Perspective

Xiao Liu, Xu Zhang, Jiulong Li and Huan Meng*

Enrichment of nano delivery platforms for mRNA-based nanotherapeutics

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Abstract: Lipid-based nanoparticles (LNP) have shown significant progress in delivering mRNA for therapeutics, particularly with the success of coronavirus disease 2019 (COVID-19) vaccines. However, there are still challenges, such as organ-specific targeting, sustained protein expression, immunogenicity, and storage that need to be addressed. Therefore, there is interest in developing additional nano drug delivery systems (DDS) to complement LNP technology. Some of these include polymer, lipid-polymer hybrid, organic/inorganic hybrid nanostructure, and inorganic nanoparticle. In our opinion, LNP technology may not be suitable for every disease scenario in categories such as infection disease, cancer, pulmonary disease, autoimmune disorders and genetic rare disease (among others). This is because different diseases may require distinct administration routes, doses, and treatment durations, as well as considerations for biological barriers that may lower the efficacy and/or exert safety concern. In this perspective, we will highlight the need and potential for enhancing the diversity of nano delivery platforms for mRNA-based nanotherapeutics.

Keywords: high throughput screening; mRNA; nano/bio interface; nano drug delivery system; non-LNP nanocarrier; nanosafety.

Significant progress has been made using lipid-based nanoparticles for mRNA delivery, including the stunning success of mRNA coronavirus disease 2019 (COVID-19) vaccines against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic [1]. This opens a new era of designing mRNA therapeutics, which are previously challenging partially due to the lack of *in vivo* delivery mechanism and understanding of mRNA technologies. Lipid-based nanoparticles (LNP) have gained great attention as non-viral vectors due to their capability of enhancing the stability of mRNA molecules, relatively high *in vivo* transfection, good biosafety and ease-of-manufacture. The lipid design and iterative optimization principles are nicely reviewed elsewhere [1–3]. The representative examples using LNPs for mRNA delivery were summarized in Table 1. While it is popular to consider "lipids" for mRNA payload as a result of understanding of quantitative structure-activity relationship (QSAR) [2, 4], the discovery of zwitterionic ionizable lipids [5], readiness of microfluidic for good manufacturing practice (GMP) [6], and established quality control (QC) [7], it is unlikely that lipid-based technology could be utilized in every disease scenarios in the category of infection disease, cancer, pulmonary disease and genetic rare disease, which may require distinct administration routes, dose and treatment duration, consideration for biological barriers and multifunctionality design.

It could be important to contemplate additional nano drug delivery system (DDS) to enrich the family of mRNA nanocarriers. The enthusiasm originates from the major challenges that LNP technology faces, which could become the basis for developing non-LNP platforms for mRNA delivery (Figure 1). For example, this includes organ specific targeting and protein expression, immunogenicity, sustained and controlled protein expression, long-term storage, encapsulation of versatile mRNA molecules and combination, and design of multifunctional system such as co-delivery or being theranostic. In fact, a variety of new materials are being developed for mRNA delivery, particular for *in vivo* application, including polymer, outer membrane

***Corresponding author: Huan Meng**, CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety and CAS Center for Excellence in Nanoscience National Center for Nanoscience and Technology, 11 Zhongguancunbeiyitiao, Haidian District, Beijing 100190, China; and University of Chinese Academy of Sciences, Beijing 100049, China, E-mail: mengh@nanoctr.cn. https://orcid.org/0000-0001-8844-3938

Xiao Liu, Department of Gastroenterology, Beijing Hospital, National Center of Gerontology, Institute of Geriatrics Medicine, Chinese Academy of Medical Sciences, Beijing, China

Xu Zhang and Jiulong Li, CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety and CAS Center for Excellence in Nanoscience National Center for Nanoscience and Technology, Beijing, China; and University of Chinese Academy of Sciences, Beijing, China

Table 1: Representative works using LNPs for mRNA delivery.

Formulation	Usage	Major discovery
DOG-IM4:Phospholipid:cholesterol: PEG=50:10:38.5:1.5	Immunization	The ionizable lipid, DOG-IM4, significantly enhanced the thermostability of LNPs. DOG-IM4 LNPs delivering influenza HA mRNA showed strong immunization reaction in mice and macaques [8].
Ionizable lipid:DOPE:cholesterol:C14-PEG2000=35:16:46.5:2.5	Bone abnormalities	Designed an ionizable lipid conjugated with alendronate that targeted bone Ca^{2+}. The LNPs enhanced mRNA delivery and secretion of therapeutic bone morphogenetic protein-2 from the bone microenvironment [9].
COATSOME SS-OP:DOPC:cholesterol: DMG-PEG2000=60:10:30:1.5	Cross blood-brain barrier	Delivered mRNA-LNP to the brain using microbubble-assisted FUS-induced BBB opening. Exogenous proteins were produced in the brain parenchyma outside blood vessels and microglia [10].
DALs:DOPE:Cholesterol:DMG-PEG =20:30:40:0.75	Melanoma	The LNP consisting of a new ionizable lipid containing di-amino groups with various head groups (DALs) effectively delivered different IL-12 and IL-27 mRNAs to tumors. Induced infiltration of immune effector cells into tumors [11].
C14–4:DOPE:Cholesterol:PEG =35:16:46.5:2.5; cholesterol substituted by six hydroxycholesterol candidates at 12.5, 25, 50 and 100%.	Jurkat cell	Substitution of 25 and 50% 7α-hydroxycholesterol for cholesterol in LNPs strongly enhanced mRNA delivery to primary human T cells ex vivo. The LNPs increased late endosome production and reduced endosome recycling [12].
SAL lipid:DOPE:cholesterol:DMG-PEG2000=20:30:40:0.75	SARS-CoV-2	STING-agonist conjugated lipid derivatives (SALs) served as both a delivery vehicle and immune adjuvant to the SARS-CoV-2 mRNA vaccine. LNPs consisting of SAL12, delivering SARS-CoV-2 Delta spike mRNA brought robust specific IgG and neutralizing antibody (nAb) production in mice vaccination [13].
TCL053:DPPC:cholesterol:DMG-PEG =60:10.6:27.3–28.7:0.7–2.1	Duchenne muscular dystrophy	Developed a pH-dependent ionizable lipid with three hydrophobic tails and formulated it into LNP to preferentially target skeletal muscle. The LNP could be repeatedly administrated intramuscularly with dystrophin protein recovery [14].
306-N16B:306-O12B:cholesterol:DOPC: DMG-PEG2000=25:25:38.5:10:1.5	Pulmonary lymphangioleiomyomatosis	LNP exhibited efficient delivery of the mouse Tsc2 mRNA for the restoration of TSC2 tumor suppressor in tumor and achieved remarkable anti-tumor effect [15].
IC8: DOPE: Chol: PEG-20000-DMG=35:16:46:2.5; MnCl2 added in citrate buffer	SARS-CoV-2	Mn doped into LNPs as an adjuvant enhanced mRNA delivery and immunization [16].
Cholesterol, DSPC, PEGylated lipid and a cationic lipid	SARS-CoV-2	The resulting mRNA vaccine had an acceptable safety profile and induced robust immune responses among participants. Homologous boosters induced immune memory. This formulation was advanced to phase 2a study [17].

SARS-Cov-2, severe acute respiratory syndrome coronavirus; LNP, lipid-based nanoparticles.

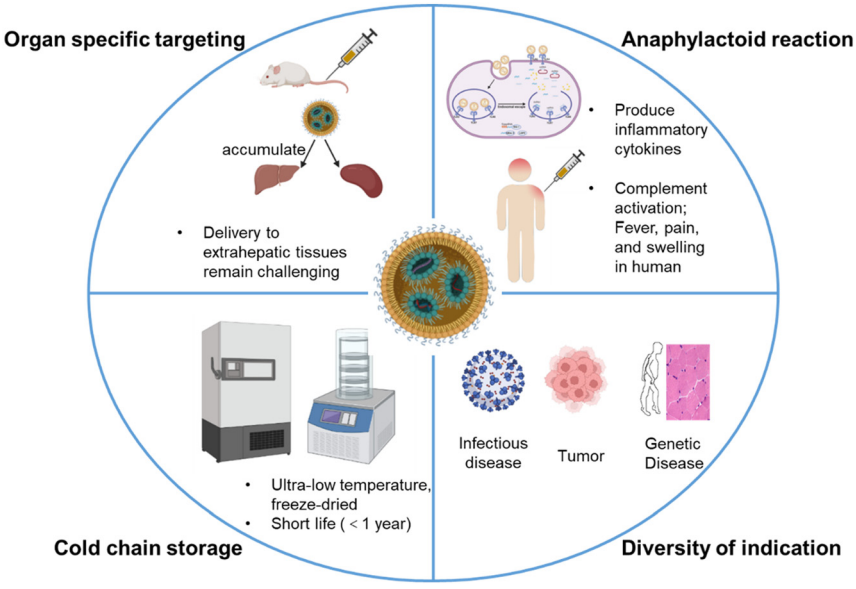

Figure 1: Major challenges of lipid-based nanoparticles (LNP) based mRNA delivery systems. Reproduced with permission from Kiaie et al. [20].

vesicles (OMV), lipid–polymer hybrid, organic/inorganic hybrid nanostructure, inorganic nanoparticle, etc. [1, 18, 19] For instance, while LNP based mRNA vaccination is an emerging new approach to manipulate and train antitumor immunity, the option for "tailored" tumor vaccine is limited. In this regard, it was possible to genetically design OMV that concurrently carries RNA binding protein (L7Ae) and lysosomal escape protein (listeriolysin O) on the vesicle surface. The resulting OMVs are capable of adsorbing mRNA antigens, followed by dendritic cell internalization and cross-presentation. This "plug-and-display" strategy could be used for tailored and/or computationally designed mRNA payload in immunologically "cold" tumors, such as melanoma and colon cancer [18]. Due to its versatile structure, our laboratory designed a biocompatible mesoporous silica nanoparticle (MSNP) based platform for mRNA delivery *in vitro* and *in vivo* (Figure 2) [19]. We established a unique assembly protocol for mRNA loading, which was achieved by premixing mRNA with positively charged polymer, then electrostatic attachment on MSNPs, which were optimized for particle size, porosity, surface topology and aspect ratio. The iterative optimization allowed us to identify the best-performing carrier to deliver a luciferase mRNA in mice, although the previous efforts mainly focus on other type of nuclear acids such as siRNAs [19].

For therapeutic purpose using mRNA-based strategy, the research tasks are way beyond chemical composition. Although it may be too early at this stage to streamline definitive blueprint of how to develop a ready-to-translate mRNA nanoplatform, we are beginning to appreciate the key components to build such platforms. The first consideration is to use iterative optimization and high throughput/content screening approach to assess large number of mRNA nanocarriers with controlled size, shape, charge, coating, N/P ratio, storage condition, colloidal stability, self-assembly sequency, treatment concentration, dose, cell type (*in vitro*), and administration route (*in vivo*), etc. The rapid and/or high-volume data generation could be used to assist decision-making and prioritization for carrying out the further study such as labor-intensive rodent and big animal experiments and ultimate human studies. Noteworthy, empirically designed or a "formulaic" design (that is usually a novel material, overlooking the roles of physicochemical properties in the sophisticated biological environment) are unlikely to succeed. Like SM-102 lipid that has been optimized for more than 10 years [21], there is no shortcut for developing new mRNA nanocarriers if both the expectation of platform development and translation are on the table.

The second consideration is the identification of disease indication with an early engagement of physicians. This point could be highly valuable in terms for future design of clinical studies and ultimate usage for patient care or disease prevention. While a new platform could be chemically novel for mRNA, critical healthcare needs, competitive advantage, freedom to operate (from the perspective of intellectual property) and the pathophysiology of an upfront defined disease seem to be critical. The impact will determine the sustainability of the mRNA research and translational program, especially beyond the proof-of-principle stage.

The third consideration is the biological barrier(s) that may negatively impact bioavailability and ADME (absorption, distribution, metabolism, and excretion) profile of mRNA nanocarriers. Escape from acidic cellular components, which are intensively studied in the field and primarily relies on so-called "proton sponge effect", is just one obstacle in the long journey of mRNA in the biological systems. Additional biological barriers [22], which exist at organ/tissue (e.g., skin, air-blood lung barrier, barriers in circulation, blood-brain barrier, reticuloendothelial system, fibrinogen deposition, enzymes in the interstitium space) and cellular levels (e.g., cell membrane receptor, intracellular enzymes), could be important. These factors seem to be critical, especially for the delivery of mRNA beyond the scope of vaccine and liver diseases. Moreover, introduction of mRNA nanocarriers *in vivo* is capable of interacting with various biological molecules, such as proteins, lipids, and sugars, which surely impact on ADME, even safety evidenced by complement activation mediated pseudo allergic reactions in humans received LNP mRNA vaccine [23]. In certain applications such as delivering a mRNA that encodes a tumor suppressor protein, it is necessary to address the heterogeneity of these carriers at tumor site as it may determine the long-term efficacy, including the possible development of drug resistance. Moreover, for certain administration route and biological barrier, such as in the gastrointestinal tract, mRNA delivery is very challenging. In this case, mRNA molecules are highly susceptible to degradation by enzymes in the gastrointestinal tract and have limited absorption across the gut epithelium. The negatively charge making the payload difficult to cross biological membranes. The acidic environment of the stomach and the presence of bile salts and enzymes in the small intestine can also negatively affect mRNA stability and reduce its effectiveness. All of these has become a big challenge that requires further research and innovation.

The fourth consideration is chemistry, manufacturing, and controls (CMC) and QC of mRNA pharmaceutical product. Particularly for mRNA nanomedicine, one can foresee that the field is rapidly moving into translation in which nano-CMC is one of the major engineering hurdles that is not

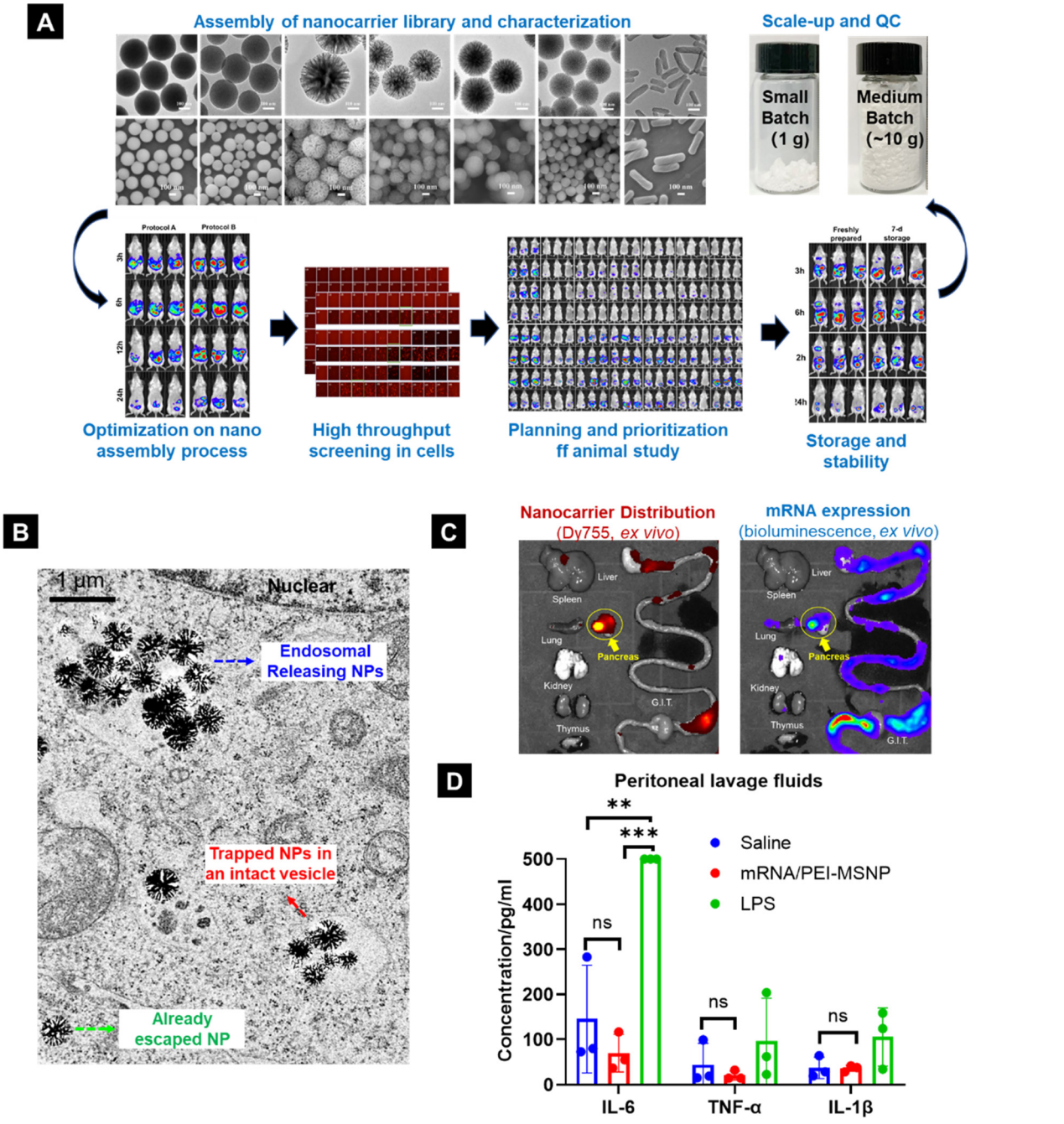

Figure 2: Use of an iterative optimization approach to design a MSNP based nanocarrier. (A) Assembly of a MSNP library with precisely controlled physicochemical properties. After comprehensive characterization, we optimized the nano assembly protocol by altering the addition sequence of mRNA, cationic polymer and silica nano cores. Various mRNA nanocarriers were subjected to *in vitro* cellular screening and protein expression experiment in mice. We also evaluated the stability of the mRNA nanocarrier after storage at different condition. The multi-round of comparative analyses allowed us to identify the best-performing MSNP candidate, which was manufactured at different batch sizes for *in vivo* evaluation in mouse (about 20 g body weight) and rat (about 250 g body weight). (B) Use of high-resolution cellular TEM microscopy to visualize a representative biological barrier (i.e., intracellular vesicle) that mRNA nanocarrier may encounter. (C) Combined use of nanoparticle imaging (NIR dye) and reporter system (Luciferase mRNA) to reveal the distribution and protein expression in mice. This includes the demonstration of interesting organ-specific expression (such as in pancreas) post intraperitoneal injection. (D) Safety assessment. In addition to the general safety assessment such as blood chemistry and organ histology, we pay extra attention on the proinflammatory cytokine release that may be associated with the administration of mRNA nanocarrier *in vivo*. Reprinted with permission from Dong et al. [19]. QC, quality control; MSNP, mesoporous silica nanoparticle; NP, nanoparticles; **, $P < 0.01$; ***, $P < 0.001$.

easy to overcome. Therefore, a successful mRNA nanomedicine requires early considerations on raw materials (including their FDA readiness), green chemistry, synthetic route, cost/yield analysis, scale-up setup and equipment, batch sizes, stability, sterility, analytical methods, and documentation, etc., all of which should meet new drug application (NDA) specifications and FDA requirements [24].

In addition to the carriers, mRNA technologies including the understanding of rational design of mRNA molecules, manufacture, modification and purification are equally important. New RNA designs are rapidly emerging such as self-amplifying mRNA and circular RNA, which the former is a mean to reduce payload dose due to the presence of an alphavirus-based replicon capable of amplifying an indicated protein target, and the latter exerts high stability [1, 2]. Payload structure factors such as molecule weight, sequence and 2D/3D structure, charge density and stability are key determination in terms of the choice of an appropriate delivery mechanism for mRNAs.

In addition to the COVID-19 vaccine, we are witnessing that a big wave of mRNA therapeutics that target different diseases are rapidly coming. To name a few, these include mRNA therapeutics to treat immunologically "cold" tumor, new infection disease, lung disease, and Duchenne Muscular Dystrophy. Interestingly, unlike to the direct injection of mRNA nanocarrier, it is possible to inject *ex vivo* treated cells (such as T-cell or stem cell), which could be theoretically processed by nano devices (such as "nanostraw" arrays and electroporation system [25]), as a new option for cell therapy. No matter what delivery mechanisms are proposed, it is essential to ensure nanosafety, in which the intensive knowledge about predictive nanotoxicology and nano/bio interface could be helpful [26]. Thanks for the development of multi-omics methods, artificial intelligence and data mining, providing a powerful to identify the non-obvious biological outcome of mRNA therapy.

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