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FORMULATION MATTERS

RNA Nanoparticle Formulation, Process and Characterization

R.K. DeLong, M.S., PhD and Eli Kraus PhD

Introduction

It is well documented that ribonucleic acid (RNA) plays an important role in molecular level regulation and control within cells and tissues. The role of RNA, especially messenger RNA (mRNA), in personalized medicine and drug development has been rapidly evolving. However, the journey of using RNA as a treatment option began as early as 1978 with the first delivery of mRNA to mouse cells using liposomes [1]. The first clinical application of using mRNA to treat disease was in 2017, where Sahin et al first used RNA-based poly-neo-epitope approach to mobilize immunity for melanoma was first accomplished [2].

Due to the pandemic, public awareness of mRNA and its potential use in applications such as vaccine treatments has increased. RNA-based drugs are becoming more and more important in the field of personalized medicine. As these new drug options advance, the delivery systems for these RNA therapeutics are continuing to evolve to provide higher loading, enhanced stability, and targeting capability. The intent of this white paper is to discuss the current state-of-the-art with respect to formulating RNA nanoparticles for pre-clinical and clinical development.

Nanoparticle Chemistries

While a variety of nanoparticles have been used for RNA delivery, they can be broadly classified into three main categories: lipid nanoparticles (LNP), polymer nanoparticles (PNP) and metal nanoparticles (MNP). RNA can be encapsulated within, associated to or conjugated to the surface, depending on the nanoparticle type as illustrated in **Fig. 1**.

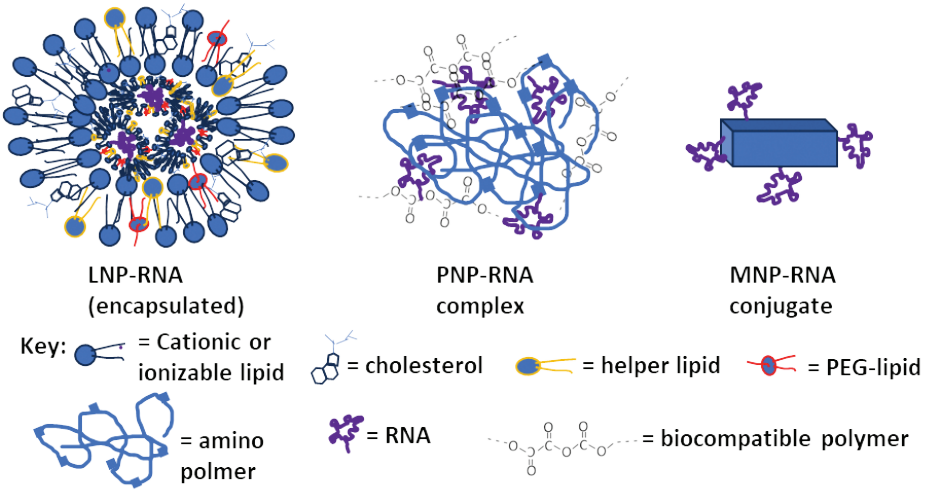
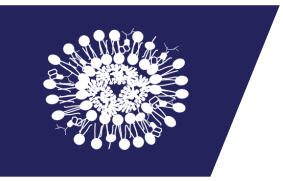


Figure 1: Artist's rendition of LNP-RNA, PNP-RNA or MNP-RNA where the RNA is encapsulated, associated, complexed or conjugated to the surface.

As shown in **Fig. 1**, RNA is encapsulated within the interior of LNP. For PNP typically the RNA is associated to the surface and partially internalized. Whereas for MNP, the RNA can either be complexed or chemically conjugated to the surface. The status of each different type of nanoparticle, its advantages and challenges are summarized in **Table 1** below.

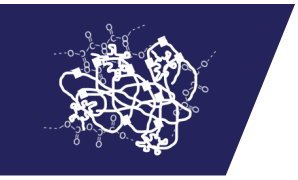
Table 1: Main Types of Nanoparticles: Their Advantages, Challenges and Current

Type	Advantages	Challenges and Limitations	Status
LNP	Established	Liver clearance, formulation process forces RNA into LNP interior lowering RNA payload and temperature stability, PEG-lipid may induce antibody response **[3]**	Commercial
PNP	Well-established for drug delivery, may increase tissue distribution	Less well established for mRNA, formulation process requires standardization	In Clinic
MNP	RNA can be associated or conjugated, increasing RNA payload and temperature stability, may increase tissue distribution, physiologically-based composi-tions may increase safety	RNA must be conjugated or associated to the surface, minimal data for mRNA, repeated dosing may be limited to physiologically-based metals, formulation processes not well established	In Clinic



LNP

LNP have proven themselves in the clinic and are now used commercially **[4].** The first mass market demonstration of their safety and efficacy was during the COVID pandemic where mRNA of the COVID spike protein were packaged within an LNP. However, one known downside of current LNP-RNA formulations are their limited application to certain disease indications due to clearance of LNPs by the liver. This limits most mRNA/LNP applications to protection against infectious agents like virus and hepatic diseases. However, encouraging new work suggests biodistribution may be improved somewhat dependent on lipid composition and ratios **[5, 6]**. As new formulations are developed wider use for LNP as a mechanism to deliver mRNA will be applied for other types of RNA therapeutics. Another disadvantage of LNP based delivery systems is the temperature sensitivity of these vaccines. The only mitigations are expensive cold storage or lyophilization (freeze drying). For the COVID mRNA vaccines this led to issues with shipping and storing around the world especially in 3rd world countries.



PNP

While not as advanced as LNP-RNAs, PNP-RNA have reached the clinic **[7,8]** and are under evaluation for delivery of mRNA **[9,10]** and other types of RNA therapeutics **[11]**. Surface-functionalized PNPs have demonstrated to protect RNA from RNase/nuclease degradation and to enhance intracellular uptake **[12].** Whereas PNP materials for drug delivery are well-established, at present it is unclear whether concerns about liver clearance and potential inflammatory responses associated with LNP [3] may also limit PNP in conjunction with RNA. While PNP systems may have fewer formulation components than LNP, control processes for their manufacturing may require further development. That said, some recent evidence suggests that PNP may enable RNA delivery beyond the liver into other tissues depending on the PNP chemistry and formulation **[13].** Further, lipid-polymer hybrid nanoparticle may also enable alternative routes of delivery **[14].**

MNP

MNP are well-established for RNA delivery to cells in culture but much less work has been accomplished in vivo or in the clinic **[5].** Cationic surface modification of gold nanoparticles has recently been shown to increase cytoplasmic mRNA delivery to cells in culture **[15].** Similarly early work by Delong et al, suggested that cationic surface modification of gold or other MNP can also increase RNA payload and enhance RNA stability and resistance to RNase degradation **[16, 17]**. A major advantage of MNP is that the chemistry is amenable to direct chemical conjugation of the RNA to the surface of the particle **[18, 19].** One major advantage of this conjugation is the increased level of protection of the mRNA which can improve stability at higher storage temperatures. This could allow for easier storage requirements and allow for shipping any vaccines developed with this technology around the world. There is also some recent evidence that synthesis of nanocluster core spherical nucleic acids, MNP with gold nanoparticle core may achieve broader biodistribution, in that these have shown prolonged blood circulation, reduced liver clearance and increased uptake into tumor in mouse models **[20]**. Clearly the synthesis of the MNP and its surface composition will be important in determining critical factors such as RNA payload, RNA stability and the delivery of RNA into tissues in vivo (patent pending).

Formulation

As depicted in **Fig. 1**, the commercial LNP-RNA contains cationic or ionizable lipid (blue), cholesterol (black), helper lipid (yellow) and PEG-lipid (red). While the commercial formulations have very similar molar ratios of each of the different lipid components **[2, 5]** these ratios are not fixed (the molar ratios are very similar or percentages of cationic:cholesterol:helper:PEG-lipid). Similar to LNP, PNP formulations typically comprise a cationic amino-containing polymer such as polyethyleneimine (PEI), poly(L-Lysine) (PLL), polyamidoamine (PAMAM), poly(β-amino esters) (PBAE) or chitosan used to complex the RNA. Often a second biocompatible polymer such as poly(lactic-co-glycolic acid) (PLGA), Poly[2- (dimethylamino)ethyl methacrylate] (PDMAEMA), cyclodextrin, poly(ethylene glycol) (PEG) or others is included which can impart other desirable properties such as physico-chemical stability, sustained release, etc. For MNP, in addition to amino-surface functionalization a variety of surface chemistries have been described for attaching RNA onto gold nanoparticles **[7, 21]**. MNPs containing small amounts of physiological metals on their surface have also been shown to form amino-based conjugates to RNA **[19, 22]**.

Process

Preparation of LNP-RNA is performed using turbulent flow micro-mixation with a dual channel device combining the lipids dissolved in ethanol and the RNA dissolved in aqueous buffer. This results in the RNA being internalized or encapsulated within the interior surrounding by stabilized lipid coating. For PNP, various formulation processes have been used for preparing PNP-RNA including; emulsion/solvent evaporation method, emulsion/solvent diffusion method, emulsion/reverse salting-out method, and nanoprecipitation **[8-11]**. Multi-channel mixing devices have also been recently described for the preparation of PNP-DNA or lipid-polymer-DNA hybrid systems **[14].**

Characterization

Maintaining and controlling product quality is critical in using these advanced therapies in the clinic and commercially. Part of the development process is the characterization of these particles to identify and understand the critical quality attributes of these nanoparticles. Some of these critical quality attributes include size, zeta potential (ZP), shape, polydispersity index (PDI), loading efficiency and stability of load. For LNP and PNP the lipid or polymer distribution and final formulation makeup is also critical to monitor. **[23]**. The hallmark early work of Chan and colleagues, demonstrated the importance of the size and shape or morphology of nanoparticles. From their work nanoparticles less than 100 nanometers (nm) and nanorod shaped showed maximal biodistribution, tissue and tumor uptake **[24]**. However, recent work by the Mirkin group suggests ultrasmall spherical nucleic acid (u-SNA) nanoparticles may show further biodistribution **[20]**. Nanoparticle size is typically measured by light scatter techniques. Zeta potential or effective surface charge is another critical parameter. If the particles are too close to neutral they will aggregate, but too positive or too negative can lead to untoward surface interactions, especially with serum proteins. Some instruments such as the Malvern Zetananosizer are capable of measuring both size and ZP, as well as the polydispersity index or PDI. PDI is essentially a measure of RNA nanoparticle heterogeneity. Typically, PDI is less than 0.1, meaning that > 90% of the particles are within a certain size limit specification. It is also essential to quantify the RNA payload or encapsulation efficiency. For LNP and some PNP, this can be done by the classic ribogreen assay, a sensitive fluorescence-based assay capable of measuring the amount of RNA incorporated or associated with the nanoparticle versus that portion which remains free in solution after formulation. For MNP, RNA can be removed by an appropriate elution buffer and measured by UV or fluorescence-based assays. The integrity of the particle associated RNA after formulation and processing and its stability is typically analyzed by gel or capillary electrophoresis, with HPLC-based assays in development. Looking ahead to the preclinical to clinical translation, similar to lipid analysis in LNP, polymer analysis in PNP is important, measured also by LC/MS. In our experience for MNP, metal analysis and quantification can be conducted by ICP/MS without any interference from the RNA **[22]**.

Look Ahead

In conclusion, an advanced understanding of RNA nanoparticle formulation, process and characterization of LNP, PNP and MNP is critical for their success in the clinic. First generation systems may give rise to hybrid systems, such as LNP-PNP, LNP-MNP or PNP-MNP to achieve increased RNA payload and RNA temperature stabilization and may also improve biodistribution. For example, recent evidence suggests that at least for self-amplifying replicon RNA (repRNA), lipid inorganic nanoparticle (LION) systems may have an improved safety profile with less systemic inflammatory response in comparison to LNP **[25]**. Further, a recent report suggests that relatively few mRNA molecules are packaged per LNP **[26]**. This seems likely to limit the LNP formulations for repRNA and longer RNA constructs. Potentially combining the advantages of LNP and/or PNP such as improved intracellular delivery and/or sustained mRNA expression, with increased RNA payload, stability and biodistribution of the MNP, may favor composite or hybrid systems. On the horizon, other biologically derived systems, such as exosomes may emerge. These new systems have the promise for tissue specific delivery capabilities. **[4, 7, 27]**. Finally, MNP materials and composites will allow for conjugation to the RNA but also potentially increase biological activity and/or the incorporation of targeting agents **[4, 7, 19, 22]**

Summary

The success of the mRNA vaccines in protecting against severe COVID disease has propelled RNA therapeutics to the forefront today. Despite commercialization of LNP-mRNA, RNA payload, RNA stability and tissue distribution is limited for these systems requiring further innovation in formulation and process to fully unlock the therapeutic potential of mRNA and other larger macromolecular RNAs. It seems likely that second generation RNA nanoparticles may combine the advantages of lipid, polymer and metal nanoparticles, in terms of increasing the RNA payload, temperature stability and distribution of RNA beyond the liver to other diseases tissues including cancer. Whereas turbulent flow micromixation has been used for these composite or hybrid systems, it remains to be seen whether the same uniform and high- quality nanoparticles established for LNPs can be generated for these more complex systems.

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LANDMARK BIO: 300 North Beacon Street, Watertown, MA 02472
info@landmarkbio.com | WEBSITE: https://www.landmarkbio.com

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