ISIS@MACH ITALIA

Science and Technology Facilities Council
ISIS Neutron and Muon Source

Experiment Proposal

Experiment number GP2024015

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Experiment title	Cryo-TEM characterization of fluorinated lipid nanoparticles
MRF Instrument	**Cryogenic Electron Microscopy**
Access Route	Direct Access
Science Areas	Biology and Bio-materials
Sponsored Grant	None
Grant Title	-
Start Date	-
Similar Submission?	-
Industrial Links	-

Days requested: 3
Previous GP Number: N/A
DOI: -
Sponsor: -
Grant Number: -
Finish Date: -

Non-Technical Abstract

Since the successful use of mRNA vaccines against SARS-CoV-2, nucleic acid (NA) therapeutics have gained renewed and growing interest. The highly charged nature of NAs prevents efficient cell entry. The development of biocompatible vectors for NAs delivery is a top priority. Nanoformulations based on lipids and polymers are the most used and developed nonviral vectors. Only lipid nanoparticles (LNPs) have been used in clinics to deliver RNAs. Even if LNPs have proven their versatility and efficacy, their cellular internalization, intracellular fate, and targeting mechanism are far from being understood and the delivery percentage into the cytosol (1-2%) is still very low. To fill this gap, we will develop fluorinated LNP for NA delivery with improved therapeutic efficacy by a systematic approach, where the investigation of their internal structure as a function of lipid composition in relation to their transfection efficacy in vitro will be the guiding principle.

Publications -

Sample record sheet

Principal contact Professor Emiliano Fratini, CSGI - Università Degli Studi DI Firenze, ITALY
MRF Instrument **Cryogenic Electron Microscopy** **Days Requested:** 3
Special requirements:

SAMPLE

Material	cholesterol, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) DMPE-PEG2000, 1,2-Distearoyl-sn-glycero-3-phosphocholine DSPC (18:0/18:0), D-Lin-MC3-DMA and fluorinated lipid F-LIP, double strand siRNA	-	-
Formula	cholesterol (C27H46O); DMPE-PEG2000 (C125H251N2O55P); DSPC (C44H88NO8P); D-Lin-MC3-DMA (C43H79NO2); F-LIP (C59H86F18N7O6) or (C61H90F18N7O6)	-	-
Forms	Liquid		
Volume	1 cc		
Weight	2 mg		
Container or substrate	eppendorf	-	-
Storage Requirements	The samples have to be stored at 4 °C	-	-

SAMPLE ENVIROMENT

Temperature Range	298 - 310 K	-	-
Pressure Range	- mbar	-	-
Magnetic field range	- T	-	-
Standard equipment	Water Bath	-	-
Special equipment	-	-	-

SAFETY

Prep lab needed	Yes	-	-
Sample Prep Hazards	no	-	-
Special equip. reqs	no	-	-
Sensitivity to air	No	-	-
Sensitivity to vapour	No	-	-
Experiment Hazards	no	-	-
Equipment Hazards	-	-	-
Biological hazards	No	-	-
Radioactive Hazards	no	-	-
Additional Hazards	-	-	-
Additional Details	-	-	-

Sample will be	Removed By User	-	-

Background and Context. Gene alteration is responsible of several complex illnesses like cancer, cardiovascular diseases, and neurological disorders. Therefore, gene therapy, working on altering, modifying or silencing defective or mutated genes, has emerged as a promising therapeutic strategy[1]. Due to low stability and high degradability of nucleic acids (NA), genes need a vector to reach cells and fulfil their therapeutic function. Gene vectors can be divided in two main categories: viral and non-viral vectors. Viral vectors have been extensively used for NA delivery; however, their immunogenicity, mutation risks, and complex preparation processes induced researchers to find non-viral ways to drive genes. The most clinically mature nonviral NA nanovector (NV) is represented by the lipid nanoparticles (LNP) for the treatment of liver pathologies[2] and, as vaccines[3], for immunization against SARS-CoV-2 virus. In particular, the use of cationic ionizable lipids (CIL) with unsaturation in the tail region in LNP formulations promoted the formation of nonlamellar phases facilitating endosomal membrane disruption and NA release. Despite this undeniable success, genetics drugs still have a very low efficacy (1-2%) in delivering NA intracellularly and releasing them into the cytosol, meaning that most of the cargo remains unused. A valuable chemical strategy for enhancing intracellular delivery of NA is fluorination. Fluorination of NV (F-NV) strongly improved their cellular uptake and NA endosomal escape[3]. This incredible effect was related to the physical-chemical features of F-NV such as high hydrophobicity and lipophobicity and intrinsic resistance towards protein binding. Fluorination strategies investigated so far used either -CF_3 groups or long linear perfluoroalkyl chains, but a tailored design of the fluorinated groups could generate more sustainable derivatives, which can also endow the NVs with imaging functions. To pursue the root of biocompatibility, we selected branched fluorinated moiety bearing perfluoro-tert-butoxyl (PFTB) groups to use as tags for developing ionizable fluorinated lipids (F-CIL). In fact, the use of short and branched fluorinated moieties is preferable in terms of biodistribution, bioaccumulation and sustainability to perfluoroalkanes (PFAs) with chain lengths ≥ 6 carbon atoms. Thus, we synthesized two F-CILs bearing as polar head either a primary ammine or a tertiary ammine, but same hydrophobic backbones functionalized with a PTFB group (Figure 1).

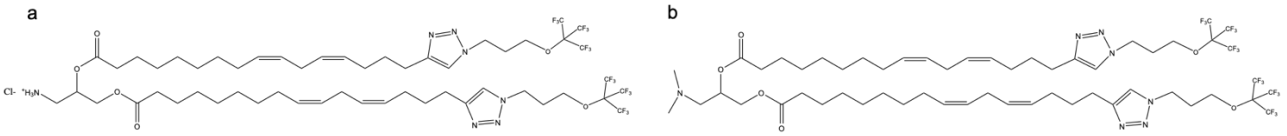

Figure 1. Molecular structure of the synthesized fluorinated ionizable cationic lipids: a) F-CIL1 and b) F-CIL2.

The synthesized lipids are amphiphilic and dispersible in aqueous solutions at different pHs. In particular, we studied F-CILs' self-assembling behavior at 0,4 mM and 1 mM in phosphate buffer at pH=7,5 and acetate buffer at pH=5. Dynamic light scattering experiments showed that F-CIL1 formed colloidal stable self-assemblies at both pHs of hydrodynamic sizes of about 100-200 nm with PDI of 0.2-0.3. On the other hand, FCIL2 formed self-assembled structures evolving overtime and reaching a colloidal stability after about 24 h. These assemblies were characterized by higher hydrodynamic sizes at physiological pH (less ionized with lower surface charge) and higher concentrations. Cryo-TEM analysis of these dispersions will be critical to understand the morphology of these assemblies and will be also supported by SAXS analysis (in-house) which is now in progress.

We have also produced by microfluidics LNPs based on the molar composition typical of the clinical formulations used for mRNA delivery: CIL:DSPC:cholesterol:DMPE-PEG (50:10:38.5:1.5) substituting CIL with F-CIL at different molar percentages (FCIL 10% and 25% mol). These formulations were studied by DLS and fluorinated LNPs showed a similar colloidal stability and slightly higher hydrodynamic sizes than unmodified LNPs (about 100 nm).

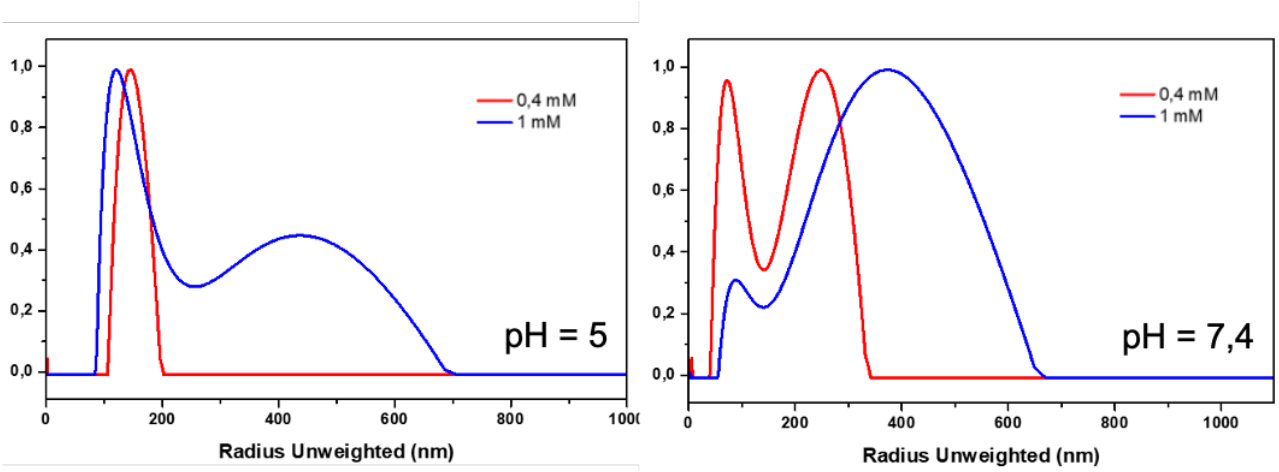

Figure 2. DLS intensity-weighted size distributions obtained by CONTIN analysis for FCIL2 dispersions at 0,4 mM and 1 mM in acetate buffer at pH=5 (left) and phosphate buffer at pH=7.4 (right) at 24 h after preparation.

The internal structure of these LNPs will be studied by SAXS (in-house), but to have information about LNP morphology, Cryo-TEM analysis is mandatory[5]. In fact, TEM analysis is not suitable as lipid-based assemblies are very sensitive to the drying procedure necessary for this kind of measurements. Moreover, we are now preparing siRNA loaded LNPs and F-LNPs, which will be further analyzed by DLS and SAXS and in terms of siRNA loading. This structural analysis will be important for our future transfection studies on *in vitro* cell lines in collaboration with Fondazione Istituto Neurologico Carlo Besta (FINCB) to relate the biological response to the structural features.

References

1. Akinc, A. *et al. Nat Nanotechnol* 14, 1084 (2019)
2. Polack, F. P. *et al. N. Engl. J. Med.* 383, 2603 (2020)
3. Cullis, P. R. *et al.*, *M. J. Mol. Ther.* 25, 1467 (2017)
4. Lv J. *et al. Acc. Chem. Res.* 55, 722 (2022)
5. Arteta, M. Y. *et al. Proc. Natl. Acad. Sci. U. S. A.* 115, E3351 (2018)

Proposed Experiment and justification of instrument time request. Here we propose to analyze by Cryo-TEM F-CIL1 and F-CIL2 dispersions 0,5 mM both in phosphate buffer and acetate buffer, fresh and after 24 h from preparation (8 samples). Moreover, we propose to analyze control unmodified LNPs and F-LNPs with 20% molar percentage of F-CIL1 and F-CIL2, respectively, empty and siRNA loaded (6 samples). Thus, we propose to measure 14 samples and we ask for 3 days.