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# Novel nanostructured lipid carriers dedicated to nucleic acid delivery for RNAi purposes

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## ABSTRACT SUMMARY

The specific down-regulation triggered through interference RNA (RNAi) provides a means to determine the gene functions and their contributions in an altered phenotype. In this way, high throughput screening (HTS) has emerged as a potent automated tool to study a large number of genes for identification of new biomarkers and therapeutic targets. However, the siRNA-mediated gene knock down requires that siRNA can reach cytoplasm compartment where RNAi occurs. Unfortunately, the siRNA is relatively vulnerable in the extracellular environment due to the presence of degradation enzymes and its high molecular weight associated to its anionic charge limit considerably its cell incorporation across the plasma membrane. Thereby, HTS requires generic carriers with highly efficient siRNA transfection. To overcome these obstacles, multifunctional nanoparticles comprising an imaging contrast agent are emerging as an original and promising approach in the improved, controlled and monitored delivery of siRNA.

## INTRODUCTION

Small interfering RNA (siRNA) is double stranded RNA from 19 to 24 base pairs which induces sequence specific down-regulation by triggering the cleavage of a targeted messenger RNA (mRNA) in the cytoplasm of mammalian cells [1]. Since its discovery in 1998 by Fire and Melo, this evolutionarily conserved mechanism, called RNA interference (RNAi), has generated a great deal of interest in understanding and treating dysregulation occurring in pathogenesis due to selective knock-down ability of a gene of interest. Although siRNA is a promising nucleic acid tool in basic and clinical research, several barriers hinder its distribution to its intracellular sites of action. To overcome the delivery obstacles, progress has been made regarding the design of non-viral vectors (inorganics, lipids, polymers, peptides [2]) to enhance efficacy and specificity of the delivery.

Among the wide range of nanocarriers, the resort to cationic nanostructured lipid carrier (cNLC) to form polyelectrolyte complex present an attractive option: (1) they can be designed from already approved human-use surfactants and inexpensive natural ingredients; (2) they can be manufactured by solvent-free up-scalable techniques; (3) their lipid core constitutes an ideal

reservoir for the high loading of hydrophobic molecules (drugs, fluorophores) [3]; (4) Specific targeting by grafting antibodies on the shell is possible.

In our study, proof of concept is performed by formulating nanoemulsion droplets with 1,2-dioleoyl-sn-glycerol-3-trimethylammonium-propane (DOTAP) as a cationic emulsifier to promote complexation with siRNA. The efficient and active transfection of lipid nanocarriers/siRNA complexes is demonstrated.

## EXPERIMENTAL METHODS

Cationic lipid nanoparticles are prepared by emulsion templating through ultrasonication based on a process described by Delmas et al [4] [5]. Both aqueous and lipid phase contain a blend of solid (Suppocire NC<sup>TM</sup>) and liquid (Super refined Soybean oil) glycerides with phospholipids (Lipoid S75-3<sup>TM</sup>) and DOTAP, while the aqueous phase is composed of PEG surfactant (Myrj 52<sup>TM</sup>) dissolved in 154mM NaCl aqueous buffer. 80  $\mu$ L of a 10 mM lipophilic cyanine derivatives solution (DiD) are eventually poured in oil premix to allow dye-labelling of the lipid nanocarrier and solvent is evaporated under vacuum. The amounts of the various constituents were determined according to a Design Of Experiment (DOE) to isolate compositions allowing stable and effective siRNA transfection. After homogenization at high temperature, both phases are crudely mixed. Then, sonication cycles are performed during a 10 min period (VCX750 Ultrasonic processor, 3 mm probe, Sonics, France; sonication power 20%). Non encapsulated components are separated from nanoparticle dispersions by gentle dialysis overnight in 154 mM NaCl against 1,000 times their volume (MWCO: 12,000 Da, ZelluTrans). Before use, nanoparticle dispersions are filtered through 0.22  $\mu$ m cellulosic membrane (Millipore). PC3 prostatic cells are seeded in 96-well plates. After 24 hour incubation at 37°C, different concentrations of lipid nanoparticles, from 1 to 500  $\mu$ g/mL, are added to the culture medium for 24 hours. Cytotoxicity is assessed 24 hours following the nanoparticle removal using the WST-1 assay (soluble formazan derivative reagent). WST-1 reagent (Roche) is added (10%) to the culture medium and kept in the incubator for 3 hours. Cells without nanoparticles and cells incubated with a solution of H<sub>2</sub>O<sub>2</sub> 10 mM are respectively used as negative and positive

controls. Absorbance is then recorded at 450 nm (soluble formazan titration) and 690 nm (background subtraction) using a microplate reader (Tecan).

To study transfection efficiency, PC3 prostatic cells are seeded overnight at 37°C. They are incubated for 1 hour at 37°C in the presence of lipid nanoparticles encapsulating DiD at a concentration of 0.61µM DiD and 125 µg/mL of total lipids complexed with Alexa488-siRNA (20 nM). They are subsequently rinsed with PBS and fixed with paraformaldehyde 1%. Nuclei are labeled with DAPI for fluorescence microscopy or treated by flow cytometry (FACS LSR2, Becton Dickinson, France).

To validate the down-regulation efficacy, PC3 prostatic cells overexpressing Green Fluorescent Protein (GFP) are developed. Chemically synthesized GFP siRNA is used to down-regulate GFP expression. Cells are seeded 24 h prior to experiments. Lipid nanoparticles/siRNA complexes containing typically 0.65 µg (100 nM) siRNA are added to each well and cells are allowed to grow further for 72 h. Lipid nanoemulsion/siRNA complexes formulated in the same conditions but with siRNA which has no homology to any known mammalian gene instead of siGFP are used as negative control. Cells are analyzed by flow cytometry and imaged by videomicroscopy.

## RESULTS AND DISCUSSION

Toxicity induced by cationic lipid nanoemulsions is studied on prostate cells. The studied particles present an IC<sub>50</sub> higher than 500 µg/mL (Fig.1.) which demonstrates a good tolerance. For these cationic nanoparticles, concentration of approximately 10 µg/mL is used in transfection, that is equivalent to cell viability upper 98% (Fig.1.).

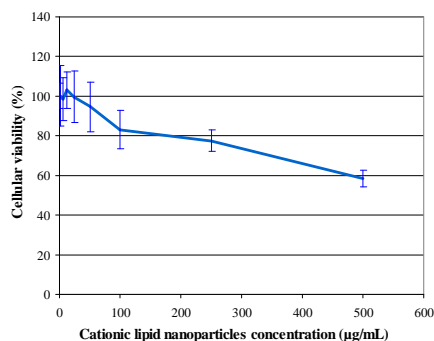


Fig.1.: Toxicity assay with cationic lipid nanoparticles on prostate cells PC3.

To study transfection efficiency, internalization of both DiD-entrapping nanoparticles and Alexa 488-labelled siRNA is visualised by fluorescence and analyzed by flow cytometry (Fig.2.). After only 30', 96.8% of cells are positives for siRNA (FITC) and nanoparticles (APC). After 3h, all cells are positive for the complexes.

Relevant lipid nanoparticles formulations are selected to transfection test with GFP siRNA to down-regulate the

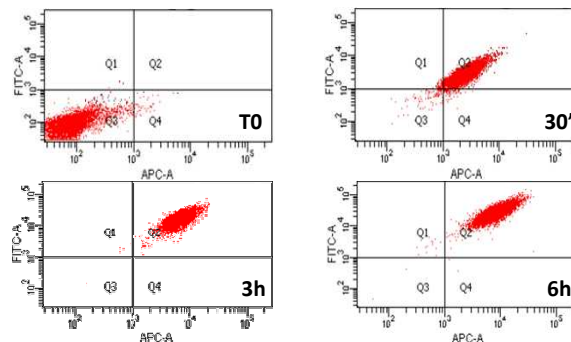


Fig.2.: FACS analysis of PC3 cells before after incubation of complexes cationic lipid nanoparticles/siRNA overtime.

GFP expression. FACS analysis reveal that lipid nanoparticle-based complexes may induce a significant inhibition of the targeted GFP expression (around 75%), demonstrating thus a relatively efficient transfection. This efficiency is visualized with videomicroscopy where GFP expression is down-regulate after 26 hours (Fig.3.).

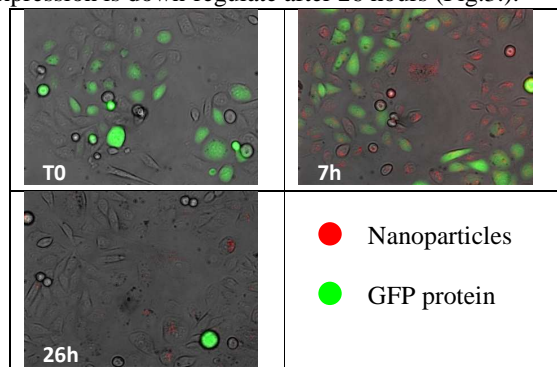


Fig.3.: Epifluorescence imaging of prostatic cells overexpressing GFP protein (T0) after internalization of cationic Lipidot/siRNA complex (7h) and after specific down-regulation of GFP protein by siRNA (26h).

## CONCLUSION

Cationic lipid nanoemulsions developed in our laboratory constitute a promising approach to transfect siRNA and down-regulate gene expression. Actually, cationic nanoparticles present high colloidal stability and transfection efficiency without inducing cytotoxicity. First results have also demonstrated improved transfection on primary cells compared to commercially available lipoplexes. Next steps will include the improvement of transfection efficiency and the surface functionalization of particles with specific ligand in order to target cells hard to transfect using commercially available agents.

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