

# Reverse Lipofection for Spatially Defined Transfection with DNA/Cationic Lipoplexes

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## ABSTRACT

In this work a new reverse transfection methodology is proposed. It is likely to allow the combination of cell array technologies with the advantages of cationic lipid-based gene delivery. In this new method, lipoplexes are immobilized onto the substrate taking advantage of the ligation avidin-biotin before cell plating.

In a first approach, the immobilization of lipoplexes was tested and some exploratory assays were performed. Secondly and by means of Response Surface Methodologies (RSM) five variables (lipid concentration, DOTAP:DOPE proportion, initial number of cells, pDNA concentration and liposome size) known to influence transfection efficiency were allowed to vary. In this way, not only the effect of each variable was obtained but also the effect of the interaction between variables in the response variable.

A maximum 63.3% transfection efficiency was obtained and DOTAP:DOPE proportion and Lipid Concentration were observed to be the variables that have the most significant effect on transfection efficiency. Although it was concluded that the experimental region under study was far from the ideal region for an optimum transfection, further experiments can be drawn from the conclusions here taken.

**Keywords:** reverse transfection, cationic lipids, lipoplexes immobilization, RSM, lipid concentration, DOTAP:DOPE proportion

## Introduction

Although naked pDNA is able to transfect *in vivo*, packing pDNA with cationic molecules and other chemicals can facilitate the uptake and the transfection both *in vivo* and *in vitro*. These chemicals among others are able to protect DNA inside the cell and prevent its degradation by nucleases and serum components<sup>1</sup>. Moreover, they promote a less negative surface charge and can be tailored with other molecules to promote cell targeting<sup>2</sup>. Therefore, these systems are the most widely studied and are subject of exhaustive investigations to increase its gene delivery efficiency.

One example of such cationic molecules is cationic lipids. The sources of interest on cationic lipids are the fact that they are very simple to use and synthesize, while showing high transfection rates and presenting relatively low toxicity comparing to other systems<sup>3</sup>. A neutral lipid (so-called helper or fusogenic lipid) is also typically included together with cationic lipids in the lipid mixture<sup>4</sup>. DOTAP (1, 2-dioleoyloxy-3-trimethylammonium propane) and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) are examples of cationic lipids and helper lipids, respectively, and the ones used in this work.

Both cationic lipid and helper lipid form a complex with DNA that is capable of efficiently delivering DNA - the lipoplex. This happens because of 3 properties: (1) spontaneous electrostatic interaction between the positively charged

headgroup of liposomes and the negatively charged phosphate groups of DNA, which results in an efficient condensation of the nucleic acids; (2) an overall net positive charge of the complex lipid-DNA that promotes their association with the negatively charged cell surface and (3) the fusogenic properties exhibited by the cationic liposome formulation that can induce fusion and/or destabilization of the plasma membrane and/or endosomal membrane thus facilitating the intracellular release of complexed DNA<sup>5</sup>.

### *Current Methods for Reverse Transfection*

Traditionally in chemical delivery methods, after the complexation of the DNA with the reagent, the solution with DNA complexes is applied to previously grown cells with a confluence between 70-90% to ensure maximum transfection and minimum toxicity effects. In opposition to the traditional methods, in reverse transfection, the DNA-reagent complexes are first immobilized onto a surface where cells are allowed to grow. In this section, a review of current reverse transfection methods is carried out before presenting the proposed reverse lipofection technique.

A reverse transfection method is patented. In this method DNA, cDNA or RNA with known sequences are trapped inside gelatin discs to form spots in a given substrate. Then this gelatin is allowed to dry and a lipid transfection reagent and cells are sequentially added to the spots. Following a period of time for transfection and

protein expression to occur within the cell, experiments can be carried out<sup>6,7</sup>.

Several methods of substrate delivery have been described and are mainly applied for tissue engineering with therapeutic purposes. In such systems, the plasmid is trapped inside a polymeric system allowing one of these two cases: a polymeric release where the DNA is released from the polymer, or a substrate-mediated delivery, where the DNA is just retained on top<sup>2</sup>.

Segura et al (2003) described a method of immobilization of DNA/polymer complexes that allows controlling of the immobilization region and the amount of complex immobilized. PLL and PEI were modified with biotin groups. The resulting complex with DNA is then attached to a neutravidin coated surface<sup>8,9</sup>. Transfection was observed only in the locations where the complex was bound suggesting the possibility of spatially defined DNA delivery<sup>8</sup>.

Regarding the immobilization of liposomes, during the development of a method for reverse transfection of non-adherent cells with pDNA deposited on biocompatible anchor for membrane (BAM)-modified glass slides, Kato et al (2003) suggested that the oleyl group in the BAM is targeted for lipid bilayers and so promotes liposomes immobilization<sup>10</sup>.

## **Aim of the Work and Organization**

### *Aim*

The aim of this thesis project is the development and optimization of a novel reverse lipofection technique.

In this work, we propose a new transfection method. Instead of applying the bulk chemical to the cells, the lipoplexes are previously immobilized onto the surface of the culture substrate due to the ligation avidin-biotin: the biotin is incorporated in the lipoplexes as a biotinylated lipid and avidin is attached to the surface of the culture substrate. The binding of avidin to biotin is specific and about four order of magnitude stronger than typical antigen-antibody ligation<sup>11</sup>. Avidin is able to attach to the substrate and therefore is able to immobilize biotin ligated compounds. Avidin coated surfaces for the immobilization of several biomolecules containing biotin is a well-established tool with different areas of application<sup>12</sup>.

Cells are then seeded on top of the lipoplexes and allowed to grow. The current research for efficient transfection methods is mainly focused on methods to be applied for therapeutic purposes. On other hand this proposed method is likely to

allow the combination of cell array technologies with a simple transfection methodology. Furthermore, the use of immobilized lipoplexes may improve the existing reverse transfection methods in terms of ease and readiness for use.

### *Novelty and Advantages*

Although immobilization of vesicles taking advantage of the affinity between biotin and avidin/neutravidin is not a new approach, it is a novelty when applied to immobilize lipoplexes for reverse gene delivery to cells. This way, one can combine the advantages of lipid-assisted transfection with the advantages of lipoplex immobilization. This immobilization is likely to allow for spatially defined DNA delivery, for example in a patterned surface. In this way, high-throughput analyses of multiple genes can be performed using the same surface and thus reducing the error and variation between samples. On the other hand, even when the same surface is not required and the analysis have to be carried out in well-plates, the uniform coating of avidin and specificity of ligation with biotinylated lipids in the lipoplexes, is expected to allow for more uniform transfection conditions from well to well, thus reducing the error. One advantage of reverse transfection with cationic lipids is that it may have a reduced toxicity when compared to the traditional method and the need for a high confluence is abolished. Furthermore, the concentration of cationic lipids applied is lower and since cells are seeded on top of lipoplexes there might be a continuous release of the DNA into the cells. Also, since cells are all seeded in a single event, they are subjected to the DNA at the same time and likely at the same cell cycle phase. Otherwise, in traditional bolus delivery, cells are seeded and allowed to grown for a given time before exposure to transfection agents, and so cells going through different cell cycle phases coexist in the same sample.

### *Organization*

By means of experimental design using Response Surface Methodologies (RSM), the optimization of the reverse lipofection methodology was performed. Five variables known to influence transfection efficiency were considered: lipid concentration, DOTAP:DOPE proportion, initial number of cells, pDNA concentration and liposome size.

RSM consists of a set of statistical methods that can be used to improve and optimize bioprocesses. It is typically used in situations where several factors influence one or more

desired response variables, in this case the transfection efficiency<sup>13</sup>.

## Materials and Methods

### Plasmid DNA

Plasmid DNA used was pVAX1GFP (3697bp) which contains GFP gene. The vector also contains the human cytomegalovirus immediate early promoter (CMV promoter), bovine growth hormone polyadenylation sequence (BGH PolyA) a kanamycin resistance gene for selection in *E.coli* and a pMB1 origin (pUC derived). Plasmid DNA was replicated in strain DH5 $\alpha$  of *E.coli* in 500 mL overnight culture and purified with a Qiagen Kit according to manufacturer instructions (QIAGEN® HiSpeed Plasmid Maxi Kit). Plasmid DNA concentrations were obtained using Nanovue Plus Spectrophotometer (GE Healthcare).

### HEK Cell Culture

Medium used for HEK cell culture was Dulbecco's Modified Eagle Medium (DMEM) (Gibco ©) supplemented with 10% Fetal Bovine Serum (FBS) heat inactivated (Gibco © Lot 1176955) and 1% PenStrep (Gibco ©) containing 10000 Units/mL of Penicillin and 10000  $\mu$ g/mL Streptomycin. Poly-L-Lysine (PLL) was used to promote cell adherence onto the solid substrate. For this, a 10% (v/v) of PLL 0,1% (Sigma ®) in PBS was used. For cell and substrate washing and dilutions a Phosphate Buffer Solution (PBS) 1x pH 7.4 (Gibco ©) was used. For cell fixation before flow cytometry assays, a 2% PFA (Paraformaldehyde) was used.

Cells were grown in T-flasks of 25cm<sup>2</sup> with vented caps (Falcon BD) in 5 mL culture (37°, 5% CO<sub>2</sub>, humidified environment) until 70-80% confluence. When this confluence was reached, cells were replated. For detachment of the cells 2ml of TrypLE reagent was used (Protease, EDTA and Inorganic Salts, Gibco©) and incubated at 37° for 5 minutes. Then, 3mL of complete medium were added and the suspension centrifuged for 5 minutes at 1500 rpm for cell pelleting. After this, the cell pellet was resuspended in 1mL of complete medium and cells were plated at 4000 cells/cm<sup>2</sup> into a new flask with fresh medium.

### Lipid Vesicles Preparation

The liposomes used in this work were prepared using the cationic lipid 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) and the zwitterionic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). A biotinylated lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) (DOPE-Cap-biotin) was also used in all liposome formulations to promote the immobilization of the lipoplexes onto the substrate (via ligation with avidin). Biotinylated lipid incorporated in lipid mixtures at a ratio of 1 biotin molecule to 1x10<sup>6</sup> lipid molecules (1: 1x10<sup>6</sup>)

When labeling of the liposomes or lipoplexes with fluorescent dye was required, 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-Rho) was used. This molecule was incorporated at lipid mixtures at a ratio of 1 DOPE-Rho molecule for 200 lipid molecules (1:200).

Three different formulations were used in this work regarding DOTAP:DOPE proportions, 1:1, 1:3 and 3:1, and all of them were prepared in chloroform for a final concentration of 1mM in PBS.

To this end, the desired amount of lipid was first measured from lipid stock with glass syringes. Lipid formulations were then dried under N<sub>2</sub> stream and left in vacuum overnight to ensure chloroform exhaustion. This results in a thin layer of lipids in a film that was kept at -20°C until further use.

For preparation of liposomes, the lipid film was suspended in PBS to the final lipid concentration of 1mM. Three cycles of

heat (60°C) and vortex and five freeze-thaw (60°C- liquid N<sub>2</sub>) cycles were performed to homogenize the lipid mixture.

Finally, the lipid vesicles were tailored according to the experiment. To obtain Small Unilamellar Vesicles (SUV's) (+/- 50nm), sonication was performed at room temperature for two minutes. To obtain Large Unilamellar Vesicles (LUV's), an extruder, LiposoFast Basic (Avestin) was used with 100 nm and 400 nm-pore-size polycarbonate membranes. During extrusion, the lipid mixture is forced to pass through a pore of determined size which homogenizes the liposome dimensions to the one of the pore. The lipid solution was extruded 21 times.

### Reverse Transfection Setup Assemble

Assays were performed in 24-well plates

The first step was the coating to promote cell adherence to substrate. For this, 400 $\mu$ L PLL solution was used as previously stated. PLL was allowed to adhere for 1h. After PBS washes, Avidin (from egg white, Sigma ®) 400  $\mu$ L 0,1mg/mL solution (in PBS) was applied in each well and allowed to adhere for 3h. After, the same washing step was performed. Both incubations were carried out at room temperature inside a flow chamber.

For preparation of lipoplexes, three different lipid concentrations (in PBS) were used: 3.6, 7.2 and 14.4 ng/ $\mu$ L. 5 minutes after dilution of the lipid vesicles to the desired concentration, 0.5, 2 or 4  $\mu$ g of plasmid DNA were added to 500  $\mu$ L of lipid solution. This results in pDNA final concentrations of 1, 4 and 8ng/ $\mu$ L. Since DNA has to interact electrostatically with the cationic lipid a 20 minute incubation time was used. Only after this period, lipoplexes were added to the wells for immobilization. This was also performed at room temperature inside the flow chamber for 1h.

During the one hour allowed for lipoplexes immobilization, cells were prepared for plating. Different initial number of cells were used: 17000, 25000 and 34000 cell/cm<sup>2</sup>. Cells were then allowed to grow for 72h at 37°C in humidified environment with 5% CO<sub>2</sub>. Also, for this assays, only cells between passages 5 and 10 were used. Before cell seeding wells were washed with PBS to remove non-adherent lipoplexes.

### Flow Cytometry Assay

After 72 h of reverse transfection, cells were washed carefully with PBS to avoid detachment and then pelleted as described before. After centrifugation, the supernatant was discarded and cell pellet was resuspended in 600  $\mu$ L PBS supplemented with 2% PFA and kept at 4°C until analysis.

The equipment used was a FACScan Scalibur (Becton-Dickinson) that recorded the forward scatter (FSC), side scatter (SSC) and green fluorescence (FL1) in each run. Therefore, for each sample, cells were isolated from the debris due to their characteristics of FSC versus SSC, which defined a gate that distinguished cells from debris that were outside the gate. Background autofluorescence of non-transfected cells was taken into account to determine transfection efficiencies, considering the difference between total cell population inside the gate, and the background autofluorescence of non-transfected cells, indicated by FL1 parameter<sup>14</sup>. This established the M1 and M2 parameters, corresponding to non-transfected and transfected cells with green fluorescence, respectively.

Data was analyzed and green fluorescence intensity corresponding to GFP expression level, histograms and dot plots were generated with CellQuest Pro Software © (Becton Dickinson).

### Data Treatment

Four independent replicates of each 30 set of assays were obtained. For a flow cytometry assay to be statistically significant, a minimum of 1000 events must be measured.

Therefore, assays with less than 1000 events were not considered. For this reason, for some conditions only 2-3 (out of 4) replicates were used.

Statistical analysis of flow-cytometry data was carried out after normalization of transfection efficiencies of each 30 assays to the maximum transfection efficiency obtained within 30 assays set. This accounted for some variation observed in assays performed in different days. After this, mean values and standard deviations for replicates were calculated and introduced in RSM.

#### Surface Response Methodologies for Process Optimization

Aiming at the optimization of the reverse transfection technique, the effect of total lipid concentration, DOTAP:DOPE proportion, cell initial number, pDNA concentration and liposome size in transfection efficiency was assessed using a Central Composite Face Centered (CCF) design with the assistance of STATISTICA software (StatSoft). Furthermore, it was also possible to assess relations between these variables and the effects that each one has on the others.

For the design setup, three different coded levels for each variable were used- low (-1), center (0) and high (+1) (Table 1) according to what was obtained in preliminary assays, with four repetitions at central point. The response variable was the percentage of transfection measured as the fraction of cells efficiently transfected (expressing GFP) within the entire population of cells measured by flow cytometry.

Table 1 Coded and uncoded values for the five factors and levels

Factors	Description	Levels		
		Low (-1)	Center (0)	High (+1)
X <sub>1</sub>	Lipid concentration (ng/μL)	3.7	7.2	14.4
X <sub>2</sub>	DOTAP:DOPE proportion	0.33	1	3
X <sub>3</sub>	Initial cell number (cells/cm <sup>2</sup> )	17000	25000	34000
X <sub>4</sub>	pDNA concentration (ng/μL)	1	4	8
X <sub>5</sub>	Liposome size (nm)	50	100	400

A CCF design allows the estimation of a full quadratic model with the following general description: number of experiments ( $n$ ) =  $2^{k-p} + 2k + cp$  where  $k$  is the factor (variable) number,  $p$  is the fractionalization number and  $cp$  is the center points required for curvature estimation which gives a planned design of 30 experiments ( $30 = 2^{5-1} + 10 + 4$ ) listed in Table 2.

#### Model Building, Fitting and Evaluation

A quadratic model that included linear and quadratic main effects plus two-way interactions was fitted to the data as follows:

$$\begin{aligned} \% \text{ Transfection} = & b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 \\ & + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{55}X_5^2 \\ & + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 \\ & + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 \\ & + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5 \end{aligned}$$

The statistical significance of the full quadratic model predicted was evaluated by the analysis of variance (ANOVA) and least squares technique. Also the significance and the magnitude of the effects estimates on each variable were determined. By means of ANOVA all the factors were tested

to determine which ones had an effect statistically significant in the response variable. Effects with less than 95% of significance, that is, effects with a p value higher than 0.05, were discarded and pooled into the error term (residual error) and a new ANOVA was performed for the reduced model.

The significance of the model can be evaluated by considering either the F-values or the p-values of the model and the lack of fit.

A final step of ANOVA was to perform a Lack of Fit (LOF) test to compare the residual error and the pure error from replication. This is achieved by estimation of the LOF F-value statistic (by the ratio of the mean square of the LOF to the mean square of the pure error) and the corresponding probability (p-value). If, in fact, the residual variability is significantly larger than the pure error variability, then one can conclude that there is still some statistically significant variability left, and hence, there is an overall lack of fit of the current model and another model may be more appropriate.

The regression model was accepted when the p-value of the model was lower than 0.05 and the lack of fit higher than 0.05. However, if any of these conditions was not fulfilled, the model was only accepted when the model correlation coefficient ( $R^2$ ) was higher than 0.90 which means that 90% of the data was explained by the model.

## Results and Discussion

### Experimental Design

Response Surface Methodology (RSM) was used for the optimization of the proposed technique. Each variable was studied at three different levels. In the context of this methodology they are called the independent variables. The transfection efficiency is the response variable.

Five different variables were studied: total lipid concentration, DOTAP:DOPE proportion, liposome size, pDNA concentration and initial number of cells

The first step was to choose an appropriate design within the experimental region under study. The design chosen was a Central Composite Face Centered Design (CCF). It allows for a quantitative estimation of effects and interactions of each variables on the transfection efficiency by measuring the differences on the response variable as the independent variables are changed from low (-1) to high (+1) values. Table 2 represent the design of the assays performed and the results. Results presented are for mean of replicates. Four independent replicates were performed for the 30 assays, and in each set results were normalized to the value of maximum transfection efficiency within each set.

Also represented in Table 2 are the lipoplexes charge ratio (+/-) used in each assay.

### Model Building

Before model building, the first step was to determine the effect estimates for each factor. This represents the improvement in the response

Table 2 Experimental design based in a CCF design and replicates mean for relative transfection efficiency. It is also represented the charge ratio of the lipoplexes used in each assay.

Assay	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	X <sub>4</sub>	X <sub>5</sub>	Transfection Efficiency	Lipoplex Charge Ratio (+/-)
1	-1	-1	-1	-1	1	0.07	0.39
2	-1	-1	-1	1	-1	0.05	0.05
3	-1	-1	1	-1	-1	0.29	0.39
4	-1	-1	1	1	1	0.13	0.05
5	-1	1	-1	-1	-1	0.52	1.25
6	-1	1	-1	1	1	0.11	0.16
7	-1	1	1	-1	1	0.29	1.25
8	-1	1	1	1	-1	0.12	0.16
9	1	-1	-1	-1	-1	0.21	1.55
10	1	-1	-1	1	1	0.18	0.19
11	1	-1	1	-1	1	0.14	1.55
12	1	-1	1	1	-1	0.13	0.19
13	1	1	-1	-1	1	0.72	4.99
14	1	1	-1	1	-1	0.83	0.62
15	1	1	1	-1	-1	0.51	4.99
16	1	1	1	1	1	0.26	0.62
17	-1	0	0	0	0	0.20	0.21
18	1	0	0	0	0	0.48	0.83
19	0	-1	0	0	0	0.18	0.19
20	0	1	0	0	0	0.17	0.62
21	0	0	-1	0	0	0.12	0.42
22	0	0	1	0	0	0.15	0.42
23	0	0	0	-1	0	0.51	1.67
24	0	0	0	1	0	0.24	0.21
25	0	0	0	0	-1	0.25	0.42
26	0	0	0	0	1	0.16	0.42
27 (C)	0	0	0	0	0	0.22	0.42
28 (C)	0	0	0	0	0	0.21	0.42
29 (C)	0	0	0	0	0	0.20	0.42
30 (C)	0	0	0	0	0	0.35	0.42

variable that is to expect as each variable setting is changed from low to high. This can be visualized in the Pareto chart present in Fig. 1. In this chart, the absolute value of magnitude of the standardized effect estimate (i.e. the effect estimate divided by the corresponding standard error) is represented for each factor (linear and quadratic effect) and for the interaction between factors sorted by their absolute sizes. Each factor or interaction is also compared to the 95% confidence minimum for statistical significance (represented by the dashed line that set  $p=0.05$ ). Factors and interactions that were not statistically valid (meaning the ones that not cross the 95% confidence threshold) were removed and pooled into the error term. The exceptions were the linear effects of liposome size and initial number of cells, so all linear effects of each variable would be included in the model to avoid possible inconsistent results during optimization. Furthermore, initial number of cells plays a great effect in interaction with DOTAP: DOPE proportion. Quadratic effects (except for pDNA concentration) and interactions that do not cross the 95% confidence were removed and pooled into the error term.

The response variable was then expressed as a function of the independent variables that were included in the model. The model coefficients were estimated by a least square fitting to the experimental results.

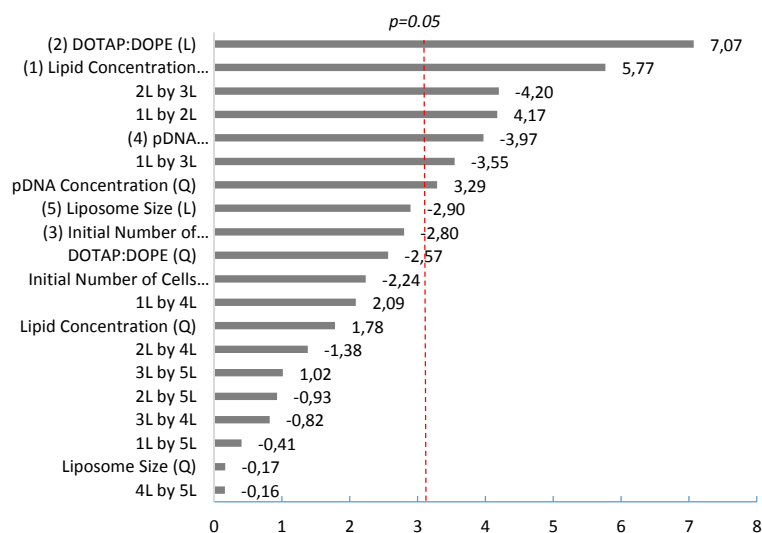


Fig. 1 Pareto Chart of standardized effects estimates obtained for the response variable. Dashed line for a confidence of 95% which corresponds to an F-value of 3.18. Effects that do not cross 95% confidence were removed and pooled into error term (except for the linear effects of liposome size and initial number of cells)

$$\begin{aligned} \% \text{ Transfection} = & -0.083243(\pm 0.135304) \\ & + 0.092405(\pm 0.028239)X_1 \\ & + 0.152121(\pm 0.046035)X_2 \\ & + 0.000017(\pm 0.000005)X_3 \\ & - 0.120291(\pm 0.045121)X_4 \\ & - 0.0003(\pm 0.000092)X_5 \\ & + 0.018214(\pm 0.009618)X_4^2 \\ & + 0.026218(\pm 0.006179)X_1X_2 \\ & - 0.000004(\pm 0.000001)X_1X_3 \\ & - 0.000006(\pm 0.000002)X_2X_3 \end{aligned}$$

### Model Validation

The statistic validation of the reduced model was conducted by means of analysis of variance (ANOVA) as described in Materials and Methods. This allows the comparison between residual error to pure error from replication.

F-values and p-values for the model, error and LOF are summarized in Table 3. By observation of the table, one can conclude that the model is statistically valid (p-value|Model < 0.05, 0.01770) and explains 79% of the observed variance ( $R^2 = 0.7987$ ).

Table 3 ANOVA outcome for the reduced model showing the three main sources of variation including discrimination of the pure error and lack of fit

Source	SS	df	MS	F-value	p-value
Model	0.85	9	0.09	18.43	0.01770
Error	0.21	20	0.02		
Lack-of-Fit	0.19	17	0.01	2.23	0.27882
Pure Error	0.02	3	0.01		
Total	1.06	29			

SS- Sum of Squares; dF- degrees of freedom; MS-Mean Square

In addition, there is no evidence of LOF at the 95% confidence level (p-value|LOF >0.05; 0.27882), meaning that the model is explaining the observed differences in the response variable.

### Optimization

The relationship between variables and response can be easily visualized by means of RSM. Based on the mathematical model previously presented, response surfaces were generated by representing the response variable as a relation of two independent variables.

Liposome size was one of the variables that had a smaller effect on the response variable. It have a negative effect on the response variables, meaning that the lower value for this variable promoted higher transfection efficiencies. For this

reason, in the next analysis here presented, liposome size was set to 50nm.

The initial number of cells also had a limited (not statistically valid) impact on transfection efficiencies. But in opposition to the previous discussed variable, two of its interactions (with the proportion of DOTAP:DOPE and with lipid concentration) were statistically valid. Both interactions have a negative effect in transfection efficiency and both might be explained by cytotoxicity of cationic lipids. In this context, assays where higher proportions of DOTAP were used added with the higher lipid concentrations promote higher levels of toxicity. Still, experiments carried out with a lower fraction of DOTAP and lower lipid concentrations, promoted lower transfection efficiencies, despite lower levels of cationic-lipid related toxicity.

The toxicity of cationic lipids is well-established and was previously referred in Introduction of this work<sup>5</sup>.

Graphs (A), (B) and (C) in Fig. 2 illustrate the previous observation. Liposome size was set to 50 nm and pDNA concentration at 1ng/μL. Lipid concentration was gradually increased.

In this sense, it can be seen that when higher lipid concentrations are used, higher transfection efficiencies were obtained with lower cell numbers. Nevertheless, when lipid concentrations are lowered, a higher cell number is more advantageous. Nevertheless, the first situation promotes the highest transfection efficiency observed.

Furthermore, it is well established that actively dividing cells promote higher cationic liposomes gene delivery<sup>15,16</sup>. Taking this into account it might be expected that a low Initial Number of Cells promote cell division and hence higher gene delivery. Thermo Fisher proposed protocol for Lipofectamine ® 2000 suggests the plating of 25000-62500 cells/cm<sup>2</sup> the day before transfection so an optimum result can be obtained. In the case of this work, plating fewer cells at the time of transfection promoted the best results.

One can go into higher detail in the interaction between the proportion of DOTAP:DOPE and lipid concentration. These isolated variables have the greatest impacts in the response variable and their interaction has the fourth greater effect. Also, pDNA concentration will be discussed along DOTAP:DOPE and lipid concentration. Since these 3 factors together influence lipoplex stability and transfect efficiency, it makes no sense to discuss them separately. Generally the impact of both higher DOTAP:DOPE proportion and lipid

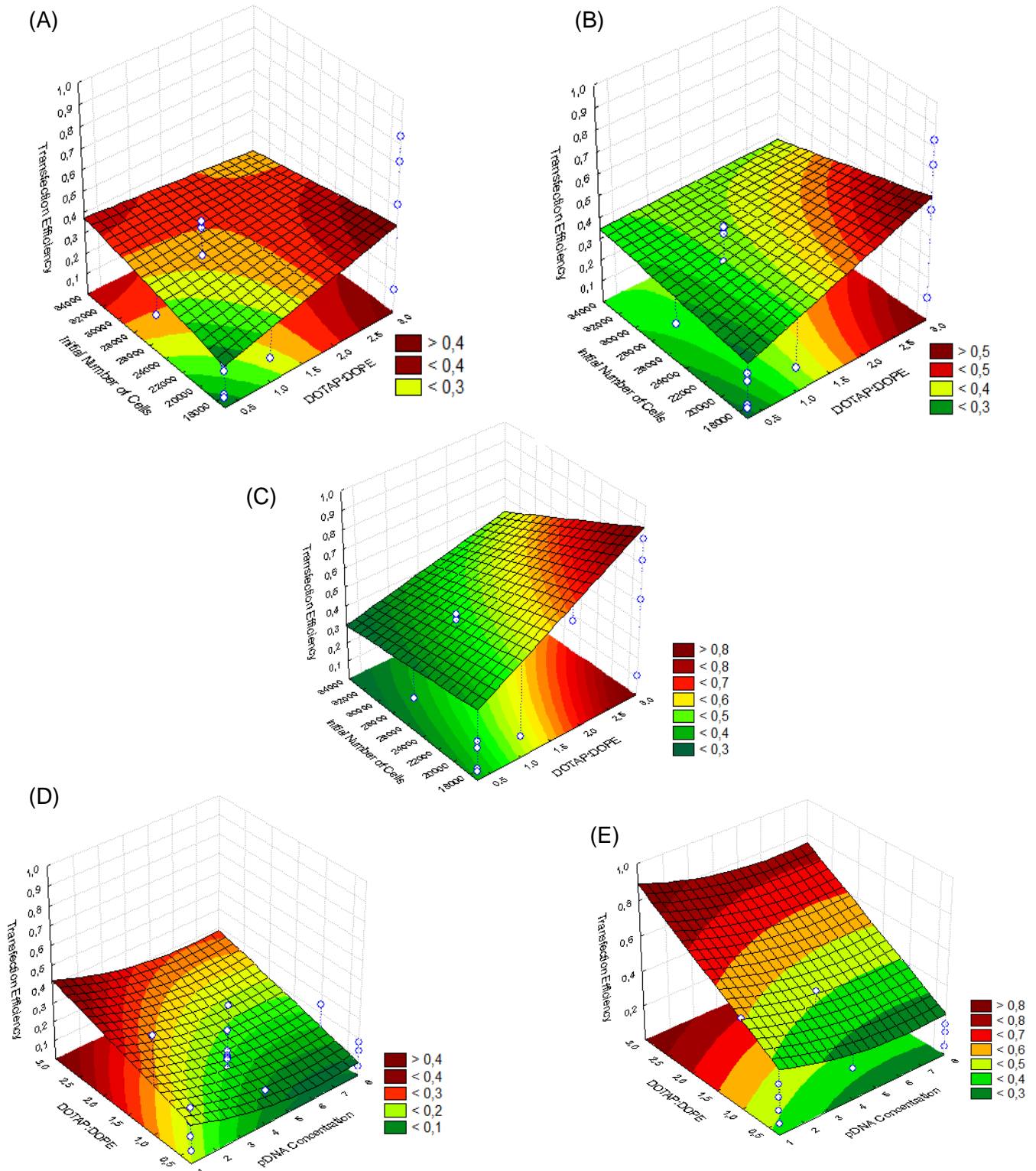


Fig. 2 (A), (B) and (C): Effect of initial number of cells and DOTAP:DOPE proportion on relative transfection efficiencies as lipid concentration is increased: 3.6ng/μL (A), 7.2 ng/μL (B) and 14.4ng/μL (C). pDNA concentration was set to 1ng/μL and liposome size to 50nm. A low initial number of Cells promote higher transfection efficiencies but lipid concentrations plays a major role in this effect, as when lipid concentrations decrease, a higher initial number of cells promote a relative transfection efficiency comparable to the one obtained with a low number of cells. (D) and (E): Effect of DOTAP:DOPE proportion and pDNA concentration on relative transfection efficiency as Lipid concentration is increased from 3.6ng/μL (D) to 14.4ng/μL €. Initial number of cells and liposome size values were set to 17000 cells/cm<sup>2</sup> and 50nm, respectively. When Lipid concentration increases the negative impact of pDNA concentration is mitigated.

concentration is positive, meaning that higher values for both variables promote higher transfection efficiency. Regarding the first variable, this result was expected because in conditions where an excess of zwitterionic lipid is used, complexation with pDNA is impaired, or complexes are actually formed but not stable and pDNA becomes susceptible to degradation. On the other hand, when an excess of cationic lipid is present, it forms more stable complexes with DNA. These observations are easily identifiable in graphs (D) and (E), Fig. 2.

Regarding maximum transfection efficiency, three main observations can be withdrawn from these graphs: (1) higher transfection efficiencies were observed at the highest lipid concentrations and DOTAP:DOPE proportions used, (2) higher pDNA concentration lead to a decrease in transfection efficiencies (3) In the presence of higher lipid concentrations the impact of the other variables is mitigated. The last observation is particularly clear in Fig. 2 (E) where higher pDNA concentrations led to a transfection efficiency comparable to the one promoted by lower concentrations. This is likely to occur because an optimum range (for transfection) of lipid/DNA ratio exists, and at high lipid concentrations this ratio is always within this range, unlike what is observed in the presence of lower lipid concentrations, where the lipoplexes are saturated with DNA, decreasing their efficiency.

#### *Further Considerations*

Much of the behavior here discussed might be explained by the charge ratios (+/-) of the lipoplexes used. A neutral charge ratio (+/-) is typically avoided because it results in the formation of large aggregates (>1 $\mu$ m). Lipoplexes prepared at positive and negative charge ratios likely represent structures with different lipid-DNA and DNA packaging<sup>17</sup>. Additionally charge ratios have a major impact in lipoplex size as reviewed by Zhang et al (2012)<sup>18</sup>.

Madeira et al. (2007), observed by gel retardation assays that at a given lipid concentration, for lipoplexes without DOPE at charge ratios (+/-) of 4 and 6 there were still free pDNA molecules, but by including DOPE in the lipid mixture, the DNA became fully protected at charge ratios (+/-) down to 2. For lipoplexes (with both DOTAP and DOPE) with charge ratios ( $\pm$ )  $\geq 4$ , the pDNA complexation efficiency was 100%. For lower charge ratios (+/-), DNA complexation efficiency decreased to 94 and 30% for charge ratios ( $\pm$ ) of 4 and 0.5, respectively.

As can be seen in Table 2 lipoplexes charge ratios (+/-) used in this work vary from 0.05 to 4.99 meaning that the majority of the lipoplexes used are far from the ideal 100% complexation efficiency. In fact, for example assay 13 present a charge ratio (+/-) above 4: lipid concentration= 14.4ng/ $\mu$ L; pDNA concentration=1ng/ $\mu$ L and DOTAP:DOPE proportion of 3:1. Although some of the highest transfection efficiencies were observed for this sample, the lipoplex charge ratios were not the most important factor in dictating transfection success, since the highest relative transfection efficiency (0.83) was observed for assay 14, where the lipoplex charge ratio (+/-) was 0.62. Assays 13 and 14 had in common the same lipid concentration (14.4ng/ $\mu$ L) and lipid composition, and were obtained for the same initial concentration of cells (17000 cells/cm<sup>2</sup>), while the DNA concentration differences accounts for the different charge ratios. This suggests that lipid concentration is a much more important predictor of transfection efficiency than the charge ratio. However, it is unclear if this is due solely to an increase in lipoplex numbers, or to the importance of the total lipid concentration (independently of DNA concentration) in defining the extent of DNA complexation, or alternatively if it is due to more sophisticated mechanisms occurring during intracellular lipoplex trafficking.

Although charge ratios are clearly not the crucial parameter in defining transfection efficiency, they do seem to correlate with transfection efficiency to some extent. Since the majority of the lipoplexes used had a charge ratio (+/-)  $\leq 1$  it is possible that the optimization presented here did not fully cover the expected variable space of maximized transfection efficiencies.

#### **Concluding Remarks and Future Work**

In this work, a new reverse lipofection assay was proposed and developed. The novelty of this methodology is the combination of cationic lipid-mediated gene delivery with intact lipoplexes and a reverse transfection approach, which could potentially offer the combined advantages of both technologies, namely high transfection efficiencies, the possibility of spatially defined transfection, as well as increased success in transfection of hard to transfect cell lines (as previously observed in other reverse transfection approaches).

Here, we focused on the optimization of transfection efficiencies. As a new reverse lipofection method and regarding transfection efficiency, expectations were exceeded, since



63.3% transfection was achieved in some conditions. But some work is still required to guarantee reproducibility of the results. RSM assays showed that higher transfection efficiencies are expected for higher lipid concentrations at the highest DOTAP:DOPE proportion tested, (3:1). Liposome size had limited impact and a lower Initial Number of Cells promoted higher transfection efficiencies. Higher pDNA concentrations limited transfection efficiencies at low lipid concentrations, but only had a small effect in the presence of higher lipid content.

RSM proved to be a crucial tool in this work. As an optimization tool it was possible to estimate the effect of each variable in the response variable and above all it was possible to understand the relationship between independent variables that wouldn't be possible with conventional one-variable-at-a-time optimization. Ultimately it was possible to perceive that the experimental region under study might be outside the optimum region in what concerns the transfection efficiency. This is particularly evident for the relation between lipid concentration and pDNA concentration (translated in charge ratio (+/-), as mostly low charge ratios were explored. In this sense, future optimization work must consider the change of the experimental region under study in a way where higher lipoplex charge ratios (+/-) are considered. However, is highly likely that increasing lipid concentration will compromise cell viability to some extent. In this context, cytotoxicity assays are needed to prove that an increase in lipid concentration would not be excessively toxic for the cells. Also, cytotoxicity assays are needed for a better understanding of the impact of DOTAP:DOPE proportion on cell viability, as well as on the mitigation of toxicity by the presence of a higher initial number of cells. Finally, once the reverse lipofection method itself is optimized, one can go further into spatially defined transfection. Since the avidin-biotin system has proven to efficiently immobilize lipoplexes, it is possible to create a surface differentially coated with avidin that spatially immobilizes lipoplexes by means of, for instance, photolithography. Such a system may also be very useful in microfluidic devices and in cell array technologies.

## References

1. Escoffre, J. M., Teissié, J. & Rols, M. P. Gene transfer: How can the biological barriers be overcome? *Journal of Membrane Biology* **236**, 61–74 (2010).
2. Pannier, A. K., Shea, L. D. Controlled release systems for DNA delivery. *Molecular Therapy* **10**, 19–26 (2004).
3. Rao, N. M., Gopal, V. Cell biological and biophysical aspects of lipid-mediated gene delivery. *Biosciences Reports* **26**, 301–324 (2006).
4. Tros de Ilarduya, C., Sun, Y., Düzgüneş, N. Gene delivery by lipoplexes and polyplexes. *European Journal of Pharmaceutical Sciences* **40**, 159–170 (2010).
5. Lima, M. C. P. De, Simões, S., Pires, P., Faneca, H., Düzgüneş, N. Cationic lipid – DNA complexes in gene delivery : from biophysics to biological applications. *Advanced Drug Delivery Reviews* **47**, 277–294 (2001).
6. Sabatini, D. M. Reverse Transfection Method. International Publication Number WO 01/200015, filed 18 September 2000, published 22 March 2001 (2001).
7. Ziauddin, J., Sabatini, D. M. Microarrays of cells expressing defined cDNAs. *Nature* **411**, 107–110 (2001).
8. Segura, T., Volk, M. J., Shea, L. D. Substrate-mediated DNA delivery: role of the cationic polymer structure and extent of modification. *Journal of Controlled Release* **93**, 69–84 (2003).
9. Segura, T., Shea, L. D. Surface-Tethered DNA Complexes for Enhanced Gene Delivery. *Bioconjugate Chemistry* **13**, 621–629 (2002)
10. Kato, K., Umezawa, K., Funeriu, D. P., Miyake, M. Immobilized culture of nonadherent cells on an oleyl poly (ethylene glycol) ether-modified surface. *BioTechniques* **35**, 1–6 (2003).
11. Birkert, O., Haake, H.-M., Schütz, A., Mack, J., Brecht, A., Jung, G., Gauglitz, G. A streptavidin surface on planar glass substrates for the detection of biomolecular interaction. *Analytical Biochemistry* **282**, 200–208 (2000).
12. Yeo, D. S. Y., Panicker, R. C., Tan, L.-P., Yao, S. Q. Strategies for immobilization of biomolecules in a microarray. *Combinatorial Chemistry and High Throughput Screening* **7**, 213–21 (2004).
13. Azevedo, A. M., Rosa, P.A., Ferreira, I.F., de Vries, J., Aires-Barros, M.R. Downstream processing of human antibodies integrating an extraction capture step and cation exchange chromatography. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* **877**, 50–58 (2009)
14. Carapuça, E., Azzoni, A. R., Prazeres, D. M. F., Monteiro, G. A., Mergulhão, F. J. M. Time-course determination of plasmid content in eukaryotic and prokaryotic cells using Real-Time PCR. *Molecular Biotechnology* **37**, 120–126 (2007).
15. Mortimer, I., Tam, p., MacLachlan, I., Graham, R.W., Saravolac, E.G., Joshi P.B. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. *Gene Therapy* **6**, 403–411 (1999).

16. Tseng, W. C., Haselton, F. R. Giorgio, T. D. Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. *Biochimica et Biophysica Acta - Gene Structures and Expression* **1445**, 53–64 (1999).
17. Segura, T. Formulations and delivery Limitations of nucleic-acid-based therapies. In Gad, S.C. *Handbook of Pharmaceutical Biotechnology* 1013–1059 (John Wiley & Sons, Inc., 2007).
18. Zhang, X.-X., McIntosh, T. J., Grinstaff, M. W. Functional lipids and lipoplexes for improved gene delivery. *Biochimie* **94**, 42–58 (2012).