Annual report: 3rd year of the FCT Individual PhD Grant, 2021/2022

Celso Joel Oliveira Ferreira

MAP-fis Student

Braga, 4th of September 2022

In this project we intend to produce Cationic Liposome (CL) – Nucleic Acid (NA) nanoparticles, i.e. lipoplexes, in a controlled microfluidic environment, in order to obtain control over their size. The lipoplex size plays an important role both on the pharmacokinetics and on cell uptake. Hence controlling it precisely will open new opportunities in gene therapeutics. One of the main challenges encountered so far, is the fact that, when the liposomes and DNA meet at the microfluidic junction, significant precipitation is observed, with CL-DNA deposits forming on the top and bottom walls of the device. This is problematic to the goal of obtaining particles of controlled size. The problem has been identified as arising from the slower flow near the top and bottom device walls, which leads to deposition of DNA there and subsequent aggregation. Hence, it is important to detach the DNA stream from the top and bottom walls by flow-focusing it in the three spatial dimensions. In this way, when the DNA and liposome streams meet, DNA will be fully engulfed by liposomes, away from the channel walls, leading to controlled nanoparticle formation.

The first approach to achieve this involved the use of a Dean-flow type device, which we designed and produced. However, by the end of the second year of the PhD project, we observed that the Dean vortices in the device were insufficient to form lipoplexes in a controlled manner. Namely, the required reagents volume and incapacity to reproduce the exact same flow conditions demonstrated that it was not possible to continue with this assembly strategy as a viable option. Hence, we moved to a different design that is no longer dependent on Dean Vortices, but instead on the fundamental properties of hydrodynamic flow focusing.

We started by adapting a cross-section device used to hydrodynamically focus the DNA stream in 2D (along the horizontal direction), by adding two extra inlets to focus also the DNA stream in the vertical direction (hence, in three-dimensions). Using this adapted setup, we were able to inhibit aggregation, which confirmed that 3D-focusing is suitable to avoid this issue. However, given the adapted nature of this device, the range of available flow conditions was limited and we were not able to reach flow regimes where we could manipulate the particle's final size. Hence, we designed from scratch a new device implementing 3D focusing, but with the capacity to probe a much wider range of flow conditions. The design stage of this new device is concluded and at the moment it is being fabricated in the INL clean room facilities.

In parallel to the microfluidic activities, the PhD candidate has also embarked in a deeper training of characterization techniques, required to analyse the structure of the CL-DNA lipoplexes that will be produced. Namely, the candidate has been trained, and is now mastering Fluorescence Cross-Correlation Spectroscopy (FCCS). This technique enables a more quantitative analysis on the lipoplex formulations since in addition to a more precise measure of the lipoplex size, it also allows quantifying the number of DNA plasmids and lipid bilayers inside each lipoplex. This is important to optimize formulation development. Moreover, mastering this technique allowed the promotion of new collaborations with other students from both the University of Minho, University of Porto, and other institutions from abroad. The candidate is also being trained in Small-Angle X-ray and Neutron Scattering (SAXS and SANS, respectively), to be able to determine the internal structure of the lipoplexes. Besides the SAXS measurements that are being conducted at INL, the candidate is, at the moment, participating in a summer course on neutron scattering organized by the JCNS Laboratory in Julich and Munich. This is a course fully dedicated to Neutron Scattering techniques that allows its participants to visit and work at the Forschungsreaktor München II (FMR II). By attending this course, the candidate will be much better suited to perform detailed measurements

and deduce the particles' internal structures in the remaining project. In addition, these skills will be important to the candidate's future, since they are in high demand and will promote his unique researcher profile.

Regarding dissemination activities, the PhD candidate participated, in February 2022, in the Spanish Portuguese Local Chapter of the Controlled Release Society (SPLC-CRS) with a poster presentation. At the end of September, the candidate will also join the fifth edition of the Italian Soft Days in Bari, again with a poster participation.

In conclusion, despite the expected challenges involved in obtaining precise microfluidic conditions to allow controlled formation of lipoplexes, the works are proceeding at an encouraging rhythm. The candidate has already achieved lipoplex production in microfluidic devices without precipitation, and the new device is expected to allow the ambitioned size control. Hence, the main PhD project goals are expected to be achieved by the end of the fellowship. In addition, the skills that the candidate is acquiring are also promoting a unique profile that will allow the conclusion of the project in a timely fashion and will make him an important member of the research community in the future. Hence, and overall, we consider that the work developed by the candidate thus far is of high quality and look forward to a successful completion of the main research goals.