A Big Future for Small Delivery: The State of the Art of RNAi Research

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The strong potential for double-stranded interfering RNAs as therapeutics has driven significant research since the initial discovery that siRNAs could induce sequence-specific gene silencing. This review provides an overview of some of the current challenges and successes in siRNA research and the advantage of sharing research reagents and data for advancement of the field.

With a limited number of companies capable of synthesis and scale-up of customizable small interfering RNAs (siRNAs), along with the growing interest in evaluating siRNA therapeutics in a wide variety of cell and tissue models, the importance of increasing the ease of access to these oligonucelotide reagents becomes increasingly important. This mini-review seeks to detail the state of the art of RNA interference (RNAi) research, summarize high-impact findings in nanoparticle-siRNA studies, and identify the potential challenges that participating biotechnology companies and academic researchers may face due to the considerable overlap of siRNA delivery technologies. Finally, we seek to illustrate how increased ease of reagent sharing and material transfer agreements may facilitate the clinical development of these candidates by avoiding research disputes and time lost to redundant studies.

Since the landmark discovery that synthetic double-stranded interfering RNAs (siRNAs) could induce sequence-specific gene silencing, the potential for siRNAs as therapeutics has piqued the interest of scientists, and this was supported further by the award of the Nobel Prize in 2006 for RNAi to Andrew Fire and Craig Mello (1, 2). If delivered correctly, siRNAs enter cells and utilize the RNAi pathway by a coordinated separation into single strands, one of which binds to its complementary single-stranded messenger RNA (mRNA) encoding the protein of interest, thus preventing its translation through a post-transcriptional "interference" (3-5). This ability to accurately silence a target gene through a consistent and controlled mechanism gives siRNAs tremendous promise as potential drug candidates.

However, administering these nucleic acids in their "free" form to interfere with mRNA-protein translation has proven to be immensely challenging, both in *in vitro* and *in vivo* animal models for several reasons. Passive uptake of siRNA by target cells is generally very low (6, 7). After intravenous administration, a significant amount of the siRNA dose is rapidly removed from circulation by hepatic and renal clearance. In addition, siRNAs are subject to degradation by endogenous nucleases and lysozymes. Beyond these hurdles siRNA must reach the cytoplasm to effectively interfere with mRNA translation, and since they are commonly taken up by endocytotic mechanisms, the siRNAs must avoid becoming trapped in subsequent endosomal compartments where they are tagged for lysosomal degradation (8, 9). Recent research has commonly referred to overcoming this obstacle as improving the "endosomal escape" (8, 10).

The delivery of siRNA therefore represents a rate-limiting step for scientists in the already laborious process of bringing new therapies to large-animal models, clinical trials, and eventually therapeutic use and commercialization. To this end, a variety of nanoparticles (NPs) have been developed to increase local and systemic delivery by encapsulating siRNA and protecting it from clearance or degradation. In addition, siRNAs themselves may be chemically modified to increase their stability, or to further optimize their encapsulation with NPs. Next generation NP-siRNAs could be functionalized with targeting ligands which increase specificity, thus limiting vehicle-associated side effects (i.e. liver toxicity due to passive uptake of NP-siRNAs in the liver). Finally, NP-siRNAs should be developed such that the maximum amount of siRNA cargo escapes the endosomes and reaches the cytoplasm to

effectively interfere with mRNA-protein translation. Together these issues comprise much of the safety and efficacy guidelines required by the FDA and thus undoubtedly represent key components for the development of future therapies. Indeed, biotechnology companies seeking new therapeutics have applied for and obtained hundreds of patents directed to the goal of safe and effective siRNA delivery through siRNA chemical modifications and lipid NPs (LNP)-siRNAs (11).

While several methods of NP delivery are currently being investigated worldwide, including recombinant lipoprotein NPs (12, 13), chemically modified siRNAs (14), and metal-core NPs (15), this review will focus on the state of polymeric LNPs. This family of LNPs has shown arguably the most consistent and promising therapeutic results thus far and furthermore can be precisely synthesized using copolymer blending and lipid chemistry (16, 17).

Pre-clinical research in LNP-siRNA therapeutics has generally fallen into the following areas:

a) High-throughput studies of "libraries" of LNP-siRNAs and their ability to silence a target gene at the mRNA and protein level in parallel *in vitro* studies. In the context of polymeric lipid NP-siRNAs, precise engineering of different compounds can be achieved through polymer chemistry, creating discrete differences along a common theme.

b) Evaluating successful particles in these initial high-throughput studies, and using this information in a "feed-forward" system to rationally design more focused LNP-siRNA candidate libraries. For instance, an initial broad evaluation of LNP-siRNAs found that successful candidates commonly had cationic lipid components (18). This information was used to design a second-generation study of LNPs carrying variations of functional cationic lipid components, along with investigation into what mechanisms were responsible for the increased effectiveness (19). Indeed greater levels of gene and protein silencing were achieved at lower doses than previously used.

c) *In vivo* therapeutic efficacy studies in relevant pre-clinical disease models with goals of reducing specific disease components, along with consideration for biodistribution of LNPs and off-target organ effects.

Therapeutic studies are long-term and generally seek high efficacy in silencing target proteins through appropriate organ disease models (i.e. atherosclerotic plaque reduction or restoration of insulin sensitivity in diabetes) at the lowest possible doses, primarily due to the fact that administration is frequently intravenous. Furthermore, progression of therapeutic studies requires the characterization of the mechanism of uptake, among other things, as a means of understanding and minimizing off-target effects. These criteria are the hallmarks of FDA approval and thus must be satisfied if clinical testing can occur.

Several high-impact publications using polymeric LNP-siRNAs have been released both in academic and industry laboratories in the last few years, highlighting the remarkable progress made in the relatively short time since RNAi discovery. By no means an exhaustive list, the following papers represent key points in the progression of these technologies:

1. Wolfrum C et al, *Nature Biotechnology 2007*. In collaboration with Alnylam Pharmaceuticals, researchers from Switzerland and Rockefeller University demonstrate how and why lipophilic molecules and LNPs can be used to deliver siRNA, and how chemical modifications of the siRNA itself can increase its stability (14).
2. Frank-Kamenetsky et al; *PNAS 2008.* A highly effective liver-targeting LNP-siRNA candidate from a previous screening study published in Nature Biotechnology (18, 20) shows remarkable effectiveness in delivery of PCSK9 siRNA. PCSK9 is an enzyme recently discovered to have a key role

in cholesterol homeostasis, and treated subjects demonstrated lower total plasma cholesterol specifically by decreasing LDL or "bad cholesterol", without affecting HDL levels in rodent, primate, and human models. This method of low-dose, long lasting delivery represents a landmark study, as PCSK9 is currently in late-stage clinical trials with huge interest for development as highlighted by a recent article in the New York Times *(Rare mutation leads to race for cholesterol drug)*. This study was done primarily in-house at Alnylam Pharmaceuticals in collaboration with MIT (21).

3. Semple SC and Akinc A; *Nature Biotechnology 2009*. Moving forward with the effective cationic LNPs, a feed-forward design of cationic LNPs to create more "stable" nucleic acid particles (SNALPs) was performed. Results indeed show ability of these SNALPs to silence *in vivo* at very low doses of 0.01 mg/kg in rodents and 0.1 mg/kg in primates. In addition, a proposed model for how cationic lipids allow endosomal escape of NP-siRNAs and thus explain their increased efficacy is presented. Interestingly, this study was conducted by both Tekmira Pharmaceuticals and Alnylam Pharmaceuticals, who became involved in an extended legal dispute wherein Alnylam agreed to a settlement in 2011 of more than $65 million after being accused of sharing RNAi trade secrets without consent from Tekmira (19).
4. *Love and Mahon et al; PNAS 2010.* While gene silencing appears to be feasible using LNP-siRNAs, authors sought to expand on the efficacy of siRNA delivery by developing LNPs designed for maximal silencing at lowest dosage, to make them more feasible and attractive candidates for human use. The design of a library of "lipid-like" materials created using epoxide chemistry showed greater than 70% silencing at doses as low as 0.03 mg/kg in nonhuman primates. Notably this is more than 300 times less carrier material compared to the previous studies (22, 23).
5. Leuschner F et al; *Nature Biotechnology 2011.* An extensive therapeutic study done in collaboration between laboratories at MIT and Harvard, using materials licensed from Alnylam. Authors show success of four different LNP-siRNAs to reduce therapeutic endpoints in four different critical disease models: atherosclerosis, myocardial infarct, type I diabetes, and tumor size in lymphoma (24).

Intriguingly, these publications illustrate that siRNA therapy is limited by the need for unique and specific delivery for each protein, cell, or tissue. While all studies use lipid-like materials of similar size (80-100nm), different libraries of targeting moieties are uniquely effective in liver cells and circulating monocytes. This has naturally resulted in a wide variety of overlap in siRNA therapeutic research and patent applications, as investigators seek to produce results in their respective areas of expertise which often have shared themes, i.e. atherosclerosis, liver/kidney disease, or immunology. For instance, Alnylam currently owns over 700 patents related to siRNA and LNPs based on their early research. Tekmira Pharmaceuticals similarly owns an array of patents for LNP/SNALP therapies as well as the methods of production used.

Since the process of RNAi therapeutics is such that they must be unique to different proteins or tissues (i.e. targeting fibroblasts in kidney vs. targeting inflammatory monocytes in circulation), there is not likely to be a "one size fits all" siRNA delivery product. For academic scientists, this provides the opportunity for exciting pre-clinical research, as laboratories may seek to develop new products in their area of expertise, or even augment work they are doing which involves siRNA knockdown. However this requires licensing and transfer agreements to obtain the reagents needed, and thus with the limited number of companies capable of synthesis/scale-up of customizable siRNAs or NP vehicles, material transfer agreements (MTAs) become critically important. They can either help facilitate a dramatic increase in collaboration between researchers through access to reagents, or rather conflict and litigation (as previously illustrated) due to the desire to keep potentially lucrative findings private. In the review we have identified a small subset of companies in the broad field of siRNA research (Alnylam and Tekmira) who currently own hundreds of closely related patents with LNPs/siRNAs which are

licensed to companies such as Merck, Roche, and Genzyme, and are shared through MTAs with academic institutions (MIT, NYU, etc.). As one can imagine, this can potentially lead to several problems:

1) Extended negotiations for MTAs for LNP-siRNA reagents, which may be individually required for each fairly similar reagent due to its patent specifications.
2) Extensive delays in publication due to the review period that most MTAs involve.
3) Reduced incentive for academic scientists to improve on current LNP-siRNA research due inability to publish or present findings because of restrictions in confidentiality clauses and loss of ability to generate intellectual property (IP) on their inventions.
4) Reduced awareness and collaboration between academic laboratories due to confidentiality specifications. Other laboratories may be more adept at solving particular issues or have more expertise in a specific area of interest, but confidentiality restrictions prevent consulting with researchers not under the MTA.

While it is intuitive that researchers and institutions would gain certain advantages by restricting access to their research to maintain trade secrets and protect patent rights, it should be noted that Alnylam and Tekmira have arguably been among the most successful companies active in the current siRNA landscape, as both companies have multiple RNAi products in stage 2 and 3 clinical trials (25, 26). They have also been among the most active in sharing reagents with academic institutions such as MIT, Harvard, Mount Sinai, and NYU through MTAs. It is thus reasonable to attribute a considerable amount of this success in advancing their products through the clinical trial stage to these participations with academia, particularly since all early stage published discovery research was done in collaboration with academic institutions.

Work produced by these collaborations has spurred new research from other laboratories that can build on previous discoveries using their specific expertise, such as inflammatory immune response (27, 28) or cell uptake and trafficking (10, 29-31), to advance the field in a way that would not be possible with the limitation of reagents. For example, researchers at Brigham and Women's Hospital have developed two drugs currently in clinical trials through the sharing of information and reagents between MIT, Alnylam (itself an MIT spinoff), and Harvard/Brigham and Women's.

In addition to the positive advancements made possible by increased ease of material transfer, it is also important to recognize the benefits of increased collaboration in expediting studies with *negative* results. For example, high-density lipoprotein (HDL, commonly known as "good cholesterol") was thought to provide an ideal, natural vehicle for siRNA delivery to reduce atherosclerosis. HDL's function is to remove excess cholesterol from peripheral sites such as atherosclerotic plaques, primarily by removing cholesterol from plaque macrophage cells. Since HDL naturally "targets" these lesions and can bind modified siRNAs, it was thought to be an excellent candidate for delivery of anti-atherogenic siRNAs to the plaque specifically. However a variety of studies were performed by our lab and others (32, 33) which demonstrated HDL nanocarriers for siRNA were in fact completely ineffective at siRNA silencing of mRNA in macrophages due to receptor-specific cell uptake mechanisms.

The total span of these research findings can conservatively be estimated as from 2006 to 2013. While these research projects are similar in theme, studies were performed at laboratories located across the United States (Texas, University of Chicago, Alnylam) and siRNA reagents were obtained through vastly different methods: commercially purchased, MTAs with companies (themselves involved in similar research), and in-house fabrication. Thus these negative findings took significantly longer than necessary, as studies were essentially performed in "series" based on published work, instead of in parallel based on shared information. This resulted in a significant cost of grant funds and time spent by talented scientists.

While therapeutic siRNA development is fairly advanced, a significant benefit can be obtained by the scientific community through working with institutions such as Kerafast that can expedite and

facilitate reagent sharing, thereby increasing the rate of research and publications, improving clarity of agreements involving intellectual property, and increasing global awareness of relevant scientific findings. Furthermore, the increased ease of reagent sharing through such institutions would provide greater diversity of NP-siRNA research by allowing rapid access to laboratories with specific research interests and expertise and thus more focused studies. Certainly this is an idea that is not unique to the field of NP-siRNA therapy, and can most likely be applied to other developing therapeutics as well.

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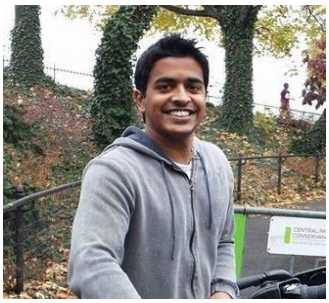

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