Expert Opinion

Challenges in the pharmaceutical development of lipid-based short interfering ribonucleic acid therapeutics

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Introduction: Harnessing RNA interference as a therapeutic approach has the potential to significantly expand the druggable target space, offering new hope for treatment of diseases that cannot be addressed with existing classes of drugs. A number of siRNA therapeutics have already progressed into preclinical and clinical development. Of these, lipid-based systems have emerged as one of the most mature classes of systemic delivery technologies. Despite tremendous advances in development, a number of significant challenges must still be addressed to enable commercialization of a lipid-based siRNA pharmaceutical product.

Areas covered: This review addresses specific challenges inherent to the pharmaceutical development of lipid-based siRNA therapeutics. Focus is placed on the development of a robust manufacturing process, the setting of appropriate product specifications and controls, development of strategies to assess and ensure product stability, and the evaluation of product comparability throughout development.

Expert opinion: Discovering and developing a lipid-based siRNA therapeutic that can be commercialized requires engineering a particle that selectively and efficiently delivers the cargo to the target tissue and cellular compartment. The particle assembly must be strictly controlled and physical properties thoroughly characterized to successfully develop an understanding of particle attributes that influence *in vivo* pharmaceutical properties. Correlation of particle physio-chemical properties to product performance is the foundation for advancements in discovery and assuring quality in a commercial drug product. Although difficult, we believe these development challenges can be addressed with appropriate scientific resources and that the industry will continue to progress siRNA therapeutic candidates through clinical development.

Keywords: assembly, biocomparability, lipid, lipid analysis, nanoparticle, pharmaceutical, siRNA, siRNA analysis, therapeutic

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1. Introduction

1.1 RNA interference

The discovery of RNA interference (RNAi) in 1998 and the recognition of its value as a research tool over the ensuing several years was one of the most significant discoveries in biology of the last several decades. This culminated in the award of the Nobel Prize in Physiology or Medicine to Andrew Fire and Craig Mello in 2006 [1,2]. RNAi is a post-transcriptional self-regulating mechanism in eukaryotic cells in which double-stranded RNA is processed by the enzyme Dicer to produce short interfering RNA (siRNA). These are typically 21 – 23 nucleotides in length,

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Article highlights.

- RNA interference has emerged not only as a paradigm-changing tool for the elucidation of gene function and for target validation, but also as a promising new therapeutic modality with the potential to address previously undruggable targets.
- Lipid-based nanoparticle formulations are among some of the most mature siRNA delivery systems in terms of progression into the clinic, but are also highly complex.
- In-depth biophysical design and characterization capabilities, as well as engineering expertise, are required to build the fundamental understanding needed for successful clinical development. This is currently fueling a large amount of academic and industrial research.
- To speed progression into the clinic and through development, lipid-based siRNA delivery systems require tools for assessing efficacy and toxicity beyond those typically required for conventional drugs, including liposomal products.

This box summarizes key points contained in the article.

and associate with specific cytosolic proteins to form the RNA-induced silencing complex (RISC). The antisense strand of the siRNA molecule, incorporated into RISC, binds to the complementary sequence on the target mRNA, which is then cleaved by RISC, preventing translation of the corresponding protein. RNAi can be harnessed to knock down expression of virtually any protein of interest by designing synthetic siRNAs against the target gene and transfecting them into the target cell.

1.2 siRNA therapeutics

RNAi was rapidly used as a research tool in studies of gene function, but was also quickly recognized as a potential new therapeutic modality. Successful development of RNAi as a therapeutic approach would allow for significant expansion of the druggable target space, offering new hope for disease targets that cannot be addressed with existing classes of drugs [2]. The technology could be disruptive and revolutionize the drug discovery model if its full potential for validating targets in the clinic is realized. Successful delivery to multiple tissues of therapeutic interest remains the key barrier to unlocking this potential [3,4].

Several siRNA therapeutic delivery systems are currently in preclinical and clinical development, some of which were originally developed for delivery of other nucleic acid therapeutics, such as gene therapy, antisense and vaccines. Local delivery, particularly for targets in the eye and lung, has been an early focus given the relatively lower number of barriers compared to systemic delivery [5-7]. Still, systemic delivery enables a much larger universe of applications as well as simultaneous delivery to many more target cells distributed across larger and spatially segregated regions of the body. For these reasons, systemic delivery remains a major focus of current research. The most advanced systemic siRNA delivery systems are lipid- and polymer-based [8,9]. Other approaches include complexes and covalent conjugates with a variety of functionalities including peptides, cyclodextrin derivatives and lipophilic moieties [10-12]. Inorganic systems, such as calcium phosphate, layered double hydroxides and quantum dots, are also being pursued [13,14]. Many of these systems also contain target ligands, such as mAbs, to promote retention and cellular uptake at specific target sites [15].

1.3 Lipid nanoparticles

Lipid-based siRNA delivery systems, such as cationic liposomes and their complexes with nucleic acids, termed lipoplexes or lipid nanoparticles (LNPs), were originally developed for transfection and gene therapy applications, and are currently the most mature of the systemic siRNA delivery approaches [16]. The Stable Nucleic Acid Lipid Particle (SNALP) technology, developed by Tekmira, has progressed into multiple clinical studies, and the AtuPlex system (Silence Therapeutics) is currently in clinical development for solid tumors (**Table 1**). These LNPs are typically composed of cationic and neutral lipids, often with PEGylated lipids, to confer improved physical stability (**Figure 1**). The cationic lipid is critical to the efficient encapsulation of polyanionic siRNA and is hypothesized to play a key role in delivery across the cell membrane either by facilitating escape of the siRNA cargo from endosomes after endocytosis [17,18] or through other mechanisms, such as direct fusion with the cell membrane [19,20], Neutral lipids, such as phospholipids and cholesterol, can be selected to modulate the fluidity and phase behavior of the particle [21,22]. LNPs can exhibit a range of phase behavior, including lamellar (single and multiple), inverted hexagonal and cubic phases, sometimes coexisting within the same population of particles. Because lipid structure has been suggested to play a critical role in cargo delivery and escape, the rational design of lipid phases is currently an area of substantial research [23-27].

Although not the focus of this paper, substantial effort has gone into optimizing the composition and assembly of lipid-based siRNA delivery systems to improve their efficacy and tolerability. Specifically, lipid components, such as the cationic and PEGylated lipids, have been optimized to improve potency [28] and modulate biodistribution [29-31]. In addition, these systems have been shown to elicit immune and inflammatory responses *in vivo* (Tekmira Pharmaceuticals Press Release, 23 March 2010), and delivery vehicle design and optimization must be approached with the goal of minimizing these responses.

1.4 Development challenges

The remainder of this review addresses specific challenges inherent to the development of lipid-based systems for therapeutic siRNA delivery. Many of these are common to conventional liposomal drug delivery systems [32], although some are unique to nucleic acid delivery. The underlying theme

Table 1. Ongoing RNAi clinical trials using lipid-based delivery systems.

Drug and company	Development phase	Gene target	Indication	Cell type
Atu-027 (Silence)	Phase I	PKN3	Solid tumors	Tumor
TKM-ApoB (Tekmira)	Phase I	ApoB	Dyslipidemia	Hepatocyte
ALN-VSP (Alnylam)	Phase I	VEGF, KSP	Solid tumors with liver involvement	Tumor
ALN-TTR (Alnylam)	Phase I	TTR	TTR amyloidosis	Hepatocyte
TKM-PLK1 (Tekmira)	Phase I	PLK1	Solid tumors	Tumor

RNAi: RNA interference.

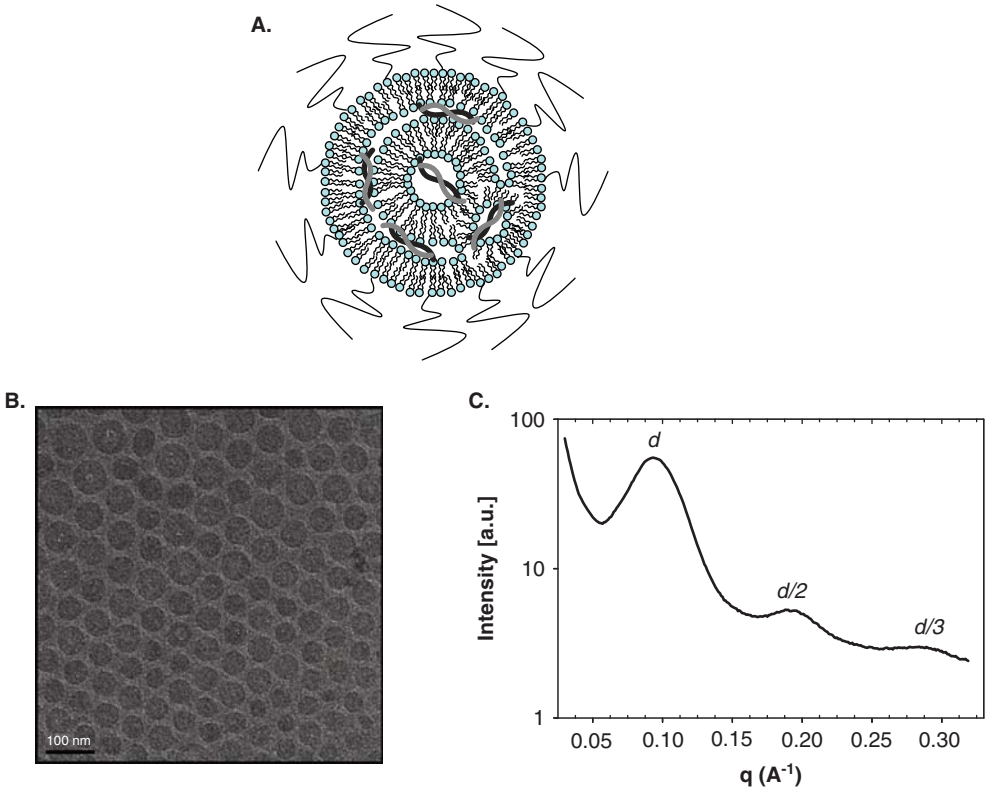

Figure 1. (A) Schematic representation, (B) cryogenic transmission electron microscopy micrograph (C) and small angle X-ray scattering curve of cationic lipid nanoparticles encapsulating siRNA cargo. In (C), the log of the scattering intensity is plotted as a function of the scattering vector, *q*. d, d/2 And d/3 correspond to the first-, second- and third-order reflections, respectively, indicating a multi-lamellar structure.

throughout is one of building mechanistic understanding and ensuring control over key parameters. This translates to an early adoption of a quality by design (QbD) approach when developing these drug delivery systems. The sections that follow address some of the challenges that must be met to consistently supply patients with a stable, high quality drug product. As an illustrative example, we showcase application of QbD to develop a manufacturing process based on solvent-shifting precipitation. The example highlights the importance of understanding engineering and lipid physical chemistry fundamentals to ensure critical quality attributes of the LNP product are consistently met.

2. siRNA LNPs via solvent-shifting precipitation

The majority of methods used for the manufacture of siRNA LNPs are based on traditional techniques of liposome preparation. These include detergent dialysis [33], reversed phase evaporation [34], freeze-thaw cycling [35], rehydration of lipid films [36] and destabilization of preformed vesicles in the presence of nucleic acids [37,38].

While successful for preparation of nanoparticles at a small scale (sub-gram), adaptation of these methods to commercial manufacturing can be challenging. These techniques often

rely on mixing of bulk phases, which can lead to heterogeneous chemical and/or mechanical conditions during the formation process and result in particles of polydisperse size, morphology and structure. A comprehensive discussion of advantages and disadvantages of these preparation techniques is outside the scope of this review. However, the interested reader is directed to an excellent book series on liposomal technology [32].

A manufacturing process that has potential to address scalability limitations is solvent-shifting precipitation. In this technique, a solute initially dissolved in a solvent is precipitated when mixed with a miscible anti-solvent. Under such conditions, particles are precipitated from a homogeneous monophase, eliminating bulk heterogeneity. Industrially, the solvent-shifting route has been used to manufacture submicron particles of many organic actives, including vitamins [39], dyes [40] and drugs [41,42]. The method has also been used to prepare stabilized drug nanoparticles, where amphiphilic polymers or lipids encapsulate the drug [43-45]. Jeffs *et al.* [46] used solvent-shifting precipitation to prepare LNPs encapsulating plasmid DNA, termed stabilized plasmid lipid particles. Similarly, Zimmerman *et al.* [47] used the technique to generate LNPs encapsulating siRNA (SNALPs).

2.1 QbD and the solvent-shifting process

While straightforward in operational execution, implementation of the solvent-shifting technique in a manner that ensures reproducible manufacture of LNPs is highly complex. The multicomponent nature of LNPs, coupled with the sensitivity and complexity of individual assembly steps, can result in significant variability in particle physiochemical properties and, consequently, therapeutic outcomes.

The QbD approach aims to provide a mechanistic understanding of individual assembly forces to enable better control of the product. In this respect, LNP formation can be decomposed into several steps: i) homogeneous micromixing of lipid and siRNA components, ii) electrostatic capture and precipitation of siRNA with cationic lipids, iii) nucleation and growth of nanometer-sized precipitates and iv) stabilization of precipitates to form the siRNA LNPs.

2.2 Homogeneous mixing

In the solvent-shifting process, LNP formation is initiated by the combination of an organic solvent in which all lipid components are dissolved with an aqueous buffer containing the siRNA. Because particle assembly relies on interactions of solutes initially segregated in individual solutions, the process is highly sensitive to mixing conditions, and variability in mixing efficiencies results in particles of diverse physical properties. Thus, control of flow characteristics during mixing is of critical importance.

A number of mixing devices have been developed to achieve mixing on the micro-scale. The most common of these is the T-chamber mixing geometry [48-50]. In this configuration, two linear, coplanar jets collide at a 180° angle inside a small mixing volume. The collision between jets creates a region of high energy dissipation in the impingement zone. The higher the rate of energy dissipation, the faster is the micromixing.

In practice, micromixing in a T-mixer of specific dimensions is most easily tuned via control of impinging fluid flow velocities. When flow velocities are sufficiently high, the necessary turbulence for rapid micromixing is generated. Rapid mixing ensures that particle precipitation commences from a state of molecular homogeneity. This has direct implications on the properties of the resulting LNPs. **Figure 2** demonstrates the influence of impinging fluid flow rates on particle size. It is shown that as flow rate (or Reynold's number) increases, the mean particle diameter decreases. Beyond a critical value, further increases in flow velocity yield little impact on particle size, and the influence of mixing in homogeneity on properties of precipitated LNPs is minimized.

2.3 Nucleation and growth

Upon mixing of siRNA and lipid solutions, LNP assembly begins via formation of initial precipitates. The aqueous siRNA stream is typically a low pH buffer (to protonate the cationic lipid), while the organic is a polar protic solvent, such as ethanol. When mixing takes place in a T-mixer, the requirement of matched stream momenta [51,52] generally restricts the effluent to an organic solvent concentration in the range of 50 v/v%. Under these conditions, a combination of electrostatic and hydrophobic interactions contributes to a lowering of siRNA and lipid solubilities, resulting in a supersaturated solution and precipitation.

For conditions of high supersaturation, precipitation proceeds through processes of homogenous nucleation and growth. The relative magnitudes of nucleation and growth rates play a critical role in dictating LNP properties, such as size, polydispersity and compositional homogeneity. The nucleation rate, defined as the number of nuclei which grow to a critical size per unit time and unit volume, can be described as [53]:

$$J = A\exp\left[-\frac{B}{S^2}\right], \quad (1)$$

where A and B are coefficients that depend on temperature and surface energy, and S is the supersaturation, defined as $S = \frac{C(r)}{C^*}$, where $C(r)$ denotes the solubility of a particle with radius r and C^* is the equilibrium solubility. Thus, the nucleation rate depends very strongly on temperature, supersaturation and interfacial energy, and can be appropriately manipulated by variations in these parameters. Once nuclei are formed, particle growth commences and occurs concurrently with nucleation. While nucleation and growth are essentially inseparable processes, they can proceed at very different rates [54].

The ability to modulate LNP properties via control of nucleation and growth rates is exemplified in **Figure 3**. LNPs

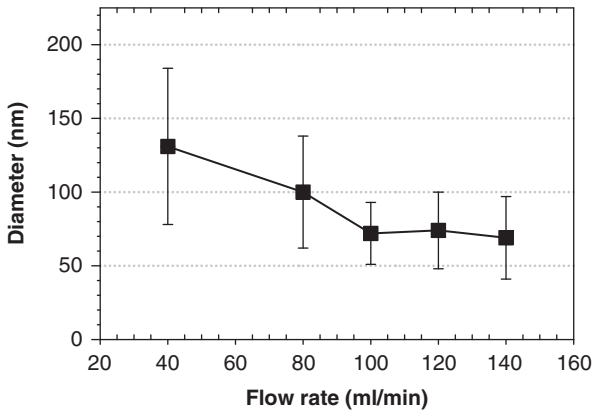

Figure 2. Effect of impinging fluid flow rate on diameter of siRNA–lipid nanoparticles prepared via solvent-shifting in a T-mixer. The intensity-averaged particle diameter, as measured by dynamic light scattering, is reported. Data shown represent the average of two independent experiments, with s.d. at each condition represented by error bars.

composed of an ionizable cationic lipid, cholesterol, phospholipid, PEG-lipid and encapsulating siRNA targeted for apoB gene were prepared via solvent-shifting precipitation in a T-mixer. As shown in **Figure 3A**, increasing the total concentration of lipids and siRNA, C, up to a level signified by C^*, results in particles with progressively smaller mean diameters, consistent with a nucleation-dominated regime. The trend is similar for particles assembled at high cationic lipid amines (N) to siRNA phosphate (P) charge ratio (N:P = 6) or low ratio (N:P = 2). Supersaturation can also be modulated by tuning the solvent composition. As shown in **Figure 3B**, decreasing the ethanol level from 50 to 40 v% in the effluent after T-mixing results in an ~ 70 nm reduction in LNP diameter. Further lowering of solvent level has little impact on size. Beyond particle size, a number of other physical properties can be impacted by changes in supersaturation during particle assembly. For example, values of the particle distribution index (PDI), measured by dynamic light scattering, for data of **Figure 3** ranged between 0.06 and 0.25, with particles generated under conditions of high supersaturation generally yielding PDI values at the lower end of the reported range (data not shown). Similarly, siRNA encapsulation ranged between 70 and 90%, with particles prepared at higher ethanol v/v% generally yielding higher siRNA encapsulation, consistent with previous reports on similar nucleic acid–lipid systems [38,55]. Additional assembly parameters can also impact particle physical properties. These include polarity of the medium, solution pH, ionic strength, buffer counterion and cationic lipid pKa.

2.4 Stability during assembly

Precipitates will continue to grow until solute concentrations reach their respective equilibrium solubilities. To ensure that nanometer-sized particles are obtained, the growth process must be kinetically arrested. This can be accomplished by way of steric stabilization, typically via adsorption of a protective polymer layer onto surfaces of growing particles. The most common polymer used for this purpose is PEG, which is typically conjugated to a lipid anchor and used at molar fractions between 1 and 10 mole% relative to total lipids [38,56]. PEG-lipids of variable PEG molecular mass, typically in the range of 1000 – 10,000 g/mole, can be used to provide a steric boundary layer of appropriate dimension [57,58]. Properties of the lipid anchor can also be important [59]. For example, influence of the anchor on the critical micelle concentration may be of relevance, especially in relation to dictating the rate of adsorption relative to other lipid components [60,61].

2.5 Post-assembly processes

Despite a protective polymer layer on particle surfaces, the high concentration of organic solvent after initial mixing can continue to facilitate colloidal instability. Particle growth can occur via aggregation or fusion due to attractive van der Waals force, or via Ostwald ripening, driven by solubility and surface curvature effects at the nanometer length scale [62]. To address these instabilities, the nanoparticle suspension after initial mixing is typically further diluted with aqueous buffer. Dilution may be effected by way of immediate introduction into a controlled amount of buffer, or by mixing with a controlled flow rate of buffer in a second mixing region. Additional post-assembly steps are typically also performed to generate the final pharmaceutical product. These may include: i) removal of unencapsulated siRNA via ion exchange chromatography, ii) exchange of residual organic solvent with compendial buffer, iii) concentration of particle suspension and iv) sterile filtration.

3. Specifications and controls

A manufacturer's ability to supply patients with valuable medicines depends on their capability to rapidly, reliably and reproducibly deliver a pharmaceutical product of high quality (safe, pure and potent) to clinical shelves. These requirements are the basis for establishing specifications and controls. With a clear scientific understanding of critical product attributes that impact performance, analytical specifications can be established to assure a product is of consistently high quality. This same understanding also enables the implementation of appropriate process and raw material controls to support large scale manufacturing.

Early adoption of a QbD approach can help the initial definition of design and normal operating spaces. As development proceeds and the knowledge space can be expanded, design and operating spaces can also take firmer meaning. To be effective at such an evaluation requires knowledge of the particle properties that impact *in vivo* performance and the analytical capability to enable accurate, precise, selective and sensitive measurements of these

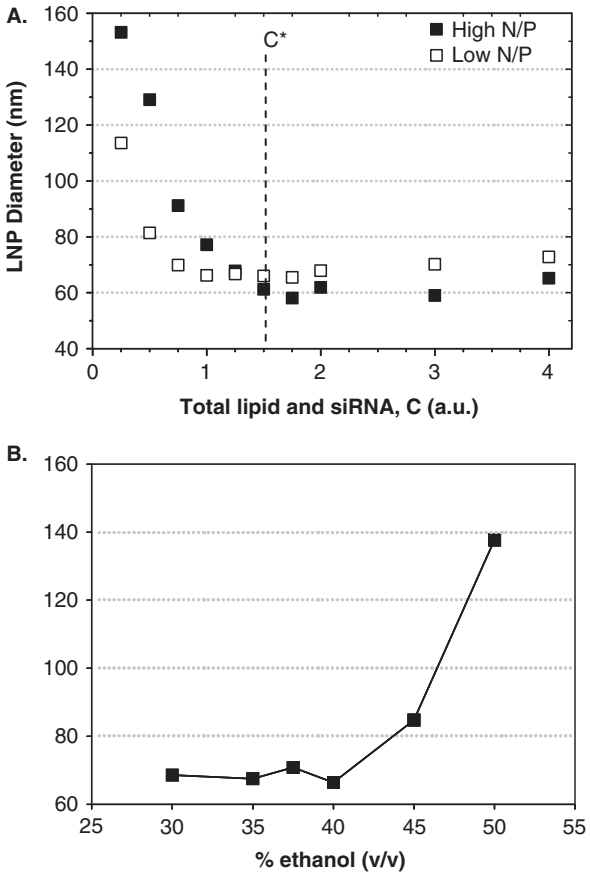

Figure 3. Influence of supersaturation on intensity-averaged diameter of lipid nanoparticles prepared via solvent-shifting. Particles are generated by mixing a solution of lipids in ethanol with an aqueous solution of siRNA in a T-mixer. Supersaturation is manipulated by varying (**A**) the total lipid and siRNA concentrations, *C*, in feed solutions or (**B**) % ethanol (v/v) in product stream. Values of *C* are presented in arbitrary units. Symbols represent the average of two independent experiments. Error bars, representing the s.d. in measured diameter, are smaller than symbols and are omitted for clarity.

properties. **Table 2** provides a summary of the techniques we have frequently used to characterize siRNA lipoplexes. Particle chemical composition, morphology, particle size, particle surface properties, polydispersity and serum stability are all important in the overall delivery vehicle design. All of these aspects of the delivery vehicle can affect interactions with blood and cellular components, cargo presentation and unpackaging, and ultimately can impact product performance [32,63,64]. As a result, analytical assays and corresponding specifications for all these properties may be necessary. Because most techniques are bulk measurements that represent an average value for a lot, the use of orthogonal methods and particle separation techniques is critical to enable accurate characterization of the product. Particle size, density, surface charge and electrophoretic mobility are all physical properties that can be utilized as a basis for particle separations and batch dispersity evaluation.

The FDA's Draft Guidance for Industry on Liposome Drug Products [65] clearly articulates the importance of maintaining extremely high standards of purity and consistency in raw material functional excipients. It is important to understand that impurities in raw material, if retained in the final product, will be delivered systemically and with high specificity to target tissue based on the delivery vehicle design. Raw material variation can also result in product variation by impacting particle assembly as well as particle chemical and physical stability. In our experience, some of the most critical aspects of raw material control include minimizing organic hydroperoxides and metals which are potentially strong oxidizers commonly found as impurities in PEG and naturally sourced lipid excipients. Other impurities that can influence particle assembly nucleation kinetics include residual solvents and nano-particulates such as silica.

Processing and formulation conditions can further impact particle properties and ultimately final product therapeutic performance. In particular, LNP physiochemical properties are determined by a delicate interplay between various factors. These can include the nature of components and their compositions, solution pH, ionic strength, buffer ion, organic solvent, cationic lipid:nucleic acid phosphate charge ratio, overall concentrations of components, kinetics of mixing (i.e., mixing geometry and flow rates) and order of operations. Energy input through temperature, shear or ultrasonics can have a large impact and sensitivities at all points of the process should be understood to enable establishing tolerable thresholds and process specifications. In-process measurements of temperature and pressure, for example, can be useful tools for understanding variability on flow rate or energy.

4. Drug product stability

Drug product stability is a key chemistry, manufacturing and controls consideration due to potential effects on efficacy, toxicity and overall product quality. While all products are constantly changing, the key is to control the rates of change to a tolerable level such that these fluctuations do not impact quality. Establishing a maximum shelf-life through which product performance is unaffected is typically desirable from a development and commercial perspective. The complexity of LNP manufacture, high cost of goods and very limited preclinical and clinical biocomparability data increase the value of achieving long shelf-lives for these materials. Strong analytical capabilities and ongoing efforts to establish *in vivo* to *in vitro* correlations are essential to be effective at establishing a shelf-life. Strong formulation capability and a mechanistic understanding of degradation pathways are also critical to solving stability challenges. Given the complexity of LNP composition, assembly and the potential for nonlinear degradation kinetics, stability studies should be initiated on multiple lots and as early as feasible during preclinical development. The

Table 2. Critical product attributes that impact product quality.

Product attribute	*In vivo* impact	Key considerations	Analytical techniques	Property target and/or range
Composition	PK, PD	Content Uniformity Component Disposition	HPLC-UV, CAD, MS, fluorescence methods	Control weight % of all formulation components within 5% of target Maintain the highest possible control on component purity and understand impact of new impurities > 0.1% on final formulation properties Encapsulation of siRNA > 85%
Size	PK, PD, immunostimulation	Mean Polydispersity Aggregates	DLS, SLS, MFI, electron microscopy	Control mean diameter within 10 nm of target Control PDI within 25% Lipid particles with mean diameter of 30 – 300 nm are suitable for siRNA delivery
Morphology	PK, PD, cargo unpackaging	Coherence of lipid arrangement Homogeneity	DSC, SAXS, NMR, electron microscopy	Amorphous, uni-lamellar and multi-lamellar particles are all suitable for siRNA delivery Understand impact of morphology on serum and plasma stability and endosomal escape properties Develop a particle with stable morphology under intended packaging and storage conditions
Particle surface properties	Specific and nonspecific interactions with blood and cellular components	Surface uniformity Surface charge Surface hydrophobicity	CE, ion exchange chromatography, isoelectric point, ζ potential, fluorescence methods	ζ Potential (-30 to +30 mV) suitable for lipid particle delivery Control ζ potential ± 20 mV of target
Serum/plasma stability	PK, PD	Half-life	*In vitro* incubation	Serum/plasma stability profile should complement delivery goals > 50% of the dose serum/plasma stable after 30 min incubation
Batch polydispersity	PK, PD, variability	Subpopulations Representation of bulk	SEC, FFF, CE, ion exchange chromatography	Understanding and controlling polydispersity is critical Most formulation analytical data represent a bulk average of particle properties Develop particle separation techniques to determine if the averages accurately describe the formulation properties
Cell uptake and endosomal escape	Potency	Capability of formulation to reproducibly deliver to target tissue and present cargo to RISC	*In vivo* or *in vitro* cell EC_{50} *In vitro* lysis of cells or endosome mimetics	Batch reproducibility within ½ log

PD: Pharmacodynamic; PDI: Particle distribution index; PK: Pharmacokinetic; RISC: RNA-induced silencing complex.

stability of oligonucleotide-active pharmaceutical ingredient, functional excipients and particle characteristics can all be inter-related [66], and can all be important in assuring quality in a pharmaceutical product. Through such a study, an understanding of key product stability liabilities and links to product performance can be established. This understanding enables effective strategies for controlling instability through formulation, storage condition and packaging, while achieving a maximum shelf-life that assures product quality.

Degradation pathways that limit shelf-life are always product specific; however, there are some well described oxidative [67-69], hydrolytic [70,71] and physical pathways [72,73] detailed in the literature for antisense oligonucleotides [74], pharmaceuticals and liposomes [75-77] that we have also frequently encountered. These pathways can affect: i) oligonucleotide chemical structure, ii) functional excipient chemical structure, iii) component disposition and iv) delivery particle properties such as size, surface charge, or component organization and particle

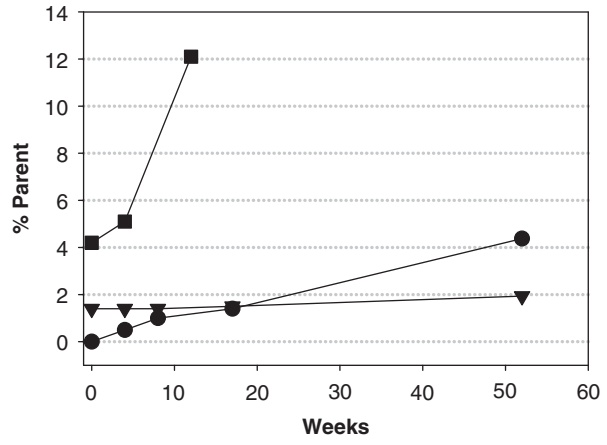

Figure 4. Representative degradation kinetics for common components of siRNA lipid nanoparticles stored at 5°C. Quantitation of all degradates is expressed as percent of parent, where (●) is phosopholipid hydrolysis degradates, (▼) is total cationic lipid oxidative degradates and (■) is total siRNA oxidative and hydrolysis degradates. Quantitation of lipid hydrolysis and oxidation degradates is determined by reverse phase HPLC–CAD analysis. Quantitation of siRNA hydrolysis and oxidation degradates is determined by ion pair reverse phase HPLC analysis.

morphology [66]. The two most common oligonucleotide degradation pathways are hydrolysis and oxidation. Both reactions can result in strand cleavage and a loss of potency. In the case of oxidation, we have also observed desulfurization of phosphorothioates [74] at rates that could be shelf-life limiting (**Figure 4**). Functional excipient identity and purity, as well as trace metal levels, are key factors that influence oxidation rates.

The functional excipient chemical composition, particle physio-chemical properties and component disposition are all critical to the function of the delivery vehicle. Commonly used and 'reactive' functional groups in LNPs include amines, esters [70,71] and unsaturated hydrocarbons [67-69]. Lipophilic amines are a common lipoplex component which drives self-assembly of the particle. In aqueous formulations, the presence of alkoxy radicals can lead to 2-electron oxidation of amines and formation of functionally stripped N-Oxides [78]. PEG-functionalized lipids and phospholipids are other common components which frequently contain esters. Ester hydrolysis rates of these components have been observed at rates which greatly exceed the rate of hydrolysis for the lipids dispersed alone under equivalent pH conditions [71,79]. Particle surface charge and PEG shielding are two critical product attributes that can be affected by these hydrolysis reactions.

The most commonly encountered physical instabilities of lipoplexes include aggregation, phase separation, particle growth or ripening, and phase transition changes [80]. These phenomena can all be dependent upon delivery vehicle chemical composition, assembly process, formulation and storage condition. These dependencies, if well understood, provide an opportunity for control. Any of these physical changes can have a major impact on efficacy and toxicity. Early analytical detection of these pathways can be very challenging and may require particle separation techniques with in- and off-line characterization.

Frozen or lyophilized formulations present a tremendous opportunity for controlling the substantial physical and chemical degradation liabilities described in this section. This may be necessary for commercialization of certain formulations depending on the degradation kinetics. Success in this approach is not necessarily assured [81] and can be specific to the delivery vehicle. Control of formulation pH, tonicity [72] and employment of anti-oxidants [76] present as other opportunities for controlling chemical and physical liabilities.

5. Evaluating comparability

Demonstrating biocomparability is particularly challenging when the delivery system actively directs or controls release of the drug after administration, as in the case of LNPs. During the course of development, it is typically necessary to demonstrate that changes in component specifications, composition, manufacturing process or manufacturing location do not adversely affect the quality and performance of the product (efficacy or safety). These assessments occur on a continual basis, and regulatory agencies expect that the comparability exercise will become more comprehensive as the product progresses through development.

The FDA Draft Guidance for Industry on Liposome Drug Products [65] and the ICH Q5E guidance (Comparability of Biotechnological/Biological Products) [82] can provide useful perspective on the topic of comparability. The comparability exercise should be informed by an analysis of the potential consequences of the change, based on the existing understanding of the product and manufacturing process. Establishing comparability on the basis of analytical testing may be an appropriate strategy early in development, when there is limited characterization of efficacy and toxicity, or later in development, if the relationship between analytical measures and bioperformance has been clearly established. Biological assays may be deemed necessary when there is an insufficient understanding of the relationship between analytical measures and bioperformance. Biological assays can be developed to assess both efficacy and toxicity, and may include plate-based binding assays, cell-based potency assays, cytokine release and complement activation assays. As an example of a biological assay directed toward assessing toxicity, Tekmira Pharmaceuticals reported at the 2010 DIA Oligonucleotide-Based Therapeutics Conference on a laboratory assay based on primary human immune cells that replicated immune stimulation observed in one subject in its apoB SNALP Phase I clinical study (Tekmira Pharmaceuticals Press Release, 23 March 2010). The company intends to use this assay to screen new RNAi drug candidates.

Non-clinical studies may be required when analytical and biological assays are insufficient to assess efficacy and safety,

particularly when there is a major change in the delivery system. Some examples of changes that might warrant non-clinical evaluation could include: i) a change in the chemical ID of a functional excipient, ii) a major change in a physical property (e.g., > 20% change in particle size, a morphological change or phase transition temperature shift of > 5°C) and iii) modest changes to multiple composition or physical properties for which the impact on potency and efficacy is poorly understood. A key element in the design of the non-clinical study is establishing appropriate comparability criteria. Measures could include pharmacokinetic (PK) parameters, pharmacodynamic (PD) response, or direct measures of efficacy or toxicity. For conventional small molecule drugs, assessing comparability is often done on the basis of PK parameters, such as C_{max} or AUC. For conventional liposomes, the FDA guidance recommends PK analysis of encapsulated and free drug [65]. In the case of lipid-based nucleic acid delivery systems, where the delivery system plays a central role in biodistribution, cellular uptake and endosomal release, plasma pharmacokinetics may be an insufficient means to determine comparability. If an appropriate biomarker of efficacy or toxicity exists, PD measures may be appropriate to demonstrate comparability. Otherwise, efficacy and/or safety studies may be necessary. It is important to establish the relevance of the non-clinical animal model to performance in higher species when designing the non-clinical study. It is also critical to understand the variability in the response(s) being measured and to design the study with sufficient sample size to ensure statistical power.

6. Expert opinion

RNAi has tremendous potential as a powerful new tool in drug discovery and as a unique new class of therapeutics that may address significant unmet medical need. Owing to the numerous physiological barriers that must be overcome in order to introduce synthetic siRNA into the target cells, safe and efficacious delivery has been among the most difficult challenges to overcome in realizing the potential of RNAi. This is particularly true in the case of systemic delivery. LNPs have emerged as one of the most mature class of systemic delivery technology, with several products progressing into early clinical studies. To achieve effective delivery, these systems must perform a number of functions. They must encapsulate the nucleic acid and protect it from degradation, transport it through the circulation to the target tissue, interact with and enter target cells, and release the siRNA cargo into the cytosol. They are, therefore, inherently complex, and this complexity presents a major challenge to successful pharmaceutical development.

As multi-component, self-assembled complex systems, LNPs must be carefully designed, their assembly must be strictly understood and controlled, and they must be thoroughly characterized in order to ensure product quality. The interactions with biological systems that drive both efficacy and toxicity are very sensitive to particle properties, such as particle size, surface charge and phase behavior. It is, therefore, important to tailor the properties of the delivery system to the specific therapeutic application, For example, to reach hepatocytes, particles must be able to pass through the liver endothelial fenestrae and should be < 100 nm in size [83-87]. Likewise, surface charge plays a critical role in the interaction with plasma opsonins [29,63,88-90]. A neutral surface charge at physiologic pH can help to minimize opsonization and uptake by phagocytic cells of the RES [29]. It is important to recognize that there is no one-size-fits-all profile and that ideal particle properties will vary according to the route of administration, target tissue and cell type, and desired PK/PD profile, and must be optimized around these needs.

A deep understanding of the physical chemistry and engineering governing LNP assembly is, therefore, required in order to optimize composition and process. We have reviewed here in detail the steps involved in assembly and characterization of LNPs, but there is also a significant body of knowledge around their interaction with blood [32], cells and cell compartments such as the endosome [91,92], which is important for enabling successful development. Establishing the biocomparability of batches is one example of where this knowledge becomes important. To develop LNPs effectively requires multidisciplinary subject matter expertise in diverse areas such as synthetic chemistry, biophysics, engineering, biology, pharmacokinetics and toxicology.

We also believe that the challenges inherent to discovering and developing LNP delivery vehicles can be solved given the appropriate resources, and that the industry will continue to progress candidates through preclinical and clinical development. It is important to recognize that the siRNA therapeutics field is still young, and that the body of knowledge is still rapidly expanding. However, the ultimate successes in translating other complex pharmaceuticals, such as vaccines and protein therapeutics, into commercial products are relevant examples of what can be achieved [93].

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Declaration of interest

All authors are employees of Merck and Co., Inc.

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