



High-throughput manufacturing of size-tuned liposomes by a new microfluidics method using enhanced statistical tools for characterization



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ABSTRACT

Microfluidics has recently emerged as a new method of manufacturing liposomes, which allows for reproducible mixing in milliseconds on the nanoliter scale. Here we investigate microfluidics-based manufacturing of liposomes. The aim of these studies was to assess the parameters in a microfluidic process by varying the total flow rate (TFR) and the flow rate ratio (FRR) of the solvent and aqueous phases. Design of experiment and multivariate data analysis were used for increased process understanding and development of predictive and correlative models. High FRR lead to the bottom-up synthesis of liposomes, with a strong correlation with vesicle size, demonstrating the ability to in-process control liposomes size; the resulting liposome size correlated with the FRR in the microfluidics process, with liposomes of 50 nm being reproducibly manufactured. Furthermore, we demonstrate the potential of a high throughput manufacturing of liposomes using microfluidics with a four-fold increase in the volumetric flow rate, maintaining liposome characteristics. The efficacy of these liposomes was demonstrated in transfection studies and was modelled using predictive modeling. Mathematical modelling identified FRR as the key variable in the microfluidic process, with the highest impact on liposome size, polydispersity and transfection efficiency. This study demonstrates microfluidics as a robust and high-throughput method for the scalable and highly reproducible manufacture of size-controlled liposomes. Furthermore, the application of statistically based process control increases understanding and allows for the generation of a design-space for controlled particle characteristics.

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1. Introduction

Liposomes are well established as delivery systems and immunological adjuvants and there are a wide range of methods employed in their production. For example, multilamellar vesicles (MLV) can be formed by the dispersion of a dried lipid film and small unilamellar vesicles (SUV) can then be produced by sonication (Lapinski et al., 2007; Maulucci et al., 2005), extrusion (de Paula Rigoletto et al., 2012; Olson et al., 1979), or high-pressure homogenization (Barnadas-Rodriguez and Sabes, 2001; Pupo et al., 2005). However, sonication may lead to sample contamination by metallic residues from the probe tip, lipid degradation and lack of scalability (Wagner and Vorauer-Uhl, 2011). Homogenization

techniques, shear or pressure induced size reduction, circumvent protein or lipid degradation and are frequently used to reduce the size and lamellarity of MLV (Wagner and Vorauer-Uhl, 2011). Maintenance of constant temperatures throughout these processes can be difficult, with restrictions to relatively small working volumes and quantities; however, continuous and heat controlled homogenization techniques have been developed to help overcome some of these problems (Riaz, 1996; Wagner and Vorauer-Uhl, 2011).

As an alternative to these methods, microfluidics is a relatively new area of liposome synthesis, where the small dimensions in a micromixer allow for fast mixing, dominated by diffusion or convection (Whitesides, 2006). Microfluidics refers to fluid handling methods in a controlled volume, typically below millimeter scales, which allows for implementation of the mixing process into planar chips (Squires and Quake, 2005). The application of microfluidics for liposome synthesis in novel lab-on-a-chip based devices dramatically reduces time for sample

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preparation as well as costs associated with experimental work and may additionally be fully software controlled to aid process robustness and reproducibility (van Swaay, 2013). Various micromixers have been designed and applied for the manufacturing of liposomes based on different channel layouts (Pradhan et al., 2008) including flow focusing (Davies et al., 2012; Jahn et al., 2004), droplet based (Teh et al., 2008), and T- or Y- shaped mixers (Kurakazu and Takeuchi, 2010). In this study, a staggered herringbone micromixer (SHM) (Stroock et al., 2002) which induces chaotic advection, is used. The chaotic advection mixing profile allows for stretching and folding of fluid streams over the channels cross-sectional area, increasing mass transfer together with the herringbone type structures on the channel floor (Stroock et al., 2002). Here, a SHM was used together with the automated mixing platform NanoAssemblr™ (Precision NanoSystems, Inc.). This system enables rapid, reproducible and scalable manufacture of homogeneous next-generation nanoparticles and liposomes (Belliveau et al., 2012; Zhigaltsev et al., 2012). Lipid dissolved in solvent is pumped into one inlet and aqueous buffer into the other inlet of the microfluidic mixing cartridge (Fig. 1). It has been suggested that a nanoprecipitation reaction results in the formation of nanoparticles (Karnik et al., 2008; Zhigaltsev et al., 2012). This reaction takes place at the interface of the solvent and aqueous streams. Liposome formation is based on polarity alterations throughout the chamber and an increase in the surface area of the fluid interface occurs, as the fluids are folded over on top of each other aided by the channel design and grooves on the channel floor (Fig. 1, small). The rate of polarity increase and the subsequent following the formation of liposomes is user-controlled by alterations in flow rates of the separate streams as well as the ratios of aqueous to solvent stream as demonstrated for liposomes (Bally et al., 2012; Zhigaltsev et al., 2012) and polymeric nanoparticles (Bally et al., 2012). Furthermore, the option of parallelization of the mixing cartridges allows for scalability as a high throughput method (Belliveau et al., 2012).

The development and optimization of new processes and methods can be a time consuming task, especially when applying the traditional one-factor-at-a-time (OFAT) method, where only one factor is optimized while all other factors remain constant. Adopting this approach may also result in the optimum process or formulation being overlooked as well as possible factor-

interactions (Montgomery et al., 1997). An alternative approach is to adopt design of experiments (DoE), a statistical optimization method, favorably used in pharmaceutical and biopharmaceutical process development and optimization (Lawrence, 2008; Singh et al., 2011; Vandervoort and Ludwig, 2002). DoE is a systematic approach of creating structured experiments, measuring or detecting the effect of changes to a pre-defined response. Product quality, as well as process understanding is maximized with a minimal number of experiments performed. In DoE, the factors are defined as the variables in a process and selected responses define the properties of the system that is investigated. Factors are the tools used for manipulation of the system, which following influence the responses. The aim is to connect the variation in the factors to the resulting responses, and link the information using a mathematical model. DoE does not only investigate statistical significant factors involved in a process (main effects), it also identifies interactions between factors and respective influence on the desired output variable (Eriksson, 2008; Mandenius and Brundin, 2008). A second statistical tool, multivariate data analysis (MVDA), allows for the analysis of more than one statistical variable at a time by reducing dimensionality in a data set by its transformation (Wold et al., 2001a,b). MVDA is used for identifying patterns and relationships between several variables simultaneously (Eriksson, 2006). It predicts the effect of changing one variable to other variables and is applied for data analysis, data mining, classification (e.g., cluster analysis or outlier detection), regression analysis and predictive modeling, frequently used in pharmaceutical and biopharmaceutical processes (Eriksson, 2006; Pasqualoto et al., 2007; Rathore et al., 2011). Both tools, DoE and MVDA, are statistical-based, process understanding and optimization tools that build and describe knowledge around a specific application, which ultimately supports the development of confidence and enhanced understanding, as well as robustness of a process.

This present study first investigated microfluidics as a new method for manufacturing of cationic liposomes using the NanoAssemblr™. To achieve this 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were used to formulate liposomes. This combination of the fusogenic lipid DOPE with the cationic lipid DOTAP, is a frequently used composition due to its high *in vitro*

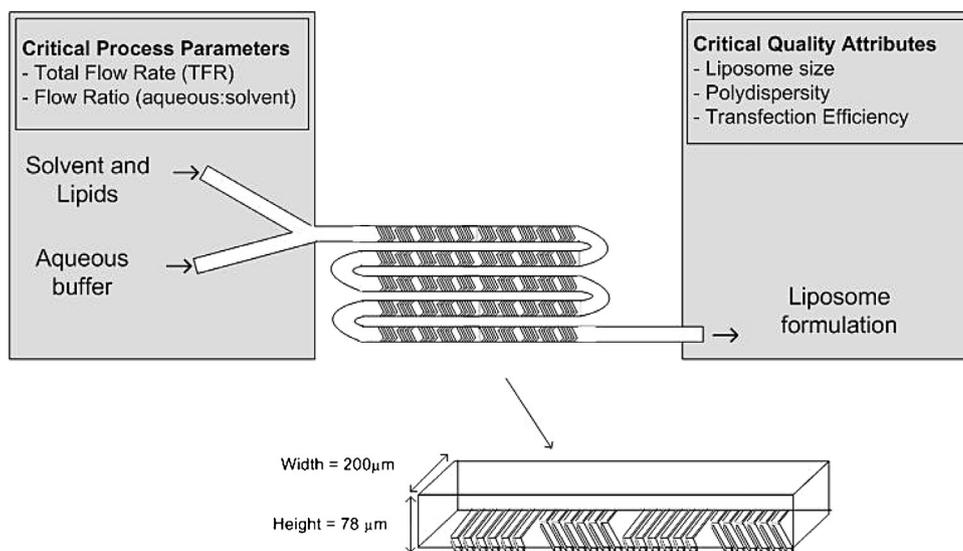


Fig. 1. Schematic of liposome formulation process. Lipids dissolved in ethanol and an aqueous buffer are injected into separate chamber inlets. Mixing takes place in the chamber (small picture), designed with grooves on the channel floor to aid chaotic advection between both streams. Depicted are the critical process parameters and the critical quality attributes.

transfection efficiency and optimal immune response (McNeil et al., 2010; Liu and Huang, 2002) and was therefore chosen to allow correlation of the systems produced via this new production method with previous studies.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids, Inc., (Alabaster, AL) (purity >99%). Ethanol and chloroform (all HPLC grade) were purchased from Fisher Scientific (Leicestershire, UK). Lipofectin™ reagent was obtained from Invitrogen Life Technologies and the luciferase assay kit and CellTiter 96® Aqueous One Solution Cell Proliferation Assay were both obtained from Promega (Madison, WI). Serum free and antibiotic free medium (opti-MEM), Dulbecco's modified Eagles medium (DMEM), L-glutamine/penicillin-streptomycin and foetal bovine serum (FBS) were purchased from Gibco-Invitrogen Ltd. (Paisley, UK) (all cell culture grade). gWiz™ Luciferase was obtained from Genovac GmbH, Germany. COS-7 cells (GMP grade) were purchased from European collection of cell cultures (ECACC), a Health Protection Agency Culture Collection (Salisbury, UK).

2.2. Micromixer

The micromixer was obtained from Precision NanoSystems Inc., with molded channels of 200 μm in width and 79 μm in height with herringbone features of 50 \times 31 μm in poly(dimethylsiloxane). Connections of disposable 1 mL syringes to the two inlet streams to the chip was done by fluid connectors. Liposome formulations using the micromixer were performed on a benchtop NanoAssemblr™ instrument (NanoAssemblr™, Precision NanoSystems Inc.). The two inlet streams comprised lipids dissolved in ethanol and aqueous buffer (Tris, 10 mM, pH 7.4), syringe pumps allowed for controlling the flow rates and the flow ratios between the two inlet streams.

2.3. Liposome preparation

DOPE and DOTAP (8:8 μmol) were dissolved in ethanol. Here, an equal molar lipid ratio was used, a standard ratio in cationic liposome-DNA transfection studies as reported previously (Felgner et al., 1994; Moghaddam et al., 2011). The ethanol-lipid solution was injected into the first inlet and an aqueous buffer (Tris 10 mM; pH 7.4) into the second inlet of the microfluidic mixer (Fig. 1). During initial studies, the TFR of aqueous buffer and lipid phase were varied from 0.5 mL/min to 2 mL/min and the FRR of the solvent and aqueous phases was varied from 1:1 to 1:5. Values of TFR and FRR were extrapolated from previous reported nanoprecipitation methods using a SHM design with a channel diameter of 200 μm (Zhigaltsev et al., 2012) as well as based on preliminary screening prior to this work. The resulting aqueous dispersions of liposome formulations, as formed by the mixing of the two adjacent streams, were collected from the outlet stream and subsequently dialysed over night against Tris buffer (10 mM; pH 7.4) to remove any residual solvent.

2.4. Liposome characterisation

The dynamic light scattering (DLS) technique was used to report the intensity mean diameter (z-average) and the polydispersity of all liposome formulations (Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcs., UK)). The measurements of vesicle size and polydispersity were carried out at 25 °C in Tris buffer (1/10 dilution;

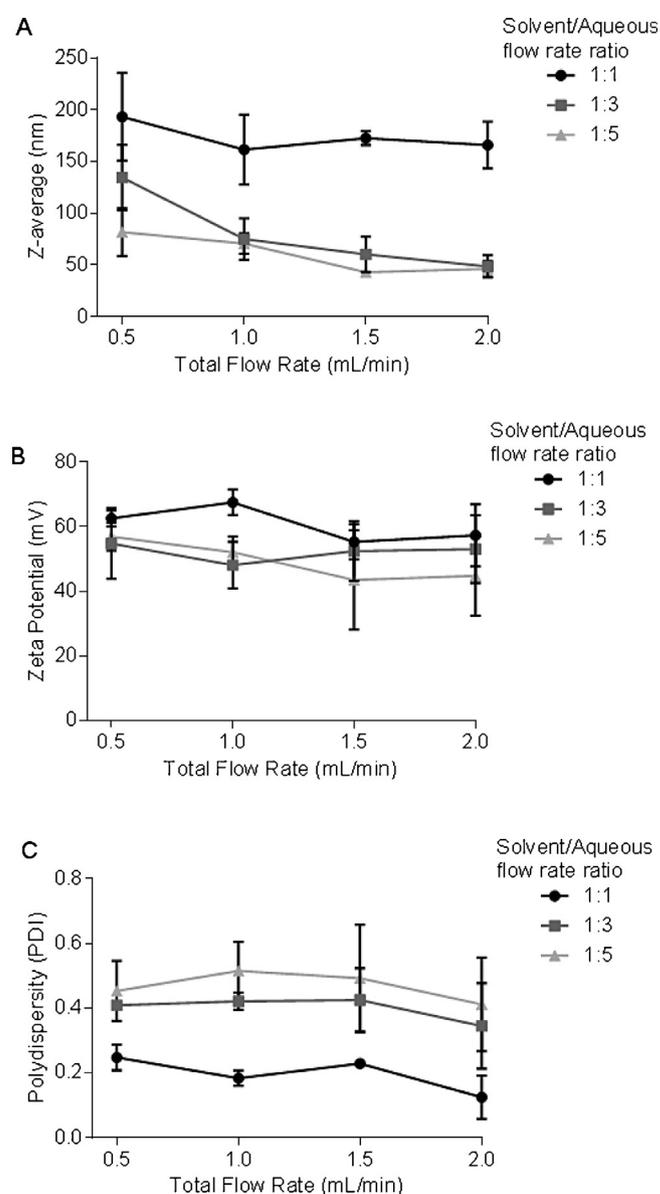


Fig. 2. Liposome characteristics. (A) Vesicle size (z-average), (B) zeta potential and (C) polydispersity of DOPE:DOTAP formulations manufactured by microfluidic mixing. Results are the mean of triplicate formulations \pm SD.

1 mM, pH 7.4). Liposome zeta potential was measured in Tris buffer (1 mM, pH 7.4) using the Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcs., UK). All measurements were undertaken in triplicates.

2.5. HPLC

Lipid quantification of the liposome formulations was carried out using an Agilent 1200 series HPLC connected to an SEDEX 90 evaporative light scattering detector (ELSD). A Phenomenex® Luna 5 μm C18 (2) 100 A 150 \times 4.6 mm column was used. An isocratic flow method was employed with 85% methanol and 15% 0.1% TFA water at a flow rate of 1 mL/min. The ELSD temperature was set at 52 °C. The total run time was 20 minutes.

2.6. DNA lipoplex preparation for *in vitro* transfection

To perform *in vitro* studies, lipoplexes were prepared by diluting 17.5 μl of SUV solution (16 μmol) to 0.35 mL with Opti-MEM, and

then incubated for 40 minutes at room temperature. After incubation, 0.35 mL of Opti-MEM containing 3.5 μg plasmid DNA was added, mixed with liposome solution and incubated again for a further 15 min at room temperature. The resultant lipoplex mixture was then diluted to a final volume of 3.5 mL with Opti-MEM. The lipid/DNA charge ratio for *in vitro* study was +1.7/1.

2.7. In Vitro transfection of COS-7 Cells

African green monkey kidney cells (COS-7 cells) were cultured at 37 °C under 5% CO₂ in Delbecco's modified Eagles medium (DMEM). Medium was supplemented with 4 mM L-glutamine, 10% (v/v) foetal bovine serum (FBS), penicillin (100 $\mu\text{g}/\text{mL}$) and streptomycin (100 $\mu\text{g}/\text{mL}$). 24 h prior to transfection, the COS-7 cells were plated at a cell concentration of 1×10^5 cells/mL in 1 mL of medium in a 12-well plate and were incubated overnight. Cells were washed with 1 mL of Opti-MEM before lipoplexes were added to the cells. 1 mL of the SUV-DNA solution (0.0078 μmole total lipid content containing 1 μg plasmid DNA) was added to each well. Each transfection was performed in triplicate. After 5 h of incubation time at 37 °C in 5% CO₂, the medium was replaced with growth medium (DMEM) containing 10% FBS and the cells were incubated for further 48 h. The transfection efficiency of each formulation was measured by determination of the percentage of luciferase activity in each sample to the control. In this study this value is reported as luciferase activity (%) and Lipofectin was the control transfection reagent.

2.8. Cytotoxicity study

Lipoplex formulations used in the cytotoxicity study were same as described above. COS-7 cells were transferred on a 96-well plate and incubated for 24 h at 37 °C in DMEM medium. 20 μL of MTS reagent (CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay) was added to each well. The MTS reagent is bioreduced by the cells into a red formazan product, which indicates the presence of metabolically active cells. After 4 h incubation at 37 °C, in a 5% humid CO₂ atmosphere, the quantity of produced formazan was measured on microplate reader (Thermo Scientific Molecular Spectrum plate reader) at A490, with the absorbance reading being directly proportional to the number of living cells in the medium. In this study, cell viability was calculated and expressed as a percentage to the positive control (i.e., cells and medium).

2.9. Statistical analysis

All experiments were performed in triplicates with calculation of means and standard deviations. Statistical significance was

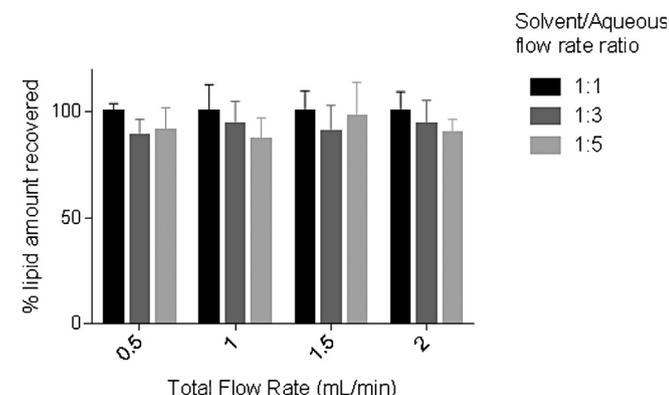


Fig. 3. Quantification and recovery (%) of lipids (DOPE + DOTAP) by HPLC. Results are the mean of triplicate formulations \pm SD.

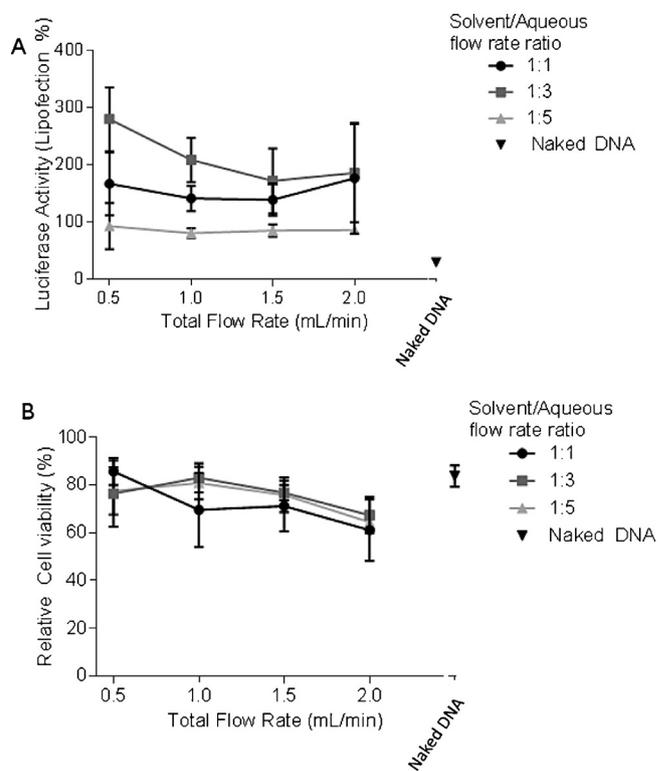


Fig. 4. (A) Comparison of transfection efficiency of cationic nanoparticles. Liposomes were complexed with gWiz plasmid DNA expressing firefly luciferase. (B) Relative cell viability of nanoparticles formulated with distilled water. Results denote mean \pm SD, $n = 3$.

determined by a one-way analysis of variance (ANOVA) on all data, and determined to 0.05 confidence intervals ($p < 0.05$).

2.10. Design of experiments

The significance of the factors TFR (0.5–2 mL/min) and FRR (1:1–1:5) on liposome size, polydispersity and transfection efficiency were investigated in a design of experiments (DoE) study (MODDE version 10.0, Umetrics). We used multiple linear regressions (MLR), which fits one response at a time, based on the assumption that the responses are independent. A quadratic response surface model (RSM) was performed. The collected data was used to estimate the coefficients of the model and assess for statistical significance. The sum of squares of the residuals was minimized in the model. The aim was to obtain small variation for the coefficients and minimize the prediction errors, which was achieved with least square regression analysis. Prediction plots (response surfaces) were used for model interpretation and assessment of optimal regions in the model prediction. Models were validated by analysis of variance (ANOVA), which identified the goodness of fit and prediction (R^2 and Q^2) and the significance of each factor in the model. Regression model significance test identified the validity of a model by dividing the mean squares of the regression by the mean square of the residual, which allowed for determination of the probability value p . With $p < 0.05$, the

Table 1

Coefficient list for the responses size, zp and PDI. Coefficients were determined as statistically significant ($p < 0.05$).

Response	Significant coefficients
Size (nm)	TFR, FRR, FRR*FRR
PDI	FRR, FRR*FRR
Transfection efficiency	FRR, FRR*FRR

model determined was good. Lack of fit (LOF) test was performed to investigate the model error and the replicate error. A model showed no lack of fit when a sufficiently small model error and a good data fit were obtained, indicated by a p -value larger than the critical reference 0.05.

2.11. Multivariate data analysis

Principal Component Analysis (PCA) and Partial Least Square (PLS) regression analysis was performed (SIMCA version 13.0, Umetrics) in order to analyse more than one variable at a time. The relationship between the variables TFR and FRR and the responses (liposome size, polydispersity and transfection efficacy) was displayed in a loading plot, using all experimentally obtained raw data in this study. Weights were selected to maximize the correlation. For interpretation, a line from a selected variable was drawn through the origin and X- and Y-variables were projected on the line. Variables opposite to each other were determined as negatively correlated, positive correlation was determined with variables adjacent to each other.

3. Results and discussion

3.1. Liposome manufacturing by microfluidics—vesicle size can be in-process controlled.

Liposomes consisting of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-

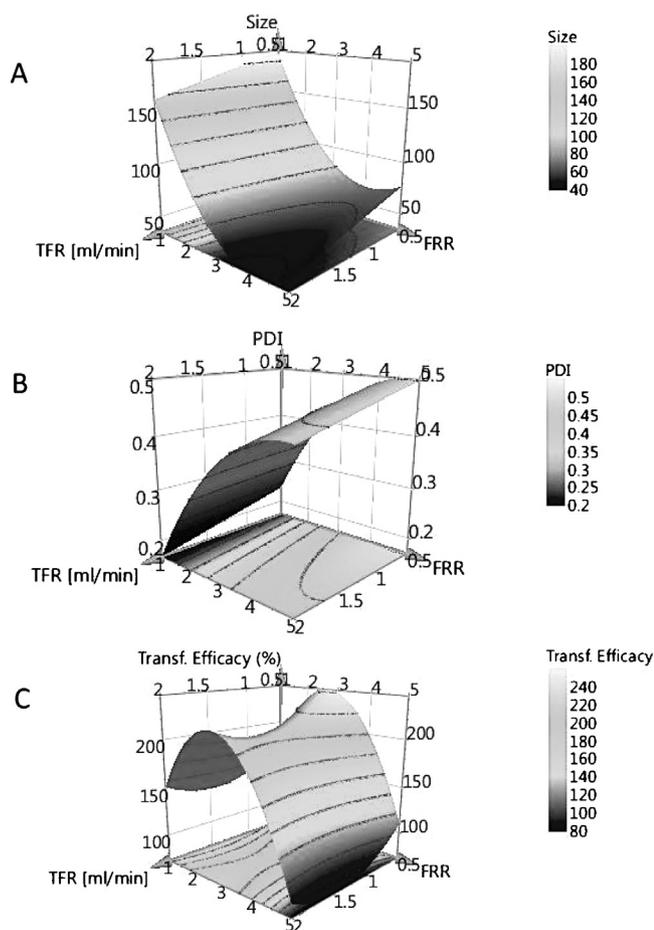


Fig. 5. The response surface plots in the DoE study for the responses size (A), PDI (B) and transfection efficacy (C) as a function of flow rate ratio and total flow rate. All three models were determined as statistical significant in an ANOVA analysis.

Table 2

ANOVA for the responses size, z and PDI. The p -statistics were analysed as well as the Lack-of-fit (LOF), together with fit power (R^2) and predictive power (Q^2).

ANOVA	Size	PDI	Transfection efficiency
Regression p	0.000	0.001	0.001
LOF p	0.255	0.973	0.585
R^2	0.989	0.885	0.889
Q^2	0.963	0.789	0.522
Model significant?	Yes	Yes	Yes

phosphoethanolamine (DOPE) were formulated using the microfluidics method with a SHM design. In this study, the aim was to optimise parameters to control particle size by varying the TFR from 0.5 mL/min to 2 mL/min and varying the FRR of the solvent/aqueous phases from 1:1 to 1:5. It can be seen from Fig. 2A that as the aqueous/ethanol FRR was increased, a reduction in liposome size was detected. However, increasing the TFR from 0.5 mL/min to 2 mL/min did not significantly affect the vesicle size for the FRR of 1:1, 1:3 and 1:5 (Fig. 2A). Liposomes formed at 1:5 solvent/aqueous formulation were smaller in size and around 50–75 nm compared to the 1:1 solvent/aqueous formulation (175–200 nm; Fig. 2A). The FRR strongly affects the polarity increase throughout the chamber as well as the final solvent concentration. At higher FRR (1:5), the final solvent concentration is reduced, thus reducing the production of larger liposomes due to particle fusion and lipid exchange (Ostwald ripening) after complete mixing is achieved. Previous work using hydrodynamic flow-focusing techniques have also reported the decrease in liposome size with the increase in FRR (Jahn et al., 2010; Zook and Vreeland, 2010), in agreement with results in this study. The zeta potential of the liposomes formed using this method was maintained despite alterations in flow rates and ratios with the liposomes had a positive zeta potential of around 45–60 mV (Fig. 2B). This is in agreement with data previously reported for DOPE:DOTAP prepared by the lipid-hydration method following sonication (McNeil et al., 2010). Furthermore, homogenous suspensions were quickly achieved using the microfluidics method as the polydispersity was around 0.2–0.5 (Fig. 2C); the increase in FRR had the highest impact on resulting PDI.

Overall, vesicle size was shown to be in-process controlled through the aqueous/ethanol flow rate ratio. The TFR was shown to have no significant effect on the liposome size, zeta potential and polydispersity indicating the potential of the microfluidics system to work at higher volumetric flow rates and higher production outputs, which represents a key advantage of the microfluidics-based manufacturing of liposomes.

3.2. Lipid content quantification by ELSD

To investigate the lipid recovery of formulations manufactured at different TFR and FRR in the NanoAssemblr™, we quantified the lipids in the liposome formulations. Lipid composition is usually quantified via high performance liquid chromatography after extraction of the lipids in an organic phase. Here, we used an evaporative light scattering detector (ELSD); a mass analyzer that allows for quantification of lipids based on light scattering. We quantified the lipid content (DOPE and DOTAP) in each formulation separately and related to it the initial lipid amount present in the solvent stock. The liposome formulations were prepared in the NanoAssemblr™ at flow rates from 0.5 mL/min to 2 mL/min and FRR of 1:1, 1:3 and 1:5 (solvent: aqueous ratio). Lipid recovery was above 87% for all formulations, with no significant differences ($p > 0.05$) within all experiments (Fig. 3). This suggests that lipid content remains independent of flow rates and flow ratios in the NanoAssemblr™ and confirms the suitability of the microfluidics method for producing small liposomes with high lipid recovery.

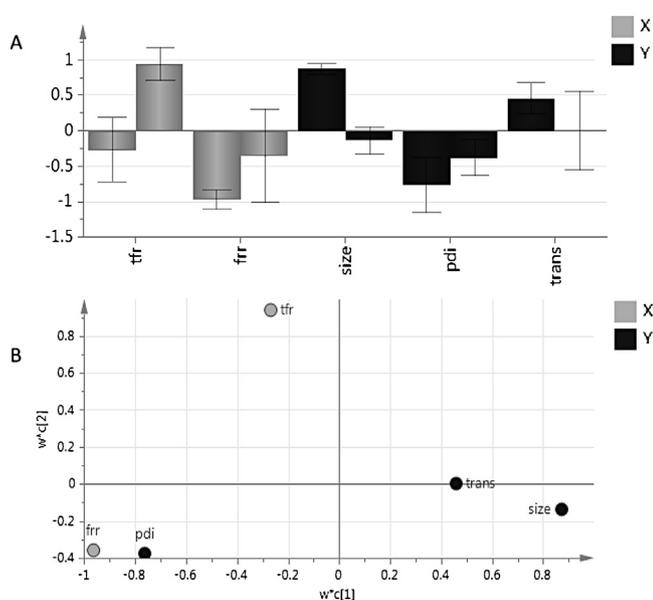


Fig. 6. Results from the PLS regression analysis colored according to model term. (A) Coefficient plot including 95% confidence interval for the two principal components. (B) The loading scatter plot indicating significance of the factors (X) and responses (Y) to each other.

3.3. Biological activity of liposomal systems – In vitro transfection efficiency

To consider the efficacy of the liposome systems prepared using microfluidics, their ability as transfection agents was tested using a standard *in vitro* assay. The commercially available Lipofectin™ was used as a control since it has been extensively used to transfect a wide variety of cells (Fortunati et al., 1996; Malone et al., 1989) and a plasmid containing the luciferase gene (gWiz™ Luciferase) was used. The transfection efficiency of each formulation was determined by measuring the percentage of luciferase activity in each sample to the control (Lipofectin™) reported as luciferase activity (%) (Fig. 4A). Whilst in general the liposomes prepared at a solvent/aqueous flow rate of 1:3 gave the highest transfection rate, changes in the total flow rate did not significantly influence the liposomes transfection activity again demonstrating this method of liposome production is applicable for high-throughput production of liposomes (Fig. 4A). The size, charge and lipid/NDA ration have previously been shown to effect transfection efficiency (Aljaberi et al., 2007; Caracciolo et al., 2007). Given that the lipids/DNA ratio, as well as the cationic zeta potential has been constant in each lipoplex formulation, the resulting difference in transfection efficacy may be due to differences in liposome sizes (Fig. 2A) as previously investigated (McNeil et al., 2010; Esposito et al., 2006; Felgner et al., 1987; Kawaura et al., 1998).

The potential toxicity of these formulations was tested to verify that transfection efficacy was independent of cell viability and toxicity. Overall, cell viabilities remained above 60% for all experiments performed with no significant ($p > 0.05$) difference between the formulations (Fig. 4B). Neither the flow rates nor the flow ratios were shown to affect the cell viability. Any gene delivery vector should ideally be of low toxicity, and should additionally be easy to manufacture in a robust and reproducible process (Lui and Huang, 2003). Here, the microfluidics process was shown to fulfil those requirements.

3.4. Statistical significance of the factors flow rate ratio and total flow rate–design of experiment studies

Given that the liposomes prepared by microfluidics were shown to be effective gene delivery vehicles and that the process parameters adopted were shown to impact on their efficacy, the statistical significant effect of the factors TFR and FRR on liposome size, polydispersity and transfection efficiency (luciferase activity) were further investigated in a response surface modeling in a DoE study. Here, a quadratic interaction model investigated the factors TFR and flow rate ratio FRR as well as the interaction terms TFR*TFR, FRR*FRR and TFR*FRR.

The significant model terms determined in the model are shown in Table 1. The significant factors in the size model (FRR, TFR, FRR*FRR) suggested that both factors together control the liposome size manufactured with the NanoAssemblr™. The significant interaction term of FRR*FRR suggests the importance of the solvent/aqueous ratios to the overall liposome size, emphasizing the FRR to be of high importance when controlling the liposome size in a microfluidics method. The response surface plots (Fig. 5) shows the combinatorial effect of alterations in FRR and TFR in the NanoAssemblr™ process to the liposome size, polydispersity and transfection efficacy. The model predicted minimal vesicle sizes of 60 nm for high flow rates (2 mL/min) and at high flow rate ratios (1:5). This underlies the theory of liposome formation by microfluidic mixing in the NanoAssemblr™. The increase in aqueous phase (flow and volume) increases the amount of polar phase available and thus enhances the rate of polarity increase, shown by the significant interaction term FRR*FRR (Table 1). This affects the nanoprecipitation reaction, as smaller vesicles should be generated with a higher amount of polar phase available, emphasizing the theory of nanoprecipitation reaction and liposome formation in the microfluidic mixing method. In the ANOVA analysis (Table 2) we could identify the statistical significance of the models generated, where all three models (size, polydispersity and transfection efficacy) generated were determined as statistical significant.

The predictions for the PDI model identified the coefficient FRR as the only significant model term (Table 2). The mathematical model confirmed statistical significance for the factor FRR as the only impact to the liposome PDI. Low PDIs were predicted for low FRRs (1:1) (Fig. 5B), the increase in FRR, which lead to an increase in PDI was already observed above (Fig. 2C) and confirmed that the PDI will inevitably increase once the FRR will be increase in the process. The model for the transfection efficiency further confirmed the significance of the factor FRR to resulting luciferase activity. Luciferase activities above 180% were predicted for FRR between 1:2 and 1:4, independent of the TFR used (Fig. 5C). These predictions allow for targeted selection of flow properties in the micromixer depended on desired vesicle characteristics and transfection efficiencies anticipated. These findings further underline the suggestions that the alterations of the TFR mainly lead to an increase in productivity by enhancing the throughput in the method.

3.5. Correlation of factors in the microfluidics process to biological responses and particle characteristics – multivariate data analysis

Multivariate analysis tools are frequently used to find relationships amongst variables (X) and response (Y). Partial least square (PLS) analysis deals with X and Y variables, and is used for regression modeling of X and Y. It can be used to predict Y from X and reveals how the variables and responses are related to each other. Principal components (PC) are fitted through the multidimensional data set in order to generate coordinates of each data

point, which are used to plot the data set onto a plane in a loading plot, which can be subsequently used for data interpretation.

In this study, two PCs were added in the PLS analysis, which were depicted in the loading scatter plot in order to evaluate the effect of factors (TFR and FRR) to the responses (liposome size, PDI and transfection efficacy). The coefficient plot (Fig. 6A) reveals the significance of the factors as well as the responses for the two principal components fitted to the data set. Here, the factor TFR was the only factor significant in the second PC. The factor FRR, as well as the responses transfection efficacy and size were shown to be highly statistical significant in the first principal component (Fig. 6A). The response PDI was significant in both principal components. The loading scatter plot (Fig. 6B) indicated that the TFR was in the upper left quadrant, opposite to the response liposome size. The coefficient plot (Fig. 6A) identified that the factor TFR and the response size were significant in different PCs, which indicates no correlation. Furthermore, the response PDI was the only further response significant in the second PC, which suggests that the factor TFR is independent of liposome size and transfection efficiency. Furthermore, the FRR factor was shown to directly correlate to the liposome polydispersity (Fig. 6B), both highly significant in the first PC, which has been previously seen in the DoE model (Table 1). Thus, the analysis predicts an increase in polydispersity in a liposome formulation once the FRR is increased. The correlation between the responses size and transfection efficiency indicated, as both responses are situated closely adjacent to each other in the loading plot, both significant in the first PC, a direct correlation (Fig. 6B). This indicates that the increase in liposome size results in a higher transfection efficiency, which has been seen in the above DoE model and gives a mathematical proof of previous findings; larger particles correlate with greater level of transfection efficiency than smaller complexes at constant lipid/DNA ratio (McNeil et al., 2010; Esposito et al., 2006; Felgner et al., 1987; Kawaura et al., 1998).

The factor FRR was shown to have the highest impact to the responses, indicated by a very small 95% confidence interval in the coefficient plot (Fig. 6A). As seen in the DoE study, the FRR was shown to be highly significant in the size, PDI and transfection efficiency model. Therefore, we can conclude that FRR needs crucial optimization in a formulation in order to develop a method with not only desired particle characteristics (size and PDI) but also in the case of this formulation the anticipated transfection efficiencies for *in-vitro* gene delivery and application of lipoplexes. Overall, the results indicate that the FRR in the microfluidic process has a strong relevance to the formation of size-controlled vesicles with MVDA studies confirm the significance of FRR in the microfluidics process for the formation of liposomes.

The systematic application of statistical based process control and optimization requires not only fewer experiments to find a local optima, it also it reveals factor interactions and can be used for process simulations. Overall, it will lead to better understanding of a process, which assists in development and scale-up. It is a cost-effective method providing deep understanding in a process (Singh et al., 2005). Gabrielsson et al., 2002 reviewed multivariate methods in pharmaceutical applications, which range from factorial designs to multivariate data analysis and regression analysis, where studies reported improved process and product quality. Where DoE is frequently used to find local optima, PCA and PLS are mainly applied to gain deeper understanding and information about a process and the effect of how factors influence the responses. In this study, we have developed a statistical valid regression model, which allows for prediction of liposome sizes, polydispersity and transfection efficiencies as a function of variables in the microfluidics-based manufacturing method. Furthermore, the application of MVDA allowed for deeper understanding of process settings that will lead to increased

process control with a defined product quality outcome. The combination of multivariate methods and experimental design in any pharmaceutical or biopharmaceutical process development strategies is a powerful tool towards developing new processes and finding optima within a defined region of factors by speeding up a developing process.

4. Conclusion

In this paper, we have used a microfluidics-based liposome manufacturing method and varied the process parameters total flow rate and flow rate ratio to produce liposomes of defined size. Using microfluidics, homogenous liposomes suspensions can be prepared in a high throughput method setup. Liposomes manufactured by this method were shown to give reproducible transfection results in standard transfection protocols. The application of statistical-based methods (design of experiments and multivariate data analysis) revealed the mathematical relationship and significance of the factors total flow rate and flow rate ratio in the microfluidics process to the liposome size, polydispersity and transfection efficacy. We show that the here applied methods and mathematical modeling tools can efficiently be used to model and predict liposome size, polydispersity and transfection efficacy as a function of the variables in the microfluidics method. Furthermore, the advantages of microfluidics as a bottom-up liposome manufacturing method have been shown, anticipating microfluidics and associated lab-on-a-chip applications will become the choice of liposome manufacturing in future. With these studies, we have demonstrated the advantages of incorporating additionally statistical based methods into a development process. Application of statistical based process control and optimization tools like DoE and MVDA will enhance the reproducibility in a process and aid for generation of a design space. This will increase the understanding and confidence in a process setting and allow for predictive and correlative comparisons between the critical process parameters and their effect on desired critical quality attributes, leading to a desired and robust product quality

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpharm.2014.10.030>.

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