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Research Paper

Inter-nanocarrier and nanocarrier-to-cell transfer assays demonstrate the risk of an immediate unloading of dye from labeled lipid nanocapsules

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ABSTRACT

Release studies constitute a fundamental part of the nanovector characterization. However, it can be difficult to correctly assess the release of lipophilic compounds from lipid nanocarriers using conventional assays. Previously, we proposed a method including an extraction with oil to measure the loading stability of lipophilic dyes in lipid nanocapsules (LNCs). The method indicated a rapid release of Nile Red from LNCs, while the loading of lipophilic carbocyanine dyes remained stable. This method, although interesting for a rapid screening of the fluorescence labeling stability of nanocarriers, is far from what happens *in vivo*, where lipid acceptor phases are nanostructured. Here, lipophilic dye loading stability has been assessed, by monitoring dye transfer from LNCs toward stable colloidal lipid nanocompartments, *i.e.* non-loaded LNCs, using new methodology based on size exclusion chromatography (SEC) and Förster Resonance Energy Transfer (FRET). Dye transfer between LNCs and THP-1 cells (as model for circulating cells) has also been studied by FACS. The assays reveal an almost instantaneous transfer of Nile Red between LNCs, from LNCs to THP-1 cells, between THP-1 cells, and a reversal transfer from THP-1 cells to LNCs. On the contrary, there was no detectable transfer of the lipophilic carbocyanine dyes. Dye release was also analyzed using dialyses, which only revealed a very slow release of Nile Red from LNCs, demonstrating the weakness of membrane based assays for investigations of the lipophilic compound loading stability in lipid nanocarriers. These results highlight the importance of using relevant release assays, and the potential risk of an immediate unloading of lipophilic fluorescent dyes from lipid nanocarriers, in the presence of a lipid acceptor nanocompartment. Some misinterpretations of cellular trafficking and *in vivo* biodistribution of fluorescent nanoparticles should be avoided.

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1. Introduction

Recent advances in nanomedicine have provided significant improvements in drug delivery and delivery of contrast agents for bio-imaging applications. Lipid vesicles, polymeric nanoparticles, and lipid nanocapsules (LNCs) are examples of nanovectors used in pharmaceutical applications [1,2]. Better selectivity, increased uptake over biological barriers, sustained release, decreased toxic side effects, and reduced chemical and enzymatic degradation of active agents, are some of the reported benefits of nanovectors compared to 'free drugs' [3]. Fluorescent dye-labeled nanocarriers are often used in *in vitro* and *in vivo* mechanistic studies. For example, cellular trafficking [4–6] and *in vivo* biodistribution [7–11] studies have been conducted using fluorescent dye labeled nanocarriers. Advantages of fluorescence techniques include simple labeling procedures, high sensitivity, no specific requirement regarding the handling of the labels, and the use of standardized analytical methods which are available in most laboratories, e.g. fluorescence spectrophotometry, fluorescence microscopy, FACS, and *in vivo* fluorescence imaging.

Dye-labeled nanocarriers are often obtained through non-covalent association between label and carriers. Consequently, one important drawback is the risk of uncontrolled release

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in vitro or during *in vivo* circulation. Before being applied, the stability of the label must be studied to ensure that it does not leak from the carrier. However, it can be difficult to correctly assess the encapsulation stability of lipophilic compounds. The nature of the dispersion medium is a key factor controlling the release. When dispersed in an aqueous phase, lipophilic compounds will obviously remain inside the lipid nanocarriers. Thus, a classical release assay, *e.g.* measuring release against water or a buffer, will not mimic the release in an *in vivo* environment where multiple lipophilic acceptor compartments are present. Therefore, in our previous work, we proposed a new methodology to investigate the labeling stability of LNCs loaded with lipophilic fluorescent dyes [12]. Briefly, dye release from LNCs toward lipophilic acceptor compartments was assessed by vortexing an aqueous suspension of labeled LNCs with an oil, followed by a separation by centrifugation.

The results showed that two dyes, Nile Red (NR) and Coumarin-6 (6-Cou), were readily transferred between LNCs and the oil, while three lipophilic carbocyanine dyes (1,1′-dioctadecyl-3,3,3′,3′-tetra methylindocarbocyanine perchlorate: DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate: DiD and 3,3′-dioctadecyloxacarbocyanine perchlorate: DiO) did not transfer. These two opposite properties could be explained by different localizations of the dyes inside the nanocarriers. For NR, a similar rapid release was demonstrated when LNCs were incubated in serum, using a Förster Resonance Energy Transfer (FRET) assay [13]. Assuming that the vortex with the oil has no direct effect on the stability of the label, the results highlight the possibility of a very rapid release of lipophilic compounds from intact lipid nanocarriers.

However, the nature of the oil acceptor phase in the extraction assay is relatively far from the nature of acceptor compartments present under *in vivo* conditions. Indeed, in systemic circulation, lipid nanoparticles such as lipoproteins can be found, as well as the lipid nanocompartments of cells (phospholipid bilayer of endothelial cells, monocytes, etc.), and not free macroscopic oil phase. New methodologies, in which the acceptor consists of stable colloidal lipophilic nano-compartments, need to be investigated.

The aim of this study was to investigate the loading stability of fluorescent dyes (NR and lipophilic carbocyanine dyes) in LNCs, when the loaded nanoparticle suspension was in contact with non-loaded LNC suspension (as lipid acceptor phase), instead of a single oil phase. We did not primarily want to use a dialysis assay, as it was believed that the membrane could have an influence on the measured release rates, and is far from an *in vivo* situation. Instead, we first studied dye transfer between LNCs of different sizes, using a new method based on a separation by size exclusion chromatography (SEC). In a second step, transfer was investigated by monitoring the FRET signals between encapsulated dyes, for LNCs with identical sizes. Thirdly, for a comparison of methods and to test our assumption regarding the dialysis membrane, we also performed a dialysis assay. In a final step, dye transfer was investigated from LNCs toward THP-1 cells (monocyte cell line). This was already showed with HEI-OC1 cells (auditory cell line) [12] and we wanted to confirm the behavior was not cell dependent, and could occur with model for circulating cells. Dye transfer was also investigated between THP-1 cells, and from THP-1 cells back to non-loaded LNCs, as these events could be observed in an *in vivo* situation.

2. Materials and methods

2.1. Chemicals

Lipoïd® S75-3 (soybean lecithin: 69% of phosphatidylcholine and 10% of phosphatidylethanolamine) and Kolliphor® HS15 (mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were kindly supplied by Lipoïd GmbH (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. Labrafac® WL 1349 (caprylic-capric acid triglycerides) was generously provided by Gattefossé S.A. (Saint-Priest, France). NaCl was purchased from Prolabo (Fontenay-Sous-Bois, France). Disodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from VWR International SAS (Fontenay-Sous-Bois, France). Deionized water was obtained from a Milli-Q plus® system (Millipore, Billerica, MA). Nile Red (NR) and Sepharose CL4b were purchased from Sigma–Aldrich (St. Quentin Fallavier, France). DiI, DiD and DiO were provided by Molecular Probes® (Eugene, OR). 4′-Dioctylamino-3-octyloxyflavone (F888) was synthesized as described [13].

2.2. Lipid nanocapsule formulation

Non-labeled and fluorescent-labeled LNC formulation processes were based on a phase-inversion property, which has been described elsewhere [2,12,14]. Briefly, the quantities of Labrafac (Lab) (oil phase), water, NaCl (aqueous phase), Kolliphor (Kol) and Lipoid (Lip) (surfactants) for each formulation were precisely weighed. For 30 nm LNC (LNC30), $m_{Lab} = 0.846$, $m_{Kol} = 1.934$, $m_{Lip} = 0.075$, $m_{Water} = 2.055$ and $m_{NaCl} = 0.103$ g; for 60 nm LNC (LNC60), $m_{Lab} = 1.028$, $m_{Kol} = 0.846$, $m_{Lip} = 0.075$, $m_{Water} = 2.962$ and $m_{NaCl} = 0.148$ g; for 120 nm LNC (LNC120), $m_{Lab} = 1.209$, $m_{Kol} = 0.484$, $m_{Lip} = 0.075$, $m_{Water} = 3.143$ and $m_{NaCl} = 0.157$ g. The mixtures were heated to 95 °C at a rate of 5 °C min^{-1} under magnetic stirring followed by cooling at the same rate to 50 °C. This cycle was repeated three times and the LNC suspensions were returned to room temperature at the end of the last cycle. The LNC suspensions were only purified using filtration through a 0.45 μm syringe filter before use.

To obtain fluorescent-labeled LNCs, the various dyes were dissolved in Lab prior to the formulation procedure described above. For size-exclusion chromatography, dialysis experiments and *in vitro* cell assays, NR and/or DiO were added at a concentration of 1 mg g^{-1} (weight ratio dye/Lab), to obtain NR-LNC, DiO-LNC and NR/DiO-LNC. For FRET experiments, NR and/or F888 were added at a concentration of 5 mg g^{-1} (weight ratio dye/Lab), to obtain NR-LNC, F888-LNC and NR/F888-LNC. In addition, DiI and/or DiD were added at concentrations of 3.75 and 2.90 mg g^{-1} (weight ratio dye/Lab), respectively, to obtain DiI-LNC, DiD-LNC and DiI/DiD-LNC. All the dye-loaded LNCs were prepared at the 3 sizes described for the non-labeled LNCs.

2.3. Hydrodynamic diameter, polydispersity index and derived count rate measurements

The hydrodynamic diameter (*Z*-ave), polydispersity index (PdI) and derived count rate (DCR) of LNC formulations were determined by quasi-elastic light scattering using a Zetasizer® Nano Series DTS 1060 (Malvern Instrument Ltd., Worcestershire, UK). The instrument is equipped with a 4 mW Helium–Neon laser, with an output wavelength of 633 nm, and a scatter angle fixed at 173°. All measurements were performed at 25 °C on LNC suspensions diluted by a factor of 60 (*v*/*v*).

The correlation functions were fitted using an exponential fit (Cumulant approach) for *Z*-ave and PdI determinations for LNC suspensions. DCR measurements were performed to confirm the presence of LNC in suspensions. DCR can be calculated from the measured count rates of the scattered light on the detector and the attenuation factor (linked to the attenuator value) according to the following relation:

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