Cationic lipid nanoemulsions for RNAi screening

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ABSTRACT

Since the discovery of RNA interference, many efforts have been made to broaden perspectives of small interfering RNA (siRNA) in research and clinical work. siRNA shows a specific and effective gene silencing activity through a sequence specific down-regulation of the complementary messenger RNA as well as a tremendous potential as therapeutic agent for treatment of numerous diseases. However, the siRNA-mediated gene knock down requires that siRNA can reach the cytoplasm, where mRNA interference mechanism 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. To overcome these obstacles, multifunctional nanoparticles comprising an imaging contrast agent are emerging as an original and promising approach in the improved and monitored delivery of siRNA.

Keywords: RNA interference, cationic lipid nanoparticles, transfection, siRNA

1 INTRODUCTION

Small interfering RNA (siRNA) is double stranded RNA from 19 to 24 base pair 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 in nanocarriers (viral vectors, inorganics, lipids, polymers and 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] (3) their lipid core constitutes an ideal reservoir for the high loading of hydrophobic molecules (drugs, fluorophores) [4] (4) specific targeting by grafting antibodies on the shell is possible. In our study, proof of concept is performed by formulating nanosized lipid droplets with 1,2-dioleoyl-sn-glycrol-3-trimethylammonium-propane (DOTAP) as a cationic emulsifier to promote electrostatic bonds establishment with anionic nucleic acid, especially siRNA.

2 MATERIALS AND METHODS

2.1 Materials

Suppocire NB™ is purchased from Gattefossé (Saint-Priest, France). Myrj 52™, polyethylene glycol 40 stearate, and Super Refined Soybean Oil are from Croda Uniqema (Chocques, France). DID dyes are purchased from Invitrogen. Lipoid S75-3 (soybean lecithin) is from Lipoid (Germany). DOTAP is purchased from Avanti Polar Lipids (Alabaster, AL, USA). For transfection assay, commercial transfection reagent Lipofectamine RNAimax is from Life Technologies (Carlsbad, CA, USA) and others products are purchased from Qiagen (Hilden, Germany).

2.2 Cationic lipid nanoparticles formulation

Cationic lipid nanoparticles are prepared by emulsion templating through ultrasonication as already described in Delmas et al [3]. Both aqueous and lipid phase contains a blend of solid (Suppocire NC™) and liquid (Super refined Soybean oil) glycerides with phospholipids (Lipoid S75-3™) and DOTAP, while the aqueous phase is composed of PEG surfactant (Myrj 52™) dissolved in 154mM NaCl aqueous buffer. Amount of various constituents was 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 10 min period (VCX750 Ultrasonic processor, 3 mm probe, Sonics, France; sonication power 20%). Non encapsulated components are separated from nanoparticles dispersions by gentle dialysis overnight in 154mM NaCl against 1,000 times their volume (MWCO: 12,000 Da, ZelluTrans).

Before use, Nanoparticles dispersions are filtered through 0.22 µm cellulosic membrane (Millipore). For fluorescence experiments, 80 µL of a 10 mM lipophilic cyanine derivatives solution (DID) are poured in oil premix and solvent is evaporated under vacuum, generating thus fluorescent particles.

2.3 Size and zêta potential measurements

The hydrodynamic diameter and zeta potential of the lipid nanoparticles are measured with a Zeta Sizer Nano (Malvern Instrument, NanoZS, UK) in 0.15mM NaCl using Zeta Sizer Nano cells (Malvern Instrument). Before measurement at 25 °C, samples are left to equilibrate for 2 min. Average hydrodynamic diameter and polydispersity are extracted from cumulant analysis of the autocorrelation function on an intensity basis. Each measurement is performed in triplicate. Stability and integrity of the nanoparticles are studied in biological medium by monitoring hydrodynamic diameter following times from 0 to 24 hours.

2.4 Cytotoxicity assay

PC3 Prostatic cells ($2x10^5$ cells/ mL) are seeded in 96-well plates (Nunc). After 24 hour incubation at 37°C, different concentrations of lipid nanoparticles, from 1 to 500 µg/mL, are added for 24 hours to the culture medium. Each group has sixplicate wells. Cytotoxicity is assessed 24 hours following the nanoparticle removal using the WST-1 assay (soluble formazan derivative reagent), analog to the MTT (3-(4, 5- diMethylThiazol-2-yl)-2, 5-diphenylTetrazolium bromide reagent) assay. 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_2O_2 10 mM are respectively used as negative and positive controls. Absorbance is then recorded at 450 nm (soluble formazan titration) and 690 nm (background substraction) using a microplate reader (Tecan). The absorbance difference (450 nm–690 nm) is directly proportional to the number of viable cells. The percentage cell viability is determined using the following equation: Viability (%) = ((AS–APC)/ (ANC–APC_) × 100; where AS, APC and ANC represent absorbances of the sample, the positive control (cells with H_2O_2 10 mM) and the negative control (only cells) respectively.

2.5 Studying the binding of siRNA to lipid nanoemulsions

siRNA are added to an aqueous solution of lipid nanoemulsions to N/P ratios (w/w) from 1:1 to 16:1. The resulting complexes are diluted in 5mM Hepes buffer up to a final concentration of 25 µg siRNA/mL before subjection to electrophoresis on a 1,5 % agarose gel for 1h30 at 100 V. The bands were observed with transilluminator.

2.6 Cellular uptake assay

3T3 murine fibroblast cells are grown on coverslips overnight at 37°C. They are incubated for 1 hour at 37 °C on the presence of lipid nanoparticles encapsulating DID at a concentration of 0,61µM DID and 125 µg/mL of total lipids. They are subsequently rinsed with PBS and fixed with paraformaldehyde 1%. Nuclei are labeled with DAPI for fluorescence microscopy.

2.7 Transfection

To validate the transfection efficacy, PC3 human prostatic cells overexpressed Green Fluorescent Protein (GFP) are developped. Chemically synthetized GFP siRNA is used to down-regulate GFP expression. Cells are seeded in 12-well plates (Nunc) 24 h prior to experiments at a density of 25.10^3 cells per well in growth medium. Prior to adding the lipid nanoparticles-siRNA complexes, the cells are washed and briefly incubated with 300 µL/well of DMEM 6,8 % FBS at 37 °C and 5 % CO_2. Next, 200 µL lipid nanoparticles-siRNA formulation containing typically 0,65 µg (100 nM) siRNA are added to each well in 12-well plates and cells are allowed to grow for further 72 h. As a blank we use lipid nanoemulsions-siRNA complexes formulated in the same conditions but with siRNA which has no homology to any known mammalian gene instead of siGFP. Cells are analyzed by flow cytometry (FACS LSR2, Becton Dickinson, France).

3 RESULTS

3.1 Cationic lipid nanoparticles formulation

Stragegies to improve electrostatic bonds establishment was illustrated in Fig.1.: DOTAP is added in the shell composition of the lipid particles to bring positives charges. A design of experiment is performed to isolate relevant composition to transfection without altering clloidal stability or trigger toxicity.

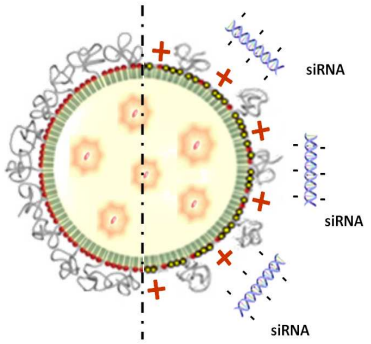

Fig.1.: structure of cationic nanostructured lipid carrier (cNLC) before (left) and afer (right) incorporation of cationic compounds.

Various formulations offer interesting results allowing to have a range of possibility in terms of hydrodynamic diameter and zêta potential. Table 1. illustrates two types of particules (approximatively 50 et 80nm diameter) which are obtained from basic formulations (with negatives charges bewteen -20 and -10 mV) to cationic formulations (with positives charges between 25 and 35 mV).

	Hydrodynamic diameter (nm)	Zêta potential in 0.15 mM NaCl buffer (mV)
Basic particles A	47,03 ± 1,04	-12,34 ±0,97
Cationic particles A	44,85 ± 4,70	26,04 ± 2,67
Basic particles B	84,88 ± 2,06	-18,89 ± 1,68
Cationic particles B	75,44 ± 2,93	31,97 ± 0,36

Table 1.: physical-chemical characterizations of nanostructured lipid carrier before (basic) or after (cationic) modifications to obtain cationic particles.

3.2 Stability and toxicity

Lipid nanoparticles have to be stable several hours in cell culture medium in order to be incorporated by cells.

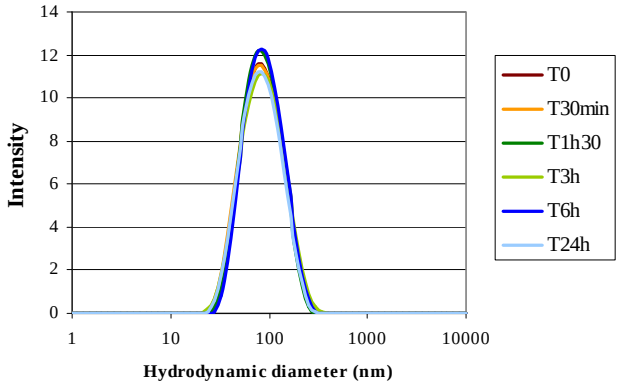

Fig. 2.: Dynamic light scattering (DLS) measurement of particles in cell culture medium following times

According to Fig.2., medium use to transfection does not alter the integrity of the cationic nanostructured lipid carrier, in term of hydrodynamic diameter, during at least 24 hours. Cationic lipid nanoparticles do not undergo coalescence or breakdown and can be used in culture media.

Toxicity induced by cNLC is studied on prostate cells. Lipid particles studied present an IC_{50} upper to 500 µg/mL (Fig.3.) which demonstrates an outstanding tolerance. For these cationic nanoparticles, concentration of approximatively 50 µg/mL is used for transfection purpose, that is equivalent to cell viability upper 95% (Fig.3.).

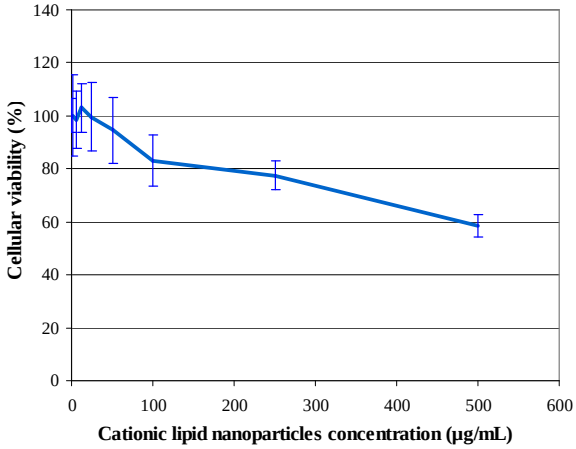

Fig.3.: Toxicity assay with cationic lipid nanoparticles on PC3, human protate cell line.

3.3 Complexation with siRNA

Complexation between cNLC and siRNA is visualized by gel retardation assay (Fig.4.): lipid nanoparticles have an important molecular weight which prevent them from migration. On the contrary, free siRNA have a molecular weight and a negative charge which promote their migration (band visualized on the gel retardation assay Fig.4.). By increasing the amount of cNLC, with a constant siRNA concentration and from the N/P ratio 8/1, all siRNA stay on the top of the gel. That demonstrates an effective complexation with cationic nanoparticles.

Fig.4.: Gel retardation assay showing efficient complexation of siRNA on lipid nanoparticles.

3.4 Incorporation into the cells

To obtain an efficient transfection, cNLC need to reach and interact with cells. A visual indicator is allowed

with DID-entrapping nanoparticles, emitting thus at 667 nm (Fig.5.).

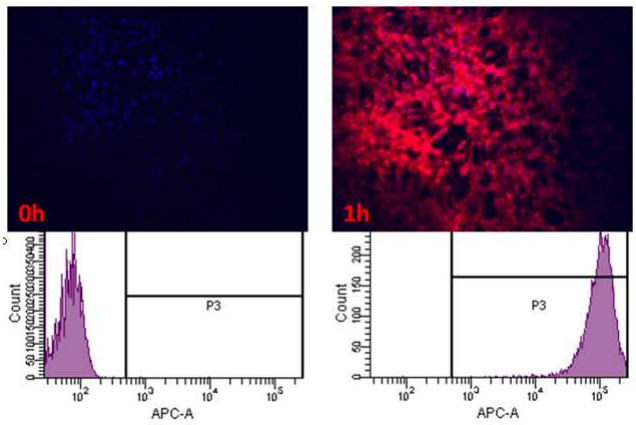

Fig.5.: Fluorescence microscopie of 3T3 murine fibroblasts before (top left) and after 1 hour incubation (top right) and FACS analysis associated (bottom)

After 1 hour incubation, cells show a red fluorescence (670nm) due to lipid nanoparticles indicating a strong interaction. That result is confirmed with FACS analysis: after 1 hour incubation, all the cells are positive for DID expression.

3.5 Transfection efficiency

Relevant lipid nanoparticles formulations are selected to transfection test with GFP siRNA to down-regulate the GFP expression. Cells are analyzed by FACS after 72 hours of transfection and results are compared to results obtained with commercial transfection reagent (Fig.6.).

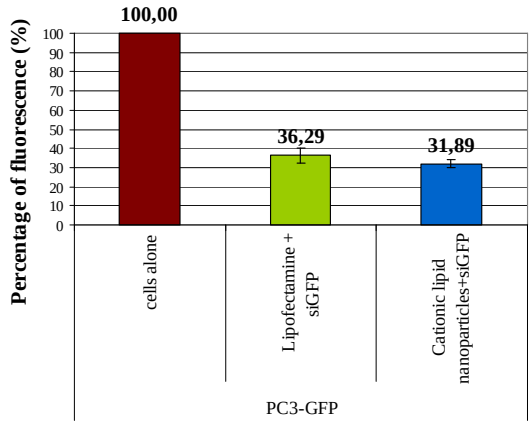

Fig.6.: Transfection efficiency with cationic lipid nanoparticles compared to commercial lipoplex reagent.

FACS analyses reveal that lipid nanoparticles-based complexes may induce a significant inhibition of the targeted GFP expression, demonstrating thus a relatively efficient transfection.

4 CONCLUSION

Cationic nanostructured lipid carriers (cNLC) designed in our laboratory are promising approach to transfect siRNA and down-regulate gene expression. These cationic nanoparticles are very stable, with an outstanding cellular tolerance and allow an effective transfection of siRNA at least comparable to the commercial reagent. However, such lipid nanoparticles offer several key advantages compared to the commercially available lipoplexes, like the monitoring of their cellular incorporation through the encapsulation of lipophilic dye into their lipid core, or again the possibility either to co-deliver another drug into the same carrier, and/ or to specifically reach cellular targets by grafted ligands onto their surface.

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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