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Counterion-enhanced cyanine dye loading into lipid nano-droplets for single-particle tracking in zebrafish

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ABSTRACT

Superior brightness of fluorescent nanoparticles places them far ahead of the classical fluorescent dyes in the field of biological imaging. However, for *in vivo* applications, inorganic nanoparticles, such as quantum dots, are limited due to the lack of biodegradability. Nano-emulsions encapsulating high concentrations of organic dyes are an attractive alternative, but classical fluorescent dyes are inconvenient due to their poor solubility in the oil and their tendency to form non-fluorescent aggregates. This problem was solved here for a cationic cyanine dye (DiI) by substituting its perchlorate counterion for a bulky and hydrophobic tetraphenylborate. This new dye salt, due to its exceptional oil solubility, could be loaded at 8 wt% concentration into nano-droplets of controlled size in the range 30–90 nm. Our 90 nm droplets, which contained >10,000 cyanine molecules, were >100-fold brighter than quantum dots. This extreme brightness allowed, for the first time, single-particle tracking in the blood flow of live zebrafish embryo, revealing both the slow and fast phases of the cardiac cycle. These nano-droplets showed minimal cytotoxicity in cell culture and in the zebrafish embryo. The concept of counterion-based dye loading provides a new effective route to ultra-bright lipid nanoparticles, which enables tracking single particles in live animals, a new dimension of *in vivo* imaging.

1. Introduction

Fluorescent nanoparticles have been a dramatic surge in the recent years due their unique properties, placing them far ahead of the classical fluorescent dyes in the field of biological imaging. Thus, quantum dots [1,2], dye-doped silica nanoparticles [3,4], and nanodiamonds [5,6] show significantly higher brightness than fluorescent dyes together with possibility of multiple surface modifications for specific targeting. They were particularly important for techniques that require super-sensitive detection, namely, single-particle tracking (SPT) and *in vivo* animal imaging. SPT with fluorescent nanoparticles was successfully used for monitoring diffusion of biomolecules in live cells and flow in biological fluids [7–11]. However, it remains a challenge to perform SPT in live animals due to the insufficient brightness of the nano-objects and the strong light absorption and scattering of the tissues. The experiments in animals reported so far deal with *in vivo* imaging of large populations of nanoparticles [12–15], so that behavior of individual nanoparticles was not addressed. One rare example showed the possibility to monitor the pathway of individual quantum dots *in vivo*, to clarify the mechanism of their interaction and internalization into tumors [16]. It is obvious that SPT experiments require ultra-high brightness. Moreover, *in vivo* applications request biodegradability, which is not the case for the well-established inorganic nanoparticles. An attractive alternative is offered by organic nanoparticles, which being composed of organic materials, lipids or polymers, can be intrinsically non-toxic and biodegradable [12,17–19]. Moreover, they can encapsulate a large quantity of organic dyes, so that particles of exceptional brightness, close or even superior to quantum dots can be obtained [20–24].

In this respect, lipid nano-emulsions, composed of nano-droplets with liquid-core, are of particular interest for biological

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imaging [21,25–27]. Firstly, nano-emulsions are composed of non-toxic components, which are biodegradable and/or readily eliminated from the animal body. Secondly [28–31], the oily core of nano-droplets is a perfect reservoir for encapsulation of lipophilic dyes, in contrast to the solid-core particles, where the distribution and fluorescent properties of the dyes are difficult to control [32]. The other advantage is their simple and rapid preparation based on spontaneous nano-emulsification [28,30,31], allowing a sharp control of the size and composition of the nano-droplets. However, examples of highly fluorescent lipid nano-droplets and their application for bioimaging are limited. In one report, Texier et al. prepared lipid nanocarriers encapsulating cyanine dyes bearing long hydrophobic chains [20]. The authors encapsulated up to 53 molecules per particle of 35 nm diameter, which corresponded to a 3.9 mM concentration of the dye in the droplets and showed their successful application for cellular and *in vivo* animal imaging [25]. Recently, we designed lipophilic fluorescent derivatives of 3-alkoxyflavone and Nile Red. For the non-planar 3-alkoxyflavone dye, 170 mM loading was achieved with no sign of self-quenching, which corresponds to ~830 dyes per 40-nm droplet. In contrast for the Nile Red derivative, self-quenching was observed already above 17 mM, indicating that its planar structure favors its aggregation at high concentrations [21]. Achieving high encapsulation efficiency for cyanine dyes, as for the 3-alkoxyflavone, would be of particular importance, because they show ~10-fold larger absorption coefficient compared to 3-alkoxyflavones. However, the cationic nature of cyanines limits their solubility in the apolar oils of the nano-emulsions. Moreover, at high concentrations, cyanines tend to form non-emissive π-stacked structures, so-called H-aggregates [33], which are responsible for strong self-quenching. We hypothesized that these problems could be solved by replacing a small hydrophilic counterion (i.e. perchlorate) of a cationic cyanine dye with a bulky hydrophobic tetraphenylborate (TPB) anion (Fig. 1). Salts of TPB are known to be soluble in organic solvents and used as ionophores in polymer matrix, to generate ionic sites in cation-sensitive electrodes [34,35]. Moreover, a recently proposed "ion-association" method uses TPB derivatives to precipitate water soluble cationic organic dyes into fluorescent nanoparticles [36].

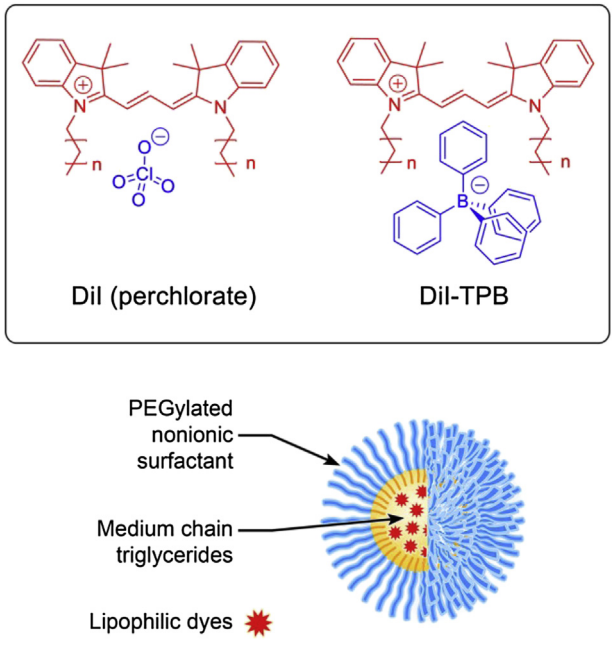

Fig. 1. Chemical structures of DiI perchlorate and DiI-TPB and schematic presentation of nano-droplet encapsulating them.

However to date, this method has never been applied for increasing the encapsulation efficiency of cationic dyes into lipid nanocarriers.

In the present work, we used fluorescent nano-emulsion as a nanocarrier of hydrophobic cyanine dye 1,1′-dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine (DiI) and proposed an innovative approach to drastically improve the dye encapsulation efficiency by replacing its perchlorate counterion with a bulky hydrophobic TPB (Fig. 1). After detailed characterization of the obtained nano-droplets of different size in solutions, they were studied in the blood circulatory system of zebrafish at the single-particle level. This work opens a new route to biodegradable organic nano-objects of exceptional fluorescence brightness, which enable single-particle tracking *in vivo*.

2. Materials and methods

All chemicals and solvents for synthesis were from Sigma–Aldrich. Nonionic surfactants (Cremophor ELP®) are of parenteral grade, and consist of a PEG chain (M_w = 1500 g/mol^{-1}) grafted onto a triglyceride (castor oil). They were obtained from BASF (Ludwigshafen, Germany) kindly gifted from Laserson (Etampes, France). Medium chain triglycerides (Labrafac CC®) were obtained from Gattefossé (Saint-Priest, France). Ultrapure® water was obtained using a Milli-Q filtration system (Millipore, Saint-Quentin-en-Yvelines, France). Culture reagents were obtained from Sigma (St. Louis, USA), Lonza (Verviers, Belgium) and Gibco-Invitrogen (Grand Island, USA).

2.1. Synthesis of DiI-TPB

100 mg of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) perchlorate (Life Technologies) was mixed with 3.6 g (100 mol eq) of sodium tetraphenylborate in ethyl acetate, which dissolved readily both salts. The formation of the desired salt was confirmed by thin layer chromatography, where the product moved much faster than the starting DiI perchlorate (dichloromethane/methanol, 95/5). After solvent evaporation the product (DiI-TPB) was purified by column chromatography (dichloromethane/methanol, 95/5).

2.2. Formulation and characterization of nano-emulsions

Nano-emulsions were prepared by spontaneous nano-emulsification. Briefly, DiI-TPB was dissolved in Labrafac CC®. Then, Cremophor ELP® was added and the mixture was homogenized under magnetic stirring at 35 °C. Nano-emulsions were formed by adding ultrapure (Milli-Q) water. Two sizes of nano-droplets were prepared by varying the proportions between the different components (Table 1). Formulation A, giving nano-droplets of *ca* 30-nm diameter contained 20 mg of Labrafac CC®, 80 mg of Cremophor ELP® and 230 mg of water. Formulation B, giving nano-droplets of *ca* 90-nm diameter contained 55 mg of Labrafac CC®, 45 mg of Cremophor ELP® and 230 mg of water.

The size distribution of the nano-emulsions was determined by dynamic light scattering on a Zetasizer® Nano series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK) using the following specifications: medium viscosity, 0.8872 cP; refractive index (RI) medium, 1.33; RI of nano-droplets 1.454; scattering angle, 90°; temperature, 25 °C.

2.3. Fluorescence spectroscopy

Absorption and fluorescence spectra were recorded on a Cary 4 spectrophotometer (Varian) and a Fluorolog (Jobin Yvon, Horiba) spectrofluorometer, respectively. Fluorescence emission spectra were recorded at room temperature with

Table 1
Characterization of the nano-droplets encapsulating cyanine dyes by DLS and fluorescence measurements.

% surfactant	Dye	[Dye], wt%	Size, nm	PDI	Dyes/droplet	QY
70	–	0	27	0.07	–	–
50	–	0	87	0.17	–	–
70	DiI-ClO4	0.1	36	0.11	8	0.50
70	DiI-TPB	0.1	23	0.13	2	0.49
70	DiI-TPB	1	28	0.02	33	0.31
70	DiI-TPB	4	24	0.19	75	0.17
70	DiI-TPB	8	31	0.13	384	0.13
50	DiI-TPB	8	87	0.16	12,052	0.14

% surfactant is the concentration of Cremophor (wt%) in the mixture with Labrafac, used in the formulation; *[Dye]* is the dye concentration (wt%); *size* is the hydrodynamic diameter of nano-droplets (nm); *PDI* is the polydispersity index; *Dyes/droplet* is estimated number of dyes per droplet; *QY* is the fluorescence quantum yield.