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Lipid nanocapsules maintain full integrity after crossing a human intestinal epithelium model

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ABSTRACT

Lipid nanocapsules (LNCs) have demonstrated great potential for the oral delivery of drugs having very limited oral bioavailability (BCS class II, III and IV molecules). It has been shown previously that orally-administered LNCs can permeate through mucus, increase drug absorption by the epithelial tissue, and finally, increase drug bioavailability. However, even if transport mechanisms through mucus and the intestinal barrier have already been clarified, the preservation of particle integrity is still not known. The aim of the present work is to study *in vitro* the fate of LNCs after their transportation across an intestinal epithelium model (Caco-2 cell model). For this, two complementary techniques were employed: Förster Resonance Energy Transfer (FRET) and Nanoparticle Tracking Analysis (NTA). Results showed, after 2 h, the presence of nanoparticles in the basolateral side of the cell layer and a measurable FRET signal. This provides very good evidence for the transcellular intact crossing of the nanocarriers.

1. Introduction

The oral route is the most common route for drug delivery. However, oral bioavailability is influenced by drug solubility and permeability. For drugs belonging to the Biopharmaceutics Classification System class II (high permeability, low solubility), class III (low permeability, high solubility), and class IV (low permeability, low solubility) [1], drug encapsulation in nanocarriers provides an alternative solution to enhance bioavailability. Indeed, knowledge of the relationship between physico-chemical characteristics of nanocarriers (size and nature of wall material, surface charge, ligands, *etc.*) and the gastrointestinal physiology (acidic pH, digestive enzymes of gastrointestinal media, mucus and intestinal barrier), allowed drug bioavailability [2] to be enhanced. In the literature, various nanocarriers (liposomes, micelles, polymeric and lipid-based nanoparticles) are described for the oral delivery of chemotherapeutics, proteins, peptides, vaccines, *etc.* [3].

Lipid nanocapsules, developed by our group, have also proved to be very interesting for oral administration [4]. These nanoparticles have a size range from 20 to 100 nm and are prepared by a well-known low-energy emulsification process: the phase-inversion temperature method [5–7]. They have already shown promising properties for the oral delivery of paclitaxel [8,9], Sn38 [10], fondaparinux [11] and miltefosine [12]. Indeed, LNCs have demonstrated *in vitro* stability in simulated gastrointestinal media [13]. They have also shown their stability and diffusion in intestinal mucus [14]. Furthermore, in a previous study by Roger et al. [15], it was shown that LNCs were taken up by Caco-2 cells mainly *via* active endocytic and more particularly *via* clathrin-dependent and caveolae-dependent transport mechanisms; this active transport was not size-dependent. Comparing the incubation of paclitaxel-LNCs at 4 °C and 37 °C, an important decrease of paclitaxel permeability at 4 °C was observed (more than 10-fold for the larger LNCs) and so, very few passive transport was evidenced. Moreover, tight junctions were not disrupted by LNCs and consequently paracellular transport was not possible. Finally, using transmission electron microscopy (TEM), nano-objects were observed on the basolateral side of the Caco-2 cell monolayers when LNCs were applied on the apical side, which probably allow the formulation of a transcytosis hypothesis [15]. Additionally, these nanocarriers have demonstrated a direct effect of P-gp on their endocytosis [16]. In view of these results, LNCs have the ability to enhance the bioavailability of class II and IV drugs [8].

Besides, another challenge of drug delivery *via* the oral route is to target pharmacological receptors after absorption, like nanocarriers injected *via* the intravenous route (I.V.). For this purpose, it is important to design nanocarriers that are able to be absorbed while keeping their integrity, and thus able to behave as circulating nanocarriers. Nevertheless, even if the mechanisms of transporting nanocarriers through the

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intestinal barrier have been well characterised, the fate of these nanocarriers after oral delivery is still unknown [3]. This lack can be explained by the difficulty to find relevant methods to confirm the real integrity of the carrier after absorption [17]. Indeed, many physicochemical characterisation techniques such as dynamic light scattering (size and charge analysis), microscopy (TEM, AFM, confocal) and spectroscopy (IR, UV, FTIR, NMR) characterise the overall carrier structure or its individual components but not its integrity - *i.e.* is the cargo still inside the carrier? In this way, new tools are needed to determine how nanocarriers are absorbed and eliminated.

FRET is the most common fluorescence spectroscopy technique used to monitor the proximity at the nanoscale level [17]. It is based on the interactions between spatially-close (2 to 10 nm) donor and acceptor dye molecules. FRET occurs when the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. The excitation energy of the donor is transferred to the acceptor whose subsequent emission can be detected. FRET is highly sensitive to donor-acceptor distances and consequently it indicates a preserved nanoscale environment. This technique is widely used to study biological phenomena such as interactions between proteins or proteins and nucleic acids, conformational changes, lipid membrane dynamics, host-pathogenic interactions, *etc.* [18]. More recently, FRET has been used to study the integrity of nanocarriers *in vitro* in different media, after cell internalisation, or *in vivo* after I.V. administration [19–28]. Importantly, it enables quantification of nanocarrier integrity *in vitro* and *in vivo* [19]. Only 3 studies involving FRET and oral administration have been reported. Firstly, Groo et al. [14] demonstrated the integrity of LNCs, using 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) as FRET pairs, after contact with mucus. Lu et al. [29] used fluorescein isothiocyanate (FITC) and Rhodamine B pairs to monitor the assembly of nanocomplexes while pH evolves in the intestine. More recently, Li et al. [30] developed a FRET assay with DiO and DiI pairs to evaluate nanostructured lipid carrier (NLC) structural integrity in cell uptake; they observed a FRET signal in Caco-2 cells, and they speculated that NLCs could maintain their structural integrity after crossing intestinal cells, but they did not prove it. To our knowledge, no published work has clearly highlighted the integrity of nanocarriers after their transport through the intestinal barrier.

In order to use the FRET technique to study nanocarrier transport across enterocytes with a Transwell® set-up, it is important firstly to reach an efficient encapsulation load for both dyes leading to acceptable FRET efficiency [31] and, secondly, to measure with high sensitivity the FRET signal, since a high level of dilution is expected in the basolateral compartment. Moreover, to avoid the immediate partitioning of dye from the carrier to subcellular lipid compartments, which could decrease FRET efficiency even if nanocarriers are still intact; it is crucial to achieve very stable dye encapsulation with no significant leakage. According to Bastiat et al. [32], loading lipophilic carbocyanine dyes (DiO, DiI and DiD) is recommended since, unlike other dyes, they remain inside the nanocapsules in the presence of lipid acceptor compartments.

Furthermore, to increase the loading of carbocyanine dyes into the lipidic core of the LNCs and to obtain a significant FRET signal, a new concept of counterion-based dye was introduced by Kilin et al. [33]. They substituted the perchlorate counterion for the bulky and highly hydrophobic tetraphenyl borate (TPB) which improves dye solubility in oil and consequently the dye loading capacity (Fig. 1).

In this context, we encapsulated two lipophilic cyanine dyes (DiD and DiI) bearing TPB counterions inside 50 nm LNCs. We optimised dye loading to achieve an efficient FRET signal. Then, the transport of the obtained ultrabright FRET-loaded LNCs was studied with a well-established intestinal epithelium model (Caco-2 cells) [34,35]. To complete this approach, NTA was also performed to measure the size distribution of nanoparticles and their concentration. The objective of the present study is to investigate the fate of LNCs after crossing the intestinal barrier, focusing on nanoparticle integrity.

2. Materials and methods

2.1. Materials

All the chemicals and solvents for synthesis were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Captex® 8000 (tricaprylin) was a gift from Abitec Corp. (Colombus, Ohio, USA). Lipoid® S75-3 (soybean lecithin at 70% of phosphatidylcholine and 10% of phosphatidylethanolamine) and Kolliphor® HS15 (mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were purchased from Lipoïd GmbH (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. NaCl was purchased from Prolabo VWR International (Fontenay-sous-Bois, France). Ultrapure water was obtained from a Milli-Q® Advantage A10 System (Merck Millipore, Darmstadt, Germany). Culture reagents were obtained from Sigma (St. Louis, USA) and Lonza (Verviers, Belgium).

2.2. Preparation of DiI-TPB and DiD-TPB

DiI-TPB was synthesised as described elsewhere [33]. To synthetize DiD-TPB, 100 mg of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD, Life Technologies, USA) was mixed with 0.71 g (20 mol eq.) of sodium tetraphenylborate in ethyl acetate, which dissolved both salts easily. The formation of the desired salt was confirmed by thin-layer chromatography, where the product moved much faster than the starting DiD perchlorate (dichloromethane/methanol, 95/5). After solvent evaporation, the product (DiD-TPB) was purified by column chromatography (dichloromethane/methanol, 95/5).

2.3. Formulation and characterisation of lipid nanocapsules (LNCs)

LNC formulation was based on a phase-inversion process and has been thoroughly described [5,6]. Briefly, the fluorescent dyes were solubilised in Captex® 8000. Then, 150 mg of Captex® 8000 (oil phase), 225.0 mg of water, 9.1 mg of NaCl (aqueous phase), 8.4 mg of Lipoid® S75-3 and 125 mg of Kolliphor® HS15 (surfactants) were precisely weighed. After this, three cycles of heating and cooling between 70 and 90 °C were carried out and the suspension of LNCs was obtained

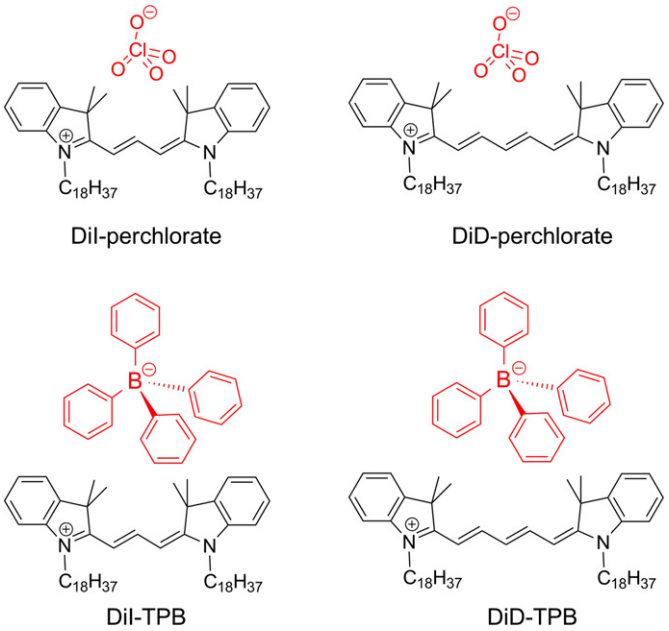

Fig. 1. Chemical structures of DiI and DiD dyes with perchlorate and tetraphenylborate (TPB) counterions.