Editorial

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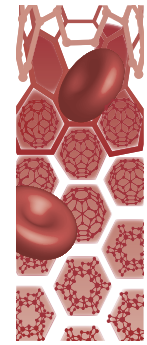

Microfluidic generation of diverse lipid nanoparticle libraries

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"Since small variations in compositional space result in large ensemble changes in LNP structure and function, it is essential to formulate them in the most controlled, reproducible manner for screening studies."

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Over the past few years, discoveries in the formulation space of nucleic-acid-loaded lipid nanoparticles (LNPs) have shown tremendous promise in delivering therapeutically active cargo for the treatment of numerous obdurate pathologies beyond COVID-19. Besides their nucleic acid cargo, LNPs generally comprise four primary lipidic components: cholesterol or a sterol analog to increase bilayer stability; a 'helper' phospholipid which largely defines the membrane curvature of the LNP; the ionizable lipid, which allows for the release of the nucleic acid cargo within the acidic environment of the late endosome; and the polyethylene glycol (PEG) lipid, which is the primary delimiter of particle size and main inhibitor of particle aggregation by means of sterics [1]. Beyond this system, additional components such as a charged fifth lipidic component [2] have been incorporated into formulations to imbue unique pharmacodynamic tropisms. The variation in the concentrations of different lipidic components alone generates a high-dimensional design space, within which small compositional variations can generate LNPs with markedly different physicochemical properties and resultant therapeutic properties [3].

To explore this chemically diverse design space, many groups have adopted the approach of generating and screening LNP libraries comprising chemically distinct lipidic components [4]. This approach involves identifying lead ionizable lipid structures for a given biological outcome, then tailoring the remainder of the LNP formulation around the best-performing iteration of that lead compound. Since small variations in compositional space result in large ensemble changes in LNP structure and function, it is essential to formulate them in the most controlled, reproducible manner for screening studies. LNP formation – the process where lipoplexes nanoprecipitate from ethanol into nucleic-acid-bearing acidic buffer [5] – is highly contingent on the local mixing dynamics of different fluids. By using a mixing process with high reproducibility, the resultant biological screening data can be directly attributable to formulation conditions. Additionally, when formulating and screening large numbers of formulations during the discovery phase, the generation of smaller volumes of LNPs is preferable to large volumes needed for subsequent clinical studies. However, given the contingence of LNP formation on mixing dynamics, changing mixing architectures or local flow rates to produce large batches of LNPs may result in fundamentally different particles from those generated during the discovery phase, even if both particle batches are identical in the relative concentrations of the lipidic components. Mixing architectures that are consistent and effective, yet which can scale to higher throughput using multiple parallelized units while maintaining the same local mixing environment across production scales, are ideal for nanoparticle screening [6].

Microfluidic platforms can consist of precise and highly controlled mixing devices which can be operated in parallel on a single chip. Chaotic mixing architectures such as the staggered herringbone mixer (SHM) and bifurcating mixer [7] have been used to generate LNPs in a controlled and repeatable fashion. Rather than turbulent mixing devices, these chaotic devices rely on the repeatable stretching and folding of different fluids into nanoscale

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lamina where advective mixing can occur rapidly [8]. Microfluidic devices have been designed that employ hundreds of these SHM microchannels operating in parallel to generate quantities of LNPs on the order of liters per hour, which is the scale relevant for clinical studies [9]. These chaotic architectures further pose an advantage over millifluidic devices such as impinging jet or multi-inlet vortex mixers [10] in that they are able to generate small LNP batches with minimal device dead volume, which is a marked advantage when generating hundreds or thousands of formulations using expensive nucleic acid reporters and custom-made lipids. Additionally, recent literature employing small-angle x-ray scattering as a means of characterizing the internal structure of LNPs demonstrates that particles with a more highly-ordered lamellar internal structure, a property which is desirable for the release of nucleic acids in the late endosome, arise more using chaotic mixing than turbulent mixing [11].

Microfluidic mixing devices have proven essential for the advancements in high-throughput *in vivo* methods for screening tens to hundreds of LNP formulations in a single animal, allowing for large amounts of biological data to be extracted across the formulation space [12]. Given the importance of LNP structure to performance and the poorly elucidated correlation to individual lipid structure and composition, data analysis of the biological response to these different formulations may prove useful for understanding meaningful structure–function relationships. The combination of these high-throughput *in vivo* screening techniques with *in silico* approaches to understand both mixing and final LNP structure [13] as well as the future implementation of machine learning techniques currently used in self-assembling solid drug [14] and polymeric nanoparticles [15] may help to explain interesting relationships between individual particle components and the biological effects of the entire LNP.

Both initial compound synthesis and the biological testing of formulations are occurring at a rate much higher than the currently rate-limiting step of individually formulating the particles. While groups have found large success in individually formulating LNPs for specific applications, such as T-cell transfection [3] or organ-specific delivery [2], the individual generation of particles still appears to be the primary limitation in the shift upwards in orders of magnitude of unique formulations screened per study. There are currently certain products being offered commercially which allow for automated screening of formulations on the order of tens every few hours [16], yet the rates at which these formulations can be uniquely generated seem to lag behind the rate at which combinatorial libraries of lipids can be synthesized using liquid handlers [17], or at which different formulations can be combinatorially pooled for testing in mouse models, on the scale of hundreds per single mouse experiment [18].

While the rate of formulation generation using microfluidics is currently limited, the post-processing requirements of LNPs, including solvent exchange and filtration, can be performed on chip in line with the mixing architectures [19]. There exists the potential to further modify LNP formulations on-chip via surface conjugation reactions to targeting ligands in flow [20]. Such an on-chip methodology would result in the ability to scale up the development of multiple different LNPs using a single device without the need for additional technology off-chip, drastically reducing the time and footprint necessary to formulate libraries comprising multiple LNPs. Multiple iterations of these mixing devices and in-line processing units could populate a single 4″ or 6″ silicon wafer to generate multiple LNPs at a given time. While challenges exist in the interfacing of different micron-scale mixing architectures to the millimetric or centimetric scale of external liquid handlers, the potential to generate and process multiple formulations using this methodology may ultimately result in significant improvements to the overall rate of LNP formulation and subsequent testing. If the timescale of formulation generation is reduced sufficiently such that generating large libraries of LNPs meets the scale of combinatorial synthesis and *in vivo* testing, contemporary machine learning techniques may prove exceedingly useful in the identification of structure–function relationships, as has been shown in the polymeric nanoparticle field. Using microfluidics, the precise amount of each individual formulation necessary to perform downstream characterization can be generated, then using the exact same mixing architecture and thus local nucleation conditions, formulations can be scaled up to perform clinical trials [9]. Such a workflow not only allows for high precision between formulations synthesized within a single study, but provides a modular, parametrizable means for other groups to replicate study results and to expound upon existing formulations to iteratively optimize for an even broader spectrum of therapeutic outcomes.

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