Nanoemulsion with tuneable lipid shell dedicated to targeted delivery of small- and biomacro-molecules

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ABSTRACT

Based on oil-in-water template emulsion, lipid nanoemulsion has been developing with monitoring both the lipid-shell and lipid-core composition imparting outstanding long-term stability of nanoparticles. Taking advantage of its colloidal behavior and high tolerability, we modulated the surface droplet to tailor make relevant nanocarriers and also transport various molecules such as large drugs, biomacromolecules. Cationic and anionic nanoparticles were prepared by incorporation of either hydrophobic-tailed polysaccharides or cationic lipids within the shell lipids. Such cationic formulations are suitable for the complexation of nucleic acids such as DNA or RNA. Moreover, large biomacromolecules, such as proteins, peptides or antibodies are chemically grafted on nanoparticle surface via bioconjugation techniques using "home-made" PEGylated surfactants. Also, the fine-tuning of shell of lipid nanoemulsion offers great opportunity to adress new applications in targeted delivery field (e.g. vaccination or large biomolecules transport).

Keywords: Nanoemulsion, targeted delivery, shell decoration, biomacromolecules.

1 INTRODUCTION

Nanoemulsions as delivery systems possess numerous advantages including the possibilities of controlled drug release and drug targeting, and the incorporation of a wide range of therapeutic and contrast agents. These lipid-based particles are composed of commercally available, mostly low cost lipid and biocompatible surfactants; their manufacturing process is versatile and easily up-scalable.

Based on oil-in-water nanoemulsion, lipid nano-particules are developed and dedicated to the encapsulation of lipophilic molecules [1-3]. As nanocarrier, the core/shell architecture relies on amorphous lipid core surrounded by a monolayer of phospholipids and PEGylated surfactants (Fig. 1). The formulation was optimized by design of experiment and these lipid nanoparticles display droplet size tunable from 20 to 200 nm with long-term shelf colloidal stability in aqueous buffer. Modulating Lipidot® core composition allows the internal physical state of the nanoparticles providing crystalline- or amorphous-, viscous- or liquid-core particles [1]. Based on shell/core location of active molecules with various lipid core blends, the encapsulation efficiency and release behavior can be modulated and predicted [2]. Enlarging the delivery functionalities of these nanocarriers is investigated by fine-tuning the lipid shell while keeping constant colloidal integrity of nanoparticles. As illustrated in figure 1, different strategies have been developped for decorating the shell of these lipid nanoemulsions. The present report summarizes the results obtained so far and the future prospects for transporting and then targeting amphiphilic and hydrophilic small- and biomacro-macromelcules.

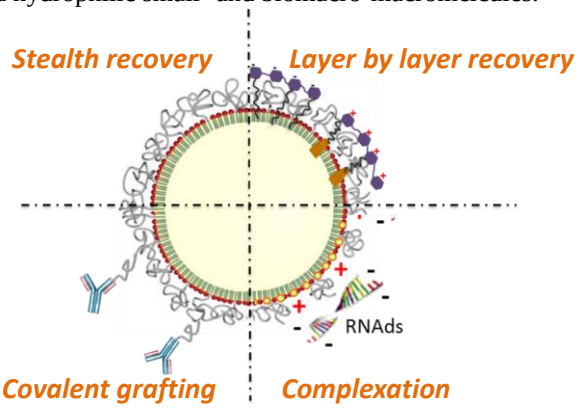

Figure 1: Strategies for lipid shell decoration of nanoparticles

2 RESULTS AND DISCUSSION

2.1 Steatlh recovery of nanoemulsion

The shell of the conventional lipid nanoemulsion developed is composed of phospholipid (lecithin) and PEGylated surfactants, both FDA approved for human-use. The PEG chain moieties of the Myrj surfactant include more than 40 units thereby ensuring stealth properties to the nanoparticles by composition itself. By fluorescence microscopy, we demonstrated a low uptake by macrophages of the doped-dye nanoparticles for 5h incubation time compared to free dyes, diffusing quickly into cell membranes (Fig 2).

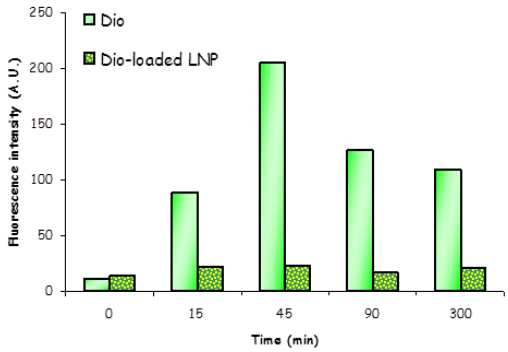

Figure 2: Macrophage Uptake of doped-DiO nanoemulsion

Near-infrared dye-doped nanoparticles are designed as nanotracers and biodistribution studies in healthy mice and dogs, and tumor-bearing mice are monitored using *in vivo* imaging. In both mice and dogs, the biodistribution of the nanotracers was identical (Fig. 3). The nanoparticles are taken up by the liver, organ involved in the lipid metabolism, and cleared by the hepatobiliary pathway (inducing fluorescent signal in the intestine) with a minimal clearance by the renal pathway for dogs (intermediate signal in kidneys). No signal is observed in spleen, lungs and other organs, known for macrophage homing, as well as heart, brain and lungs (analyzed post-mortem).

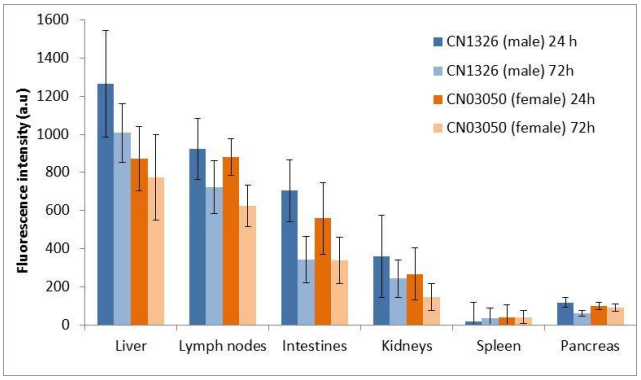

Figure 3: Fluorescence biodistribution in dogs at 24h and 72h post-injection

Dense PEGylated coating of conventional lipid nanoemulsions allows them to impart a good *in vivo* stealthiness and prevent their massive macrophage uptake as already reported for other nanoparticle carriers [6].

2.2 Surface charge modification for complexation of nucleic acids

Cationic vectors have emerged as potential delivery vehicles for nucleic acids such as genes, DNA or RNA. Because of their size and negative charge, these small molecules can not easily reach their intracellular sites of action. To overcome cellular barriers, complexation with cationic nanocarriers is appeared as attractive strategy.

Substitution of PEGylatd surfactants by cationic DOTAP lipids in nanoemulsion formulation allows to obtain cationic nanoparticles. Design of Experiments (DOE) is performed to assess the suitable replacement of cationic lipids while keeping colloidal integrity of cationic nanoparticles. As example of complexation with nucleic acids, siRNA are mixed with cationic nanoparticles in different ratio. Through electrostatic interaction with negative charges of nuclei acids and cationic nanoparticles stable complexes can be formulated. This complexation efficiency is demonstrated by gel retardation assay (Fig. 4). While its high molecular weight hinders cationic lipid nanoparticle to migrate down the gel, free siRNA goes down due to its negative charge. Effective ratio nanoparticle to siRNA is reached from N/P to 8/1.

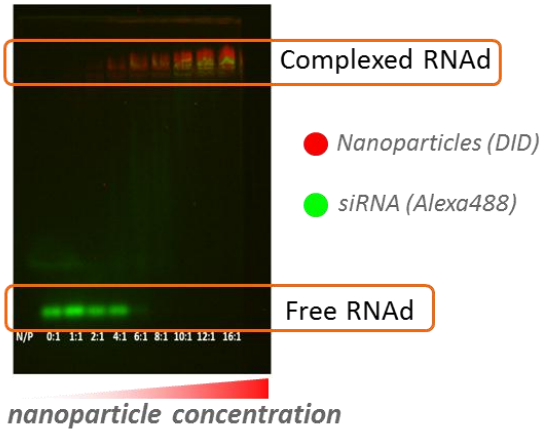

Figure 4: Gel retardation assay showing efficient complexation of siRNA with cationic nanoparticles

These cationic nanostructured lipid carriers are promising aproach to transfect siRNA and down-regulate gene expression.

2.3 Polymer Coating for Layer by Layer encapsulation

Polyelectrolytes and nanoparticles can be utilized to form the ultrathin multilayer structures using the LbL self-assembly technique [7]. The technique is based on the alternating adsorption of oppositely charged components, such as synthetic and natural polyelectrolytes, proteins and nanoparticles, which saturate the available surface. While nanoparticle is used as template, very thin LbL shells (three to four polyelectrolyte layers with a total thickness of 5–10 nm) can be coated on surface and offer a tiny container for drug encapsulation based on electrostatic interlayer bonding and hydrogen bonding [8]. Lipid nanoemulsions were coated with hydrophobic-tailed polysaccharides bearing negative and positive charges thereby forming cationic and anionic nanoparticles. In this context, chitosan and hyaluronic acid polyelectrolytes are chemically modified by alkyl and cholesteryl chain as pending moieties promoting insertion into lipid shell. Their incorporation was studied

using *in situ* insertion and post-insertion methods. Depending on insertion way, the coated-polelectrolytes nanoparticles exhibit different surface charge and particle size properties, evidenced a difference of anchoring patterns. What ever insertion methods applied, colloidal stability of nanoparticles is preserved with larger particle size observed for post-insertion pattern (Fig. 5A).

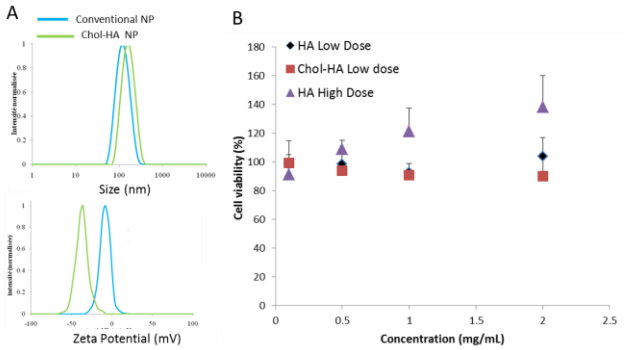

Figure 5: A) Physical characterization (size and zeta potential measurements) of anionic nanoparticles, B) Cytotoxicity assays on 3T3 fibroblast cells.

Cytotoxicity of anionic alkyl and cholesteryl-modified hyaluronic acid polymer is assessed by WST-1 assay using fribrosblasts cells (Fig. 5B). Since no toxicity is observed for polymer concentration up to 2.5 mg/mL (~100µM), a good tolerabilty is expected for polyelectrolyte-coated nanoparticles.

2.4 Grafting ligands by covalent bonding

For better controlling the density and orientation of targeted ligands on surface, chemical binding is more suitable between attached ligands and nanocarriers [9].
The coupling strategy for large biomacromolecules described here is based on useful Thiol/Maleimide conjugation between biomolecules and nanoparticles to form thioether bond, as already applied on polymeric nanoparticles [10] as well as liposomes [11]. This nanoparticle decoration required also prior functionalization steps of both nanocarriers and proteins. For that purpose, "home-made" PEGylated surfactants have been prepared bearing suitable and activated ending groups. Coupling conditions were optimized such as reaction time, addition rate/order and density of proteins grafting over nanodroplet surface, in order to keep the integrity (size, charge, colloidal stability) of these novel nanocargos. As example, the anti-EGF.R antibody Cetuximab is grafted onto nanoparticle surface. Flow cytometry experiments performed in EGF.R overexpressed cell line CAL33 reveal that the Cetuximab-grafted particles are extensively taken up by cells compared to appropriate controls, i.e the ungrafted particles (Caped doped-dye nanoparticles) or non specific antibody grafted particles (Levu-(DiD)-nanoparticles) (Fig. 6). A prior incubation with the free Cetuximab reduces significantly this uptake, confirming an EGF.R-mediated interaction and demonstrating therefore an active targeting of the particles for this receptor.

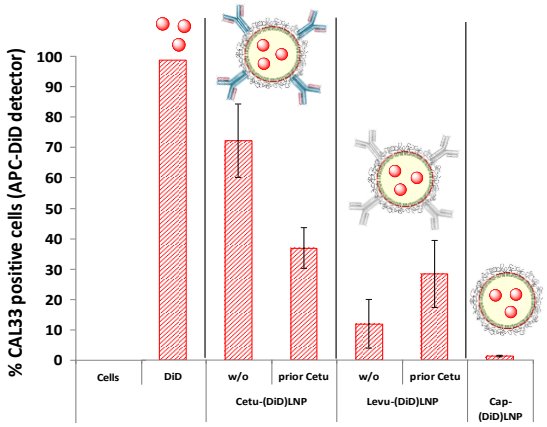

Figure 6: Flow cytometry experiment in EGF.R overexpressed CAL33 cell line showing percentage of positive cells, i.e., having interacted with DiD particles after incubation at 4°C for 30 min.

Several biomacromolecules such as anti-CD44, anti-CXCR4 antibodies, ovalbumin protein, and small peptides and oligosaccharides have been grafted onto surface nanoparticules. Moreover depending on chemical functions available on ligand for coupling, nanoparticles can bear either maleimide or active thiol groups for functionalization. Whatever selected pattern, biological activity of targeted ligands has been maintained, evidenced the suitability of this couling strategy for surface decoration of lipid nanoparticles.

3 CONCLUSION

Different strategies of modulating the lipid shell of nanoemulsion have been illustrated in this work. Decoration of nanoparticle surface requires great knowledge of core/shell architecture of nanoparticules while preserving colloidal stability and outstanding cellular tolerance. These physical and chemical modifications open new opportunities to enlarge the delivery of small and biomacromolecules.

4 EXPERIMENTAL SECTION

4.1 Preparation of lipid nanoparticles

The formulation of conventional nanoemulsion has previously described elsewhere [1-4]. Cholesteryl-tailed hyaluronic acid polyelectrolytes has been synthesized by Michael addition between thiocholesterol on maleimide-actived hyaluronic polymer. Alkylation of chitosan backbone is carried out by reductive amination using sodium cyanoborohydride or non-toxic α-picoline borane as reducing agents. The home-made PEGylated surfactant is prepared by introduction of a protective group as N-Succinimmidyl-3-(2-PyriDylthio)-Propionate (SPDP) is

introduced as thiol-activated entity at the ending of surfactant. Once synthesized, this surfactant, is then easily incorporated at desired amounts into nanoparticles during their formulation. Following the reduction of pyridyldithiopropionate (PDP) group using reducing reagents such as dithiothreitol (DTT), thiol-modified nanoparticles are ready-to-use to couple to maleimide–activated ligands. Thiolated nanoparticles (645 µl, 10 % w/w of dispersed phase) earlier cooled by ice bath were added dropwise to a gently stirred solution of maleimide-activated ligand (340 µg in 1 mL PBS 1X) and mixture reactionc stirred during 2 h up to room temperature. Finally, an end-capping step is carried out with excess of 1-(2-hydroxyethyl)-1H-pyrrole-2,5-dione solution (Mal-EtOH) (18.2 mg/mL in 1X PBS) for 1 h. Ligand-targeted nanoparticles were separated from the ungrafted ligands by size exclusion chromatography (packed-Superdex 200 column). Cationic nanoparticles are obtained by incorporation of DOTAP as surfactant by replacement PEGylated surfactant. The proportion of DOTAP is rationally adjusted to obtain stable cationic nanoparticles with desired particle sizes.

4.2 Cellular uptake assays

J774A-1 cell lines are grown on coverslips overnight at 37°C. They are incubated for 1 hour at 37 °C on the presence of lipid nanoparticles encapsulating DiO /DiD at a concentration of 0,61µM DiO/DiD and 125 µg/mL of total lipids. They are subsequently rinsed with PBS and fixed with paraformaldehyde 1%.

4.3 Cytotoxicity assays

NIH 3T3 fibroblasts 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-diphenyl-Tetrazolium 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.

4.4 Binding Assays

Lipid nanoemulsion and siRNA are mixed together in range of 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. Bands were observed using UV-transilluminator.

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