific neuronal mRNA targets in multiple regions of the peripheral and central nervous system[100, 101, 103].

The application of unmodified siRNA for therapeutic use has been demonstrated in certain tissues; however, for more general use the transfection efficiency of unmodified siRNA is too low (see above discussions). Besides, unmodified siRNA is susceptible to attack from serum enzymes and is apt to induce off-target effects. As mentioned earlier, siRNA modifications to improve transfection or a delivery vehicle are required to overcome the issues. siRNA bearing 3'overhangs of two P=S 2'-O-(2-methoxyethyl)-ribonucleotide and targeting the pain related cation-channel $P2X_3$ intrathecally administered to rats resulted in pain relief demonstrated by inhibition in the neuropathic pain response[104]. siRNA stability in the extracellular and intracellular environments can be enhanced by modifying the oligo backbone and replacing individual nucleotides with analogues. Administration of this chemically modified siRNAs resulted in silencing of the apolipoprotein B (apoB) messenger RNA in the liver and jejunum, decreased plasma levels of apoB protein, and reduced total cholesterol[37].

The ease of formulation and administration using direct delivery of naked siRNA to tissues make this an attractive therapeutic approach. However, the naked uncomplexed siRNA dose that is typically required for target silencing in these studies is high and siRNA uptake is uneven increasing the risk of off-target effects and toxicity. Moreover, systemic delivery is more feasible where the disease site is not easily accessible and intravenous or intraperitoneal (i.p.) treatments are required. Therefore, it is sometimes essential to formulate siRNA with a transfection reagent or a specialized delivery system.

"AB" system - based delivery

AB particles are generally formed by the condensation and/or encapsulation of nucleic acids using cationic polymers (**B**-layer). When lipids condense with nucleic acids to form shapeless particles, for example, they are named as lipoplexes. Lipoplexes are formed by simply mixing siRNAs with most commercial transfection reagents, including Lipofectamine™ 2000 (Invitrogen) and Transit-TKO® (Mirus). Intranasal administration of siRNAs complexed with/without the transfection agent Transit-TKO® against viral genes decreased the viral load of respiratory syncytial virus (RSV) and parainfluenza virus (PIV)[105]. It suggests that, if properly designed, low dosages (as low as 70μg per

animal) of inhaled siRNA might offer a fast, potent and easily administrable antiviral regimen against respiratory viral diseases in humans without any adverse events. Moreover, a rational approach to deliver siRNA has been recently designed using cationic lipids[169]. The **AB** formulation was started with the ionizable cationic lipid 1, 2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA), a key lipid component of stable nucleic acid lipid particles (SNALP) as a vector[169]. The results have shown that this DLinDMA-based formulation achieved superior *in vivo* siRNA delivery capacity using either the rodent model or the nonhuman primate models[169].

In addition, protein transduction domains (**B**-layer) are short amino acid sequences that interact with the plasma membrane of a cell in a receptor-independent manner that results in efficient cellular uptake[106]. Fusiogenic peptides based on those found in the fusion domains of the Influenza A virus have been synthesised and shown to be effective in the *in vitro* delivery of siRNA, the enhancement of endosomal escape and the silencing of the oncogene EGFR[106]. Only short sequences of primarily positively charged amino acids (arginine and lysine) are responsible for the translocation through the membrane. Cell penetrating peptides (CPPs) may be used to transfect a range of different cell types with high efficiency[107].

"AD" system - based delivery

Conjugation of siRNAs to a targeting molecule (**D**-layer) has been used to delivery siRNA into cells and can be employed for cell-specific targeted delivery. Conjugation is particularly attractive for siRNA-based therapeutics since only one of the strands in the duplex is functional and conjugates can be attached to either the sense or antisense strand without compromising the activity of the silencing. A peptide mimetic of insulin-like growth factor 1 (IGF1) conjugated to siRNA and directed to insulin receptor substrate 1 (IRS1) protein, increased the *in vitro* cellular uptake of siRNA in comparison to unconjugated siRNA[108].

Aptamers, peptide molecules that bind to specific target molecules, can be synthesised and subsequently utilised for the cell specific delivery of siRNAs[109, 110]. Both components can be chemically synthesised so aptamer-siRNA conjugates have the potential to be applied to a wide range of diseases. Chu *et al.*[109] demonstrated that an anti-prostate specific aptamer (PSMA) coupled via a streptavidin bridge with siRNA against lamin A/C, could be used to specifically and efficiently deliver functional siRNA to PSMA positive cells *in vitro*. Similarly PMSA conjugated siRNA targeting the survival gene PLK1 has been used *in vivo* to induce regression of PMSA expressing LNCaP induced prostate tumours[110].

Wolfrum *et al.*[111] demonstrated that cholesterol-conjugated siRNA is taken up in murine models by a lipoprotein-dependent mechanism. Delivery of the siRNA conjugates depended upon interactions with

lipoprotein particles, lipoprotein receptors and transmembrane proteins. siRNA delivery was directed towards the liver, gut, kidney and steroidogenic organs by high density lipoproteins whereas low density lipoproteins targeted siRNA to the liver.

A particularly challenging area is delivery to the brain across the blood brain barrier. Kumar et al.[112] showed that a short peptide derived from rabies virus glycoprotein (RVG) enables the transvascular delivery of siRNA to the brain. To enable siRNA binding, a chimeric peptide was synthesized by adding nonamer arginine residues at the carboxy terminus of RVG, which specifically bound to the nicotinic acetylcholine receptor expressed by neuronal cells[112]. After intravenous injection into mice, this system delivered siRNA to neuronal cells, resulting in specific gene silencing within the brain and robust protection against fatal viral encephalitis in mice[112].

It has recently been shown that siRNA synthetically conjugated to a CpG oligonucleotide agonist of the TLR9, targeted and silenced the signal transducer and activator of transcription 3 (STAT3, an immune suppressor gene) in TLR9 (+) myeloid cells and B cells[113]. STAT3 has been reported to orchestrate the expression of immunosuppressive and angiogenic factors, contributing to a tumour environment characterized by a lack of tumour-specific cytotoxic T cells and an inhibition of T helper 1 (T_H1) cells[113]. Silencing of STAT3 using CpG-linked siRNA by either local delivery at the tumour site or intravenously injection in a mouse model led to activation of tumour-associated immune cells, triggering of antitumour immune responses and eventual inhibition of tumour growth[113].

"ABC" system - based delivery

Due to the **C** component, the addition of stealth/biocompatable polymers, formulations of this type appear to be very promising for systemic applications and gene therapy due to improved serum stability in comparison with **AB** systems[95]. **ABC** particles are especially beneficial for passive targeting delivery, in light of the leaky endothelial wall (~400nm) in inflammatory and tumour sites resulting in the EPR effect as described above. Kim and colleagues[114] utilized PEG-conjugated VEGF siRNA (siRNA-PEG) and PEI to spontaneously form nanoscale polyelectrolyte complex micelles, having a siRNA/PEI inner core with a surrounding PEG shielding layer. Intravenous administration of this formulation successfully inhibited VEGF expression and tumour development in a tumour-bearing mouse model. In addition, a systemic siRNA delivery approach based on a novel lipid-like material ($98N_{12}$-5(1)), cholesterol, PEG-lipid and modified siRNA has been recently developed[115] . This **ABC** system was able to target the liver (>90% distribution) and induce reversible, long-lasting gene silencing without any loss in activity following repeated administration, possible orchestrated by the fact that

smaller particles would have efficient access to hepatocytes via the fenestrated endothelium of liver (~100nm).

However, as PEGylation may shield the cationic surface of formulations, the EPR effect achieved results in good permeability but poor retention for small particles without a targeting ligand. One approach that has been developed to combat this is the addition of a novel pH-sensitive benzoic imine linker between the PEG group and the polymer (e.g. poly-l-lysine)[116]. The linkage is stable in aqueous solution at physiological pH (pH ~7.4) but cleaves in acidic conditions such as the extracellular environment of solid tumour (pH ~6.8) and in endosomes (pH ~5.5). When this formulation arrives at the tumour sites via EPR effect, the cationic surface becomes exposed. This is driven by the reversible detachment of the shielding PEG chains due to the processing of the imine linkage, resulting in binding of vectors to the tumour tissues by electrostatic interaction and enhancement of endosomal escape[116, 117].

"ABD" system - based delivery

Some attractive examples of **ABD** particles, in addition to those described in the cell-specific targeting section above, have recently emerged and these appear to be particularly effective for in *vivo* studies, although they can be somewhat irregular in formulation[95]. Liposomes for instance, can fuse with biological membranes and enhance drug delivery into cells. Liposomes employed for therapeutic delivery are composed of many components, including a variety of lipids, such as cationic and/or fusogenic lipids, cholesterol and helper lipids[54]. Liposomes with targeting moieties have been developed to improve cell specific uptake of siRNA[118, 119]. In this context, Vitamin A-coupled liposomes successfully delivered therapeutic siRNA targeting gp46 (a rat homolog of human heat shock protein 47 which is crucial for cellular stress response) to prolong the survival in a rat model of lethal liver cirrhosis[118]. The efficacy of this approach has been expanded to both acute and chronic models of liver fibrosis and suggests its therapeutic potential for reversing human liver cirrhosis[118].

In the context of CNS delivery, siRNA was administered into the brain *in vivo* with the combined use of a receptor-specific monoclonal antibody delivery system, and in this case liposome technology was replaced by avidin-biotin technology[119]. It suggests that brain delivery of siRNA following intravenous administration is possible with siRNA and appropriate targeting vectors.

Moreover, a newly developed **ABD** system has been shown successfully to deliver siRNA to macrophages through oral administration, resulting in suppression of inflammation responses and increased survival rates in mice[120]. In this study, siRNA was condensed with PEI and the "siRNA-PEI" layer was encapsulated inside β 1, 3-D-glucan particles[120]. These particles directed phagocytosis by macrophages and dendritic cells by means of the dectin-1 receptor (a major beta-glucan receptor) and

perhaps other β 1, 3-D-glucan-receptor-mediated pathways[120]. This technology offers an exciting and novel strategy for oral delivery of siRNA to attenuate inflammatory responses in human disease.

"ACD" system - based delivery

Using a previously designed **AD** delivery system appended with a PEG moiety[110], a novel **ACD** particle has recently been successfully developed[121]. Researchers have enhanced the silencing activity and specificity of the aptamer-siRNA chimera by incorporating modifications that enable more efficient processing of the siRNA by the cellular machinery. These complexes resulted in significant inactivation of PSMA-expressing tumours in an athymic mouse model by intravenous administration. More importantly, the *in vivo* circulating half-lives of the chimeras were dramatically increased from <35min to >30hour by the addition of the PEG group[121].

"ABCD" system – based delivery

For most systemic applications and gene therapies, the entire **ABCD** class of particles is possibly the most desired strategy. The number of novel **ABCD** systems is increasing despite the different technical barriers in developing reproducible and scalable formulations of an **AB** core coupled with the controlled and consistent association of **C** and **D** layer components. As these **ABCD** formulations are nearly neutral or even negative in charge, the remarkable gene silencing data when administered systemically must be a result of active cell-specific targeting. Li and colleagues developed a nanoparticle formulation for successfully systemic delivery of siRNA into xenograft[122, 80] and metastatic tumours[78, 79] which produced anti-cancer effects. In this system, the nucleic acids (siRNAs and carrier DNA), a polycationic peptide (protamine) and a cationic liposome, were initially prepared in a condensed core. The resulting core was then modified by PEG-lipid containing the tumour targeting ligand, anisamide (see above) [78-80]. The resulting targeted nanoparticles silenced the epidermal growth factor receptor (EGFR) in the tumour and induced ~15% tumour cell apoptosis without any indication of immunotoxicity[122]. In a mouse model of metastasis, a mixture of siRNA against the cancer associated proteins MDM2, c-myc, and VEGF, co-formulated in the targeted formulation, administered intravenously caused simultaneous silencing of each of the genes. Importantly, the targeted nanoparticles at the therapeutic dose used, showed little local and systemic immunotoxicity, body weight alterations or damage to the major organs[78].

In addition, as described earlier Peer et al. [69] developed a strategy for selectively silencing CyD1 in leukocytes *in vivo*. Liposomes were grafted with a spacer namely hyaluronan (a high-molecular-mass polysaccharide) to the outer surface, thus stabilizing the particles both during subsequent siRNA entrapment and during systemic circulation *in vivo*. The resulting particles were successfully modified

with a targeting ligand by covalently attaching a monoclonal antibody to hyaluronan. Targeted stabilized nanoparticles (tsNPs) were eventually loaded with CyD1-siRNA. The antibody was specific to direct particles to β_7 integrins overexpressing in gut mononuclear leukocytes. Systemic application of β_7 integrin-tsNPs silenced CyD1 in leukocytes and reversed experimentally-induced colitis in mice[69]. This research further identifies CyD1 as a potential anti-inflammatory target, and suggests that the application of similar modes of targeting of siRNA may be applicable in other therapeutic settings and for further target validation efforts.

Some of the most novel areas of development of **ABCD** vectors are those employing cyclodextrins[72, 123-125]. Cyclodextrins are natural cyclic oligosaccharides composed of 6, 7, or 8 D(+)-glucose units known as α, β and γ respectively. The geometry of cyclodextrins gives a hydrophilic outer surface and hydrophobic inner cavity into which it is possible to fit insoluble molecules to form inclusion complexes. Davis and colleagues have developed a novel **ABCD** system based on i) condensation of siRNA with cyclodextrin-containing polycations (CDPs), and, ii) modification of the CDP-siRNA by forming inclusion complexes with adamantane (a hydrophobic molecule) which in turn is utilised for the attachment of the functional groups PEG-transferrin . This formulation has indicated efficacy in the delivery of the siRNA against oncogene to inactive the tumour growth in a murine model of metastatic Ewing's sarcoma[124]. Three continuous daily doses of the CDP-siRNA-adamantine-PEG-transferrin formulation carrying two types of siRNAs specific for the gene encoding ribonucleotide reductase subunit M2 (RRM2) slowed tumour development, whereas non-targeted formulations were dramatically less effective when administered under the same conditions[72].

An alternative strategy uses cyclodextrins as potential core molecules which can be modified directly with various functional groups including; polycations, lipophilic chains (amphiphilic vectors), PEG chains and targeting ligands[126-129]. Amphiphilic cyclodextrins have been shown to form vesicles with the potential to encapsulate siRNA inside the aqueous core[126, 130, 131]. The structural concept of cyclodextrin:siRNA vesicles is illustrated in Figure 4. There are many advantages associated with this approach, as oligosaccharides they have low immunogenicity and the availability of multiple sites for attachments of functional groups confers flexibility to accommodate the targeting requirements for a range of therapeutic applications. In essence, this technology has the capacity to form nanosized, neutral, cell specific transfection systems compliant with the 'ideal' properties as outlined in Figure 2.

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Insert Figure 4 about here

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In addition to the **ABCD** concept described above, general summaries of the major delivery strategies including disease, gene target and delivery method used *in vitro* and *in vivo* are given in Table 1 and Table 2 respectively.

Clinical Trials

A number of siRNA therapies have advanced into clinical trials, a summary of these on-going trials is provided in Table 3. Given the ease of access to the eye and that the use of naked siRNA is effective in these cases, the most progress to date is in the treatment of eye diseases[132]. Age-related macular degeneration (AMD), a progressive condition leading to impaired central vision is currently the subject of several clinical trials. The condition is characterised by the growth of abnormal leaky blood vessels beneath the macula that damages the area of the eye essential for fine-detail vision. All trials involve the direct injection of siRNA into the macular of the eye. However, Allergan halted development of its AGN211745, a chemically modified siRNA after the drug failed to meet a key efficacy endpoint in a phase II study (www.allergan.com). No safety issues were associated with AGN211745 targeting VEGF-1. But since the drug did "not meet its efficacy hurdle", Allergan opted to halt its development (www.allergan.com). Additionaly, OPKO Health, Inc. has decided to terminate its Phase III clinical study of Bevasiranib (a naked siRNA) for the treatment of AMD (www.opko.com). Although there were no systemic safety issues identified and local ocular safety was generally unremarkable, review of the data indicated that the trial, as structured, was unlikely to meet its primary end point (www.opko.com).

QPI-1002, the first systemically administered siRNA drug developed by Quark Pharmaceuticals, Inc., is currently being evaluated in a Phase I/IIa clinical trial study for the prevention of acute kidney injury (AKI) and also in a Phase I/II clinical trial for the prophylaxis of delayed graft function (DGF) following renal transplantation (www.quarkpharma.com). Treatment of the respiratory disease respiratory syncytial virus (RSV) using nebulised delivery of naked siRNA has progressed to Phase II. The highly contagious RSV causes infections in both the upper and lower respiratory tract. Interim reports suggest that subjects receiving the drug ALN-RSV01 administered by nebulisation and targeting the RSV nucleocapsid experienced a 38% reduction in infection rates (www.alnylam.com). This was in conjunction with an increase in the number of subjects who remained free of infection (www.alnylam.com). Pachyonychia congenital, a rare keratin disorder that affects the nails, skin, oral mucosae, larynx, hair and teeth has been associated with mutations in the N171K gene. Patients can suffer from painful blisters and keratoderma with no disease specific treatment currently available[133]. In association with the

Pachyonychia Congenita Project, TransDerm are performing a clinical trial into the effectiveness of TD101, an siRNA targeting mutations in N171K and more specifically the keratin K6a[133]. Initial trials involve the local injection of siRNA into the foot with a view to developing a gene cream for topical application (www.transderm.org).

An **ABCD** delivery system currently undergoing clinical trials utilises the RONDEL™ (RNAi/Oligonucleotide Nanoparticle Delivery) technology developed by Calando Pharmaceuticals. Their targeted nanocomplex, composed of transferrin targeted cyclodextrin containing polycations and anti RRM2 siRNA (CALAA-01), yielded promising results in mouse models[72]. CALAA-01 has advanced to phase I clinical trials in the treatment of solid tumours to determine the safety and maximum tolerated dose of the 'drug' when administered intravenously to patients. It was announced recently that in addition to CALAA-01, pre-clinical development of a second siRNA oncology therapeutic is underway (www.calandopharma.com). CALAA-02 uses the same delivery system as CALAA-01, but will target hypoxia inducible factor-2α (HIF-2α), which is over-expressed in many solid tumours and has a role in tumourigenesis[134].

An interventional clinical trial that is for Metastatic Melanoma and absence of CNS metastases conditions was started in January of 2008 in Duke University. Transfection with siRNA targeting the immunoproteasome alters proteasome-mediated antigen processing by the dendritic cell, resulting in a reduction of enhanced anti-melanoma immune responses (www.cancer.duke.edu). This Phase I trial, open to subjects with metastatic melanoma, will assess the safety of vaccination with melanoma tumour associated antigen-encoding RNA-transfected mature dendritic cells derived from monocytes that have been either untreated, transfected with control siRNA, or transfected with siRNA targeting the inducible immunoproteasome (www.cancer.duke.edu). As a secondary objective, this Phase I study will also assess the anti-melanoma immune responses, as well as clinical responses, induced by vaccination with this dendritic cell-based product.

Conclusion

The use of synthetic siRNA as a therapeutic to knockdown the expression of a gene or protein has generated huge interest in both the scientific and business communities. It has the potential to be utilised in a diverse range of disease states, including cancer, cardiovascular disease, inflammatory conditions, viral infections and CNS disorders. Despite the identification of numerous gene targets for these

conditions (Table 1 and Table 2) the main impediment to the use of siRNA therapeutically is the achievement of safe and efficient delivery.

The rate of progress in the delivery dilemma has nonetheless been remarkably rapid and the future is likely to reside with the development of non-viral systems due to the decreased risk of toxicity compared to viral-based systems. However, as can be seen from the current clinical trial studies described above most involve naked or chemically modified siRNA, the Calando trial is the only formulated product, it is the first and so far the only trial ongoing for treatment of solid tumours. Consequently, opportunities exist to develop further innovative delivery technologies to satisfy the enormous unmet need in this field. Given the requirement for cell-specific delivery of therapeutic siRNA, justifiable consideration is being given to the design and formulation of targeted, non-immunogenic and efficient delivery technologies, such as described by the **ABCD** vector strategy. By driving this promising approach forward the gap between concept and clinic will potentially be bridged heralding a new era of joy, the silent era resulting in the availability of gene-based medicines.

Table 1. A summary of studies on the *in vitro* delivery of siRNA, including the delivery method, the target gene and the therapeutic target.

Delivery system	Therapy	Target(s)	Comments	References
Conjugated siRNA	LNCaP cells (Prostate Cancer)	Lamin A/C	Aptamer:streptavidin:siRNA conjugates	[109]
Conjugated siRNA	LNCaP cells (Prostate Cancer)	Bcl-2 PLK1	Aptamer:siRNA chimeras	[110]
Conjugated siRNA	CEM cells (HIV)	Anti tat/rev	Anti gp120 aptamer:siRNA conjuates	[135]
Peptide	MCF-7 cells (Breast Cancer)	IRS-1	IGF1 peptide mimetic conjugated to siRNA	[108]
Peptide	HS-68 cells (fibroblasts) HeLa cells (Cervical Cancer)	N/A fluorescent siRNA and Luciferase (reporter)	Cell penetrating peptide	[107]
Peptide	Dorsal root ganglion cells	$p75^{NtR}$	Histidine rich reducible polycations	[136]
Peptide	A431 cells (epidermoid carcinoma)	EGFR	Influenza derived fusogenic peptide diINF-7/Lipofectamine/siRNA	[106]
Peptide	C26 cells (Colon Cancer)	K-ras	Influenza derived fusogenic peptide diING-7/Lipofectamine/siRNA	[106]
Liposomes	PANC-1 (Pancreatic Cancer) MDA-MB-435 (Breast Cancer)	HER-2	Immunoliposomes	[64]
Liposomes	SK-MES-1 cells (Lung Cancer)	HDM2	Liposomes modified with arginine octamer molecules	[137]

Liposomes	Hepatic Stellate cells (Liver Cirrhosis)	gp46 (rat homolog of human HSP47)	Lipotrust cationic liposomes coupled with vitamin A	[118]
Lipoplexes	Hela cells SW756 cells (Cervical Cancer)	E6	Oligofectamine	[138]
Lipoplexes	HeyA8 and SKOV3ip1 cells (Ovarian Cancer)	IL-8	RNAiFect	[139]
Lipoplexes	Fibroblast like synoviocytes (Rheumatoid Arthritis)	PARP-1	DharmaFECT 1	[140]
Lipoplexes	Human Mesenchymal Stem Cells	EGFP (reporter)	Lipofectamine 2000, RNAiFect, RiboJuice, GeneEraser and siPORT	[141]
Lipoplexes	Parkinson's disease	LRRK2	Nucleofection Kit (Amaxa)	[142]
Lipoplexes	Vascular Smooth Muscle Cells (Cardiovascular Disease)	Ki-ras2A	Lipofectamine Plus	[143]
Lipoplexes	HEK293 (Alzheimer's Disease)	TMP21	Cells stably transfected with PS2, siRNA transfected using Lipofectamine 2000	[144]
Lipoplexes	HeLa cells (Polio)	siC (capsid) siP (viral polymerase)	Lipofectamine 2000	[145]
Nanoparticles	HeLa cells (Cervical Cancer)	EGFP (reporter)	Quantum dots conjugated to peptide and siRNA	[146]
Nanoparticles	H1299 cells (Lung Cancer)	EGFP (reporter)	Chitosan/siRNA nanoparticles	[147]

Nanoparticles	Cardiomyocytes	ERK1/ERK2	Single walled carbon nanotubes	[148]
Nanoparticles	PC-3 (Pancreatic cancer)	VEGF	Thermally sensitive cationic polymer nanocapsules	[149]
Nanoparticles	FL5.12A IL-3 dependent pro B cells D1 IL-7 dependent cells	Survivin	Bacteriophage phi29 pRNA nanoparticles	[150]
Cyclodextrins	HeLa cells (Cervical Cancer)	Luciferase (reporter)	Cyclodextrin containing polycations	[151]
Cyclodextrins	N2A cells (Neuroblastoma)	RRM2	Transferrin targeted cyclodextrin containing polycations	[72]
Polymers	MCF-7 cells (Breast Cancer)	sCLU	PEG-PEI –siRNA polymer complexes	[152]
Polymers	PC-3 cells (Prostate Cancer)	VEGF	VEGFsiRNA-PEG-PEI	[114]
Gel-based medium	(Colorectal Cancer)	Bcl-2	Agarose/liposome/siRNA	[153]

Table 2. A summary of studies on in vivo delivery of siRNA, including the delivery method, the target gene, the therapeutic target and the route of administration.

Delivery system	Therapy	Target(s)	Comments	Route of Administration	References
Naked siRNA	Pulmonary Alveoli	GAPDH (reporter)	Naked siRNA	Administered intranasally	[154]
Naked siRNA	Limbs	Luciferase (reporter)	Naked siRNA	Luciferase siRNA administered via the great saphenous vein of mouse	[155]
Naked siRNA	Liver and Limbs	GFP (reporter)	Naked siRNA	Catheter-based injection	[99]
Modified siRNA	Chronic Neuropathic Pain	$P2X_3$	Naked siRNA	Administered intrathecally	[104]
Conjugated siRNA	LNCaP tumours (Pancreatic Cancer)	PLK-1	Aptamer:siRNA chimeras	Administered intratumourally	[110]
Conjugated siRNA	HIV	CCR5, Vif, Tat	Anti-CD7 antibody conjugated to siRNA	Administered via i.v.	[67]
Protein	Bone metastasis	EZH2 p110-α	Atelcollagen/siRNA complexes	Administered via the tail vein	[156]
Peptide	Eye	N/A Lissamine conjugated peptide	Peptide for ocular delivery (POD) complex	Administered topically to the eye	[157]
Peptide	Encephalitis (Brain)	Anti viral FvE[1]	Rabies virus glycoprotein derived peptide/siRNA	Administered via i.p.	[112]
Liposomes	HeyA8 or SKOV3ip1 tumours (Ovarian Cancer)	EphA2	DOPC liposomes	Administered via i.v.	[158]

Liposomes	Capan-1 tumours (Pancreatic Cancer)	N/A fluorescent siRNA	Cationic DOTAP:DOPE liposomes (transferrin targeted)	Administered via i.v.	[63]
Liposomes	HeyA8 or SKOV3ip1 tumours (Ovarian Cancer)	FAK	Neutral DOPC liposomes	Administered via i.p. injection	[158]
Liposomes	HeyA8 or SKOV3ip1 tumours (Ovarian Cancer)	IL-8	Neutral DOPC liposomes	Administered via i.p. injection	[139]
Liposomes	Hepatitis C	GB-virus B	Cationic Liposomes	Primate model of disease	[159]
Liposomes	Bladder Cancer	PLK-1	Cationic Liposomes	Administered via a catheter	[160]
Liposomes	Arthritis	TNF-α	DOPE/RPR209120 cationic liposomes	Administered via i.v.	[161]
Liposomes	Liver	Ubc13 (endogenous)	Galactosylated cationic liposomes	Administered intraportally or i.v.	[81]
Liposomes	Liver (Hepatitis C)	Anti HCV	Lactosylated cationic liposomes	Administered via i.v.	[82]
Lipoplexes	Hep3B (Liver Cancer)	Rec QL1 DNA helicase	Jet-PEI or Cationic liposomes	Local and systemic delivery	[162]
Lipoplexes	Inflammatory Bowel Disease	TNF-α	Lipofectamine	Administered Intrarectally	[163]
Nanoparticles	B16F10 tumours (Lung Cancer)	Luciferase (reporter)	siRNA-Liposome-PEG-Anisamide particles	Administered via i.v.	[79]
Nanoparticles	B16 tumours (Lung Metastasis)	MDM2/c-myc/VEGF	siRNA-carrier DNA-peptide-cationic liposome	Administered via i.v.	[78]
Nanoparticles	H460 tumours (Lung Cancer)	EGFR	siRNA-Liposome-PEG-Anisamide particles	Administered via i.v.	[80]

Nanoparticles	Lung	EGFP (reporter)	EGFP-transgenic mice used	Chitosan/siRNA particles administered intranasally	[147]
Nanoparticles	Inflammatory Bowel Disease Intestinal Inflammation	CyclinD1	Liposome based integrin targeted nanoparticles	Administered via i.v.	[69]
Cyclodextrins	N2A tumours (Neuroblastoma)	RRM2	Transferrin targeted cyclodextrin containing polycations	Administered via the tail vein	[72]
Polymers	N2A tumours (Neuroblastoma)	VEGF	Peptide-PEG-PEI	Administered via the tail vein	[70]
Polymers	PC-3 tumours (Prostate Cancer)	VEGF	VEGFsiRNA-PEG-PEI	Administered intratumourally and i.v.	[114]
Polymers	Systemic Inflammation	TNF-α/ Map4k4	siRNA - PEI - β 1, 3-D-glucan particles	Oral administration	[120]
Polymers	Various to determine Biodistribution	EGFR	PLGA microspheres Sustained release	Administered via i.v. subcutaneously	[164]
Polymers	S-180 tumours (Sarcoma)	VEGF	VEGFsiRNA-PEI, PLGA microspheres	Administered intratumourally	[165]

Table 3. A summary of clinical trials ongoing using siRNA including the delivery system, the route of administration, the disease target, the pharmaceutical company and the current status of the trial.

siRNA	Delivery System	Route of Administration	Disease	Target	Company/ Institution	Status
AGN211745	Naked siRNA	Intavitreal Injection	Wet Age-related Macular Degeneration	VEGF receptor	Allergan www.allergan.com	Phase II (Halted)
Bevasiranib	Naked siRNA	Intravitreal Injection	Wet Age-related Macular Degeneration	VEGF	Opko Health Ltd www.opko.com	Phase III (Terminated)
PF-4523655 (RTP801i-14)	Modified siRNA	Intravitreal Injection	Diabetic Macular Edema; Wet Age-related Macular Degeneration	Hypoxia-inducible gene	Quark www.quarkpharma.com	Phase II
QPI-120	Modified siRNA	Intravenous Injection	Acute Kidney Injury	P53	Quark www.quarkpharma.com	Phase I/IIa
QPI-120	Modified siRNA	Intravenous Injection	Delayed Graft Function	P53	Quark www.quarkpharma.com	Phase I/II
ALN-RSV01	Naked siRNA	Intranasal	Respiratory Syncytial Virus (RSV)	RSV nucleocapsid	Alnylam Pharmaceuticals www.alnylam.com	Phase IIb
TD101	Naked siRNA	Injection into foot	Pachyonychia congenital (PC)	PC keratin K6a	TransDerm www.transderminc.com	Phase I
CALAA-01	Transferrin targeted cyclodextrin nanoparticles	Intravenous Infusion	Cancer	RRM2	Calando Pharmaceuticals www.calandopharma.com	Phase I
N/A	Proteasome siRNA and tumour antigen RNA-transfected dendritic cells	Intradermal Injection	Metastatic Melanoma	Immunoproteasome beta subunits LMP2, LMP7 and MECL1	Duke University www.cancer.duke.edu	Phase I

Figure Legends

Figure 1. Knockdown of endogenous mRNA using the RNAi pathway. (A) longer dsRNAs can be transfected into the cytoplasm, follow Dicer processing and are recognized by RISC, (B) synthetic siRNAs can be transfected into cells and loaded into RISC directly for the RNAi pathway, and (C) miRNA blocks translation with a less perfectly matched sequence. (Based on Ref 168)

Figure 2. Systematic approach to the design, formulation and assessment of an "ideal" non-viral construct for cell specific targeted delivery of siRNA.

Figure 3. Schematic of the proposed ABCD concept using cationic polymeric vectors as a model. A=siRNA, B=cationic polymer, C=PEG and D=targeting ligand.

Figure 4. The structural concept of cyclodextrin:siRNA vesicles. Uncharged targeting (i.e. galactosylated) amphiphilic cyclodextrins can form a hydrophilic interior. This water interior acts as hosts to possibly encapsulate siRNA inside (Adapted from Ref 126).

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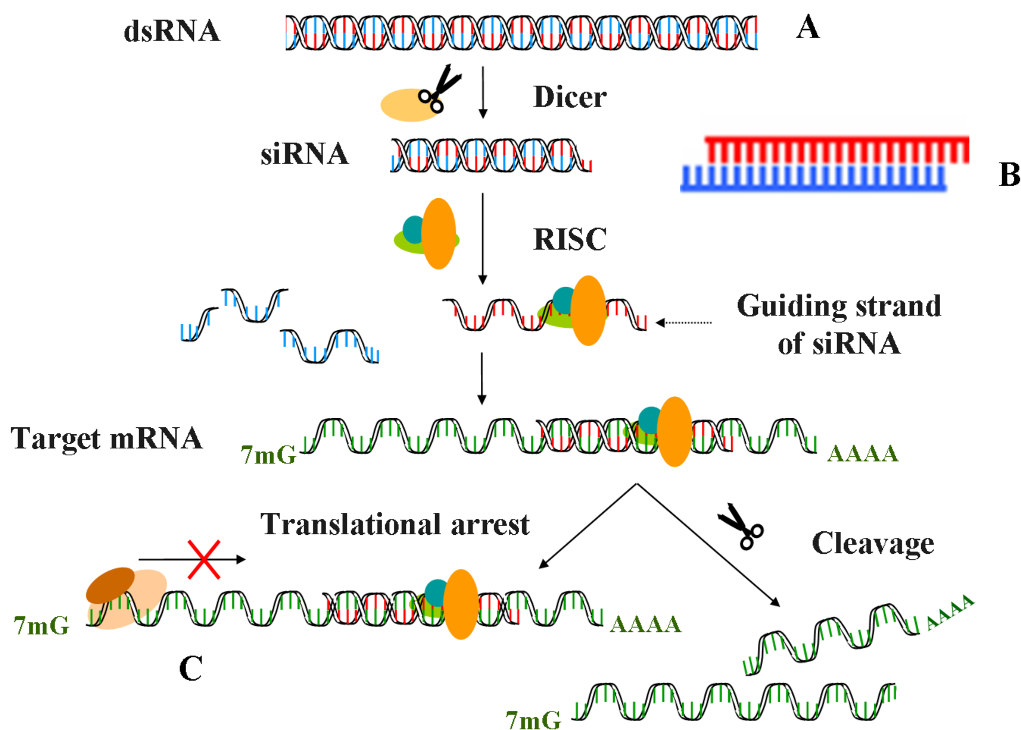
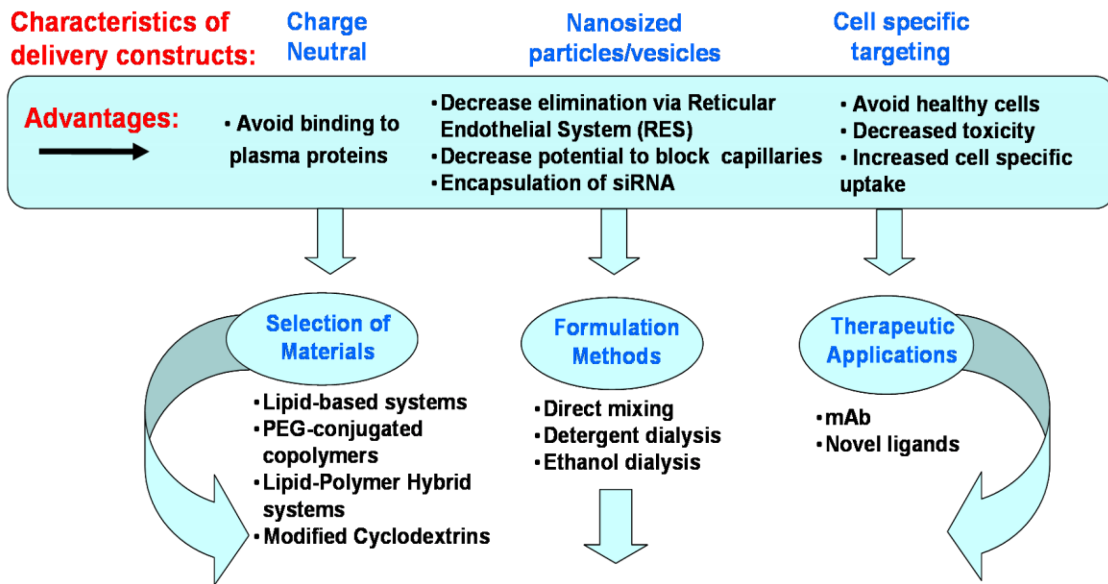
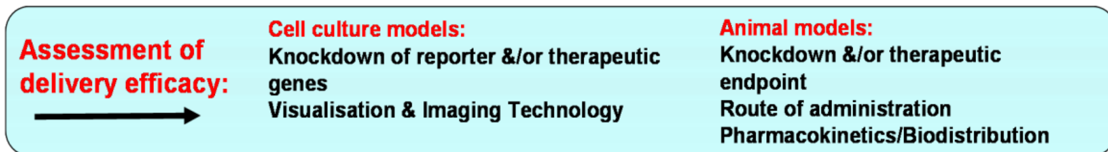


Figure 1.





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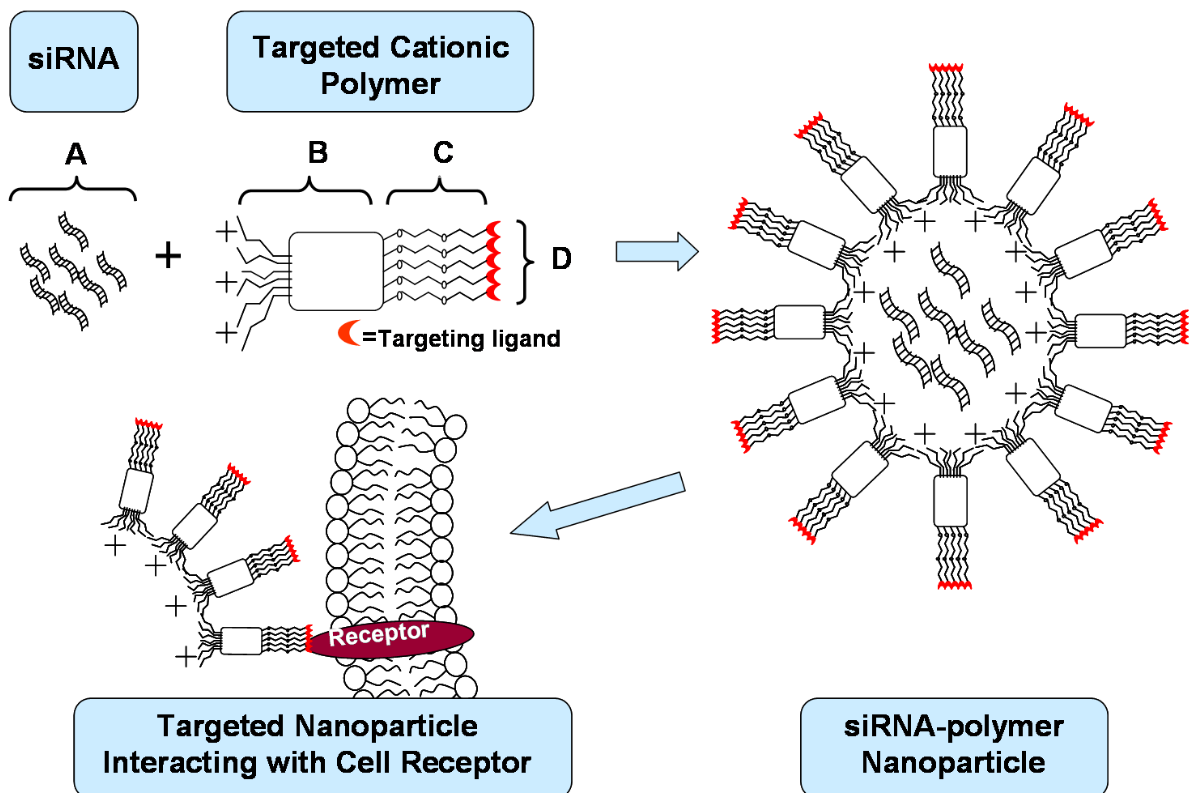

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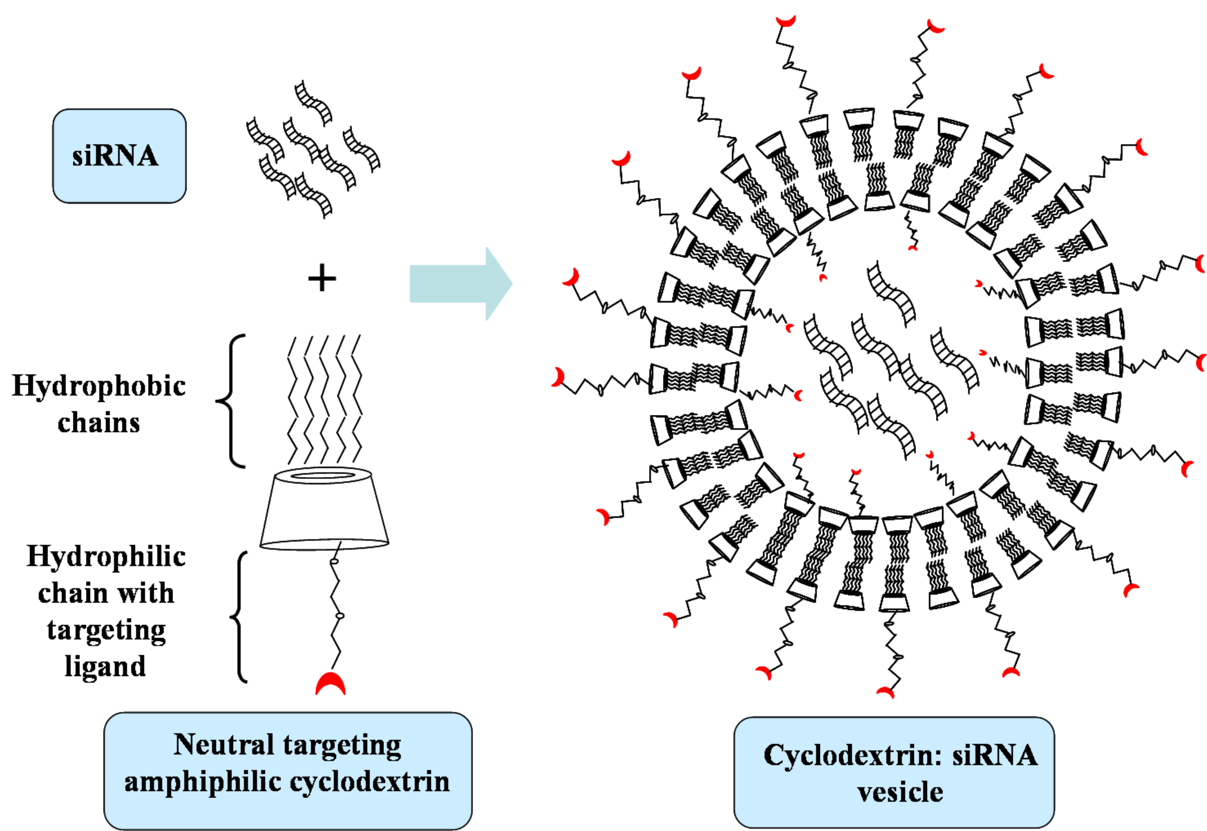

Figure 4.