EDITORIAL

Focus on RNA interference: from nanoformulations to \textit{in vivo} delivery

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Focus on RNA interference: from nanoformulations to in vivo delivery

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Almost eleven years ago, Andrew Z Fire at Stanford University, California, USA, and Craig C Mello at the University of Massachusetts Medical School in Worcester, USA shared the 2006 Nobel Prize in Physiology or Medicine for their discovery that double-stranded RNA (dsRNA) triggers suppression of gene activity in a homology-dependent manner, a process named RNA interference (RNAi) [1, 2].

RNAi is a natural cellular process by which double-stranded RNA (dsRNA) suppresses the expression of specific genes bearing complementary nucleotide sequences, a process mediated by the RNA induced silencing complex (RISC) [3]. RNAi can be activated exogenously by expressing short hairpin RNA and micro RNA (miRNA) duplexes with viral vectors, or by incorporating synthetic small interfering RNAs (siRNAs), dicer substrate RNA (dsRNA), double stranded miRNA mimetics molecules, and anti-miRNA oligonucleotides (antagomirs) directly into the cell cytoplasm.

siRNAs, the most investigated form of RNAi and the most advanced in the clinic are chemically synthesized RNA duplex molecules of 19–23 nucleotides long that are substrates of the RISC in the cytoplasm, similarly to endogenous RNAi components [4]. The RISC separates the two siRNA strands and keeps only the guide strand conferring the affinity towards targeted mRNA molecules through base pairing. This process is followed by the attachment of guide RNA–RISC complex to mRNA and gene inhibition via mRNA cleavage by RISC endonucleases. Next, the guide RNA–RISC complex is once again free to bind and degrade a new copy of target mRNA. The sequence-dependent mechanism of gene inhibition by siRNAs enable these molecules to target virtually any gene of interest, including those traditionally considered as ‘undruggable’ (proteins with structural homology to important cell factors, or lacking ligand binding domains), thus siRNA emerged rapidly as a highly promising therapeutic platform in personalized medicine [5].

On 20th October 2017, Alnylam Pharmaceuticals in Cambridge, MA USA, together with Sanofi in Paris reported on a successful Phase III clinical trial of Patisiran™. Patisiran™ is an investigational intravenously administered RNAi therapeutic drug targeting transthyretin for the treatment of hereditary ATTR amyloidosis with polyneuropathy. The clinical trial met all endpoints and it is likely it will be approved by the FDA as the first RNAi drug. It took more than 15 years to advance a scientific discovery made in 2001 by Elbashir et al [4] before it was successfully translated to the clinic; small RNAs can induce potent gene silencing in mammalian cells.

Utilizing RNAi for therapeutics is not a trivial task. Due to the large molecular weight (13 kDa), the net negative charge and their hydrophilicity, the efficiency with which naked molecules of siRNA cross the plasma membrane and enter the cell cytoplasm is very low [6, 7]. When injected intravenously, in addition to rapid renal clearance and susceptibility to degradation by RNAses, unmodified naked siRNAs are recognized by Toll-like receptors. This often stimulates the immune system, hence provoking an interferon response as well as complement activation,
cytokine induction, and coagulation cascades. Besides the undesired immune activation, these effects can suppress gene expression globally, generating off-target and misinterpreted outcomes \cite{7,8}. Most of these obstacles are relevant for other naked RNA molecules such as dsRNA, miRNA mimetics and antagonirs. Therefore, there is a clear need for appropriate delivery systems for RNAi, all of which have to utilize cellular mechanisms for internalization, cargo release (from the carriers and escape from the endosomes), cargo appropriate delivery system for RNAi molecules would protect them from rapid clearance or degradation, minimize their potential immunogenicity and allow their release into the cell cytoplasm.

Since the discovery of RNAi's therapeutic potential, numerous RNAi delivery designs have arisen, which have tried to address the delivery challenges \cite{9}. Lipid based nanoparticles are the most advanced delivery platform to date for systemic administration of RNAi therapeutics \cite{10} (Patisiran™ is based on LNPs). LNPs are formed by a controlled mixture of different lipids that self-assemble into 20–200 nm diameter particles when mixed together with nucleic acids.

The LNP formulation includes cationic lipids, which provide efficient encapsulation of the anionic RNAi molecules. In addition, the cationic lipids provide the LNPs a positive surface charge that promotes interaction with the negatively charged plasma membrane, resulting in enhanced cellular uptake via endocytosis \cite{11}. Furthermore, cationic lipids have been hypothesized to facilitate siRNA endosomal escape into the cytoplasm following cellular internalization, thanks to the formation of ion pairs with anionic lipids in the endosome, which disrupt both the LNP and the organelle membrane \cite{12}. Nevertheless, the use of cationic lipids has major drawbacks. Particles with a positive charge are easily cleared by macrophages of the RES system and consequently have a short circulation time. In addition, toxicity can be manifested by hepatocellular necrosis, leukopenia, and potent inflammatory responses such as complement activation and cytokines’ induction \cite{13,14}.

To address these disadvantages, new generations of lipids were developed. These are termed ionizable lipids \cite{15–17}. They possess an amine in their headgroup and have an apparent pKa less than seven. The use of ionizable lipids allow efficient encapsulation of siRNAs in acidic buffer, while their neutral charge in physiological conditions provide long circulation times and low toxicity \cite{11}.

Ionizable lipids that exhibit exceptional siRNA encapsulation and silencing efficacy were developed. The first drug that is likely to be approved after a successful phase III Patisiran™ include a special cationic ionizable lipid named Dlin-MC3-DMA, whose potency has been demonstrated in hepatocytes \cite{15–19}. So now that the delivery of RNAi into the liver has progressed clinically, it is time to go beyond the liver.

Several strategies utilize MC3 cationic ionizable lipids as the core of the LNPs and are their surface of the LNPs is decorated with targeting ligands such as hyaluronan that targets CD44 expressing tumors, for example in glioblastoma multiform or that target lymphocytes (T or B cells using specific monoclonal antibodies targeting CD4 expressing T cells or CD38 expressing B cells or hematological B cells such as Mantle Cell Lymphoma or Multiple Myeloma) \cite{20–22}.

In this RNAi collection we focus on novel strategies to target cells beyond the liver.

Ernst Wagner and colleagues show in vitro an elegant approach to target EGF receptor expressing tumors using T-shaped lipo-oligomer 454 that was used to complex RNA into a core polyplex, which was subsequently functionalized with the targeting peptide ligand GE11 via a polyethylene glycol (PEG) linker. To this end, free cysteines on the surface of the polyplex were coupled with a maleimide-PEG-GE11 reagent (Mal-GE11). Resulting particles with sizes of 120–150 nm
showed receptor-mediated uptake into EGFR-positive T24 bladder cancer cells, MDA-MB 231 breast cancer cells and Huh7 liver cancer cells. Furthermore, these formulations led to ligand-dependent gene silencing [23].

Erik Luijten and colleagues have shown a novel method to enhance the condensation of siRNA with PEGylated linear polyethylenimine (PEI) using organic solvents and have prepared smaller siRNA nanoparticles with a more extended PEG corona and consequently higher stability. As a proof of principle, they demonstrated improved gene knockdown efficiency resulting from reduced siRNA micelle size in mice livers following intravenous administration [24].

Kenneth A Howard and colleagues investigated the effect of a specific cysteine site-selective covalent attachment of a factor IXa anticoagulant aptamer on aptamer functionality and human FcRn (hFcRn) engagement using recombinant human albumin of either a wild type or an engineered hFcRn high binding variant (HB). This work describes a novel albumin-based aptamer delivery system whose hFcRn binding can be increased using a HB engineered albumin [25].

Vincent Rotello and his team report on nanoparticle-stabilized capsules (NPSCs) as a platform for the co-delivery of survivin-targeted siRNA and tamoxifen. These capsules feature an inner oil core that provides a carrier for tamoxifen, and is coated on the surface with positively charged nanoparticles self-assembled with siRNAs. The NPSC co-delivery of tamoxifen and survivin-targeted siRNA into breast cancer cells disables the pathways that inhibit apoptosis, resulting in enhanced cell death [26].

Finally, Olivia Merkel and her team investigate micelleplex made from tri-block copolymer PEI-polycaprolactone-PEG (PEI-PCL-PEG), and their in vitro and in vivo fate following microfluidic preparation of siRNA nanoparticles compared to the routinely used batch reactor mixing technique. The use of microfluidic mixing techniques yields an overall smaller and more uniform PEG-PCL-PEI nanoparticle that is able to more efficiently deliver siRNA in vivo. This preparation method may be useful when a scaled up production of well-defined polyplexes is required [27].

These studies may open new avenues for improved formulations, encapsulation and cell-specific targeting toward diseased cells while leaving healthy cells untouched.

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