NANOPARTICLE DELIVERY OF siRNA AS A NOVEL THERAPEUTIC FOR HUMAN DISEASE

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Introduction

RNA interference (RNAi) is a naturally occurring gene-silencing mechanism that holds great promise for the treatment of human disease. A major advantage of this process is its high degree of specificity and potential to silence any gene of interest. However, a number of challenges need to be overcome to allow gene-silencing therapeutics to become a reality. Nanotechnology has come to the forefront in the area of RNAi delivery with non-viral nanoparticles being developed as highly efficient vehicles for short-interfering RNA (siRNA), micro-RNA (miRNA) mimics and inhibitors. Several nanoparticle-siRNA therapies are in human clinical trials to assess their safety and efficiency. This review will focus on siRNA delivery using nanoparticles and their application for the treatment of human disease.

Mechanism of RNA Interference in Mammals

RNA interference was first described in the nematode *Caenorhabditis elegans* in the late 1990s by Fire and Mello (1). The introduction of exogenous, long double-stranded RNA (dsRNA) into the nematode caused potent suppression of a gene (encoding a myofilament protein) by inducing the degradation of a homologous host messenger RNA (mRNA) (1). This process, termed post-transcriptional gene silencing, involves the cleavage of the long dsRNA into small RNA fragments (21 to 22 nucleotides long) termed short-interfering RNA (siRNA). The siRNA is incorporated into a multiprotein RNA-induced silencing complex (RISC) (2). In this complex, the double-stranded siRNA is unwound and the sense strand discarded. The antisense (guide) strand attached to the RISC complex (activated RISC) then binds to the target mRNA with perfect complementarity (2). This in turn allows the endonuclease argonaute 2 (ago 2) protein (part of the RISC complex) to cleave the mRNA exactly 10 to 11 nucleotides downstream from the 5′ end of the antisense strand of the siRNA, causing mRNA degradation and gene silencing (2). The activated RISC complex is then recycled to cleave additional mRNA targets.

MicroRNA

MicroRNAs (miRNAs) were also first discovered in *C. elegans* (3,4), however, it was not until 2001 that the significance of this class of RNA were recognised following the identification of numerous endogenously expressed miRNAs in worms, flies and mammals (3,5,6). miRNAs are naturally occurring, small, non-coding single-stranded RNAs (approximately 21 base pairs long). Unlike mRNA, which acts as a template for DNA translation to protein, miRNAs are unique in that they have the capacity to directly regulate post-transcriptional gene silencing by controlling the translation of mRNA into protein (7) [synthesis of miRNAs and their mode of action has been extensively reviewed elsewhere (9)]. Importantly, one miRNA is capable of targeting multiple mRNAs and/or signaling networks. Since their discovery, there has been a significant advancement into the understanding of their role in regulating cellular function. For example, it has been reported that the human genome contains approximately 300 conserved miRNA genes and that miRNAs are estimated to regulate the translation of more than 60% of all protein-coding genes (8). Hence, it not surprising that their dysregulation (under- or overexpression) is thought to play a major role in the development and progression of human disease. Collectively, harnessing the power of RNAi has enormous potential as a therapeutic to treat a host of human diseases ranging from viral infections to cancer.

Nanoparticles as Delivery Vehicles for siRNA

Despite the promise of RNAi inhibitors, there are a number of obstacles that need to be overcome before they can reach their full potential. For example, the large size (approximately 13,400 g/mol) and net negative charge of these molecules under physiological conditions means that these cannot enter a cell without the aid of a delivery vehicle (10). Furthermore, it is well established that systemic administration of naked unmodified siRNA is prone to rapid degradation by RNAses present in serum as well as elimination by the reticulo-endothelial system (10). Moreover, siRNAs present in the circulation are exposed to cells of the innate immune system, hence initiating a potential off-target immune response (11). To overcome these challenges, there has been an intense research effort to develop a wide range of non-viral nanoparticles (ranging in size from 100–400 nm) capable of delivering siRNA to its target cell without eliciting toxic or immune-mediated side effects (**Fig. 1**). To date, there are many nanoparticle systems, which possess differing chemical and pharmacokinetic properties for siRNA delivery. However, for this review, only a select few that have been used to deliver therapeutic siRNA in pre-clinical models of human disease or are in human clinical trials will be discussed.

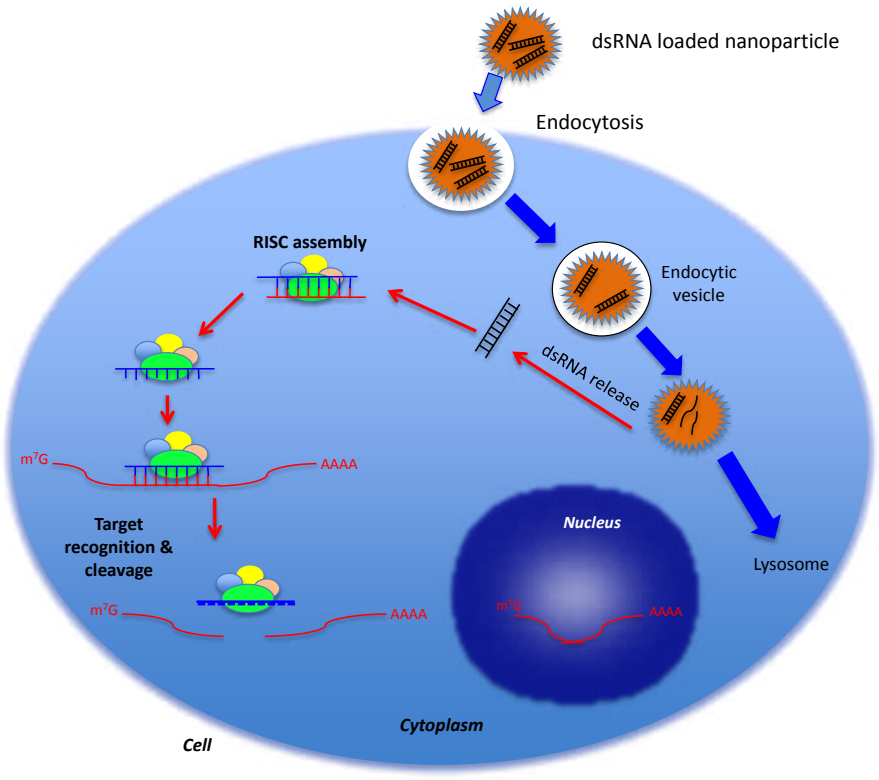

Fig. 1. Intracellular delivery of RNAi.
Nanoparticles usually enter cells by endocytosis. Before the endosome is degraded and the remaining nanoparticle removed via lysosomes, the synthetic double-stranded RNA (dsRNA) (small interfering RNA [siRNA] or microRNA [miRNA]) needs to escape the acidic environment of the endosome. Upon release of the dsRNA, it incorporates into the RNA-induced silencing complex (RISC), resulting in RNA interference by either translational repression or mRNA cleavage.

Liposomes and Lipid Nanoparticles

Liposomes and lipid nanoparticles (LNPs) are some of the best characterised and developed systems for the delivery of RNAi inhibitors. Liposomes are spherical structures that typically comprise a phospholipid bilayer with an aqueous core that can encapsulate siRNA (12–14). These vehicles are often synthesised with a mixture of lipids with different surface charges (cationic, neutral and ionic) and properties to help in the uptake and release of siRNA into the cell. To date, there have been numerous published reports that have described the use of liposomes to deliver siRNA to silence the expression of genes involved in regulating human disease. In some cases, these genes have proved to be difficult to target using conventional chemical inhibitors. Initial proof-of-principle studies have focused on cationic liposomes such as DOTAP, DOTMA (lipofectin) and DOSPA (lipofectamine) due to the ease with which these lipids can interact with negatively charged siRNA (via an electrostatic interaction) to form nanoparticles (12–14). Indeed, many of these systems proved to be highly efficient in delivering siRNA to a variety of different cell types. However, using these nanoparticles in preclinical models of human disease has been hampered by a number of factors, including toxicity (due to excess positive charge on the surface of the nanoparticle), aggregation with negatively charged serum proteins and non-specific/off-target side effects (12–14). To overcome these obstacles, liposomes have been designed that incorporate polyethylene glycol (PEG) (12). This hydrophilic peptide provides an aqueous barrier around the surface of the nanoparticle (liposome), thereby significantly reducing its interaction with serum proteins and contact with immune cells and allowing increased residence time within the bloodstream (15). In addition, liposomes have also been tailored to target a specific organ or cell type by modifying their surface with targeting moieties (antibodies or small peptide fragments). Examples of targeted liposome-siRNA complexes are given below.

In 2010, Kim *et al.* (16) reported the use of a surface-modified liposome composed of soybean phosphatidylcholine, DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), and cholesterol (approximate size of 100 nm) to deliver therapeutic siRNA to all of the major immune cell types involved in the pathogenesis of human immunodeficiency virus (HIV). To target the liposomes to these cells, they conjugated an antibody to its surface directed against the lymphocyte function-associated antigen-1 integrin (LFA-1), which is expressed on leukocytes. The targeted liposomes were able to specifically deliver siRNA to human leukocytes and potently silence a gene (chemokine receptor type 5 or CCR5) that functions in most strains of HIV as a co-receptor for entry to its target cell. Importantly, they were able to demonstrate protection against HIV infection using these targeted liposomes in BLT mice (mice that contain all of the major human hematopoietic lineages, including T and B cells, macrophages and dendritic cells). In another study, vitamin A-coupled liposomes were used to selectively deliver siRNA to hepatic stellate cells (the principal cell type responsible for producing fibrosis) in the liver of rats with established cirrhosis (17). In addition to their role in cirrhosis, hepatic stellate cells actively take up vitamin A from the circulation via receptor-mediated endocytosis for processing and storage. To take advantage of this property, Sato *et al.* (17) developed vitamin A-coupled liposomes (approximate size of 150 nm) composed of cationic lipids and cholesterol to deliver siRNA to hepatic stellate cells to silence a gene involved in regulating the synthesis and secretion of collagen (a major component of fibrous tissue). Systemic administration of low clinically relevant doses (0.75 mg/kg) of the vitamin A-liposome-siRNA complex

to rats reversed liver cirrhosis and increased survival (17). Moreover, delivery of this therapy twice weekly was non-toxic and non-immunogenic. Collectively, both studies highlight the potential of liposomes to deliver siRNA to silence key genes involved in regulating or promoting human disease.

One family of LNPs that has achieved great success and is seen as the gold standard in siRNA delivery is the family of stable nucleic acid lipid particles (SNALPs). These nanoparticles are uniform in size (typical size of 100 nm) and can encapsulate and deliver siRNA to the liver with very high levels of efficiency. Morrissey *et al.* (18) were the first to show that SNALPs could deliver low doses of siRNA to silence hepatitis B virus (HBV) RNA. Importantly, gene knockdown was potent and lasted for up to seven days in a mouse model of HBV infection. In 2006, Zimmermann *et al.* (19) demonstrated the further potential of SNALPs by delivering siRNA against apolipoprotein B (apo B) to non-human primates. After a single dose of siRNA, apo B mRNA and protein levels were reduced by greater than 80%, which in turn resulted in a significant decrease in total cholesterol. Importantly, administration of the nanoparticle complex produced long-lasting knockdown of apo B (11 days) and was non-toxic, therefore adding to its potential as a novel anti-cholesterol therapeutic. Finally, SNALPs have also demonstrated their versatility as a potential anti-cancer agent by delivering siRNA to suppress a gene involved in regulating and promoting human cancer cell growth in an established orthotopic mouse model of liver cancer (20). Taken together, the success of SNALPs as a vehicle for siRNA in pre-clinical models has boosted their human clinical evaluation for the treatment of a number of diseases, including hypercholesterolemia, Ebola virus infection and liver cancer.

Chitosans

Chitosan is a naturally occurring cationic polysaccharide which is biocompatible and non-toxic and has been widely used as a delivery vehicle for gene therapy (21). The protonated amine groups allow it to interact with siRNA and form self-assembled nanoparticles. In 2009, Howard *et al.* (22) used chitosan to form an ionic (charged) complex with siRNA which was targeted against TNF-α. This nanoparticle complex was then administered via the intraperitoneal route to deliver siRNA to macrophages in a mouse model of rheumatoid arthritis. This cell type is important in the pathogenesis of this disease and releases TNF-α as part of the inflammation cascade. The chitosan-siRNA complex was taken up by macrophages and was able reduce TNF-α levels by greater than 60% when compared to control mice treated with chitosan and non-silencing siRNA. This decrease correlated with a reduction in joint swelling in a collagen-induced arthritic mouse model. More recently, a tumour-homing poly-siRNA chitosan complex was used to deliver siRNA to cancer cells in a subcutaneous tumour mouse model (23). For this study, the authors used chemically cross-linked (multimeric) siRNA by introducing a disulfide linkage at the 5′ end of each siRNA strand. The purpose of this was to increase the stability of the chitosan complex when under physiological conditions (23). When administered systemically, the chitosan-siRNA complex was able to potently silence VEGF levels in tumour tissue and reduce tumour growth (via an inhibition of angiogenesis) by 80%.

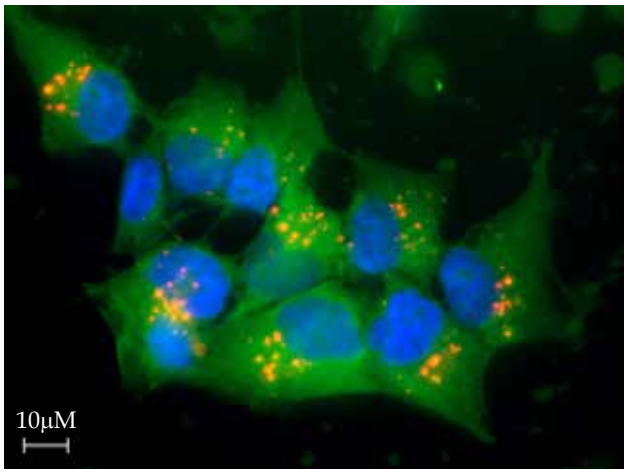

Fig. 2. A fluorescent image showing efficient uptake of fluorescently labelled siRNA (yellow) complexed to a modified poly-L-lysine dendrimer (iNOP-7) in SKNBE2 neuroblastoma cells. Green = stably expressing GFP; blue = nucleus; yellow = fluorescent siRNA. Image courtesy of Dr Rafael Erlich.

Dendrimers

Dendrimers are synthetic, well-defined, spherical polymers that are synthesised in a step-by step fashion. Each repeated sequence represents a higher generation molecule, which produces a precise highly branched structure. Poly(amidoamine) (PAMAM) dendrimers possess cationic amine groups on their surface that allow for excellent binding affinity to any negatively charged macromolecule, including DNA or RNA. In addition, they also contain numerous tertiary amines in their interior, which become protonated in the acidic endosomes, leading to endosomal disruption and release of its contents within the cell. Hence, there have been numerous reports showing their ability to deliver gene therapy agents to a diverse array of cell types. However, like most cationic nanoparticles, toxicity and immunogenicity are common. To overcome these problems, dendrimers synthesised with biocompatible components with various lipid or targeting moieties on their surface have been developed. In 2007, Baigude, McCarroll *et al.* (24), synthesised a fourth-generation poly-L-lysine dendrimer with lipid (oleic acid) chains on its surface. The lipid moieties increased hydrophobicity of the dendrimer as well as facilitated cellular uptake. Interestingly, the authors found the optimal number of oleic acid lipid molecules on the surface of the dendrimer to be seven. This nanoparticle, termed interfering nanoparticle-7 (iNOP-7), was shown to be non-toxic and non-immunogenic in mice (24). More importantly, iNOP-7 was able to deliver siRNA with high levels of efficiency to a range of different cell types including mouse liver hepatocytes and human cancer cell lines (**Fig 2.**). The authors also demonstrated that iNOP-7 could deliver siRNA at clinically relevant concentrations

(1–5 mg/kg) to mice and silence a gene in the liver that is involved in regulating cholesterol metabolism (24). To further demonstrate the ability of iNOP-7 to deliver RNAi inhibitors, a recent study showed that iNOP-7 could complex with a miRNA inhibitor targeting miR-122. This miRNA is known to play an important role in modulating the expression of multiple genes involved in regulating cholesterol metabolism. Delivery of the iNOP-7-miRNA inhibitor was able to suppress miR-122 levels by greater than 80% in the liver of mice, resulting in a significant decrease in total cholesterol (25). More recently, dendrimers have been used to deliver a siRNA cocktail targeted against both viral and cellular transcripts in a humanised mouse model of HIV infection (26). The dendrimers were able to deliver siRNA to cells that are traditionally known to be very difficult to transfect using non-viral vectors. Administration of the dendrimer-siRNA cocktail suppressed HIV-viral loads by an average of three logs of magnitude when compared to controls (mice injected with control or non-functional siRNA). The suppression of virus production lasted for up to three weeks in mice treated with the dendrimer-siRNA complexes (26). Importantly, in mice in which the viral loads had become elevated after the initial treatment, re-treatment with the nanoparticle complex resulted in re-inhibition of the HIV viral levels. The initial or continued treatments were not toxic. Taken together, this study highlights the potential of dendrimer-siRNA complexes as a novel treatment strategy for treatment and/or long-term management of HIV.

Nanoparticles and siRNA in Clinical Trials for the Treatment of Human Disease

Since the initial discovery of RNAi, there have been over 30 clinical trials assessing the potential of siRNA as a novel therapeutic. To date, a number of trials are assessing intravenous administration of siRNA using SNALP technology (developed by Tekmira Pharmaceuticals) as the delivery vehicle. One of the first studies involving SNALPs (TKM-ApoB) was designed to silence apo B for the treatment of hypercholesterolemia (11). A Phase 1, controlled, single-blinded, dose-escalating study was undertaken to assess safety, tolerability and pharmacokinetics. No liver toxicity was observed, however, one patient who received the highest dose reported flu-like symptoms that were consistent with an immune response. Importantly, two patients showed a 20% reduction in apo B protein and LDL-cholesterol levels after a single administration when compared to placebo controls. However, the study was terminated due to the design of a new generation of SNALPs that have higher efficiency for siRNA delivery and reduced immunostimulatory effects. The same company has also commenced a Phase 1 trial to assess the efficacy of its lead oncology SNALP-siRNA compound, TKM-PLK1. For this study, SNALPs are complexed with siRNA targeting Polo-like kinase-1 (PLK-1). This gene plays an important role in regulating cell growth and is increased in a number of different cancers (27). The initial study enrolled 52 patients with advanced solid tumours. Most recently, the company released interim results showing that the treatment was well tolerated and displayed therapeutic activity, with one patient demonstrating a partial response and one patient achieving stable disease. Based on these data, patient enrollment is continuing at a dose of 0.75 mg/kg (28).

Nanoparticles designed to actively target the cell of interest are also in clinical trials. For example, Calandro Pharmaceuticals has developed a tumour-targeting nanoparticle to deliver siRNA to silence the M2 subunit of ribonucleotide reductase (RRM2) in relapsed or drug refractory human cancer (29). To direct the nanoparticle to the tumour cells, the authors coated the nanoparticle with a transferrin protein to enable binding to the transferrin receptor (which is expressed at high levels on the cell surface of tumour cells). Notably, this study was the first to demonstrate the ability of a targeted nanoparticle to deliver siRNA into a human patient with a solid tumour (29). Moreover, siRNA-induced cleavage of the target mRNA and knockdown at the mRNA and protein level was confirmed in a tumour biopsy sample after treatment.

Concluding Remarks

In slightly more than a decade, there have been significant advancements made in our understanding of RNAi and its mechanism of action. In the laboratory setting, the use of siRNA and/or miRNA inhibitors/mimics complexed to non-viral nanoparticles has led to the discovery of a host of new gene targets as potential therapeutics for human disease. As a result of these exciting discoveries, there are an increasing number of therapeutic siRNAs using non-viral nanoparticles in human clinical trials, thus illustrating the high hopes held for this technology as a therapeutic agent. However, there are still significant challenges that need to be addressed before RNAi therapeutics become a reality in the clinic, namely, delivery of the nanoparticle-siRNA complexes to their target cell, while avoiding off-target or immune-stimulated side effects. Future studies will also need to consider the role of the microenvironment of the disease and how this may influence the delivery of nanoparticle complexes to their target. Also, biomarkers (preferably non-invasive) to validate RNAi-directed activity against the target mRNA need to be developed to confirm that any effect on the disease is due to the RNAi agent. Collectively, the ever-expanding development of nanoparticles tailored for the targeted delivery of siRNA and improved modifications to siRNA to increase their specificity and potency give us hope that together these technologies will become a powerful therapeutic for human disease in the not too distant future.

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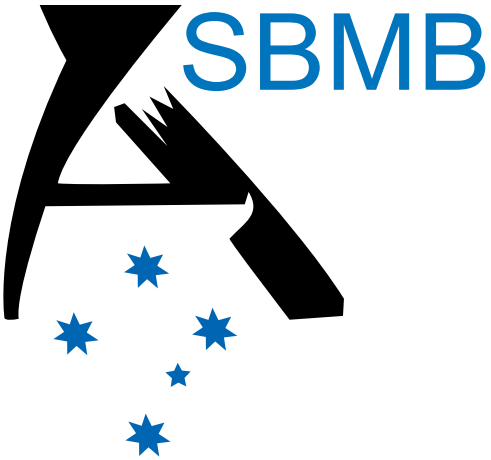

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