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White Paper

Strategies for Successful Formulation Development of Lipid-Based RNA Delivery and Vaccines

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Nucleic acid therapy using RNA and DNA as the active pharmaceutical ingredient (API) has the potential to cause a paradigm shift in the way diseases are addressed. This innovative therapy targets the source of the disease at the genetic level and can be used to modulate the expression of one or more proteins simultaneously – a unique advantage over conventional biologics or small molecules. Given this mode of action, RNA and DNA therapeutics are a powerful means to treat, and in some cases cure, diseases that could not be addressed by other approaches.[1]

RNA can be used for many applications including protein production, gene silencing and activation, enzyme replacement therapy, and gene editing. The diverse set of applications warrants the use of RNA as APIs against cancer, pulmonary diseases, metabolic diseases, gene therapy and as vaccines (Figure 1).

In 1998, the first therapeutic nucleic acid, a DNA oligonucleotide, was approved for clinical use. The potential of RNA as a therapeutic was made clear in 2006 when the Nobel Prize in Physiology was awarded for the discovery of gene silencing by RNA interference (RNAi), followed by the approval of the first RNAi drug in 2018.[2] RNA continues to make news – this time as the approach for creating vaccines against SARS-CoV-2. Moderna, BioNTech, Curevac and many other companies are waging war against the COVID-19 pandemic, having translated the genetic sequence of the novel SARS-CoV-2 virus to messenger RNA (mRNA) vaccine candidates at record speeds. As of October 2020, there are at least 24 RNA based therapeutics and vaccines under development for COVID-19.[3]

Recognizing the potential of RNA-based therapeutics, this whitepaper focuses on lipid-based RNA therapeutic development.

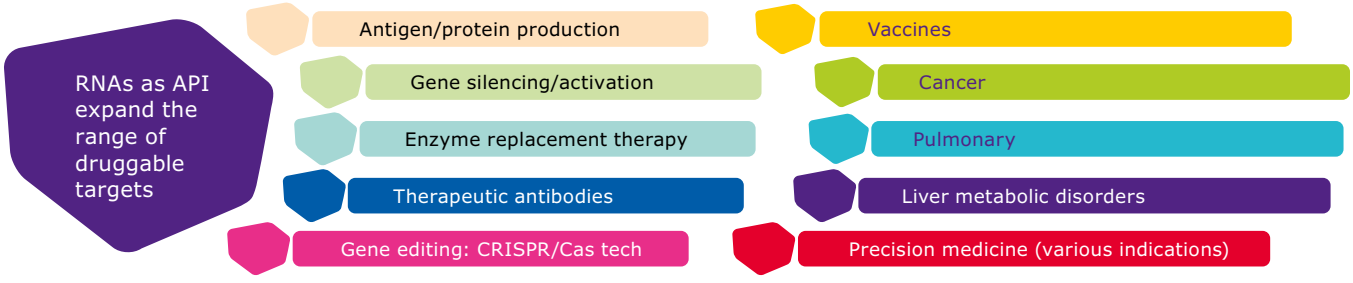

Figure 1. RNAs as active pharmaceutical ingredients can be used for a variety of applications and therapeutic modalities.

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Strategies to Leverage RNA as API – Including in the Fight Against COVID-19

Figure 2 summarizes the strategies by which RNA could be used to address diseases such as COVID-19. These approaches include:

- **mRNA of antigenic proteins:** The mRNA of viral protein(s) can be delivered to produce these proteins in the body, functioning as a vaccine.
- **mRNA of therapeutic proteins:** A therapeutic approach could be to deliver the mRNA of a therapeutic protein such as a neutralizing antibody that can prevent virus binding to cells.
- **RNA interference:** RNAi can be used to target key viral gene sequences, halting viral replication.

The success of these strategies against COVID-19 would act as a template for vaccines and drug development for a wide range of diseases and conditions, revolutionizing the field and expanding the potential of RNA-based therapeutics.

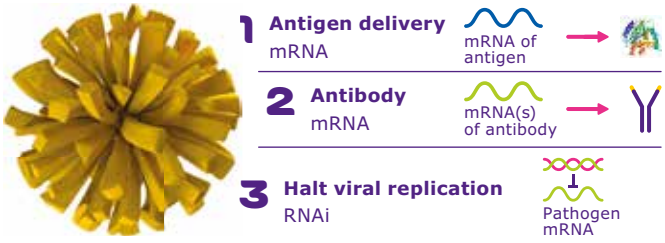


Figure 2. Possible RNA therapeutics approaches to COVID-19: 1. mRNA delivery of antigen proteins | 2. mRNA delivery of therapeutic proteins | 3. Use of RNAi to halt viral replication.

Design Principles for RNA Therapeutics

The critical first step when designing an RNA therapeutic is to select an appropriate gene target. Some diseases, such as cancer, have complex alterations in protein expression levels of several proteins, and hence, the choice of the gene target becomes critical. Based on the target, the RNA sequence can be designed based on whether the goal is RNA interference/activation via short interfering RNA (siRNA), microRNAs (miRNA) or antisense oligonucleotides (miRNA, ASOs), protein expression using mRNA, or gene editing using single guide RNA (sgRNA) and mRNA.

Following sequence optimization, decisions must be made related to the delivery of the RNA API.[4] Foreign RNAs can be easily recognized by the immune system and attacked by nucleases rendering them ineffective as an API. RNA APIs must also be able to traverse the negatively charged cell membrane to reach the site of action in the cell, such as the cytoplasm. For short RNAs, such as siRNA, miRNA, and ASOs, chemical modification of the bases and/or the backbone can provide in vivo stability and resistance to nucleases. Such molecules can also be conjugated to targeting molecules for targeted delivery; an example of this is N-Acetylgalactosamine (GalNac) which can direct the RNA to the liver. However, such approaches are not suitable for delivery of large RNAs such as mRNA. A more universal approach to RNA delivery, applicable to any size of RNA, is encapsulation within a viral or non-viral particle composed of lipids, polymers or inorganic material.

Abbreviations used: RNA – Ribonucleic acid; DNA – deoxynucleic acid; (siRNA) – short interfering RNA; miRNA – microRNA; ASO – antisense oligonucleotides; mRNA – messenger RNA; GalNac – N-Acetyl-D-galactosamine; RNAi – RNA interference; API – active pharmaceutical ingredient; DOPE – 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine; R-DOTAP Cl – R-1,2-dioleoyl-3-trimethylammonium-propane (chloride salt); (DSG PEG 2000) – Distearoyl-rac-glycerol-PEG2K

Lipids as RNA Delivery Vehicles

Non-viral drug delivery systems have several advantages over viral methods including better safety profiles, lower costs of production and the flexibility of cargo that can be delivered.[5] As such, non-viral delivery methods are more commonly used for RNA delivery (Figure 3). Of the non-viral approaches, lipid-based particles such as lipid nanoparticles (LNP) are the most advanced and the most frequently used delivery method.

Several features make lipid-based delivery an attractive system for RNA therapeutics. Lipid-based drug delivery is an established method with several approved drugs for both hydrophobic and hydrophilic molecules.[6] This delivery method is also suitable for multiple RNAs to be delivered at the same time, which can allow multiple antigen sequences to be delivered in a single vaccine injection. Lipids also allow co-delivery of small molecule drugs, such as immunosuppresants, along with the RNA. Lipid-based systems allow for a plug and play model, where different diseases could be addressed by changing the RNA sequence while keeping the same carrier. Some lipids, such as 1,2-dioleoyloxy-3-(trimethylammonium) propane chloride (R-DOTAP Cl) have immunostimulant properties and can function as adjuvants[7], reducing the number of components in the vaccine formulation, lowering the costs and effort required for regulatory dossier preparation.

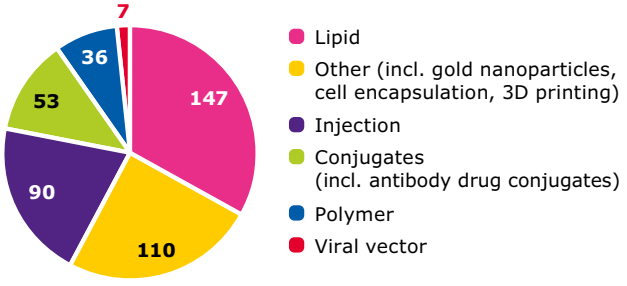


Figure 3. RNA pipeline showing number of preclinical and clinical trials based on delivery method employed (Source: Pharmacircle, August 2020). Note figure does not include information on ASOs.

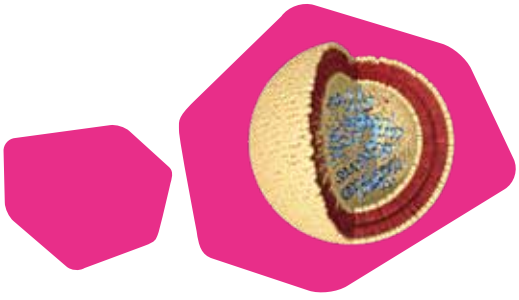
Parameters Defining the Performance of Lipid-Based Formulations

The performance of a formulation is mainly dependent on the composition of the drug delivery vehicle, the quality and characteristics of the raw materials, and the formulation process parameters. Each of these factors is described below.

Composition of the Delivery Vehicle

LNPs are usually composed of four different lipids, each of which play an important role (Figure 4).[8]

- **Cationic/ionizable lipids** are required for encapsulating the RNA via electrostatic interactions. Delivery to the hepatocytes (for boosting or silencing of protein expression) requires ionizable lipids (passive targeting, endosomal release) whereas uptake by immune cells is much easier and works also with strong cationic lipids. This lipid is also responsible for efficient release of the API into the cytoplasm. The structure of the cationic lipid has a major impact on the activity of the LNP, its toxicity, and its biodistribution, which also has an influence on potential toxicity effects in the body.
- **Polyethylene glycol (PEG) lipids** provide colloidal stability and prevent protein binding to the particle, thereby shielding it from the immune system and achieving longer circulation. The length of the PEG chain and fatty acid chains determine the circulation lifetime and fusogenicity, or how well the particle can fuse with the endosomal membrane, of the LNP. If the goal is prolonged circulation, longer fatty acid chains can be used, such as polyethylene glycol-distearoylglycerol (DSG PEG 2000).
- **Neutral/anionic lipids** provide structural stability and play a role in defining the fusogenicity and biodistribution. For example, a recent study showed LNPs containing dioleoyl phosphatidyl ethanolamine (DOPE), which plays an important role in endosomal release, led to enhanced delivery of mRNA to the liver as compared to 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC).[9] Recent studies suggests that these helper lipids also assist in stable encapsulation of the RNA.[10]
- **Cholesterol** is used to modulate the bilayer density and fluidity and uptake (raft formation) of the LNP. While there are animal-derived and synthetic versions of cholesterol available in the market, synthetic cholesterol offers several advantages including higher purity, lack of animal derived molecules such as prions, scalability, and highly consistent quality.



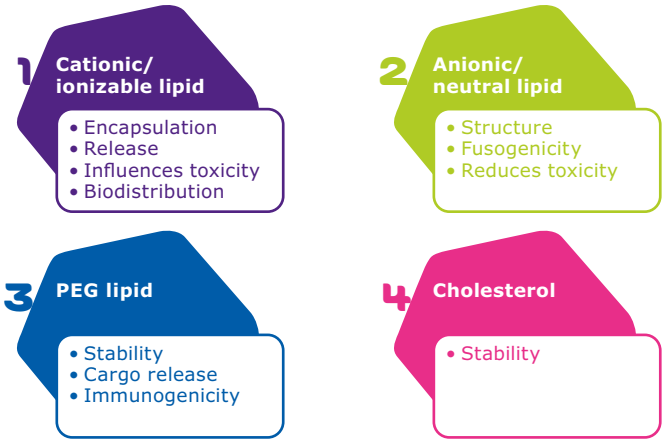


Figure 4. Key components of a lipid nanoparticle for RNA delivery.

Lipids should be chosen based on the delivery route in mind to achieve maximum efficacy and optimal biodistribution. In addition to the choice of lipids, the ratio between the individual lipids is an important component to fine-tune, as this has a direct impact on the bilayer fluidity and the fusogenicity of the LNP.

Quality and Characteristics of Raw Materials

The quality of raw materials has a major impact on the convenience of manufacturing, reproducibility, stability, and the drug release profile of formulation. Lipids should have high purity as impurities such as trans fatty acids can affect the bilayer stability and the drug release profile. Certain impurities that can cause side reactions or oxidation, and the presence of impurities such as nucleases would be detrimental to the lipids and API stability. In addition to high purity, consistent quality is essential for reproducibility and has a direct impact on the cost of development; inconsistent quality may require additional work, such as bridging toxicity studies, and a greater likelihood of regulatory challenges.

Material characteristics such as crystallinity are important considerations as they influence the solubility, stability and flowability of the lipids. This, in turn, determines the ease and reproducibility of the downstream formulation process.

Refinement of the manufacturing process is required to achieve high lipid quality. As an example, improvements in the process used to manufacture DOPE resulted in a free-flowing powder that is much easier to weigh and has superior dissolution when compared to the lumpy, wax-like consistency (Figure 5).

Wax-like DOPE
- Lumps, gel & foam
- Limited solubility even after lyophilization

Powder DOPE
- Free flowing powder
- Fast and complete dissolution

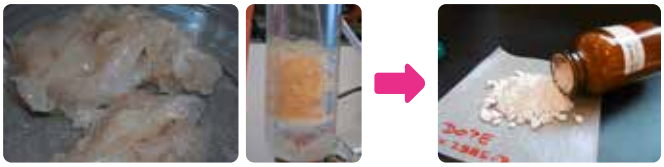


Figure 5. Improvements to the process used to manufacture DOPE enabled production of a free-flowing powder.

Steps to Improve Lipid Synthesis

- Reduce the number of GMP synthesis steps, which simplifies the process and reduces costs
- Avoid reaction conditions that lead to isomerization
- Consider scalability, economy of scale, batch size early in drug development
- Develop suitable analytics from the earliest stages

Formulation Process Parameters

The process conditions used during the formulation process have a major impact on the characteristics, stability, and the performance of the final formulation. A typical formulation workflow is shown in Figure 6.

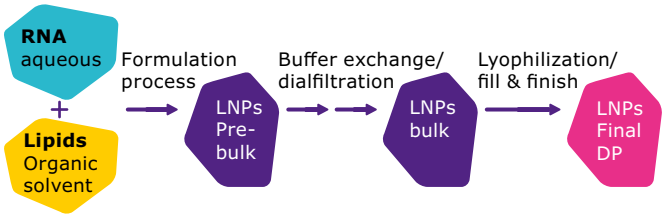


Figure 6. Workflow of manufacturing a lipid-based RNA formulation.

Typically, the workflow begins with preparation of a low pH aqueous solution of RNA. while lipids are dissolved in an organic solvent such as ethanol or chloroform, depending on the downstream formulation process used. A variety of formulation techniques can be used to achieve LNPs ("pre-bulk") that need to be purified, buffer exchanged, and concentrated to achieve the bulk LNPs. The bulk LNP solution is then either lyophilized or down-filled under aseptic conditions to achieve the final drug product (DP).

Some of the important parameters that should be controlled in the formulation process are:

- **Lipid concentration:** The lipid concentration impacts the size and polydispersity of the LNPs. Size is an important feature to control as it impacts the targetability/biodistribution of the LNP.
- **Lipid to RNA ratio:** It is important to adjust the ratio between the cationic/ionizable lipid and the RNA for maximum encapsulation of the RNA.
- **Formulation technique:** Different formulation techniques yield different sizes and polydispersity of particles (Figure 7).[11] The selected formulation process also affects the cost, speed, reproducibility, scalability, and stability of the formulation. For example, techniques such as high-pressure homogenization can lead to lipid oxidation and hydrolysis. Owing to its scalability and ability to achieve high encapsulation efficiencies, ethanol injection is the most commonly used formulation technique for manufacturing LNPs. When using this approach, one must consider the flow rate and the injection hole size. The mixing speed must be adjusted and the process temperature monitored as RNAs are prone to degradation.

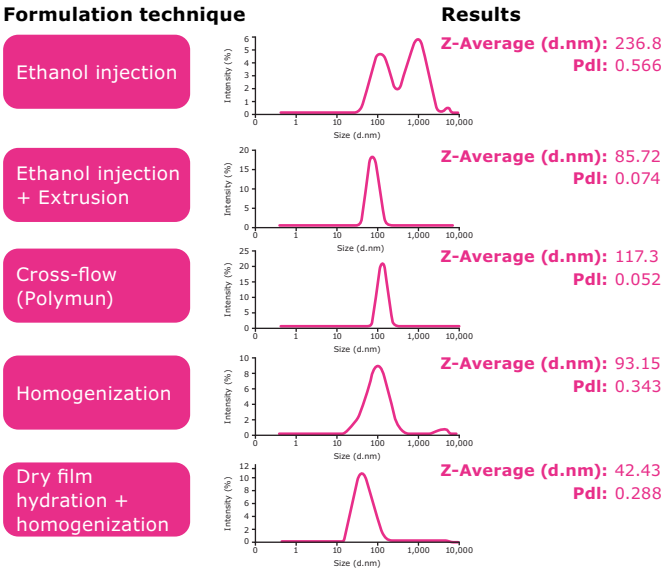


Figure 7. Effect of different formulation techniques on size and polydispersity of the resulting LNPs. Data from Dr. Finn Bauer, Dr. Michael Platscher with Polymun, Austria.

- **Diafiltration parameters:** Diafiltration is used to adjust the pH of the "pre-bulk" LNP solution from low pH to neutral pH. The duration of this process is very important because lipids can be hydrolyzed at low or high pH. Lipid hydrolysis leads to the formation of lysolipids, glycerphospho compounds, and free fatty acids. These impurities can adversely affect the permeability of the lipid bilayer and thereby the stability of the formulation and the drug release characteristics. Degradation of lipids can also increase the particle size, polydispersity, and lead to aggregation. It is also important to consider the selection of hollow fiber modules as they are available in different lengths and pore sizes; hollow fiber modules should be compatible with the rest of the equipment. In this step, the LNPs are being subject to high shear forces and as such, the variable parameters of permeate flow, concentration factor, and the inlet pressure must be controlled.
- **Sterilization/filtration:** If filtration is used for sterilization, the maximum capacity of the filter must be calculated by determining how much volume of the solution can be passed through a filter before blocking it completely. Sterilization of the final and intermediate products, however, cannot always be accomplished using 0.2 µm filtration. Heat sterilization by autoclaving, or high-pressure sterilization, and γ-irradiation are possible alternatives in some cases.
- **Filling:** Depending on the final dosage form of the formulation, the LNP solution is either lyophilized and stored as a solid or dispensed as a liquid solution into vials. If the final dosage form is liquid, key considerations include buffer conditions, pH, cryoprotectants and antioxidants, storage temperature, storage under inert gas. The vial size and the filling volume are also important to consider beforehand, to account for extraction losses and requirements for analytical tests.

Analytical Testing is Essential Throughout Manufacturing

The suite of analytical tests expected by regulatory authorities is described in the guidelines FDA issued in 2018 for liposomal drug products.[12]

Appropriate and robust analytical methods are required to identify the critical parameters that can affect the performance of the formulation. For liposomal drug products, full physicochemical and biological characterization is required before regulatory submission. The lipid identity, quantity, and impurities must be identified; the identity tests should be capable of distinguishing the intended lipid component from lipids with similar structures. Chemical analysis is required to identify and quantify drug product components and the degradation products. Any possible leftovers from the manufacturing process, such as metal, residual solvents, and catalysts should also be checked. Parameters such as the encapsulation efficiency, the API integrity, and the drug release profile need to be evaluated.

The LNP structure can be assessed by spectroscopic or other analytical methods such as measuring the size, polydispersity, and the zeta potential. The presence of particulate matter should also be tested.

The level of endotoxins should be evaluated whenever possible via chemical (non-animal derived LAL test alternatives) or biological tests. Bioburden, the presence of microorganisms, should also be tested in the lipids, API and final formulation.

A Proactive Approach is Essential

As the drug development process is a long and costly process, a proactive approach is essential for success. Product development of new formulation components, such as novel lipids, should be carried out in parallel with the drug development process (Figure 8). After fixing the composition of the formulation, the lipids to be included, and the formulation technique during the initial research phase, the next step is process research. This stage involves synthetic route finding to produce material of appropriate quality and develop analytical methods capable of identity and purity testing. In this phase, the formulation process conditions need to be evaluated.

By the time preclinical studies are initiated, the process of raw material synthesis and formulation should be optimized to get deliver the desired economic yields. Major safety aspects need to be determined and the process adjusted accordingly. By this point, reliable analytical methods should be in place and initial product stability studies initiated, along with GMP manufacturing.

Scale up of the manufacturing process and the formulation are then completed along with a set of other critical activities, while keeping in mind the commercial quantities needed for later clinical steps. For example, the equipment must be adjusted with cleaning aspects in consideration, analytical methods must be implemented including corresponding in-process controls, packaging requirements must be defined, raw material providers need to be qualified and product stability studies should be ongoing.

Finally, during process maturation, analytical methods and manufacturing processes are validated by defining the operating and acceptable ranges of the critical process parameters.

Ongoing dialogue with the raw materials supplier and the manufacturing partner is critical to ensure that the product development is progressing in line with the drug development program timelines.

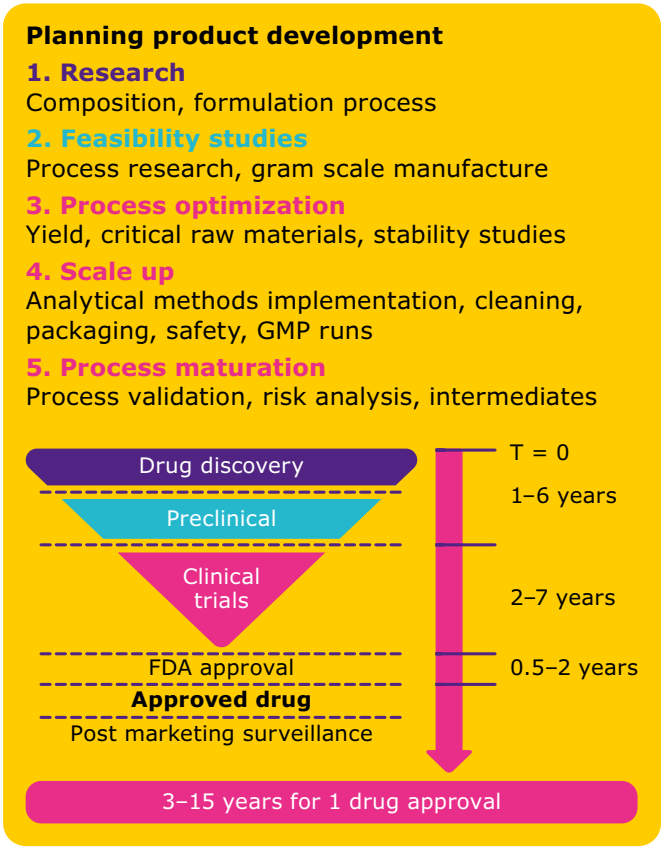


Figure 8. Novel formulation raw materials can be developed along with drug development.

The Drug Approval Process can be Streamlined

To ensure a smooth and streamlined drug approval process, all clinical data and detailed documentation regarding raw materials and the manufacturing process are needed. A description of each unit operation used in the manufacturing processes of the raw materials and the final drug product is required. The drug loading process should be clearly explained as well as the methods to remove the unencapsulated drug. Any new manufacturing steps or packagings require an in-depth description. All impurities that could be present in the formulation that could lead to toxicity or structural changes also need to be analyzed and listed. It is important to note that the availability of raw material chemistry, manufacturing

and control (CMC) data is more critical than the availability of a drug master file (DMF). If the CMC section of the drug delivery system has already been reviewed as part of another drug approval process this can help to shorten the timelines.

Consistency in the quality and source of the raw materials can shorten timelines because any changes made between the clinical phases must to be described in detail along with their clinical relevance. Consistent quality also means reproducible data which in turns means more confidence in the results.

Completing all steps of product development, such as validated methods, the manufacturing process, and at least three stability studies of each lipid and the final drug product, prior to the NDA submission, is of utmost importance. Collaborating with an experienced partner to ensure inclusion of all data required for regulatory submission is advantageous.

Selecting a CMO Partner

An experienced contract manufacturing organization can provide invaluable support for RNA therapeutic and vaccine formulation, quality lipids and LNP manufacturing. Selecting the right partner, early in your process, can provide a competitive advantage and accelerate development and manufacturing.

Merck is well-versed in the development and manufacturing of GMP lipids and has a strong technical and regulatory team with more than 85 years of combined experience. Developers of RNA-based therapeutics can select from an extensive portfolio of lipids or opt for custom manufacturing. Merck is experienced in the manufacture of ionizable lipids, PEGylated lipids, and targeted lipids, with manufacturing sites around the world that are regularly audited by regulatory authorities and customers.

Conclusions

RNA therapeutics have the potential to be a powerful means to tackle diseases that can't be addressed with traditional small molecule therapeutics or biologics. The approval of an RNA vaccine for COVID-19 will represent a remarkable breakthrough and redefine our approach to vaccines and infectious diseases.

Lipids are the most widely used delivery system for RNAs therapeutics and LNPs offer a plug and play system for RNA therapeutics, where the same vehicle can be used to target different diseases. For successful development of lipid-based RNA therapeutics, however, it is crucial to plan ahead. Creative and thoughtful design of the formulation is needed, as the choice of raw materials and the formulation process are as important as the drug delivery method itself. Ensuring quality of raw materials and the formulation is critical, as inconsistent quality implies irreproducible results, regulatory hurdles, high costs, and wasted resources. Similarly, selection of a supplier with a robust supply chain is also critical to ensure the necessary product quality and reliable delivery timelines.

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