Report: 1st and 2 sd year of the FCT Individual PhD Grant, 2019/2022

Celso Joel Oliveira Ferreira

MAP-fis Student

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Report

The importance of lipid-nucleic acid nanoparticles, also known as lipoplexes, for the delivery of genes has been demonstrated with the development of the COVID-19 vaccines from Moderna® and Pfizer®-BioNTech®. Despite this huge leap forward in the technology, these nanoparticles are still not suitable for many applications as in general they tend to present a low transfection efficiency (TE) of the gene[1]. In part this low efficiency can be attributed to the poor understanding of the interactions of lipoplexes with biological barriers, which by itself is also hampered by a low control on the particles size [2]. To circumvent this problem, this project aims to develop a methodology based on microfluidics to produce lipoplexes of controlled size. This will allow to decouple the influence of particle size from other factors, facilitating the rationalization of efficiency, and allowing a smarter design of efficient lipoplexes. Conventional batch-like methods for lipoplex preparation results in polydisperse populations. Conversely, by tuning precisely the conditions of mixing between the nanoparticle components, microfluidic devices can be used to produce particles of tuneable size [3].

This report is a summary of the work developed throughout the last 2 years during my PhD.

During the first year of this project, it was proposed to start task 1, namely the development of a microfluidic methodology to tailor with high precision the size and structure of lipoplexes for gene delivery. Also, the first year corresponds with the beginning of the MAP-fis courses (Table 1) where I was able to provide an oral communication in the Jornadas CF-UM-UP (2019) on the work developed during my master and beginning of the PhD. This was focused on how to control the lipid particles size using hydrodynamic focusing in microfluidic devices

Table 1: Curricular Units (UC) from the MAP-fis doctoral program.

Course	Mark	Observation
Entrepreneurship	17	Completed
Nanomedicine	19	Completed
Biophotonics: sensing and imaging	17	Completed
Advanced Materials Preparation and Characterization	18	Completed
Topics in Advanced Physics II	---	Ongoing
Essay	17	Completed

The first year was concluded when the Essay Curricular Unit, whose evaluation was the delivery and defence of the PhD project in front of a scientific jury of the Doctoral Program, was successfully completed.

For the second year of this project, it was proposed to advance with the development of a microfluidic methodology to produce cationic liposome (CL)-DNA complexes (lipoplexes). And so, the beginning of the year was initiated with the development of the Dean Flow Focusing device, a device whose geometry permitted the formation of Dean vortices that aligned a fluid stream at middle high of a microfluidic channel by focusing it on the vertical direction. The intent was to hydrodynamically focus the DNA stream in 3 directions, avoiding the proximity of the complexation region from the channel walls as this would lead to the precipitation of both lipid and DNA.

With the Dean device, we were able to screen many of the CL-DNA conditions. From it, regions where no precipitation of CL-DNA occurred were detected, and where the collected lipoplexes were complexed into a monodisperse population. However due to the difficulty in using this device, it is hard to obtain reproducibility, and so, we are developing a new device that does not have the same drawbacks. This new device is inspired both on the works developed by Whitesides [4] and Karnik [5].

Size and membrane charge density are two of the keywords of this project, as the size component is being developed with the exploration of other geometries and fluid dynamics using microfluidics, so is the membrane charge density. The use of the Dean device allowed for a better understanding of the conditions for which the membrane charge density could be relevant when assembling these particles. Nonetheless, we proceeded with the first transfection efficiency assays using a well known bulk methodology described by Safinya [6]. From this I acquired the first skill set on cell culture maintenance while also verifying the transfection efficiency of some formulations.

The recent advances in the Fluorescence Cross – Correlation Spectroscopy (FCCS) technique within our group as led to one published article [7] and a few others already under submission. As this technique allows to determine the co-localization in time and space of two or more components, lipoplexes have also been characterised using this technique. As such, for now we have produced lipoplexes with different membrane charge densities following a bulk methodology, these will be later used to transfect different cell lines in order to understand some of the dynamics that the membrane charge density can have on the internalization and release of the nucleic acids (NA). This is an ongoing work that provided me the knowledge in a technique that has a significant impact in my project, not only that but it also enabled me to collaborate with other researchers on the same field.

As part of a previous work developed in the group on the formation of Monoolein cubosome dispersions formed using a microfluidic device. Throughout the last year, Phytantriol was also included and this work is now ready for submission.

The International Iberian Nanotechnology Laboratory (INL) possess a wide range of research areas while promoting collaborations between its researchers. Thanks to it, we were able to collaborate on the now submitted work of Vieira A. by analysing lipid particles with Small Angle X-Ray, a technique that is crucial for our work and that I have been trying to master.

Work Plan

Throughout the work, along with TE measurements, a thorough structural investigation will also be carried out to better rationalize good and bad TE regimes as a function of particle structure (besides size). For the structural characterization, dynamic light scattering (DLS) will be the first and rapid method to evaluate the size of the particles after they have been prepared in the microfluidic device. Small-angle x-ray scattering (SAXS) and cryo-TEM will be used to characterize their internal structure [7]. Fluorescence microscopy and Fluorescence Cross Correlation Spectroscopy (FCCS) will be used to evaluate co-localization of the DNA with lipids.

Task 1. Develop a microfluidic methodology to tailor with high precision the size and structure of lipoplexes for gene therapy

T1.1. Design of a 3D-flow focusing microfluidic device for controlled lipoplex assembly.

While we have already developed through the candidate's thesis suitable microfluidic devices to start the work [8], we intend to dedicate some time to improving the device, namely developing a new 3D-flow focusing device, which is expected to result in even better performance and narrower particle sizes [9]. Since when using a 2D-flow focusing microfluidic device the boundary conditions have a direct influence on the velocity flow profile of the lipid stream, the 3D-flow focusing permits to avoid the contact between the lipid or DNA stream and the channel walls thus reducing this influence [10].

This work will be carried out in INL, by designing new devices and using both the clean room and microfluidics laboratory facilities, to produce SU-8 masters, and PDMS-based chips following the procedure described by Whitesides et al. [11]. This task will be performed in parallel with T1.2.

Contingency: In case we cannot manage to build our own 3D microfluidic devices, other strategies will be applied. Companies such as microfluidic ChipShop sell microfluidic devices made from other materials besides PDMS, 3D printing can also be explored, and fluid manipulation inside 2D devices can also lead to 3D focusing systems. Dean vortices allows to squeeze a fluid stream horizontally between vortices in the outer fluid, this method is usually applied to focus and sort particles but we think it can also be applied to produce liposomes by the same principle as in conventional hydrodynamic focusing [12,13].

T1.2. Manipulation of flow conditions and assembly strategy to achieve lipoplex formation with controlled size.

In this task we will develop the microfluidic-based methodology to produce lipoplexes of controlled size. The most promising self-assembly strategies to be employed are illustrated in Figure 1.

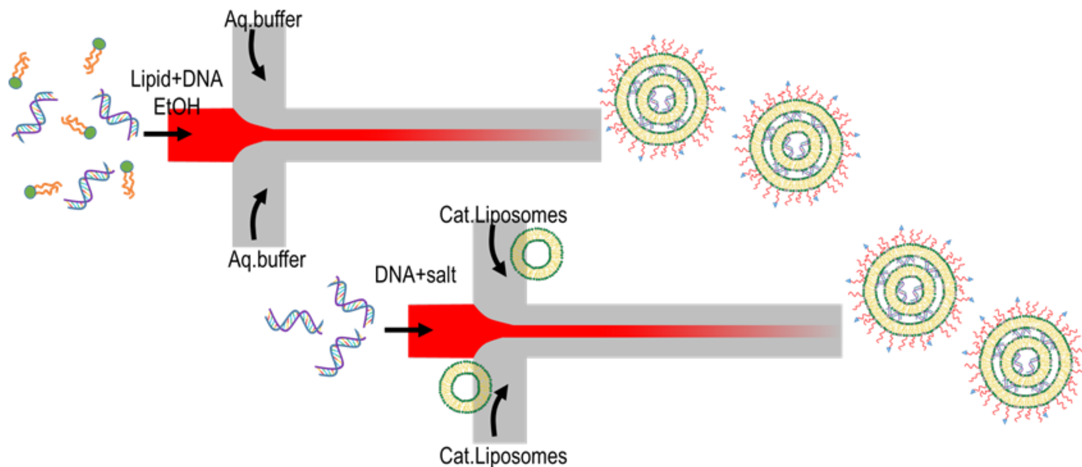

Figure 1: Possible schemes for size-controlled lipoplex assembly. **Top**: both DNA and lipids are dissolved in ethanol and flowed into the microfluidic device through the main inlet. When mixing with buffer flowing from the sides, the ethanol-lipid-DNA is focused and rapid solvent exchange between ethanol and buffer leads to formation of lipoplexes. **Bottom**: a DNA solution is flowed through the central inlet and focused by a liposome solution flowing from the sides. The mixing of the two solutions brings the cationic liposomes and anionic DNA together, leading to the formation of lipoplexes. By manipulation of the flow conditions and salt concentration, lipoplexes of tuneable size can be produced.

One approach will be based on the solvent-exchange method. Both DNA and lipids are dissolved in ethanol and flowed into the microfluidic device through the main inlet. When mixing with buffer flowing from the sides, the ethanol-lipid-DNA is focused and rapid solvent exchange between ethanol and buffer leads to formation of lipoplexes. In the other, we will use pre-assembled liposomes flowing from the sides, and a DNA solution flowing through the central inlet. The mixing of the two solutions brings the cationic liposomes and anionic DNA together, leading to the formation of lipoplexes. In both approaches, the channel dimensions and speed of mixing (controlled by the flow rate ratio between the side fluids and central stream) will be crucial at determining lipoplex size. In case the assembly is too fast to be controlled, we can modulate the assembly partially by adjusting the ionic strength (i.e. by adding salt) before mixing [14].

The task will start using the 2D hydrodynamic-focusing devices [8] that we previously developed. This will be ideal to pre-screen the conditions for controlled assembly. We expect that the 3D-device developed in T1.1 will result in even-narrower size distributions.

The conditions that will be manipulated are: (i) device geometry; (ii) flow conditions, namely the flow-rate ratio; (iii) liposome composition (membrane charge density and inclusion of special lipids); and (iv) amount of salt in the medium.

The standard liposome composition will be DOPC and DOTAP, but monoolein is also expected to be tested.

Contingency: Since the remaining tasks rely on achieving a strategy to assemble lipoplexes with customized size, this is the most critical task. The inclusion of two strategies already accounts for this risk. If none of these strategies succeeds in creating lipoplexes with custom sizes, we will explore other assembly routes.

Task 2. Elucidation of the influence of lipoplex size on the universal transfection efficiency curve

T2.1. Determine transfection efficiency as a function of size and membrane charge density: TE measurements in DMEM.

The aim of this task is first to investigate how the size of lipoplexes influences in a direct manner the universal transfection curve, and in particular, if there is an optimal size where TE is improved. The transfection efficiency assay will be based on luciferase (following transfection of the pGL3 plasmid) and GFP in cultured cell lines. Different cell lines will be tested to look for general trends that do not rely on specific characteristics of particular cell types.

To determine pGL3 TE, cells will be seeded in 24-96 well-plates such that confluency at transfection is 60-80%. Cells are incubated with lipoplexes for 6 h. Then, the transfection medium is removed, cells are rinsed with PBS and then incubated in supplemented DMEM for 18 h. Cells are harvested in Passive Lysis Buffer and subjected to one freeze-thaw cycle. Luciferase expression is measured with a microplate reader. An analogous procedure will be employed for transfection with GFP plasmids, evaluating the results through confocal fluorescence microscopy and flow cytometry.

Contingency: In this task, provided that we have custom-sized particles from T1.2, the risks are low. Determination of TE is a well-established procedure. If the TE is low, we will test the formulations in a different cell-type to assess whether size indeed influences TE or not.

T2.2. Influence of Serum - Determine transfection efficiency as a function of size and membrane charge density: TE measurements in serum.

Although these experiments are in-vitro, prior to cell uptake, nanoparticles will be incubated in bovine fetal serum to better simulate blood circulation conditions from in-vivo and better assess the potential efficacy of these formulations for future in-vivo applications. These experiments will reveal the indirect effect of particle size and charge combined in TE, through their effect on the protein corona structure and composition.

T2.3. Effect of PEGylation - Determine transfection efficiency as a function of size and membrane charge density: TE measurements of acid-labile PEGylated lipoplexes in Serum.

We are currently synthesizing an acid-labile PEG-lipid through the FCT-funded project POCI-01-0145-FEDER-032520. The evaluation of lipoplexes prepared employing these kind of lipids should allow the selection of structures that significantly hinder protein adsorption, but which would still enable endosomal escape by fusion with the endosomal membrane after PEG-detachment.

T2.4. Effect of targeting ligands (RGD) - Determine transfection efficiency as a function of size and membrane charge density: TE measurements of RGD-tagged acid-labile PEGylated lipoplexes in Serum.

We are currently synthesizing an acid-labile PEG-lipid with RGD-tagging through the FCT-funded project POCI-01- 0145-FEDER-032520. The effect of lipoplexes prepared employing these lipids will provide insights into the effect of the previously mentioned physico-chemical nanoparticle characteristics on TE, but employing actively-targeted structures with enhanced uptake by cancer cells overexpressing integrins. Control experiments with cells not-overexpressing such receptors will also be performed.

Contingency: If these specialized acid-labile PEG-lipid-RGD molecules are not able to be synthesized, we will obtain commercial acid-labile PEG lipids without the RGD moiety.

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