



Rapid Communication

Dialysis is a key factor modulating interactions between critical process parameters during the microfluidic preparation of lipid nanoparticles



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ABSTRACT

Manufacturing lipid nanoparticles through microfluidic mixing can be approached from a Quality by Design perspective. Research involving critical process parameters seems to focus on the total flow and flow rate ratio, thus other process variables, such as dialysis, are underestimated. This study used a Design of Experiments to identify the influence of critical process parameters on particle size, polydispersity index, and zeta potential. A response surface Design of Experiments modeled the influence of: total flow (400 to 4000 $\mu\text{L min}^{-1}$); flow rate ratio (3 to 9) and dialysis (yes/no). Results suggest that dialysis is a crucial parameter that strongly influences particle size and zeta potential and moderately affects polydispersity index. The flow rate ratio's relevance decreases when dialysis is performed. As the purification method can change the influence of other process parameters, it should be an integrated part of the microfluidic manufacturing of lipid nanoparticles instead of an extra step.

1. Introduction

Lipid nanoparticles (LNPs) are versatile drug delivery systems with a wide range of therapeutic and diagnostic applications [1–4]. In fact, LNPs are the most clinically advanced nonviral vector for nucleic acid delivery [5]. Microfluidic mixing methods (MFMs) have been developed in recent years and have become among the most effective methods for LNPs manufacturing [5–9]. Through MFMs, small volumes of ethanolic lipid solutions are continuously mixed into aqueous buffered solutions inside a microscale chip, coupled to a flow control mechanism [4,6,10,11]. This process causes rapid and homogeneous lipid

nucleation, generating small and uniform LNPs [6,12] with high encapsulation efficiency and exhibiting high scale-up possibilities and high batch-to-batch reproducibility [7,13]. The development of LNPs using MFMs has previously been performed from the perspective of Quality by Design (QbD) [14–19]. According to QbD, the quality profile of a product must result from the establishment of a design of space since the early stages of conceptualization in pharmaceutical development. This design of space provides an extensive understanding of how the critical quality attributes (CQA) of a product are affected by the critical material attributes, and the critical process parameters (CPP) and their interactions [20,21]. Although the enhanced application of QbD in the

Abbreviations: CART, Classification and regression trees; CPP, Critical process parameter; DoE, Design of Experiments; CQA, Critical quality attribute; FRR, Flow rate ratio; LNPs, Lipid nanoparticles; MFMs, Microfluidic mixing methods; N/P ratio, Nitrogen to phosphate ratio; PDI, Polydispersity index; PSD, Particle size distribution; QbD, Quality by design; QTPP, Quality target product; TF, Total flow.

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pharmaceutical development process of new medicines is not mandatory, it represents some regulatory advantages, especially in terms of flexibility for postapproval changes [22,23]. The financial and scientific advantages of QbD implementation are recognized by regulatory authorities and pharmaceutical companies worldwide [24]. Design of Experiments (DoE) is among the most commonly used QbD tools for exploring the design of space and optimizing the response of LNPs manufacturing by MFMs [7,17,25].

CQA normally reported for LNPs are those related to their ability to penetrate target cells and exhibit a therapeutic effect, such as: particle size distribution (PSD), polydispersity index (PDI), morphology, surface charge, encapsulation efficiency, stability, drug release [26–28], cell permeability and targeting efficacy [21]. On the other hand, the academic attention on CPP for MFMs seems to be focused on only the following parameters: total flow (TF) and the aqueous to lipid flow ratio, or flow rate ratio (FRR), as exemplified in Fig. 1.

Despite the extensive academic production that applies DoE to LNPs manufacturing, several authors state that QbD principles must be further implemented from a manufacturing perspective [21,25,29–31]. In particular, some authors identified an insufficient control and understanding of parameter interactions [7,17]. Although TF and FRR are important variables in the process of MFMs, the approach shown in Fig. 1 seems to underestimate other process variables that could affect the quality of LNPs, as nanoparticle properties are highly dependent on the possible interactions between variables [21]. Whereas those less studied parameters must be somehow controlled or defined in LNPs manufacturing by MFMs, their exclusion in experimental designs could antagonize the QbD philosophy. This could lead to the establishment of design spaces that exclude areas that may significantly contribute to the variability of effects. Among others, Fig. 1 points to the dialysis, or any other purification process, as an input variable not typically considered CPP.

Dialysis (as a purification technique) is usually necessary for the clinical or experimental application of LNPs obtained by MFMs [9,16]. In particular, purification is necessary to remove ethanol from the medium and thus allow the preparation to be used in cell cultures or in studies with laboratory animals [32]. Despite the importance of the process, a systematic underestimation of dialysis as CPP is suggested, as shown in Fig. 1. The lack of consideration of dialysis in experimental designs has previously attracted the attention of several authors. It has been suggested that dialysis should not be seen as an isolated operation, specifically for changes in PSD after the purification process [9,33–35]. Although some published works include the application of dialysis as a

fixed factor [17,33,36,37], only a few authors include this process as a variable in their experimental design. For example, Terada *et al* showed an increase in the PSD after dialysis and claimed that the neutralization method is important to achieve their target dimensions of the nanoparticles [16]. More drastically, Kulkarni *et al* concludes that the LNPs size is more affected by the conditions of the dialysis process (particularly pH) than the mixing conditions in the previous step [35]. Nevertheless, from an interactions point of view, the study remains an opportunity to broaden the knowledge and understanding of CPP's integrated impact on the MFMs of LNPs.

This study seeks to determine the influence of the dialysis process in describing the CQA (PSD, PDI and zeta potential) of LNPs from a QbD perspective. Our approach seeks to understand the direct effect of dialysis and its possible interactions with TF and FRR. We used DoE to describe and model the response and quantify the relative influence of each CPP in the description of variability. The inclusion of the dialysis process in the experimental design yielded new evidence of how it can model the influence of TF and FRR, typically tested manufactured conditions. With the aid of the regression model, we demonstrate the importance of the purification process as a CPP that should be incorporated into experimental designs since the early stages of formulation development.

2. Experimental section

2.1. Materials

DOTAP [1,2-dioleoyl-3-trimethylammonium-propane, chloride salt] was purchased from Nanosoft Polymers. DSPC [1,2-distearoyl-sn-glycero-3-phosphocholine] was purchased from Avanti Polar Lipids. Cholesterol was purchased from Sigma Aldrich. DSPE-mPEG2000 [1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]] was purchased from MuseChem.

2.2. Lipid nanoparticle preparation

LNPs were prepared following the ethanol injection method in a microfluidic system [5,16,38]. Specifically, we mixed ethanol and aqueous phases using a microfluidic polycarbonate chip with the aid of a pressure flow controller. We used a staggered herringbone chip with a channel depth of 200 μm (FLUIDIC 187, Chipshop, Germany) and a pressure flow controller (OB1 MK3+, Elvesys, France / Inside Therapeutics, France). The TF tested was in the range between 400 and 4000

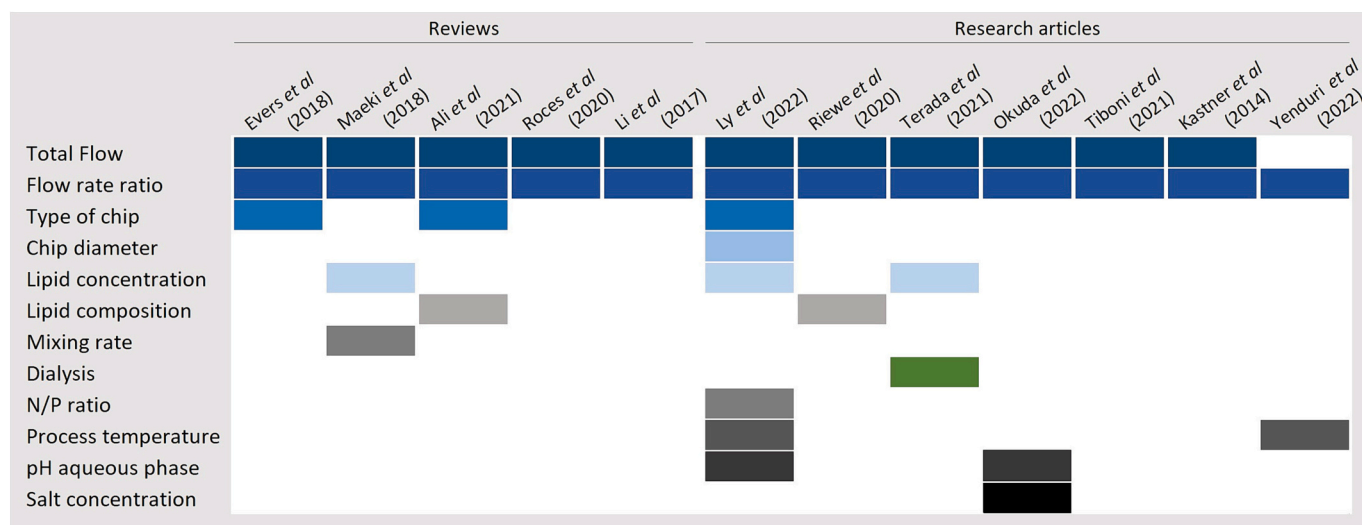


Fig. 1. Process variables in microfluidic mixing preparation of lipid nanoparticles. Reported as critical process parameters in reviews, or included with at least two levels in experimental designs in research articles. N/P ratio = nitrogen to phosphate ratio.

mL min⁻¹, while the FRR was between 3 and 9.

For the ethanol phase, lipids were dissolved in ethanol at total lipid concentration of 2,5 mM. The lipid proportion was 40/10/48.5/1.5 (DOTAP/DSPC/cholesterol/DSPE-mPEG2000). The aqueous phase consisted of 1 mM citrate buffer (pH 5.3).

When needed, ethanol removal was performed using a 0.5–3 mL dialysis cassette with a 10,000 molecular weight cutoff (Slide-A-Lyzer®, Thermo Scientific, USA). Each dialysis cassette was filled with 3 mL of LNPs suspension and dialyzed against 400 mL of 0.1 M KCl for at least 18 h under continuous stirring at room temperature. The dialysis was conducted in three consecutive steps with renewal of the purification fluid in between. LNPs were dialyzed for 2 h in the first and second steps and continued overnight for the third step.

2.3. LNPs characterization

PSD and PDI were determined from the hydrodynamic diameter measured by dynamic light scattering on a Zetasizer Nano ZS90 (Malvern Instruments, UK). Surface charge (zeta-potential) was measured by laser Doppler microelectrophoresis in a Zetasizer Nano-Z (Malvern Instruments, UK).

2.4. DoE design and statistical analysis

A response surface central composite design of experiments modeled the influence of the selected variables of operation as follows: total flow (400, 2200 and 4000 $\mu\text{L min}^{-1}$); flow rate ratio (3, 6 and 9) and dialysis as a categorical variable (yes or no). The fixed variables were 2.5 mM lipid content; an aqueous buffer concentration of 0.01 mM; and 0.1 M KCl dialysis solution. All experiments were executed at room temperature. The experimental design consisted of 1 base block and 22 base runs in 3 replicates, for a total of 66 runs in 3 blocks: 24 cube points, 24 axial points and 18 center points. The experimental data were analyzed with the statistical software Minitab19.1 (Minitab Inc. USA). $P < 0.01$ was considered to indicate statistical significance. When necessary, Johnson transformations were applied to data that were not normally distributed.

Multiple regression analysis with least-squares assessment and analysis of variance allowed us to identify the statistical significance of factors (TF, FRR and dialysis) describing the variability of PSD, PDI and zeta potential of empty LNP. The regression model enabled the prediction of optimal parameter configuration as well as response prediction for hypothetical scenarios. The goodness-of-fit and predictive accuracy of the model were evaluated using residual plots, and R^2 and R^2 -predicted values. To further evaluate the variance and correlations of data, we used Minitab 19.1 to obtain classification and regression trees (CART) [39], and to perform a principal components analysis [40].

3. Results and discussion

The Design of Experiments conducted in this study allowed us to model the influence of the operational variables on some physico-chemical attributes of lipid nanoparticles produced by microfluidic mixing. The surface response experimental design described and modeled the impact of total flow, flow rate ratio and dialysis process on the particle size distribution, zeta potential and polydispersity index. The correlations among variables are also supported by principal component analysis presented in the supplementary information (Figs. S1 and S2) and the CARTs presented in Fig. 5, and figs. S10 and S11 (supplementary information). The results suggest that the role of dialysis in the modeling of critical quality attributes could be more relevant than demonstrated in the traditional approach and thus, highlight its importance as a critical process parameter.

3.1. Effects on particle size distribution

In our experimental conditions, dialysis caused a reduction in the

analytical outcome of particle size distribution. The contour plot presented in Fig. 2 shows a greater area for mean particle sizes lower than 36 nm when dialysis is conducted (right side of the chart); in addition, mean sizes higher than 60 nm are not observed when the purification process is applied.

Notably, Fig. 2 provides insight into the interaction between the dialysis process and the FRR. When comparing the contour plot with and without dialysis, the contour lines almost verticalize. Therefore, at specific TF values, the observed mean particle size is constant regardless of the FRR when dialysis is conducted, contrary to the system behavior at the same TF values without dialysis. Thus, Fig. 2 allows a better understanding of the FRR influence within the design of space. One possible implication of this result could be that including dialysis from the early screening stages of LNPs development might prevent the FRR contribution from being overrated.

More broadly, Fig. 3A compiles the combined effect on particle size distribution of all variables of operation included in the experimental design. Consistent with reported data in the literature, the size of nanoparticles tended to reduce at higher TF and FRR values. The reason for that influence is commonly attributed to higher mixing rates, which lead to a minimal size based on the lipid constituents. Additionally, the error bars in Fig. 3A and Fig. S4 (supplementary information) suggest less variability between purified samples. This observation suggests that dialysis could play a role in enhancing process repeatability, which is consistent with our observation for the preliminary screening tests. In those previous experiments, the PSD in 60 observations of LNPs manufactured with the same TF and FRR showed a relative standard deviation of 7.33% for unpurified samples, and 4.61% for purified samples (data not shown).

The statistical relevance for the differences and interactions mentioned above are supported by the regression model ($R^2 = 0.87$) results summarized in Table S1, the data in Fig. 4 and the verifications detailed in table S2, and Figs. S5, S6 and S7 (supplementary information). TF, FRR, and dialysis exhibited a statistically significant effect on particle size distribution ($p = 0.000$). According to the adjusted mean squares and the chart of standardized effects (Fig. 4A), TF provides the greatest contribution when describing the variability of PSD. The dialysis process is the second relevant factor, above the contribution of the FRR. This observation contrasts with the traditional approach to control TF and FRR above any other process variable.

Consistent with the observations in Fig. 2, the ANOVA results for PSD also showed a significant interaction between dialysis and FRR, as well as TF and FRR. To our knowledge, this is the first study to model and demonstrate those interactions. According to the interaction plot shown in Fig. 4B-iii, the expected mean particle size at the highest FRR tested is comparable with and without dialysis, but there is a difference at lower FRR, with higher mean sizes for unpurified samples. This demonstrates that including dialysis as an extra step (detached of any optimization process) could cause the FRR effects extracted from the previous optimization experiments to be misinterpreted.

The TF*FRR interaction is less evident and joint interpretation with the p -value in Table S1 and Fig. 4A is necessary to derive conclusions about their statistical significance, which is the lower than the other factors. Moreover, Fig. 3A shows a change in the FRR effect for the dialyzed samples with 400 mL min⁻¹ and 2200 mL min⁻¹ (the gray and light blue bars in the right part of Fig. 3). As the TF*FRR interaction is more marked in dialyzed samples, it is possible that some authors did not notice the interaction, and it probably offers one explanation of why the design of spaces in MFMs does not usually explore areas of interaction.

In a complementary way, we also used CART to illustrate and evaluate the correlations between factors. Utilizing CART allows a more intuitive representation of the design of spaces, providing a graphic interpretation of the data effects and order of contribution. Fig. 5 provides an additional illustration for the reduction in FRR relevance with the dialysis process and TF. A comparison of nodes 6 and 9 with node 4

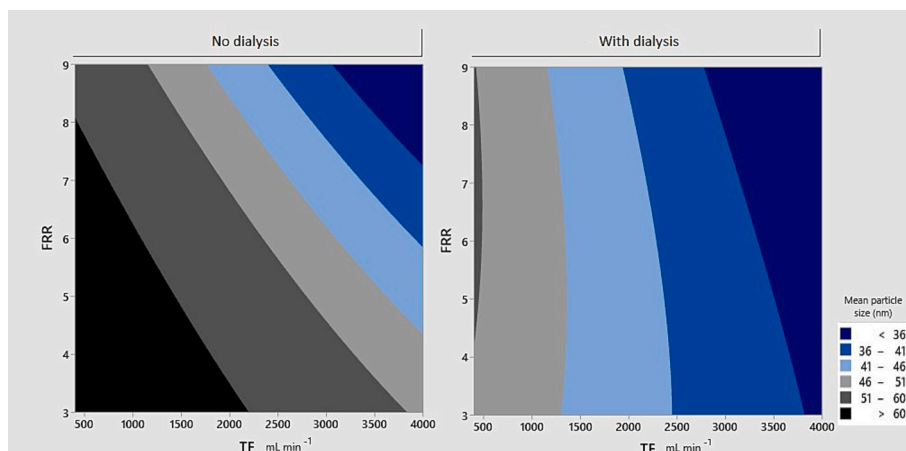


Fig. 2. Contour plot of the particle size distribution of lipid nanoparticles. The introduction of dialysis changes the combined effect of total flow (TF) and flow rate ratio (FRR) on the size of the nanoparticles. The purification process involves lower mean particle sizes and a reduced influence of the FRR value.

shows how the high TF values change the way in which dialysis reduces the FRR effect. The CART on PSD also supports the above-discussed observations on how dialysis could be a key to reduce the process variability, as fewer nodes were observed for dialyzed branches, with smaller standard deviation values.

One possible mechanism that explains why the dialysis impacts the size of LNPs is related to the phenomenon of Ostwald ripening [15,41]. Nanoparticles undergo this mechanism as the smaller particles fuse after the rapid-mixing procedure [35]. The presence of ethanol decreases the stability of lipid membrane, facilitating nanoparticle fusion and high PSD when dialysis is not performed [9]. Furthermore, exchanging the medium with one that contains a higher ionic content will increase the electrostatic repulsion LNPs, reducing the possibility of particle fusion, following the Derjaguin-Landau-Verwey-Overbeek theory regarding the stability of hydrophobic colloid dispersions [33]. In our study, PSD showed a decrease with dialysis, similar to the results obtained by Hibino *et al* [34]. On the other hand, Terada *et al* reported an increase in the size of nanoparticles with dialyses. They hypothesized that the neutralization of cationic lipids decreases nanoparticle repulsion [16]. As both the cationic lipid and the dialysis medium were different in the Terada *et al* and ours, the difference in the direction of the dialysis effect suggests that other variables, such as the type of lipid, and dialysis medium composition could also play a transcendental role in the mechanism of particle fusion. These observations should be considered in studies that involve a further expansion in the design of space.

3.2. Effects on zeta potential

The zeta potential results point to dialysis as the most critical process parameter for that CQA, and show an interaction between the purification process and the FRR. Fig. 3B suggests that the dialysis process exerts a strong influence on the increase in zeta potential, while the FRR showed opposing trends depending on whether the purification process was conducted. In a supportive way, the regression model summarized in Table S1 ($R^2 = 0.83$) indicates statistical significance for dialysis and for the previously mentioned interaction between FRR and dialysis ($p = 0.000$). The adjusted mean squares from Table S1 and Fig. 4C highlight dialysis as the factor that considerably explains most variation, followed by the interaction of dialysis with FRR.

Table S1 and Fig. 4C also indicate a statistically significant effect on zeta potential for TF ($p = 0.000$), although its effect may not be evident in Fig. 3B. This is probably because TF exhibits an adjusted mean square 16 times lesser than that of dialysis, which means its contribution to describing the variability of zeta potential is considerably less significant. However, it appears that TF caused a slight reduction in the zeta potential value as the flow increased, as shown in Fig. 4D. This trend is

also more evident in the main effects plot on zeta potential (Fig. S3—B, supplementary information), and by charting the average zeta potential only by total flow (Fig. S8, supplementary information). In addition, CART in Fig. S10 illustrates the TF relevance in dialyzed and low-FRR conditions.

An additional analysis of Fig. 4D could emphasize the importance of parameter interactions in zeta potential values. There are minimal differences in zeta potential in Fig. 4D-i, in which the dialysis effect is not considered. On the other hand, there are considerable differences in the mean zeta potential values by TF and FRR values when the dialysis effect is analyzed. In particular, the FRR*dialysis plot (Fig. 4D-iii) shows opposite effects for FRR with and without dialysis. Those opposite effects are averaged in the complete design, which is why FRR shows no significant effect in Table S1 and Fig. 4C. The effects shown in Fig. S10 (supplementary information) could clarify any possible confusion regarding the relevance of FRR on zeta potential, specifically of the clarification occurs through observing the splitting after nodes 2, 3, 4 and 6 (4 out of 7 split nodes). In addition, the analysis of only the dataset including dialysis (represented in Fig. S9, supplementary information) showed significant effects for FRR on zeta potential, with a higher contribution than that of TF. The opposite effects of FRR also support the theory that if dialysis is added as an extra step, the knowledge gathered in optimization studies without dialysis may become insignificant.

The strong influence of dialysis on the zeta potential could be explained by the change in dialysis medium composition. During dialysis, ethanol and the original aqueous buffer were substituted by a new medium. This medium could change the zeta potential, as it contains different saline composition and exhibits different ionic strength and pH values [42]. To increase DoE robustness, further studies should be carried out, including studies with different levels of composition and concentrations of dialysis medium. In addition, the changes in the zeta potential could contribute to variation in PSD due to the electrostatic repulsion and particle fusion modulation theory previously mentioned [33,42].

3.3. Effects on polydispersity index

The data for the polydispersity index differ from those observed for the PSD and zeta potential. The most important difference is the nonlinear behavior evidenced by the significant effect for the quadratic factor FRR*FRR shown in Table S1 and Fig. 4E, and the low value for the R^2 of the regression model (0.50). The lack of linearity is also shown in the curvature of each individual effect line from the interaction plot for PDI in Fig. 4F, and in Fig. S3—C (supplementary information). This behavior could be a consequence of PDI being a parameter of relative dispersion, which is affected by the standard deviation of data and the

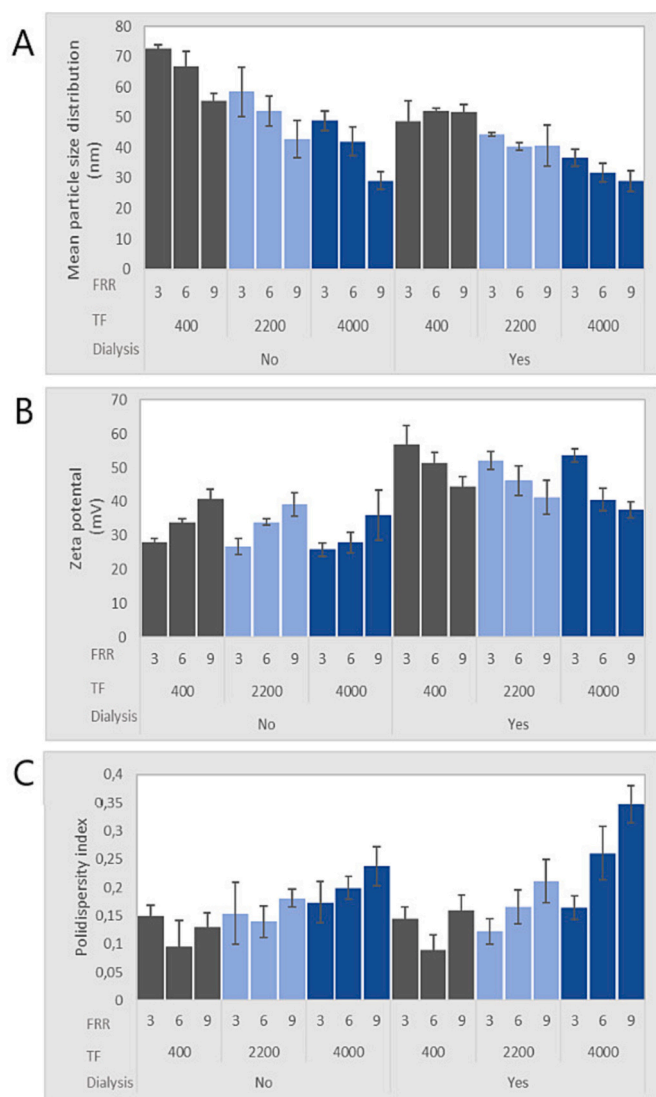


Fig. 3. Effect of each variable of operation on the mean values of the critical quality attributes assessed. Gray bars correspond to the 400 mL min⁻¹ samples, light blue to 2200 mL min⁻¹ and dark blue to 4000 mL min⁻¹. A) The particle size distribution (PSD) showed a tendency to decrease with higher total flow (TF) and flow rate ratio (FRR) values. Dialyzed samples showed lower PSD and less variability. B) The dialyzed samples showed higher zeta potential values. Flow rate ratio (FRR) differences showed opposite trends with and without dialysis; while total flow (TF) exhibited a less evident effect on reducing zeta potential (additional representation in Fig. S10). C) Polydispersity index (PDI) changes on the flow rate ratio (FRR) showed a different trend at 400 mL min⁻¹, compared to 2200 and 4000 mL min⁻¹, suggesting an interaction between those two variables. Fig. S3A in supplementary information provides additional visualization of main effects for each quality attribute. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mean particle size values [28,43,44]. Due to the lack of linearity, the interpretation of the PDI outcomes shown in Fig. 3C might not be as intuitive as the PSD and zeta potential results.

However, based on the DoE results from Fig. 4E, Fig. S11, and Table S1, we determined that TF is the factor with the highest contribution when describing PDI variability, followed by the FRR and TF-FRR interactions. Thus, the traditional approach that focuses on TF and FRR might provide a better fit for describing the behavior of PDI, unlike the observed for PSD and zeta potential. Regarding the interaction, Fig. 4F-i shows that the differences in PDI among FRRs impact the PDI values at

high TF values, which are expected to cause smaller particle sizes. Thus, it is possible that the increase in PDI influenced by the high FRR is more due to the decrease in the mean particle size than by the dispersion itself.

The effects of dialysis illustrated the CART presented in Fig. S11 (supplementary information) could also support the hypothesis that PDI is affected by smaller particle sizes, instead of the dispersion itself. The dialyzed terminal nodes show the highest PDI values of their respective subbranches, and it was previously discussed that the dialysis process shows a strong influence decreasing PSD with relatively low standard deviation on particle size. More studies are needed to determinate the mechanisms of PDI correlations; this is especially true for relatively low particle sizes, which could lead to higher PDI values even with similar ranges of dispersion. To perform a deeper evaluation, additional DoE with more levels are needed to include nonlinear relationships and clarify the integrated effect on PDI. It is possible that the conclusions for PDI could change as more description and correlation are added to the model. In particular, due to the presence of standardized effects close to the line of significance (Fig. 4E) and the low accuracy of the regression model ($R^2 = 0.50$).

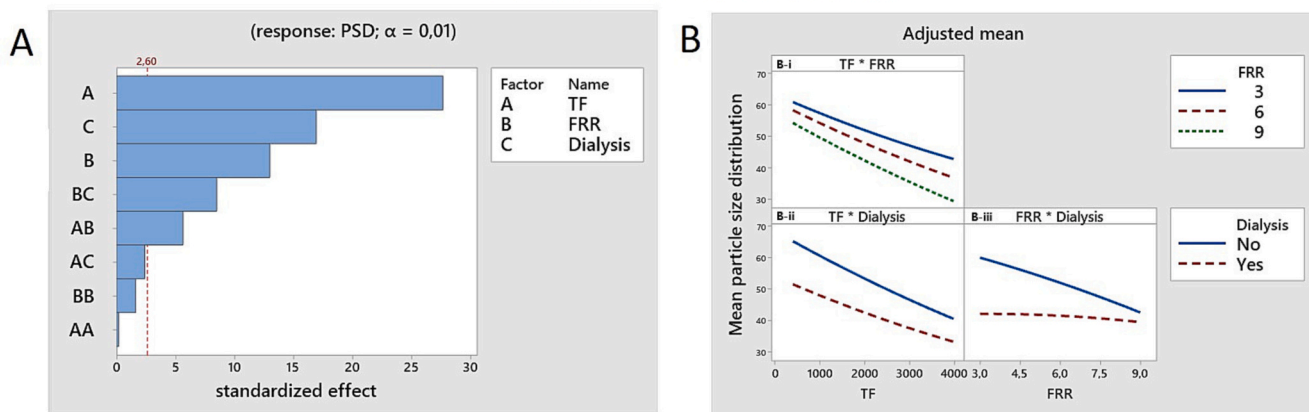
3.4. Implications for the quality by design development of lipid nanoparticles

When performing a QbD approach, the purification requirement should be defined from the very first step in the process, i.e., specification of the quality target product profile (QTPP) [45]. As the necessity to include a purification method depends on the biological application of LNPs and the incompatibility of ethanol content, it is arguably possible that some authors could include the purification step only when the formulation process has moved toward biological evaluation; this possibly occurs after screening or optimization experiments including TF and FRR. This could be particularly reasonable given the limited attention that purification has received as CPP in the QbD literature. However, the evidence of direct and interactive effects of dialysis revealed in this study suggests that such latter inclusion could lead to additional testing and adjustments in the development process. In the best cases, the quality profile of the LNPs would result from chance and not through knowledge of how CPP affects CQA. Thus, excluding the dialysis effect in the design of space exploration could be considered a quality-by-testing approach.

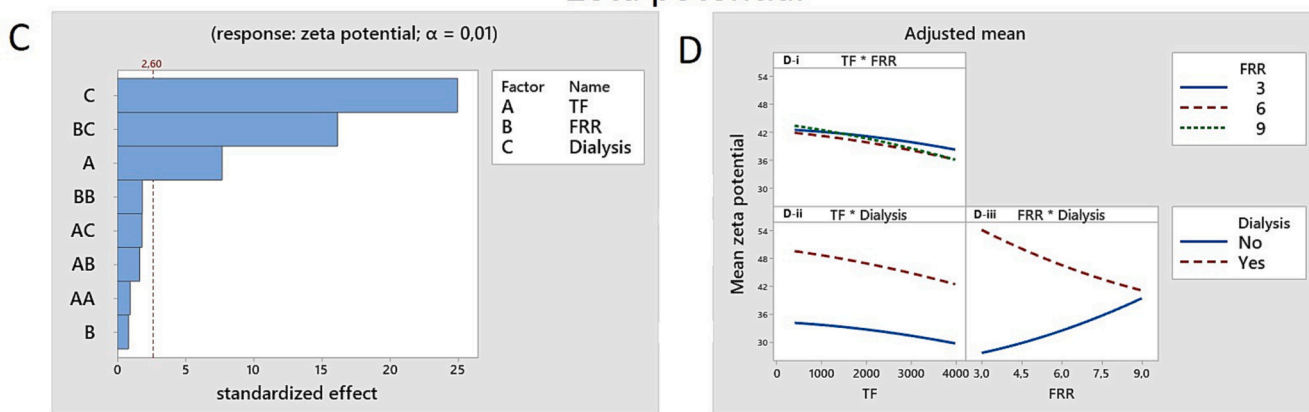
By considering our formulation in a hypothetical design of space without the dialysis data, the possible implication on LNPs design is illustrated. Table 1 represents a response optimizer for two different approaches. The first design of space (DS1) corresponds to the complete design shown in this study. The second one (DS2) is a hypothetical and more limited design of space, as if only TF and FRR were evaluated without considering dialysis for this exploratory stage.

Table 1 illustrates how the same formulation and a relatively similar experimental procedure (except for the inclusion of dialysis) could lead to two different LNPs systems. From the manufacturing point of view, the formulation from DS1 would have a higher lipid concentration than that obtained from DS2 (as a high FRR implies that the ethanolic phase is more diluted). Additionally, both hypothetical formulations will show different PDI and zeta potential values. Moreover, the formulation from DS2 probably must undergo dialysis at some point. It is not possible to predict the PSD, PDI and zeta potential values if only the DS2 regression model is considered. The effect of this subsequent change will be a source of uncertainty until the change is executed and tested. On the other hand, having the complete data set and the regression model of DS1 allows us to predict the results for the inclusion of dialysis to the configuration of TF = 2181.82 mL min⁻¹ and FRR = 9. The PSD for that inclusion of dialysis would be 39.51 ± 8.48 nm, PDI of 0.239 ± 0.071, and zeta potential of 41.15 ± 8.16 mV. All CQA attributes are different from those expected for any two proposed scenarios. As previously discussed, the knowledge gained from the optimization studies without any purification method may become irrelevant after performing

Particle size distribution



Zeta potential



Polydispersity index

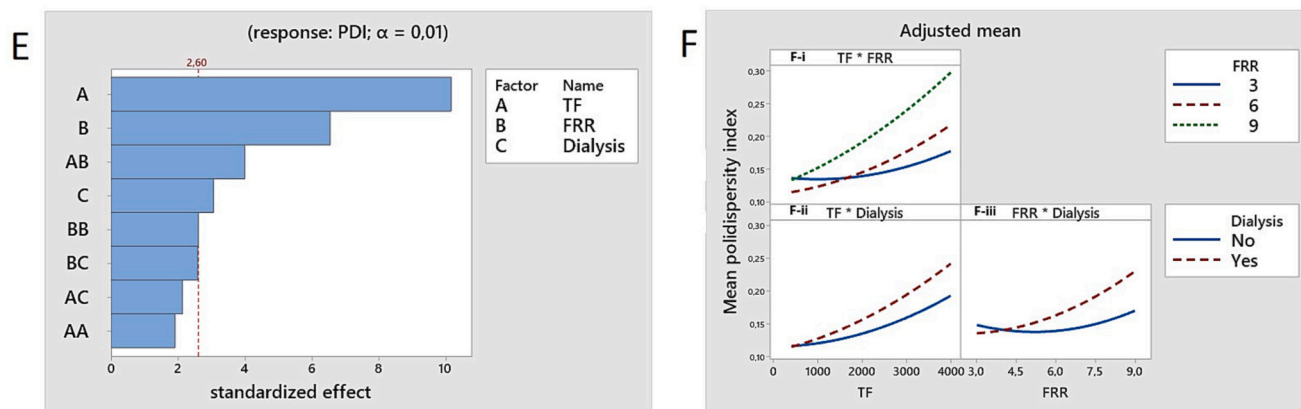


Fig. 4. Pareto charts for standardized effects and interaction plots of each critical quality attribute from the surface response design. A) Standardized effects for particle size distribution (PSD) show significant effects for total flow (TF), dialysis, flow rate ratio (FRR) and the interactions between FRR*dialysis and TF*dialysis (in descending order of contribution). B) Interaction plot for PSD. The nonparallel lines in the bottom right panel (B-iii) represent the strong interaction between FRR and dialysis. The red lines in B-iii show almost no difference between FRR values, while the blue lines show a decrease in PSD as the FRR values increase. To interpret the less evident interactions for B-i and B-ii joint observation with the results shown in Fig. 4A and Table S1 must be performed. C) The standardized effects of dialysis, the dialysis*FRR interaction and TF showed significant effects on zeta potential (in descending order of contribution). D) The lines in D-iii (converging at high FRR values) show a strong interaction as the FRR effects depends on the dialysis process. Panels D-i and D-ii are almost parallel representing no interaction between factors. E) The standardized effects for the polydispersity index (PDI) shows significant effects for TF, FRR, the TF*FRR interaction, dialysis and the quadratic effect of FRR (in descending order of contribution). The value of the standardized effect for FRR*FRR is 2.6117 (significance limit at 2.600), while the value for the dialysis*FRR interaction is 2.59281. F) Panel F-i shows a strong interaction between TF and FRR. In particular, the more evident differences can be seen at an FRR of 3. Although panels F-ii and F-iii may suggest an interaction for TF*dialysis and FRR*dialysis, joint observation with Fig. 4E and Table S1 results are necessary for interpretation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

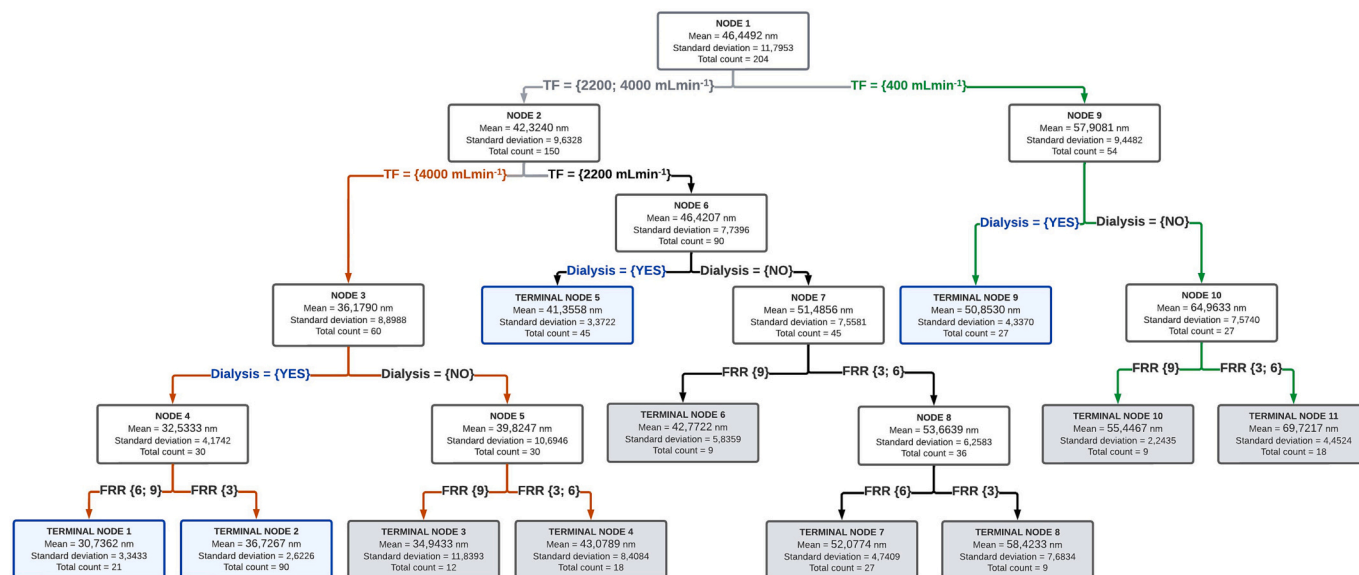


Fig. 5. Classification and regression tree for the particle size distribution. The effects of total flow (TF), dialysis and flow rate ratio (FRR) are shown. The orange, black and green lines represent the branches from the 4000 mL min⁻¹, 2200 mL min⁻¹ and 400 mLmin⁻¹, respectively. The primary splitting by TF indicates that TF provides the highest contribution when describing the variability in PSD data. Each TF branch splits into dialyzed and undialyzed subbranches. Notably, performing dialysis led to a terminal node for low and mid TF values, regardless of the FRR value. Significant differences by FRR are seen only at the highest TF (4000 mL min⁻¹) and for unpurified samples. The light blue boxes (the dialyzed terminal nodes) show lower standard deviation values than those of the gray boxes (unpurified terminal nodes). Additional CART for zeta potential and polydispersity index are presented in supplementary information. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Response optimization minimizing PSD and PDI for two scenarios with and without the inclusion of dialysis as part of the optimization studies. Optimization was performed to minimize the PSD and PDI, and maximize the zeta potential.

Scenario	Variable configuration			Predicted response		
	TF (mL min ⁻¹)	FRR	Dialysis	PSD (nm)	PDI	Zeta Potential (mV)
DS1. Optimize with dialysis (full design) ¹	1829,72	3	Yes	43,35 ± 1,70	0,129 ± 0,071	54,72 ± 8,16
DS2. Optimize without dialysis ²	2181,82	9	Not included	42,64 ± 1,66	0,169 ± 0,014	39,46 ± 8,15

Note: 1- Regression model for DS1 includes all the data shown in Table S3 (supplementary information). 2- DS2 only includes half of the Table S3 data ("No" dialysis data).

dialysis as an additional step. In similar cases, any subsequent modification in the process after dialysis is performed will require additional testing and resources to predict, evaluate, and address process variability in accordance with QbD.

QbD must identify, explain and manage all sources of variability [46]. As dialysis effects have now been identified and explained in terms of their interaction with TF and FRR, our suggestion is that dialysis or any other suitable purification method should be included in the formulation process from the early stages, even if the biological applications are postponed until later development stages.

Dialysis is a widely used technique for nanoparticle purification despite some disadvantages such as: potential loss of the loaded drug, batch-size dependency or being a time-consuming method. The impact of dialysis and its variables should be carefully monitored to minimize any possible negative effect. Particularly, the selection of the dialysis membrane, time, and volume of dialysis buffer should be optimized in terms of their effect in the loading capacity and stability. We acknowledge that alternative purification techniques, such as ultracentrifugation, size-exclusion chromatography or filtration techniques could be preferred for a given formulation depending on the specific balance of advantages or disadvantages. Regardless the selected method, we encourage other authors to apply such purification methods with caution in order to not underrate any potential interactions, as we have

stated in our study.

Regarding other possible variables on LNPs preparation, it is highly possible that some other process variables excluded from this study could also exert an interactive effect similar to dialysis, given the high data interconnection observed in the DoE and the principal component analysis. Thus, the parameter interactions could be underestimated, as previously discussed. Even our conclusions could change if higher contributions appear, as more parameters are included in more than one level.

Therefore, more research work that includes more process parameters is needed. More knowledge about interactions would provide a more robust design of spaces. The work of Ly *et al* [17] is among the most parameter-extensive studies included in Fig. 1. Interestingly, they did not include purification method as a factor in the experimental design. However, their approach in two iterations DoE could be used as a reference for reducing the number of experimental runs that are needed as more factors are incorporated. Our observations could be used as references for further evaluations, as well as the observations on lipid content from Terada *et al* [16] and lipid composition from Tiboni *et al* [37].

There is also a research opportunity to focus on the dialysis variables. From a process perspective, dialysis involves several variables that might be sources of variation. Consequently, it is possible to establish an

even broader design of spaces considering different levels of: dialysis medium composition or concentration, different dialysis times and the effect of latency times before dialysis. Further research is needed including different methods of purification such as ultracentrifugation, size-exclusion chromatography or filtration techniques.

Future research, including the encapsulation of a model drug, could shed more light on how the working parameters and their interactions could impact the release properties of lipid nanoparticles. On the other hand, studies that include nonlinear relationships in the description of PDI are also needed, as previously mentioned.

4. Conclusions

In this study, dialysis was identified as a critical parameter in microfluidic mixing during the process of manufacturing lipid nanoparticles. The influence of dialysis on the particle size distribution and zeta potential is stronger than the influence of the flow rate ratio. In contrast to the traditional approach centered on total flow and flow rate ratio as the key critical process parameters, our results suggest that the effect of FRR in PSD seems to be nearly negligible when the dialysis process is performed.

Any knowledge gained from the design of space explorations without dialysis might become irrelevant after performing dialysis as an additional step. Consequently, dialysis or any other purification method should be considered as an integrated part of microfluidic manufacturing. Its integration with the process and interactions with other variables should be evaluated from the early stages of pharmaceutical development by Quality by Design.

A broader design of space explorations would lead to a greater understanding of microfluidic mixing manufacturing of lipid nanoparticles. The time and cost of lipid nanoparticle developments should be reduced as a consequence of knowledge growth based on more variables and more levels of interaction, increasing the clinical translation of lipid nanoparticle developments.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting the conclusions of this study are available in the article. Raw data is available upon request.

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Appendix A. Supplementary data

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