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# A simple methodology to test the fluorescence-labeling stability of a nanocarrier

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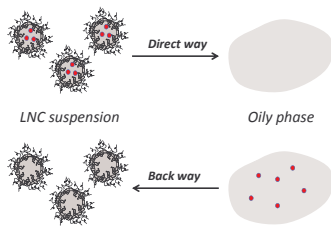
## ABSTRACT

Dye release from loaded- lipid nanocapsules (LNC) to dye-free oil medium (direct way) or from an oily phase containing dye to non-loaded LNC (back way) was performed in various conditions, by changing the nature of the dye and the oil, the oil volume and the LNC dilution. Results showed that the encapsulated dyes inside the LNC core (proved by surface tension) were able to be released, according to a partition coefficient behavior. In contrast, when the dyes were entrapped in the surfactant shell of LNCs, no release was observed. Dye-loaded LNC were put in cell culture and dye diffusion was clearly observed from LNC to cells, without LNC uptake.

## INTRODUCTION

Since biological fluids are hydrophilic, *in vitro* release of drug or dye from nanoparticles against water or buffers is a classical experiment to test formulations. Nevertheless, with encapsulated hydrophobic compounds, to our knowledge, no precise protocol was described for *in vitro* release studies. When release was operated against water or buffer, only the stability of the vector was assessed, and the hydrophobic compounds did not leave the nanoparticles due to thermodynamic considerations.<sup>1</sup> An original process was developed to study the release of amiodarone from nanoparticles to liposomes or non-loaded particles.<sup>2</sup> The results were not easy to extrapolate, due to the presence of the dialysis membrane separating the two media. Here, we proposed a new and simple method to study the *in vitro* release of dyes from nanoparticles to lipophilic media, without any medium separation. *In vitro* release of various dyes was studied and new insights about dye localization in nanoparticle were brought. Finally, the release properties observed *in vitro* were illustrated with cell culture, showing the possibility of misinterpretation if proper characterization was not previously performed.

## Objectives



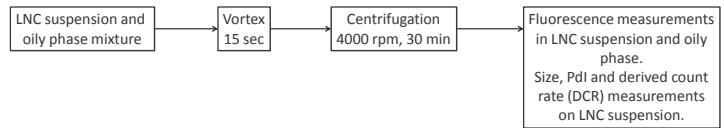
## Parameters:

- Dye nature : Nile Red, 6-Coumarine, DiI, DiO and DiD;
- Dye concentration : 1, 0.5 and 0.1 mg.g<sup>-1</sup>;
- R (ratio of oil in LNC / oil in oily phase) : 1/1, 1/2 and 2/1;
- LNC hydrodynamic diameter : 25, 50 and 100 nm;
- Oily phase nature : Labrafac®, Captex® 200, Captex® 300, ethyl oleate, PFCE;
- LNC suspension factor dilution : 1, 2, 3, 5;

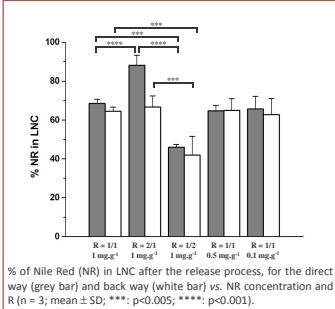
## LNC formulation

Based on a phase inversion process of an oil-in-water emulsion.<sup>2</sup>

## Protocol

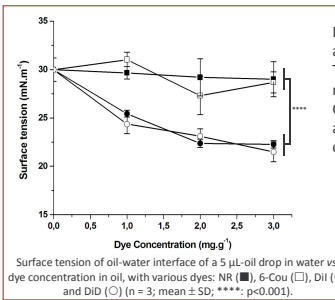
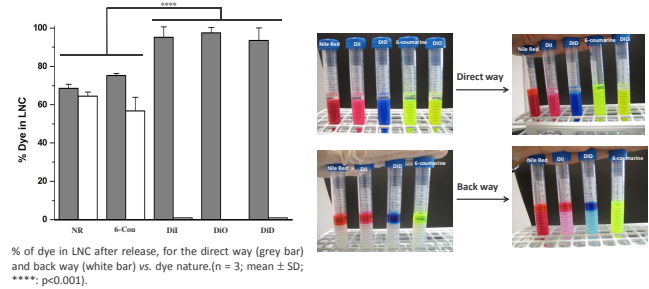


After separation, for each experiment, LNC size, Pdl and DCR values were the same that before the addition of oily phase, showing a good separation by centrifugation with a constant LNC concentration in LNC suspension.

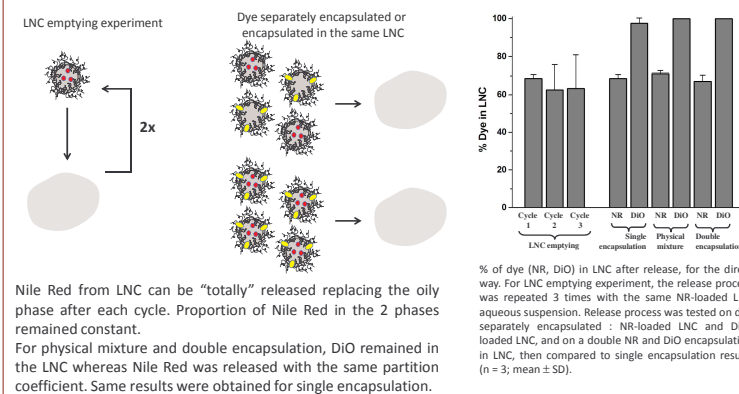
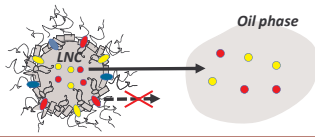


With Nile Red-loaded LNC, dye can be released from LNC to an oily phase. With Nile Red-loaded oily phase, dye can enter inside LNC from the oily phase. A partition coefficient was observed between LNC and the oily phase : after the separation protocol, about 65% of Nile Red was inside LNC and 35% in the oily phase, whatever the direct or back way. Nile Red proportion in each phase did not depend on the concentration of Nile Red but depended on R: the volume ratio of oil in LNC and oil in the outer phase, as for a partition coefficient. No effect of size was observed. Oily phase nature modified the partition coefficient values. LNC suspension dilution affected the partition coefficient (data not shown). A partition coefficient was also observed with 6-Coumarine.

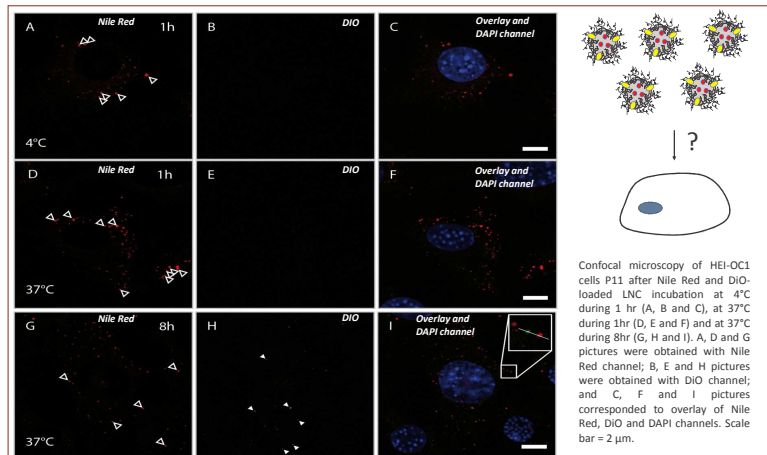
For DiI, DiD and DiO, an opposite effect was observed, with no release from dye-loaded LNC and no entry in LNC from the oily phase containing the dye. These dyes remained inside the LNC whatever the concentration and R (volume ratio of oil in LNC and oil in the outer phase).



DiI, DiD (and DiO) were tensioactive at the oil-water interface and participated to LNC formation, as other surfactants. These dyes were trapped in the LNC shell and can not be released from LNC to the oily phase. Nile Red and 6-Coumarine were not tensioactive at the oil-water interface and were encapsulated in the core of the LNC. These dyes could be released from the LNC to the oily phase.



Nile Red from LNC can be "totally" released replacing the oily phase after each cycle. Proportion of Nile Red in the 2 phases remained constant. For physical mixture and double encapsulation, DiO remained in the LNC whereas Nile Red was released with the same partition coefficient. Same results were obtained for single encapsulation.



At 4°C, when endocytosis is not occurring, Nile Red staining inside the cells (intracellular lipophilic structures) was observed after 1 hr incubation while DiO fluorescence was absent. Nile Red presence in the cells was due to a diffusion process, and its proportion increases with temperature, from 4 to 37°C. A 8h-incubation at 37°C allowed the LNC to be internalized by endocytosis : distinct DiO and NileRed positive structures were observed. However, DiO and Nile Red were not associated in the same structures (See insert in I), indicating that Nile Red was associated with lipid bodies and DiO stayed confined within the LNCs in endo-lysosomes.

## REFERENCES

- 1 - Lamprecht, A.; Bouligand, Y.; Benoit, J. P. *J. Control. Release* **2002**, *84*, 1-2, 59-68.
- 2 - Heurtault, B.; Saulnier, P.; Pech, B.; Proust, J. E.; Benoit, J. P. *Pharm. Res.* **2002**, *19*, 6, 875-880.

## CONCLUSION

A new protocol was developed to evaluate the release of hydrophobic active ingredients from nanocapsules to lipophilic media. As proof of concept, hydrophobic dyes were used but it could be applied to hydrophobic drugs, to obtain information on their release pattern (immediate or sustained profile). It will allow improvement of interpretation about cellular uptake and *in vivo* cell distribution.

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