Payload Capacity and Distribution of mRNA Lipid Nanoparticles

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Keywords: mRNA; lipid nanoparticles; payload capacity; cylindrical illumination confocal spectroscopy (CICS); single-particle analysis.

Statement of Purpose: mRNA lipid nanoparticles (LNPs) have gained worldwide attention as an effective prophylactic vaccine formulation and hold great potential as therapeutic delivery vehicles. The packaging characteristics of mRNA in these vehicles, including its conformation, payload distribution and capacity, and dynamic behaviors during the manufacturing process are under active investigation with the goal of elucidating the structure-property-function relationship to guide the development of future therapeutic LNP formulations. We systemically investigated the payload capacity (*i.e.*, mRNA copy per LNP) and payload distribution of mRNA LNP formulations, which were difficult to assess by conventional LNP characterization methods, such as cryo-EM, light scattering, and NMR. The findings of this study would greatly enhance the mechanistic understandings of the assembly of mRNA LNPs.

Methods: We used cylindrical illumination confocal spectroscopy (CICS) to examine LNPs loaded with fluorescently tagged mRNAs (Cy5-mRNA) and helper lipids (TMR-PC) on a single-nanoparticle level. A benchmark mRNA LNP formulation was investigated, with dosage of the ionizable lipid DLin-MC3-DMA, cholesterol, the helper lipid DSPC and DMG-PEG2000 at a molar ratio of 50: 38.5: 10: 1.5.[1] The LNPs were formulated through rapid mixing in a T junction at pH 4.0 (acetate buffer), and subsequent dialysis against phosphate-buffered saline at pH 7.4. Multiple lasers were assembled in this CICS setup (**Fig. 1**) to identify unencapsulated mRNAs or empty LNPs *via* fluorescence analysis (**Fig. 2A**). The payload capacity was resolved numerically through deconvolution[2] of the Cy5 intensity profile of Cy5-loaded LNPs in reference to that of free individual mRNAs (**Fig. 3A**).

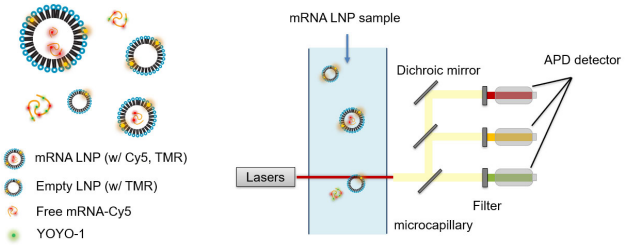

Figure 1. Multi-laser cylindrical illumination confocal spectroscopy to assess mRNA LNP composition with various species of interest.

Results: As shown in **Fig. 2B**, distinct populations were clearly identified when plotting TMR signal intensity against Cy5 intensity. An average of 96.3% of the signals from the Cy5 channel were identified as mRNA LNPs (*i.e.*, with coincidence with a positive TMR signal), and the rest 3.7% were identified as free mRNA (absent of a TMR signal). In the TMR channel, mRNA-loaded LNPs accounted for 23.5% of all the LNP events, while empty LNPs account for 76.5%. This is the first-ever statistical measurement of empty LNP% in the LNP formulation. The sensitivity of the method was validated through optimization of fluorescent dose and excitation voltage to yield around one order of magnitude of signal separation.

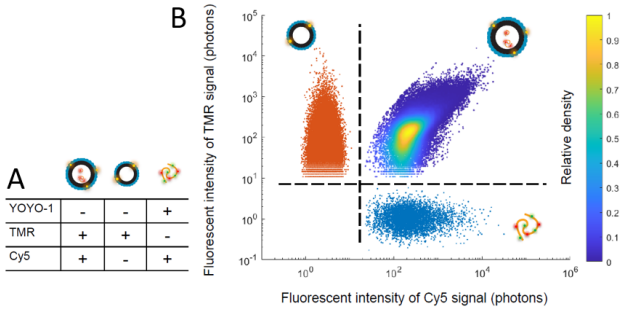

Figure 2. (A) Gating strategy to sort (from left to right) mRNA-loaded LNPs, empty LNPs, and unencapsulated mRNAs; **(B)** Sorting of the benchmark mRNA LNP formulation.

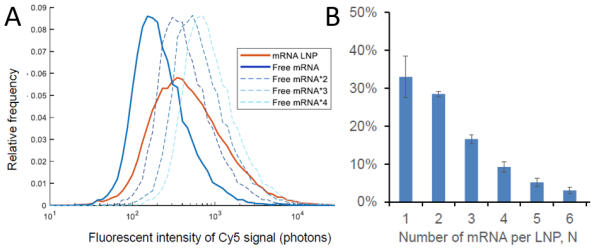

Figure 3. (A) The Cy5 fluorescent intensity profiles of single, individual mRNA molecules (the blue solid line), theoretical multiplexes (dashed lines), and the benchmark mRNA LNP formulation (the red solid line). **(B)** The estimated mRNA payload distribution in the benchmark LNP formulation.

The deconvolution algorithm resolved the Cy5 signal intensity profile of the formulation against the theoretical distributions of single or multiplexed mRNAs (**Fig. 3A**) that gave rise to the most possible distribution of mRNA payloads (**Fig. 3B**). Notably, more than 60% of LNPs contain only 1 or 2 mRNAs per LNP; while the number average was 2.6 ± 0.3 mRNAs per LNP.

Conclusions: The multi-laser CICS technique captured fluorescent signals from mRNAs encapsulated in LNPs and resolved the correlated payload capacity and distributions. This method offers a sensitive approach to further examine the effect of assembly conditions and lipid compositions on the payload characteristics of mRNA LNPs.

References

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