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Overcoming cellular barriers for RNA therapeutics

Steven F Dowdy

RNA-based therapeutics, such as small-interfering (siRNAs), microRNAs (miRNAs), antisense oligonucleotides (ASOs), aptamers, synthetic mRNAs and CRISPR–Cas9, have great potential to target a large part of the currently undruggable genes and gene products and to generate entirely new therapeutic paradigms in disease, ranging from cancer to pandemic influenza to Alzheimer's disease. However, for these RNA modalities to reach their full potential, they first need to overcome a billion years of evolutionary defenses that have kept RNAs on the outside of cells from invading the inside of cells. Overcoming the lipid bilayer to deliver RNA into cells has remained the major problem to solve for widespread development of RNA therapeutics, but recent chemistry advances have begun to penetrate this evolutionary armor.

Because of the high selectivity for their target RNA or DNA, over the past decade RNA-based therapeutics have witnessed an explosion of interest in academia and industry. These drugs offer a wide ability to selectively drug the undruggable human and viral genomes to knock down gene expression, to alter mRNA splicing, to target trinucleotide repeat disorders, to target non-coding RNAs (ncRNAs) involved in transcriptional and epigenetic regulation, to upregulate target genes, to express genes and to edit the genome. These are all forms of therapeutic intervention that we could never dream of ever achieving with small-molecule inhibitors or antibodies. RNA-based therapeutics are also the only modality with the ability to 'pharmacoevolve' to keep pace with, for example, cancer mutations and pandemic viral infections. The development pathway timeline of RNA-based therapeutics closely parallels the development of other groundbreaking therapeutic modalities, such as antibodies, where hyperbolic inception is followed by grand failure and ultimate success after technology maturation, as new generation molecules overcome the limitations of pioneer drug molecules. Similarly, second-generation RNA chemistries have vastly improved the stability of RNA therapeutics, reduced unintended off-target effects, and maximized on-target pharmacologic activity. For example, by developing and incorporating new chemistries into their ASOs, Ionis Pharmaceuticals (Carlsbad, CA, USA) is treating multiple central nervous system (CNS) indications for which no other therapeutic option existed previously[1,2]. Likewise, RNA interference (RNAi) biotech companies, such as Alnylam Therapeutics (Cambridge, MA, USA) and Solstice Biologics (San Diego), are seeing single-dose pharmacodynamic (PD) effects (gene knockdown) in excess of six months on liver target genes using second-generation enhanced stabilizing RNA chemistries (ESC)[3] and third-generation phosphotriester RNA chemistries, respectively (C. Bradshaw, personal communication). These new chemistries confer drug-like attributes to RNAs that have turned the poorly performing field of ten or more years ago into the almost-ready-for-prime-time, must-have therapeutic platform of tomorrow.

However, for all the enthusiastic upside surrounding the potential of RNA-based therapeutics, there continues to be an equal and opposite downside that started more than a billion years ago, namely the delivery problem. Life started on this planet ~4 billion years ago when the primordial RNA and macromolecular soup became encapsulated by a lipid bilayer that allowed chemical reactions to take place inside without interference from RNAs and macromolecules on the outside[4–7]. Lipid bilayers allow small neutral, slightly hydrophobic molecules <1,000 Daltons (Da) to passively diffuse across them, while preventing large, charged molecules, like RNAs, from crossing them[7,8]. Thus, the lipid bilayer was both fundamental in creating life and in protecting it from invading RNAs. Layered on top of this billion-year-old barrier are a series of evolutionary defenses designed to further protect metazoan cells from invading RNAs, including RNases and the innate immune pattern recognition toll-like receptors (TLRs) 3, 7 and 8, present on the outside of cells, and double-stranded RNA receptors PKR, retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated protein 5 (MDA-5) on the inside of cells[9]. Furthermore, naked charged RNAs are rapidly cleared from the blood by the kidneys[10] and scavenger receptors on liver hepatocytes[11]. Consequently, to successfully deliver RNA-based therapeutics, we need to tackle a billion year's worth of evolutionary defenses (**Table 1**).

Of all these barriers, delivery across lipid bilayers remains 'the problem' to solve. Small-molecule inhibitors for the most part have low-molecular weight (<1 kDa), low to no charge and enough hydrophobicity (logP value) to allow them to gently slip across the cell membrane's lipid bilayer[12]. In contrast, all RNA-based therapeutics are large and/or highly charged macromolecules that have no ability to cross lipid bilayers, and range in size from 4–10 kDa for single-stranded ASOs, to ~14 kDa for double-stranded siRNAs, to ~200 kDa for CRISPR–Cas9 sgRNAs to 700–7,000 kDa for self-replicating mRNAs (**Fig. 1**).

RNA-based therapeutics are macromolecules taken up by endocytosis[11,13], but remain trapped inside of the endosome, behind the lipid bilayer and as such, are outside of the cytoplasm and nucleus. The analogy is that of your recently masticated lunch, which is inside your stomach (and you), but remains biologically outside of your bloodstream and other tissues. Although there are many types of endocytosis, including clathrin, caveolae, phagocytosis, macropinocytosis and others[13], the problem of getting across the endosomal lipid

Department of Cellular and Molecular Medicine, University of California, San Diego, School of Medicine, La Jolla, California, USA. Correspondence should be addressed to S.F.D. (sdowdy@ucsd.edu).

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Table 1 Challenges erected by evolutionary barriers to RNA therapeutic delivery

Feature	Challenge for delivery
Oligonucleotide size and charge	Too large or too charged to passively diffuse across the lipid bilayer
RNase susceptibility	Rapid degradation by blood and tissue RNases.
Reticuloendothelial system	Rapid clearance from the blood by the kidneys and liver scavenger receptors
Immunogenicity	Oligonucleotides activate extracellular and intracellular innate immune responses
Endocytosis	Oligonucleotides are taken up, but trapped inside endosomes

bilayer remains the same for all forms of endocytosis. Thus, getting RNA-based therapeutics out of the endosome and into the cytoplasm in a non-toxic manner is the key technological problem to solve before we can tap into the full potential of RNA-based therapeutics (for a detailed review on delivery issues, see ref. 14).

Delivery of single-stranded ASOs

The field of single-stranded ASOs started in 1978 in Paul Zamecnik's laboratory with the synthesis of a 13-mer ASO containing a phosphodiester backbone[15,16]. Unfortunately, phosphodiester linkages are highly charged, hydrophilic and susceptible to rapid degradation by nucleases, so these ASOs quickly hit all of the delivery barriers listed in **Table 1**. Fortunately, prior work in 1966 by Fritz Eckstein[17] on modification of mononucleotides where one oxygen of the phosphate group was substituted with a sulfur atom, termed a phosphorothioate (**Fig. 2**), resulted in a significantly increased resistance to phosphodiesterases and a serendipitous increase in hydrophobicity. In 1984, Stec, Zon and Egan[18] synthesized the first fully phosphorothioate ASO, which resulted in a dramatic increase in stability and deliverability that was reproduced by others[19–21]. Phosphorothioate backbone ASOs also avidly bind serum proteins such as albumin that serves as a carrier protein to greatly increase the ASOs pharmacokinetics (PK) profile[21] as well as binding to intracellular proteins[22,23]. In contrast, phosphodiester ASOs bind poorly to albumin, are rapidly degraded by RNases and are cleared by the kidneys. Consequently, it cannot be overstated how important the incorporation of phosphorothioate chemistry into oligonucleotides is for the ASO field.

Because phosphorothioates are chiral centers, a 21-mer ASO is actually a mixture of ~10^6 stereoisomers[21]. In a bid to increase potency, Stec *et al.*[24] investigated aspects of phosphorothioate stereoisomers on ASO function. This was followed by synthesis of stereospecific phosphorothioate ASOs by Wada's group[25], which served as the basis for starting WaVe Life Sciences (Singapore). Others have, however, reported that a mixture of phosphorothioate ASO stereoisomers maintains optimal activity[26]. This remains an important area for further investigation.

Additional important chemical modifications to improve the potency and pharmacologic properties of ASOs include modifying the 2′-hydroxyl (OH) to 2′-*O*-methyl (O-Me), 2′-fluoro (F), 2′-methoxyethyl (MOE) or bicyclics that contain a 2′,4′-*O*-methylene bridge[27] (otherwise known as locked nucleic acid (LNA); **Fig. 2**). The use of bicyclic LNAs increases the binding affinity to target mRNA (measured as melting temperature (Tm)) and thereby reduces the overall length of the ASO. Collectively, these chemical modifications serve to further improve ASO stability, binding avidity to the target RNA and decrease the ASO length, thereby aiding with delivery. Not surprisingly, most, if not all, ASOs in pre-clinical development and clinical trials today contain fully phosphorothioate backbones with extensive 2′ modifications.

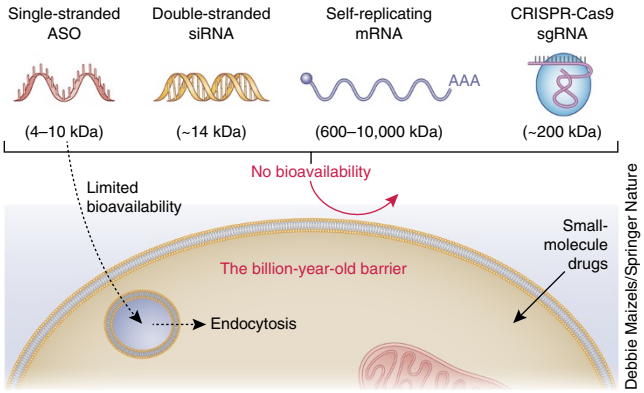

Figure 1 The four-billion-year-old lipid bilayer protects cells from invading RNAs. Unlike small-molecule drugs that can slip across the lipid bilayer, with the exception of some single-stranded phosphorothioate ASOs that can productively enter cells, the vast majority of RNA-based therapeutics are too charged and/or too large to enter cells, and require a delivery agent.

Like all macromolecular therapeutics, naked (unconjugated) ASOs are taken up into cells by endocytosis[13]. However, unlike naked double-stranded siRNAs that have no ability to escape across the endosomal lipid bilayer barrier, due to the increased hydrophobicity of the phosphorothioate backbone, ASOs slowly cross the lipid bilayer to escape endosomes into the cytoplasm and nucleus by an unknown mechanism, termed gymnosis[28–31]. The importance of this process is that ASOs enter the cytoplasm of cells without a delivery agent[30]. This substantial advantage has resulted in numerous clinical trials with ASOs targeting liver and the CNS[32].

For multiple reasons, including direct blood access due to the liver's architecture and rapid endocytosis, hepatocytes are highly receptive to ASO uptake. However, to enhance ASO hepatocyte delivery, Seth, Henry and collaborators[33,34] conjugated a tris *N*-galactosamine (GalNAc) targeting domain to ASOs and observed a tenfold enhancement of activity with a broad range of ASOs. The GalNAc targeting domain was initially developed in the late 1990s in Erik Biessen's laboratory[35] and binds to trimeric asialoglycoprotein receptors (ASGPRs), which are highly abundant on hepatocytes (>10^6/cell)[36]. These observations build on the improvements of second-generation ASO chemistries and point to the potential of ASO therapeutics conjugated to targeting domains.

ASOs have no ability to cross the blood-brain barrier (BBB). However, Smith *et al.*[37] discovered that an intrathecal ASO administration into the cerebrospinal fluid (CSF) enabled broad distribution of the drug throughout the brain parenchyma where it was taken up by neurons, and other cell types, and escaped from endosomes into the cell body[37]. Following up on this observation, multiple academic groups have used ASOs to treat pre-clinical models of Huntington's disease (HD)[38,39], amyotrophic lateral sclerosis (ALS)[40–42] and spinal muscular atrophy (SMA)[43,44]. Not surprisingly, there are currently three ongoing commercial CNS clinical programs on HD, ALS and SMA. Following the publication of papers with encouraging results from a phase 2 SMA study[1,2] and interim data analysis from a phase 3 study on infants with SMA (ENDEAR), the US Food and Drug Administration (FDA) recently approved Biogen (Cambridge, MA, USA)/Ionis' nusinersen (Spinraza), a splice-switching oligonucleotide (SSO), and the drug is also awaiting a decision at the European Medicines Agency (London). Although these clinical successes are encouraging for CNS applications of ASO, the delivery

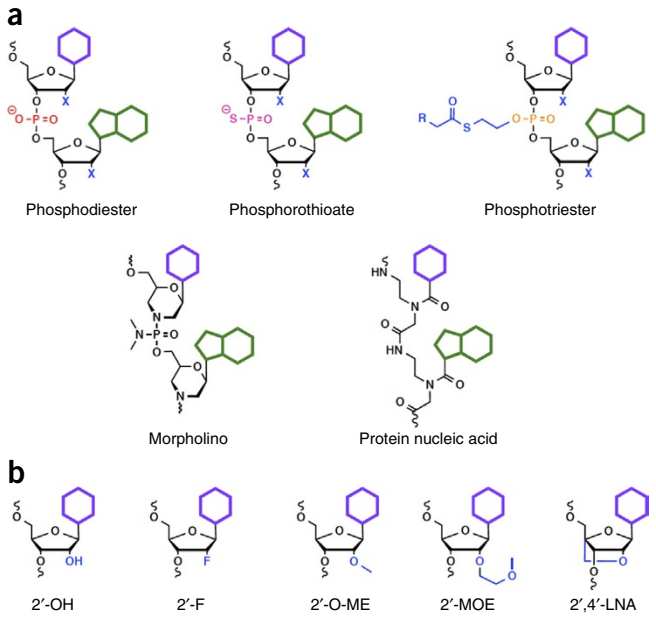

Figure 2 Common ASO and siRNA modifications. (**a**) Phosphate backbone modifications: native, anionic charged phosphodiester (achiral phosphorus atom); charged phosphorothioate (phosphorus atom is chiral); neutral phosphotriester (phosphorus atom is chiral, but becomes achiral after intracellular conversion to charged phosphodiester); neutral morpholino backbone (PMO) and peptide nucleic acid (PNA) backbones align nucleobases with native mRNA nucleobase spacing. (**b**) Common 2′ modifications of the sugar: native 2′-hydroxyl (OH), 2′-fluoro (F), 2′-hydroxymethyl (O-Me), 2′-methoxyethyl (MOE) and 2′,4′-bicyclics that contain *O*-methylene bridge or locked nucleic acid (LNA).

mechanism that these drugs use to cross from the CSF barrier into the brain parenchyma remains entirely unknown. Therefore, further efforts to unravel the ASO uptake mechanism may lead to even greater opportunities for enhancing the application of phosphorothioate ASOs in the CNS.

Two alternative antisense chemistry approaches substitute the charged phosphate linkage and ribose sugar backbones for a neutral phosphorodiamidate morpholino oligomer (PMO)[45] or peptide nucleic acid (PNA)[46] backbone that maintains the correct nucleobase spacing (**Fig. 2**). The net result is highly stabilized molecules that selectively and avidly bind their target mRNA. However, because of the absence of a phosphodiester backbone, PMOs and PNAs are unable to activate RNase-H (required for gene knockdown) and are instead, used as steric blockers and as SSOs for exon-skipping[47]. Several groups have used PMOs and PNAs as splice skipping and/or correction agents in pre-clinical mouse models of Duchenne muscular dystrophy (DMD) resulting in expression of a truncated, but partially functional dystrophin protein with correction of the disease[48–50]. Because of the absence of dystrophin, the muscle cell membranes are highly unstable and somewhat permeable, allowing enhanced levels of delivery[48]. This is an unusual example where delivery of the PMO into the target muscle cells is greatly facilitated by the disease state itself. Based on strong pre-clinical results Sarepta Therapeutics (Cambridge, MA, USA) developed a 30-mer PMO, eteplirsen (Exondys 51), to treat DMD. Although the results in a limited cohort of DMD patients were much less spectacular than the pre-clinical results, resulting in only a minimal increase in patient dystrophin expression (http://www.fda.gov/AdvisoryCommittees/Calendar/ucm490665.htm), the FDA nevertheless decided to grant eteplirsen conditional approval.

Collectively, ASO delivery successes to date are a result not only of new oligonucleotide chemistries that effectively address some of the challenges outlined in **Table 1**, but also of opportunism in exploiting ASO PK and the unique properties of diseased tissues. However, many, if not most, tissues and cell types remain *de facto* unreachable by current ASO delivery approaches. The dramatic success of the GalNAc conjugates points the phosphorothioate ASO field in the direction of conjugating cell-type-specific targeting domains.

Delivery of double-stranded siRNAs

The field of double-stranded, short-interfering RNA (siRNA)-induced RNAi responses in mammals exploded onto the scene in 2001 with work from Tuschl's laboratory[51] that followed up on Fire and Mello's Nobel-prize-winning discovery of RNAi in 1998 in worms[52]. Unlike ASOs that directly bind to their cognate mRNA target in an unaided fashion and can thereby incorporate exotic chemistry modifications, siRNAs are inactive until loaded by trans-activation responsive (TAR) RNA-binding protein (TRBP) into their catalytic counterpart, Argonaute (Ago2)[53]. After loading into Ago2, the sense or passenger strand is removed and the antisense or guide strand is retained. TRBP contains three double-stranded RNA binding domains (DRBDs) that bind A-form RNA by making contacts with the 2′-OH in the minor groove and charged phosphodiester backbone in a sequence-independent manner[54]. Ago2 binds the 5′ end of the guide strand by a strong mid-domain binding plus multiple contacts to the charged phosphate backbone and 2′-OH down the central groove, and PAZ domain binding to the 3′ terminal hydroxyl[55,56]. Consequently, all siRNA chemical modifications must maintain or mimic a double-stranded, A-form RNA structure[57,58]. Not surprisingly, this dependency on intracellular enzymes severely restricts the type and extent of siRNA chemical modifications. Fortunately, 2′-F and 2′-O-Me modifications (**Fig. 2**) closely mimic the biophysical properties of 2′-OH, are highly tolerated by the RNAi machinery, serve to stabilize siRNAs from RNAses and prevent loading into and activation of innate immune receptors (TLR, RIG-I, MDA-5)[9,14,57,58]. In fact, all therapeutic siRNAs in clinical trials today contain most, if not all, 2′-F/O-Me modifications[3,14,57].

Reducing the overall charge on siRNAs while maintaining activity is critical to improving siRNA deliverability and stability. Taking a page from the ASO chemistry playbook, Segal *et al.*[3,59] at Alnylam incorporated phosphorothioates onto the ends of the siRNA strands. Although phosphorothioates do not mimic a fully charged phosphodiester backbone, because TRBP binds the siRNA in the middle of the molecule in opposing minor grooves[54], phosphorothioates are well tolerated on the ends of each strand. This type of directed chemical modification has greatly improved the stability, potency and duration of RNAi responses *in vivo*. Similarly, taking advantage of the mechanics of TRBP binding to siRNAs, several groups have reduced the passenger strand length by eliminating nucleotides from the 5′ end coupled with placing 6–8 phosphorothioates on the extended 3′ single-stranded guide strand tail[60,61]. This results in a net reduction of overall charge and increased local delivery when conjugated to sterol, which embeds itself into the lipid bilayer to aid with delivery[62].

Beyond phosphorothioates, the siRNA's charged phosphodiester linkage has been largely recalcitrant to chemical manipulation. My laboratory[63,64] took an alternative approach to reduce the overall charge on siRNAs by working out the synthetic chemistry of neutral bioreversible phosphotriester oligonucleotides. These prodrugs were termed short-interfering ribonucleic neutral (siRNN) molecules. They represent a Trojan horse approach to delivery that masks the negative charge of the drug and molecularly sculpts the siRNN surface

to essentially mimic a protein surface (**Fig. 2**). Once inside cells, ubiquitous cytoplasmic enzymes cleave the phosphotriester bond that initiates a rapid intracellular two-step conversion of the neutral siRNN into a native negatively charged phosphodiester siRNA that induces a robust RNAi response[63]. Importantly, for treating systemic disease where an extended PK is required to achieve as many shots on goal as possible, phosphotriester neutralization of the backbone is critical to avoid kidney clearance, prevent liver scavenger receptor absorption (via binding of negatively charged RNAs), and increase stability by avoiding RNase recognition (RNases cleave at charged phosphodiester linkages). Working within the limitations of having to fit into the cellular RNAi enzymatic machinery, the incorporation of next-generation RNA chemistries has greatly improved siRNA drug-like properties over the past ten years.

Although chemical modifications are important for enhancing stability, reducing innate immune responses and reducing overall charge, siRNAs are ~14 kDa macromolecules that have no bioavailability to traverse the lipid bilayer and enter cells. Consequently, unlike naked ASOs, siRNAs need a delivery agent. Early siRNA delivery approaches adapted lipid nanoparticles (LNPs) and synthetic nanoparticles that were originally designed for DNA-mediated gene therapy[65]. These LNPs were retooled and optimized for siRNA delivery using ionizable lipids that effectively lowered the dose 100-fold from ~1 mg/kg to 0.01 mg/kg for liver target genes[66–68]. Alnylam currently has a phase 3 clinical trial targeting liver transthyretin amyloidosis (TTR) with a LNP siRNA formulation (http://www.alnylam.com/product-pipeline/hereditary-attr-amyloidosis-with-polyneuropathy/).

However, for all their enhanced delivery power, LNPs come with substantial costs[57,69,70]. First, LNP synthesis generally involves the addition of four to five components at different ratios with different toxicity profiles for each component[14]. Maintaining size in solution is also problematic as LNPs shed components. Second, while a single LNP can potentially deliver a hundred siRNAs, a LNP 100 nm in diameter is ~100 megaDa in size or some 5,000 × larger than the 14 kDa siRNA drug being delivered. The LNP size results in an inescapable poor diffusion coefficient and poor PK that, due to the hepatocyte's space of Disse architecture, predominantly targets liver. Third, in oncology, LNP delivery of siRNAs to solid tumors is predicated on the enhanced permeability and retention (EPR)[71,72] effect where blood vessels are thought to be sufficiently disorganized in solid tumors to allow accumulation of nanoparticles. Although the EPR effect is clearly part of the biology of pre-clinical subcutaneous rodent tumor models that grow at a rapid pace, it remains unclear to what extent the EPR effect occurs in the vast majority of human solid tumors. Taken together, although LNPs solve many of the siRNA delivery barriers listed in **Table 1**, they also have major and inescapable liabilities for use outside of normal liver or local delivery.

A vastly simplified and significantly smaller-molecular-weight alternative siRNA delivery approach to target liver hepatocytes was spearheaded by Manoharan's group[59,73] at Alnylam and involves conjugation of a tris-GalNAc domain directly to a naked, but highly stabilized siRNA. As discussed above for ASOs, the GalNAc-siRNA delivery has proven so successful that Alnylam currently has seven GalNAc-siRNA clinical programs for liver genes (http://www.alnylam.com/product-pipeline/), which also utilize a second-generation ESC RNA stabilization chemistry that reduces the dose and toxicity concerns, while maintaining single-dose durations of >6 months in patients[3]. Derivatives of this approach are also being developed by other RNAi biotechs, including Arrowhead Pharmaceuticals (Pasadena, CA, USA), Dicerna Pharmaceuticals (Watertown, MA, USA) and Solstice Biologics. Unlike complex LNP formulations, GalNAc–siRNA conjugates are readily synthesizable on a solid-state RNA synthesizer[74] and can be chemically defined by mass spectrometry. For optimal siRNA hepatocyte delivery, conjugation of a tris-GalNAc is required to efficiently engage the trimeric ASGPR[35,73] (see below).

For extra-hepatic delivery, antibodies rise as a premier targeting domain; however, antibodies do not escape from endosomes into the cytoplasm. Early attempts to target siRNAs with antibodies[75] were fraught with difficulties of antibody expression and aggregation. Moreover, a recent *tour de force* with antibody and site-selective conjugation resources by scientists at Genentech (S. San Francisco, CA, USA; now part of Roche) resulted in minimal activity of antibody–RNAi conjugates[76]. Even so, given the potential of such conjugates to target specific cell types with repurposed antibodies, this area of research deserves further investigation. Overall, with GalNAc–siRNA conjugates on the verge of being anointed bona fide drugs, this success clearly points the RNAi therapeutics field away from large LNPs to small, targeted siRNA conjugates.

Delivery of synthetic mRNAs and CRISPR–Cas9 guide RNAs

Various groups have developed approaches to capitalize on mRNA to express genes going back to 1990 (ref. 77) and more recently for vaccine development, but only with the arrival of Moderna Therapeutics (Cambridge, MA, USA) has a highly capitalized commercial developer advertised its intention to pursue the therapeutic potential of the mRNA approach. Unlike DNA-based gene therapy approaches that invite concerns over genomic integration, synthetic mRNA-based therapeutic approaches use a hit-and-run strategy to transiently express a therapeutic protein after which the mRNA is degraded. Although improving the 5′ capping and inclusion of pseudouridine and 5′-methylcytidine nucleotides to avoid activating the innate immune system[78,79], mRNA therapeutics, which can range in size from 2-kb mRNAs (~660 kDa) to 20-kb self-replicating RNAs (7,000 kDa)[80], are highly charged and have no bioavailability. For systemic delivery of RNA molecules of this size and charge, the only avenue available to address the systemic delivery problem is via nanoparticles, primarily ionizable LNPs[81]. However, mRNA treatment of chronic diseases will require repeated LNP mRNA exposure where the toxicity associated with LNPs or synthetic NPs remains a significant barrier[14]. In contrast, for local intramuscular delivery of RNA vaccines where hitting cells in a relatively small area results in a strong stimulation of the immune system, effective delivery approaches range from LNPs to cationic nanoemulsion (CNE) to electroporation[82–84]. So while delivery of RNA vaccines have demonstrated their potential, systemic delivery of mRNAs to produce therapeutic proteins remains a high-barrier work in progress and is currently locked into liver-only expression.

When the CRISPR–Cas9 genome editing machinery exploded onto the scene in 2012 by work from the Charpentier, Doudna[85], Church[86] and Zhang laboratories[87], the problem of how to deliver CRISPR components into humans in a clinical context instantly rose to the top of the list. CRISPR has a double delivery problem because two macromolecules are required for a functional therapeutic: one, the *Streptococcus pyogenes*160-kDa catalytic Cas9 recombinase and the other, a ~150-nucleotide (~50 kDa) tracer/single guide RNA (sgRNA)[85–88]. Although these components will invariably get somewhat smaller by use of lower molecular weight CRISPR recombinases from other species, like *Staphylococcus aureus* saCas9 at 125 kDa[89], and shorter chimeric sgRNAs[87,90], from a delivery perspective, these are all very large and charged problematic macromolecules.

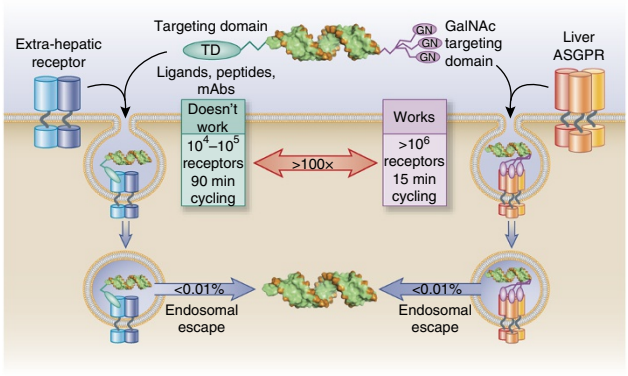

Figure 3 The numerology of endosomal escape. *Tris*-GalNAc binding to liver ASGPR (~10^6/hepatocyte) induces endocytosis (~15 min) where a small fraction of the siRNA or ASO cargo escapes into the cytoplasm to induce selective RNA drug responses. In contrast, targeting non-hepatic cell surface receptors (10^4–10^5) that have a much slower rate of endocytosis (~90 min) has proven extremely difficult. Assuming there is no endosomal escape advantage in ASGPR endosomes, ASGPR brings in ~100-fold more siRNAs/ASOs into hepatocytes than is mathematically possible in any other ligand–receptor pair. Consequently, development of next-generation RNA-based therapeutics needs to incorporate new chemistries, materials and/or mechanisms of enhancing endosomal escape ~100-fold.

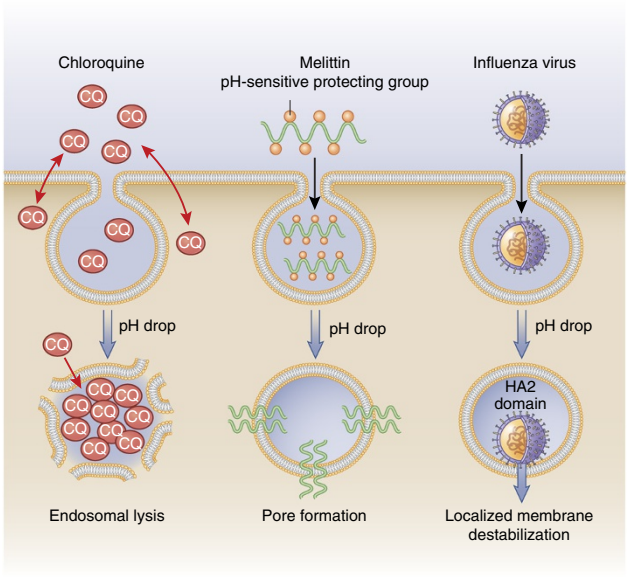

Figure 4 Endosomal escape agents. Protonation of small-molecule chloroquine (CQ) traps it in the endosome, resulting in a dramatic increase in its concentration and lysis of the endosome (left panel). Pore-forming melittin peptide from bee venom contains pH-sensitive protecting groups that are removed as the endosomal pH drops resulting in endosomal lysis (middle panel). Influenza virus contains a pH-sensitive fusogenic hemagglutinin-2 protein domain (HA2) that inserts into the endosomal membrane to locally destabilize it in a non-toxic manner to facilitate virus entry into the cytoplasm (right panel).

On top of the delivery problem is the fidelity issue of in-human DNA editing where the patient's genomic DNA is altered for life, with all the concomitant ethical issues[91,92]. This essentially excludes the use of DNA-based vectors on humans that would result in constitutive Cas9 expression. Consequently, early CRISPR clinical applications will likely focus on delivery of CRISPR components into *ex vivo* cells and replacement of the cells into the patient. *Ex vivo* CRISPR gene-editing components can be delivered by a combination of Cas9 mRNA and sgRNA or recombinase protein preloaded with sgRNA by electroporation, LNPs or protein transduction approaches[93–97]. The University of Pennsylvania has applied for and received permission from the NIH Recombinant Advisory Committee (RAC) to perform *ex vivo* CRISPR knockout of the genes encoding PD1 and TCR alpha/beta in cancer patient's isolated T cells for reintroduction into the patient (http://osp.od.nih.gov/under-the-poliscope/2016/06/emerging-biotechnologies-and-role-nih-rac). However, the first clinical trial of a CRISPR-edited gene was performed on a single lung cancer patient by Lu You's group at Sichuan University by deleting the *PD1* (also known as PDCD1) gene from the patient's T cells and then reintroducing them into the patient to fight the cancer (https://clinicaltrials.gov/ct2/show/NCT02793856?term=crispr&rank=4). For CRISPR, it is far too early in the developmental timeline to predict which of these delivery approaches, target cells and diseases will demonstrate the full clinical potential of CRISPR-mediated genome editing in the clinics.

Escape, escape, escape

In the world of delivering RNA-based therapeutics into cells, all roads eventually lead to the endosomal escape abyss (**Fig. 3**). Although ASO and siRNA delivery to the liver has been solved by ASGPR-targeted GalNAc-siRNA conjugates (and perhaps ASO delivery to the CNS) and LNP-mediated mRNA and CRISPR delivery to the liver looks promising, an effective, clinically ready, extra-hepatic systemic delivery approach for RNA-based therapeutics remains across the abyss.

For unknown reasons, ASGPR has properties that are uniquely suited for macromolecular drug deliver to hepatocytes. As its name implies, ASGPR binds to asialoglycoproteins (glycoproteins minus a terminal sialic acid) in the blood, pulls them into clathrin-coated endosomes and traffics them to the lysosome for degradation[36,98]. ASGPR bound asialoglycoproteins are not thought to escape into the cytoplasm (**Fig. 3**). However, hepatocytes express millions of copies of ASGPR on their cell surface, which cycle at an astonishingly rapid rate of every 10–15 min[36]. A maximum RNAi response appears to require as few as 5,000 siRNAs (or less)[99]. So it's possible that GalNAc engagement of the ASGPR could result in an extremely rare localized membrane destabilization event that with millions of siRNAs coming into ASGPR endosomes every 15 min and an escape rate as low as <0.01%, GalNAc delivery would easily exceed the 5,000-molecule mark over the course of a day.

Unfortunately, there is no other ligand–receptor system that expresses receptors at this level or cycles into endosomes this rapidly. In fact, the vast majority of cell surface receptors are expressed in the 10,000–100,000 range (or lower), and caveolin and clathrin-mediated endocytosis typically recycle every 90 min[14]. In theory, delivery approaches that target receptors could bring ~100,000 or more siRNAs into endosomes of cells every two hours or so. But with a presumed endosomal escape rate of <0.01%, reaching even the exceedingly low threshold of ~5,000 siRNAs escaping has remained elusive. Fortunately, multiple groups are working on solutions to the endosomal escape problem.

The classic approach has been to use small-molecule endosomolytic agents that disrupt or lyse endosomes, with chloroquine being the prototypical molecule[100] (**Fig. 4**). Chloroquine passively diffuses across the cell membrane and into endosomes where, as the pH drops, it becomes protonated and trapped inside the endosome resulting in a dramatic increase in its endosomal concentration. Chloroquine is thought to insert a hydrophobic motif into the lipid bilayer and at a

critical concentration, lyse the endosome[100]. Similar types of small-molecule endosomolytics have recently been described by both the Juliano and Khvorova groups[101,102]. However, at the effective concentration, these endosomolytic agents invariably lyse not only the endosome containing the siRNA cargo, but many, if not most, other endosomes inside the cell resulting in substantial and unacceptable toxicity. Consequently, although these small-molecule endosomolytic enhancers have greatly aided the field and pointed it in the right developmental direction, they currently have too small of a therapeutic index for clinical use.

An alternative endosomal escape approach is to conjugate endosomolytic peptides and/or molecules directly to the RNA, which will strictly limit their action to endosomes containing the RNA therapeutic. Arrowhead Pharmaceuticals developed a two-molecule dynamic polyconjugate (DPC) system[103]. The siRNA is conjugated to cholesterol, which forms a large aggregate (low-density lipoprotein) in blood that is transported to the liver and taken up by endocytosis into hepatocytes. The second molecule is a derivative of the pore-forming melittin peptide (derived from bee venom) that lyses membranes[104]. To tame melittin, Arrowhead synthesized it with pH-sensitive protecting groups and conjugated it to GalNAc. In the low pH of hepatocyte endosomes, melittin is deprotected, becomes active and lyses the endosome to release the siRNA into the cytoplasm (**Fig. 4**). Unfortunately, owing to toxicity likely from the melittin, Arrowhead had an FDA clinical hold placed on their lead program and decided to drop all three clinical programs that rely on melittin to escape the endosome[105]. The inclusion of an endosomolytic molecule as powerful and potentially toxic as melittin shows the degree of difficulty required to address the endosomal escape problem.

Work from my laboratory[106] has also focused on synthesizing small hydrophobic, endosomal escape domain (EED) peptides that enhance escape of macromolecular cargo into the cytoplasm by five- to eight-fold in a non-toxic manner. A different approach, taken by the Liu laboratory[107] is to focus not on hydrophobic residues, but on a highly charged Aurein 1.2 peptide that enhances endosomal escape fivefold. Although these EEDs have the right characteristics of low toxicity, an increase in payload delivery of fivefold is insufficient to develop systemic RNA-based therapeutics. What is needed are non-toxic endosomal escape enhancers that are ~100 times more efficient.

Conclusions

RNA-based therapeutics offer three major advantages over traditional small-molecule and antibody therapeutics. First, once delivery to a specific cell type or tissue has been devised (e.g., siRNA/ASO delivery to hepatocytes; ASO delivery to the CNS), it is highly likely that every disease-promoting gene in that cell type can be targeted. Second, RNA therapeutics can selectively target single genes and can be readily engineered to keep away from off-target genes, whereas small-molecule inhibitors often hit multiple targets and have unknown off-target binding. Third, unlike static small molecules and antibodies, RNA therapeutics can pharmacoevolve their sequence at the same pace as disease, be it cancer or pandemic influenza. These attributes give RNA-based therapeutics considerable potential to treat undruggable human diseases (once delivery is solved).

Now that ASO, RNAi and mRNA chemistries have advanced to a stage that enables enhanced stability and avoidance of the innate immune system, while at the same time maintaining high on-target activity profiles, further work is needed to (1) target these molecules to specific cell types or tissues and most importantly to (2) devise non-toxic endosomal escape agents.

On the targeting front, the GalNAc sugar approach for RNAi and ASOs clearly steers the field in the direction of conjugating targeting domains to RNA therapeutics. But endosomal escape remains the central problem for research to solve—a problem that applies to all RNA-based therapeutics. The field, and especially the academic funding agencies, needs to place a much higher priority on enhancing endosomal escape by several orders of magnitude by developing new chemistries and materials, and furthering our understanding of the mechanisms of escape.

Lastly, keeping fully intact RNA-based therapeutics in the blood with extended PK by avoiding kidney clearance or liver absorption with phosphorothioate, PMO and PNA backbones for ASOs and neutral phosphotriester backbones for RNAi will likely become more critical to allow as many shots on goal to extra-hepatic tissues that have a much lower receptor number and much slower rate of receptor-mediated endocytosis compared with ASGPR in the liver.

Looking at the big picture, the recent successes of ASOs, PMOs and GalNAc-siRNAs in clinical trials, along with the therapeutic potential of mRNAs and CRISPR, argues not only that we are at the threshold of a new era of RNA-based therapeutics; but that if and when delivery to tissues other than the liver is solved, these drugs have the potential to dominate the future therapeutic landscape.

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The author declares competing financial interests: details are available in the online version of the paper.

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