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アプリケーションノート

Automated Preparation of Oligonucleotide-Loaded Lipid Nanoparticles Using Andrew+™ Pipetting Robot for High-Throughput In-Vitro Screening

Isabell Kroth, Michael Karimov, Ryan Karongo

BAYER AG

本書はアプリケーションブリーフであり、詳細な実験方法のセクションは含まれていません。

Abstract

The LNP formulation technology is usually a time-consuming and cost-intensive process, where the need of microfluidic devices makes the process difficult to streamline many samples. The following work comprises the development of an automated high-throughput method using Andrew+ pipetting robot.

Benefits

- Automated preparation of LNP formulations for high throughput screening purposes allowing the preparation of up to 96 formulations in two hours
- Comparable results to manual pipetting and microfluidics-based methods regarding LNP characterization parameters
- Automated pipetting shows adequate well-to-well repeatability (n=8) for LNP preparation

Introduction

The progress in RNA-based therapies has been significant, marked by the approval of pioneering treatments like patisiran in 2018 and the recent development of COVID-19 vaccines utilizing Lipid nanoparticle (LNP) formulations.[1–3] To optimize the effectiveness of these therapies, researchers have leveraged the electrostatic complexation of anionic RNA, which not only enhances cellular uptake but also safeguards the RNA, *i.e.* from degradation by RNAse enzymes. These LNPs, usually consist of a composition of cationic and ionizable lipids, combined with phosphatidylcholine (PC), cholesterol, and polyethylene glycol (PEG) lipids.[4,5] The process of particle formation hinges on swift mixing procedures, with precise control of flow rates being essential to produce uniform particles. Enhancing mRNA delivery efficiency necessitates exhaustive screening of formulation components, often employing methods such as design of experiment approaches.[6] High-throughput screening, facilitated by robotic automation in a 96-well plate format, has emerged as an efficient method, outperforming microfluidic approaches in terms of material consumption.[7–9] In the following, a protocol to automate LNP formulation for screening purposes was established, which is based on the pipette mixing method from *Wang et al*. and utilizes the Andrew+ liquid handling platform, demonstrating results comparable to the current state-of-the-art methods.[10]

Experimental

Materials

mRNAs encoding green fluorescent protein (CleanCap EGFP mRNA) were purchased from TriLink Biotechnologies (San Diego, USA). Ionizable lipids (D-Lin-MC3-DMA) and helper lipids (DSPC, cholesterol, and DMG-PEG_{2000}) were purchased from Avanti Polar Lipids (Birmingham, USA). All lipids were solved in pure ethanol (D-Lin-MC3-DMA: 40 mg/mL, helper lipids: 20 mg/mL each). Phosphate buffered saline (PBS) was prepared by dissolving of PBS tablets (Merck KGaA, Darmstadt, Germany) in nuclease-free water. Citrate buffer (10 mM, pH=3) was prepared by dissolving 27.6 mg sodium citrate dihydrate and 174.1 mg citric acid in 100 ml nuclease-free water. All chemicals used for the formulation were of analytical grade.

Automated liquid handling steps were performed using Andrew+ (Waters – Andrew Alliance, Switzerland) equipped with a 10 µL and 300 µL single channel pipette, a 1200 µL 8-channel pipette and Peltier+ for heating. The setup further comprised the corresponding dominos for the following labware: 1.5 mL Eppendorf Safe Lock Tubes (Eppendorf, Hamburg), 10 mL multichannel reservoirs purchased from INTEGRA (Integra Biosciences AG, Switzerland) and a twin.tec® 96-well skirted LoBi plate (Eppendorf, Hamburg) used as the target labware

(Figure 1).

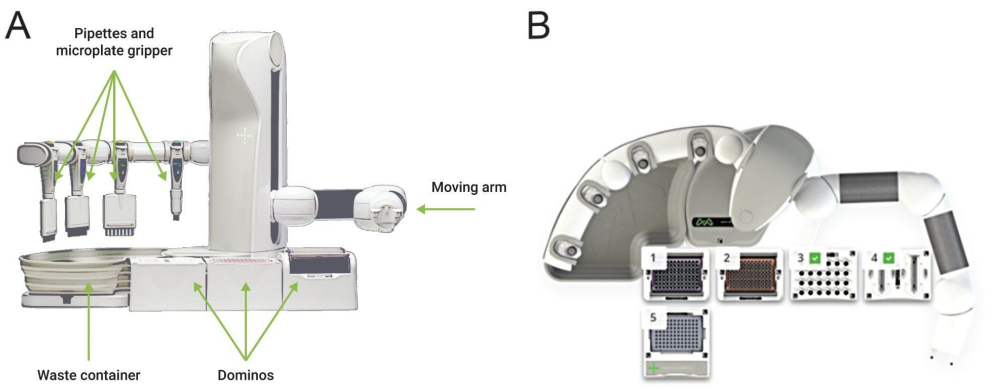

Figure 1 A. Front view of Andrew+ B. Top-down view of the Dominos positioned for the LNP protocol.

Preparation of mRNA Loaded LNPs

Microfluidic Method

LNPs were prepared by mixing appropriate volumes of a lipid mixture (molar composition 50:10:38.5:1.5) in ethanol with an aqueous phase (10 mM citrate buffer) containing mRNA using a microfluidic micromixer (NanoAssemblr® Ignite™) at a 1:3 organic to aqueous volume ratio. Amicon Ultra Centrifugal Filters (Merck Millipore, 30 kDa molecular weight cutoff) were used to remove ethanol and citrate buffer.

Manual Pipetting Method

The procedure involves preparing LNPs using a 96 well plate format. Initially, each constituent of the lipid mixture (6.2 µL D-Lin-MC3-DMA, 3.1 µL DSPC, 5.8 µL cholesterol, 1.5 µL DMG-PEG) is introduced into one well. In another well, a combination of citrate buffer (58 µL, 10 mM, pH 4) and mRNA (2 µL, 1 mg/mL) is created and thoroughly mixed. The resulting mRNA-buffer dilution (60 µL) is rapidly pipetted into the lipid mixture-ethanol solution in a volume ratio of 3:1 between the aqueous and ethanol phases. Quick and vigorous pipetting for 20–30 seconds is crucial to ensure proper LNP formation; failure to do so may result in polydisperse LNPs and insufficient encapsulation rates. After 15 minutes of incubation at room temperature, optional dialysis can be performed using a dialysis tube (*i.e.*, Pur-A-Lyzer Midi 3500) and 1× PBS to remove ethanol and acidic buffers. The solution is then transferred to an RNase-free tube and the volume adjusted to 200 µL using 1× PBS to achieve a final concentration of 10 µg mRNA/mL. The resulting solution can then be stored at 4 °C for several

days before use.

Automated Pipetting Method

The final protocol involves 12 steps to create LNPs (Table 1). Initially, the well plate temperature was set to 20 °C. The lipid components were pre-mixed in an Eppendorf tube (#1). Separately, citrate buffer (48 µL × number of wells) is transferred into another Eppendorf tube (#2), then mRNA (2 µL × number of wells) is added. Following, 50 µL of diluted mRNA are added to each well, and subsequently, 16.6 µL of the lipid mixture is rapidly pipetted into each well, followed by immediate mixing through fast up and down pipetting (six times). The plate is then incubated for 15 minutes, after which the heating is stopped. Finally, each well is supplemented with 152 µL of PBS, completing the LNP formation process.

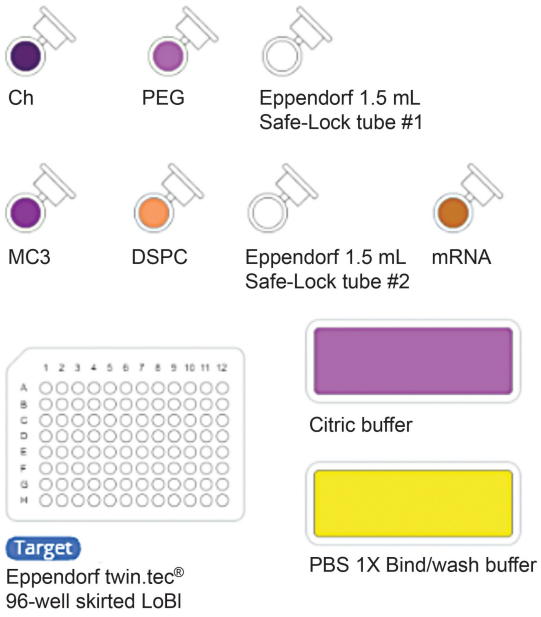

Figure 2. Screenshot of OneLab™ bench at start of the protocol.

Step	Device	Action	Volume [µL]	Source	Destination	Additional action(s)
1	Peltier +	Start heating	–	–	–	20 °C
2	Single channel, 10–300 µL	Pipetting	124	MC3	Eppendorf tube 1.5 mL #1	Pipetting: forward, blow-out Air cushion: Air bottom cushion
3	Single channel, 10–300 µL	Pipetting	62	DSPC	Eppendorf tube 1.5 mL #1	Tip position: Source – with respect to liquid, Destination – on the fly
4	Single channel, 10–300 µL	Pipetting	116	CH	Eppendorf tube 1.5 mL #1	Tip choice: Change tip at beginning, change tip between pipetting steps
5	Single channel, 10–300 µL	Pipetting	30	PEG	Eppendorf tube 1.5 mL #1	Mixing: 3 times, normal, 30 µL
6	Single channel, 50–1000 µL	Pipetting	960	Citric Buffer	Eppendorf tube 1.5 mL #2	Air cushion: No air cushion Tip position: Source – with respect to liquid, Destination – with respect to liquid Mixing: none
7	Single channel, 10–300 µL	Pipetting	40	mRNA	Eppendorf tube 1.5 mL #2	Air cushion: Air bottom cushion Mixing: 3 times, normal, 300 µL
8	Single channel, 10–300 µL	Pipetting	50	Tube 1.5 mL #2	96 well plate, A:H1, A:H2	Air cushion: No air cushion Mixing: none
9	Single channel, 10–300 µL	Pipetting	16.6	Eppendorf tube 1.5 mL #1	96 well plate, A:H1, A:H2	Air cushion: No air cushion Mixing: 6 times, fast, 60 µL
10	Timer	–	–	–	–	15 minutes
11	Peltier +	Stop heating	–	–	–	20 °C
12	Eight channel, 10–300 µL	Pipetting	133.4	1× PBS	96 well plate, A:H1, A:H2	Mixing: 1 time, slow, 133.4 µL

Table 1. Step by step description of the protocol.

For all pipetting destinations, the tips were positioned with respect to liquid in all sources and either with respect to liquid or on the fly at destinations. An air bottom cushion was used for solutions with low viscosity, namely the lipids, and the mRNA. Mixing steps were performed as stated in Table 1. After each dispense, a "Blowout" was carried out. The speed for aspirating and dispensing was set to normal in the protocol except for once when mRNA and lipids were mixed, for which fast mixing was applied.

LNP Characterization and High-Throughput in vitro Screening

LNP Characterization

The hydrodynamic particle size and polydispersity index (PDI) of the nanoparticles were measured using dynamic light scattering in 1× PBS (Zetasizer, Malvern Instrument). Diameters are reported as the largest intensity mean peak average, which constitutes >95% of the nanoparticles present in the sample. To calculate the nucleic acid encapsulation efficiency, a Quant-iT RiboGreen RNA assay (Invitrogen) was used according to the manufacturer's instructions.

In vitro Transfection Efficiency

HepG2 cells (American Type Culture Collection, USA) were cultured at 37 °C and 5% CO_2 in high glucose Dulbecco's Modified Eagle Medium with GlutaMAX supplement (ThermoFisher) containing 10% fetal bovine serum and 1% penicillin-streptomycin. For *in vitro* transfections, 10,000 cells per well were seeded into 96-well plates one day prior to transfection. Next day, the particles were pipetted to the cells at a final particle concentration of 100 ng mRNA/well (n=8). Manually prepared nanoparticles were used as a positive control. After 24 h incubation, eGFP fluorescence intensity was quantified using a flow cytometer (MACSQuant Analyzer 16; Miltenyi Biotec, Germany).

Results and Discussion

Automated Preparation of mRNA Loaded LNPs

The main goal of this work was the development of an automated high-throughput method using Andrew+ pipetting robot, based on the pipette mixing method from *Wang et al*..[10] The method and LNP composition were modified to be compatible with the Andrew+ pipetting robot and applicable for screening various LNP formulations.

Initially, preliminary experiments were conducted to automate the manual pipetting procedure. However, the rapid evaporation of ethanolic lipid solutions hindered a precise protocol transfer. Following, a gravimetric assessment of the ethanol evaporation rate was performed, revealing a significant dependency on room temperature, which in turn restricts the calculation and correction of the volume loss. Subsequently, the pipetting sequence was modified. Instead of introducing the diluted mRNA into the lipid mixture prepared individually in each well, the lipid mixture was pre-prepared in an Eppendorf tube beforehand to minimize the overall liquid surface area for evaporation. This pre-prepared lipid mixture was then introduced into each well containing diluted mRNA. Further, the 96-well plate was maintained at 20 °C during the whole process, to guarantee the temperature optimum required for the formulation. The arm motion speed during the whole protocol was set to fast, to reduce the overall protocol duration, and minimize the evaporation of solvents.

LNP Characterization and High-Throughput in vitro Screening

For comparison, manually prepared fresh D-Lin-MC3-DMA particles formulated through either the manual pipetting method or microfluidic technology, were utilized. After preparing the formulation with mRNA encoding green fluorescent protein (GFP), first particle size, polydispersity index (PDI), and encapsulation efficiency were characterized. It was observed that particles prepared by Andrew+ show a slightly larger average particle diameter (~200 nm) compared to manually prepared formulations (~160–180 nm). Relatively

slower mixing rates, compared to the manually mixing method may result in larger particles. However, both methods were observed to have a low PDI (below 0.2), indicating that there was a consistent range of particle sizes, independent of the preparation method. More importantly, both methods showed a high mRNA encapsulation efficiency (above 97%), irrespective of the preparation method (Table 2).

	Microfluidics	Manual pipetting	Automated pipetting (Andrew+)
Size [nm]	90 +/- 2.3	176.1 +/- 1.5	207.08 +/- 18.88
PDI	0.08 +/- 0.01	0.11 +/- 0.01	0.17 +/- 0.04
mRNA encapsulation efficiency [%]	>97	>97	>97

Table 2. LNP characterization and comparisons across preparation methods.

To assess the transfection efficiency of both methods, HepG2 cells were transfected at a final concentration of 100 ng per well. Both formulations exhibited excellent biocompatibility and demonstrated a high transfection efficacy 24 hours post-transfection (Figure 3). Finally, we also noted that the high-throughput pipetting method using Andrew+ shows adequate well-to-well repeatability (n=8), resulting in a high precision for the investigated characterization parameters. These results indicates that the formulation of the particles by Andrew+ is perfectly suitable for preparation of many samples and thereby can be used for the high-throughput screening of various LNPs.

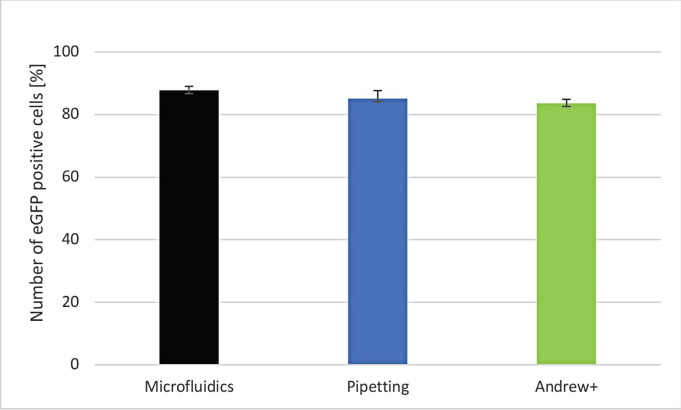

Figure 3. Comparison of transfection efficiency into HepG2 cells of LNPs prepared using different methods.

Conclusion

Proceeding from a manual preparation procedure, an automated method for LNP formulation using the Andrew+ pipetting robot was developed to allow the high-throughput screening of various lipid nanoparticle formulations. By pre-preparing the lipid mixture in Eppendorf tubes and maintaining a constant temperature, the initial hurdles, including the rapid evaporation of ethanolic lipid solutions, and the dependency of evaporation rates on room temperature, were overcome, thereby significantly improving the precision of the protocol. While we observed slightly larger particle sizes when compared to microfluidics and manually pipetted formulations, the consistency of particle size and high mRNA encapsulation efficiency remained consistent across all methods. Further, the transfection efficiency into HepG2 cells was comparable to both manual pipetting and microfluidics based LNP preparation methods. In essence, this demonstrated the feasibility of streamlining LNP formulation using Andrew+ and thereby and advancing the development of mRNA-based therapies.

References

1. J.H. Erasmus, A.P. Khandhar, J. Guderian, B. Granger, J. Archer, M. Archer, E. Gage, J. Fuerte-Stone, E. Larson, S. Lin, R. Kramer, R.N. Coler, C.B. Fox, D.T. Stinchcomb, S.G. Reed, N. Van Hoeven, A Nanostructured Lipid Carrier for Delivery of a Replicating Viral RNA Provides Single, Low-Dose Protection against Zika, *Molecular Therapy* 26(10) (2018) 2507–2522. https://doi.org/10.1016/j.ymthe.2018.07.010 < https://doi.org/10.1016/j.ymthe.2018.07.010> .

2. D. Adams, A. Gonzalez-Duarte, W.D. O' Riordan, C.-C. Yang, M. Ueda, A.V. Kristen, I. Tournev, H.H. Schmidt, T. Coelho, J.L. Berk, K.-P. Lin, G. Vita, S. Attarian, V. Planté-Bordeneuve, M.M. Mezei, J.M. Campistol, J. Buades, T.H. Brannagan, B.J. Kim, J. Oh, Y. Parman, Y. Sekijima, P.N. Hawkins, S.D. Solomon, M. Polydefkis, P.J. Dyck, P.J. Gandhi, S. Goyal, J. Chen, A.L. Strahs, S.V. Nochur, M.T. Sweetser, P.P. Garg, A.K. Vaishnaw, J.A. Gollob, O.B. Suhr, Patisiran, an RNAi Therapeutic, for Hereditary Transthyretin Amyloidosis, *New England Journal of Medicine* 379(1) (2018) 11–21. https://doi.org/10.1056/NEJMoa1716153 < https://www.nejm.org/doi/10.1056/NEJMoa1716153> .

3. K.S. Park, X. Sun, M.E. Aikins, J.J. Moon, Non-viral COVID-19 vaccine delivery systems, *Advanced Drug Delivery Reviews* 169 (2021) 137–151. https://doi.org/https://doi.org/10.1016/j.addr.2020.12.008 < https://www.sciencedirect.com/science/article/pii/S0169409X20302799?via%3Dihub> .

4. A.K. Blakney, P.F. McKay, B.I. Yus, Y. Aldon, R.J. Shattock, Inside out: optimization of lipid nanoparticle formulations for exterior complexation and in vivo delivery of saRNA, *Gene Therapy* 26(9) (2019) 363–372. https://doi.org/10.1038/s41434-019-0095-2 <https://www.nature.com/articles/s41434-019-0095-2> .

5. J.A. Kulkarni, S.B. Thomson, J. Zaifman, J. Leung, P.K. Wagner, A. Hill, Y.Y.C. Tam, P.R. Cullis, T.L. Petkau, B.R. Leavitt, Spontaneous, solvent-free entrapment of siRNA within lipid nanoparticles, *Nanoscale* 12(47) (2020) 23959–23966. https://doi.org/10.1039/D0NR06816K < https://pubs.rsc.org/en/content/articlelanding/2020/NR/D0NR06816K> .

6. Y. Fan, Z. Shi, S. Ma, S.Z.A. Razvi, Y. Fu, T. Chen, J. Gruenhagen, K. Zhang, Spectroscopy-Based Local Modeling Method for High-Throughput Quantification of Nucleic Acid Loading in Lipid Nanoparticles, *Analytical Chemistry* 94(25) (2022) 9081–9090. https://doi.org/10.1021/acs.analchem.2c01346 < https://pubs.acs.org/doi/10.1021/acs.analchem.2c01346> .

7. Y. Fan, C.-W. Yen, H.-C. Lin, W. Hou, A. Estevez, A. Sarode, A. Goyon, J. Bian, J. Lin, S.G. Koenig, D. Leung, K. Nagapudi, K. Zhang, Automated high-throughput preparation and characterization of oligonucleotide-loaded lipid nanoparticles, *International Journal of Pharmaceutics* 599 (2021) 120392. https://doi.org/https://doi.org/10.1016/j.ijpharm.2021.120392 < https://www.sciencedirect.com/science/article/abs/pii/S0378517321001964?via%3Dihub> .

8. V. Francisco, C. Rebelo, A.F. Rodrigues, J. Blersch, H. Fernandes, L. Ferreira, A high-throughput screening platform to identify nanocarriers for efficient delivery of RNA-based therapies, *Methods* 190 (2021) 13–25. https://doi.org/https://doi.org/10.1016/j.ymeth.2020.12.002 < https://www.sciencedirect.com/science/article/pii/S1046202320302644?via%3Dihub> .

9. D. Ulkoski, M.J. Munson, M.E. Jacobson, C.R. Palmer, C.S. Carson, A. Sabirsh, J.T. Wilson, V.R. Krishnamurthy, High-Throughput Automation of Endosomolytic Polymers for mRNA Delivery, *ACS Applied Bio Materials* 4(2) (2021) 1640–1654. https://doi.org/10.1021/acsabm.0c01463 < https://pubs.acs.org/doi/10.1021/acsabm.0c01463> .

10. X. Wang, S. Liu, Y. Sun, X. Yu, S.M. Lee, Q. Cheng, T. Wei, J. Gong, J. Robinson, D. Zhang, X. Lian, P. Basak, D.J. Siegwart, Preparation of selective organ-targeting (SORT) lipid nanoparticles (LNPs) using multiple technical methods for tissue-specific mRNA delivery, *Nature Protocols* 18(1) (2023) 265–291. https://doi.org/10.1038/s41596-022-00755-x <https://www.nature.com/articles/s41596-022-00755-x> .

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