Commentary

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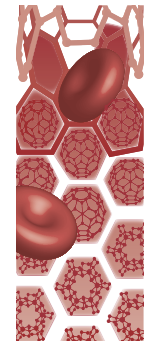

High-throughput synthesis and characterization of next-generation lipid nanoparticles for enhanced *in vivo* performance

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"These recommendations may expedite the emergence of next-generation nucleic acid-LNPs for regenerative medicine applications."

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Next-generation lipid nanoparticles (LNPs) have the capacity to dramatically improve the intracellular delivery and release of functionally active, nucleic acid-based therapeutics. LNP therapeutics that incorporate entities such as mRNA, sa-RNA, miRNA, siRNA, pDNA, antisense oligonucleotides (ASOs) and so on, can enhance the production of therapeutic monoclonal antibodies, expand the arsenal of critically needed vaccines and increase the effectiveness of genome- and DNA base-editing technologies. In particular, mRNA-based therapeutics can be easier and faster to manufacture compared with protein and viral therapeutics because they can be developed in a cell-free environment via *in vitro* transcription. Selective delivery of mRNA to specific cell types is enabled by the ability to decorate the surface of LNPs with targeting molecules. Encapsulation of mRNA therapeutics in LNPs is expected to facilitate the efficient targeting of undruggable and/or poorly druggable protein targets, thus augmenting the capacity and production of immunotherapies [1,2]. In addition, by encapsulating mRNA into LNPs, the delivery and uptake of mRNAs, as well as other nucleic acid drugs, are protected from enzymatic degradation. Encapsulation in LNPs also prevents the induction of mRNA immunogenicity. Currently, there exists only a small number of approved mRNA-based LNP therapeutics on the market, but, fortunately, the numbers and types of mRNA- and other nucleic acid-based therapeutics in clinical trials are rising rapidly [3]. Furthermore, polymeric [4] and hybrid polymer-lipid [5] nanoformulations under development show promise for mRNA delivery, as complements to the LNPs; potential benefits include structural stability and chemical flexibility. There are several potential factors contributing to the low number of approved mRNA-based therapeutics, such as the lack of available automated platforms for combinatorial syntheses of mRNA-LNPs linked to *in vitro* and *in vivo* high-throughput screening (HTS) systems (critical need 1), mRNA and/or LNP reference materials to facilitate measurement assurance, method development and technology innovation (critical need 2) and robust test methods and protocols linked to consensus documentary standards that appropriately describe the manufacture and syntheses of mRNA-LNPs, as well as characterization of the physicochemical and biological quality attributes (QAs) of the nucleic acid and drug delivery component of these therapeutics (critical need 3). For the purposes of this commentary, we describe potential solutions that will address the limitations of the current mRNA-LNP therapeutic development process. These recommendations may expedite the emergence of next-generation nucleic acid-LNPs for regenerative medicine applications.

We concentrate primarily on systems that may enhance the efficient development and characterization of LNP delivery vectors. Regarding critical need 1, concerted efforts to create combinatorial synthetic (CS) libraries of

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LNP structures were previously developed and optimized for the delivery of siRNA [6–8]. In general, CS techniques that are based on solid-phase synthesis, Michael addition, click chemistry and so on, can be used to facilitate the development of a large variety of LNP structures. LNP diversity is built-in by varying molecular factors such as lipid type, tail length, charge and structure, as well as overall LNP size. Similar CS approaches can be brought to bear for the development of LNP libraries to deliver mRNA [9]. CS LNPs can be prepared using automated robotic systems implementing solvent injection or microfluidic techniques in a 96- or 384-well plate format. The appropriate use of multifactorial analyses that implement design-of-experiment strategies for LNP library design/development is required to obtain optimal benefit from the application of CS techniques [3]. Furthermore, using CS techniques, it is estimated that LNP libraries containing $1 \times 10^8 - 2 \times 10^{11}$ structurally diverse LNPs [9] can be created using robotic systems. It is worth emphasizing that picking the proper LNP structures can facilitate efficient mRNA cellular uptake and endosomal escape, two of several LNP QAs that are critically important for establishing the efficacy of mRNA LNP therapeutics [10]. Sorting, purification and optimization of the crude CS library using *in vitro* HTS techniques for relevant physicochemical QAs must be methodically conducted [6]. Selecting suitable LNPs for further development and formulation into therapeutics is an arduous task as *in vitro* testing data are notoriously incongruent with *in vivo* testing data. To further complicate the selection of therapeutically viable LNPs, the *in vivo* biodistribution of LNP therapeutics is not correlated with their biofunctionality at the delivery site [11]. However, one highly innovative path for the quantitative evaluation of biodistribution and biofunctionality in tandem is to encapsulate either DNA or mRNA molecular barcodes [12,13] into the LNPs during the initial CS step. Molecular barcoding allows for HTS of the respective LNPs upon binding to their designated targets at the cellular and tissue levels. Quantification of LNP biodistribution is subsequently based on target DNA sequence amplification via PCR followed by next-generation sequencing (NGS). Several researchers have demonstrated the practical benefits of encapsulating DNA, mRNA or ASO molecular barcodes into structurally diverse LNP libraries followed by quantitative HTS studies in *in vivo* models [11–14]. Quantification of LNP delivery biofunctionality either *in vitro* or *in vivo* can potentially be addressed via the use of flow cytometry techniques after labeling the LNPs with appropriate fluorescent probes [15,16].

The need for both mRNA and/or LNP reference materials (critical need 2) has been described by industry, government regulators and academic subject matter experts. This has been debated for a number of years in the general nanomedicines field from the regulatory science perspective [17], and, lately, has been emphasized in the RNA therapeutics field [18]. One important challenge for reference material development in RNA therapeutics is the stability of the reference material itself during storage and handling; this applies both to the chemical integrity of mRNA and lipids and to the colloidal stability of the LNPs, which are both affected by environmental conditions such as elevated temperature. For mRNA-LNPs, this is strikingly reflected in the stability challenges faced by the COVID-19 vaccines [19]. In terms of molecular stability, unmodified RNA is inherently much less stable than DNA, for example, largely owing to the presence in RNA of a reactive 2′-OH group on the ribose units, as well as abundant and ubiquitous degradative RNAses in the environment. LNPs are composed of endogenous and synthetic lipids that are stable as isolated compounds, but their assembly into the (noncovalently) bonded, multicomponent nanoparticles introduces challenges. Our lab has observed (unpublished data) severe instability upon extremely high dilution, even at short time scales (minutes to hours). Another complicating yet defining feature of LNPs is the function of RNA as not only the active pharmaceutical ingredient but also as a crucial structural component of the LNP through its electrostatic complexation with the ionizable lipids. This inherently and fundamentally challenges the capacity to produce any reference material for the carrier LNP as such without a biologically (pseudo-)active mRNA inside. Finally, it has been recently shown that minor impurities originating from the ionizable lipids – before or after LNP formulation – can lead to covalent lipid–nucleoside adducts, which render the mRNA untranslatable [20]. This impurity formation mechanism likely applies to virtually all ionizable lipids currently used, has probably been severely underdetected and requires highly targeted and sensitive analyses, typically by chromatography coupled to mass spectrometry (LC-MS/MS), to detect. The latter aspects point to one of the critical applications for reference materials in this area, which is to help develop and qualify the robustness of analytical methods.

Translation of these emerging nanomedicines into the global healthcare market is challenged on many levels by the lack of standardized methods and consensus documentary standards (critical need 3) for characterizing the cytoplasmic delivery, endosomal release, particle size, particle size distribution, lipid composition, stability, purity and identity of LNPs. Although robust methods like field flow fractionation exist to measure nanoparticle-specific QAs like size and size distribution of liposomes, LNP-specific method adaptations are frequently required

(e.g., [21]). The need to adapt and optimize characterization methods originally developed for liposomes and other lipid-based nanoparticles stems from the fact that LNPs contain structural components, such as ionizable lipids, that interact with RNA to influence not only the morphology but also the stability of the nanoparticle and its propensity to interact with other entities. Yet, the most pressing need is arguably for methods to assess the mRNA component, its interaction with the LNPs and its translocation into and translation inside the cytoplasmic compartment. Without robust standardized methods for LNP QAs, these LNP therapeutics cannot be accurately and reproducibly characterized either within or across laboratories. Therefore, ASTM International has recently initiated a Standard Guide work item (WK75607) [22] to address the need for mRNA-LNP characterization, which includes participants from regulatory authorities, standardization bodies, academia and the pharmaceutical industry. High-level evaluation of well-defined QAs may lead to the production of mRNA-LNP therapeutics that demonstrate enhanced *in vivo* performance characteristics (safety and efficacy).

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