Report on the outcomes of a Short-Term Scientific Mission[1]

Action number: CA17103

Grantee name: Laura Taina González

Details of the STSM

Title: **Identifying new delivery systems for nucleic acid therapeutics**

Start and end date: 25/05/2022 to 11/07/2022

Description of the work carried out during the STSM

Description of the activities carried out during the STSM. Any deviations from the initial working plan shall also be described in this section.

(max. 500 words)

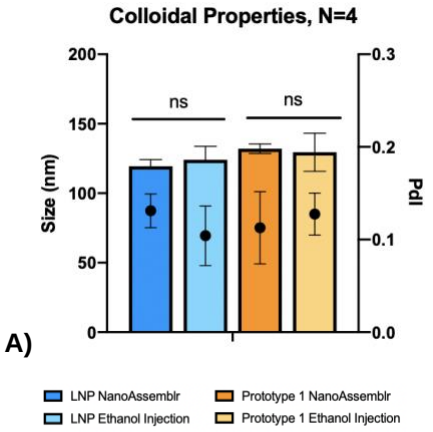
As explained in the application report, one of the main aims of this STSM was to assess how the formulation method impacts Lipid Nanoparticles (LNPs) properties. To do so, the grantee explored two formulation procedures: 1) **Microfluidics mixing**, specifically using NanoAssemblr (NA) IGNITE instrument (Precision Nanosystems), widely known and utilized for LNPs preparation, both in industry and academia laboratories; and 2) **Ethanol Injection** (EI) method, a well-established and characterized methodology used for the preparation of different types of nanoparticles such as nanoemulsions, liposomes and newly, LNPs [1]. To carry out this research, the grantee compared a well-established formulation for mRNA delivery, containing MC3, cholesterol, DSPC and DMG-PEG-2000 formulated with both methods. In addition, the grantee aimed to compare one of the prototypes (Prototype 1) developed in the initial laboratory (Santiago de Compostela, Spain). To achieve these goals, the grantee followed the established protocols at the host institution in University of Copenhagen, including nanoparticles' isolation, mRNA quantification by Ribogreen Assay, toxicity, and transfection studies.

Methods. Nanoparticles formulation. mRNA loaded nanoparticles formulation is based on the mixing between two phases: an ethanol phase, containing the desired lipids at adequate ratios, and an aqueous phase composed of 10 mM citrate buffer at pH 3, containing the mRNA. For the purpose of this research, the mixing of these two phases was performed either by using microfluidics or by direct injection of the ethanol phase to the aqueous phase. For both methods, the total lipid and mRNA content in the final formulation was maintained constant, as well as the ethanol: aqueous ratio, 1:3 v/v. For the microfluiding mixing method, NanoAssemblr IGNITE instrument was used at 10 mL/min flow rate ratio, with a total final formulation volume of 1,24 mL. For the ethanol injection method, 0,3 mL of ethanol phase were injected to 0,9 mL of aqueous phase, obtaining 1,20 mL final formulation. Upon mixing, nanoparticles

[1] This report is submitted by the grantee to the Action MC for approval and for claiming payment of the awarded grant. The Grant Awarding Coordinator coordinates the evaluation of this report on behalf of the Action MC and instructs the GH for payment of the Grant.

were left at RT for 30 to 40 minutes to self-assembly, after which buffer exchange was performed using 10kDA or 30 kDA 4 mL Amicon® Ultracentrifugation Filter Units. For each formulation, 2 washes of a total of 8 mL DPBS 10mM were performed. Upconcentration of the formulations was performed at the same time, leaving final volumes from 0,150 mL to 0,350 mL. **Nanoparticles quantification.** Ribogreen assay was performed according to manufacturer's instructions. Two calibration curves, in both 1X TE and 2%Triton (TX) were performed to assess unencapsulated mRNA (TE) and total mRNA per formulation (TX). Encapsulation efficiency was calculated by subtracting the unencapsulated mRNA to the total mRNA. All formulation and quantification experiments were performed in quadruplicates (N=4).

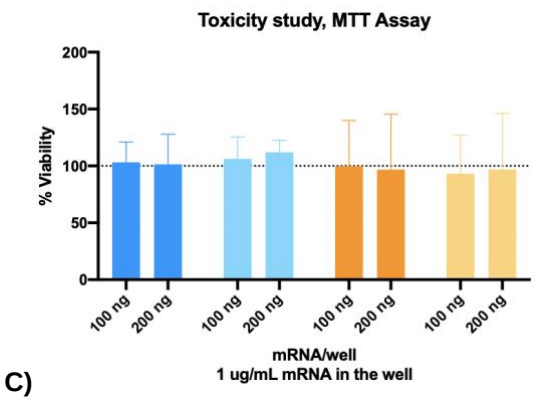
***In vitro* studies:** To assess the *in vitro* performance of nanoparticles, HEK293 human cell line was used. For both toxicity and transfection experiments, 20.000 cells/well were seeded In a P96 well-plate, and the mRNA concentration was maintained constant to 1 ug/mL. MTT assay was performed to assess **toxicity of the nanoparticles** upon 24 h incubation. After this time, nanoparticles were removed and 20 uL of 0,5 mg/mL MTT substrate were added and incubated for 2 h. The absorbance was measured at 630 nm. Cell viability was calculated as a percentage of the untreated cells. **Transfection studies** were performed on a white-flat-bottom P96 well plate. Nanoparticles were added to each well and incubated for 24 h to allow the formation of Firefly Luciferase protein. After this time, 25 uL of One-Glow® substrate were added for lysis and activation of the protein. Results were expressed as a ratio between the mean signal and the untreated cells. All experiments were performed in triplicates (N=3) with three different formulation batches, except for Prototype 1 – NanoAssemblr, which was performed in duplicates (N=2).



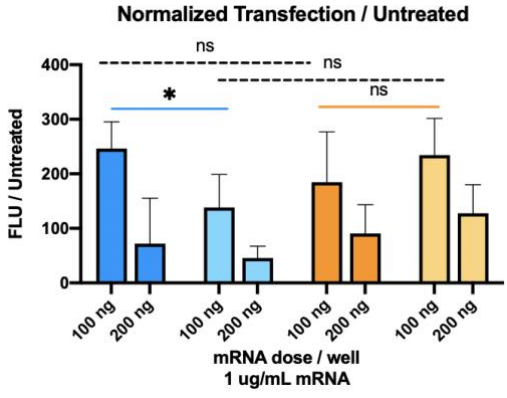
A)

B)

Formulation	Initial Dose	Encapsulation Efficiency (EE%)			
LNPs NanoAssemblr	5 ug	96,1	94,6	95,4	88,1
LNPs Ethanol Injection	5 ug	92,9	96,4	93,6	93,2
Prototype 1 NanoAssemblr	5ug	93,1	95,7	93,3	93,9
Prototype 1 Ethanol Injection	5 ug	94,5	96,7	95,1	93,8



C)



D)

Figure 1. **A)** Size (nm) and Polidispersity Index (PdI) of performed formulations. **B)** Encapsulation Efficiency (EE) in terms of percentage of the dose of mRNA per formulation. **C)** Toxicity study performed on HEK293 cells. D) Transfection activity, expressed as a ratio of fluorescence units and untreated cells. One-Way ANOVA was performed in all cases.

1. Cheng, Q. *et al.* Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR–Cas gene editing. *Nature Nanotechnology* **15**, 313–320 (2020).

Description of the STSM main achievements and planned follow-up activities

Description and assessment of whether the STSM achieved its planned goals and expected outcomes, including specific contribution to Action objective and deliverables, or publications resulting from the STSM. Agreed plans for future follow-up collaborations shall also be described in this section.

(max. 500 words)

The main objective of the Delivery Strategies Working Group is to **create consensus and identify gaps in the current knowledge** on the mechanisms that are involved in **Antisense Oligonucleotides** (**ASOs) delivery** and on the different approaches currently tested to optimize the process. ASOs can be delivered making use of the already developed technology of LNPs for mRNA and siRNA. For this reason, it is important to further investigate LNP technology, and, among it, formulation method.

In this STSM, the grantee has contributed to the investigation of the LNPs formulation process and how it can impact nanoparticles' colloidal properties and *in vitro* performance. A head-to-head comparison between the two formulation methods (*i.e.:* microfluidics mixing and ethanol injection method) was performed, obtaining preliminary results indicating there is no statistically significant difference between both methods when referring to size, PDI, surface charge, encapsulation efficiency, toxicity, and transfection efficiency. These findings indicate that the ethanol injection method performed with the described volumes might be enough to encapsulate the mRNA, and that good size and PDI values can be achieved without the need of the microfluidics instrument. Despite these findings, it is important to mention that more replicates as well as other techniques such as high-resolution microscopy (i.e.: Cryo-TEM) would be needed to further confirm these results as well as to study the morphology of the nanoparticles.